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The Role and Control of Growth Suppression in the Transition from Diffuse to Apical Meristematic Growth in *Physcomitrella patens* (Funariaceae)

by

Madeline Micceri Mignone

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

2001

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Abstract

The Role and Control of Growth Suppression in the Transition from Diffuse to Apical Meristematic Growth in *Physcomitrella patens* (Funariaceae)

by

Madeline Micceri Mignone

Adviser: Dominick V. Basile

"The Suppression Hypothesis", proposed by Basile and Basile states, in part, that the interrelated actions of auxin, ethylene, and hydroxyproline containing arabinogalactan proteins (AGPs) comprise a correlative control system involved with the place-dependent suppression of cell proliferation. They further proposed that this correlative control system plays three important roles in plant morphogenesis. Of the three roles, this study concentrated on the first, the mediation of the transition from diffuse to apical growth. Using *Physcomitrella patens* as a model system, it was ascertained that the hypothesized interrelated action of auxin, ethylene, and arabinogalactan proteins regulated the transition from a protonematal stage, characterized by a diffuse pattern of cell proliferation to a leafy gametophore stage, characterized by strictly apical growth. Antagonists of each component of the chemoregulatory system (i.e., auxin, ethylene, and hydroxyproline proteins[AGPs]) were used to determine whether each is involved in bringing about the changes from a primary chloronematal stage to that of the gametophore bud stage. 2-[(1-Naphthalenyl-amino)carbonyl]benzoic acid (ALANAP) and 2,3,5 triiodobenzoic acid (TIBA) were the antagonists used against auxin transport, aminoethoxyvinylglycine (AVG) as an antagonist to ethylene synthesis, and hydroxy-L-proline (Hyp) and 3,4 dehydroproline (3,4 D) as antagonists of hydroxyproline synthesis. When compared to the control, each of these antagonists successfully prevented the suppression of diffuse cell proliferation and thereby the transition from the chloronematal stage to the gametophore stage.

Comparative analysis of AGPs revealed significant differences in the quantity of protein extracted from equal amounts of diffusely growing protonema, desuppressed tissue and the apically grown leafy gametophore (suppressed) tissue. Electrophoretic studies also revealed a difference in the protein profile between the chloronematal and gametophore tissue.

That auxin and ethylene promote the transition was evidenced by the finding that 1-naphthaleneacetic acid –NAA (auxin) and or 1- aminocyclopropane-1 carboxylic acid –ACC (an ethylene precursor) accelerated the transition from protonemata to bud formation.

Further physiological studies provided evidence that the suppression of gametophytic tissue could be reversed by exogenous treatments of antagonists to auxin transport, ethylene production, and AGP synthesis (the same antagonists cited above). This indicates that the apical dominance countermanded, thus providing a reversion back to a desuppressed state.

Acknowledgments:

At this time I would like to thank Dr. Dominick V. Basile, my mentor and friend, for all the support, patience, and encouragement that he gave me during my tenure as his student. I also wish to give special thanks to Dr. Margaret R. Basile for all the help she gave me during these past years. She has been my teacher and friend, and like Dominick, she always found time in her busy schedule to give encouragement. To both of the Basiles I will always be grateful for the honor of working under their guidance. It has been a privilege to work with you.

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Finally I would like to dedicate this thesis to my family--my husband, children, and parents. First and foremost I am most grateful to my husband, Nino, and my four beautiful and patient children: Roberto, Joseph, Francesco, and Stefania. I know these past several years have been difficult for you, but you have been more than understanding. I never would have succeeded if it weren't for your continuous tolerance and sacrifice.

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Table of Contents

Table of Contents

List of Tables

List of Illustrations, Charts, Diagrams, Micrographs

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 6:

Chapter 1: Introduction

The capacity of cells to divide and thereby reproduce is one of the defining properties of life. In the estimated three billion years between the evolution of cellular life on Earth and the origin of the earliest land plants (embryophytes), this capacity has continued to be "expressed" in the cells of virtually all pre-embryophytes. In the great majority of the existing taxa of Chlorophytes, the putative pre-embryophytes, this capacity is still expressed in most of their somatic cells in the course of their development.

Although not possible to demonstrate, it seems reasonable to infer that in the earliest known embryophytes, the Rhyniophytes, this capacity to proliferate was suppressed in most of the somatic cells. The inference is based on what is known about the morphogenetic process that results in the strict dichotomous branching which occurs in extant plants such as *Psilotum nudum* . After an apical cell is differentiated, all proliferative divisions are restricted to a small number of meristematic cells found at the apex of each developing axis. In *P. nudum* plants, proliferation becomes completely suppressed in an ever-increasing majority of the living cells during the course of their growth and development. Inferential evidence strongly suggests that morphogenesis in the dichotomously branching Rhyniophytes was like that of *Psilotum*, both developing according to a common pattern: cell proliferation being "expressed" at the very apices only and "suppressed" everywhere else.

The foregoing observations led to the proposition that the evolution of a morphoregulatory mechanism by which some cells could become "dominant' by virtue of suppressing the proliferation of neighboring cells was a prerequisite for establishing apical growth (Basile and Basile, 1993). Furthermore, since strict apical meristematic growth characteristic of embryophytes but not characteristic of presumptive pre-embryophytes, the establishment of this regulatory system may be viewed as a critical event in the origin and diversification of land plants from their aquatic ancestors.

Although it cannot be proven that the transition from a diffuse pattern to a highly localized pattern of cell proliferations marked the "phylogenetic transition" from pre-embryophytes to embryophytes, it can be demonstrated that such a transition regularly occurs during the course of ontogeny in all extant land plants, with the exceptions of the sporophytes of liverworts and the gametophytes of heterosporous taxa. These exceptions are widely interpreted as being the result of "regressive" or "reductive" evolution. In all other embryophytes, including the gametophytes of liverworts and the sporophytes of heterosporous taxa, whether starting from spore or zygote, initial development proceeds through an early "embryonic" stage in which the plant body is generated by a more or less diffuse pattern of cell divisions (Wardlaw, 1955). In most taxa, the period characterized by a diffuse pattern of cell divisions is relatively short-lived after which most cells are suppressed with only one or more subpopulations being able to continue to express their innate capacity to divide.

There should be no doubt that suppression of cell proliferation plays a pivotal role in the transition from a diffuse to a polar, apical meristematic growth that is characterisitic of one or both generations of all land plants. The question posed by this hypothesized basis for the transition is, "by what means do the proliferation of some cells of a developing plant become suppressed?" Insight as to a possible mechanism that could be responsible for mediating the suppression of cell proliferation was obtained from a series of experiments conducted with species belonging to five families of leafy liverworts, the results of which have been thoroughly reviewed (Basile, 1990; Basile and Basile, 1990, 1993). Briefly what these experiments revealed was that, first antagonists of hydroxyproline protein synthesis, then antagonists of ethylene synthesis or action, and finally antagonists of auxin transport or action could act to prevent the suppression of cell proliferation in certain leaf and branch primordia. Since antagonizing any one of the three components produced the same results, it was hypothesized that auxin, ethylene, and certain hydroxyproline-containing proteins (tentatively identified as arabinogalactan proteins or AGPs) constitute a correlative control system. This is responsible for the place dependent suppression of leaf and branch primordia of leafy liverworts (Basile and Basile, 1993). It was further hypothesized that this correlative control system may not be peculiar to the suppression of leaf or branch development in leafy liverworts. Rather, it was suggested that this system provides one of the means by which place-dependent suppression can be achieved in any plant part or region and in any or all land plants (loc.cit). This hypothesis has been termed 'the suppression hypothesis" (Basile and Basile, 1993).

The objective of the research reported herein was to test this hypothesis. Specifically, the goal was to determine whether the same correlative control system that operates to suppress cell proliferation in certain leaf and branch primordia could also be involved in the suppression of cell proliferation that is correlated with the transition from diffuse to apical, meristematic growth and development in a plant other than a leafy liverwort.

The plant chosen for this purpose was *Physcomitrella patens* Hedw., a moss in the Funariaceae, Bryales. This plant was chosen for a number of reasons. Like most species included in the Bryales, the initial developmental stage following spore germination in *P. patens* in charactaerized by an extended period of diffuse cell proliferations that result in a much-branched filamentous body, a protonema. The transition from a diffuse to a highly localized pattern of cell proliferation is signaled by the differentiation of numerous apical initials at various positions on the protonema. Moreover, the regulation of development of *P. patens* has been the subject of numerous investigations resulting in an important body of literature to which the present study will be later related.

This study was conducted in three stages. The first stage consisted of a series of experiments in which antagonists of auxin, ethylene, and hyroxyproline-protein synthesis and/or action were used to determine whether the suppression of protonematal development and onset of apical development would thereby be delayed. The second stage consisted of comparative chemical analyses to determine whether the switch from the diffuse to the localized pattern of cell proliferation could be correlated with either qualitative or quantitative changes in hydroxyproline-containing arabinogalactan-proteins (AGPs). The third stage consisted of experiments in which an auxin and a precursor of ethylene synthesis were used to determine whether these components of the putative correlative control system could induce a precocious transition from protonematal (diffuse) to apical (localized) cell proliferation. The results of all these studies support the hypothesis that auxin, ethylene, and some arabinogalactan-protein(s) can act in some presumably interrelated way to bring about the transition from diffuse to localized cell proliferation by virtue of locally suppressing cell proliferation.

Chapter 2: Literature Review

Pertinent literature review:

The literature review will be divided into sections that include background information on: apical bud development, the "Suppression Hypothesis", auxin and ethylene and how their pathways relate to moss development, the significance of the family of AGPs and their relationship to plant development, and related research pertaining to *P. patens* and related moss (Funariaceae) development.

1. The importance of apical bud development:

Morphogenesis of the moss *Physcomitrella patens* proceeds characteristically with the transition from a diffuse stage of growth, to that of suppressed growth as charactererized by restricted apical meristematic growth.

Gifford (1983) asserted that the establishment of apical growth and apical dominance were important events in the evolution of green land plants. The apical cell is essentially the ultimate source of all cells for the moss gametophyte and likely plays a regulatory role in development (Gifford, 1983). The idea of an apical cell as an apical initial has arisen because the apical cell is solely situated to generate all other cells of the shoot presuming of course that the apical cell grows and divides. In mosses, the pattern of cell walls in the shoot tip indicates that the derivative of an apical cell forms a leaf. In essence, the apical cell is the only cell left for proliferation of the apex (Paolillo, 1984).

In most of moss taxa, the complete protonema is filamentous. There is a distinct differentiation within the protonematal stage of the chloronematal and the caulonematal cells. It is from these protonematal filaments (either from the chloronemata or caulonemata), that pyramidal apical cells arise. The bud development emanates from complex physiological events that involve highly refined phytochrome, cytokinin, auxin, and protein interactions (Crandall-Stotler, 1980).

The apical growth of the moss shoot occurs from a single tetrahedral (also described as a pyramidal) apical cell usually with three cutting faces. The gametophytic histogenesis of both liverworts and mosses begins with regular, slow, mitotic division of the single pyramidal apical initial (also known as derivatives, segments, or merophytes) (Crandall-Stotler, 1980; Puri, 1973). In this three-faced apical cell, segments are demarcated regularly from each face. Each segment divides periclinally (Puri, 1973).

The apical cell of mosses varies in shape from obovoid in the acrocarps (erect mosses) to narrowly fusiform in pleurocarps (prostrate mosses). In transverse section the apical cell appears to be from three to five sided. The variations in the appearance of the apical cell in cross-section is due to the obliquity (angling) of segmentation that characterizes most mosses (Crandall-Stotler, 1980). Per Crandall-Stotler (1980), the phragmoplast of the developing derivatives extends from a point of intersection of two sides of the apical cell to a given location on the wall opposite the point of intersections rather then extending in a plane parallel to an existing wall. This obliquely located derivative produces a triangular outline of the apical cell when it is viewed at the surface, with the base of the triangle lying in a plane contiguous with the long axis of the next formed derivative (see figure 2.1 [apical cell]) (Crandall-Stotler, 1980). In almost all mosses (see figure 2.1 [apical cell]), the first division wall of the derivative extends from one lateral wall to the other in an approximately periclinal plane and divides into inner and outer initials. The three inner daughter cells or initials, go through rapid longitudinal and transverse divisions, and form the inner tissues of the stem, (Puri, 1973), that is the cortex and stele initials (Crandall-Stotler, 1980). The three outer daughter cells of the segments contribute to the three leaves, the subtending outer portion of the stem and to the lateral branches (Puri, 1973). From the foliar initial, the leaf apical cell will be formed by two oblique divisions of the foliar initials. The first division will generally occur in the anodic portion of the initial, whereas the stem (caulogenic) initial after a period of dormancy, will form a branch and internodal epidermal tissue. Only the internodal epidermal tissue or caulogenic tissue regenerates. As the undivided leaf begins to grow from regular segmentations of the single leaf apical cell, a filamentous, chloronema-like mucilage hair is formed from the anodic basal leaf cell, and differentiation of a multilayered costa, often containing special water-conducting cells (called hydroids) begins at the leaf base. Further development of cellular differentiation proceeds acropetally (Crandall-Stotler, 1980).

In *Funaria hygrometrica*, a tetrahedral apical cell is differentiated from certain caulonematal cells. This apical cell gives rise to three series of derivative cells that soon become organized as minute buds. Each bud may form young leafy gametophytes (Bold et al.,1987). In mosses, once the bud has been formed, it first grows to a moruloid stage, through divisions of the apical cell. The basal part of the bud develops rhizoids with positive geotropism (Knoop, 1984). In a group of mosses with distichous or bilateral symmetry, exemplified by *Fissidens*, the foliar initials are cut from only two faces of the apical cell (Gifford, 1983; Crandall-Stotler, 1980; Bold et al., 1987).

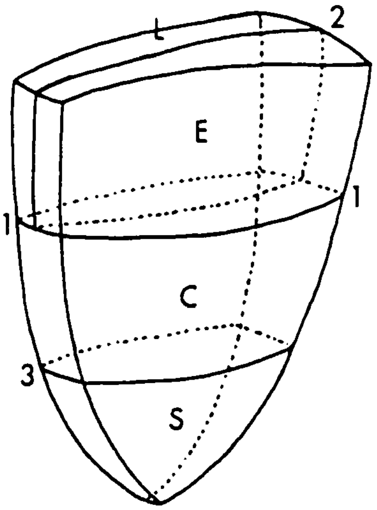


Figure 2.1. A tetrahedral apical cell. (Crandall-Stotler, 1980)

The apical pole forms a kind of meristem in that the daughter cells of the apical cell continue to divide, achieving a higher frequency of cell division than the central cell itself, thus forming a mound around it. It is from this mound that leaf primordia are derived. Each of the daughter cells of the apical cell finally gives rise to one primordium, permitting the expression of the regular divisions of the apical cell involving temporal and spatial patterns (Knoop, 1984). The apical cell therefore apparently controls the direction and velocity of apex development. In turn the derivatives, which are smaller in size with a shorter cell cycle, take over the production of stem and leaf tissue (Knoop, 1984).

Hébant (1978) reported that during an active growth period, the apical cell of *Polytrichum commune* divided on the average of two times every three days. He also found a difference in the polarity of the apical cell during the quiescent and active growth periods. During the quiescent period, the nucleus is located in the upper part of the cell with a zone of dense cytoplasm existing. During mitosis, the nucleus is located more centrally, and the bulk of cytoplasm moves laterally. After division occurs, the apical cell contains dense cytoplasm and the derivative can be identified by the more vacuolated cytoplasm. It is conjectured that perhaps the numerous microtubules found in the apical cell during interphase are involved in these protoplasmic movements (Gifford, 1983).

Per Hèbant et al. (1978) apical cell enlargement, accompanied by organelle migration, follows each segmentation and reestablishes the polarity necessary to produce the next oblique division. The angles produced by such a segmentation cause the phyllotaxy characteristic of the species and what has been termed by some researchers as apical torsion.

The most common phyllotaxies in mosses are 2/5 and 3/8. In the 2/5 phyllotaxy a 2 + 3 contact parastiches exists and in a 3/8 phyllotaxy, a 3 + 5 contact parastiches exists (Hèbant et al., 1978).

The *Polytrichum commune* morphogenesis study by Hèbant et al. (1978) is an excellent model and example of apical cell and meristem formation. From these studies it has been established that the outer cell (surface) divides and produces two cells. The upper cell forms a projection that will give rise to a bilateral apical cell of the leaf primordium. The lower cell, through successive divisions, contributes to the formation of the leaf base tissue and cortex. The original inner cell of the first segment will produce the central hydroids, leptoids, and parenchyma-like cells. The apical cell is essential in giving rise to derivatives which are then responsible for leaf development and the formation of the gametophytic axis (Gifford, 1983).

In essence, apical meristems may have evolved as a consequence of the evolution of a correlative control system that functions to suppress the inherited capacity (i.e., totipotency) that all intact cells originally had or still presently do have--that is, the ability to undergo proliferative cell divisions (Basile and Basile, 1993). This brings me to the discussion of the significance of the "Suppression Hypothesis" as it pertains to the transition from diffuse growth to apical mersitematic growth (Basile and Basile, 1993).

2. Suppression Hypothesis:

The "Suppression Hypothesis" as proposed by D.V. Basile is a working hypothesis based on over thirty years of observations and experimentation (Basile 1967, 1979, 1980, 1990; Basile and Basile 1980, 1982, 1984, 1990, 1993, 1994). In these years, as more experimental data became available, this hypothesis has been refined. This hypothesis proposes that auxin, ethylene, and hydroxyproline-containing proteins (specifically arabinogalactan proteins [AGPs]) make up a correlative control system in the place-dependent suppression of cell proliferation. The hypothesis states that the strongly directional transport of auxin creates a higher concentration of auxin in a given location of the developing plant, and its effective concentrations is the component of this control system that is most likely responsible for spacial ordination. This effective concentration of auxin is responsible for triggering ethylene production which, in turn, is critical in establishing the spacial component of the spacio-temporal regulation of growth suppression. Ethylene provides the environment that determines whether and/or how suppression occurs by permitting the binding (or deposition) of one or more AGPs to plant cell surfaces (Basile, 1990; Basile and Basile, 1993, 1994).

Of the three components involved in the interrelated correlative system, AGPs may contribute most directly to the subtle differences in the make-up of plant cell surfaces (Basile, 1990; Basile and Basile, 1993, 1994).

The components of the control system regulating growth suppression have been observed to play at least three critical roles in leafy liverwort morphogenesis and phylogeny. First this suppression is responsible for bringing about the transition from the protonematal stage to the apical growth stage. Secondly, it is responsible for the localized suppression of primordia. The recurrent trend in taxa toward reductive evolution is a trend that has been characterized by what is now termed an "apparent loss" in the capacity to produce a given number of parts and/or kind of parts. Finally, this suppression is responsibile for the modulation of size and shape of organs having a determinable mode of development thus generating the form and size that a given plant will have (Basile, 1990; Basile and Basile, 1993, 1994).

3. Auxin

Auxin is a phytohormone that promotes cell elongation in plants. It has been observed that the highest concentrations of auxins are found in the growing tips of plants -- that is, in coleoptile tips, buds, and growing tips of leaves and roots (Devlin and Witham, 1983; Taiz and Zeiger, 1991). There are two general categories of auxins in plants: free and bound.

Free and Bound Auxins:

Free auxins include diffusible auxins which move out of the tissue readily and auxins which are extractable in various solvents (Devlin and Witham, 1983).

The most abundant form of free auxin is indole-3-acetic acid (IAA). Other forms of free auxins can be found in the form of indole-3-acetaldehyde (IAAD), indole-3-acetonitrile (IAN), indole-3-pyruvic acid, and indole-3-ethanol (Taiz and Zeiger, 1991). The chemical formula of IAA is seen in figure 2.2.

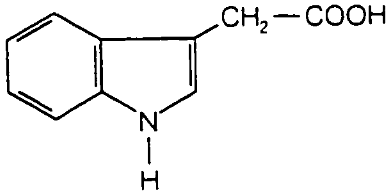


Figure 2. 2 The Chemical formula of Indole-3-acetic acid. (Devlin and Witham, 1983; Taiz and Zeiger, 1991)

Bound auxins are those that are not readily extractable, and can only be released from plant tissue after they are subjected to hydrolysis, autolysis, or enzymolysis (Devlin and Witham, 1983). These are also called conjugated auxins (Taiz and Zeigler, 1991). Bound auxins are found in reserve or storage forms. These auxins are combined with substances in the cell, so that they are not easily extractable. They are also formed in detoxification forms that are a result of excess IAA or the presence of high levels of synthetic auxins that may exogenously be introduced into plants. One example of endogenous bound auxin is auxin glucosyl esters, which are abundant in seeds, and which are inactive until the IAA is released through enzymolysis (Devlin and Witham, 1983). IAA has been conjugated to both high and low molecular-weight compounds. For example, low molecular-weight bound auxins include the previously mentioned esters of IAA with glucose or myo-inositol conjugates such as IAA-N-aspartate. High molecular weight conjugates include IAA-glucan and IAA-glycoproteins found in cereal seeds (Taiz and Zeiger, 1991).

b. Auxin Biosynthesis.

The auxin (Indole-3-Acetic Acid) biosynthesis pathway is produced by converting tryptophan via several enzymatic steps. The pathway of IAA synthesis from tryptophan is shown below in Figure 2.3.

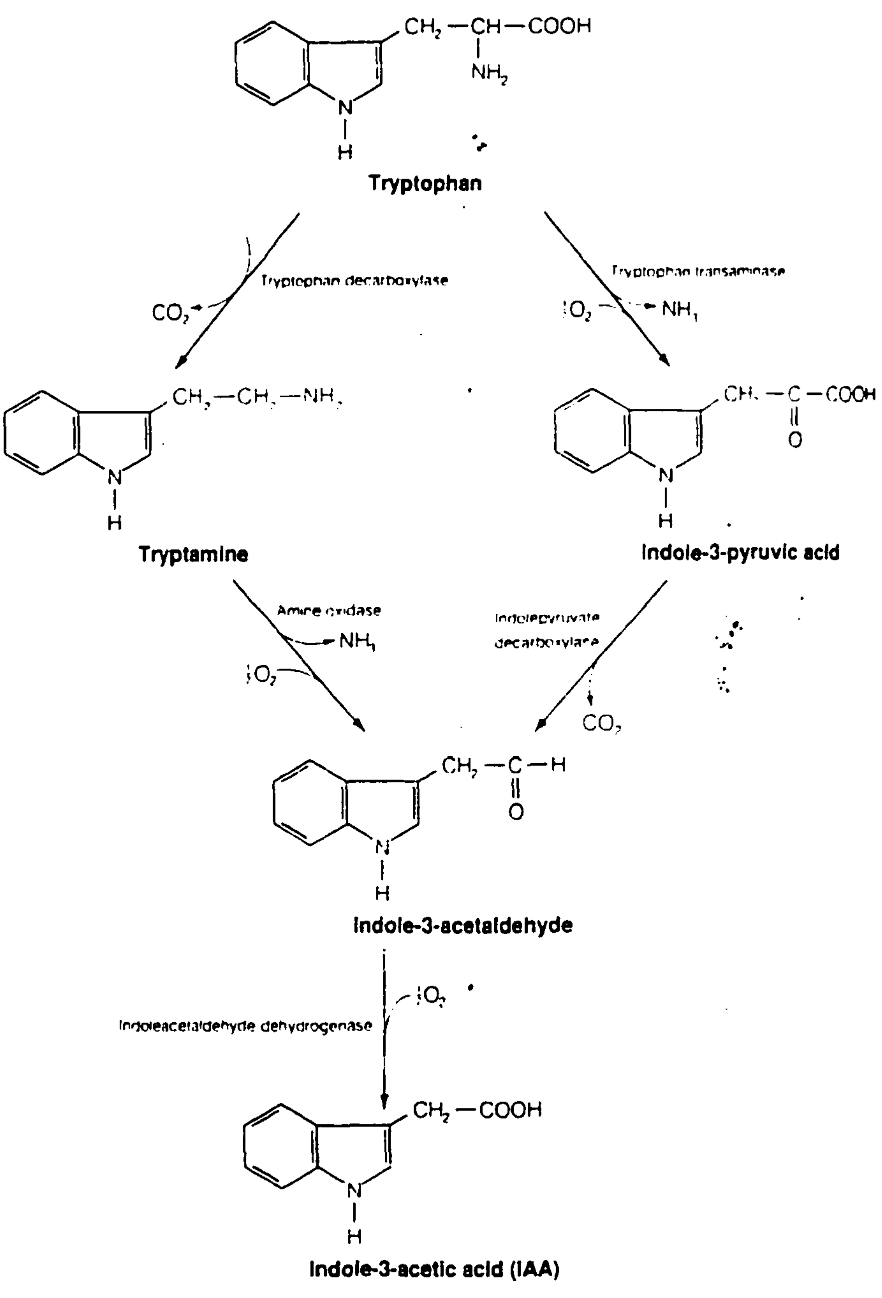


Fig. 2.3 There are two principal pathways for IAA biosynthesis in highter plants. The first. tryptophan to tryptamine yielding IAA. and the second. tryptophan to indole-3-pyruvic aicd yielding IAA.(Taiz and Zeiger. 1991: Devlin and Witham. 1983).

Note there are several known pathways from tryptophan to the final production of IAA, of which one is relevant to mosses and will be discussed later in this chapter (Taiz and Zeiger, 1991).

The exact mechanism of IAA biosynthesis is not completely understood although IAA is known to be synthesized from tryptophan in most plants. In general, IAA biosynthesis within a plant is associated with apical meristemic activity of shoots, young leaves, and developing fruits, in other words, in sites where rapid cell division takes place (Taiz and Zeiger, 1991).

Auxin metabolism includes the synthesis, degradation, and deactivation of auxin. These processes control the steady state of auxin level or auxin concentration, and in essence, influence all the physiological processes that are effected by auxin (Taiz and Zeiger, 1991; Devlin and Witham, 1983).

c. Polar Transport of Auxin:

IAA transport plays an important role in the regulation of plant cell growth and differentiation. Studies have found that the nature of IAA transport may depend in the development stage of the plant as well as the plant organ or tissue in question. There are at least two basic systems for IAA transport in higher plants:

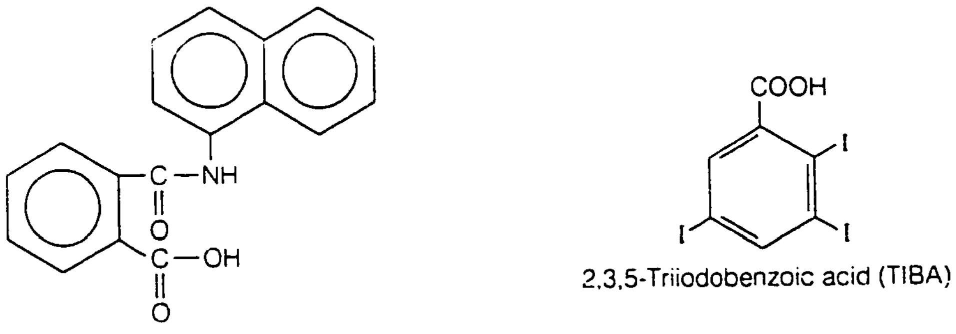
1. an energy requiring, unidirectional polar transport system, and
2. a passive non-polar transport system via the phloem. (Taiz and Zeiger 1991; Devlin and Witham, 1983).

In the shoot tissues which have been studied (coleoptiles, stems, hypocotyls, petioles, and flower-stalks), auxin moves more rapidly basipetally then acropetally. Auxin transport in roots also appears to be polar, but depending on the root region, the preferred direction may either be acropetal or basipetal (Wareing and Phillips, 1981).

The polar movement of auxin is an active process that requires energy. Poisons such as dinitrophenol (DNP) which inhibit ATP synthesis also inhibits polar IAA transport (Taiz and Zeiger, 1990). Polar auxin transport moves auxin at a slow rate of approximately 1 cm./hour (Taiz and Zeiger, 1990). Auxin probably does not travel by way of the symplast, but apparently crosses both the plasma membrane and cell wall in moving from cell to cell (Taiz and Zeiger, 1990). In contrast, auxin synthesized by mature leaves is transported via the phloem in a non-polar transport. Auxin is moved from these leaves up or down the plant at velocities much higher than those of polar transport. Phloem transport is largely passive, not directly requiring energy (Taiz and Zeiger, 1990; Devlin and Witham, 1983).

The currently accepted model of IAA transport is the chemiosmotic model. According to this model, the plant cells expend metabolic energy to maintain a pH gradient and electric potential across the plasma membrane to drive polar IAA transport (Taiz and Zeiger, 1990; Wareing and Phillips, 1981).

It has been found experimentally that the synthetic compound naphthylphthalamic acid (NPA) also known as 2[(1-Naphthalenyl-amino)carbonyl] benzoic acid (ALANAP or Naptalam) blocks polar auxin transport, probably by interfering with the auxin anion efflux step and causing a net accumulation of IAA$^-$ in the transporting cells. NPA does not directly compete with auxin, but it does bind to a protein in the plasma membrane. NPA binds to either the auxin anion transport protein (at the site other than the IAA$^-$ binding site) or another plasma membrane protein that interacts with the IAA$^-$ transporter. Another antagonist which is also a synthetic inhibitor, 2,3,5 triiodobenzoic acid (TIBA), also blocks polar auxin transport. The structures of auxin-transport inhibitors are shown in the following diagram (Taiz and Zeiger, 1991).



Naphthylphthalamic acid (NPA)

2,3,5-Triiodobenzoic acid (TIBA)

Fig. 2.4. Structure of auxin-transport inhibitors (Taiz and Zeiger, 1991).

Natural polar auxin transport inhibitors may be found in a group of flavonoids including quercetin, apigenin, and kaempferol that can bind to the specific receptors where auxin is known to bind, therefore are considered excellent candidates for natural regulators of polar auxin transport in plants (Jacobs and Rubery, 1988).

d. Physiological Effects of Auxin

Auxin is known to have a wide variety of effects on both plant growth and morphogenesis. The varied effects of auxin depend on a number of factors. Some factors include the developmental stage of the tissue or organ, the concentration of auxin, the type of auxin (synthetic or natural), the involvement of other hormones, and the use of intact versus excised tissue for experiments (Devlin and Witham, 1983; Taiz and Zeiger, 1991).

I will restrict this discussion of auxin's physiological effects to those of morphogenesis and apical dominance. Auxin works in association with other hormones and plays a part in the correlative control of the plant's growth process (Raven et al., 1986; Devlin and Witham, 1983; Wareing and Phillips, 1981). Auxin is a growth-promoting hormone, and at this point, I wish to discuss the influence of auxin in conjunction with other variables in initiating organogenesis. In what are now classical axenic culture experiments, callus tissue (undifferentiated homogenous parenchyma cells lacking morphological and physiological organization) was exposed to auxin and other chemicals and thus differentiation was promoted (Raven et al., 1986).

In 1963, Wetmore and Rier showed that by introducing the proper concentrations of auxins and sucrose, formation of vascular tissue could be induced in lilac callus cultures. They also noted in their experiments that by varying concentrations of sugar and maintaining the same concentration of auxin, one could alter the proportion of xylem formation to phloem. For example, at low concentrations of sucrose (1.5-2.5%) xylem tissue development was favored. At middle concentrations of sucrose (2.5-3.5%) both xylem and phloem tissue development was equally favored. Finally, at high concentrations of sucrose (3.5-4.0%) phloem histogenesis was favored (Wetmore and Rier, 1963).

This experiment pointed out several important factors. First, auxin seems to work synergestically with other components in promoting histogenesis. There is always an interaction or synergistic interplay, such as with sucrose, or phytohormones such as cytokinin, gibberillic acid, and ethylene (Wareing and Phillips, 1981).

