ABSTRACT

Title of Dissertation: THE ROLE OF AUXIN ON THE EVOLUTION OF EMBRYO DEVELOPMENT AND AXIS FORMATION IN LAND PLANTS

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This thesis examined the role of auxin in the evolution of land plants. Several approaches were used to study how auxin regulates the development in the bryophyte sporophytes. The altered growth of isolated young sporophytes exposed to applied auxin (indole-3-acetic acid) or an auxin antagonist (*p*-chlorophenoxyisobutyric acid) suggested that endogenous auxin regulates the rates of axial growth in all bryophyte divisions. In the hornwort *Phaeoceros personii*, auxin moved at very low fluxes, was insensitive to an auxin-transport inhibitor (N-[1-naphthyl]phthalamic acid), and exhibited a polarity ratio close to 1.0, implying that auxin moves by simple diffusion. The liverwort *Pellia epiphylla* exhibited somewhat higher auxin fluxes, which were sensitive to transport inhibitors but lacked any measurable polarity. Thus, auxin movement in liverwort sporophytes appears to result from facilitated diffusion. In the moss *Polytrichum*

*ohioensis*, auxin movement was predominantly basipetal in young sporophytes and occurred at high fluxes exceeding those measured in maize coleoptiles. In older sporophytes, acropetal auxin flux had increased beyond the level measured for basipetal flux in the specimens observed in several, but not all, seasons. The evidence from both inhibitor treatments and isolated tissues is consistent with the interpretation that the cortex carries out basipetal transport in both younger and older sporophytes, whereas the central vascular tissues carries out basipetal transport in younger sporophytes and acropetal flux in older sporophytes. Given the significant differences in fall rainfall in the collection years, the purported sensitivity of vascular tissue development may account for the seasonal variation observed in these experiments. Auxin regulators and polar transport were also used to study the regulation of the embryogenesis of the fern *Marsilea vestita*. Auxin biosynthesis inhibitors affected initial cell proliferation resulting in the formation of aborted embryos, *p*-chlorophenoxyisobutyric acid delayed growth and development in all stages of embryogenesis while α-naphthaleneacetic acid mediated rapid cell proliferation that caused enlarged disorganized embryos. Polar auxin transport inhibitors caused no significant abnormalities, which suggested a limited role for polar transport in fern embryogenesis. In conclusion, this evidence suggests that auxin is ultimately involved in the establishment of the body plans in all land plant sporophytes.

# THE ROLE OF AUXIN ON THE EVOLUTION OF EMBRYO DEVELOPMENT AND AXIS FORMATION IN LAND PLANTS

by

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## DEDICATION

This dissertation is dedicated to my mother, Donna Belle Craig, who gave me everything necessary to succeed in the world. Without her continual support, unconditional love, and devotion none of this would be possible. Everyone needs a Momma Bear like mine! The work is dedicated to my entire family, thank you.

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## TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# CHAPTER 1

## GENERAL INTRODUCTION

The goal of this Chapter is to summarize our current knowledge about the current phylogeny of land plants, the general features of auxin (indole-3-acetic acid, IAA), the metabolism and transport of auxin in land plants, and the role of auxin in land plant development. This information is essential to understand the significance of my observations on the role of auxin transport and auxin action on the evolution of land plants.

### The current phylogeny of land plants

Photosynthetic eukaryotes appear to have independently evolved into multicellular growth forms at least three times giving rise to the phaeophytes (brown plants), rhodophytes (red plants), and viridiphytes (green plants). The rhodophytes and viridiphytes are closely related sister groups, and they appear to have acquired their chloroplasts by primary endosymbiosis of a single cyanobacterium or a group of related cyanobacteria (Baldauf et al., 2000; Chu et al., 2004). The pheaophytes (brown plants) originated via secondary endosymbiosis of engulfed rhodophytes that were transformed into chloroplasts (Delwiche, 1999; Cooke et al., 2002). This dissertation will focus on the viridiphyte group known as the land plants that are thought to evolve from the

charophycean green algae (charophyte) lineage within the viridiphytes. Because I anticipate that the dissertation will be read by a wide range of plant biologists, I shall use a more colloquial terminology for describing the different divisions of land plants (Table 1-1). I recongnize that I am sacrificing some rigor in order to communicate with a wider audience.

The charophycean green algae are thought to be the green algal class most closely related to the land plants since they share many specialized characteristics, such as cell division mechanism, sperm ultrastructure, and photorespiratory enzymes (Graham, 1993; Graham et al., 2000). Karol et al. (2001) used modern phylogenetic methods to resolve the evolutionary relationships among the simplest charophytes and found that the order Charales, including the genera *Chara* and *Nitella*, were the closest living relatives to the land plants.

The most ancient division of extant land plants, Bryophyta *sensu lato* was originally thought to be monophyletic (Schuster, 1966) but currently it is suggested that the bryophytes have a polyphyletic origin that is best represented by three divisions: Hepatophyta (liverworts), Anthocerotophyta (hornworts), and Bryophyta (mosses) (Crandall-Stotler, 1980; Kenrick and Crane, 1997). Plants in these divisions are primarily non-vascular gametophytes, but they have short-lived sporophytic generations with the sporophytes being very simple structures (for more details on sporophyte structure in the three divisions, see Chapter 3). Among the bryophytes *sensu lato*, it is unresolved if the liverwort or the hornwort lineage is the first-divergent lineage of earliest land plants. Several morphological and cellular features hint that the hornworts are the closest living relative to the green algae. For example, hornwort cells have a single large

chloroplast like those found in algae, not the multiple discoid shaped chloroplasts found in most other plant cells. However the presence of stomata on hornworts, mosses, and vascular plants and its absence on liverworts, had initially created the controversy as to whether the liverworts or the hornworts lineage is more ancient. Moreover, some morphological, cell, and molecular data (Doyle et al., 1994; Mishler et al., 1994; Pryer et al., 1995; Lewis et al., 1997), including the observation that liverworts lack four mitochondrial introns present in most mosses, hornworts, and all vascular plants (Qiu et al., 1998; Qiu and Lee, 2000; Dombrovska and Qiu, 2003), suggest that liverworts are basal to the rest of the land plants. However, other data suggest that the hornworts are more basal. Nickrent et al. (2000) used parsimony and likelihood methods to analyze a 6,095-character data set of four genes (cloroplast rbcL and small-subunit rDNA) from all major land plant lineages. If the rbcL third-codon-position transitions were either excluded or downweighted, then hornworts were placed as the sister to all other land plants. The mosses are generally found to be the sister group to the vascular plants in most analyses (Mishler et al., 1992; Kenrinck and Crane, 1997; Lewis et al., 1997; Qiu and Lee, 2000; Dombrovska and Qiu, 2003).

Similar work is being done to resolve the phylogenetic relationships among the vascular land plants. Using morphology and four genes, Pryer et al. (2001) developed phylogenetic analyses for 35 representative land plants. They show three monophyletic groups of extant vascular plants: 1. lycophytes, 2. seed plants, and 3. a clade including equisetophytes (horsetails), psilotophytes (whisk ferns), and all eusporangiate and leptosporangiate ferns with maximum-likelihood analysis showing the horsetail and fern clade as the closest relatives to seed plants.

| **Formal name of major divisions** | **Derived name of major divisions** | **Colloquial name of major divisions** | **Colloquial name of evolutionary grade** |
|---|---|---|---|
| Hepatophyta | hepatophytes | liverworts | brophytes (basal land plants) |
| Anthocerophyta | anthocerophytes | hornworts | |
| Bryophyta | bryophytes | mosses | |
| Lycophyta | lycophytes | club mosses | pteridophytes (seedless vascular plants) |
| Equisetophyta | equisetophytes | horsetails | |
| Pterophyta | pterophytes | Ferns | |
| Pinophyta | pinophytes | conifers | seed plants (seeded vascular plants) |
| Magnoliophyta | magnoliophytes | angiosperms | |

**Table 1-1:** Explanation of names used in the thesis

**General features of auxin**

The German botanist Julius von Sachs proposed that chemical messengers are responsible for the formation and growth of different organs. He did not know the identity of these messengers, but his thoughts led to other efforts to research this concept. The term hormone, developed in animal research, refers to chemical messengers in low concentrations that are synthesized at one site and mediate intercellular communications at another site. In the plant world, this term is used similarly, except the hormone may also act at the synthesis site.

The activity of the plant hormone that we refer to today as auxin was first revealed by Ciesielski (1872) but it was popularized by Charles Darwin and his son while investigating plant tropisms, especially phototropism (Darwin & Darwin, 1892). They showed that when the tip of the coleoptile was covered, the characteristic bending of the coleoptile toward light did not occur. Darwin concluded that a signal was produced in the tip, traveled to the subapical growth zone, and caused the unequal growth necessary for bending. Went (1926) collected this "growth promoting substance" into gelatin blocks from the excised coleoptile tips of *Avena sativa* (oat). The blocks were then tested for their ability to restore growth by being placed on top of decapitated coleoptiles. If the blocks were set asymmetrically then that side bent toward the light. This work has led to the coleoptile-bending assay used for quantitative auxin analysis because the "growth-promoting substance" did restore elongation on a dosage-dependant basis to coleoptiles and the assay was specific to auxin. Work done by F. Kogl and A. J. Haagen-Smit (1934 and in 1954) in Holland, and K. V. Thimann (1935) in the United States revealed that the

principal auxin in higher plants was indole-3-acetic acid (IAA) (Taiz and Zeiger, 2002), which is generally thought to be synthesized from the amino acid tryptophan in actively growing regions of the plant (see later section of this chapter). Even though other auxins are found in higher plants, IAA is the most abundant and most often studied in physiological research. For example, no mutants lacking auxin have been found suggesting that auxin is required for viability.

What are our current concepts about the nature of auxins? Cleland (1995) defined auxin as"[a] *compound that has a spectrum of biological activities similar to, but not necessarily identical with those of IAA. This includes the ability to: 1) induce cell elongation in isolated coleoptile or stem sections, 2) induce cell divisions in callus tissues in the presence of a cytokinin, 3) promote lateral root formation at the cut surfaces of stems, 4) induce parthenocarpic tomato fruit growth, and 5) induce ethylene formation* (p. 214-227)." Chemically, an auxin needs an aromatic ring of a certain size range and a carboxyl group separated from the ring by at least one carbon (Katekar and Geissler, 1980). The distance between the negative charge on the carboxyl group and the partial positive charge on the ring seems critically important for detectable activity. The optimal distance appears to be about 0.5 nm (Porter and Thimann ,1965; Farrimond et al., 1978). Edgerton et al. (1994) suggested a series of molecular requirements for auxin activity. Auxin binding protein 1 (ABP1), a putative auxin receptor, needs three essential regions for binding: a planar aromatic ring-binding platform, a carboxylic acid-binding site, and a hydrophobic transition region that separates the two binding sites. This definition will no doubt continue to change as we learn more about the actions of this hormone.

**Auxin metabolism in land plants**

The metabolite indole-3-acetic acid (IAA), the major auxin in plants, has been studied in detail for the last 70 years, but only recently have plant biologists started to understand the broad features of its regulation. IAA is ubiquitous in the plant with highest concentrations located in meristematic regions and actively growing organs. Cells in older leaves, stems, and roots do not synthesize comparable amounts of IAA; therefore, the type of tissue and its state of growth are important factors for determining IAA concentrations found at various locations within a plant during development. For example, the endosperm of a germinating maize seed was measured to contain 308 pmoles of IAA while its shoot contained 27 pmoles of IAA (Epstein et al., 1980). In general, a hormone is an effective chemical messenger only if it has a limited lifetime in the target cell in order to maintain its dynamic regulatory functions. Therefore the amount of hormone in a cellular pool must be closely regulated and maintain a rapid rate of metabolic turnover.

Auxin levels are regulated through three main processes: several biosynthetic pathways, several pathways for the conjugation of IAA to sugars and/or to amino acids, and several degradation pathways (Normanly, 1997; Bartel, 1997; Slovin et al., 1999). Figure 1-1 presents a simplified diagram of steady state levels of free IAA. The homeostatic control of the active auxin pool is a complex system with many metabolic, physiological, and developmental considerations.

The shikimic acid pathway is the only known source of benzene ring compounds found in nature. This pathway produces tryptophan, an abundant amino acid known to

form IAA through several pathways (Cohen, 1983; Gaspar and Hofinger, 1968; Sembdner et al., 1981; Zazimalova and Napier, 2003). Recently two genetically discrete pathways from tryptophan to the intermediate indole-acetaldoxime have been found, reiterating further redundancy in auxin biosynthesis (Cohen et al., 2003). Traditionally, synthesis is normally studied by feeding the plant radiolabeled tryptophan, carrying carbon ($^{14}C$) or tritium ($^{3}H$), and then the subsequently labeled IAA and its intermediates are quantified via GC-MS technology. This technology has shown that the tryptophan pool is typically at least three orders of magnitude larger than the IAA pool (Epstein et al., 1980). However, low rates of IAA pool labeling via tryptophan have caused researchers to investigate other possible routes of biosynthesis. For example, using *Lemna gibba*, Baldi et al. (1991) examined the ability of D- and L- tryptophan to label endogenous IAA pools. While both forms were taken up quickly, only a small amount of L-tryptophan was able to label the IAA pool, thereby suggesting that IAA was not made solely from tryptophan. Wright et al. (1991) studied a maize *orp* (*orange pericarp*) mutant that is a double recessive for both tryptophan synthase β genes and is therefore unable to synthesize tryptophan. However, *orp* seedlings produce 50-fold higher levels of IAA than wild type seedlings. Because the precursor $^{15}N$-anthranilate was readily converted to IAA but not to tryptophan, they concluded that a tryptophan-independent pathway for IAA biosynthesis was operating in maize seedlings. Normanly et al. (1993) showed similar findings in *Arabidopsis thaliana*.

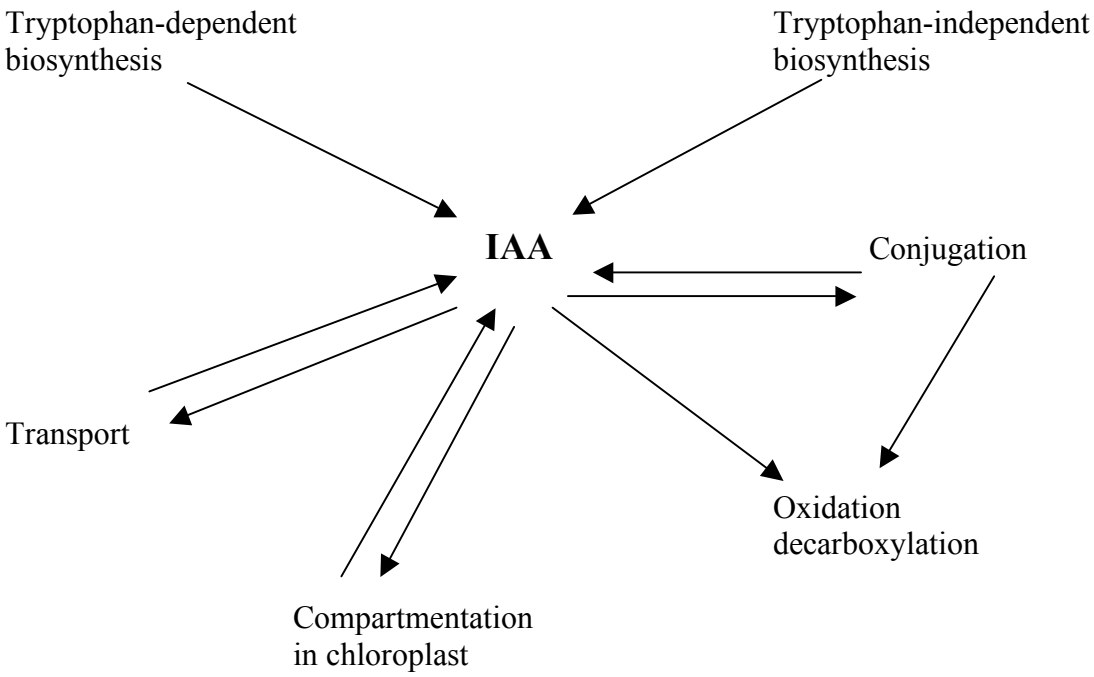



**Fig. 1-1**: A simplified diagram of the steady-state levels of free IAA in plant cells (modification of Normanly et. al., 1997)

Excised organs, tissue sections, cultured cells and cell-free preparations can utilize tryptophan-dependent pathways to carry out IAA biosynthesis (Wildman et al., 1947; Sembdner et al., 1981; Nohebel et al., 1993); this was further supported by analytical work on isolated axes of germinating bean seedlings (Bialek et al., 1992; Sztein et al., 2001), embryogenic cells in carrot cultures (Michalczuk et al., 1992), and excised maize coleoptiles (Koshiba et al., 1995). Two tryptophan-independent pathways appear to be the predominant IAA biosynthetic pathways operating in the intact plants examined: *Lemna* (Baldi et al., 1991; Rapparini et al., 1999), globular carrot somatic embryos (Michalczuk et al., 1992), and wild type *Arabidopsis* seedlings (Normanly et al, 1993). In all experiments, labeled precursors common to both the tryptophan-dependent and – independent pathways resulted in much greater enrichments of the IAA pool than the enrichment observed with labeled tryptophan. It is now thought that the tryptophan-independent pathway predominates over the tryptophan pathway in ordinary vegatative tissue (Bandurski et al. 1995).

Native auxins exist as free acids and conjugated forms. During developmental processes, the active form of IAA is in its free state but most IAA metabolites accumulate as conjugates. Conjugates of IAA come in two main types: amide conjugates that link IAA to amino acids or peptides (e.g. IAA-aspartate or IAA-peptide), and ester conjugates that link IAA to sugars and other molecules (e.g. IAA-glucose) (Cohen and Bandurski 1982). The conjugates are thought to serve as: (1) reserve forms for homeostatic control of hormone concentration, (2) transported forms for the seed to shoot transport, and (3) stable forms for protection of IAA mostly against oxidation (Cohen and Bandurski 1982).

Therefore, conjugates are the predominant forms of auxin found in most plants and are thought to act as short-term intermediates that can be hydrolyzed to release free IAA (Cohen and Bandurski, 1982; Normanly, 1997). One example is that 20-60% of the IAA requirement of a germinating maize shoot is met by the hydrolysis of IAA conjugates supplied by the endosperm (Epstein et al., 1980). Once the bound hormone is released, it is transported to the necessary location (for further discussion on auxin transport, see next section within this chapter). Conjugation/hydrolysis balance appears to regulate the free IAA levels along with degradation via decarboxylation and oxidative pathways.