Schiavone and Cooke (1987) added NPA and TIBA to the globular stage of embryo development of carrots, and in doing so prevented their transition to the next stage of its development. The globular and oblong embryos continued to increase in size with only a few oblong embryos succeeding to develop into heart embryos. When the above antagonists were added to the heart stage, all embryos failed to reach the torpedo stage, but instead became abnormal embryos. Schiavone and Cooke demonstrated the important influence auxin played on somatic embryogenesis. Since the antagonists used were auxin-transport inhibitors, the researchers asserted that a similar transport could also occur naturally in developing carrot embryos.

Torrey (1957) studied auxin control of the vascular pattern formation in regenerating pea root meristems. In this *in vitro* experiment, he found that the vascular patatern in pea root regeneration could be influenced by the concentration of exogenous auxin that is added to the basal medium. Torrey demonstrated that an increase is exogenous IAA to the culture medium caused a change from the control roots which produced a triarch vascular bundle. Those roots treated with the higher concentrations of IAA produced a pentarch, hexarch vascular bundle, etc. with each increase of IAA concentration. He showed that there was a direct correlation of auxin concentration to that of the morphological complexity of the vascular bundle ultimately produced (Torrey, 1957).

Nozeran et al. (1966) studied the influence of auxin gradients involved in the formation of the plant *Phyllanthus amaris*, Euphorbiaceae. They observed that the gradients resulted from the activity of the axial meristem, which starts its development with the seed. After development of cotyledons, more advanced development is exemplified by the formation of erect orthotropic stems, i.e., the stems which grow upward with axial symmetry. Under the influence of this orthotropic branch, a suppression occurs and plagiotropic stems (horizontal stems with bilateral symmetry) are formed. If the suppression of the orthotropic branch is altered, such as by cutting away of the original orthotropic branch, then the second stem grows with axial symmetry. Banchilon (as cited in Nozeran et al., 1966) provided evidence of apical dominance's ability to suppress the expression of plagiotropic stems, which he attributed to the presence of auxin. If exogenous auxin is placed on the cut orthotropic stem, then the original suppression is reinstated (Nozeran et al., 1966). It is an accepted fact that, in most higher plants, the growth of lateral (axillary) buds is inhibited by the growing apical bud. This phenomenon is called apical dominance (Taiz and Zeigler, 1991). By removing the shoot apex (the apical meristem) results in the growth of one or more lateral buds (Devlin and Witham, 1983).

The apical bud of a shoot usually grows much more vigorously than the axillary buds. There is a great deal of variability between species with respect to the degree of dominance of the apical bud over the lower axillary buds. For example, in some species such as *Helianthus annuus*, the dominance is complete and extends over almost the whole length of the stem. In other species, such as *Lycopersicon escuelentum*, the apical bud is weaker, and lateral shoots tend to grow out, thus resulting in a bushy shoot system (Wareing and Phillips, 1981).

In addition, in many species the dominance of the shoot tip becomes weaker as the plant ages. Examples of this is seen in plants such as *Acer pseudoplantanus* or *Fraxinus excelsior*. The early years of growth are characterized by strong growth of a leading shoot, whereas, as the plant ages, a branching habit is seen (Wareing and Phillips, 1981).

As seen from Banchilon's experiment (as cited by Nozeran et al., 1966), if a shoot is decapitiated, that is, when the apical bud is cut off, then the growth of axillary buds is promoted. Usually one of the outgrowing laterals become dominant over the others, exerting an inhibitory influence over their growth. It is the uppermost of the axillary buds that becomes the dominant shoot, and exerts an apical dominance or inhibits the growth of the lateral shoots (Wareing and Phillips, 1981).

Early experiments by researchers studying the phenomenon of apical dominance and in particular the period in which the auxin (IAA) molecule was discovered, led to investigations of the possibility that transmission of IAA from the apical bud is responsible for the apical dominance phenomena. Thimann and Skoog (as cited by Wareing and Phillips, 1981) found that the application of exogenous IAA could substitute for the apical bud in maintaining inhibition of axillary buds in bean plants. Further investigations confirmed Thimann and Skoog's findings. Therefore, the synthesis of IAA in the apical region of the shoot, probably in the young expanding leaves, and its transport down the stem constitute a basic component of the mechanism of correlative inhibition (Wareing and Phillips, 1981).

Several theories have been proposed on how apical dominance occurs. One of the first theories was proposed by Thimann (as cited by Wareing and Phillips, 1981) and called the "Direct Theory". This theory states that the optimal auxin concentration for bud growth is lower than for stems, and that the bud is inhibited solely by the "supra-optimal auxin concentration" present in the stem as a result of young leaves synthesizing auxin and its basipetal transport from there. This theory is currently doubted, as it has been observed that auxin does not exert its inhibitory effect on lateral buds in such a direct manner (Wareing and Phillips, 1981).

Another theory is the "Nutritive Theory", which basically asserts that since the apical meristem is the first-formed shoot meristem in the germinating seedling, it would continue to command a preferential supply of metabolites as these moved along their concentration gradients (Wareing and Phillips, 1981). Thus, the shoot apex acts as a sink for nutrients, diverting them from the lateral buds, possibly because the vascular connections to the lateral buds are incomplete (Taiz and Zeiger, 1991).

Further investigations of the "Nutritive Theory" did not explain why auxin could substitute for the apical bud in correlative inhibition of axillary buds. Further studies, including those using ^{32}P-phosphate and ^{14}C-sucrose, have demonstrated that nutrients accumulate in regions of high exogenous auxin concentration. The "auxin-directed" transport of metabolites indicates that auxin production in the apical bud and its basipetal transport induce movement of available nutrients towards the region of highest auxin concentration, that is, to the apical bud itself. It is important to note, however, that direct application of nutrient solutions to axillary buds fails to reverse apical dominance (Taiz and Zeiger, 1991).

Investigations of inhibited buds have revealed that these buds appear to be served by well formed xylem and phloem connections. Though contradictory data have come from these investigations, the accumulating evidence argues against the idea that lack of bud growth is attributable to deficiency in their vascular supply (Wareing and Phillips, 1981).

Cytokinins have been implicated in possibly playing a role in apical dominance. It has been found that exogenous application of cytokinin directly onto a correlatively inhibited bud can release that bud from inhibition in an intact plant. Therefore there is a suggestion that the lack of cytokinin inhibits buds from developing. There is a possibility then that the role of auxin from the apical bud is to direct the transport of root synthesized cytokinins so that the apical bud receives preference over the axillary buds (Wareing and Phillips, 1981).

The plant hormone abscissic acid has been found in dormant lateral buds in intact plants. When the shoot apex is removed, the ABA level in the lateral buds decrease. High levels of IAA in the shoot may help to keep ABA levels high in the lateral buds. Removing the apex removes a major source of IAA which may allow the bud growth inhibitor levels to fall (Taiz and Zeiger, 1991).

It is a well known fact that auxin promotes elongation growth in young stems and coleoptiles. When auxin is removed from these tissues, the growth rate decreases. When auxin is restored, growth usually resumes within ten to twenty minutes. The optimal auxin concentration is typically 10^{-5} to 10^{-6} M for young stems and coleoptiles. High (supraoptimal) levels of added IAA may actually inhibit growth. This effect is usually attributed to auxin-induced production of the plant hormone ethylene that suppresses elongation growth (Taiz and Zeiger, 1991).

Abeles (1973) stated that the early investigations of the Boyce-Thompson scientists suggesting that auxin-induced ethylene may account for the ability of auxin, especially that applied to a plant, to induce certain responses, has been confirmed and extended by others. Rates of ethylene production were correlated with the levels of endogenous auxin in tissues.

Russel and Thimann (1988) used *Vicia faba* and *Phaseolus vulgaris* in several experiments to see if:

6. low-physiological levels of ethylene could duplicate the inhibition caused by auxin;
7. the inhibition caused by auxin could be nullified or decreased under reduced pressure to draw off ethylene; and
8. the inhibitors of ethylene action or formation (i.e., AVG or norbornadiene) would nullify the inhibition caused by auxin.

They decapitated the stem of*Vicia faba* one centimeter above the third node or one centimeter above the primary leaves of *Phaseolus vulgaris* and made daily meaurements of bud length. On some plants auxin was applied at a range of concentrations, and renewed applications were made daily. In other experiments ethylene was applied via injection to a level of 15 ppm. These several experiments were repeated numerous times (Russell and Thimann, 1988).

The results of Russell and Thimann's (1988) experiments were rather clear. Ethylene at 15 ppm inhibited the outgrowth of the buds on decapitated stems. Under reduced pressure conditions, 200 µM of auxin (IAA) applied to the cut surface under reduced external pressure can relieve most of the resulting inhibition.

When auxin-supplied decapitated plants grown under normal pressure were injected with 5 µM of AVG, lateral bud growth was shown. The same results were also noted with similar treatments using norbornadiene (Russell and Thimann, 1988).

As with angiosperms, when the apex of the leafy gametophore of bryophytes is removed, lateral buds grow out, forming a new plant tip and side branches. Therefore, as in higher plants, apical control in the intact apex of bryophytes suppresses growth in lower meristems (Knoop, 1984). For example, in the decapitated gametophores of *Splachnum ampullacem* Hedw. the inhibitory influence of the apex can be replaced by an agar block containing

1 mg/ ml of IAA (Von Multzahn. 1959). In *Plagiomnium cuspidatum*. bud development can also be released from apical dominance in intact plants when endogenous IAA transport is antagonized by exogenous 2,3,5 triiodobenzoic acid (TIBA) (Nyman and Cutter, 1981).

Filamentous organ regeneration follows a different gradient from that of bud release. Filaments first appear and generate mainly at the base of decapitated gametophores. whereas. buds first develop in the more apical part of the stump. Growing apical buds reestablishes apical dominance and inhibits the growth of plant organs. and even filaments situated below the buds (Gay, 1971; Nyman and Cutter, 1981; Bopp, 1984).

The relationship of endogenous concentrations of auxin has been correlated to the production of ethylene, thus there is evidence of a coupled relationship between auxin and ethylene. Ethylene probably provides the environment by which the binding of some AGP epitope to the cell surface may occur thus determining whether and how suppression occurs (Basile and Basile. 1993).

4. Ethylene

Ethylene is a small unsaturated hydrocarbon ($CH_2=CH_2$). Since 1901 ethylene has been known to regulate plant growth and development. Its ability to promote abscission was discovered in 1913. and its ability to cause fruit ripening in 1924. and since 1934. it has been recognized as a product of plant tissue (Ho and Varner. 1975). Early observations made by Crocker and Zimmerman of ethylene-induced epinasty. and that of ethylene-evoked inhibition of the lateral transport of auxin by Crocker. led to ethylene being considered a phytohormone (Ho and Varner. 1975). Ethylene is a powerful inhibitor of root and stem growth.

The inhibitory effect of auxins at high conentrations is believed to be entirely due to auxin-stimulated ethylene synthesis (Devlin and Witham. 1983). Ethylene is known as a potent inhibitor of bud growth, and in this respect, may have a controlling influence on apical dominance. Ethylene seems to be more prevalent in meristematic tissue where auxin is produced. In the mature light grown plant, lateral bud growth seems to be suppressed by IAA -

induced ethylene formation in the nodal regions as a result of auxin transported there from the apical bud and leaf blades (Devlin and Witham, 1983).

The amino acid methionine is the precursor of ethylene. Ethylene is synthesized through a series of reactions in which methionine is converted to ethylene:

Methionine--->S-adenosylmethonine (SAM) --->
1 aminocyclopropane-1-carboxylic acid (ACC) →Ethylene
(Taiz and Zeiger, 1991)

As seen from the above reactions, the immediate precursor of ethylene was found to be ACC. It was discovered by Lieberman and Mapson (1964) that under anaerobic conditions, ethylene was not produced from the [^{14}C] methionine and labeled ACC accumulated in the tissue, but on exposure to oxygen, a surge of ethylene production took place. The labeled ACC was rapidly converted to ethylene by various plant tissues, suggesting that ACC is the immediate precursor of ethylene in higher plants (Lieberman and Mapson, 1964).

It is believed that auxins have the ability to promote ethylene synthesis by enhancing the conversion of SAM to ACC. Some examples of this possibility have been observed by Abeles, 1973. In some instances, auxins and ethylene have been observed causing similar plant responses, such as induction of flowering in pineapple and inhibition of stem elongation (Abeles, 1973).

These observations suggest that some responses previously attributed to auxin are actually controlled by ethylene produced in response to auxin. It has also been observed that inhibitors of protein synthesis block both ACC-synthesis and IAA-induced ethylene synthesis, indicating that the synthesis of ACC synthase caused by auxins brings about the marked increase in ethylene production (Imaseki et al., 1982; Yang et al., 1982).

Law et al. (1985), using high performance liquid chromatography (HPLC) with electrochemical detetection and GCMS, determined that *Plagiochila arctica* endogenously produced IAA. This group of experiments also provided evidence that IAA can promote ethylene production by these liverworts. One milliliter (1 ml) of exogenous IAA (57 µM) was added to axenic cultures of *P. arctica* and one milliliter of nutrient medium was added to the control. One millilter (ml.) of gas samples were withdrawn just after treatment of IAA and a second sample was withdrawn twenty-four hours later. The initial gas samples showed ethylene levels increased 2.8 fold, when compared to the control. In the twenty-four hour samples, IAA induced a 1.9 fold increase in ethylene production over those of the controls (Law et al., 1985).

Sato and Theologis (1989) measured the levels of mRNA encoding ACC synthase of zucchini (*Curcubita pepo*) fruits using recombinant DNA techniques. The amount of ACC synthase mRNA in the fruits increased in response to both wounding and IAA treatment. The question still remains whether this increase in ACC synthase mRNA is caused by an enhanced rate of transcription or simply a decrease in turnover rates (Taiz and Zeiger, 1991).

In the studies of ethylene, specific inhibitors have made it possible to discriminate between actions of auxin and ethylene. This is especially helpful since ethylene mimics high concentrations of auxins by causing stem growth inhibition and epinasty (Taiz and Zieger, 1991). Studies by Beyer et al. (1984) showed that ethylene is the primary effector and that auxin is indirectly involved by causing a substanatial increase in ethylene production.

Aminoethoxyvinylglycine (AVG) and aminooxyacetic acid (AOA) are inhibitors that block the conversion of SAM to ACC. Since AVG and AOA are known to inhibit enzymes that use the cofactor pyridoxal phosphate, this suggests that the ACC synthase is a pyridoxal phosphate enzyme (Beyer, 1979). Cobalt is also an inhibitor of the ethylene biosynthetic pathway blocking the conversion of ACC to ethylene, thus inhibiting ethylene biosynthesis. In addition, silver ions applied as silver nitrate ($AgNO_3$) or as silver thiosulfate are potent inhibitors of ethylene action. Silver is very specific, and the inhibition it causes cannot be induced by any other metal ion. Carbon dioxide at high concentrations (range of 5 to 10%) even though it is less efficient then silver, inhibits many effects of ethylene, such as fruit ripening induction (Taiz and Zeiger, 1991). Figure 2.5 shows the effects of antagonists on ethylene synthesis.

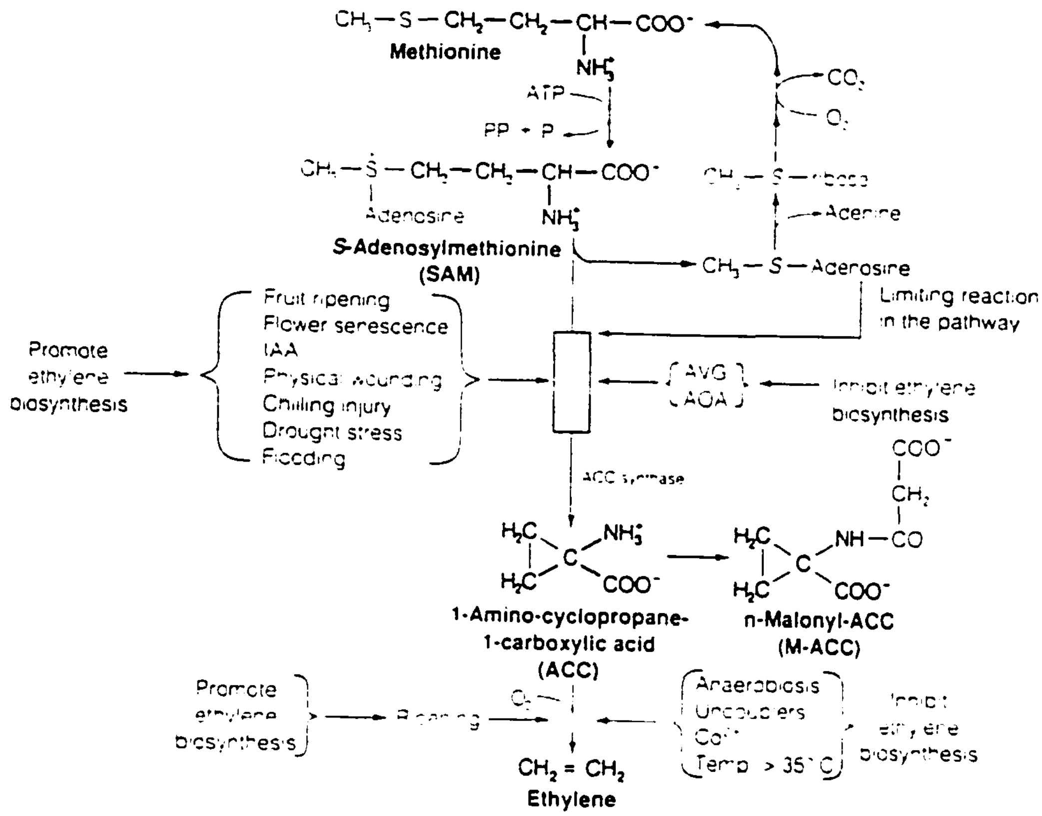


Fig. 2.5. Note how various antagonists act specifically within the ethylene biosynthesis pathway. AVG and AOA block the conversion of SAM to ACC. Cobalt blocks ACC conversion to Ethylene. Silver nitrate or silver thiosulfate are potent inhibitors of ethylene action. (Taiz and Zeiger, 1991).

Ethylene can be measured either in bioassays or by gas chromatography. Neljubow (as cited by Taiz and Zeiger, 1991) produced a bioassay based on the triple response of etiolated pea seedlings, which is still considered a reliable bioassay because of its specificity, sensitivity, low concentrations, and rapidity. When plant tissues are exposed to various concentrations of ethylene (0.1µl/L and higher) in a sealed environment, inhibition of stem elongation, increased lateral growth (swelling) and horizontal growth of the epicotyl are observed. The magnitude of the response is proportional to the ethylene concentration in the sample. Tissue exposed to known amounts of ethylene are used as standards and the actual concentration is determined by comparing the response of the sample to this standard (Taiz and Zeiger, 1991).

Though other bioassays also exist and include epinasty of tomato leaves and leaf abscission, these bioassays have been observed to be less sensitive than the triple response of Neljubow's bioassay (Taiz and Zeiger, 1991).

All bioassays are qualitative tests for the presence of ethylene, but none of them permits quantification over a large range of ethylene concentrations. Gas chromatography coupled with mass spectrophotometry is a means by which quantification of ethylene can be measured (Taiz and Zeiger, 1991).

Gas chromatography has proved to be the most sensitive and accurate method of ethylene detection. As little as two parts per billion (ppb) of ethylene can be detected and the analysis time is only one to four minutes. The basic protocol used in gas chromatography permits the ethylene produced by a plant tissue to be accumulated in a sealed vial and the sample is withdrawn with a syringe. The sample is injected into a gas chromatography column in which different assays are separated and deluted by a flame ionization detector (Taiz and Zeiger, 1991).

There have been several studies on the effects of auxin and ethylene on the development of moss protonema. It has been established that ethylene is synthesized in higher plants from methionine via S-adenosyl-methionine and ACC. It has also been established that microorganisms can also use other substrates such as 2-ketoglutarate and glutamate as ethylene precursors (Bhatla and Dhingra-Babbar, 1990). Rohwer and Bopp (1985) set up experiments to obtain information about the precursors for ethylene synthesis in the protonema of *Funaria hygrometrica*. The possible ethylene precursors of methionine, ACC, oxoglutarate, and glutamate were individually provided in the nutrient medium. Ethylene production by the moss protonemata raised on this media was significantly increased only by ACC. Both methionine and glutamate had no effect on ethylene production, and oxoglutamate actually slightly decreased with ethylene production.

The effect of ACC together with observations of ACC concentration in the tissue indicated that ethylene synthesis in the moss protonema occurs via the methionine-ACC pathway (Rohwer and Bopp, 1985).

Rohwer and Bopp (1985) also observed that the protonemata of *F. hygrometrica* produce more ethylene on IAA containing medium. When protonema, cultivated on basal medium for three weeks, were transplanted onto fresh medium containing IAA (10^{-6}), the ACC content of the tissue analyzed twenty-four hours later was nearly ten time higher (1.36 *n*M/ACC mg/protein) as compared to the protonemata transplanted onto IAA-free medium (0.16 *n*M ACC mg/protein) indicating the similarity of ethylene synthesis in higher plants and the protonema of *Funaria*..

The increasing amount of ACC and ethylene during protonematal growth and development may be an argument for ethylene being involved in the morphogenesis of the protonema, especially in transition from the chloronema stage to the caulonema stage. Rohwer and Bopp (1985) noted that from a developmental point of view ACC content and ethylene production coincided with progressing differentiation of caulonema. It is well documented that caulonema differentiation of *Funaria* and *Physcomitrella patens* protonema is regulated by auxin (Johri and Desai, 1973; Sood and Hackenberg, 1979; Lehnert and Bopp, 1983; Ashton et al., 1993). Therefore there is evidence pointing to a direct link between the effect of auxin concentration and the enhancement of ethylene formation (Rohwer and Bopp, 1985).

In addition, Rohwer has observed that protonemata older than twenty-five days are characterized by the formation of "tmema" in younger protonemata. "Tmema" are cells developed during stressed environment that have no cellular content but serve as a point in which filaments can break off. This allows vegetative growth of new filaments. Therefore in *Funaria* the high natural production of ACC and ethylene in older cultures may demonstrate that ethylene may also act as a kind of senescence hormone in moss protonema (Bhatla and Dingra-Babbar, 1990; Rohwer and Bopp, 1985).

Though Rohwer and Bopp (1985) experimentally established strong evidence of a ACC-mediated ethylene synthesis, Osborne et al. (1996) using only random and very small samplings from the ferns, mosses, and liverworts, concluded that "the regulative ethylene synthesis route of higher plants arose late in plant evolution."

The final and most direct component of the correlation control system is a family of highly glycosylated proteins called AGPs. The insertion of AGPs into the cell membrane/cell wall most directly contributes to the subtle differences in the make up of the cell surface, thus promoting suppression. In *P. patens* this promotes the transition of diffuse protonemata growth to that of apical meristematic growth.

The following section will provide a basic background of AGP and a summary of reviews of how its structure may be linked to function, and how AGPs are possibly essential in the determination of ontogeny and morphology.

5. Arabinogalactan proteins (AGPs):

a. Introduction:

Arabinogalactan proteins (AGPs) form a very large and diverse group of macromolecules in plants, and have previously been either classified as proteoglycans or glycoproteins (Fincher et al., 1983; Showalter and Varner, 1988; Basile and Basile, 1990). Proteoglycans are molecules in which the protein component carries polysaccharide components (Clarke et al., 1979), and glycoproteins possess covalently attached mono-or oligosaccharide units (Fincher et al., 1983). AGPs have a typical molecular weight of 60-300 KDa. AGPs usually consist of 10% protein and 90% carbohydrates, although some variations have been noted, and have been assigned to the proteoglycan class (Nothnagel, 1997). Some AGPs or AGP-like molecules have been characterized which have a high protein content in the range of 30-65% (depending on the AGP), and have been classified as glycoproteins (Nothnagel, 1997). The most abundant aminacyl residues are hydroxyproline (Hyp), alanine (Ala), serine (Ser), thronine (Thr), and glycine (Gly). They are also classified as making up one of two classes of hydroyxyproline proteins, the other class being hydroxyproline rich glycoproteins (HRGPs), otherwise known as 'extensins' (Clarke et al., 1979; Fincher et al., 1983;). The difference between AGPs and HRGPs will be addressed later in this text. These proteins have been detected in plants at all taxonomic levels (Fincher et al., 1983) including liverworts (Basile and Basile, 1990).

The significant functions of these proteins are still being investigated, but results from research have included the possible roles of morphoregulatory function as well as wounding response, cell adhesions, drought resistance, and potential for two types of major interactions, that being, macromolecule-small ligand interaction, and macromolecule-macromolecule association (Fincher et al., 1983; Clarke et al., 1979). Since AGPs have a potential for the latter interactions, it is possible to predict non-covalent intermolecular interactions between individual AGPs, between AGPs and other cellular macromolecules, especially those found in the cell plate, the cytoskeleton, and the cell wall (Bacic et al., 1996). Per Bacic et al. (1996), macromolecule-macromolecule interactions may occur through specific ligand-receptor associations in which various epitopes of the AGP surface would be recognized by complementary (protein) receptors possessing specific binding sites. The ability of AGP to bind with ß-glucosyl Yariv's reagents gives evidence of potential macromolecule-small ligand interactions (Bacic et al., 1996). AGPs are antigenic and are capable of generating monoclonal antibodies with reactivities inhibitable by L-arabinose,D-galactose, and/or associated disaccharides (Knox et al., 1989).

b. Structure:

The determination of the AGP structure evolved through numerous studies, one of the pioneers being Aspinall (Fincher et al., 1983). Studies of *Vicia faba* suggested that in addition to the specific cell wall protein extensin, there were a number of other and different classes of cell constituents which contain hydroxyproline. These studies emphasized purification and characterization of materials soluble in phenol. The leaf hydroxyproine soluble in phenol could be categorized into different groups (Pusztai and Watt, 1969). The first were those comprised of glycoproteins containing more than 90% hydroxyprolines (now referred to as HPRGs) (Pusztai and Watt, 1969; Showalter and Varner, 1988), and the second glycolipid-rich materials containing less than 10% of the hydroxyprolines (Pusztai and Watt, 1969).

Arabinogalactans were originally classified by Aspinall as either type I or type II (as cited by: Clarke et al., 1979; Fincher et al., 1983; Nothnagel, 1997). Type I arabinogalactans have a linear (1—>4) ß-D-galactan backbone with arabinose oligosaccharide side chains. Type II arabinogalactans make up a highly branched framework consisting of a (1—>3)-ß-D galactan backbone with (1—>6)-ß-D galactan side chains attached at carbon (C) atom 6 of some of the galactosyl residues making up the backbone. In a type II arabino-galactan, most of the arabinosyl residues are attached to C-atom-3 of some of the galactosyl residues found in the side chain (Nothnagel, 1997).

Clarke et al. (1979) described arabino-3,6-galactans as type II arabinogalactans. These structures have been detected in seeds, leaves, roots, fruits, xylem, gum exudates, sap, cells filtrates of plant cells, and cells of callus cultures. The arabino-3,6- galactan from plant tissues and tissue cultures are typified by the *Acacia senegal* gum endosperm arabinogalactan protein, and its proposed structures is shown fig 2.6 (Clarke et al., 1979).

Several investigations have shown that type II arabino-galactans may vary in average size. Kawasaki (1998) estimated that tobacco has an average glycan chain that ranged in size from 46-140 residues with 70% of these sugars attached to Hyp. Gum arabic's sugar residue chain is made up of approximately 30 sugars, and *Nicotiana alata* stigmas and styles are made up of approximately 95 sugar residues (Kawasaki, 1998; Nothnagel, 1997). In studies of *Lolium multiflorium*, Van Holst and Fincher (1984) confirmed that arabinogalactan proteins predominantly exhibit an extended polyproline II type helix via circular dichrometry. It had been determined that only these conformations exhibit negative ellipticity near 200 nm, whereas ∂-helices, β -sheets, and β -turns all exhibit positive ellipticity at 200 nm.

Studies of the sugar content of *L. multiflorum*and *N. alata* found that the AGPs contained carbohydrate portions almost totally made up of galactosyl and arabinosyl residues in approximately 2:1 Molar ratio with only trace amounts of neutral sugars present and no uronic acids prsent. Other AGPs have been found to contain some other sugar including L-rhamose, D-mannose, D-xylose, D-glucose, L- fucose, D-glucosamine, the uronic acids, D-glucuronic acid, and D-galacturonic acid (Nothnagel, 1997; Tsumuraya et al., 1988; Fincher et al. 1983; Clarke et al., 1979).

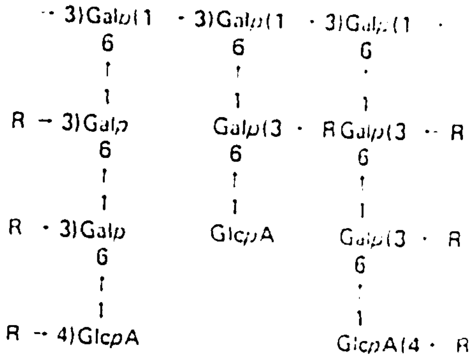


Figure 2.6 Partial core strucutre of Acacia senegal gum (gum arabic) where:
R= Rha*p*(1, Ara*f*(1,Gal*p*(1→3)Ara*f*(1,Ara*p*(1→3)Ara*f*(1) (Clarke et al., 1979).

Serpe and Nothnagel (1994) found a large amount of glucuronic acid that exceeded the amount of arabinose in AGPs extracted and isolated from rose. Due to the high quantities of glucuronic acid, the AGP was renamed glucuronogalactan protein (GGP) (Serpe and Nothnagel, 1994; Nothnagel, 1997).

The arabinogalactan protein structure, which was proposed by Fincher et al. (1983), is a wattle blossom type structure. In this basic moiety, there may be twenty-five hydroxyproline (Hyp) residues, each of which may bear an arabinogalactan substituent, as demonstrated from the diagram below (fig. 2.7) (Fincher et al., 1983 and Showalter and Varner, 1988).

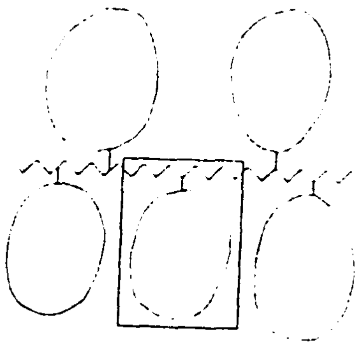


Figure 2.7 Wattle blossom model. Note how the AG substituents branch off the protein backbone with linkages to Hyp residues.

The molecule, as a whole, is spheroidal with kinked regions of arabinose or 6-substituted galactose. These latter regions are periodate sensitive or susceptible residues. There may be twelve residues in each stretch of the backbone of the galactan framework between periodate-susceptible linkages,and possibly ten or more galactan stretches per substituent (Fincher et al., 1983; Showalter and Varner, 1988). This is diagrammed in fig. 2.8.

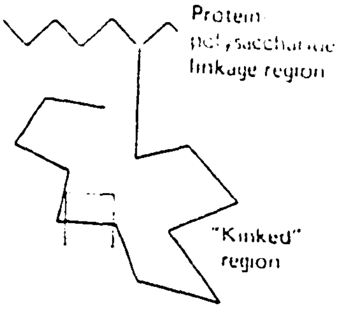


Fig. 2.8 AGP molecule. Note kinked regions of arabinose or 6-substituted galactose which are periodate sensitive or susceptible residues.

Thus, the "wattle blossom" model for AGP, and gum arabic predicts a few large polysaccharide substituents along a polypeptide backbone of a spheroidal macromolecule (Qi et al., 1991).