Understanding the importance that hormones might play in the evolution of developmental mechanisms, Sztein et al. (1995, 1999, 2000) performed a comprehensive survey of IAA metabolism in the major divisions of land plants. They showed that IAA biosynthesis in representative non-seed plants is carried out through a tryptophan-independent pathway. For example, the apical regions of both charophytes and liverworts synthesize IAA via a tryptophan-independent pathway, with IAA levels being regulated via the balance between the rates of IAA biosynthesis and IAA degradation. Liverworts, mosses and vascular plants can be differentiated by the total amount of IAA metabolites, the proportion of free and conjugated IAA, the chemical nature of their IAA conjugates, and their IAA conjugation rates. Liverworts appear to employ a biosynthesis-degradation strategy for the regulation of free IAA levels, in contrast to the conjugation-hydrolysis strategy apparently used by mosses and vascular plants. The apical regions of all land plants utilize the same class of biosynthetic pathway, but mosses and vascular plants have greater potential to utilize IAA conjugation and conjugate hydrolysis reactions to achieve more precise spatial and temporal control of IAA levels (Baldi et al.,

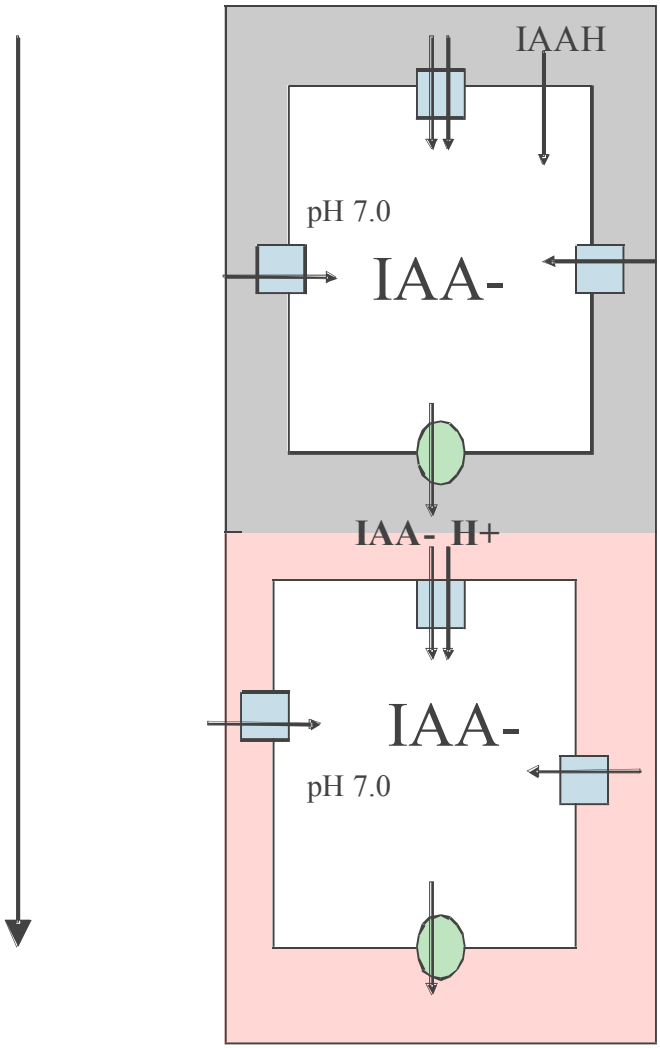
1991; Wright et al., 1991; Michalczuk et al., 1992; Normanly et al., 1993; Sztein et al., 1995, 1999, 2000).

**The basic information on auxin transport**

There are three types of intercellular auxin transport: lateral, vascular, and polar transport. *Lateral auxin transport* is movement associated with tropic responses (as with the Darwin's seedling response). For example, during phototropism IAA moves from the illuminated side of the organ to the dark side, which causes the differential elongation necessary for organ bending. Long distance transport is commonly referred to as *vascular transport* since it occurs via the phloem. Auxin velocities in the phloem have been measured to range between approximately 10 and 20 mm/h. This type of long-distance transport is crucial for apical dominance and other processes in whole plant development.

*Polar transport* is defined as movement of a substance in a specific direction and generally takes place over a short distance, for example, within an apical region. According to the chemiosmotic model (Fig. 1-2), the total proton motive force across the plasma membrane drives the uptake of auxin (influx) and the polar distribution of the efflux carriers results in auxin movement in a polar fashion from cell-to-cell through shoot and root tissues (Rubery and Sheldrake, 1974). Auxin must therefore enter the cell either through passive diffusion across the membrane (Rubery and Sheldrake, 1974), or via a protein symport $2H^+/IAA^-$ carrier (Lomax et al., 1995). The passive permeability of the membrane to auxin depends on the interaction between the pKa of auxin (4.75) and the ambient pH of the cell wall outside the plasma membrane. The lipophyllic protonated form of IAA can readily diffuse from the cell wall (pH 5) across the lipid bilayer into the

**Fig. 1-2**: The chemiosmotic model of polar auxin transport between two plant cells (modified from Lomax et al. 1995). Auxin travels from the apex to the base (large black arrow). Influx carriers are represented by blue boxes and efflux carriers are represented by the green ovals.



cytosol (pH 7). By contrast, the dissociated form of IAA in the cell wall does not cross the membrane unaided and requires the aid of an import carrier. Evidence for a proton symporter comes in part from the ability of a specific inhibitor napthoxyacetic acid (1-NOA) to inhibit auxin uptake (Bennett et al., 1996; Imhoff et al., 2000). Once inside the cytoplasm the IAA dissociates into the anionic form due to the more basic environment. Efflux of IAA must again be carrier aided but this time the carrier needs to be located basally only in order for polarity to occur. PIN is involved in the basipetal efflux of auxin from a cell (Galweiler et al., 1998) and GNOM, an ADP-ribosylation factor G protein(ARF GEF), is thought to coordinate the polar localization of the auxin efflux carrier PIN1 via GNOM-dependent vesicle trafficking (Steinmann et al., 1999; Muday et al., 2003). This influx/efflux mechanism establishes the directionality of auxin transport.

Basipetal polar auxin transport occurs primarily in stems and leaves in the vascular parenchyma tissue. Coleoptiles are the exception because basipetal polar transport occurs in the nonvascular tissues. Bi-directional auxin transport occurs in roots (Scott and Wilkins 1968; Davies and Mitchell, 1972; Rashotte et al., 2003) with the majority of auxin reaching the root tip via phloem (Goldsmith et al., 1973; Cambridge and Morris, 1996). However, acropetal polar transport is associated with the xylem parenchyma of the stele (Palme and Galweiler, 1999) while basipetal auxin transport in the root tip occurs in the epidermal and cortical tissues (Young and Evans, 1996; Rashotte et al., 2000; Swarp et al., 2001; Rashotte et al., 2001; Friml et al., 2002a, b)

One is able to manipulate the model of polar auxin transport with transport inhibitors to examine the role of auxin transport in the establishment of cellular polarity and overall development. The inhibitors 1-N-naphthylphthalamic acid (NPA), 2,3,5-

triiodobenzoic acid (TIBA) and α-(p-chlorophenoxy) isobutyric acid (PCIB), affect auxin transport differently due to mechanistic differences between them. Auxin transport inhibitors prevent polar auxin transport by blocking auxin efflux; however, the mechanism for this differs between a phytotropin and a non-phytotropin. According to the conventional theory, NPA, a phytotropin, may bind to a protein that interacts with another protein at the efflux carrier while TIBA, a non-phytotropin, may bind directly to the auxin-binding site on the efflux carrier (Lomaxet al., 1995). NPA can inhibit lateral transport associated with tropisms but TIBA cannot. Also, NPA is neither transported nor contains any auxin activity while TIBA is transported polarly and has weak auxin activity. Another model for polar auxin transport suggests that it is not a carrier-mediated flux across the plasma membrane but rather a neurotransmitter-like secretion since auxin transport is mediated by regulated vesicle trafficking (Baluska et al., 2003). With that in mind, NPA does interfere with the secretory process. PCIB acts as a weak auxin that inhibits the effects of endogenous auxin. PCIB may compete for auxin receptors insofar as PCIB inhibition can be overcome by the simultaneous application of exogenous auxin.

Higher plants such as maize, bean, pea, and cucumber have been the historical focus for auxin transport work. The auxin transport in lower plant literature is not extensive but transport has been measured in most groups, from liverworts to ferns, and tends to focus on the gametophytic structures. Tables 1-2 and 1-3 give a summary of the data available on basal land plants in relation to auxin transport. For example, it appears that *Chlorella vulgaris,* a single cell alga, does not posses auxin carriers while the multicellular green alga *Chara vulgaris* has influx and efflux carriers in its thallus (Dibb-Fuller and Morris 1992). *Chara globularis* rhizoids also have both carriers and are

strongly inhibited by NPA, suggesting phytotropin receptors (Klambt et al. 1992). The gametophyte of *Marchantia polymorpha* appears to possess auxin transport carriers and phytotropin receptors (Maravolo 1976, 1980; Gaal et al. 1982) while the setea of *Pellia epiphylla* seems to lack polar auxin transport (Thomas, 1980). But these liverwort results are highly questionable. Maravolo (1976, 1980) and Gaal et al. (1982) sandwiched the thallus between agar so that different cell types are exposed to the auxin and therefore, one can not distinguish between uptake differences and transport differences in their experiments. The setae results lack transport inhibitor information, so a complete analysis cannot be made. The moss *Funaria hygrometrica* shows classic influx and efflux carrier physiology with sensitivity to phytropins in both the protonema and rhizoids (Rose and Bopp, 1983; Geier et al., 1990). In summary, it appears that the lower plants possess influx and efflux carriers, but differ in terms of the degree of transport polarity. It is obvious that the moss *Funaria hygrometrica* and the ferns have extreme polar auxin transport, but the picture is less clear for the liverworts and lycophytes.