Even though AGPs are characterized with large carbohydrate chains as per the "Wattle blossom" model (also referred to as traditional AGPs) presented by Fincher et al., (1983), there have been reports of smaller glycan chains in AGPs and AGP-like molecules (Nothnagel, 1997). Qi et al. (1991) reported the presence of smaller glycan residues attached to Hyp in addition to the large glycan chains. These molecular variations have been documented by several recent researchers contrary to the observed characterization of gum arabic, *Acacia senegal*, etc. Research using *Acacia senegal* nodules or (authentic gum arabic), concluded the *A. senegal* AGP structure consisted of a twisted hairy rope structure (Qi et al., 1991). The data provided evidence of a rod-like molecule with numerous small poysaccharide substituents (attached to 24% of the Hyp-residues) regularly arranged along a highly periodic polypeptide backbone based, hypothetically, on a ten to twelve residue repetitive peptide motif. Kieliszewski et al. (1992) reported a His-rich macromolecule with large glycan chains characteristic of AGPs and small arabinose oligo-saccharides characteristic of extensin.

Baldwin et al. (1993), characterized an AGP from *Daucus carota* that proved to have all characteristics of a type II AGP, that is, spheroidal, reacts positively with Yariv's reagent, etc., except that no hydroxyproline was detected in the protein backbone. As the role of glycoproteins in the form and function of eukaryotes became more evident, so did the need for its characterization (Mort and Lamport, 1977). Therefore, several researchers made use of various physicochemical methodologies in their attempt to decipher the components of arabinogalactan proteins in various plant genera.

Fincher et al. (1984) presented a detailed study of an arabinogalactan peptide isolated from water extracts of wheat endosperm and its characterization using physicochemical techniques, thus determining some aspects of its chemical structure. It was determined that the arabinogalactan had a molecular weight of 22000, a peptide content of 8% w/w, and thus probably had a maximum of twenty amino acid residues present. Of these residues, three or four were hydroxyprolines. Based on this study, it was also proposed that the hydroxyprolines were substituted by polysaccharide chains. It was concluded that the possible structure of the isolated arabinogalactan-peptide consisted of a peptide core to which polysaccharide chains are attached through each of the hydroxyproline residues. This is known as the O-Hyp linkage. It was proposed that this structure may either have an unusual peptide structure, or that polysaccharide chains shielded it from enzymatic attack in light of the protein core's resistance to proteolytic enzymes. In addition, the apparent ease of removing arabinose in the presence of oxalic acid without disrupting the polysaccharide linkage, suggested that the peptide must be linked to galactose, and furthermore, due to alkaline degradation resistance, may be linked glycosidically. In addition, i.r.-absorption spectroscopy and optical rotation studies demonstrate these glycosidic linkages had a *B*-anomeric configuration. Fincher et al. (1984) also stated the possibility that other types of polysaccharide-protein linkages could be present in the AGP, that arabinose was an integral part of this molecule, and that it was present in the ∂ L-arabinofuranosyl form. Yamagishi et al. (1976) established that an O-glycosyl linkage existed between L-arabinose and hydroxyproline in rice-bran proteoglycans using the alkaline-degradation method. HF (anahydrous hydrogen flouride) reagents were also used to cleave glycosidic linkages of neutral and acidic sugars (Mort and Lamport, 1977). Akiyama and Kato (1977) used various methods including Smith-degradation, anion chromatography, and PMR spectroscopy in order to cleave, purify, and determine the configuration of two hydroxy-proline-arabinosides from tobacco cells.

Kieliszweski et al. (1992) described a His-rich extensin isolated from *Zea mays* cell-suspension cultures that also had characteristics associated with AGPs. This extensin seems to contain an AGP component. ß-Elimination studies indicated that the O-linkage was to serine rather than to the Hyp in 'traditional ' AGPs as described by Fincher et al. (1984) (Kieliszewski, 1992).

Another possible type of AGP-like molecule has been been described by Showalter (1993). This is a Hyp-rich glycoprotein isolated from *Pseudotsuga menziesii* (Douglas fir) cell suspension that contained characteristic Ser-Hyp_4 and Ala - Hyp repeat units (Stone et al., 1983).

Present research into AGP structure characterization has focused on the cloning of cDNAs that encode the core polypeptides of the AGP molecule (Nothnagel, 1997). Chen et al. (1994) used cDNA to code AGP core polypeptides. AGP isolated from suspension cultures of *Pyrus communis* (pear) cells, termed AGPc1 cDNA, was found to be made up of 145 amino acids. The protein was rich in Hyp. Ala. Ser. and Thr. and accounted for more than seventy-five percent of the total residue. The isolated protein appeared to consist of three domains (Chen et al., 1994). The three domains consisted of: an N-terminal secretion signal, a central hydrophilic domain containing all the Pro residues, and a hydrophobic C-terminal domain that Chen et al. (1994) predicted to be a transmembrane helix. The AGP Pc1 predicted molecule follows the characteristic criteria for AGPs, and thus was classified as a "Classical AGP" molecule (Chen et al., 1994; Nothnagel. 1997). Another putative molecule fitting into the classical AGP category is AGP Na1 (an isolated AGP from *Nicotiana alata* cultures).

Other studies have shown putative AGPs fitting into a non-classical AGP category, that is, the amino acid composition was rich in Asn, Gly, and Tyr residues as opposed to the Pro/Hyp, Ala Thr, and Ser residues of "classical" AGP. Examples of these non-classical AGPs would be AGP Pc2 (from pear), AGP Na2 (from *N. alata*) and AGP Na3 (from *N. alata*). AGP Pc2 differs from other cDNA putative mature molecules in that it contains four domains. The N-terminal of AGP Pc2 consisted of a signal sequence of 20 residues, the second domain was a 32 residue sequence rich in Asn residues. The third domain consisted of a 35 residue sequence rich in Pro, Ala, Thr, and Glu. The fourth domain was rich in both Asn and Gly residues (Nothnagel, 1997).

AGPNa3 was cloned from AGP isolated from stigmas and styles from flowers of *N. alata*. The putative polypeptide was 160 residues long and contained three domains. A signal sequence of 24 residues was located at the N-terminal, the central domain contained most of the Pro residues found in the whole molecule, and was 82 residues long. The C-terminal contained some Pro residues and contained all the Cys residues. These last two domains were rich in Pro, Asx, Glx, and Ala residues and Cys. Du et al. (1996) found the amino acid composition of the isolated AGP similar to the combined second and third domains predicted by AGP Na3 cDNA. The isolated AGP retained the unusual amino acid components of the putative cDNA AGP Na3 (Du et al., 1996; Nothnagel, 1997). The presence of a three domain structure in putative AGPs seems to be consistent in other AGP cDNAs found in loblolly pine and tomato (Bacic et al. 1996).

Other cDNA studies have included screening for style-specific gene expression in both *Nicotiana tabacum*. and *Nicotiana alata* (Chen et al., 1992; Cheung et al., 1993). These studies produced transmitting tissue specific-1 (TTS-1) and transmitting tissue specific-2 (TTS-2) cDNAs from *N. tabacum* and NaPRP4 cDNA from *N. alata*. Chen et al. (1993) found TTS-1 and TTS-2 to be closely related to each other, and found TTS-1 to be almost identical to NaPRP4c DNA (Cheung et al. 1993; Nothnagel, 1997).

Loblolly pine xylem-specific gene expression screening has produced PtX3H6 and PtX14A9. The cDNAs seem to encode transcripts that are the most abundant of xylem-specific RNAs. Loopstra and Sederoff (1995) suggested that these two cDNAs were AGPs based in the Ala-Pro presence which was a repeated sequence in PtX14A9, and though not directly repeated in PtrX3H6, it was found at several separate sites. This identification of these two sequences as putative DNA is tentative although both these cDNAs have domains that probably sequence for proline and a C-terminal hydrophobic domain which are characteristic of the classical AGP model. The problem lies in the high abundance of Thr predicted by the repeated sequence of PtX3H6 and the many N-glycosylation sites predicted by the sequencing of PtX14A9 which are less characteristic of known AGPs. Therefore, investigators must isolate and characterize the mature translation products of these two cDNAs before a positive AGP identification can be presented (Nothnagel, 1997).

Continued research efforts using cDNA clones have been directed on *Lycopersicon esculentum* fruit expression. These studies have produced LeAGP-1c cDNA which predicted a molecule with characteristics consistent with "classical AGPs" (Nothnagel, 1997). *Brassica rapus* was screened for stamen-specific gene expression by Gerster et al.(1996), and as a result Sta39-3 and Sta39-4 were isolated. These cDNAs are expressed late in pollen development and the sequence characteristics were similar to the properties of classical AGPs (Nothnagel, 1997).

One result of these cDNA studies and resulting predicted polypeptide sequences of confirmed AGP molecules and putative AGPs is a comparative sequence identity among the AGPs (Nothnagel, 1997). The cDNA sequence of the protein backbone showed little sequence identity among the AGPs (Bacic et al., 1996). One exception is the TTS-1 gene from *N. tabacum* and NaPrp4 from *N. alata* which shared 96.9% similarity at the amino acid level (Nothnagel, 1997). Again this lack of sequence identity has been explained as possible evolutionary interchanges of amino aicd residues due to the similarity of the codons encoding proline, alanine, serine, and threonine (Bacic et al., 1996).

Nothnagel (1997) asserted that existing homology between different AGPs has often been limited to the level of domains. Even at this level of the molecular structure, the similar sequences have been restricted to the presence of a putative signal sequence and a proline-rich domain.

In the light of present research status, it is understood that for a clearer understanding of AGP amino acid sequences, additional cDNA encoding of AGPs and putative AGP molecules must become available. Only then can sequence homologies be thoroughly studied and may become more evident. At this point, AGPs seem to represent a relatively diverse group at the level of amino acid sequence (Nothnagel, 1997).

Arabinogalactan proteins may be differentiated from the structures of hydroxyproline rich glycoproteins (also known as "extensins"). Though they each make up one of the two classes of hydroxyproline proteins, and though some features overlap between these two classes of proteins, they are physically separable and identifiable, based on their bouyant density and net charge. AGPs appear to function as morphoregulatory determinants at all levels of organization from cells to species, are heavily glycosylated, and therefore demonstrate high bouyant densities (1.50 or higher) as well as exhibiting resistance to proteolysis in its native state. They are acidic with isoelectric points (PIs) of 2-5. Extensin, in contrast, has primarily a structural function, exhibits moderate to low bouyant densities (1.44-1.38) due to their moderate to light glyscosylation. Hydroxyproline rich glycoproteins (HRGPs) are strongly basic with a PI of 10-

12 (Showalter and Varner, 1988). Extensins are also characterized by a low alanine but rich serine amino acid content (Qi et al., 1991).

Studies of AGP structures and in determining the difference of these structures with that of HRGPs, cDNA clone and mRNA studies were used by several investigators. Avery et al. (1987) used cDNA clones that hybridize to a mRNA, and that accumulates to a substantial level in germinating soybean seeds, in order to sequence the protein. The amino acid sequence was Pro-Pro-Val-Tyr-Lys. Protein extraction was performed from the walls of soy bean cell culture, which were fractionated and purified. The result was a 33-K dalton, and also tentatively a 28-K dalton protein was obtained that has an amino acid distribution similar to that of the cDNA clone. The protein lacked histidine and serine. It also contained both twenty percent hydroxyproline and twenty percent proline. In view of post translational modification, and hydroxylation, it was determined that the HRGP is distinct in both its amino acid content and in its pentomeric repeat of Pro-Pro-Val-Tyr-Lys with half of the prolines being hydroxylated (Avery et al., 1987).

Adair and Apt (1989) reported that the cell wall of *Chlamydomonas* is composed entirely of HRGPs. Regeneration in *Chlamydomonas* is accompanied by marked alterations in abundance of translatable mRNAs encoded proline-rich polypeptides. Several transcripts increased severalfold as an early response to wall removal, whereas others declined. This increase in transcription seemed to be the expression of genes of two outer wall HRGPs, thus suggesting that up regulation of HRGP mRNAs is a primary response to cell wall removal.

cDNA studies have showed repetitive motifs are characteristic of structural cell wall proteins. The Ser-Hyp$_4$ sequences are classic examples of extensin but the repetitive motifs may be made up of other peptides, as observed by Avery et al. (1987), and these peptides may be characteristic of extensins, repetitive proline-rich proteins, and glycine-rich proteins (Showalter, 1993; Nothnagel, 1997).

The ability of AGPs to produce an immune response has been linked to the polysaccharide side chaines. The properties of *Calendula officinalis* L. (Asteraceae) have been documented to be wound healing, and further reports have demonstrated a polysaccharide fraction having anti-tumor activity (Varljen et al., 1989). Varljen et al. (1989) characterized the polysaccharides. These homogenous polysaccharides were isolated by ethanol fractionation and gel filtration. Sugar and methylation analysis demonstrated that the polysaccharides contained a ((1-3)-linked *β* -) galactan backbone with branching points at C-6. The side chains were composed of short *α*-Ara*f* - (1-3) - Ara*f* , *α*- L- Rha*p*-(1-3)- Ara*f* or simple *α*-Ara*f* units. Further characterizations of the polysaccharides were performed by periodate oxidation, and Smith degradation, acid hydrolysis, and various C-NMR spectroscopic methods. This study concentrated on the polysaccharide structure, and whether these were free arabinogalactans or originally part of the arabinogalactan protein structure was not reported. It is important to note that as for the above arabinogalactan structure studied, information on its ability to produce immune responses may be quite significant.

c. AGP biosynthesis.

There is little direct evidence available about the biosynthesis of arabinogalactan proteins. It is postulated that the protein is synthesized on the rough endoplasmic reticulum. Proline seems to be incorporated into the newly synthesized protein, and in post-translational modification, is enzymatically hydroxylated to produce hydroxyproline (Clarke et al., 1979; Fincher et al., 1983). Most biosynthetic studies have relied on specifically labeling proline, and by following its progressive incorporation into a particular cell fraction (Clarke et al. 1979). The cell walls of *Acer* suspension cultures have been studied in this way. The enzymes responsible for producing the protein-carbohydrate linkage may originate from either the endoplasmic reticulum or golgi apparatus. The golgi apparatus is implicated as a site of cell wall glycoprotein synthesis on the grounds that proline to hydroxyproline conversion and the enzyme which catalyzes the transfer of arabinose from UDP-arabinose to cell wall glycoprotein are found to cosediment with latent IDPase in a fraction containing intact dictyosomes. Secretion of an arabinogalactan protein from suspension culture cells in *Acer pseudoplatanus* is limited by the rate of fusion of dictyosome derived vesicles with the plasma membrane and this process is driven by cations (Fincher et al., 1983). In addition, golgi apparatus associated arabinosyl-transferases are known in other systems, and specifically high concentrations of prolyl hydroxylase, by which peptidyl proline is hydroxylated in*Lolium multiflorum* endosperm cells, is found also to be golgi -associated (Cohen et al., 1983). Other recent investigators including Kawasaki (1981), Owens and Northcote (1981), and Rodgers and Bonell (1992), have provided evidence that arabinosyl transferase activity is localized in the golgi apparatus membranes. This location has also been reported for galactosyl transferase, the enzyme responsible for the synthesis of (1—>6)-ß-D-galactan (Nothnagel, 1997). Dashek found, that in suspension cultures of *Acer pseudoplatanus*, most hydroxyproline rich proteins is transferred via smooth membrane components (as cited in Fincher et al., 1983). Wenicke, et al., concluded in his studies of carrot disks, that synthesis of both AGPs and cell wall glycoprotein is initiated in the endoplasmic reticulum, and that both are transported via the golgi apparatus to the plasma membrane (as cited in Fincher et al., 1983). cDNAs of AGPs encode for N-terminal domains that have the characteristics expected of a secretion signal sequence or entry into the endoplasmic reticulum (Nothnagel, 1997). These signal sequences have been observed to be cleaved and missing from the mature AGP (that is the final protein product of synthesis) (Nothnagel, 1997). Once these macromolecules enter into the endoplasmic reticulum, it is believed they are then secreted to the cell surface (Nothnagel, 1997).

During AGP and cell wall biosynthesis the ferrous ion chelator a' dipyridyl has no apparent effect on the total amount of peptide bound radioactivity secreted from cells provided with C-14 and H-3 proline, even though the inhibitor completely blocks formation of hydroxyproline from proline. This provides evidence that these antagonists restrict the post-translational pathway only (Fincher et al., 1983). It has been suggested that the elongation of the carbohydrate chains could occur by sequential addition of monosaccharides to the glycosylated backbone, at which time, the protein may be considered as a primer presenting multiple sites for initiation of glycosylation and growth. The elongation of carbohydrate chains could also possibly occur by assembling the monosaccharides on some type of intermediate and added to the molecule in a block (Clarke et al., 1979).

From animal studies, there is evidence that glycosyl transferases, which are specific to the sugar being transferred, exist and are actively involved in the transfer of large blocks of sugars. It is also suggested that each terminal disaccharide unit formed in the AGPs would correspond to a glycosyl transferase specific for the transfer of the non-reducing terminal unit of the disaccharide. Differences in the terminal sequence of side chains would indicate a genetically controlled expression of a different compliment of glycosyl transferases and possibly nucleotide sugars. Therefore, glycosyl transferases, as well as the different disaccharides in the terminal sequences of the AGP may be useful as taxonomic markers (Clarke et al., 1979).

In pulse-chase experiments with *Vinca rosea* suspension cultures, C-14-Ara and Gal believed to originate from AGP, were lost from cell walls. The results may be from the turnover of cell wall bound AGP, or it may also reflect transitory extracellular AGP in the cell wall during secretion. It is interesting to note that a range of hydrolytic enzymes capable of degrading AGPs is present in plant tissues, suggesting that *in vivo* , hydrolysis of AGPs is possible (Fincher et al., 1983). Fincher et al. also state that a number of systems have an effective mechanism of incorporating free hydroxyproline. For example free hydroxyproline is able to be taken up by endosperm cells of *Lolium multiflorum* and converted to proline, which then can be incorporated into a protein, hydroxylated, and then secreted as AGP. The carrot can be characterized as another model system. The conversion of hydroxyproline to proline occurs through the intermediate 4,5 dehydroxyproline. Through this system, a high amount of free hydroxyproline is eventually converted to peptidyl-proline and peptidyl-hydroxyproline (Fincher et al., 1983). More recent pulse chase studies by Gibeaut and Carpita in 1991 used a suspension culture of millet cells. The culture was fed radioactive arabinose for two hours after which an excess of unlabelled arabinose (the chase) was added to the same culture. The result also gave evidence of an AGP turnover in that the radioactive polymers decreased with minutes of the chase introduction. Within one hour there was a 33% decrease of the radioactivity of soluble polymers (Nothnagel, 1997).

Basile et al. (1988) demonstrated the complexity of the metabolic pathway by which *Plagiochila arctica* metabolizes free 4-hydroxyproline (Figure 2.9). The pathway on the left appears to be the one used by plants to metabolize the levels of free hydroxyproline that is produced via hydroxyproline protein turnover (Basile et al., 1988) mentioned in the previous paragraph. The center pathway produces an analogue of hydroxyproline which involves both hydrogen loss and a substitution at carbon 3. Basile and Basile (1990) only found a small amount of free hydroxyproline entering this pathway when any concentration of exogenous hydroxyproline was added to *P. arctica*. Finally, the pathway on the right leads to direct incorporation of hydroxyproline into peptide linkages. This pathway occurs in several steps, and is apparently the principal pathway utilized when the level of free hydroxyproline is higher than that normally produced from the normal turnover of the extracellular matrix protein.

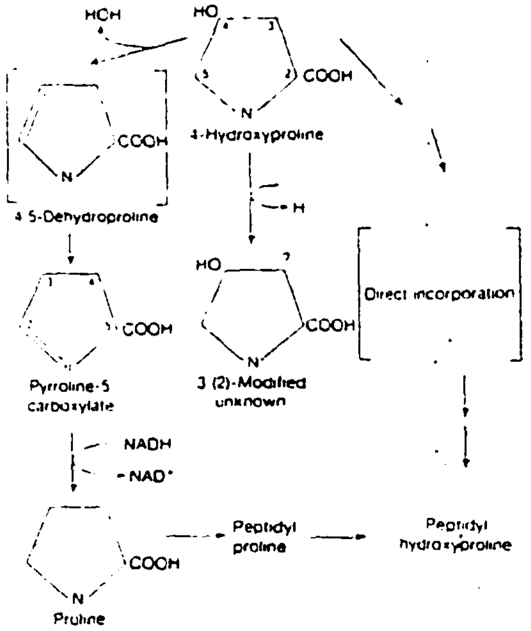


Figure 2.9. Metabolic pathway by which *P. arctica* metabolizes free 4-Hyp. Left pathway (A) used by plants to metabolize the levels of free Hyp produced via Hyp protein tunover. Center Pathway (B) produces an analogue of Hyp. The right pathway (C) leads to the direct incorporation of Hyp into peptide linkages, this is the principal pathway when free Hyp level is higher that that produced by the normal turnover of the extracellular matrix protein. (Basile e al., 1988)

d. AGP functions:

In studies thus far, a number of speculative functions have been ascribed to arabinogalactan proteins, and these functions can be inferred from their physical properties and cellular localization (Fincher et al., 1983). The conformation of the arabinogalactan protein suggests that its galactan framework may have the ability to form a heli or triple heli. This conformation thus would permit substituents to be located or oriented to the outside of the helix, and would avail several surfaces for cooperative interactions, such as the regular areas of the helix face, the helical grooves, the triple helix, and substituent chains. As previously mentioned, these interactions may be of two major types: that of macromolecule-macromolecule or that of macromolecule-small ligand interaction (Fincher et al., 1983). Macromolecules, such as antisera and lectins (galactose-specific) are known to bind to AGPs via specific interactions with particular components of the arabinogalactan chains. Other macromolecules, such as polysaccharides and proteins may also interact in a non-specific manner with AGPs causing changes in viscosity and gel forming potential of AGP solutions. Small ligands, such as flavonol glycosides and artificial β - glucosyl antigen (such as Yariv's reagent), which causes aggregation to a relatively large complex, may also bind AGPs (Fincheret al., 1983).

There is experimental evidence that the terminal disaccharides of plants and gums have taxonomic significance. These terminal disaccharides may be involved in the expression of individual plant tissue and cell types, just as the identity of animal cells is expressed by cell surface determinants, such as the blood groups, histocompatibility proteins of tissue and organs, etc. (Fincher et al., 1983). Bobalek and Johnson (1983) described a statistically designed study used to evaluate compositional variations in the β- lectin in various tissues of *Pseudotsuga menziesii* and *Pinus taeda* and their potential as developmental and chemotaxonomic markers. Two-way analysis of variance revealed that certain β lectin parameters differed significantly from one another depending on the species and developmental state of the sample, thus providing evidence of the possible significance of AGPS as chemotaxonomic markers.

AGPs have been implicated in the morphogenetic change through growth suppression and as such are strongly suggested to be determinants of cell to cell recognition (Basile and Basile, 1984, 1990).

Other studies have suggested that waterholding capacity properties of AGPs play a role in wound-healing, frost-hardiness, and drought resistance. Unfortunately, available data do not support these claims. Per Fincher et al. (1983), water-holding capacity of mucilage (possibly containing AGP) from *Opuntia ficus-indica* is considered insufficient to explain its ability to have drought resistant abilities.

Fincher et al., (1984) suggested a storage role in wheat endosperm, but concede that it may have a broader function. Per Clarke et al., (1979), gums have been thought to elicit a response to either fungal or bacterial infections. For evidence, it is known that bacteria are associated with *Acacia* gums even within the plant itself, and their presence may stimulate gum production. But in contradiction, some trees have similar gum production while in a healthy condition and the composition properties, and structures of *A. senegal* gum appears to be independent of the nature of the wound or stimulus inducing gum production. Thus it can be inferred that the gum is a product of normal metabolism, which is influenced by several environmental factors, such as stress and temperature, in addition to pathogenic agents. Also per Clarke et al. (1979), it has been suggested that the pronounced water-holding capacities of gum exudate gels permits them to form a seal, thus isolating the infected or damaged area of the plant. This would prevent both the spread of infection, and water loss at the site of damage.

AGPs may also act as a nutritional asset, as seen in the style canal of *Lilium*. When compatible pollen has been received as by the stigma, it germinates and produces a pollen tube which grows through the style canal. The canal is filled with mucilage made up of arabinogalactans which may provide carbohydrate precursors for the growing pollen tube cell wall (Clarke et al., 1979). Another possible and important function for AGPs is that of an adhesive located in the middle lamella of the extracellular matrix (EM) that functions to cement cell to cell contacts. They may also be involved in the adhesion of the callus cell clumps. For example, suspension cultures of *Prunus avium* callus cells respond to temperature increases by increasing the size of cell aggregates with a direct proportional increase in the viscosity of the culture media to the amount of secreted arabinogalactans (Clarke et al., 1979). The AGP component of the stigma surface secretion and its possible involvement in capture and adhesion of the pollen to the stigma surface has been observed (Clarke et al., 1979). cDNA studies of TTS-1 proteins seem to provide evidence of this.

It has been suggested that AGPs function to protect membranes from dessication. They may also be associated with lipids as seen in proteoglycolipids of *Vicia faba* leaves and it is very possible that they may be membrane bound as proteoglycolipids in a way analogous to the blood group glycolipid. That is, they may bind with the protein or lipid component embedded in the double lipid bilayer and the hydrophilic component exposed at the extracellular matrix face of the membrane (Clarke, 1979).

The "Suppression Hypothesis" also points to the AGP molecule as a morphoregulator. In the correlative interrelated actions of auxin, ethylene, and AGP, it is AGP that is most directly implicated in the morphogenetic change through growth suppression, since they are stongly suggested to be determinants of cell to cell recognition (Basile and Basile, 1984, 1990). AGPs are made up of complex glycan structures, which suggests a possible morphoregulatory role that they may play a part in plant morphogenesis. There is experimental evidence that they may be information-bearing molecules at the plant surface. The information that the glycan structures may provide may in turn be used to coordinate developmental processes (Knox, 1995).

Per Fincher et al. (1983), the animal proteoglycans called glycoaminoglycans (GAGs), like AGPs, are found in highest concentrations in extracellular spaces. They are stored in subcellular organelles of specialized cells and are secreted by many mammalian cell cultures into the medium. Some GAGs are associated with animal cell surfaces either embedded into the plasmalemma via hydrophobic regions or held via specific binding sites. AGPs are also located in the plasma membrane and there are glycolipids cantaining hydroxyproline, galactose, and arabinose in tobacco leaves. As previously mentioned, as AGPs may be involved in cell-cell adhesion, so evidence points to a similar function with GAGs (Fincher et al., 1983). Another suggested function for GAGs is the control of morphogenesis, and as already noted in the research of the Basiles, there is evidence of AGPs having a similar morphoregulatory role.

Edelman (1989) demonstrated the importance of a group of glycoproteins in the genesis of form in avian embryos. The group of molecules were termed CAMs (cell adhesion molecules), SAMs (substrate adhesion molecules), and CJMs (cell junction molecules). These molecules interact with each other as morphoregulators. Is there a parallel system functioning in both plants and animals? Parallel characteristics have been inferred from recent studies.

Plant development may be viewed as a process that involves both spatial and temporal organization of cell division, cell expansion, and cell differentiation all of which takes place without cell migration (Knox, 1992). Presently it is a known fact that apical meristems or embryos consist of adhered cells which eventually give rise to mature structures possessing more loosely attached cells. The degree to which there is cell separation, and intercellular space formation seems to be developmentally regulated (Knox, 1992).

In animal development, cell adhesion is an important process that involves cell recognition mediated by distinct adhesive systems. During the development of animal tissues and stuctures, diverse molecular mechanisms are utilized to promote sorting and aggregation of appropriate cells. The recognition between the plasma membranes of adjacent cells is mediated by cell-surface glycoproteins such as cadherins. Recognition also occurs between cells and the extracellular matrix and this process is promoted by adhesive glycoproteins found in the extracellular matrix such as fibronectin and vitronectin that are recognized by integrins. Integrins are plasma membrane receptors (Knox, 1992).

Conversely in plants, due to the cell wall structure, the protoplasts are contained and separated from other protoplasts. Thus the plant cell structures limit the plasma membrane to direct contact with its own wall. Therefore plant cell adhesion does not involve interactions between plasma membranes and the wall, but instead cell wall to cell wall adhesions occur

(Knox, 1992). Cell development is believed to occur at least in part as a result of cell wall modification in plants (Knox, 1992).

As in plants, proteoglycans are also common components of animal extracellular matrix and plasma membranes. Like AGPs, these proteoglycans are highly charged molecules, have a high water-binding ability, and also may play a structural role. In animals, proteoglycans also regulate cell-signalling through carbohydrate-protein and protein-protein interactions (Bacic et al., 1996).

Animal proteoglycans also share several common characteristics with AGPs:

1. In plants, the dominant glycan chains are O-glycosidically linked to hydroxyproline amino acids, and to serine and threonine in animals.
2. Both plant AGPs and animal proteoglycans share little sequence identity of their protein backbones, but do possess domain structures including O-glycosylation sites. These glycosylated domains are flanked by domains with other functions.
3. In both animal and plants, some carbohydrate chains have a broad tissue distribution whereas others involve an expression of specific epitopes during growth stages of plant and animal development (Bacic et al., 1996).

d. Localization of organ-specific AGPs:

Now that the similarities and function of AGPs have been addressed, it is important to discuss investigations in which evidence as to where the localization of organ-specific AGPS occurs.

Electrophoretic separation of AGPs in the presence of Yariv's antigen show that AGPs are expressed in a tissue-specific manner and that a given plant tissue can contain more than one kind of AGPs. Whether these AGPs differ with respect to their protein moieties, carbohydrate moieties or both, has not yet been established (Showalter and Varner, 1988). In addition, other immunological and anitbody reactions via several methods have localized the presence of specific AGPs within given plant tissues (Showalter and Varner, 1988).

Yariv's antigen is an artificial reagent that is prepared by coupling diazotized p-aminophenyl glycosides to phloroglucinol and was first used by Yariv et al. (1962, 1967) to precipitate arabinogalactans from plant extracts. Since their development, they have been widely used as precipitants and cytochemical reagents to detect arabinogalactan proteins in tissue extracts and sections. The mechanism of how Yariv's reagent reacts to precipitate arabinogalactan proteins is largely not understood, but it is known that glycosyl residues of the artificial antigen must be in the β-configuation in order for them to react positively (Hoggart and Clarke, 1984). Another structural feature found to be required for a positive reaction is that the carbon atom 2 in the sugar must have a hydroxyl group in the D-gluco configuration (Nothnagel, 1997). An important feature of Yariv phenylgycosides is their ability to form complexes with itself of ten to fifty molecules in aqueous solutions. Due to this ability of self-association Yariv's reagent has an effective molecular weight much greater than the calculated molecular formula of 972.87 (Nothnagel, 1997). Spectral and stoichemical studies have given evidence that AGPs bind to self-associated complex rather then monomeric Yariv phenylglycosides (Nothnagel, 1997). Binding of self-associated Yariv phenylglycosides is saturatable. That is, a 100 kDa AGP-molecule precipitates in a complex with about 100 molecules of Yariv phenylglycoside (Nothnagel, 1997), and the binding is proposed to occur with the carbohydrate portion of the AGP (Nothnagel, 1997). Conversely, partial degradation by various treatments (Tsumuraya et al., 1988) provided evidence that the polypeptide portion of the AGP interacts with the Yariv phenylglycosides. As seen from these studies there have not been consistent conclusions drawn from various research efforts as to what epitope of AGP is involved with Yariv phenylglycosides interaction (Nothnagel, 1997).

It is also important to note that the precipitation of the AGP-Yariv phenylglycoside complex will not occur in pure water. The presence of 1% w/v sodium chloride (NaCl) or some other solutes of equivalent or greater ionic strength is needed (Nothnagel, 1997).