**Table 1-2**: A summary of flux experiments used to characterize transmembrane IAA fluxes and the potential for polar IAA transport in green plants. IAA accumulation increase was calculated as the ratio of the difference in net IAA accumulated in inhibitor vs. control experiments over the net IAA accumulated in control experiments times 100, as reported in the cited references.

| Group | Species | Generation | Structure | Inhibitor | % Increase in IAA concentration | References |
|---|---|---|---|---|---|---|
| Algae | *Fucus distichus* | Sporophyte | Zygotes | 5 x $10^{-5}$ M NPA | 58 | Basu et al. (pers. comm.) |
| | *Chorella vulgaris* | Gametophyte | Unicells | 3 x $10^{-6}$ M NPA | 0 | Dibb-Fuller and Morris (1992) |
| | *Chara globularis* | Gametophyte | Thallus segments with rhizoids | 1 x $10^{-4}$ M NPA | 67 | Klambt et al. (1992) |
| | *Chara vulgaris* | Gametophyte | Thallus | 1 x $10^{-5}$ M NPA | 0 | Dibb-Fuller and Morris (1992) |
| | | | | 1 x $10^{-5}$ M TIBA | 0 | Dibb-Fuller and Morris (1992) |
| Mosses | *Funaria hygrometrica* | Gametophyte | Protonemal | 1 x $10^{-5}$ M NPA | 32 | Geier et al. (1990) |
| | | | Protoplasts | 1 x $10^{-5}$ M TIBA | 29 | Geier et al. (1990) |
| | | | Protonemata | 1 x $10^{-5}$ M TIBA | 27 | Rose et al. (1983) |
| | | | Rhizoids | 1 x $10^{-5}$ M TIBA | 96 | Rose and Bopp (1983) |
| Angiosperms | *Zea mays* | Sporophyte | Coleoptile | 1 x $10^{-5}$ M NPA | 140 | Sussman and Goldsmith (1981) |
| | | | Segments | 1 x $10^{-5}$ M TIBA | 64 | Sussman and Goldsmith (1981) |
| | *Cucurbita pepo* | Sporophyte | Hypocotyl | 1 x $10^{-5}$ M NPA | 80 | Hertel et al. (1983) |
| | | | Vesicles | 1 x$10^{-5}$ M TIBA | 84 | Hertel et al. (1983) |

**Table 1-3**: A summary of agar-block experiments used to characterize polar IAA transport in green plants. Polarity (B/A) ratio was calculated as the ratio of the basipetal transport over the acropetal transport rates reported in the cited reference. Transport inhibition was calculated as the ratio of the difference in basipetal transport rates in control vs. inhibited structures over the basipetal rate in control structures times 100.

| Group | Species | Generation | Structure | Polarity (B/A) ratio | Inhibitor | Transport inhibition (%) | References |
|---|---|---|---|---|---|---|---|
| Liverworts | *Marchantia polymorpha* | Gametophyte | Thallus | 5.3 | $10^{-3}$ M TIBA | 62 | Maravolo (1976); Gaal et al.(1982) |
| | *Pellia epiphylla* | Sporophyte | Seta | 0.9-1.1 | $10^{-5}$ M NPA | 0 | Thomas (1980); Poli et al. (2003) |
| Hornworts | *Phaeoceros pearsoni* | Sporophyte | Immature sporangium | 0.9 | $10^{-5}$ M NPA | 0 | Poli et al. (2003) |
| Mosses | *Funaria hygrometrica* | Gametophyte | Rhizoid | 11.4 | $10^{-4}$ M TIBA | 54 | Rose and Bopp (1983) |
| | *Polytrichum ohioense* | Sporophyte | Seta | 9.1 | $10^{-5}$ M NPA | 11 | Poli et al. (2003) |
| Pteridophytes | *Selaginella willenovi* | Sporophyte | Stem | 2.1 | None applied | -- | Wochok and Sussex (1973) |
| | *Osmunda cinnamomea* | Sporophyte | Rachis | 190.0 | None applied | -- | Steeves and Briggs (1960) |
| | *Regnellikium diphyllum* | Sporophyte | Rachis | 52.0 | None applied | -- | Walters and Osborne (1979) |
| Angiosperms | *Zea mays* | Sporophyte | Coleoptile | 609.3 | $10^{-5}$ M NPA | 99 | Poli et al. (2003) |
| | *Cucurbita pepo* | Sporophyte | Hypocotyl | 20.0 | $10^{-5}$ M TIBA | 68 | Jacobs and Hertel (1978) |

When one takes into consideration the work of Sztein et al. (1999), some potential correlations become evident. Algae and liverworts appear to regulate free auxin levels via a biosynthesis-degradation strategy, while mosses, lycophytes, and ferns use more efficient conjugation reactions to maintain auxin homeostasis. This correlation has led to several possible theories for the evolution of polar auxin transport: 1. the pattern of conjugate choices may dictate the relative polarity of auxin transport, and 2. the increased facility for regulating free auxin levels due to conjugate hydrolysis may have favored the subsequent evolution of polar transport.

**The role of auxin in angiosperm development**

There are several examples of how IAA is an important regulator of developmental processes in the seed plants. Angiosperm embryo development requires the sequential activation of two different auxin biosythetic pathways (Michalczuk et al. 1992a, b; Cooke et al., 1993; Ribnicky et al., 2002), a tryptophan-dependent pathway and a tryptophan-independent pathway. The tryptophan-dependent pathway results in high biosynthetic rates that mediate isodiametric growth in young embryos. The tryptophan-independent pathway, in conjunction with polar auxin transport, establish polarized growth of older embryos (Schiavone and Cooke, 1987; Liu et al., 1993; Steinmann et al., 1999). Establishing IAA gradients through localized synthesis and/or polar transport is essential for positioning leaf primordia on shoot apices (Meicenheimer, 1981; Lyndon, 1998; Reinhardt et al., 2000; Hay et al., 2004) and for initiating lateral root primordia on mature roots (Blakely et al., 1988; Reed et al., 1998; Casimiro et al., 2001). IAA acts as

an intercellular signal coupling environmental stimuli to growth responses in phototropism and gravitropism (Kaufman et al., 1995; Dolan, 1998; Marchant et al. 1999). Finally, vascular tissue development is also controlled by IAA with respect to primary vascular tissue induction (Roberts et al., 1988; Aloni, 1995), primary vascular bundle positioning (Sachs, 1991; Berleth et al., 2000), and vascular cambia activity (Uggla et al., 1996, 1998; Kessler and Sinha, 2004).

There have been great advances in understanding the molecular mechanisms behind the role of IAA action and transport on development (Muday and DeLong, 2001; Friml and Palme, 2002; Leyser, 2002). One interesting example for this thesis is the role of the PIN protein family of auxin efflux carriers on the distribution of auxin throughout the plant (Friml, 2003). PIN proteins mediate various developmental proceses: vascular tissue and flower development (PIN1, Galweiler et al., 1998), tropisms (PIN2, PIN3; Muller et al., 1998; Friml et al., 2002b), and patterning of the root (PIN4; Friml et al., 2002a). Asymmetrically located PIN proteins result in dynamic cellular efflux of auxin gradients especially at primordia tips. Therefore, the PIN-dependent local auxin gradients result in the formation of all plant organs (Benkova et al., 2003)

**The role of auxin in the development of other land plants**

The effects of auxin, its analogues and anti-auxins, are generally found as studies focused on a specific organism in the lower-plant development literature. The occasional review tends to focus on a particular group's hormonal regulation of a unique developmental process, for example, moss chloronema-to-caulonema transitions (Bopp, 1980; Cove and Ashton, 1984; Bhatla et al., 1996; Christianson, 1999). In order to describe the roles that IAA plays in several developmental processes that occur

throughout the land plant lineage, I surveyed the literature and classified the widespread IAA responses into somewhat arbitrary categories: tropisms, correlative interactions, and positional relationships, in order to provide a general overview of certain IAA responses in the non-seed plants.

*IAA and tropisms*

In order to thrive in a terrestrial environment, the early land plants had to evolve novel physiological processes to cope with the non-uniform distribution of essential resources. The evolution of tropistic growth responses allowed multicellular plants to be able to orientate toward light and gravity stimuli. Buoyancy mechanisms give multicellular algae the ability to upright their orientation in the water in relation to light; therefore they lack tropistic responses (Bold and Wynne, 1985) but the filamentous charophycean algae and land plant protonemata exhibit tropic responses within individual cells (Wada and Kadota, 1989; Sievers et al., 1996; Braun, 1997; Staves, 1997). The evidence reported in those papers indicates that these plants do not use IAA as the intracellular signal for either phototropism or gravitropism.

Through the phototropic investigation of the liverwort *Pellia* setae, it can be stated that IAA was recruited early in land plant evolution to serve as the intercellular signal between the apical cells and the subapical cells needed for rapid directional growth (Thomas, 1980). When the side of the setae are shaded, they become acidic prior to phototropic curvature; this acid efflux and curvature are able to be inhibited by both neutral buffers and IAA antagonists, suggesting that seta phototropism is mediated by lateral IAA transport as a result of enhanced proton efflux and wall loosening on the shaded side (Ellis and Thomas, 1985). This mechanism appears almost identical to the

mechanism proposed to operate in seed plants (Kaufman et al., 1995). An interesting difference between phototropism in *Pellia* and seed plants is the sensitivity to the polar transport inhibitor TIBA. This inhibitor blocks seta phototropism in *Pellia* (Ellis and Thomas, 1985) but not in seed plants (Lomax et al., 1995). The lycophyte *Selaginella* exhibited asymmetric IAA accumulation and phototropic curvature when light was applied asymmetrically and similar results could be witnessed when IAA was applied on either side of the stem independent of the light conditions (Bilderback, 1984). TIBA could also block the phototropic responses of these stems. Thus, it can reasonably be concluded that lateral IAA gradients act as the common intercellular signaling mechanism for phototropic responses throughout the land plant lineage; however, the differential sensitivity to transport inhibitors implies that the regulation of these gradients may differ in non-seed vs. seed plants.

*IAA and correlative interactions*

*Apical dominance* is defined in seed plants as the ability of IAA from a growing region to maintain the quiescence of preexisting meristematic regions and/or to inhibit the formation of new meristematic regions. Several species of liverworts exhibit gemmae growth that is suppressed by exogenous IAA (LaRue and Narayanaswami, 1957; Maravolo and Voth, 1966; Stange, 1971), which appears to be an apical dominance phenomenon in liverworts similar to that seen in seed plants. As another example, Davidonis and Munroe (1972) presented surgical evidence that IAA transported from the larger branch of *Marchantia* thalli inhibited the growth of adjacent smaller branches. In the moss *Sphagnum*, a genuine apical dominance example was witnessed when gametophore apices were decapitated resulting in vigorous growth of reactivated lateral

buds (MacQuarrie and von Maltzahn, 1959). The application of IAA to the cut apex suppressed bud activation, whereas cytokinin overcame the IAA inhibition of bud activation (von Maltzahn, 1959), in a manner very similar to the hormonal regulation of apical dominance observed in seed plants (Tamas, 1995). Polar transport inhibitors placed in a lanolin ring around the intact moss *Plagiomnium* gametophores led to bud activation below the ring similar to decapitated gametophore responses (Nyman and Cutter, 1981). However, the simultaneous application of IAA and a cytokinin was required to suppress all microscopic indications of bud activation in this moss.

Several ferns also show lateral bud inactivation if shoots are decapitated (Wardlaw, 1946; Croxdale, 1976). Pilate et al. (1989) quantified the levels of IAA and cytokinins in growing, quiescent, and activated buds in a fern (*Marsilea*), and in general, they observed that growing buds exhibited much higher hormone levels than quiescent buds. Fern lateral buds, similar to that of seed plants, can become activated upon apical bud removal if cytokinin precursors in those buds remain inactive (Pilate et al., 1989).

*IAA and positional relationships*

Each division of land plants can be said to exhibit a characteristic body plan based on such organizational features as axial polarity, embryo structure, meristematic activity, vascular tissue organization, positional relationships among vegetative organs, and positional relationships of reproductive organs on vegetative organs (Bold et al., 1987; Gifford and Foster, 1989). When the limited literature is examined, IAA regulates the body plans of seed plants and this trend is similar in the non-seed plants, at least with respect to the positions of absorptive structures and vascular tissue differentiation.

Numerous observations exist for IAA mediation of rhizoid formation in the non-seed plant gametophytes: liverworts (e.g., Kaul *et al.*, 1962; Maravolo and Voth, 1966; Stange, 1977; Kumra and Chopra, 1987), mosses (e.g., Nyman and Cutter, 1981; Chopra and Vashistha, 1990), and pteridophytes (e.g., Haupt, 1957; Kato, 1957; Hickok and Kiriluk, 1984). Sievers and Schröter (1971) observed increased rhizoid formation on cut sections of *Chara* thalli, which suggests that the role of IAA mediating rhizoid formation is an ancient trend that evolved before plants moved to land. But did this ancient physiological IAA role lead to vascular plant root initiation regulation?

It appears that endogenous IAA in the extant pteridophytes examined controls lateral root initiation. In ferns, IAA mediated the formation of both lateral roots on excised *Pteridium* roots (Partanen and Partanen, 1963) and adventitious roots on *Matteuccia* rhizomes (Ma and Steeves, 1992). In *Selaginella*, root formation was greatly enhanced by the application of IAA and the IAA analogue indole-3-butyric acid at the leafless cylindrical axes called rhizophores (Williams, 1937; Webster, 1969; Wochok and Sussex, 1975). Furthermore, TIBA induced all lateral meristems to develop as shoots (Wochok and Sussex, 1975).

In seed plants it has been established that vascular tissue development is primarily regulated by IAA but this topic has not been as extensively investigated in the non-seed plants. Nothing is known about the hormonal regulation of the simple vascular tissues observed in certain bryophytes (Hébant, 1977; Ligrone et al., 2000) but a few pteridophyte studies exist. When IAA was applied in place of excised *Osmunda* pinnae, final xylem maturation occurred in the leaves (Steeves and Briggs, 1960). IAA may regulate the patterning of vascular bundles in pteridophytes. Vascular tissues in

*Matteuccia* stems, generally a dictyostele, resulted into a siphonostele when the leaf primordia on the apices were suppressed. When IAA-soaked beads were applied to the suppressed apices, the stele exhibited parenchymatous leaf gaps similar to those formed in the dictyosteles of untreated plants (Ma and Steeves, 1992). Therefore, two possible explanations exist for these observations: 1. IAA acted to reduce the total amount of vascular differentiation in the suppressed apices and/or 2. IAA was involved in the positioning of the remaining vascular bundles. The latter is reminiscent of the positioning of primary vascular tissues in seed plants (Sachs, 1991; Berleth et al., 2000).

**Objectives**

What emerges from this survey is the surprising perspective that the physiological mechanisms for regulating IAA levels and many IAA-mediated responses found in seed plants are also present in charophytes and bryophytes, at least in nascent forms. The overall goal of this dissertation is to show that the hormone auxin has played a crucial role in the development and evolution of all lineages of land plants, including the bryophytes. Chapter 2 presents the different basal plants examined for potential system development and usage. Chapter 3 explores the evolutionary relationships of polar auxin transport in liverwort, hornwort and moss sporophytes. Chapter 4 further explores the mechanisms of polar auxin transport during the development of the moss sporophyte. Chapter 5 investigates the role of auxin on fern embryogenesis. Chapter 6 presents a summary of the results obtained in this work.

# CHAPTER 2

## POTENTIAL MODEL SYSTEMS: THE BAD, AND THE GOOD AND UGLY

The successful invasion of the land by the plants depended on two major evolutionary processes: 1) the evolution of various structures to facilitate the absorption, transport, and retention of water, and 2) the evolution of the sporophyte generation, including protected embryos and large post-embryonic organs such as leaves and roots, that helped these plants colonize diverse terrestrial environments. Together the cuticle, the waxy layer that covers the aerial parts of most plants, and the stomata, epidermal pores that are regulated by guard cells, aid in water retention and gas exchange. Sterile jacket cells enclosed the male and female gametangia (antheridia and archegonia respectively), and thus subsequent development of the embryo could occur protected inside the archegonium. Evolution of rhizoids, and later roots, enabled the plants to take water up from their surroundings as well as anchoring them to the soil.

As plants moved from water to land, the environment imposed selection pressures that were satisfied by the evolution of the embryo and the sporophyte. To compete for light acquisition, plants began to grow upwards rather than outwards which favored the evolution of an axis. Naked axes evolved first and only later did the axes become upright leaf-bearing stems. Evolving microphylls and then megaphylls made photosynthesis

more efficient by increasing the surface area available for light reception. Larger plant size resulted in the selection pressure for conducting tissues in order to move compounds to different areas of development and growth. Hydroids and leptoids arose in some bryophytes while more complex vascular cells evolved as plants became even larger resulting in xylem and phloem in the pteridophytes, gymnosperms, and angiosperms. All of these anatomical, morphological, and physiological changes resulted in three levels of life cycles: bryophytes have gametophyte dominant life cycles, pteridophytes have independent gametophyte and sporophyte generations, and seed plants have sporophyte dominant life cycles.

The identification of model systems for studying the evolution of plant development requires an analysis of all of the major non-seed plant groups taking into account the evolutionary significance. In order to examine any physiological phenomenon in an evolutionary context, one must manipulate plants that are not considered "model systems" since, historically, the primary criterion for establishing plant model systems are of agricultural importance (e.g. corn, wheat) and ease of genetic manipulation (e.g. *Arabidopsis*) rather than the evolutionary importance. Consequently, despite the evolutionary significance of the embryo and the sporophyte generation as a whole, we know nothing about the physiological mechanisms that may have operated in sporophyte evolution.

Several liverworts, hornworts, mosses, and ferns were examined as potential model systems for studying the evolutionary mechanisms operating in lower land plants. Based on the literature surveyed in Chapter 1, I hoped to identify model systems with the following characteristics: 1) easy manipulation in *in vitro* culture, 2) rapid progression

| **Hornworts** | **Liverworts** | **Mosses** | **Ferns** |
|---|---|---|---|
| *Anthoceros punctatus* | *Marchantia polymorpha* | *Funaria hygrometrica* cv "Duke" | *Ceratopteris richardii* |
| *Phaeoceros pearsoni* | *Pellia epiphylla* | *Funaria hygrometrica* cv "stream" | *Marsilea vestita* |
| | *Pallavicinia lyellii* | *Funaria hygrometrica* cv "Brachynema" | |
| | | *Polytrichum ohioense* | |
| | | *Sphagnum angustifolium* | |
| | | *Physcomitrella patens* | |

**Table 2-1**: Basal land plants examined as potential model systems

from fertilization to sporophyte development, 3) reliable induction of embryo formation, and 4) published evidence of auxin action, 5) putative evolutionary significance. Obviously, the selection of appropriate experimental systems must inevitably involve a compromise among these criteria. For example, it turned out that the only fern system that could be investigated with the current technology for auxin analysis is *Marsilea vestita*, which has the advantage of being also amenable to reverse genetics, but which is not an early divergent species. For a list of all species that I examined as potential model systems, see Table 2-1.