Yariv's reagent has been used as a universal indicator of AGP presence. However, there are examples of AGP molecules differing widely in how they bind to Yariv phenylglycosides. Some molecules containing many of the principal structural characteristics of AGPs bind little if at all with Yariv phenylglycosides (Nothnagel, 1997). Other molecules such as glucuronogalactan protein which has much less arabinose than the typical AGP has a tendency to bind six times more Yariv phenylglycoside than a typical AGP (Serpe and Nothnagel, 1994; Nothnagel, 1997).

Hoggart and Clarke (1984) used Yariv's antigen to study style and stigma extracts. The styles of *Gladiolus gandavensis*, *Lilium longiflorum*, and *Nicotiana alata* were used in Hoggart and Clarke's study, and the following observations were made:

1) AGPs are common and major components of styles of angiosperms.
2) Variations in the structure of the AGPs isolated from different plants existed.
3) AGPs appear in the tissues of the style, simultaneously as the stigma becomes receptive to pollen, and
4) AGPs are localized in the tissue through which the pollen tube grow.

Gell et al. (1986) found the AGPs of style and stigma in *N. alata* were different and the amount of AGP of each tissue type varied during flower development and after pollination occurred. Later investigations by Gane et al. (1995) found that even though the AGPs were different in the style and stigma, the carbohydrate portion of both *N. alata* stigma and style AGPS were similar. Other studies by Gane et al. (1995) showed that ovary AGPs were most abundant at the epidermis of the placenta, and although the overall AGP concentration remained constant, electrophoretic studies confirmed that developmental changes reflected the presence of several different types of ovary AGPs (Gane et al. 1995; Nothnagel, 1997).

Studies of TTS-1cDNA and its putative AGP revealed that the mRNAs are abundant in the stigma and style of *N. tabacum*, but were absent in fruit, ovary, sepal, petal, stamen, root, stem, and leaf (Cheung et al. 1993; Nothnagel, 1997).

Cheung et al. (1993) asserted that TTS-1 glycoproteins are important to the growth of pollen tubes in the styles. There was a direct correlation to the rate of growth to the concentration of TTS-1 glycoprotein present (Cheung et al. 1993; Nothnagel, 1997).

Wu et al. (1995) presented evidence that TTS-1 glycoprotein adhered to pollen tubes that were grown in the style or *in vitro* in medium containing TTS glycoproteins. Wu et al. (1995) also found that the interaction of TTS-1 glycoproteins with the pollen tubes caused deglycosylation of the TTS-1 glycoprotein, and seemed to be closely associated with the pollen tube's cell wall or plasma membrane (Wu et al., 1995; Nothnagel, 1997). Wu also found a glycosylation gradient. The greatest glycosylation concentration was found near the ovary, and the least concentration was near the stigma (Wu et al., 1995; Nothnagel, 1997). Wu hypothesized that this existing glycosylation gradient of TTS-1 glycoproteins may be a guide to help growing pollen tubes toward the ovary (Wu et al, 1997; Nothnagel, 1997).

Similar studies of *N. alata* styles established similar results as Wu et al. (Chen et al., 1993). But even more intriguing were studies performed by Jauh and Lord (1996). These investigators used both monoclonal antibodies LM2, MAC 207, and Jim 13-16. They found lily pollen tube tips were rich in AGP epitopes. When these tubes were exposed to pectinase treatment, the AGP epitopes for the above monoclonal antibodies were detected in bands along the lateral walls (Jauh and Lord, 1996; Nothnagel, 1997). They proposed that protein digestion either exposed and therefore increased the sites of AGPs, or AGPs may have been cleaved by the pectins thus generating a number of epitopes (Jauh and Lord, 1996; Nothnagel, 1 997).

Jauh and Lord's (1996) research provided information about the possible role AGPs play on tip growth of lily pollen tubes. When Yariv's reagent (B-D-Glc)$_3$ was added to the medium of *in vitro* grown pollen tubes, the tube growth was arrested within fifteen minutes of the treatment. In addition to the arrested growth, an abnormal morphology occurred. The tube tip became bulbous twelve hours after initial treatment. Abnormal callose deposition at the tube tip and along the length of the tube also occurred. Red staining (Yariv precipitation of AGPs) was observed only in the tube tip. When Yariv's reagent was injected directly into the style of the lily, pollen tube growth inhibition and reduced fertilization was observed. The investigators asserted that AGPs played a major role in pollen tube growth in both *in vivo* as well as *in vitro* conditions.

Jauh and Lord (1996) studies, using the LM2 antibody which binds to all cells in the style save for the transmitting tract epidermis (which lines the lily's hollow styles) and JIM 13 which only binds to the transmitting tract epidermal cells and their secretions, provided interesting information. They observed a spatial regulation of stylar AGP epitopes. In addition growing pollen tubes adhering to this transmitting tract epidermis were also observed and they hypothesized that AGP and/or pectins mediated this adhesion (Jauh and Lord, 1996; Nothnagel, 1997).

Pollen grain studies also showed distinct localization of AGP epitopes in *Arabidopsis thaliana*. Epitopes of MAC 207 antibody were detected only in the intine (the inner layer of the wall of the vegetative cell) near the plasma membrane. JIM 8 antibody, instead, only labeled the thin layer between the plasma membrane of a sperm cell and the inner plasma membrane to the vegetative cell (Van Aelst and Van Went, 1992; Nothnagel, 1997).

Samson et al. (1984) determined that more than one AGP is localized in the extracellular space of six day old *Phaseolus vulgaris* hypocotyls. Hydroxyproline in this sample showed a major hydroxyproline peak at pH 2.5 - 3 and another at pH 5 which they could not attribute to any known molecule.

Van Holst and Clarke (1986), made use of a cross immunoelectrophoresis by which AGPs were separated electrophoretically in one direction, and then were run electrophoretically into a gel containing *B*-glucosyl Yariv reagent in the second direction. This experiment established that the style, petal, leaf, petiole, stem, and callus of *Lycopersicon peruvium* (a wild tomato) each contained different and separable groups of AGPs, thus supporting the idea that AGPs may be involved in the expression of tissue identity. Cross electrophoresis was used to provide evidence that AGPs (I and II) of eight day old radish seedlings clearly differed from each other and from the AGP III and IV in older mature roots in its high molecular weight, sugar, and amino acid composition (Tsumuraya et al., 1988). In primary roots, the most prominent feature was the transient presence of appreciable amounts of L-fucose containing AGPs which promoted serological activity. Mature roots did not exhibit this ability, but were found to be rich in Hyp and 4-0-methyl glucuronic acid (Tsumuraya et al., 1988).

Earlier purification and structural characterizations of AGPs by Tsumuraya et al. (1984) using mature radish leaves yielded AGP R-I and AGP R-II. These AGPs differed both in molecular size and protein content. AGP RI contained a high Met content, whereas AGP R-II had a higher Glu plus Gln content (Tsumuraya et al., 1984; Nothnagel, 1997).

Monoclonal antibodies have been developed for use against specific organs of plants. Anderson et al. (1984) were pioneers in this line of research, who raised monoclonal antibodies to styles that were prepared from mature flowers of *Nicotiana alata* . Their goal was to produce a monoclonal antibody that would be directed to the product of the S-gene (self-incompatibility gene). This gene prevents inbreeding by arresting the pollen tube of the same S-genotype from growing within the style.

Mice were immunized with extracts of mature styles that consisted of a number of proteins including AGPs in the proteoglycans and glycloproteins configurations that corresponded to the S-genotype. These proteoglycans, as cited previously have been established as weakly antigenic, and it was a surprise to Anderson et al. (1984) that a high proportion of the hybridoma secreted antibodies directly against AGPs. These monoclonal antibodies are specific to polysaccharides that did not inhibit the complex-formation of antigen and antibody. The specific residues were α-L-Ara*f* andβ-D Gal *p* . A projected problem with the application of this hybridoma is that this monoclonal antibody would detect these groups, whether they occur in a proteoglycan, glycoprotein, or polysaccaride. The authors also stated that if the relative affinites of the monoclonal antibodies for different, but related saccharide sequences could be established, it would be possible by careful titering, to detect only a single sugar in a tissue. At present, the positive identification of some carbohydrate epitopes have been successful by using a conjugated carbohydrate hapten of defined structure as the immunogen. A more popular method is to have an AGP, or mixture of different AGPs used as the immunogen, but findings so far are vague. It has been found that the epitopes of some monoclonal antibody remain unknown or have been partially characterized only to the point of showing that carbohydrates are the important components of the epitope (Nothnagel, 1997).

Cassab and Varner (1987) made use of antibody characterization of extensin in *Glycine max*. Although the researchers did not study AGPs, they introduced an interesting method of immunocytolocation of extensin through immunogold-silver staining, and by tissue printing on nitrocellulose paper. This later methodology can be modified to accommodate monoclonal antibodies to AGPs, and through the ability of nitrocellulose to imprint the chemical integrity of the protein intact, one can visualize the cellular location of the AGP. The development of anti-AGP monoclonal antibodies provided an added tool to studying the possible role that AGP epitopes play in developmental regulation (Knox, 1995).

Bradley et al. (1988) successfully prepared a group of monoclonal antibodies against antigens of the peribacterial membranes and other components of the pea root nodules. Using three slightly different preparations of the peribacteroid as immunogens, proved to diversify the range of monoclonal antibodies specificities. Pennel et al. (1989) made use of one of the above monoclonal antibodies, specifically MAC 207, and demonstrated that it recognized and bound to an AGP secreted by carrot suspension culture cells. The MAC 207 antibody was bound to all vegetative cells in the plant, but not to progenitor cell clusters, and determined cells involved in sexual reproduction. Non-staining cells were those of the pollen sac, tapetum, sporangeous tissue, embryo sac, and nucellus. After fertilization, the zygote globular stage embryo and endosperm did not stain, but MAC 207 staining returned at the heart stage of development (Pennel and Roberts, 1990; Nothnagel, 1997). Thus the group from the John Innes Institute opened a new research tool application, by which localization of AGPs, and insight into possible functions could be inferred. The researchers via frozen section immunogold electron microscopy of onion root confirmed that the plasma membrane was the main site of antibody binding. Furthermore, fine structural analysis of labeled resin sections revealed that in carrot suspension cells and excised tissues of intact flowering plants, the principal site of MAC 207 binding was the outer face of the cell membrane (Pennel et al., 1989).

In some somatic cells of some species, the intracellular membrane showed little significant labeling, but in others such as the vegetative cell plasma membranes of sugar beet pollen was generally intense. In all instances, binding to the plasma membrane was accompanied by equally notable labeling of cytoplasmic vesicles (Pennel et al., 1989). Of interest, Pennel et al. (1989) found that the only region of the plasma membrane that seemed not to become labeled was that part forming the lining of the plasmodesmata.

Using seedlings of *Daucus carota* L., it was demonstrated that the monoclonal antibody JIM 4 recognizes an epitope common to a set of glycoproteins of the plasma membrane of a specific carrot cell line and an AGP secreted by the cell line (Knox et al., 1989). These researchers also demonstrated that, in the carrot seedling, the most abundant expression of the membrane associated proteins occurs in the vascular tissues. Examination of the root apical meristem of the carrot seedlings indicated that the expression of the epitope occurs early in the continuing development of the root meristem and seems to reflect early stages in the formation of the vascular pattern.

The various methodologies used to ascertain developmental information included indirect inflorescence, immunogold electron microscopy, immunoblotting, and ELISA (the enzyme-linked immunosorbent assay). These techniques revealed a specific and restricted distribution of the JIM4 antigens in the root apex in a region 50-100 μm from the most apical meristem cells. The most intense labelling of JIM4 occurred in two arcs of pericycle cells with oblique longitudinal cells. Weakly labeled cells were to be found in the center of the stele, which are the vessel mother cells that would produce the future xylem plate (Knox et al., 1989).

It seems that a similar pattern could be detected in other regions of the seedling and mature carrot plant. In shoot tissues of a mature plant, JIM4 antigen expression within the vascular tissues is maintained through the transition from root to the shoot tissue of cotyledons and in mature plants (Knox et al., 1989).

In later studies done on cell cultures and somatic embryogenesis of *Daucus carota*.L (Stacey et al., 1990), JIM-4 recognized an epitope (J4e) which occurred in a set of plasma membrane-associated arabinogalactan proteins (AGPs). This showed that proteins contain information in terms of epitope restrictions, to distinct tissue or groups of cells, during the early stages of plant development. In concurrence with anatomical studies of the carrot, the expression of J4e is correlated with various stages of development by its appearance in the outer surface layers of cells at the globular stage; its restriction to a defined region later at the heart-shaped stage; and its expression by cells associated with the future root-shoot junction. Finally

J4e expression was associated with provascular tissue in the future hypocotyl and then in the cotyledons.

Thus the expression of J4e provides a marker for stages of the acquisition of the major tissue patterns during carrot somatic embryogenesis. This class of AGPs does not appear to be tissue specific or concerned with specific differentiation pathways. It does, however, seem to be important in the establishment of positional distinctions between cells, and of tissue patterns within which cytodifferentiation processes can be contained (Stacey et al., 1990; Knox, 1992).

Anti-AGP antibodies have been useful in identifying the embryogenic potential of singular suspension cultured cells (Pennel et al., 1992). It is recognized that in embryogenic suspension cultures, specific surface cells can form a particular type of cell cluster termed by Halperin and Wetherell as a proembryogenic mass. It is also known that small single cells termed type I cells can form somatic embryos after a preculturing period at which time proembryogenic masses are formed (Pennel et al., 1995).

Pennel et al. (1992) used a Jim 8-anti AGP antibody and found this antibody to be a useful marker for embryogenic potential in carrot suspension cultures. They asserted that the epitope identifies a particular cell state that is transitonal between explanted tissue cells and somatic embryos. The researchers also asserted that upon entering this transitional state, the cells may either elongate with or without several cell divisions and eventually die; or they may divide and form daughter cells with a thickened cell wall and proembryogenic mass inital cell. Jim 8-antiAGP antibody recognizes the cell that is in transition, follows the phytohormone-controlled developmental pathway that is fated for cell death, but does not recognize the derived daughter cells, and the latter fate of the proembryogenic mass formation. The Jim 8 reactive cell wall epitope is therefore considered as a useful marker for an early determinative event in carrot somatic embryogensis (Pennel et al., 1992; Knox, 1995).

Other investigators provided further evidence of the potential influence AGPs have on embryogenesis. Kreuger and Van Holst (1993) exogenously added a total AGP fraction from carrot seed media and succeeded in increasing the amount of overall embryogenic cells to a moderately embryogenic carrot culture. When this same fraction was added to an old culture that no longer exhibited embryogenic potential, an increased proportion of embryogenic cells as produced (Kreuger and Van Holst 1993; Nothnagel, 1997).

In later studies monoclonal antibodies Zum 15 and Zum 18, developed by Kreuger et al. (1995), were used to fractionate carrot seed AGP by affinity chromatography. They found that carrot seed AGPS bound by Zum 15 antibodies (i.e., Zum 15 AGPs) inhibited embryogenesis when added to a moderately embryogenic carrot cell culture. Carrot seed AGPs bound by Zum 18 (i.e. Zum 18 AGPs) increased embryogenic potential of the carrot cell line (Kreuger et al., 1995; Nothnagel, 1997). Bound Zum 18 AGPs of tomato cells though electrophoretically different from Zum 18 AGPs of carrots still promoted embryogenesis in carrot cell cultures, as were carrot seed Zum 18 AGPs in *Cyclamen persicum* cultures (Kreuger et al., 1995; Nothnagel, 1997).

Serpe and Nothnagel (1994) applied Yariv's reagent (ß-D-Glc)$_3$ to suspended culture-medium of rose cells, and at a concentration of 50 µM of Yariv's reagent, a total inhibiton of growth occurred. After seven days, the cells were transferred to normal culture medium, and normal growth of these cells resumed. Further analysis of the (ß-D Glc)$_3$ treated cells showed that the Yariv's reagent was bound to the cell wall AGPs (Serpe and Nothnagel, 1994; Nothnagel, 1997).

Serpe and Nothnagel also provided evidence that cell wall AGPs mediated the effect of cell division. Studies on live rose protoplasts showed that (ß-D-Glc) $_3$ did not alter lipid diffusion but the variability of the diffusion coefficients were increased, and that there was a decrease in the mobile fractions of membrane proteins and glycoconjugates (Nothnagel, 1997). This provided evidence that the cell wall AGPs and plasma membrane AGPs could very well be involved in observed inhibition of cell division as proposed in the Basile "Suppression Hypothesis" (Basile and Basile 1993).

As can be understood from the cited researchers, it seems that modulations in the glycan components of the AGPs or the interactions at the molecular level may be involved in the development of plant anatomy (Knox, 1995). There are several thoughts on the significance of epitope modulation. Epitope modulation could reflect AGP metabolism in the wall or turnover and processing events in the Golgi Apparatus. The absence of a particular epitope may reflect the masking of an antibody-binding site through interaction of the epitope with wall components. AGP epitopes occur at the outer face of the plasma membrane, but can also appear in the cell wall (Knox, 1995).

A library of AGP-binding monoclonal antibodies is now available. This has permitted comparative studies of the binding abilities of different antibodies to various AGPs or other relevant molecules by several researchers. The results indicated that some monoclonal antibodies such as MAC 207 and Jim 13 were able to bind to a wider variety of AGPs than other monoclonal antibodies, such as, Jim 4 and Jim 14. Yates and Knox performed epitope mapping studies in which different monoclonal antibodies were tested in competition for binding to gum arabic. These studies suggested that proximity or some amount of overlapping occurred among the epitopeds recognized by Mac 207, Jim 4, Jim 13, Jim 14, Jim 15, Jim 16, and LM2 (Nothnagel, 1997).

Although monoclonal antibodies are designed for the specificty of binding, cross reactivities have been found with several exudate gums and rhamnogalacturonan I a pectic polymer. Per Nothnagel (1997), cross reactivity with exudate gums is not of widespread concern because they are abundant only in particular species, but rhamnogalacturonan I is present in the primary cell walls of many flowering plants, and may be of greater concern to researchers (Nothnagel, 1997).

6. *Physcomitrella patens* :

a. Basic taxonomy.

Physcomitrella patens (Funariaceae) has a wide distribution in the Northern Hemisphere from Europe to Siberia, and also eastern North America (Tan, 1978). *Physcomitrella patens* subsp. *patens* is widely distributed from southern Quebec west to Illinois, south to South Carolina and Louisiana, and in Europe (from Sweden south to Italy; and is known to inhabit from Ireland, to as far east as Hungary and Poland). Collections of *P. patens* have also been made in S. E. British Columbia and Oregon (Tan, 1978).

Physcomitrella patens usually inhabits exposed river banks or recent alluviums. Specimens have also been recorded at bottoms of ephemeral ponds or reservoirs and occasional specimens are recorded from edges of ditches and wheel ruts. It's habitat is frequently associated with semi-aquatic hepatics. All specimens grow on clay or silt (Tan, 1978). *Physcomitrella patens* habitat seems to be restricted to low elevations in all parts of the world (Fife, 1982). *P. patens* sproangia is generally recognized by its globose, inoperculate capsule with extremely delicate exothecial cells and absence of a basal neck, short seta, and no suggestion of an operculum at the apex of the sporophyte seen in figure 2.10 (Courtice et al., 1978; Fife, 1982). Between 2000 -4000 viable haploid spores may be contained in a mature capsule (Courtice et al., 1978).

b. Histological Development of *P. patens* (Life Cycle)

The life cycle of *P. patens* is predominantly haploid. Nakosteen and Hughes (1978) showed that under laboratory conditions, *P. patens* can complete its life cycle in twenty-one days.

Protonematal development is similar whether the plants germinate from spores, develop from single cells, from protoplasts, or as regeneration products from small pieces of tissue (as in homogenized plant tissue). They usually produce a single filament from one cell. However, from spores, a few primary filaments may form, one of which may be small primary rhizoids (Bopp, 1990). Spore germination, which takes one to three days, is followed by the protonematal or juvenile stage. The growth is characterized by irregular branching filaments (primary chloronemata) (Bhatla, 1994). Chloronematal cells contain many chloroplasts and intercellular walls that are perpendicular to the axis of the filaments (Grimsley et al., 1979). Figure 2.11 shows a micrograph of a chloronematal cell. A second cell tissue type develops which consists of long peripheral filaments (caulonemata) which radiate from the central chloronemata, which grows perpendicular to the direction of incident light. Caulonematal cells are longer and contain fewer chloroplasts than chloronematal cells.

Figure 2.10 *Physcomitrella patens* sporophyte (Zeiss, 150x)

Figure 2.11 *Physcomitrella patens* chloronema cell. Note the abundance of chloroplasts, the wide cell width, and the perpendicular cross walls (SEM, 250x)

Intercellular walls are oblique and older cells contain red-brown pigments (Grimsley et al., 1979). Caulonematal cells divide to give rise to regular branching side branches most of which develop into secondary chloronemata, a tissue which appears to be morphologically identical to primary chloronemata. Secondary chloronemata, however, respond differently to varying intensities of unidirectional light (Grimsley et al., 1979). The chloronemata and caulonemata together constitute the protonema. A few caulonematal side branches develop into buds (Grimsley et al., 1979), although bud development can also be initiated from chloronemata grown on solid agar (Reski and Abel, 1985). Figure 2.12 is a micrograph of a typical caulonematal cell.

Funaria hygrometrica (Funariaceae) shares a very similar developmental pattern with *Physcomitrella patens*. Of the two mosses, *F. hygrometrica* has been studied more extensively, and much of what is known about protonematal differentiation and cytological ultrastructure, comes mostly from investigations conducted with *Funaria* .

Studies on the chloronematal tip growing cells of *Funaria hygrometrica* and *Dawsonia superba* gave evidence that a variety of organelles are present at the tip of the chloronematal growing cells, and were also distributed uniformly over it length. The studies showed that chloroplasts, vacuoles, and mitochondria are frequently present in the terminal portion of the cell. Ribosome density is often greater in the tip region. Pairs of dictyosomes are often found close to the cell wall (DeMaggio and Stetler, 1977). Numerous vesicles are located between the plasma membrane and the cell wall, and not concentrated at the tip. Microtubules are commonly found both in the inner and outer cytoplasm of the protonema. The peripherally located microtubules are found lying close to the plasma membrane. The microtubules close to the tip appear to be randomly oriented. In regions behind the tip of the chloronematal apical cell, the microtubules are predominantly axially aligned, whereas, in the inner cytoplasm they have no specific orientation, but many are arranged along the axis of the cell (DeMaggio and Stetler. 1977).

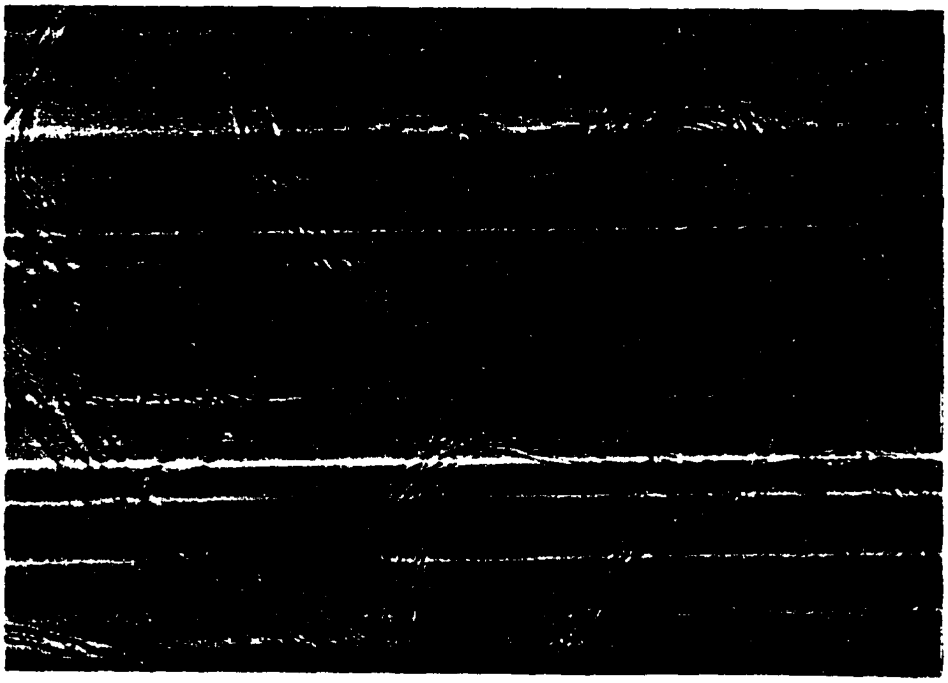


Figure 2.12 *Physcomitrella patens* caulonematal cell. Note the lack of chloroplasts, the thinner cell width, the longer length, and the oblique cross walls (SEM 250x)

Studies on the chloronema cells of *P. patens* provide evidence that microtubules run alongside the spindle-shaped chloroplasts (Abel et al., 1989). Fluorescence microscopy after staining with rhodaminyl phalloidin has shown that actin filaments form a fine network with long strands parallel to the long axis of *P. patens* chloronematal cells. The actin filaments are concentrated in the very tip and at both sides of the cross wall of the tip cells (Abel et al., 1989).

Studies of *F. hygrometrica* have shown that young caulonema have a large central vacuole surrounded by a thin layer of parietal cytoplasm. Older caulonema cells tend to be especially vacuolated. The elongated chloroplasts are located peripherally just below the plasmalemma, and the cell walls of mature caulonema show browning due to the accumulation of phenolics (Bhatla, 1994).

Caulonema cells are generally negatively phototropic under standard laboratory conditions, and show a regular pattern of branching in *F. hygrometrica* (Bhatla, 1994). At a specific distance from the growing tip cell, each cell of the caulonematal filament develops a protuberance near its apical pole, which develops into a side branch from the basal cells of the main caulonemal filament. This primary side branch usually differentiates as another caulonemal cell (Bhatla, 1994).

When the main filament of *F. hygrometrica* get older, a second branch arising near the base of the first filament also forms. The latter branches are usually made up of chloronematal cells, and are called secondary chloronema. The secondary chloronemata are similar in structure to primary chloronema, but in contrast, primary chloronema arise from the spore (Bhatla, 1994).

Caulonematal cells in *F. hygrometrica* attain a maximum length of 400 µm as compared to 200 µm or less in chloronema. The growth is restricted to the dome-shaped apex (Schmiedel and Schepf, 1980). The caulonema apical cell possesses a linear organization. Studies of *F. hygrometrica* and *Physcomitrium turbinatum* (=*P.pyriforme*) has shown that the caulonema apical cell can be divided into a terminal (distal) region, extending from the cell tip to the nucleus, and a basal (proximal) region extending from the nucleus to the basal end wall. The terminal region is further subdivided into an apical tip body which lacks chloroplasts, and a chloroplast replicating zone which extends from the base of the tip body to the nuclear zone (Jensen and Jensen, 1984).

The tip body of caulonemata appears as a clear cap. This zone is composed of golgi vesicles with electron-dense contents. Irregular particles of endoplasmic reticulum are also seen, and smooth endoplasmic reticulum occupies a considerable part of the tip body. In this zone, there is a great density of dictyosomes, which progressively decreases in the more basal region of the cell. The tip body and chloroplast replication zones suggest that their formation is correlated with the metabolic events occurring in these young regions of the tip cell (Jensen and Jensen, 1984).

The growing tip of *F. hygrometrica* caulonemata maintains a definite distance from the nucleus. Cell elongation seems to be controlled by the temporally-shifted nuclear movement (Quader, 1991).

The basal region of the tip growing cell is highly vacuolated. Evidence of investigations show that cell vacuoles increase in size through a merging of smaller vacuoles in a growing cell. The number of vacuoles decreases along an apical-basal gradient, along with a corresponding increase in vacuole size (Bhatla, 1994).

In addition to uneven distribution of organelles in the caulonema tip cell, some organelles exhibit a gradient in their differentiation. Examples are chloroplasts, vacuoles, smooth endoplasmic reticulum, and dictyosomes. Where vacuoles become more extensive in mature parts of the cell, the dictyosomes, chloroplasts, and smooth endoplasmic reticulum are more extensive in younger regions (Jensen and Jensen, 1984).

Caulonema filaments branch very regularly in *Funaria hygrometrica*. As the tip cell divides, the former second, now third cell initiates a new side branch near its distal end. In the fourth cell, the outgrowth develops further. In the fifth cell a second side branch is formed. The same cell does not produce further branches (Schmiedel and Schnepf, 1979).

Caulonema is considered the main adventitious tissue and chloronemata and gametophores are the main photosynthetic tissue of mosses (Bhatla, 1994).

Bud induction on moss protonema, and subsequent development of buds into upright leafy gametophores, represents a switch or transition from two dimensional filamentous growth to three dimensional growth (Ashton et al., 1993; Bhatla, 1994). The gametophore buds can be initiated as early as ten days after germination. Each bud develops into a gametophore with a stem, leaves, and rhizoids (Grimsley et al., 1980). The bud formed first grows to a moruloid stage through divisions of the apical cell (Knoop, 1984). This early stage of bud formation can be seen in figure 2.13.

Two different patterns of bud induction have been observed in mosses. The '*Funaria* type' in which buds form on the caulonema only. In *Funaria*, since the caulonema form a ring around the central primary chloronema, the buds also develop annularly. The second type of bud induction observed in 'true' mosses, is referred to as the '*Polytrichum* type'. Buds develop on cells immediately near the germinated spore or from the spore itself, so that even very young protonema can produce buds. Similar buds develop from chloronema in *Physcomitrium sphaericum* (Bhatla, 1994), and *Physcomitrella patens* (Abel et al., 1989; Reski and Abel, 1985).

Physcomitrella patens is useful as a simple model system for studying plant development as it provides developmental parallels to that of higher plants, possesses a similar phytohormonal regulatory action to that of higher plants, and is easily cultured axenically on defined media. The plant itself is small and possesses a short life cycle, it contains small numbers of cell types, it has been relatively well defined genetically, and provides a wide variety of mutants including morphological, phytohomonal-insensitive, and phytohormone requiring types. Lastly, the existence of established protocols that facilitates genetic analysis of this moss, makes studying *P. patens* beneficial (Ashton et al., 1993).

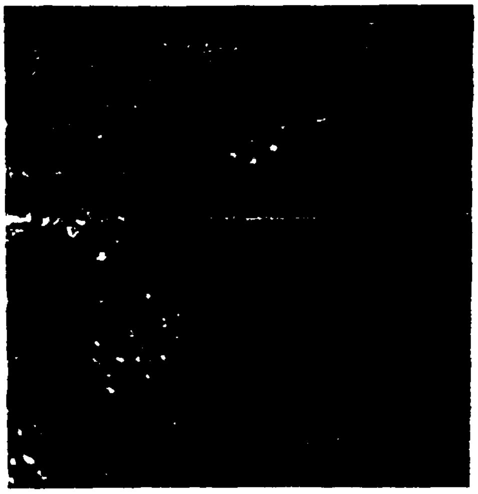


Figure 2.13 The moruloid stage of a developing bud form the protonema of *Physcomitrella patens* (SEM 150x)

6. Phytohormone Regulation of Moss Development.

Over the years, there has been much interest on the role phytohormones play in regulating moss development. In this section I will review the findings of researchers in work done on mosses. Special emphasis will be placed on developmental studies done with *F. hygrometrica* and *P. patens*.

Researchers find that the protonemata of mosses can be viewed as a "morphogenetic system," and as such can be seen from the earlier histological development discussion, this "system" has a very clear and stepwise differentiation (Bopp, 1990). Researchers have found that auxin and cytokinins are clearly involved in the morphogenetic regulation of moss protonema (Bopp, 1990; Ashton et al., 1979a, 1979 b, 1985,1990, 1993; Ashton and Cove, 1990; Featherstone et al., 1990; Cove and Ashton 1984, 1988; Knight et al., 1988; Cove et al., 1980).

a. Auxins.

For more than thirty years, researchers have recognized that auxin has several effects on moss development (Bopp, 1990). The effects include inhibition of protonema growth, stimulation of rhizoid formation, transformation of buds to filaments, torsion of young stems, and complete suppression of leaves on gametophytes (Bopp, 1990).

Johri and Desai (1973) established that auxin was a prominent morphoregulator of moss development. *Funaria hygromterica* was cultured in Knops liquid medium in ehrlenmeyer flasks. Under these conditions, the moss remained in the chloronema stage, as this method is considered a sort of "stress condition" (Bhatla, 1994). When cultures were treated with indole-3-acetic acid (IAA) or 1-naphthaleneacetic acid (NAA) the chloronema differentiated into caulonemata (Johri and Desai, 1973; Johri, 1974). It was also observed that cells of *F. hygrometrica* responded to ethyl ester of IAA. The number of caulonemata formed was dependent on both auxin concentration and the cell density within the culture. The lower the cell density, the lower the auxin concentration needed for the transition from chloronemata to caulonemata to occur. For example, at cell densities below 0.1 mg. protonema per millimeter of solution (0.1mg mm^{-1}), caulonemata appeared spontaneously. Johri and Desai (1973) reasoned that the density effect depends on the release of IAA oxidase from the protonema which degrades IAA in the medium. In further investigations, Sharma et al. (1979) observed that peroxidase and IAA oxidase have the highest activity in cultures with the highest cell density . Thus in cultures with high cell density, since there is a high auxin oxidase activity, more exogenous auxin molecules per cells are required for caulonematal production to occur. Exogenous treatments with non-auxins such as ß-NAA, cytokinins, or gibberillins have no effect on caulonema differentiation (Johri, 1974). IAA-glycine and other conjugates (-D-alanine, -L-aspartic acid, -L-phenylalanine, -D-valine, and 0L0valine) are completely inactive (Johri and D'Souza, 1990)

Johri and Desai (1973) stated that auxins at physiological concentrations (0.5 to 5.0 µM) enhance the proportion of caulonematal branches in cell suspension cultures of *Funaria*. The cellular basis of this increase is two fold: first the inhibition of chloronematal branch growth and second, the promotion of caulonematal branch initiation. Therefore, auxin may act as a possible morphoregulator at a specific time during protonema development.

Lehnert and Bopp (1983) showed that protonema grown on agar under low irradiance produced only chloronemata. When low concentrations of IAA (0.1mM) was added to cultures grown under low light intesity (1 W/ m^{-2}), the protonema formed oblique cell walls almost immediately.

Use of other types of auxin such as ß-NAA and 2.4 dichlorophenoxyacaetic acid (2,4 D) differ only quantitatively from IAA treatment (Lehnert and Bopp, 1983). The same is true for substances found as IAA precursers in higher plants.

In moss, there seems to be a variation in the indole-3-acetic acid (auxin) biosynthetic pathway. As seen in figure 2.14 the moss seems to synthesize auxin via the:

Tryptophan → indole-3-pyruvic acid →indole-3-acetaldelhyde pathway. Auxin is also naturally produced via the:

Typtophan -->Indole-3-acetamide pathway (Lehnert and Bopp, 1983). This is particularly interesting since this pathway occurs in higher plants only after the plant is infected with *Agrobacterium tumefaciens* (Lehnert and Bopp, 1983).

On the other hand, when tryptamine and indoleactonitrile were exogenously added to protonema cultures, there was no induction of caulonemata formation (Bopp, 1990). Therefore it is believed that these mosses tested did not possess the:

Tryptophan →Tryptamine pathway and the Glucobrassicin → indole-3-acetonitrile pathway (Lehnert and Bopp, 1983). These modifications in the moss' ability to synthesize auxin based on Lehnert and Bopp's experiments is shown below.

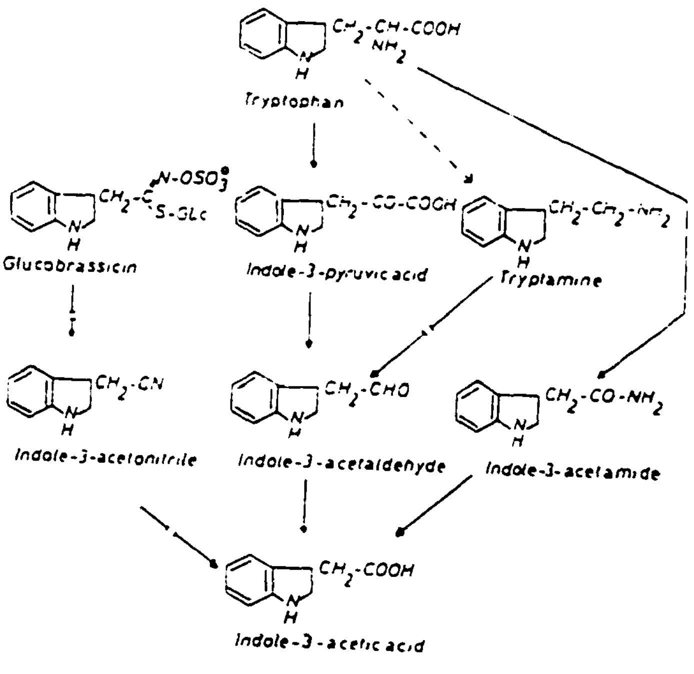


Figure 2.14 The biosynthetic pathway of auxin as synthesized by moss. (Lehnert and Bopp, 1983).

Of interest is auxin antagonist experiments performed by Sood and Hackenburg (1979), who showed that caulonema filaments revert back to chloronema when treated with (parachlorophenoxy) isobutryic acid (PCIB), an antagonist to auxin. The cell diameters become smaller and transverse cell walls are formed in the newly formed filments. When high concentrations of both PCIB and auxin are exogenously added to cultures grown in basal medium, protonema growth is inhibited, but the inhibition by both endogenous and exogneous IAA can be reduced or nullified with PCIB (Bopp, 1990; Bhatla, 1994). These results offer more evidence that endogenous auxin plays an active role in the chloronema - caulonema transition stage.

Rose et al. (1983b) found that a higher pH favors chloronematal growth and inhibits caulonematal production. IAA accumulation in the protonema is strongly pH dependent, being greatly enhanced at pH 4.0 then at pH 7.6. The low proportion of caulonema formed at pH 6 or 7 in the IAA-containing medium may be due to the reduced uptake and accumulation of auxin. The differentiation of caulonema in auxin free medium buffered at pH 5.0 can be due to a change in the sensitivity of cells to endogenous auxin or to a build up of auxin (intracellular or in the medium) above a threshold level during the lag period (Rose et al., 1983b).

If PCIB is added to the buffered medium, caulonematal formation is inhibited, and there is a prolongation of the lag period for caulonemal formation in *F. hygrometrica*. The total duration of the lag period is concentration dependent on the amount of exogenous PCIB present in the medium. At 15 µM PCIB, primary caulonema cannot be discerned. The prolongation of a lag period due to PCIB is nullified by applying IAA (Johri and D'Souza, 1990). The precise mode of action of PCIB in *Funaria* is not known. It has been observed that 10 µM PCIB reduced polar, basipetal transport in about 40% of the controls (Rose and Bopp, 1983). In maize coleoptiles and *Cucurbita* hypocotyls, PCIB competes with IAA for IAA binding sites in vitro (Dohrmann et al., 1978; Jacobs and Hertel, 1978; Bhatla, 1994). The delay in caulonema production by PCIB can be due to one of three factors:

1) the disruptionof the endogenous auxin transport system
2) the inhibition of auxin synthesis and/or accumulation
3) specific competition for auxin-binding sites (Bhatla, 1994).

The fully developed caulonema, with its side branches, represents an apical dominance system with regards to its regular branching system and regenerative behavior. The overall growth velocity of the side branches of caulonemata always remains less than that of the main tip. That is, the young side branches near the apex grow more slowly than older ones more distal from the tip. Therefore, the further the side branch is from the tip cell, the faster is its growth. If the tip cell is removed, the closest cell to the apex regenerates a new tip cell from its uppermost part and continues to grow in the old direction. If the tip cell is removed again or if the development of the new tip cell is somehow delayed, the side branches nearest to the apex increases its growth velocity and forms a new tip cell (Knoop, 1984).

The polar transport of auxin plays a most important role of chloronema to caulonema transition. Several experiments have shown that if the apical caulonematal cell is killed, all cells within that filament lose their caulonematal properties (Bopp and Knoop, 1974). When caulonematal filaments are removed from whole protonema, again there is a loss of caulonematal characteristics and the filaments reverts back to chloronematal properties (Bopp and Knoop, 1974).

It is believed that since the removal of the tip cell alone can revert the protonema to chloronematal conditions, then the auxin signal from the tip cell must maintain the caulonematal status of that cell (Bopp, 1990).

Polar auxin transport was found to occur in whole protonema fed ^{14} C-IAA (Larpent-Gourgaud, 1974; Bopp, 1990). The transport mechanism includes a pH passive auxin influx (Rose et al., 1983b), and a 2,3,5 TIBA-inhibited active efflux (Rose et al., 1983a, 1983b).

Auxin is tranported in *F. hygrometrica* from the protonema tip to its base (Bopp and Geuer, 1988). Bopp and Geuer (1988) in studies of IAA influx and IAA efflux, showed that when both 10 µM TIBA and 10 µM NPA at a pH 5.0 is exogenously added to *Funaria* cultures, the accumulation of IAA in protoplast cells are enhanced 30%. Their experiments supported the assumption that the inhibition of polar transport is a consequence of the action of efflux of IAA being inhibited at the protoplast level, and further explains why more auxin is accumulated in basal cells than in the apical cell. When this occurs, there is a gradient created along the protonema filament (Bopp, 1990). Auxins act as a signal and must be transported basipetally on a continuous basis in order for the differentiation of subapical cells into caulonema to occur. Per Rose and Bopp (1983), there appears to be a definite relationship between IAA transport and the stability of the caulonematal state. Therefore, continued caulonematal growth is dependent on the formation of apical cells de novo, either from chloronematal apical cells or from side branch initials. Since the formation of primary caulonema is not accompanied by an inhibition of chloronema, formation of primary caulonema must be under the regulation of extremely low levels of endogenous auxin (Bhatla, 1994; Johri and D'Souza, 1990).

b. Ethylene.

As discussed earlier in this chapter (Ethylene pp. 25-30), ethylene is a ubiquitous phytohormone. The effect of ethylene can be correlated with auxin concentration, and has been found to have a possible role in protonema differentiation (Thomas et al., 1983). Rohwer and Bopp (1985) established that more ethylene is produced on culture media containing IAA than on IAA-free culture medium. They also established in their studies with*Funaria hygrometrica* that when protonema was transplanted onto fresh plates with medium containing 10^{-6} M IAA after initial cultivation for a duration of three weeks on hormone free solid medium, ACC contents were found to be nearly ten times higher. In addition Rohwer and Bopp (1985) also observed a direct correlation of ethylene production to auxin (IAA) concentration. The higher the concentration of auxin added to the media, the greater the amount of ethylene produced. Rohwer and Bopp also observed the ACC content and ethylene production coincided with the progressing differentiation of caulonema from chloronema filaments. They also argued that these results give evidence of the possible involvement of ethylene in the morphogenesis of the protonema especially at that stage in which there is a transition from chloronema to caulonema.

Rohwer and Bopp (1985) also proposed a second role for the involvement of ethylene in protonema. This role is ethylene's influence on senescence within the older moss protonema, that is, protonema consisting of aging chloronema, and that is more than twenty-five days old. In these protonema, empty cells formed by unequal cell division which separate the filaments are formed. These cell types are termed "tmemata." Although these naturally appear in old moss protonema, Rohwer and Bopp found that by adding exogenous ACC to the medium, younger protonema were induced to form "tmema" cells. They therefore inferred that the observed high natural production of ACC and ethylene in older cultures may be evidence that ethylene can act as a kind of senescence hormone in moss protonema (Rohwer and Bopp, 1985; Bopp et al., 1986).

Although studies of ethylene's effect on higher plant morphogenesis is well documented, unfortunately there is little published literature available on the possible morphoregulatory role that ethylene plays in protonema morphogenesis (differentiation). Yet this provides exciting possibilites for future studies including my present studies which I will present in later chapters.

Strong experimental evidence by Basile (Basile and Basile, 1984; 1993) show that ethylene may play a suppressive role in plant development, and its production may be a result of higher localized concentrations of auxin present within the developing tissue.

c. Cytokinins.

Bauer (as cited in Bhatla, 1994) was the first researcher to isolate a cutokinin – like substance from callus that was derived from sporophytic tissue of a moss hybrid *Funaria hygrometrica* x *Physcomitrium pyriforme*. Beutelman and Bauer (1977) characterized this substance using gas chromatography-mass spectrophotometry (GC-MS) as N^6-(Δ^2 - iso-pentenyl) adenine (iP or i ^{6}Ade) occurring at the conentration of approximately 10^{-6}M. The substance had similar properties to kinetin, and therefore was called "bryokinin" (Bhatla and

Dhingra-Babbar. 1990). In addition another substance whose properties resembled an adenine derivative, was isolated by Klein. This compound was termed factor H. It has been shown to shorten the time required for bud production to occur and to increase the number of buds produced (Bopp and Knoop, 1974; Bopp, M., 1968; Bhatla and Dhingra-Babbar, 1990).

Radio immunoassay (RIA) studies have confirmed the presence of the two forms of cytokinins characterized by GC-MS. Furthermore, RIA has established the presence of a third cytokinin in moss culture medium using OVE mutants of *Physcomitrella patens* (Wang et al., 1984). The OVE mutants are ones that overproduce cytokinin, and therefore overproduce gametophore buds. This third cytokinin is probably zeatin glucoside

Physcomitrella patens OVE mutants have the ability to convert [8-14C] adenine (Ad) to N6-C-14-etc. i6Ade. This has been confirmed by thin layer chromatography (TLC), HPLC, and recrystallization to constant specific radioactivity (Wang et al., 1981a, 1981b).

Brandes and Kende (1968) gave evidence of how the phytohormone cytokinin does not act as a trigger for bud formation, but must be present during a critical period of time until differentiation is stabilized. In their studies they used *F. hygrometrica*. They observed that if benzyladenine was removed at early stages of development, the bud reverted back to the protonematal filament stage. If the hormone was withdrawn at later stages of bud development, there was less evidence of dedifferentiation.

If the cytokinin was removed during a washing period, and transferred back to nutrient agar containing benzyladenine, bud formation proceeded normally. The buds reverted to filamentous growth when put on nutrient agar without benzyladenine for forty-eight hours (Brandes and Kende, 1968).

Cytokinins exert both non-specific and specific effects on moss protonema. The non-specific effect would be that of general cell division such as the induction of branching of caulonema cells. Under normal conditions, as previously discussed, one of the characteristic features of the caulonematal stage is the very regular and spontaneous formation of side branches. Cytokinin requirement for this to occur is met by the endogenous cytokinin level

(Bhatla, 1994). Side branches of caulonema may be initiated by adding picomolar concentrations of exogenous cytokinins (Bopp and Jacob, 1986).

The specific effect of cytokinin in mosses is found in the induction of shoot buds. All cytokinins have this characteristic effect and all mosses have similar responses independent of the protonema's growth form (Bopp, 1968; Bhatla, 1994). Bud induction requires a higher concentration of kinetin than it does for protonema cell division, that is, micromolar concentrations are required (Bopp and Jacob, 1986). Soon after kinetin was identified as a cell division factor, it was observed that the formation of buds was drastically enhanced in protonema of the moss *Tortella caespitosa* when the moss reached what is now known as the "critical size" (Bopp, 1990; Sood and Hackenberg, 1979). The effect of kinetin was subsequently confirmed for many moss species with or without a typical caulonema.

Studies on *F. hygrometrica* show that the transformation process from protonema to bud starts shortly after exogenous kinetin is applied to the culture. After three hours, the growth of the caulonematal side branches stop if they are shorter than 80 µm. The apical area of the filament then becomes dome shaped and new wall material moves from the very tip region to the side of the cell forming a round "bud initial" (Bopp, 1990). In most cases, moruloid buds are formed as a consequence of relatively high cytokinin concentration applications (Conrad et al., 1986; Bopp, 1984, 1990).

Saunders and Hepler (1981) used the two effects of cytokinin -- both specific and non-specific, to study the molecular mechanism of cytokinin action by using a fluorescent Ca $^{2+}$ chelate probe chlorotetracycline (CTC), which is a lipophilic chelate probe for divalent metals. They examined changes in the temporal and spatial localization of Ca^{2+} during cytokinin-induced cell division and bud formation in the gametophyte of *F. hygrometrica*. Twelve hours after cytokinin treatment a bulge was detected on the distal end of caulonematal cells. The nucleus migrated to the distal end between 17-22 hours and by twenty-four hours, the first asymmetrical mitosis occured cutting off a small lens-shaped initial cell from the original caulonematal cell. Subsequent divisions led to the formation of a tetrahedral apical cell and an apocarpic gametophore.

Saunders (1990) proposed the hypothesis that cytokinin directs, in part, the stimulation of Ca^{2+} uptake by responsive cells (cells which seem to have more binding sites for cytokinin as, for example, in *Funaria*'s caulonema cells). She also stated that the polarized response of cytoplasmic rearrangement and assymetrical divison which occurs in *Funaria* caulonema cells after cytokinin treatment is a direct result of localized cytokinin binding to the distal end of these cells. This may have direct results on specific ion channels leading to a spatially controlled change in intracellular Ca^{2+}. The receptor location on the "target" or "response" cells may be a general mechanism needed to establish subcellular positional information essential for plant morphogenesis (Saunders, 1990).

Christianson (1998) performed a number of experiments which contradicted Saunders proposed hypothesis that presumptive target cells were involved in bud formation. That is, the regulation of bud number occurs during initial perception of cytokinin. Christianson found that cytokinin concentration during inital perception did not control the number of buds formed. Only when bud initials became permanently committed as buds did the cytokinin concentration exert a quantitative effect on bud number. Thus per Christianson (1998) a massive initial response by many cells would be followed by a limiting of responding cells during the subsequent cytokinin-requiring "Brandes and Kende" event.

Reski and Abel (1985) studied the budding effect of the cytokinin N^6-(Δ^2-isopentenyl)-adenine (i^6-Ade) in *Physcomitrella patens* that was grown in liquid culture. Important to this study, Reski and Abel acknowledged the innate ability of *P. patens* in liquid culture to undergo normal morphogenesis -- that is normal protonema differentiation including the production of leafy gametophores. On this premise, they added exogenous i^6-Ade to the medium at various concentrations. At 10^{-7} M and 10^{-6} M chloronema cells were induced into budding. The buds produced at the 10^{-6} M were larger than those induced by the 10^{-7} M. At concentrations above the limit of 10^{-5}M i^6Ade, the chloronema cells no longer produce buds, but caulonematal cells are initiated. After which buds develop on these caulonematal cells. These experiments gave evidence to the ability of *P. patens* to form buds on chloronematal cells. This contradicted Bopp's assertion that only caulonemata contain target cells for exogenous cytokinins -- a suggestion supported by the discovery of caulonematal specific proteins (CSP) (Bopp, 1974).

Sood et al. (1978) observed CSP in two mutants of *P. patens* which do not produce buds without exogenous cytokinins added (Ashton and Cove, 1977; Reski and Abel, 1985). Reski and Abel used SDS-PAGE to examine the existence of CSP on *F. hygrometrica* and *P. patens*. They could not detect any evidence of CSP, and furthermore upon personal communication with Bopp found that the experiments originally done by Bopp and his coworkers could not be reproduced. Reski's experiments gave evidence that different concentrations of the cytokinin i^6-Ade may have different qualitative effects on protonema differentiation (Reski and Abel, 1985).

Researchers also had an interest in studying nucleic acid metabolism during cytokinin induced cellular differentiation (Schneider et al., 1969), using 4.65 mM concentrations of the cytokinin 6-furfurylaminopurine (kinetin) added to Kofler nutrient medium to induce bud differentiation from protonematal cells of *Funaria hygrometrica*. When compared to the protonematal cells, the total RNA content of kinetin-induced bud cells was nearly fifteen times higher. The same increase in total RNA was found in bud cells that spontaneously developed in older cultures (Schneider et al., 1969).

Phytohormones do not act just as a trigger for each developmental stage but their continued presence is a requirement for the whole differentiation process. Kinetin is very unstable in mosses. After a short incubation period of about one hour, products of side chain cleavage appear. In pulse-chase experiments with kinetin, the response disappears after removing the kinetin, whereas 2iP or BAP response remains unchanged even if the cytokinin is no longer available (Bopp et al., 1986).

As previously discussed, Lehnert and Bopp (1983) performed a series of experiments on

F. hygrometrica grown in low light intensity resulting in solely chloronematal production. Under these conditions both exogenous treatment of the auxins IAA, NAA, and 2,4D and the exogenous treatments of auxin precursors such as tryptophan, indopyruvic acid and indoleacetaldehyde induced the formation of caulonema. They also found that auxin must be applied before cytokinin to the moss culture in order for bud formation to occur. If kinetin is applied first and then removed before a subsequent auxin treatment no bud formation will occur. Lehnert and Bopp interpreted this as a sequential interaction where auxin creates the "target cells" for cytokinin. Though, in *Funaria*, auxin is a prerequisite to the formation of caulonema cells, which are the target cells for cytokinin and bind in order for bud formation to occur (Lehnert and Bopp, 1983), this may not be the case for *Physcomitrella patens* under certain culture conditions (Bhatla, 1994).

d. *Physcomitrella patens* Developmental Studies Using Mutations.

Studies of the roles played by phytohormones in protonematal and gametophore development using exogenous auxin and cytokinin, auxin precursors, and auxin antagonists have provided valuable information on their involvement in caulonematal and gametophore differentiation in mosses (Brandes and Kende, 1968; Bopp, 1968; Bopp and Jacob, 1986; Bhatla, 1994).

Additional studies using developmental mutants of mosses have given an insight into the regulation of response by the endogenous phytohormones auxin and cytokinin. Though studies on mutants of *Funaria* exist, this discussion will be limited to the *P. patens* developmental mutants, and the findings of the research of Neil W. Ashton, David J. Cove, and their various collaborators on *P. patens* development.

Ashton, Cove and their numerous collaborators have accrued much evidence through years of both physiological studies using wild-type *P. patens* and genetic studies using *P. patens* phytohormone sensitive and phytohormone resistant mutants. With the data from these studies, these researchers have analyzed the interrelated actions of phytohormones with light and have presented a comprehensive model of moss development from its chloronemata stage to the formation of leafy gametophytes.

Ashton et al. (1985) established that *P. patens* synthesized endogenous auxin. These studies also ascertained that *P. patens* possesses a saturable IAA influx carrier in the plasma membrane. This was determined by using ^{14}C -IAA and ^{3}H-IAA in centrifugation and filtrations assays. They determined that auxin accumulation by wild-type *P. patens* has the following characteristics:

1. It is pH dependent. Auxin accumulation is greatest when the pH of the incubation medium is low.
2. Auxin accumulation is markedly reduced in the presence of 2,5 dinitrophenol (DNP).
3. *Physcomitrella patens* plants have a saturable component . This means that the accumulation of labeled IAA is reduced by the presence of an excess of unlabelled IAA.
4. IAA accumulation is increased by the auxin transport inhibitor naphthlyephthalinic acid (NPA) (Ashton, 1987; Ashton et al., 1990, 1993; Ashton and Cove, 1990).

These results are consistent with the chemiosmotic model of auxin accumulation and transport described by Rubery and Sheldrake which has been discussed earler in this chapter, and has been suggested for some higher plants by Sussman and Goldsmith (Ashton et al., 1990). Ashton et al. also found evidence that there may be two types of auxin carriers in the plasma membrane in *P. patens*. One type may be an IAA anion/proton symport responsible for the influx of auxin. The second type of auxin carrier may be an uniport which results in the efflux of IAA anions. The uniport carrier also interacts with auxin transport inhibitors of which NPA is one example (Ashton et al., 1993).

Cove et al. (1978) studied the effect of light on the development of *P. patens*. For spore germination to occur, the spores must be wet and exposed to light, and germination begins between 24-36 hours after initial illumination. Protonema grown in darkness gave evidence of increased caulonemata production and greatly decreased chloronematal growth. No new chloronematal side branches were formed in the absence of light (Cove et al., 1978).

Initial chloronematal growth in protonema shows weak phototropic response but in about eight days, chloronemata are strongly phototropic. Caulonematal filaments grow at right angles in respect to the light direction at lower intensities (0.3-3.0 w/m^2). At higher intensities above 3.0 w/m^2 caulonemata grow at a angle away from the light source (Cove et al., 1978).

Gamteophyte growth is strongly phototropic and is influenced by gravity and light. Normal growth is observed in cultures grown in low white or red light intensities. When gametophores are transferred to darkness, they continue to grow. The growth of gametophytes in dark conditions is strongly geotropic. Etiolation occurs, and leaves are reduced in size to small scales. New gametophore production does not occur in the dark. These effects can be reversed by introducing low white light intensities (0.15 w/m^2) and short day lengths (45 minute white light (10w/m^2) and higher light intensities (>15 w/m^2)) (Cove et al., 1978).

Per Cove et al. (1978), these experiments provide evidence that phytochrome plays a role in the detection of light, which is necessary to prevent the dark-development of gametophores.

In summary light promotes spore germination, primary chloronematal development, secondary chloronemata development, and gametophore bud production. Light inhibits caulonemata development, and light must be a recognized integrated factor which interrelates with both auxin and cytokinin in controlling cell differentiation and *P. patens* development (Knight et al., 1988; Ashton et al., 1993).

Paulinius Engel (1968) was the first researcher to use *Physcomitrella patens* to obtain biochemical mutants, and provided an initial protocol by which *P. patens* complete life cycle could be studied under lab conditions. Mutations were induced by treatment of either spores or protonematal cells with ethyl methane sulfonate, N-methyl-N^1-nitro-N-nitroso-guanidine and x-rays (Engel, 1968). Methods, similar to the above, were later utilized by Ashton, Cove, and various collaborators (Ashton and Cove, 1977) to genetically analyze the phytohormone action and morphogenesis of *P. patens*. The isolation and study of developmental mutants of *P. patens* by conventional mutagenic procedures has corroborated related studies using antagonists to auxin and treatments of both auxin and cytokinin. These studies have also helped to reveal key developmental transitions within *P. patens* development (Ashton and Cove, 1977, 1990; Ashton et al., 1979a, 1979b, 1985, 1990, 1993; Grimsley, et al. 1979; Cove et al., 1980; Cove and Ashton, 1984, 1988; Knight et al., 1988; Featherstone et al., 1990).

Selected mutants with abnormal devlopment were classified 'resistant' or 'sensitive'. Resistant mutations were plants that were incapable of responding to the developmental effects of the phytohormone which permitted the identification of the mutant itself (Ashton et al., 1979b). For example NAA and BA leads to a marked decrease in chloronematal production in wild-type *P. patens*. Any mutant strain which does not respond to the effects of these phytohormones will be much greener when cultured in their presence, and thus the mutant is termed resistant (Cove et al., 1980).

In wild-type specimens of *P. patens*, the gross morphology of the moss is flat with normal sequence of producing primary chloronema, caulonemata, secondary chloronemata, and gametophores. Exogenous auxin treatments increase the production of caulonemata, decrease the production of secondary chloronemata, and have no effect on gametophore production. Exogenous cytokinin decreases caulonemata production, decreases secondary chloronemata production, but increases gametophore production in the wild-type *P. patens* (Cove et al., 1980; Ashton et al., 1993).

Sensitive mutants, when exposed to exogenous treatments of NAA or BA, respond to these hormonal effects, and proceed with normal development (Ashton et al., 1993).

Mutants which are resistant to the effects of both auxins and cytokinins are termed Category I mutants. These mutants grow into plants that are more or less spherical in shape and are made up entirely of chloronemata (Ashton et al, 1979b, 1993; Cove et al. 1980). This mutant may be caused due to a lack of response to auxin and cytokinin and are known as NAR mutants (Ashton et al. 1979a). Ashton et al. (1993) also found evidence that wild type *P. patens*, when grown on medium with NPA or TIBA, produce plants with similar morphology as the NAR mutants.

Two other categories of auxin resistant mutants have been described by Ashton et al. (1979b). They are the caregory 2 and 3 mutants.

Category 2 mutants are morphologically abnormal. They produce primary chloronema, caulonema normally, but subsequently produce abnormal amounts of secondary chloronema and no buds. Though they do no respond to exogenous treatments of even high concentrations of auxins, they do respond to cytokinin treatment. When cytokinin is added to cultures of category 2 mutants of *P. patens*, normal development is promoted. Less secondary chloronema is produced and ultimately leafy gametophores are formed (Ashton et al., 1979b; Cove et al., 1980).

Category 3 mutants are not morphologically abnormal and develop normally on minimal medium. Exogenous cytokinin has the same effect on these plants as it does on wild-types, but the mutant plant does not respond to auxin except at very high concentrations. Ashton et al. (1979a) proposed that though synthesis of endogenous auxin is normal, uptake of exogenous auxin is blocked (Ashton et al., 1979a).

Five categories of BAP resistant mutants known as BAR mutants have been identified by Ashton et al. (1979a, 1979b). They are termed category 4 – 8 mutants.

Category 4 mutants produce normal primary chloronemata, abnormal caulonemata, and produce more secondary chloronemata than wild-type plants. Exogenous auxin treatment restores normal caulonemata and increases caulonemata production. This treatment also restores normal gametophore development. There is no response to cytokinin (BAP) treatment. Apparently these mutants are unable to synthesize endogenous auxin (Ashton et al., 1979a, 1979b; Cove et al., 1980).

Category 5 mutants make more numerous chloronemata then the wild type, and slightly fewer caulonemata. These mutants make few or no gametophores. They are strongly resistant to BAP, though they may be repaired by exogenous NAA. Interestingly, exogenous 500 nM NAA added to cultures produce mutants which resemble the wildtype grown on minimal medium (Ashton et al., 1979a, 1979b; Cove et al., 1980). Ashton et al. (1979a) proposed that these mutants synthesize reduced amounts of auxin.

Category 6 mutants make many more chloronemata and slightly more caulonemata than the wild-type. Few or no gametophores are formed. These plants are strongly resistant to BAP, but are sensitive to NAA. and normal gametophore development may be restored to wild-type levels using 500 nM NAA (Ashton, 1979b).

Category 7 mutants make much more chloronemata but approximately the same number of caulonemata than the wild-type. Fewer gametophores (only producing 5-10% that of the wild-type numbers) are produced. The distinctions between chloronema and caulonema cells are not clearly defined as in the wild-type. These mutants are resistant to 5 nM - 50 µM BAP, and the protonema morphology is virtually unaffected with growth and slightly inhibited (Ashton et al., 1979b). 5-50 µm BAP either does not affect or only slightly affects gametophyte bud production. The gametophore buds are callus-like buds or clusters of callus-like buds. These mutants are sensitive to NAA and most BAR mutant gametophore production is partially repaired by NAA treatment. In BAR 77, 5-12.5 µm of NAA completely repairs the ability of this mutant to produce gametophores (Ashton et al., 1979b).

BAR 161 is the only existing category 8 mutant. This mutant produces more caulonemata and many more chloronemata than the wild type (Ashton et al., 1979b). Gametophore production is normal. The protonematal growth is strongly cytokinin resistant. Both wild type and BAR 161 strains show reduction of secondary chloronemata with BAP treatment. Ashton et al. (1979b) proposes that the cytokinin resistance of BAR 161 appears to originate from its increased chloronemata and caulonemata production. BAR 161 is NAA sensitive (Ashton et al., 1979b).