**Hornworts**

The Anthocerophyta, the hornworts, include approximately 100 species in total. In general, hornwort gametophytes are thallose, and thus, they are similar in appearance to liverworts. Hornwort cells contain a single large chloroplast like those found in many green algae, but unlike the multiple discoid shaped chloroplasts found in other land plant cells. Gametophytes can be unisexual or bisexual, depending on the species chosen. Therefore one must look for the presence of sunken archegonia on the dorsal surface of the gametophyte, while the antheridia are clustered in chambers toward the edges of the thalli. After successful fertilization, sporophytes develop an embedded foot, an intercalary meristem, and a long, cylindrical green capsule that is capable of limited photosynthesis. Stomata and a cuticle are present on the sporophyte but it lacks specialized conducting tissue (Hébant, 1977) (for further information on hornwort sporophyte morphology, see Chapter 3).

The class Anthocerotopsida contains the order Anthocerotales that includes 5 genera with three genera widely distributed in the United States: *Anthoceros*,

*Phaeoceros*, and *Notothylas*. *Anthoceros* and *Phaeoceros* are in the same family (Anthocerotaceae), and they have several species that grow as winter annuals in the southern United States. Due to the wide variety of possible collection sites and the ability to collect species for a longer time frame, Anthocerotales became the order of choice for my experiments. Also both genera have embryos that are easy to dissect out of the protective gametophytic tissue, and they generate similar linear sporophytes that are substantially different than those found in the liverworts and mosses (see Chapter 3).

I was hopeful that both species, *Anthoceros punctatus* and *Phaeoceros pearsoni*, would be able to be propagated in the laboratory, in soil and/or in culture, because several reports claimed that gametophytes grown with the nitrogen fixing bacteria *Nostoc* would give rise to antheridia when exposed to photoperiods of 4-12 hours resulting in sporophytes (Ridgway, 1967). However, I could not induce either hornwort species to produce sporophytes on the bench top, in soil from its original environment, or in culture with or without its symbiont at the suggested photoperiods. Other potential propagation problems included the apparent need to adjust light and humidity conditions throughout the maturation process (Dr. Daniel Norris, personal communication). Therefore, hornworts were used only for the study of axial elongation in Chapter 3.

**Liverworts**

Hepatophyta, the liverworts, is a division that contains about 6000 species that lack specialized conducting tissue, a cuticle, and stomata making these plants the simplest of all living land plants (Hébant, 1977). The gametophytes of most liverworts are composed of an axis bearing thin leaves and grow from a single apical cell, but some are thallose and grow from an apical meristem (Bold, 1973). Leafy liverworts make up more

than 4000 of the 6000 species of this division, and they are most abundant in the tropics and subtropics. The leaves are a single layer of non-differentiated cells and lack the thickened midrib found in the leaves of moss gametophytes (Bold, 1973). In contrast, the thallose gametophytes are non-leafy and the thallus is many cells thick and differentiated into a chlorophyll-rich dorsal region and a colorless ventral portion. On the dorsal surface of some thallose liverworts are large pores that lead to internal air chambers. On both thallose and leafy gametophytes, rhizoids are single-celled and arise as two different types. One thallose liverwort, *Marchantia*, produces specialized structures known as gametophores that house the gametangia. Sporophytes are composed of a foot, seta, and a capsule that matures prior to elongation (for more information and discussion see Chapter 3).

*Marchantia polymorpha* is a very common thallose liverwort that is found worldwide. This liverwort was investigated as a potential experimental system because it grows very well in the greenhouse, on the bench top, and in culture, but gametangia production proved to be very challenging. *Marchantia* gametophytes are propagated in culture by thallus tip transfers (Maravolo, 1976; Sztein et al., 1999) or through gemmae obtained from the male and female gametophytes (Schneider et al., 1967). I utilized gemmae from a healthy and highly fertile population at the University of Maryland greenhouse to start sterile cultures. *Marchantia* can be grown vegetatively on a Knops formula medium supplemented with sugar, iron and micronutrients; vermiculite supplemented with Voth's nutrient solution; or M-5 nutrient solution, but it is photoperiod, light quality, humidity, and temperature that are critical for inducing gametangia (Klebs, 1903; Wann 1925; Voth and Hammer 1940; Voth, 1943; Chopra and

Bhatla, 1983). Gametangia induction requires longer photoperiods of at least 16 hours light daily and this light must be of a particular light quality (Chopra and Bhatla, 1983). Gametangia induction is also temperature dependent (Chopra and Bhatla, 1983); if the temperature varies within 2°C during sex-organ development, gametangia are not induced. High humidity is reported to hasten gametangia development and fertilization, but different humidity levels are required for each developmental stage.

Despite the reports in this literature, when I treated the gametophytes gametangia as above, only a few gametophytes developed gametangia in a rather asynchronous manner. I changed temperature and light conditions from 10 °C and 200 μmol/$m^2$/s to 18 °C and 150 – 200 μmol/$m^2$/s since both variations are reported to induce gametangia (Chopra and Bhatla, 1983), yet gametangia production was still low and asynchronous. I also varied the carbohydrate to nitrogen ratio by modifying the media provided in the culture dishes. A higher ratio resulted in about a 10% increase but the number of gametangia produced was still not adequate.

*Pallavicinia lyellii* was another species easy to get into culture (Sztein et al., 1999) but neither gametangia induction nor fertilization was reliable. *Pellia epiphylla,* another thallose liverwort, was tried for the same reasons as the *Marchantia* species. Increasing the photoperiod to at least 16 hours daily can induce gametangia in this species, but again, this liverwort is sensitive to both photoperiod and temperature (Chopra and Bhatla, 1983). In addition, *Pellia* will not grow or develop in an axenic culture due to its requirement for a symbiotic relationship with a bacterium (unknown at this time which species) similar to the hornworts. Therefore, *Pellia* was collected between April and May to ensure the presence of young sporophytes; the plants were stored at 0-4 °C in

darkness for up to a month without effecting additional growth and development of the sporophytes before they were used in Chapter 3 (Thomas et al., 1983).

**Mosses**

Bryophyta, the mosses, contains approximately 9500 species that are found worldwide. Gametophytes exhibit two growth phases: the initial protonema arising from a germinating spore develops into an upright leafy gametophyte. The protonemal stages are characteristic of all mosses, and they are present in some liverworts but no hornworts. Typically, the leaves of moss gametophytes are single cell thick except at the midrib. Specialized conducting tissues called hydroids (water-moving) and leptoids (sugar-moving) are found in many leafy gametophytes as well as in some moss sporophytes (for more discussion about conducting tissues, see Chapter 5) (Hébant, 1977). Rhizoids are multicellular in the mosses, in contrast to the single-celled rhizoids of liverworts and hornworts. The female gametophytes bear the sporophytes that take 6 to 18 months to reach maturity for temperate species. A foot, seta, and capsule make up the moss sporophyte (for more information see Chapter 3). The presence of stomata on the sporophytes is thought to represent one of the evolutionary links to vascular plants.

*Funaria hygrometrica* and *Physcomitrella patens*, the two systems used in genetics and molecular biology, are rather well understood and able to be manipulated in culture (for more information, see Collier and Hughes 1982; Cove and Knight, 1998). They tend to not be as dependent on day length as the liverworts in order to produce gametangia, yet mosses do exhibit variable temperature-photoperiod interactions that coincide with their natural habitats. *Funaria* was propagated vegetatively utilizing a Knop's media with the addition of 1% glucose, iron and formulation VII micronutrients

at pH 6, 30 μmol/$m^2$/s continuous light and 23°C (Sztein et al. 1999). Under our chamber conditions, *Funaria* gametangia were obtainable by lowering the temperature to 10 °C and modifying the light quality to 25 μmol/$m^2$/s. Fertilization was attempted using several methods: splashing the gametangia with water, flooding and then draining the gametangia, and by trying to collect sperm and deliver them directly to the archegonium by hand. None of the methods were completely reliable in culture, insofar as I was able to produce only a few sporadic sporophytes. Therefore, I used the species *Polytrichum ohioense* because it could be collected very easily on the University of Maryland campus during critical times during sporophyte development. Moreover, the embryo and sporophytes of this species resembles the same characteristics of *Funaria* and *Physcomitrella*.

**Ferns**

Pryer et al. (2001) utilized 136 vegetative and reproductive characters and four genes (plastid atpB, rbcL, rps4, and nuclear small-subunit ribosomal DNA) from 35 representative species to show that extant vascular plants belong to three monophyletic groups: lycophytes, seed plants and a clade including equisetophytes, psilophytes and all eusporangiate and leptosporangiate ferns. This analysis suggested that horsetails and ferns are the closest relatives to seed plants, instead of the traditional perspective that they represent the transitional evolutionary grades between bryophytes and seed plants. Therefore in order to gather a complete picture for the evolution of plant embryogenesis, I wanted to examine embryo development in the ferns.

*Ceratopteris richardii* is a model system that is used for genetic studies and molecular biology (Hickok et al., 1995). Therefore, it is easy to manipulate this species

in the greenhouse and in tissue culture. But I observed that embryo development was rather slow and asynchronous for *Ceratopteris* because it took several months following fertilization to develop conspicuous sporophytes. Considering that I was planning long-term treatments with this species, it did not seem to be an appropriate experimental system since the embryo of *Ceratopteris*, like most other ferns, arises inside the archegonium attached to a much larger gametophyte. Furthermore, it would be difficult to observe the effects of auxin-regulatory compounds on developing embryos due to compounding interactions with the maternal gametophytes. By contrast, the water fern *Marsilea* is not plagued by these problems.