In addition to the production of NAR and BAR mutants, Ashton et al.(1979a) also isolated several phenotypically distinct groups of gametophore overproducing mutants called OVE mutants. The gametophores are slightly abnormal. In some gametophores, the stem is short or absent. The leaves are smaller than those of the wild-type cultured on minimal medium. The gametophyte growth rate is slower than that of the wild-type on minimal medium (Ashton et al., 1979a). Its development is similar to that of wild-type strains cultured in the presence of high level cytokinins (Ashton et al., 1979a, 1993; Cove et al., 1980). These mutants initiate several gametophores on each caulonema. Analysis of the OVE strains have shown that they produce more cytokinin than the wild-type. IPA has been identified as the major cytokinin in these strains, and though zeatin is also present, it is present at a lower concentration (Cove et al., 1980).

OVE mutants have been classified as categories 9 and 10. When both of the mutants are grown on minimal medium, they are phenotypically similar to the wild-type cultured on medium containing respectively a high and low concentration of exogenous cytokinin.

Ashton et al. (1979a) proposed that the OVE-mutant pheno-ypes may be a result of one of several causes. The most feasible explanation would be the over production of the normal endogenous cytokinin resulting from either an increase in the rate of its biosynthesis, or a reduction in the rate of cytokinin degradation. Evidence for this explanation may be found by the extraction of (N^6-(Δ^2-isopentenyl) adenine (IPA), a cytokinin found in mosses from the medium of OVE mutants (Ashton et al., 1979a) Also, wild type *P. patens* cultured on medium treated with exogenous cytokinin produce plants that are similar to OVE mutants (Ashton et al., 1979a). Figure 2.15 is a flow chart which summarizes the roles of the phytohormones, auxin and cytokinin, and their interaction with light in controlling cell differentiation in *P. patens* as discussed in this section. The model is based on evidence accrued through both previously presented physiological experiments, and genetic studies by Ashton, Cove and their collaborators using the aforementioned mutants.

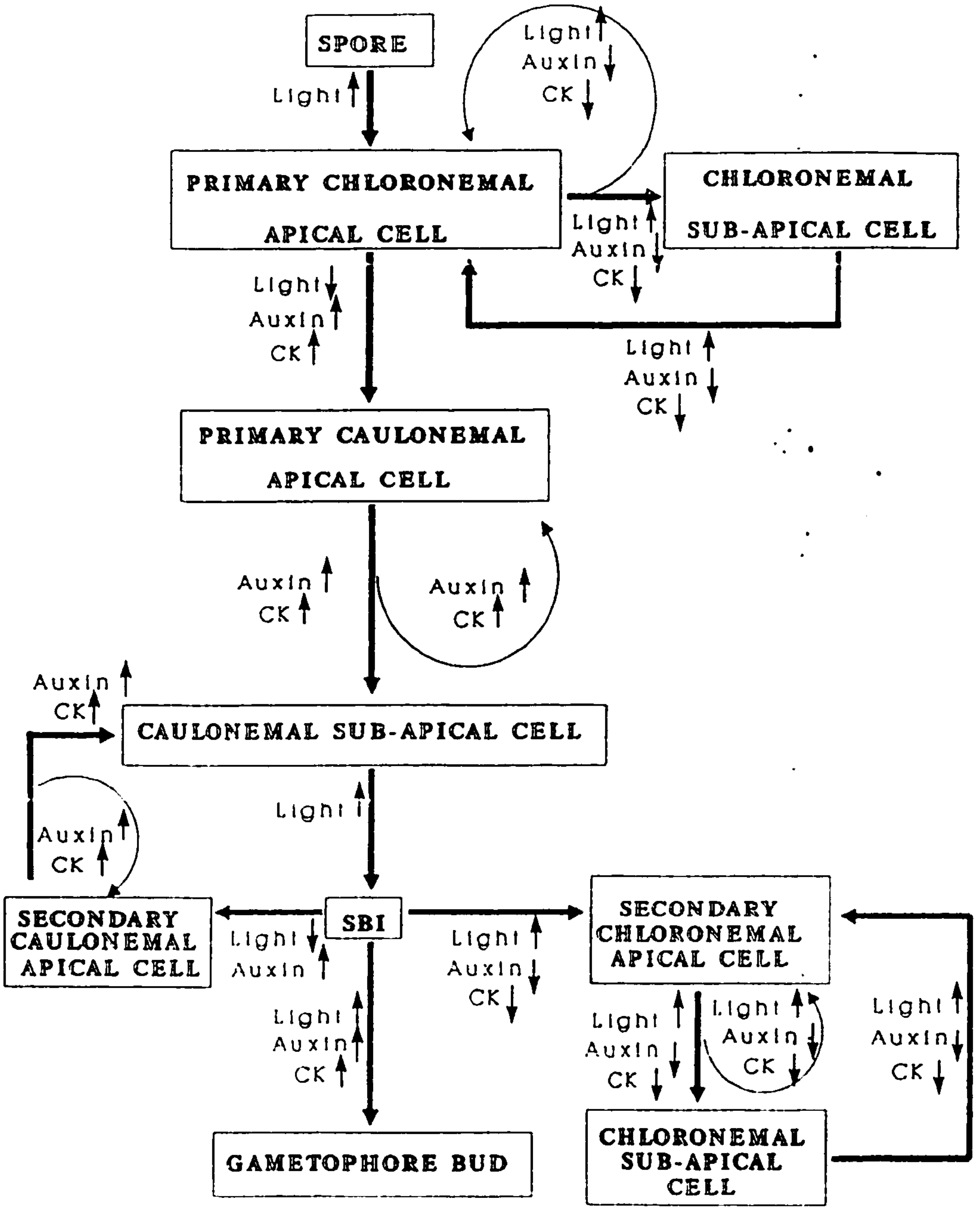


Figure 2.15. The flow chart of the elaborate model based on both physiological and genetic experiment depicting the roles of both auxin and cytokinin phytohormones, and their interaction with light in controlling the morphogenetic development of *P. patens* (from Ashton et al., 1993).
Key: CK= cytokinin,
SBI= side bud initial,
⇑= factor promotes the transition,
⇓ = factor inhibits the transition

Chapter 3: Antagonist Studies.

INTRODUCTION:

The role and control of the spatial-temporal suppression of cell proliferation and/or cell enlargement in plant morphogenesis was investigated. A prime example of this phenomenon is the suppressed development of every third leaf primordium formed in a large proportion of the species of leafy liverworts (Jungermanniales, Hepaticae). Through a series of experiments, it was found that the correlative inhibition of the development of these leaves past a primordial stage was regulated by the interrelated actions of auxin, ethylene, and certain arabinogalactan proteins (AGPs) (Basile 1990; Basile and Basile 1987, 1990, 1993, 1994). In addition, it became evident from experiments with species of *Streptocarpus*, a flowering plant (Rosenblum and Basile 1984, Rauh and Basile, 2000) that this correlative control system was not restricted to determining whether or not leaves developed in liverworts at a specific place and time. The results of over 25 years of research in this area, indicated that the auxin-ethylene-AGP system played a critical role in three important aspects of pattern formation and pattern change in leafy liverworts and probably all plants (Basile and Basile 1993). One, already mentioned, is to stop (suppress) the further development of specific organ primordia as soon as they are established. The failure of primordia to develop into mature organs is the basis for the recurrent trend in leafy liverwort families toward "reductive" or "recessive" evolution via the "loss" in the numbers (e.g. leaves) and/or kinds of parts (e.g., branching-types). A second is the modulation in the size and shapes of leaves. The third is the setting apart of the apical meristematic regions during the transition from a diffuse (e.g., protonematal, prothallial, or embryogenic) growth pattern to one that is strictly apical/polar. This transition from diffuse to polar/ apical growth is characteristic of the early stages of development of both gametophytes and sporophytes of all land plants (Basile and Basile, 1993). It is this third hypothesized role for suppression that the experiments reported here were designed to test.

For these experiments, a moss, *Physcomitrella patens* (Funariaceae), was chosen. During its relatively short life cycle, this small, easily cultured plant undergoes a series of distinct pattern changes. These pattern changes have proven to be amenable to both physiological and genetic manipulation and analysis (Engel, 1968; Ashton and Cove, 1977, 1987; Ashton et al., 1979a, 1979b, 1993; Cove and Ashton, 1984, 1988; Cove et al., 1978, 1980).

Vegetative development of *P. patens* normally proceeds through four distinct stages (Cove et al., 1980; Bhatla, 1994). The first, following spore germination, is termed the "primary chloronema stage." The cells of the branching uniseriate filaments that comprise the plant body at this stage are distinguished by their high chloroplast content and by their perpendicular cross walls. The second stage is termed the "caulonematal stage". This stage represents a somewhat more suppressed development in that the cells of the filaments formed during this stage are narrower than those of the chloronema, have noticeably less chloroplasts per cell, and are separated by oblique cross walls. The third or "secondary chloronematal stage" is distinguished from the primary chloronema by virtue of their origin as branches from caulonematal cells. More importantly, they are also distinguished by their greater tendency to give rise to the gametophore buds that mark the beginning of the fourth or "leafy gametophore stage" (Cove et al., 1978, 1980; Bhatla, 1994). According to my hypothesis, gametophore initiation is brought about by the suppression of protonematal growth. Gametophore bud development is the result of protonematal growth suppression that is brought about by the interrelated actions of auxin, ethylene and an hydroxyproline-containing protein, namely an AGP.

There is ample evidence that auxin and ethylene are naturally produced by moss protonema (Johri and Desai, 1973; Johri, 1978b). It is also well established that auxin is required before development of chloronemata can progress to gametophore bud initiation (Ashton et al. 1986, 1988, 1990; Ashton and Cove 1977, 1990; Cove and Ashton, 1988; Cove et al.; 1980; Lehnert and Bopp, 1983). Comparable data indicating an essential role for ethylene in gametophore bud induction is not yet available. Nevertheless, it is reported that an exogenous auxin and ACC can trigger ethylene production in at least one moss, as they can in a wide variety of plants (Rohwer and Bopp, 1985; Bopp, et al, 1986; Bhatla and Dhingra-Babbar, 1990).

AGPs, appear to be ubiquitous components of plants, and have been detected at the cell surfaces of representatives of most mosses (Mankiewicz and Basile, unpublished). There are early reports that the presence of exogenous hydroxyproline (Hyp) in culture media can dramatically prolong (i.e., prevent the timely suppression of) protonematal growth of several mosses (Burkholder, 1959) and one leafy liverwort species (Basile 1967). It was subsequently determined that exogenous Hyp can interfere with the normal synthesis and function of Hyp-proteins in general (Basile, 1980; Basile et al. 1987) and AGPs in particular (Basile and Basile 1987). This literature, although scattered and seemingly unrelated, nevertheless provides the earliest indication that all three of the components of the proposed correlative control system are naturally occurring in mosses and may be involved in mediating the transition from protonematal to leafy gametophore stages in mosses. The results of a series of experiments that support the proposition that the transition from diffuse growth to polar, apical growth is correlated with the suppression of protonematal growth and this suppression is regulated by the interrelated actions of auxin, ethylene and a hyp-protein, most probably an AGP is presented.

MATERIALS AND METHODS

Plant material. Axenic cultures of wild type *Physcomitrella patens* (Hedw.) Bruch and Schimp. derived from cultures kindly provided by John Wallace of Bucknell University were the source of inoculum for all experiments. Inoculum was prepared by homogenizing stock cultures of *P. patens* in a heat sterilized microblender head at 60 rpm for forty seconds. Ten drops (approximately = 1.00 ml) of the homogenized tissue was inoculated into culture vessels using sterile disposable transfer pipettes (Samco Sci. Inc. San Fernando, Ca.), in which 3 mm of the tip was removed to prevent the tissue from clogging the pipette.

Culture conditions. The basal nutrient medium used for both stock and in all experiments employing antagonists consisted of aqueous Knop macronutients supplemented with Hutner's "metals 49", micronutrients, and1% w/v glucose. Formulae for the macro- and micronutrients used are given in Basile (1978). The pH of the media was adjusted to 4.6 and 30 ml aliquots were delivered to Sigma culture ("baby food") jars and closed with Magenta caps (Sigma) before being heat sterilized for 20 min. @ 15 psi. Cultures were incubated on lighted shelves in rooms thermostatically controlled to maintain a temperature of 18 °C (with continuous illumination of 700 - 1000 lux from cool white fluorescent bulbs). The incubation period for each of the experiments employing antagonists was 6 weeks.

Antagonists. Five antagonists were individually tested for their capacity to prevent subapical growth suppression. Two, 2,3,5 triiodobenzoic acid (TIBA) (ICN Biochemicals) and2[(1-Naphthalenyl-amino carbonyl] benzoic acid (ALANAP) (a gift from the Uniroyal Co. Inc., Naugatuck, Conn.), were auxin transport antagonists, one, Aminoethoxyvinyl-glycine (AVG) (Sigma) is a specific antagonists of ethylene synthesis , and two, 3,4-dehydroproline (3,4D) (Sigma) and trans 4-hydroxy-L-proline (Hyp or Hypro) (Calbiochem) were antagonists of normal hyp-protein synthesis. All stock solutions of antagonists were cold-sterilized through 0.2 (µm) filters and added to the sterile basal medium prior to inoculation. As a result of pilot experiments, the concentrations of antagonists that were found to be effective were ALANAP, 8 µg /ml; TIBA, 11 µg /ml; AVG, 0.90 µg /ml ; 3,4-D. 8µg/ ml ; and Hyp12 µg /ml. Thirty-five (35) replicates were prepared for each treatment, and the experiments were repeated three times.

Microscopy. The tissue from each culture vessel was then harvested, and carefully examined for the presence of gametophore bud formation. Representative material from each experiment was fixed for one hour in 2% v/v gluteraldehyde in fresh basal nutrient media. The fixed tissue was then rinsed three times with cacodylate buffer. The tissue destined for light microscopy studies was prepared by being placed in 50%v/v aqueous glycerol overnight, and then transferred to 100% glycerol solution. Semipermanent slides were then made of representative material from each experimental treatment and labeled for the subsequent light microscopy studies. Photomicrographs were taken with Kodakchrome film with an Olympus OM4 camera fitted to a Zeiss Axiovert microscope. The tissue that was to be used in SEM studies was fixed in 2% v/v gluteraldehyde with fresh basal nutrient medium for one hour, was rinsed as above with cocodylate buffer. Washed material was dehydrated in a graded alcohol series, critical-point dried, mounted on copper studs, and gold coated (Sicko and Jensen, 1973). SEM micrographs were taken with Amray 1830 at 20 KV.

Preparation of AGP Extracts. AGPs were extracted from equal amounts of lyphilized samples (1.0 gram) of both suppressed (control) and desuppressed (antagonist treatment) tissue of *P. patens*. The tissue samples were pulverized after freezing with liquid nitrogen, transferred to 50 cc centrifuge tubes with 10 mls. of Barbital Buffer (Sigma Diagnostics # B6632 modified by D.V. Basile (unpublished) (0.5M $CaCl_2$(Calcium chloride): 0.02% sodium azide w/v), and 15 drops 1%

CHAPS. The extraction mixture was vortexed (SMI Multi-tube vortexer) for thirty minutes, and centrifuged at 15,000 RPM for 30 minutes. The supernatant was decanted into another 50 cc centrifuge tube, micro-waved to the boiling point, and cooled. The pellet resulting from the centrifugation was resuspended in 10 ml. of fresh modified barbital buffer, and 15 drops of fresh 1% CHAPS, and the above extraction process repeated. The cooled original supernatant and the resuspended pellet were then centrifuged at 15,000 RPM. The original supernatant was transferred to Centriprep 30 tubes (Amicon) where it was spun at 3500 RPM. The supernatant resulting from the second extraction is microwaved, cooled, and then recentrifuged at 15,000 RPM. The supernatants from the first and second extraction were pooled and desalted, using Centriprep 30 tubes which were spun at 3500 RPM until concentrated to 0.5 ml. volume. The concentrates were then filter- centrifuged through nylon membranes to remove any remaining particulates.

Radial diffusion assay. The resulting protein extract was identified as an AGP and quantitatively measured by the radial diffusion assay of Van Holst and Clarke (1985). This assay is prepared using 30 ul Yariv's reagent/ml. of 1% w/v agarose gel. Yariv's reagent (B-Glc) is a universal indicator of AGP (Yariv et al., 1962, 1967).

RESULTS:

With the culture conditions, gametophore bud formation by *P. patens* was well underway in control cultures after five weeks incubation (Fig. 3.1). Leafy gametophores were evident in all control cultures by the end of the six-week incubation period (Fig. 3.2). In sharp contrast and without exception, cultures in which the basal nutrient medium was supplemented with any one of the antagonists remained in a purely protonematal pattern of development during this period

(Figures 3.3 A, B, C, D, E). In fact, antagonist-treated cultures were never observed to procede beyond the primary chloronematal stage during the experimental incubation period. Due to the very small dry-weight mass of the tissues produced in individual cultures in six weeks, no attempt was made at a quantitative comparison of growth among and/or between the replicate cultures of each of the several experiments. Suffice it to say that no obvious growth inhibitory effects on the protonemata treated with any of the antagonists at the concentrations used. If any difference, growth of the protonematal tufts seemed more luxuriant in the presence of the antagonists than in the controls. It seems reasonable to conclude from these results that each one of the molecules being "antagonized" has some important role in bringing about the transition from diffusely proliferating, branching filaments to locally (apically) proliferating, unbranched leafy shoots.

Figure 3.1 Four week cultures of *P. patens*. Note bud formation on protonema (LM – Zeiss, 781x)

Figure 3.2: *P. patens* from six week old culture (Control). Note primordia formed from gametophore buds (SEM 352x).

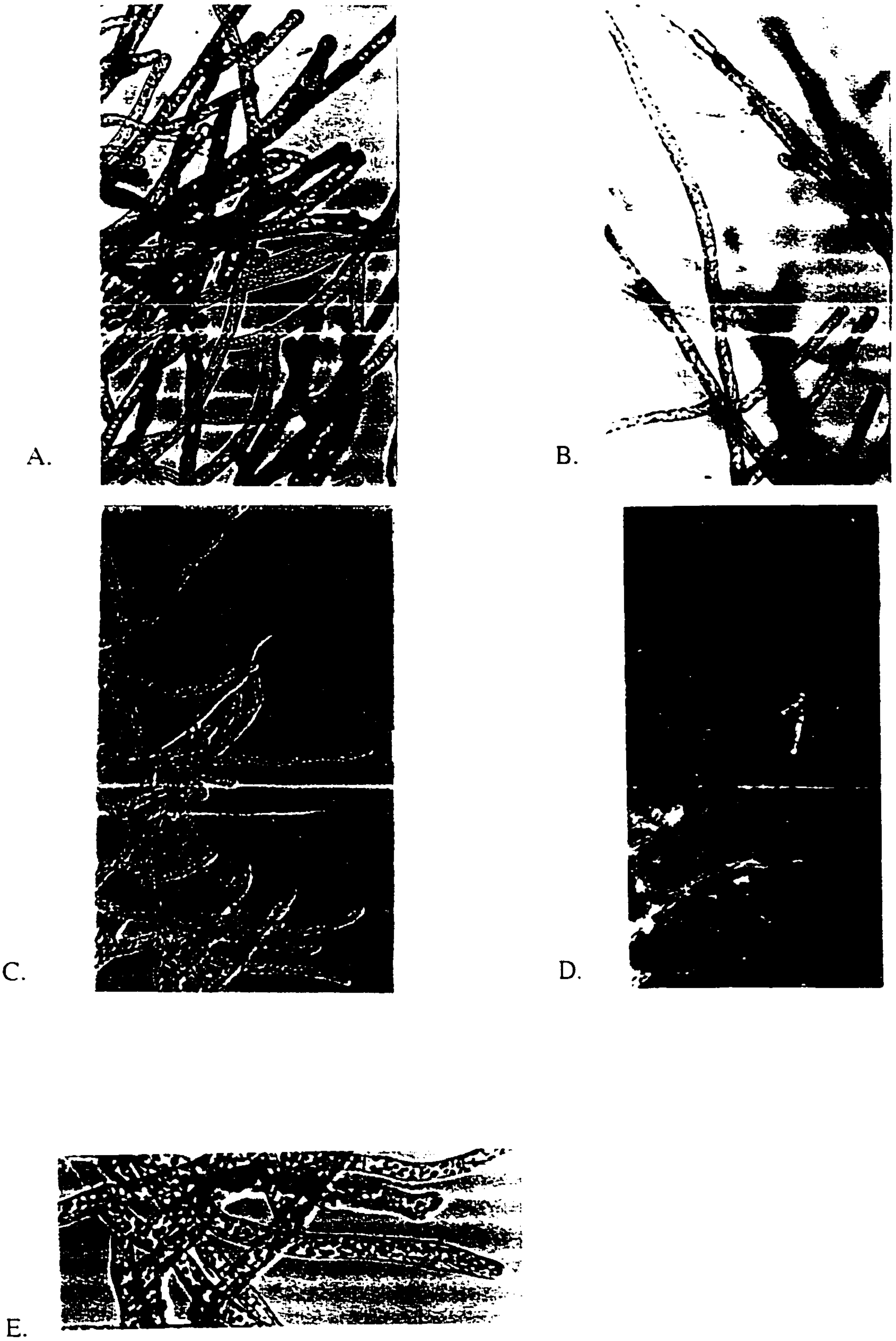


Figure 3.3 A-E: Antagonist treatments of P. patens. Note the filamentous growth, and that all cells are chloronemata. A. ALANAP treatment(430x); B. TIBA treatment(400x); C. AVG treatment (350x); D. 3,4D treatment (330x); E. Hyp (420x)

Radial diffusion assay results clearly show the presence of AGPs in both the control and antagonists. The concentration of the desuppressed (antagonistically treated) tissue showed fluctuations in concentrations when compared to the control (Figure 3.4).

DISCUSSION:

How auxin, ethylene and hyp-protein individually function to bring about this growth pattern transition from a diffuse, branching filamentous body to an apically growing parenchymatous one(s) is beyond present understanding. It seems clear, however, that each of these components plays a role in bringing about suppression since antagonizing any of them has the same results: cell proliferation continues throughout the developing protonema. By extrapolation, the establishment of a mechanism to suppress growth throughout most of the developing plant body is prerequisite to establishing highly localized growth centers (apical cells).

Ashton, Cove and their collaborators have produced strong evidence that auxin is an important component of moss development. In order for chloronemata to form caulonemata, auxin must be present (Ashton et al., 1979b). NAR mutants that are classified category I, grow into plants that are spherical in shape and are made up entirely of primary chloronemata (Ashton et al, 1979b; 1993; Cove et al., 1980). These mutants are resistant to the effects of both exogenous auxin and exogenous cytokinin treatments, and Ashton et al. (1979a) postulated that this mutation may be caused by an inability to respond to auxin.

The research of Ashton, Cove, and collaborators did not provide any evidence that auxin requirement for gamteophore production was related to the capacity of auxin to induce ethylene. It is well established that auxin can trigger ethylene synthesis. Furthermore, Rowher and Bopp (1985) found that auxin induces ethylene in a related moss, *Funaria*, and that ACC, and ethylene precursor, stimulates gametophore production.

Lehnert and Bopp (1983) experiments also provided strong evidence of the need of auxin's presence for gametophore bud formation to take place. They reported that auxin must be applied to the protonema culture before cytokinin in order for bud formation to occur.

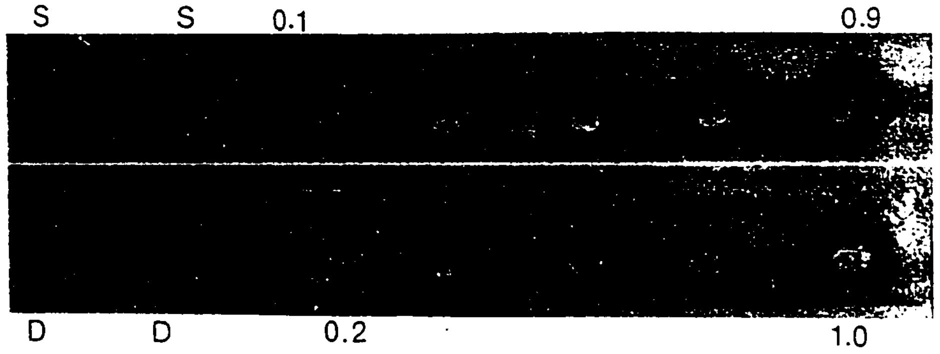


Figure 3.4 AGP radial diffusion assay of the suppressed (S) Control tissue vs. desuppressed (D) [illegible] treated tissue that remains in the protonemal stage. Numbers (mg/ml) of AGPs are given [illegible]. Note that AGPs are found in each tissue type.

These physiological experiments reported herein, provide important insights as to how *P. patens* progresses in a stepwise development from its most desuppressed chloronemata state to its transitional stage of suppressed polar apical meristematic growth.

No matter how the polar transport of auxin is arrested, whether due to the moss being unable to respond to auxin, if auxin is subject to rapid turnover leaving its physiological concentration ineffective; or polar transport is inhibited by specific auxin antagonists, the result is always constant. The plant's development is arrested in its most desuppressed stage -- that is, the chloronemata stage.

If any one component of this correlative system is blocked and cannot function, development continues in a pattern of cell proliferation, that is, the chloronemata level (fig. 3.5 flow chart). Thus through these series of experiments, using *P. patens* as a model system, evidence has been obtained that auxin, ethylene, and hydroxyproline proteins comprise a correlative control system that results in a transition from diffuse growth to strictly polar apical growth by setting apart the apical meristem.

The results of experiences using antagonists provide indirect evidence that auxin, ethylene, and AGPs have an interrelated role in suppressing cell proliferation of chloronematal cells thereby mediating the transition from diffuse to polar growth. The results obtained with auxin and ethylene antagonists complement and corroborate the findings that exogenously supplied auxin and ethylene precursors stimulated a similar transition (Rohwer and Bopp, 1985). The only evidence that hydroxyproline proteins may play a role in this transition were reports by Burkholder (1959) that exogenously supplied hydroxyproline prolonged the protonematal growth phase of *Atrichum undulatum* and *Sphagnum squarrosum*, because exogenously hydroxyproline can interfere with the normal synthesis of proline-hyroxyproline proteins.

Studies with *Funaria* and *P. patens* have provided evidence that raised auxin levels were necessary for the transition from a diffuse pattern of cell proliferation to a localized, meristematic pattern. Prior to the studies reported herein, a role of ethylene in this pattern change was only obtained in experiments with *Funaria.*

The experiments in which antagonists of auxin and ethylene were provided, *P. patens* cultures corroborate the regulatory roles for these phytohormones. In addition, experiments in which antagonists of hydroxyproline-protein synthesis were introduced into *P. patens* culture also provided evidence of the role AGPs may play as a morphoregulator.

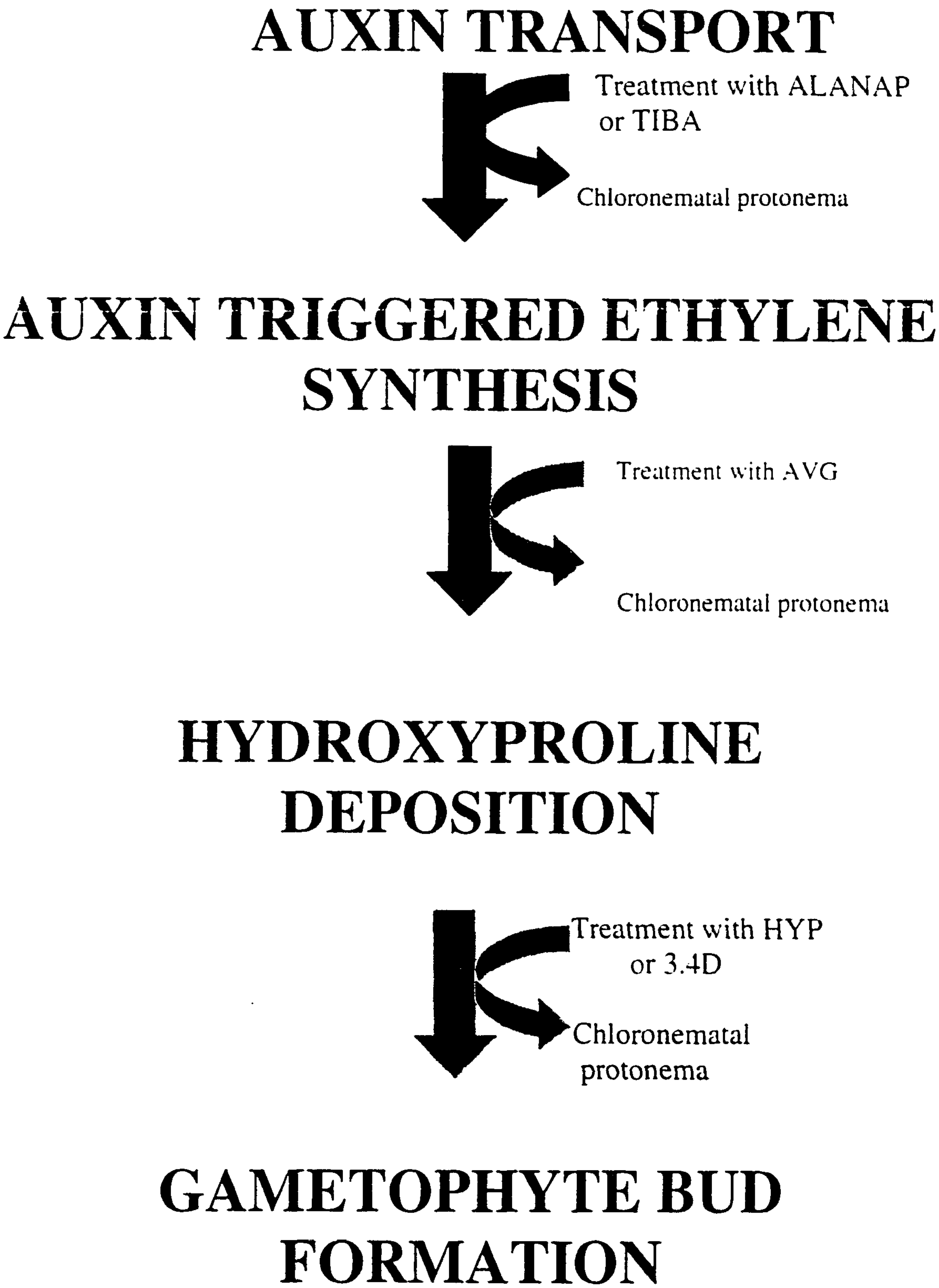


Figure 3.5: Flow chart depicting the mechanisms of the correlative control system. If any one component of this system is blocked then suppressed development is arrested to the most desuppressed level, that is, the chloronematal level.

Chapter 4: Biochemical Studies.

Introduction:

In the previous chapter experimental evidence as to the role that the correlative control system of auxin, ethylene, and arabinogalactan-proteins (AGPs) plays in the early protonematal development and transition from juvenile diffuse growth to gametophore apical mersitematic growth of *P. patens* was presented. These experiments corroborated evidence obtained by others that auxin and ethylene played some role in mediating the transition from protonematal growth to that of gametophore bud development. Using radial diffusion assays (Van Holst and Clarke, 1985), proteins extracted from suppressed (gametophores) and desuppressed (chloronemata) tissue was identified as AGPs.

With the establishment of AGPs being identified as an extracted protein of the moss *P. patens*, the biochemical studies focused on comparing the AGPs of suppressed tissue and AGPs of desuppressed tissue. Recent investigators have shown much interest in studying AGP's possible role as a morphoregulator. Experimental evidence exists that AGPs may coordinate developmental processes as information-bearing molecules (Knox, 1995). The influences of AGPs on embryogenesis was presented by Kreuger and Van Holst (1993). When Yariv's reagent was applied to suspended culture medium of rose cells, a total growth inhbition of these cells occurred (Serpe and Nothnagel, 1995). Yariv's reagent (B-D-Glc)$_3$ is known to bind and precipitate AGPS, and thus by binding the AGPs, blocked the ability of the rose cells to continue the gorowth process (Serpe and Nothnagel, 1995).

Studies by the researchers at the John Innes Center in England have provided much evidence of AGPs role as a possible morphoregulator. The use of a series of monoclonal antibodies have identified specific membrane-associated AGP epitopes to be involved in early stages of plant development. One such epitope J4e has been identified by the monoclonal antibody Jim-4. The J4e expression was associated with provascular tissue in the future hypocotyl and then in the coytledons of *Daucus carota*, L. (Stacey et al., 1990).

Some features of the AGPs present in mature gametophytes (the most suppressed state of *P. patens* development) are different than the AGPs found in chloronemata (the most desuppressed state of *P.patens* development), and therefore one may expect a comparative electrophoresis with clear differences in the protein mobility pattern. If so, then one may extrapolate (infer) the possible morphoregulatory role that AGPS play in *P. patens* development. Basile and Basile (1993) studies on *Gymnocolea inflata* have provided such evidence in electrophoretic comparative studies of normal "suppressed" pattern of leaf and branch development and "desuppresssed" pattern (the phenovariant) (Basile and Basile, 1993, 1994).

Methods and Materials:

Plant material. Two sources of plant materials were used. Axenic cultures of wild type *Physcomitrella patens* (Hedw.) Bruch and Schimp. derived from cultures kindly provided by John Wallace of Bucknell University, Pennsylvania were the source of inoculum for the first comparative series of electrophoretic studies, and for the histological assays performed. Axenic cultures of NAR 91 (auxin insensitive mutant) and OVE 102 pab (bud over-producing mutant) were kindly provided by Neil Ashton from Regina University, Canada, were used for the second comparative series of electrophoretic studies. Inoculum was prepared by homogenizing stock cultures of *P. patens* in a heat sterilized micro-blender head at 60 rpm for forty seconds. Ten drops (approximately = 1.00 ml) of the homogenized tissue was inoculated into culture vessels using sterile disposable transfer pipettes (Samco Sci. Inc, San Fernando. Ca.), from which 3 mm of the tip was removed to prevent the tissue from clogging the pipette.

NAR 91 is a category I mutant which is auxin insensitive, and develops only to the chloronemata stage. OVE Bud 102 Pab is a *P. patens* mutant which overproduces gametophore buds (Ashton et al., 1993).

Culture Conditions

1. **Culture in Knops Medium**. The basal nutrient medium used for all stock cultures and in all experimental controls consisted of aqueous Knop macronutrients supplemented with Hutner's "metals 49" micronutrients, and 1% w/v glucose. Knop medium promotes gametophore growth.
2. **Culture in Knudsens medium**. After the results of the physiological experiments using antagonists were confirmed, a quicker mode of producing protonematal tissues was tested. The tissue was homogenized as above and the tissue was placed in Knudsen medium which promotes protonematal growth. The Basiles observed that *Gymnocolea inflata* maintained its normal "suppressed" pattern of leaf and branch development when cultured on ammonium free nutrient medium (Knop), and a "desuppressed" pattern on ammonium ion-containing medium " (Knudsen) (Basile and Basile, 1980). Knudsen medium was observed to promote short term protonematal (desuppressed) growth in *Physcomitrella patens*, and thus, the tissue was grown for fourteen days in Knudsen and then harvested.

Formulae for both Knop and Knudsen macro and micronutrients are given in Basile (1978), and the basic stock formulae are provided in the appendix. The pH of the above media was adjusted to 4.6 and 30 ml aliquots were delivered to Sigma culture ("baby food") jars and closed with Magenta caps (Sigma) before being heat sterilized for twenty minutes at 15 psi. Cultures were incubated on lighted shelves in rooms thermostatically controlled to maintain a temperature of 18°C ± (with continuous illumnination of 700-1000 lux from cool white fluorescent light bulbs). The incubation period for the desuppressed tisssue (tissue that was made up of solely chloronemata) grown in Knudsens was 14 days and for the most suppressed tissues (that of mature gametophores) was 12 weeks. These tissues were freeze-dried and put aside for electrophoretic studies.

3. **Culture of Mutant *Physcomitrella patens*.** The basal medium was prepared according to Ashton et al. (1979 a, b). The pH was adjusted to 5.6 and sterilized in Erhlenmeyer flasks for 20 minutes at 15 psi. The medium was poured into sterile petri dishes which when cooled were each covered by a heat sterilized cellophane disk. The cellophane disks were kindly provided by Neil W. Ashton of Regina Universty, Saskatchwen, Canada.

Microscopy. The tissue from each culture vessel was then harvested, and carefully examined for the presence of gametophore bud formation. Representative material from each experiment was fixed for one hour in 2% v/v gluteraldehyde in fresh basal nutrient media. The fixed tissue is then rinsed three times with cacodylate buffer. The tissue destined for light microscopy studies was prepared by being placed in 50%v/v aqueous glycerol overnight, and then transferred to 100% glycerol solution. Semipermanent slides were then made of representative material from each experimental treatment and labeled for the subsequent light microscopy studies. Photo micrographs were taken with Kodachrome film with an Olympus OM4 camera fitted to a Zeiss Axiovert microscope. The tissue that was to be used in SEM studies was fixed in 2% v/v gluteraldehyde with fresh basal nutrient medium for one hour and was rinsed as above with cocodylate buffer. Washed material was dehydrated in a graded alcohol series, critical-point dried, mounted on copper studs, and gold coated (Sicko and Jensen, 1973). SEM micrographs were taken with Amray 1830 at 20 KV.

Histological Assays. The distribution of AGPs during different stages of development was determined. Whole samples containing both suppressed (gametophore buds) and desuppressed tissue (protonema) were cleared in 80% ethanol, and then stained with Yariv's reagent a known and universally accepted indicator of AGPs (Yariv et al., 1962, 1967).

Preparation of AGP Extracts. AGPs were extracted from equal amounts of lyophilized samples (1.0 gram) of suppressed, transitional, desuppressed, NAR 91 mutants, and OVE 102 Pab mutants of *P. patens*. The tissue samples were pulverized after freezing with liquid nitrogen, transferred to a 50 cc centrifuge tube with 10 mls. of barbital buffer (Sigma Diagnostics # B6632, modified according to D. V. Basile (unpublished) [10 mM Barbital Buffer, 0.1M $CaCl_2$, and .02% NaN_3 (sodium azide)] and 15 drops of 1% CHAPS. The extraction mixture was vortexed (SMI multi-tube vortexer) for 30 minutes, and centrifuged at 15,000 RPM for 30 minutes. The supernatant was decanted into another 50cc centrifuge tube, microwaved to the boiling point, and cooled. The pellet resulting from the centrifugation was resuspended in 10 ml. of fresh modified barbital buffer, and 15 drops of fresh 1% CHAPS and the above extraction process repreated. The cooled original supernatant and the resuspended pellet were then centrifuged at 15,000 RPM. The original supernatant was transferred to Centripep 30 tubes (Amicon) where it was spun at 3500 RPM. The supernatant resulting from the second extraction was microwaved, cooled, and then recentrifuged at 15,000 RPM. The supernatant was then added to the appropriate Centriprep 30 tube, and spun at 3500 RPM until concentrated to 0.5 ml. volume. The concentrates were then filter centrifuged through nylon membranes to remove any remaining particulates.

Bioassay. The concentration of the final protein extract was quantitatively determined by the radial diffusion assay of Van Holst and Clarke (1985). This assay is prepared using 30 µl Yariv's reagent/ml. of a 1% w/v agarose gel. In order to obtain equal concentrations of AGP, so that the concentration of the final AGP extract used for electrophoretic studies be a constant 1.0 mg/ml concentration of AGPs, proper dilutions were made on samples whose assays measured more than

1.0 mg/ml. In order to obtain a concentration of 1.0 mg./ml. of AGPs from the desuppressed tissue, the above protein extraction was performed three times. The resulting extracts were then pooled, and concentrated to approximately 0.5 ml. Again, the concentration of the resulting extract was measured by the radial diffusion assay of Van Holst and Clarke (1985).

Electrophoresis. A 1.6% agaorse gel was prepared using a glyoxyl agarose (NuFix ™ glyoxyl agarose FMC Bioproducts, Rockland, ME, U. S. A.) using a framed template per Basile et al. (1989). The pooled protein extracts were then diluted and assayed so that equivalent values would be placed on the gel. 20 µl of the pooled protein samples were placed in the wells, as with 1.0 mg/ml gum arabic samples (obtained from Sigma, St. Louis, MO., U. S. A.) that were used as standards. The outermost wells on each end of the gel, contained 20 µl of bromophenol blue which was used as the dye front.

The gel was run at 500 volts, 15 mamps, for approximately five hours or until the dye front reached 6 cm. from the line of origin.

The gel was immersed in 0.08M cyanoborohydride for a minimum of two hours to effect crosslinking of the AGPs to the gel. Excess cyanoborohydride reagent was removed by washing the gel in running tap water for two hours. The gel was then placed in a heat-sealable plastic food bag containing Yariv's ß-glucosyl reagent (30 µl/ml) in 50 ml. of 0.15 M NaCl solution (Basile et al., 1989). The bags were sealed and the gels were incubated in the stain for approximately three to four days.

Results.

When the results of the physiological experiments using antagonists, as discussed in the previous chapter, were confirmed, a quicker mode of producing protonematal tissues was tested. As per the results of the Basile investigations on *Gymnocolea inflata* (Basile and Basile, 1980), *Physcomitrella patens* protonematal stage was extended when cultured in Knudsen's medium. The tissue proved to be as luxuriant in growth using Knudsens medium as it was in the antagonists. Figure 4.1A shows tissue grown on Knops for twelve weeks. Note the full development of the leaf, stem, and rhizoids of *P. patens* at the stage of mature gametophore. Figure 4.1B. shows tissue grown on Knudsen's for two weeks. Note the healthy and extensive filamentous growth of *P. patens* at this juvenile stage.

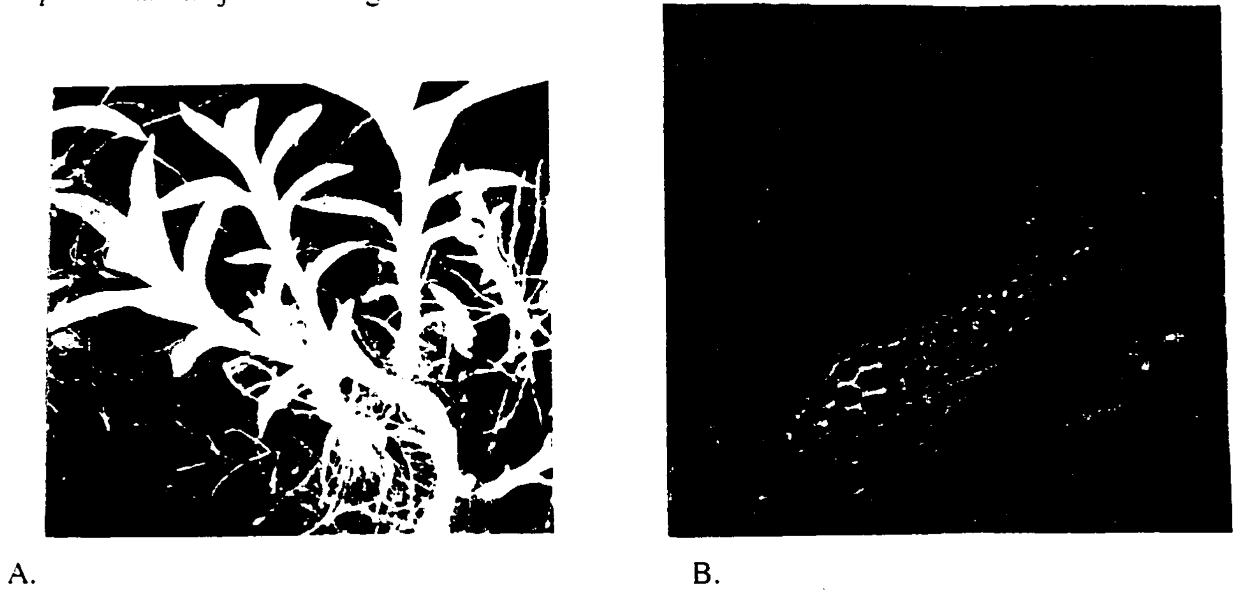


Figure 4.1 A. A. *P. patens* 12 week old cultures grown in Knops medium . Note the full development of leaves, stems and rhizoids. **B**. *P. patens* 14 day old cultures grown in Knudsen's medium. Note the healthy and extensive filamentous growth of *P. patens* at this juvenile stage.

Radial diffusion assays provided evidence of a significant difference in the amount of AGPs extracted from both the gametophore and protonematal tissue. Approximately fifteen times more AGPs were produced by the suppressed leafy gametophores than was produced by the desuppressed protonema. This is exemplified by figure 4.2.

Figure 4.2. Radial diffusion assay of AGPs extracted from Knops grown tissue (Suppressed =S) versus Knudsens grown tissue (Desuppressed =D). It was estimated that there was approximately fifteen times more AGPs produced by the suppressed tissue when compared to the desuppressed tissue.

Figure 4.3 Comparative electrophoresis of AGPs extracted from suppressed (S) and desuppressed (D) *P. patens* tissue. Note the clear difference in electrophoretic patterns between the AGPS of the suppressed tissue samples and the AGPs of the desuppressed tissue samples.

Figure 4.3 shows that when AGPs that were extracted from plants cultured on two different types of media (Knops producing gametophores or suppressed tissue and Knudsen's producing protonema or desuppressed tissue) were compared by agarose gel electrophoresis, a clear difference in the electrophoretic patterns was produced by the two samples. In the suppressed tissue, the bulk of the AGPs were to be found between 2 cm. and 3 cm. from the origin. In the desuppressed tissue, the bulk of the AGPs were found between the origin and two centimeters distance.

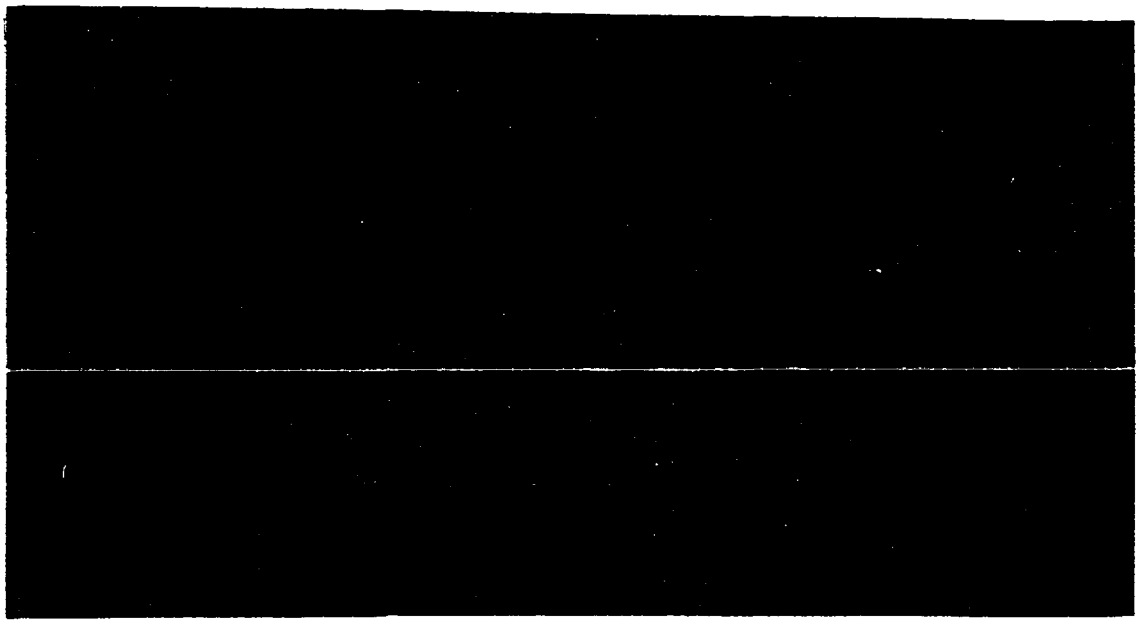


Figure 4.4: Comparative electrophoresis of suppressed(S), desuppressed(D), Nar 91 (N), and Ove bud 102 Pab (O) tissue samples. Note the similar electrophoretic patterns between Nar 91 and desuppressed (Kn grown) tissue. Also note how Ove bud includes the protein profile of both desuppressed and suppressed tissue AGPs.

When comparative electrophoresis on P. patens tissue was performed using AGPs extracted from the above plants (Knops medium grown 12 weeks and Knudsen medium grown 14 days), and AGPs extracted from the Nar 91 and Ove bud 102 Pab mutants, an interesting pattern appeared. As seen from figure 4.4, the suppressed tissue (Knops grown) AGPs were concentrated between 2 cm. and 3 cm. from the origin. The desuppressed tissue AGPs (Knudsen grown) were found between the origin and two centimeters. Nar 91 provided a similar profile as the Knudsen grown tissue (desuppressed), but OveBud 102 Pab which is a mutant that is made up of gametophore buds on protonema, demonstrated a profile that combine the profile of the suppressed and desuppressed tissue.

The tissue samples stained with Yariv's reagent exhibited a definite staining of the gametophore, and some staining in the protonema near the gametophore region. Protonema located distally from the gametophore, demonstrated no positive reaction with the Yariv's reagent (Figure 4.5).

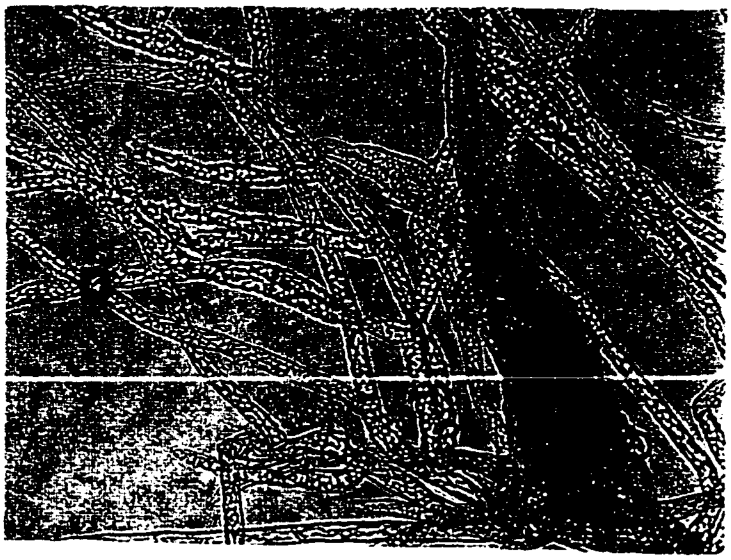


Figure 4.5. Yariv staining of tissue samples containing gametophore bud an surrounding protonema. Note that there is a definite staining of the gametophore (S), and the protonema near the gametophore region produces some evidence of staining. Protonema distally located (D) from the bud demonstrate no positive reaction with Yariv's reagent.

Discussion.

The possible morphoregulatory role played by AGPs in plant development is of current interest. Van Holst and Clarke (1985) used cross-electrophoresis to show that organ specific AGPs existed in *Lycopersicum peruvianum* (Mill). Later studies (Basile and Basile 1993, 1994) using glyoxyl agarose gels stained with Yariv's reagent provided evidence of different electrophoretic patterns between suppressed (control) tissue and desuppressed tissue (phenovariants) of *Gymnocolea inflata*. Studies of developing stigma and style AGPS of *Nicotiana alata* indicated that during the developmental process AGPs change in their charge characteristics during flower development, thus providing different electrophoretic patterns for each developmental stage (Gell et al., 1986). These findings demonstrated that AGPs may be involved in the specific expression of tissue identity (Gell et al., 1986).

Physcomitrella patens is an excellent model by which development can be studied. It is a small plant with few cell types. The stepwise development of *P. patens* from its chloronemata stage to the gametophore bud stage has been studied and both genetic and phytohormonal analysis has been accomplished (Ashton and Cove, 1977; Ashton et al., 1979a, 1979b, 1985, 1988, 1990, 1993; Cove and Ashton, 1984, 1988; Cove et al., 1978, 1980).

The biochemical studies have provided evidence that an AGP may be a morphoregulator and the chloronemata is identified by an organ specific AGP that differs from the mature gametophore tissue. The fact that the suppressed tissue possessed an AGP electrophoretic pattern that does not contain the AGP at or near the origin signified that this specific AGP ceases to be produced by the mature gametophyte. It is at this stage of growth when the protonema stage no longer persists, and the existing AGP is organ specific for suppressed growth (gametophytic growth).

Chapter 5: The Effects of 1 aminocyclopropane-1-carboxylic acid (ACC) and 1-naphthaleneacetic acid (NAA) on *Physcomitrella patens* Bud Development.

INTRODUCTION:

In chapters three and four, I have presented evidence that auxin, ethylene and arabinogalactan proteins are a correlative control system that together regulates the transition of *P. patens* protonema to mature leafy gametophores.

In chapter three, when antagonists to any one of the correlative control components were added to the culture medium, it resulted in the tissue remaining in its desuppressed state. When desuppressed (protonematal) tissue is compared to that of suppressed (mature gametophore) tissue, a distinct AGP eletrophoretic pattern is observed as documented in chapter four. In this chapter, I will present studies on the effects of ACC and NAA on *P. patens* bud development. I hypothesized that if auxin or ethylene truly influenced the transitional development of *P. patens*, then in a study in which auxin and ACC were added in specified concentrations to the culture medium, a greater number of buds should be produced within a given period when compared to the control.

As discussed in the review section of this thesis, when cultures of *F. hygrometrica* were treated with IAA or NAA the caulonematal stage of development was promoted (Johri and Desai, 1973; Johri, M., 1974; Lehnert and Bopp, 1983; Johri and D'Souza, 1990). Further studies provided evidence that under control culture conditions only chloronemata growth was promoted under low light conditions, the addition of auxin to media promoted caulonemata production (Lehnert and Bopp, 1983).

When cultures were exposed to PCIB, an antagonist to auxin, the cultures reverted from the caulonematal state to that of the chloronematal stage. The effects of PCIB on providing a lag period before caulonematal cells were once again produced was concentration dependent, but the prolongation of a lag period due to the effects of PCIB are nullified by the addition of auxin into the medium (Johri and D'Souza, 1990).

In other auxin studies, it was found that when exogenous auxin was introduced to the medium, not only were caulonema formed, but there was also a simultaneous higher level of ACC present (Rohwer and Bopp, 1985). This is an important observation since ACC is a direct precursor to the production of ethylene.

In the following group of experiments, I performed a concentration series using individual treatments of auxin and ACC in order to evaluate the role these phytohormones may play in the formation of gametophore buds in *P. patens*. The results are presented in this chapter.

MATERIALS AND METHODS:

Plant material. Axenic cultures of wild type *Physcomitrella patens* (Hedw.) Bruch and Schimp. derived from cultures kindly provided by John Wallace of Bucknell University were the source of inoculum for all experiments. Inoculum was prepared by homogenizing stock cultures of *P. patens* in an heat sterilized microblender head at 60 rpm for forty seconds. Ten drops (approximately = 1.00 ml) of the homogenized tissue was inoculated into culture vessels using sterile disposable transfer pipettes (Samco Sci. Inc, San Fernando, Ca.), fromwhich 3 mm of the tip was removed to prevent the tissue from clogging the pipettes.

Culture conditions.

1. **ACC**: The basal nutrient medium used for both stock and in all experiments employing ACC consisted of aqueous Knop macronutients supplemented with Hutner's "metals 49", micronutrients, and1% w/v glucose. Formulae for the macro- and micronutrients used are

given in Basile (1978). The pH of the media was adjusted to 4.6 and 30 ml aliquots were delivered to Sigma culture ("baby food") jars and closed with Magenta caps (Sigma) before being heat sterilized for 20 min. @ 15 psi. Cultures were incubated on lighted shelves in rooms thermostatically controlled to maintain a temperature of 18 °C (with continuous illumination of 700 - 1000 lux from cool white fluorescent bulbs). The incubation period for each of the experiments using various concentrations ACC was a total of 15 days.

1. NAA: The basal nutrient medium used for both stock and in all experiments employing NAA consisted of aqueous Knop macronutients supplemented with Hutner's "metals 49", micronutrients, 1% w/v glucose, and 0.6% w/v Agar-agar (manufacturer). Formulae for the macro- and micronutrients used are given in Basile (1978). The pH of the media was adjusted to 4.6 and 30 ml aliquots were delivered to Sigma culture ("baby food") jars and closed with Magenta caps (Sigma) before being heat sterilized for 20 min. @ 15 psi. Cultures were incubated on lighted shelves in rooms thermostatically controlled to maintain a temperature of 18 °C (with continuous illumination of 700 - 1000 lux from cool white fluorescent bulbs). The incubation period for each of the experiments using various concentrations NAA was a total of 17 days.

NAA and ACC stock solutions. Five concentrations of NAA: 10 µM, 20 µM, 30 µM, 40 µM, and 50 µM and five concentrations of ACC: 10 µM, 20 µM, 30 µM, 40 µM, and 50 µM were prepared. All stock solutions of both ACC and NAA were cold-sterilized through 0.2 µm filters and added to the sterile basal medium prior to inoculation. Each treatment was individually tested for their capacity to promote bud formation in *P. patens* and compared to the control in which *P. patens* was grown in basal medium. Both individually treated cultures and the controls consisted of fifteen replicates.

Examination of ACC treated *P. patens* . There were three examinations done of the *P. patens* tissue treated with ACC. The first analysis was scheduled five days after inoculation, and the other two examinations were each scheduled ten days and seventeen days after the inoculation respectively. For each analysis, five replicates of each treatment and of the control were opened. For each group of replicates, the total number of plantlets were counted while under an Olympus stereomicroscope. The specimen tissue was then transferred to a slide, and put under a Zeiss Axiovert microscope, where each plantlet was examined under 100x magnification for bud formation. Photomicrographs were taken with Kodachrome and Kodak black and white ISO 100 film.

Examination of NAA treated *P. patens*. There were three examinations done of the *P. patens* tissue treated with ACC. The first analysis was scheduled five days after inoculation, and the other two examinations were each completed at the tenth and seventeenth day. For each analysis, five replicates of each treatment and of the control were opened. The Agar gel was removed, and placed face-down onto a petri dish. The petri dish was then put onto the stage of a Zeiss Axiovert microscope, the plantlets were counted under 50x magnification, and then each plantlet was examined under 100x magnification for bud formation. Photomicrographs were taken with Kodakchrome and Kodak black and white ISO 100 film.

RESULTS:

As expected the ACC treated cultures showed a higher production of caulonematal tissue when compared to the control that showed little to no caulonema. Previous researchers have stated that *P. patens* when grown in aqueous media, form little to no caulonema (Johri and Desai, 1973). My own group of experiments confirmed this. Compared to the control, the growth rate of the treated plants were slower, with little chloronema compared to the caulonema. It has been my previous observations, that plants grown in aqueous media almost always produced buds from the chloronematal filaments and produced little if any caulonematal cells. Interestingly, ACC treatments of *P. patens* promoted bud production on the caulonematal filaments. More importantly, at 40 µM ACC treated cultures, *P. patens* had a significant increase in the number of buds formed when compared to the control, as seen in the *anova* values that is significant to $P \leq 0.05$, as seen in Table 5-1.

Although 10 µM ACC (2.10 ± 0.64), 20 µM ACC (0.70 ± 0.21), 30 µM ACC (2.40± 0.79) µM, and 50 µM (1.80 ±1.00) promoted some bud formation, the results were not significant at $P \leq 0.05$ on day ten (10) when compared to the control.

On day seventeen (17), the same concentrations of ACC: 10 µM (9.5 ± 1.92), 20 µM (5.7 ± 1.48), 30 µM (2.8 ± 1.28), and 50µM (0.80 ± 0.61) again did not prove to be significant at $P \leq 0.05$ when compared to the control.

The results with the NAA treated cultures showed interesting results. First there was a higher production of caulonematal tissue in treated cultures then there was with control cultures. It is important to note that previous attempts to grow cultures of *P.patens* in aqueous cultures were unsuccessful, and within ten days, the cultures would die. IAA treatments in aqueous cultures were also tested with similar results. I then tried to use a solid agar medium, and added the same concentrations in individual cultures as previously discussed. I used NAA as my preferred source of exogenous auxin based on protocols of other researchers that stated NAA was most successful in producing a higher percentage of caulonemata.

The resulting tissue growth was healthy and of the treatments in early bud promotion. 20 µM NAA was most successful. Yet, when compared to the control, 10 µM NAA produced three times as many buds as the control. At day 17, 10 µM NAA promoted a high number of buds when compared to both the control and 20μM NAA. 20μM NAA treated tissue, which had produced successful numbers of buds at day ten (10), was necrotic and showed mixed phenolic coloring with some green filamentous growth at this point. More significantly, this tissue also produced a smaller number of buds when compared to both 10 μM and the control.

For day ten (10), though buds were produced, 10 μM NAA (1.8 ± 1.32), 30 μM NAA (0.80± 0.489), 40 μM (0.60 ±0.89), 50 μM (0.20±0.20), the results were not significant at P ≤ 0.05 on day 9 when compared to the control.

For day seventeen (17), though buds were produced, 20 μM NAA (18.4 ± 6.23), 30 μM NAA (30.0 ± 4.60), 4 0 μM NAA (25.0±1.14), 50 μM NAA (14.2 ± 2.94) the results were not significant at P ≤ 0.05 on day 17 when compared to the control.

Below see tables 5.1 showing the details of the anova table for 40μM ACC treatments, 5.2 showing the statistical analysis for 10μM NAA, and table 5.3 showing the same for 20μM NAA.

Table 5-1 40 μM ACC Treatments:

The number of buds formed after treatment with 40μM ACC vs. the number of buds formed in the control group.

Day	Control (Mean ±SE)	40μM ACC (Mean ±SE)	F-ratio	df	P>F
10	2.70 ± 0.80	6.4 ± 1.61*	4.8220	1	0.0423
17	9.20 ± 1.68	28.4 ± 3.93**	21.8351	1	0.0002

*The mean is significant to the control at P ≤ 0.05.
**The mean is significant to the control at P≤ 0.01.

Table 5-2. 10 μM NAA treatments:
The number of buds formed after treatment with 10μM NAA vs. the number of buds formed in the control group.

Day	Control (Mean ± SE)	10 μM NAA (Mean ± SE)	F-ratio	d*f*	P>F
10	0.60 ± 0.40	1.8 ± 1.32	0.7579	1	0.4094
17	26.40±4.37	49.2±7.47 *	6.9497	1	0.0299

*The mean is significant to the control at P≤ 0.05.

Table 5-3. 20 μM NAA treatments:
The number of buds formed after treatment with 20μM NAA vs. the number of buds formed in the control group.

Day	Control (Mean ±SE)	20μM NAA (Mean ± SE)	F-ratio	d*f*	P>F
10	0.60 ± 0.40	3.8 ± 0.97*	9.3091	1	0.0158
17	26.4 ± 4.37	18.4 ± 6.23	1.1050	1	0.3239

*The mean is significant to the control at P≤ 0.05.