*Marsilea: a good and ugly model system for embryogenesis*

This section presents both literature data and my own observations that were used to evaluate *Marsilea vestita* as a model system for embryogenesis. In order to gain a complete picture of current knowledge about this fern, I surveyed all papers (approximately 150) investigating the species but I shall only cite here those relevant to its development as a system for studying embryogenesis.

*Evolutionary significance of Marsilea* -- All water ferns, including the Marsileales, Salviniales, and Azollales, are derived from a common terrestrial ancestor, meaning that they form a monophyletic group within the paraphyletic homosporous leptosporangiate ferns (Schneider & Pryer, 2002). Fossil records of quadripinnate fronds and presence of sporocarps, lacking *in situ* spores, have been observed for Marsileaceae from as far back as the Lower Cretaceous (Skog & Dilcher, 1992). The compressed branching systems of *Azolla* are known from the Upper Cretaceous (Bell & Hensley, 2000); however, megaspores morphologically similar to those of extant *Marsilea* are unknown (Lucia et

al., 2000). Monomegaspory is restricted to the water ferns, and therefore becomes a relevant link to the evolution of heterospory and seed habit evolution (Rothwell & Stockey, 1994; Hasebe et al., 1995; Pryer et al., 1995; Schneider, 1996; Stevenson & Loconte, 1996; Kendrick & Crane, 1997; Pryer et al., 2001).

*A closer look at the plant and its gross morphology –Marsilea*, the common water fern, was named after the Italian naturalist Count F. L. Marsigli. This plant is found in temporary ponds and shallow ditches all over the world with greater abundance in the Southern and Pacific regions of the US. When one thinks of a fern what comes to mind is a large dissected frond with brown sporangia found on the back of that frond. However, *Marsilea*, which has roots and a stolon-like stem, has compound leaves that resemble a four-leaf clover and a hard round sporocarp that produces the spores. Most species have the ability to survive in conditions of low water or cold temperatures as long as the stem is partially covered under mud. However, at least one species is xerophytic.

Leaves are inserted in two ranks on opposite sides of the stem. In general, the leaves exhibit a dichotomously branched, but laterally and marginally united leaf vein pattern. However, the leaf type reflects *Marsilea*'s ability to live in either an aquatic or terrestrial environments. Submerged leaves have smaller blades, lack a cuticle and differentiate into palisade and spongy mesophyll (Gaudet, 1964). Floating plant leaves have flaccid petioles that utilize air chambers to float the leaflets pointing the upper leaf surface stomata toward the air-water interface (Bold, 1973). In direct comparison, terrestrial leaves lack petiolar air chambers, have rigid support for leaflets, possess upper and lower epidermal stomata on both leaf surfaces, and produce the mesophyll composed of palisade and spongy cell types (Bold, 1973). The ability for *Marsilea* to change

morphologically from an aquatic to a terrestrial form is regulated by the hormone abscisic acid (ABA) (Hsu et al., 2001), which is also known for becoming active during water stress in angiosperms.

The *Marsilea* stem cortex is composed of vascular tissue and pith with the cortex interrupted by large air chambers and covered by an epidermis. Vascular tissue forms an amphiphloic siphonostele with the inner and outer phloem surrounded by a single layer of pericycle cells, which are further surrounded by a single endodermal layer (Bold, 1973). Changes in the environment will cause slight differences within the pith cells; submerged plants are parenchymatous while non-submerged plants are sclerotic. Adventitious roots are found at the nodes of the stolen-like stem. An endodermis surrounds the root stele, while the cortex is divided into a sclerotic inner layer and an air chamber-containing outer layer (Bold, 1973).

*Spore development – Marsilea* sporangia develop within specialized structures called sporocarps that are evolutionarily derived from a folded fertile pinna with united margins. Depending on the species, sporocarps develop singly or in clusters on short lateral axes of the petioles of terrestrial individuals, in response to water depletion. Sporocarps mature from a soft, green structure to a brown, nutty one. Each lateral half, within the sporocarp, contains a row of elongated sori within receptacles where an individual sorus is covered with a delicate indusium. *Marsilea* is a leptosporangiate fern producing 32-64 heterosporous spores called megaspores and microspores (Schneider & Pryer, 2002). Sporocarps can be easily maintained, once collected, by placing the dry sporocarp into plastic bags, and herbarium samples have been known to remain viable for over 100 years (Raven et al., 1999).

In order for a sporocarp to open and shed its spores in nature, the outer wall must become broken down by bacterial action (Bold, 1973) or weathered and abraded (Bell and Hemsley, 2000) to allow water to enter. Parenchymatous tissue in the outer wall is only one cell layer thick with the inner layer being similar (Lupia et al., 2000), but the process of "cracking the sporocarp" can take many years. Experimentally, sporocarps can be cut on either end using a razor blade and up to 8 sporocarps can be placed into 500 ml of water-based medium. More than eight sporocarps per 500 ml solution appear to have toxic effects on subsequent embryo development. Once the sporocarp imbibes water, it swells apart and the sorophore emerges bearing the groups of sporangia called sori. After several hours, the sporangial walls become gelatinous which results in the release of the microspores and megaspores into the soral cavities. The enclosing indusium disintegrates and the spores are released (Rice and Laetsch, 1967; Machlis and Rawitscher-Kunkel, 1967). The hydration of the enveloping gel surrounding the megaspores and microspores makes the spores buoyant (Laetsch, 1967; Rice and Laetsch, 1967; Machlis and Rawitscher-Kunkel, 1967).

Microspores (round and 0.075 mm in diameter) are many times smaller than megaspores and contain a single central nucleus with dense cytoplasm and starch grains (Rice and Laetsch, 1967). Newly released microspores will float in groups for up to one hour before separating (Schneider and Pryer, 2002). Outer layer microspore morphology is characterized by the possession of baculate perine sculpture (Lupia et al., 2002) that is divided into two sublayers, a perine and an exine, retained until the male gametophyte is fully developed (Schneider and Pryer, 2002).

Spermatids arise through a series of unequal and equal cell divisions within the microspore. Prior to the first mitotic division, the nucleus is surrounded by dozens of plastids that move to one side of the cell. Then the nucleus undergoes the first mitotic division, which creates the sole remnant of the vegetative tissue, referred to as the prothallial cell. After an additional 8 divisions, the spore is partitioned into 39 cells: 32 spermatids, 6 jacket cells, and the prothallial cell (Sharp, 1914; Klink and Wolniak, 2001). Each antheridium surrounds 16 spermatogenous cells and after hydration, the prothallial and jacket cells disintegrate and release the active and swimming sperm within 6-12 hours (temperature dependent). At an ambient temperature of 22-25º C, sperm populations are active for 3-3 1/2 hours in tap water (Rice and Laetsch, 1967; Schneider and Pryer, 2002). *Marsilea* spermatozoids are the same basic structure but are twice as long as found in those in Polypodales (Bell and Hemsley, 2000).

A megaspore (0.7 – 0.75 mm long and prolate) contains a spore cell that is filled with starch grains and has only a small amount of dense cytoplasm and a single nucleus that lies at the papilla protuberance (Machlis and Rawitscher-Kunkel, 1967). This papilla is the site of several cell divisions forming the apical archegonium containing the egg cell. Once the megaspore hydrates, a gelatinous perine layer swells rapidly and reaches maximum size about 2-4 hours after the spore's release in the water (Schneider and Pryer, 2002). Above the archegonium, between the outer gelatinous perine and the inner solid exine, is the "sperm lake". This structure, upon hydration, traps and funnels chemotaxically driven sperm toward the egg ensuring fertilization (Machlis and Rawitscher-Kunkel, 1967). The gelatinous perine layer has been shown to disintegrate

after approximately 6 hours with only a residue remaining after 20 hours in water (Schneider and Pryer, 2002).

Developmental and maturation failures are the main causes of reduced sporocarp viability, with the microspores being more susceptible to aging effects than megaspores (Bloom, 1955). Proper spore development and maturation are correlated with the outer appearance of the sporocarp, and therefore, anyone utilizing this system should use only plump, hard, and brown sporocarps for plant propagation.

*Fertilization and initial development* – At room temperature, the chemotactically responsive sperm reach the archegonium resulting in fertilization at approximately 10 hours after spore hydration (Laetsch, 1967; Rice and Laetsch, 1967; Machlis and Rawitscher-Kunkel, 1967). Cross-fertilization is highly probable since several sporocarps are put into a single volume of solution, but it requires a lot of care to ensure cross-fertilization. Within several hours following fertilization the zygotes undergo an initial cell proliferation that leads to a four-part embryo, including the quadrants for the root, leaf, foot, and shoot apex that will later generate additional vegetative organs (Gifford and Foster, 1989). Four cells constitute an epibasal hemisphere, which gives rise to the leaf and shoot apical meristem and four cells constitute a hypobasal tier that results in the root and foot (Bower, 1963). In the natural environment, successful fertilization is visually confirmed by the development of rhizoids on the archegonium. The shoot apex of the embryo gives rise to a primary leaf and root in three days time. These structures are easily maintained within a petri dish sitting on a lab bench at room

temperature with overhead lighting. Gross morphology can be observed under a common light-dissecting microscope for all stages of development.

*Marsilea vestita* spore-to-spore development takes approximately 3-4 months in a greenhouse pond with the entire reproductive process occurring in water. Spores are released, dispersed to the air/water interface for fertilization and then finally sink to the water/soil interface where the young embryo develops (Schneider & Pryer, 2002). Temperature and water supply can influence timing and developmental rates.