Note, as seen in the micrographs (Figures 5-1 ACC treatments and 5-2 NAA treatments), as the concentration of ACC was increased, so were the number of buds produced by the treated cultures. The optimum concentration ACC was 40μM. NAA was most effective at 20 μM on day 10, and at 10 μM on day 17.

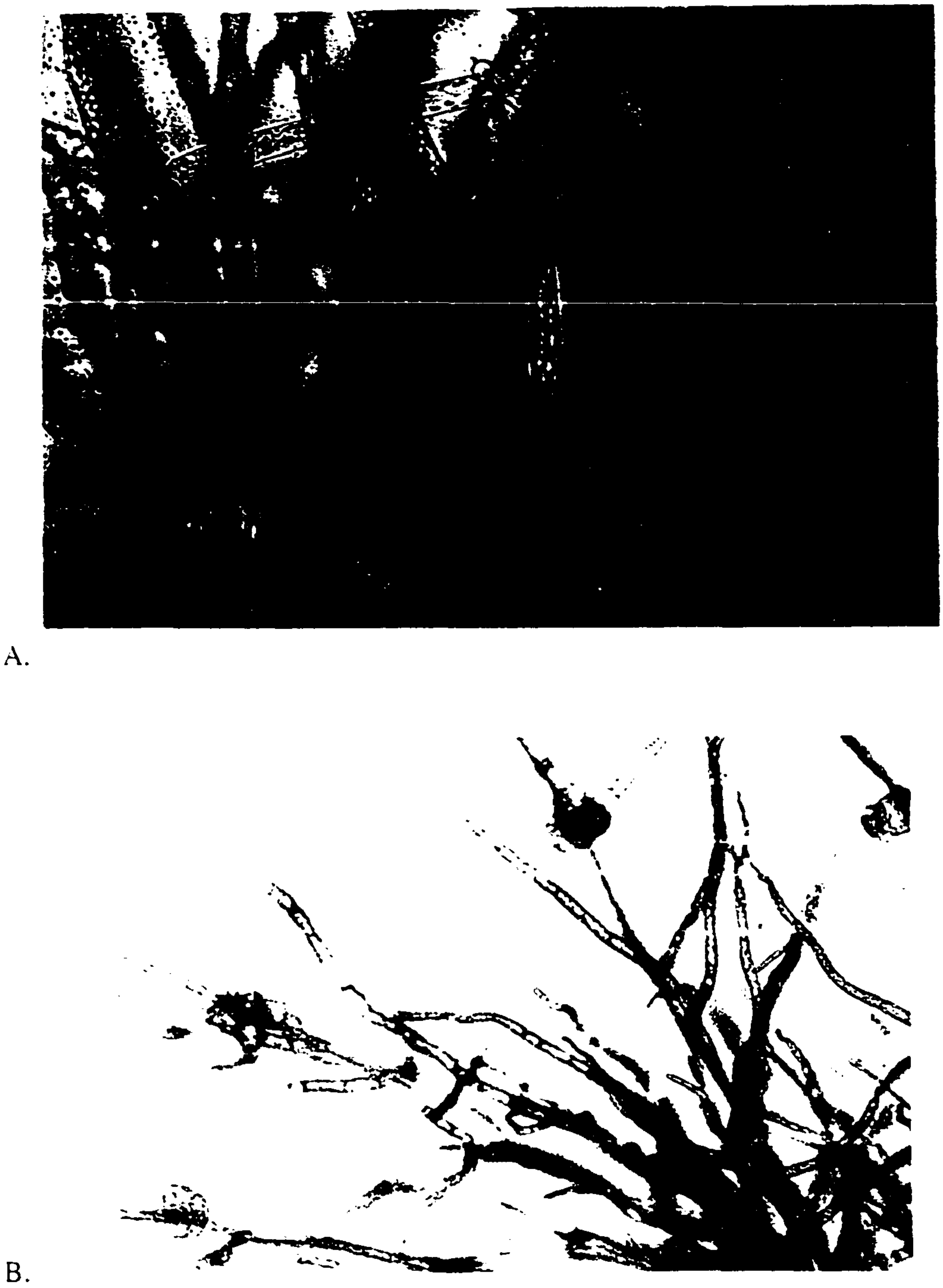
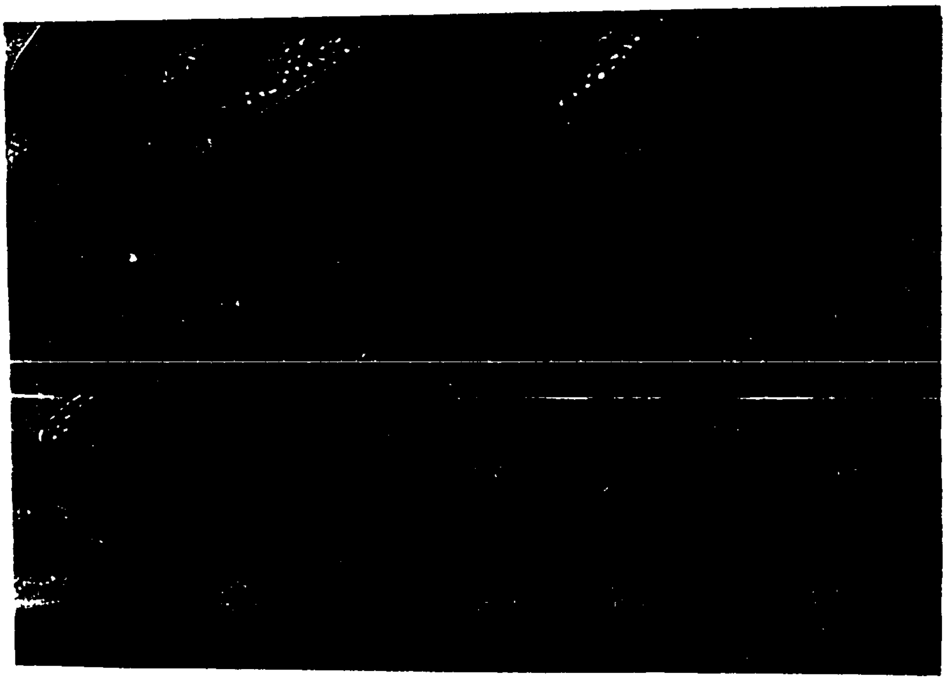
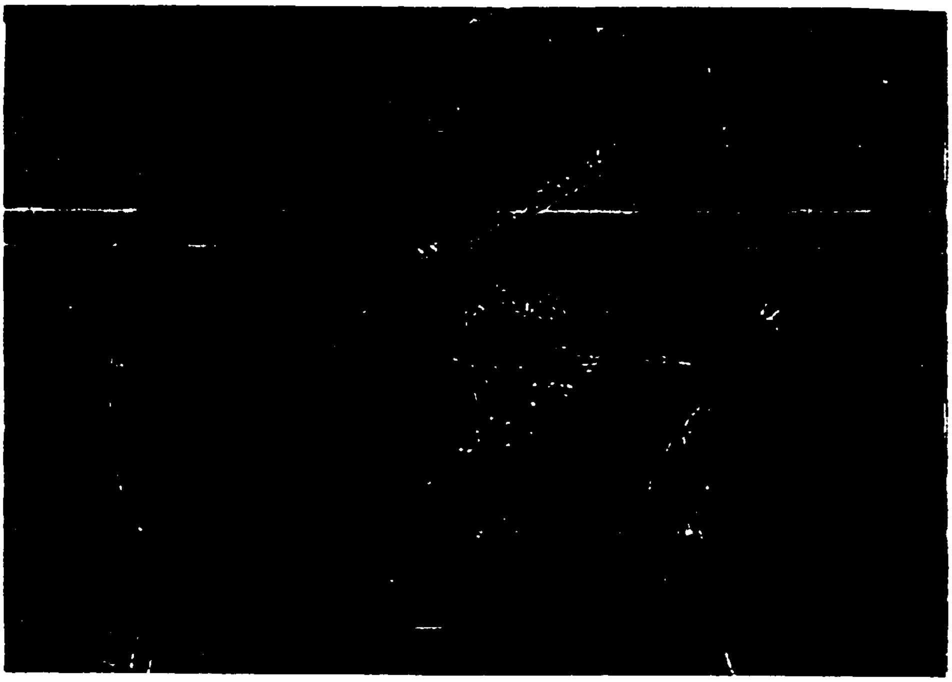


Figure 5-1(A-B) ACC Treatments. A. Control (350X) Day 17. B.40μM ACC(250x) Day 17At the 17th day, notice how the control shows no bud development. In comparison ACC has numerous large and well developed gametophore buds. Bud formation is observed as early as the tenth day, and is statistically significant when compared to the control group.



A.



B.

Figure 5-2 (A-B). NAA Treatments . A. Control (Day 17) (250x) Note the protonematal growth of the control, and the absence of gametophore buds. Though some buds could be found on the control by day 17, it is auxin that provides a significant number of buds formed. B. 20 μM NAA(Day 17) (250x) Note how well developed the buds are formed in this treatment. The size of the bud had also a tendency to be larger than that of the control.

DISCUSSION.

Both Law et al. (1985) and Lehnert and Bopp (1983) provided evidence that a specific concentration of auxin will result in the production of ethylene in bryophytes. Other researchers have also provided evidence that introduction of exogenous auxin at physiological concentrations also promotes the formation of caulonematal filaments (Johri and Desai, 1973; Sood and Hackenberg, 1979; Bhatla, 1994).

In this thesis, the "Suppression Hypothesis" and the role it plays in plant development has been emphasized. When we discuss the "Suppression Hypothesis" we must focus in how the transition of diffuse growth to that of polar apical growth is initiated. The above evidence provides possible answers. If auxin in turn promotes both the formation of ethylene and production of caulonematal filaments, then we see a coupled physiological and morphological process occurring simultaneously. Now, the results of my studies also provide further evidence that individual exogenous treatments of auxin and ethylene (via ACC addition) treatments can be interchangeably used to promote gametophore bud production.

As always in the scheme of all processes, there is no simple or direct answer. When we discuss the correlative control system as it relates to the *P. patens* development, and in particular to the transition from the diffuse protonemal growth to that of the suppressed polar apical growth of the gametophore bud, we must also understand that other factors weigh in. Again, referring to the literature review and the evidence that my first group of experiments provided, AGPS weigh in heavily as the most direct possible morphoregulator in determining fates for cells and tissue types. Although Ashton and his collaborators (1977, 1993) have stated the importance of the presence of auxins, they have also experimentally provided further evidence that light and cytokinins also play a significant role in *P. patens* development. Cytokinin experiments have provided evidence that cytokinins must be present through the whole differentiation process of gametophore bud production. If the cytokinin, which is an unstable compound in mosses (Bopp, et al., 1986), is removed during the early stages of bud formation, the bud will revert back to the protonematal filament stage (Brandes and Kende, 1968).

When assertions that auxin, ethylene and AGPs are components of a correlative control system, it is understood that the success of this system functioning as a morphoregulatory system is dependent on the active presence of each component in its place. In other words, if auxin cannot be transported and a differential concentration gradient is not produced in given cells or tissue, then differentiation cannot progress. The effects of PCIB in cultures of *Funaria hygrometrica* promoted a lag period in which caulonematal formation is suppressed, and chloronematal growth is promoted. In my antagonist studies, presented in chapter 3, TIBA and ALANAP, both antagonists to auxin tranport, promoted chloronematal filaments. In contrast the addition of NAA in this study promoted both caulonemal and bud production, thus providing further evidence of the possible role auxin may play with the correlative control system.

If ethylene is not produced, then differentiation cannot continue. This was seen by previously presented AVG (antagonist) studies. AVG is a specific antagonist to ethylene, and inhibits enzymes that use the cofactor pyridoxal phosphate (Beyer, 1979), and blocks ACC synthase (which Beyer suggests is a pyridoxal phosphate enzyme). By preventing ethylene production, *P. patens* protonema remained in its most desuppressed state. In contrast, the addition of exogenous ACC to basal media promoted bud formation, thus providing further evidence that ethylene may indeed be a component responsible for the transition of desuppressed filamentous protonematal growth to that of suppressed apical meristematic growth.

Finally, as seen from the antagonist experiments, if the proper AGP molecules are not inserted into the plasma membrane and /or cell wall, then differenitation cannot proceed. This provides evidence for the importance of each component in promoting suppressed growth in *P. patens* and thus permitting the earliest mainifestation of pattern changes as exemplified in the regulation of the transition from the primary chloronematal stage to that of the gametophore bud stage.

Chapter 6. Desuppression Studies

Introduction:

In chapter 3, using antagonists to auxin, ethylene, and arabinogalactan proteins, the components of the correlative control system were successfully blocked in promoting the spacio-temperol suppression of cell proliferation in plant development at the protonemal level. Basile and Basile (1980, 1993) provided evidence that when mature gametophytes of *Gymnocolea inflata* were transferred to Knudsen media, the plants reverted to a radial symmetry as a result of the ventral merophyte being desuppressed. The possibility that antagonists to auxin, ethylene and arabinogalactan proteins could similarly induce a reversion from the suppressed to a desuppressed pattern seemed worth investigating.

It has been shown that dedifferentiation may occur at the protonematal level, reverting caulonemata filaments to the chloronemata state, by in the introduction of antagonists to auxin such as PCIB (Sood and Hackenburg, 1979). This dedifferentiation, which amounts to desuppression may also occur from the removal of exogenous auxin from liquid media (Johri and Desai, 1973).

There is also some evidence that the stability of caulonematal filaments is dependent on the continued presence of signals eminating from the caulonematal apical cell (Knoop, 1984). At least one signal known to be needed is auxin, and there appears to be a definite relationship between IAA transport and the ongoing stability of the caulonematal state (Bhatla and Dhingra-Babbar, 1990).

Based on the evidence that dedifferentiation leading to a more desuppressed pattern was possible, I decided to conduct experiments to find out if the same antagonists that prevented growth suppression that is related to the transition from protonemal to leafy gametophyte stage would cause leafy gametophores to revert to a protonematal (desuppressed) pattern of growth. Investigations as to whether given concentrations of ALANAP, TIBA, AVG, 3,4D, and Hydroxy-L – proline would promote cell proliferative growth in mature gametophytes in *P. patens*, were carried out. Several experiments were performed to test whether each of the above antagonists: ALANAP and TIBA against auxin polar transport; AVG against ethylene production; and 3,4D and Hyp against AGP synthesis; at specific concentrations would counteract the apical dominance of the gametophore.

Methods and Materials.

Plant material. Axenic cultures of wild type *Physcomitrella patens* (Hedw.) Bruch and Schimp., derived from cultures kindly provided by John Wallace of Bucknell University were the source of inoculum for all experiments. Inoculum was prepared by homogenizing stock cultures of *P. patens* in an heat sterilized microblender head at 60 rpm for forty seconds. Ten drops (approximately = 1.00 ml) of the homogenized tissue was inoculated into culture vessels using sterile disposable transfer pipettes (Samco Sci. Inc, San Fernando, Ca.), in which 3 mm of the tip was removed to prevent the tissue from clogging the pipettes.

Culture conditions. The basal nutrient medium used for both stock and in all experiments employing antagonists consisted of aqueous Knop macronutients supplemented with Hutner's "metals 49", micronutrients, and1% w/v glucose. Formulae for the macro- and micro-nutrients used are given in Basile (1978). The pH of the media was adjusted to 4.6 and 30 ml aliquots were delivered to Sigma culture ("baby food") jars and closed with Magenta caps (Sigma) before being heat sterilized for 20 min. @ 15 psi. Cultures were incubated on lighted shelves in rooms thermostatically controlled to maintain a temperature of 18 °C (with continuous illumination of

700 - 1000 lux from cool white fluorescent bulbs). The cultures were incubated for a period of 12 weeks, thus producing luxuriant growth and fully developed gametophores. At this twelve week period, the basal Knops medium was poured off under sterile conditions within the confines of a hood, and replaced with Knops medium containing appropriate concentrations of the antagonists. The basal medium was also poured off from the control group and replaced with more Knops basal medium, and the cultures were incubated for three weeks more as per the initial protocol. There were 10 replicates per treatment. The experiment was repeated three times.

Antagonists. As with the original group of experiments (Chapter 3), stock solutions of five antagonists were prepared after individually being tested for their capacity to prevent subapical growth suppression, thereby preventing apical meristem initiation. Two, 2,3,5 triiodobenzoic acid (TIBA) (ICN Biochemicals) and 2[(1-Naphthalenyl-amino carbonyl] benzoic acid (ALANAP) (a gift from the Uniroyal Co. Inc., Naugatuck, Conn.), were auxin transport antagonists, one, Aminoethoxyvinyl-glycine (AVG) (Sigma) is a specific antagonist of ethylene synthesis , and two, 3,4-dehydroproline (3,4D) (Sigma) and 4-hydroxy-L-proline (Hyp or Hypro) (Calbiochem) are antagonists of normal hyp-protein synthesis. All stock solutions of antagonists were cold-sterilized through 0.2 (μm) filters and added to the sterile basal medium prior to inoculation. As a result of pilot experiments, the concentrations of antagonists that were found to be effective were ALANAP, 8 μg /ml; AVG, 0.90 μg /ml ; 3,4-D, 12 μg/ ml ; and Hyp 24 μg /ml. There were 35 replicates per treatment.

Microscopy. Tissue from each culture was then harvested, and carefully examined for the presence of protonematal growth originating from the gametophore.

Slides made up of the live tissue were then examined under the Zeiss axiovert microscope. Photomicrographs were taken with Kodachrome film Gold Max and Kodak black and white film ISO 100 with an Olympus OM4 camera fitted to Zeiss axiovert microscope.

Results.

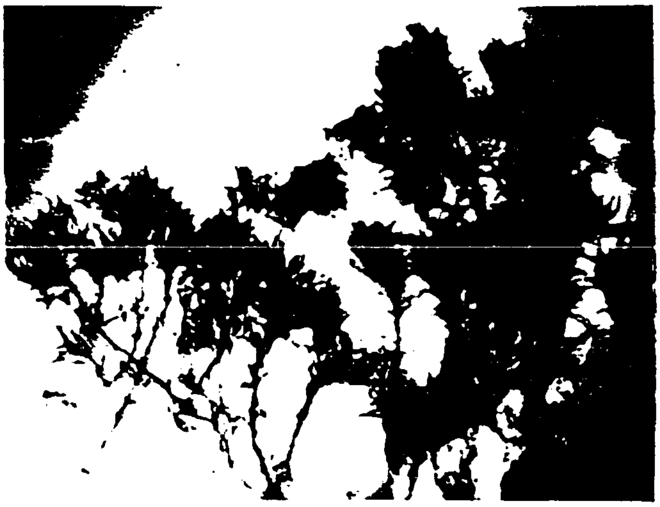
At the end of the twelve week period, the *P. patens* tissue grown in the Knops medium, were fully developed gametophores showing full rhizoid growth and distichous phyllotaxy. The apical meristem was well established, with full leaf development present from the apical to the basal stem region. There was no evidence of any protonematal growth anywhere on the gametophore, thus insuring that the protonematal stage no longer existed.

At the end of this t twelve week period, the basal Kp medium was poured off, and replaced with basal medium containing the appropriate concentrations of individual antagonists. The tissue was incubated for another two weeks before examination.

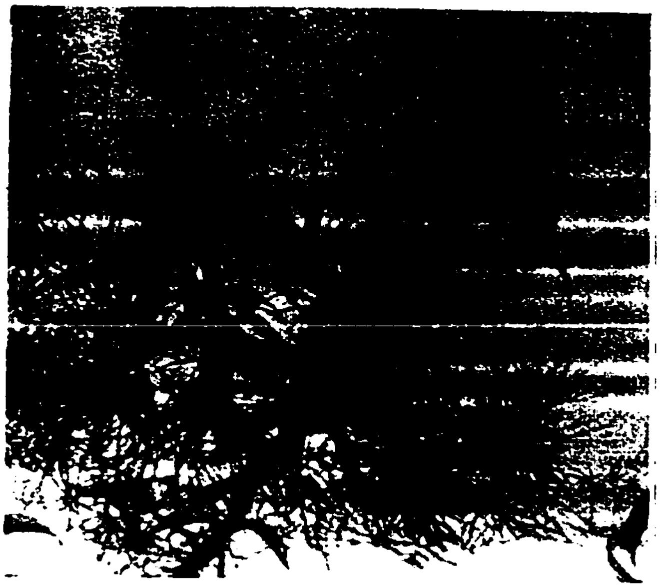
After the two week incubation in the presence of antagonists chloronematal filaments were growing at a higher density from the basal region of the gametophore to a lower concentration, more apically. When the tissue was harvested during the third week, there was a greater growth observed toward the apical region of the gametophyte originating from the stem and possible basal regions of the leaves. All protonematal growth was lush and healthy as were the gametophytes themselves.

Though all components of the correlative control system was blocked, one antagonist to auxin transport, the TIBA treatment, did not promote protonematal growth. All other antagonists were effective in promoting protonematal growth originating from the gametophytes. As seen in figure 6.1A, the control group is completely free of protonematal growth. 6.1 B-E, show that results using individual treatments of ALANAP, AVG, HYP, and 3,4D. Note the lush and healthy growth of the protonematal filaments coming from each of the treated plants.

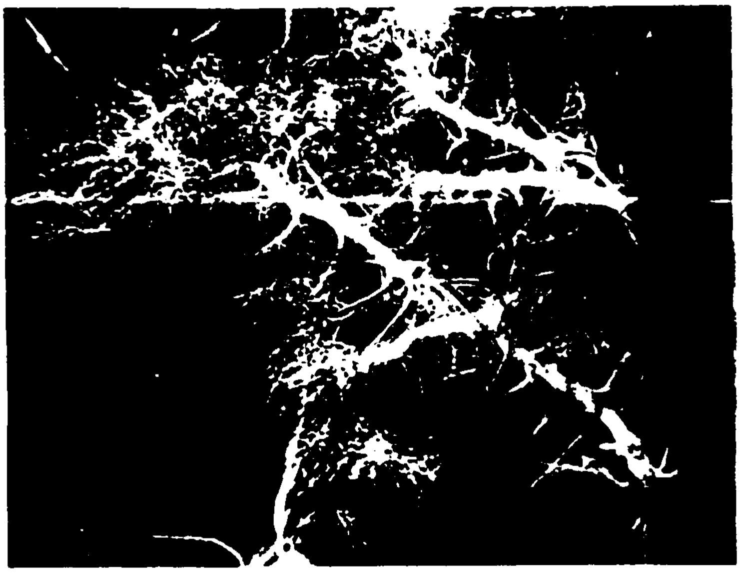
Figure 6.1 (A-D): A. Control: Note how the control is free of protonematal growth. B. ALANAP, C. AVG, D. 3.4 D, E. Hyp (B-E): Note the lush protonematal growth growing out of the gametophyte. (pages 124-125)



A.

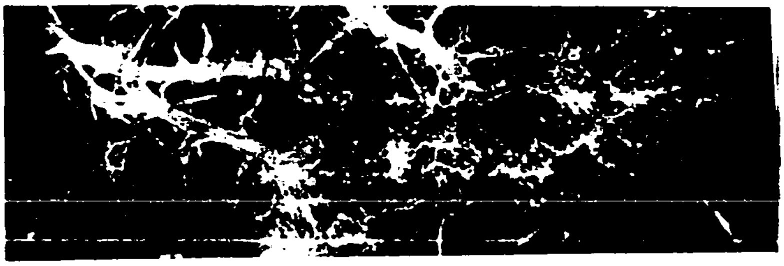


B.



C.

D.



E.

Discussion.

Basile and Basile (1980) discovered that one species of leafy liverwort, *Gymnocolea inflata*, which maintains its "suppressed" pattern of leaf and branch development when grown in nitrate-containing medium (Knops) reverted to a "desuppressed" pattern of growth in ammonia-ion-containing medium (Knudsen's medium). This discovery was quite remarkable, and therefore, it was of interest to see whether *P. patens*, once fully developed, could revert to a desuppressed state in the presence of antagonists, thus producing the diffuse growth of protonema.

Was the correlative control system still susceptible to antagonist response, by the disruption of the apical dominance of the gametophores? Each time this experiment was performed, the same results were obtained, and within days of the introduction of media supplemented with antagonists, growth of protonema began to originate from the rhizoids and basal stem regions. Within two weeks, healthy protonematal growth flourished.

The antagonists, except for TIBA, had the ability to promote desuppressed growth. In the pilot experiments described in chapter 3, TIBA successfully antagonized the polar transport of auxin, and therefore, promoted protonematal growth of the treated cultures. TIBA is a very effective antagonist, and the TIBA concentrations were kept low. Perhaps, at the low concentration, there was not enough TIBA in proportion to endogenous auxin that would successfully inhibit polar transport.

The fully developed caulonema with its side branches has been described as an apical dominance system, with regards to its regular branching system and regenerative behavior. The younger side branches of caulonematal filament grow slower and always remains less than that of the main tip. The further away the side branches are from the tip cell, the faster is its growth. If the tip cell is removed, and the development of the tip cell is delayed or if the new tip cell is removed again, then the side branches nearest to the apex grow faster that the norm, and forms a new tip cell (Knoop, 1984).

Sood and Hackenburg (1979) provided evidence that caulonematal filaments reverted back to chloronema in the presence of parachlorophenoxy-isobutyric acid (PCIB), an auxin antagonist. It had been observed that caulonema formed in liquid cultures of *Funaria* remains stable only in the presence of exogenous IAA, and if IAA is removed from the medium, there is a dedifferentiation back to the chloronemata state (Johri and Desai, 1973).

It is known that in mosses, as in higher plants, the removal of the gameophyte's apex will result in lateral buds growing out, thus forming a new plant tip and side branches. The IAA-mediated suppression of lateral bud development was demonstrated by von Multzahn (1959). Nyman and Cutter (1981) provided evidence that bud development can be released from apical dominance by using TIBA to antagonize IAA transport.

Decapitation of gametophores apices promote the regeneration of filamentous protonema. Filaments appear mainly at the base of decapitated gametophores, whereas the buds develop in the more apical part of the stump. Growing apical buds reimpose apical dominance and growth below the newly formed bud is suppressed (Gay, 1971, Nyman and Cutter, 1981).

These series of experiments have provided additional evidence that auxin, ethylene, and AGPs individually function to bring about the growth pattern transition from a diffuse filamentous state to that of suppressed apical mersitematic growth. As ALANAP, AVG, 3,4D and Hyp successfully maintained a desuppressed state in *P. patens* protonema, so have these antagonists also successfully disrupted the correlative control system that normally maintains apical dominance in a mature gametophore.

It always comes down to the basic fact, that in blocking only one component of this correlative control system, a reversion to the desuppressed state is brought about as exemplified by filamentous growth. Thus this series of experiments has provided additional evidence in support of the "Suppression Hypothesis" as it pertains to the transition between diffuse and suppressed growth.

Chapter 7: Overall Summary of Experimental Conclusions:

This investigation has shown that the regulation of the transition from chloronematal (diffuse) growth to that of the gametophore stage (restricted, apical meristem) growth, may be brought about by the interrelated actions of auxin. ethylene, and AGPs. The literature, published prior to undertaking this investigation, although scattered and seemingly unrelated, provides the earliest indication that all three of the components of this proposed correlative control system are naturally occurring in mosses; and were somehow involved in mediating the transition from protonematal to leaf gametophore stages in mosses. The results of the present study are far more extensive that any published to date and ties together the earlier and seemingly unrelated studies.

The results of the experiments employing antagonists indicate that when the actions of any one component of the correlative control system was blocked, then suppressed development is arrested at the most diffuse (desuppressed) level. Thus, the use of *Physcomtrella patens* as a model system, provided important evidence that auxin, ethylene, and hydroxyproline proteins (AGPs) act in an interrelated way for mediating growth suppression in moss. It was already fairly well established that when auxin acts to inhibit (suppress) rather than to promote growth, it is because it triggers ethylene synthesis. It is also very well established that ethylene characteristically act to inhibit growth. Unfortunately, the mechanism by which ethylene brings about growth suppression is not known. Evidence from the research conducted with leafy liverworts indicate that ethylene's inhibitory effect may influence one or more cell surface-associated AGPs. The primary reason for implicating AGPs is that changes in qualities or physical-chemical characteristics of AGPs can be correlated with changes from desuppressed to suppressed growth patterns and vice-versa.

The biochemical studies provided both qualitative and quantitative analysis of AGPs. AGPs were identified as the probable hydroxyproline proteins in the correlative control system. When equal masses of protonematal and gametophore tissue was used to extract AGP, the desuppressed tissue yielded a much smaller concentration of the protein AGP than did the suppressed tissue. Further studies provided evidence that an AGP may be a morphoregulator, and the chloronemata is identified by an organ specific AGP that differs from the mature gametophyte tissue. The fact that the suppressed tissue possesses an AGP electophoretic pattern that does not contain the AGP at or near the origin, as does the protonematal tissue, signifies that this specific AGP ceases to be produced by the mature gametophyte. It is at this stage of growth when the ptoonema stage no longer persists, and the existing AGP is organ specific for suppressed growth (gametophytic growth).

Comparative studies of electrophoretic patterns showed similar patterns between protonema (desuppressed) wildtype tissue and NAR 91 mutants. OVE bud mutants which contain both protonematal and gametophore bud tissue, and can be considered a transitional tissue, provided an electrophoretic pattern that was comparable to a composite pattern similar to that of both protonematal and gametophyte bud tissue. The mutant studies corroborate the data obtained in the original set of electrophoresis containing both the wild-type protonematal and gametophore tissue. These studies are significant, as the *P.patens* mutants have been analyzed, using phytohormone studies, and are well characterized genetically.

Additionally, the results of auxin and ACC experiments indicate that individual treatments promote an increased number of gametophore buds when compared to the control. These data provide evidence of the possible morphoregulatory role played by both auxin and ethylene in the correlative control system.

The desuppression studies, furthermore, provided evidence of the importance for all components of the correlative control system to be in place at all times, so that the morphology of the mature gametophyte may be maintained continuously. The results of these studies showed a strong correlation to the results of the antagonist studies. The data supports the needed role for the interrelated actions of auxin, ethylene, and AGPs play in maintaining suppressed growth. This final group of experiments provided further support of Basile and Basile (1990,1993) "Suppression Hypothesis" by corroborating the fact that when the action of any one component of the correlative control system is blocked and inactivated, the suppressed development is arrested to the most desuppressed level.

APPENDIX 1: KNOPS MEDIUM FORMULA

STOCK	KNOPS MEDIUM
$MgSO_4 \bullet 7H_2O$ 2.5g/ 100 ml distilled H_2O	Use 5ml/L
$Ca(NO_3)_2 \bullet 4H_2O$ 10.0g/100 ml distilled H_2O	Use 5 ml/L
KH_2PO_4 2.5 g/ 100 ml distilled H_2O	Use 5 ml/L
KNO3 1.25 g/100 ml. distilled H_2O	Use 10 ml/L
(NH4)2SO4 5.0 g/ 100 ml. distilled H_2O	----------------
Metals "49" from 1000x stock	Use 1.0 ml/L
Dextrose	10.0 g/ L

APPENDIX 2: KNUDSEN MEDIUM FORMULA

STOCK	KNUDSEN'S MEDIUM
$MgSO_4 \bullet 7H_2O$ 2.5g/ 100 ml distilled H_2O	Use 10 ml/L
$Ca(NO_3)_2 \bullet 4H_2O$ 10.0g/100 ml distilled H_2O	Use 10 ml/L
KH_2PO_4 2.5 g/ 100 ml distilled H_2O	Use 10 ml/L
KNO3 1.25 g/100 ml. distilled H_2O	----------------
(NH4)2SO4 5.0 g/ 100 ml. distilled H_2O	Use 10 ml/L
Metals "49" from 1000x stock (instructions from enclosed paper)	Use 1.0 ml/L
Dextrose	10.0 g/L

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