*Marsilea as the model system for embryogenesis* -- An ideal system for studying embryogenesis is one that has rapid development, allows for easy manipulation and collection of embryos from an in vivo system, and is easy to grow long term. *Marsilea* has all of these features, plus it allows for the applications of various treatments since inhibitors can be added directly to the culture medium and cytological effects can be easily assayed (Klink and Wolniak, 2001), for example, using a common dissection microscope to observe morphological changes.

**Summary**

It was determined that *in vitro* manipulation of basal plant embryogenesis was not reliable for most species. However, these studies were valuable in determining the best species to utilize for studying the axial development of post-embryonic sporophytes (see Chapters 3 and 4) and the developmental regulation of fern embryogenesis (see Chapter 5).

# CHAPTER 3

# AUXIN REGULATION OF AXIAL GROWTH IN BRYOPHYTE SPOROPHYTES: ITS POTENTIAL SIGNIFICANCE FOR THE EVOLUTION OF EARLY LAND PLANTS

## INTRODUCTION

Recent paleobotanical work and molecular sequence analyses have provided new insights into the evolution of early land plants (for reviews, see Graham, 1993; Kenrick and Crane, 1997; Niklas, 1997; Bateman et al., 1998; Graham et al., 2000). In particular, it is firmly established that ancient charophycean green algae gave rise to the early land plants(Graham, 1993; Karol et al., 2001). The fossil record from the Middle Ordovician shows the first microfossils, including obligate spore tetrads with sporopollenin-impregnated walls, imperforate cuticles, and narrow tubes that can tentatively be attributed to land plants (Gray, 1985; Edwards and Wellman, 2001; Graham and Gray, 2001). The presence of obligate spore tetrads is consistent with the interpretation that the earliest land plants exhibited a bryophyte-grade of structural organization, at least with respect to spore morphology (Gray, 1985; Graham and Gray, 2001). Furthermore, available molecular sequence information supports the perspective that the three extant bryophyte lineages (hornworts, liverworts, and mosses) diverged earlier than the monophyletic lineage evolving into extant vascular plants (Qiu et al., 1998; Nickrent et

al., 2000; Renzaglia et al., 2000; Karol et al., 2001). However, the macrofossil record for putative bryophytes is too fragmentary at present to confirm this perspective (Kenrick and Crane, 1997; Niklas, 1997; Bateman et al., 1998; Edwards, 2000; Goffinet, 2000; Kenrick, 2000).

Living charophycean algae have haplobiontic life cycles, with a dominant haploid gametophyte and a diploid phase solely consisting of the zygote that undergoes meiosis to produce four haploid zoospores (Graham and Wilcox, 2000); therefore, it is often proposed that the first land plants evolved a multicellular diploid embryo through the intercalation of mitotic divisions in the zygote prior to sporic meiosis (e.g., Graham, 1993; Hemsley, 1994; Graham and Wilcox, 2000). The origin of the embryo, and its subsequent elaboration into a complex axial sporophyte, was accomplished by several innovations critical to the widespread colonization of terrestrial environments: (1) jacketed sporangia capable of producing numerous meiospores and (2) erect axes that grow above the gametophyte and are well suited for aerial dispersal of those spores. In all extant nonseed plants except for a few genera of specialized aquatic liverworts, the sporophyte is elevated above the prostrate thallus, low-lying clump, or subterranean axis of the gametophyte. Moreover, complex sporophytes with erect axes containing vascular tissue have almost completely dominated fossil terrestrial flora ever since the early Devonian period ca. 400 million years ago (my BP) (Taylor and Taylor, 1993; Kenrick and Crane, 1997). Despite the obvious evolutionary significance of erect sporophytes, the botanical literature is silent about plausible physiological mechanisms that might have acted to generate the sporophytic axes of early land plants (Cooke et al., 2003).

For several reasons, an examination of the axial sporophytes of extant bryophytes should reveal useful information for considering the evolutionary origins of these structures in the early land plants. First of all, because bryophytes represent the earliest divergent lineages of extant land plants, one bryophyte lineage may have retained the ancestral process that these plants used to generate axial sporophytes. Secondly, bryophyte embryos first develop as spherical or oblong structures, almost all of which will eventually undergo polarized growth to form the tripartite axes characteristic of mature bryophyte sporophytes (Smith, 1955; Bold et al., 1987; Crum, 2001). Bryophyte embryogenesis can thus be said to consist of an initial stage of spherical growth and a subsequent stage of axis elongation, which parallels the most plausible scenario for the evolution of early land plant sporophytes (Graham, 1993; Hemsley, 1994; Niklas, 1997). Finally, each bryophyte division utilizes unique morphological processes for generating its axes (Doyle, 1970; Crum, 2001), which raises the possibility that further investigation may disclose the common origin of the sporophytic axes of one bryophyte lineage and the vascular plants. Of particular interest is the opportunity to use developmental evidence in order to evaluate alternative hypotheses concerning the phylogenetic relationships among bryophyte lineages and other related lineages (Kenrick and Crane, 1997; Bateman et al., 1998; Qiu et al., 1998; Goffinet, 2000; Nickrent et al., 2000; Renzaglia et al., 2000; Karol et al., 2001; Delwiche et al., 2004).

Because the embryos of all land plants, including angiosperms, have similar spherical and axial stages (Bold et al., 1987; Gifford and Foster, 1989; Cooke et al., 2003), it is appropriate to utilize current knowledge about auxin regulation of axis

elongation during angiosperm embryogenesis as the starting point for designing working hypotheses about the same process in young bryophyte sporophytes. In angiosperms, the hormone auxin (indole-3-acetic acid) regulates both phases of embryo development through several mechanisms such as alternative pathways for auxin biosynthesis, homeostatic control over auxin levels, and auxin concentration gradients (Cooke et al., 2003; Ljung et al., 2002; Ribnicky et al., 2002). For example, a pronounced surge in free auxin levels appears to mediate the rapid cell proliferation during the initial stage of carrot zygotic embryogenesis (Ribnicky et al., 2002). Specific inhibitors of polar auxin transport are reported to block or alter subsequent polarized growth in the developing embryos of many angiosperms (Schiavone and Cooke, 1987; Liu et al., 1993; Fischer et al., 1997; Hadfi et al., 1998). Recent molecular investigations have substantiated the interpretation from physiological experiments that auxin acts as the key regulator of axis elongation during angiosperm embryogenesis (Souter and Lindsey, 2000; Hamann, 2001). For example, in *Arabidopsi*s, *gnom* mutant embryos develop into enlarged spherical structures unable to initiate a polarized growth axis. The molecular basis of the *gnom* phenotype appears to be that the embryos fail to localize the auxin efflux carrier PIN1 in the proper position for carrying out polar auxin transport (Steinmann et al., 1999).

Unfortunately, few researchers have investigated auxin biosynthesis, movement, or action in bryophyte sporophytes (for review, see Cooke et al., 2002). In liverworts, auxin-treated setae elongate at more than twice the rates observed in control setae (Schnepf et al., 1979; Thomas, 1980). In addition, an auxin antagonist markedly reduced elongation rates of *Pellia* setae, which suggests very strongly that seta elongation is

principally regulated by endogenous auxin under normal conditions. Nevertheless, agar-block studies involving long-term equilibration resulted in similar levels of auxin accumulation in both donor and receiver blocks, regardless of seta orientation (Thomas, 1980). These results hinted at the possibility that axial auxin movement is not polarized in *Pellia* setae; however, lateral auxin movement did appear to mediate phototropic curvature of these setae (Ellis and Thomas, 1985). The only observations available on the auxin responses of moss sporophytes come from the work of French and Paolillo (1975a), who observed that high levels of exogenous auxin could slightly increase the elongation of intact *Funaria* sporophytes growing attached to the gametophytes and could partially compensate for the inhibitory effect of apical decapitation under the same growth conditions. There is no current evidence of polar auxin transport being involved in axial growth of bryophyte sporophytes.

By contrast, in many structures of vascular plant sporophytes, auxin movement often occurs by polar transport in which auxin moves in a specific, generally basipetal, direction over a short distance through transporting cells (Goldsmith, 1977; Lomax et al., 1995). In the chemiosmotic model, the electrochemical $H^+$ gradient across the plasma membrane is the ultimate driving force for polar transport (Raven, 1974; Rubery and Sheldrake, 1974; Goldsmith, 1977). Apoplastic indole-3-acetic acid (IAA) in the cell wall (pH 5) is thought to cross the plasma membrane passively as protonated indole-3-acetic-acid (IAAH) (pKa 4.7) or via the IAA-influx carrier AUX1 acting as a proton symporter (Bennett et al., 1996) at the apical ends of transporting cells (Swarup et al., 2001). Indole-3-acetic acid in the cytosol (pH 7) is transported back into the apoplast via IAA-efflux carriers encoded by the PIN genes in *Arabidopsis* (Muller et al., 1998;

Steinmann et al., 1999). Therefore, the polarity of auxin transport is usually attributed to the asymmetric localization of auxin carriers at opposite ends of the transporting cells (Jacobs and Gilbert, 1983; Estelle, 1998; Palme and Galweiler, 1999; Swarup et al., 2000).

Both the influx and efflux carriers are sensitive to several inhibitors. The compounds N-(1-naphthyl)phthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) have traditionally been used to inhibit the efflux component of the polar auxin transport mechanism (Thomson et al., 1973). The NPA acts as a phytotropin to inhibit both lateral and axial auxin transport, but the inhibitory effects of TIBA are restricted to axial transport (Lomax et al., 1995). The influx carrier is sensitive to (1-napthoxy)acetic acid (NOA) (Imhoff et al., 2000; Parry et al., 2001). Although these inhibitors were originally thought to act directly on the auxin carriers (Thomson et al., 1973; Lomax et al., 1995), more recent work has suggested that the efflux-carrier inhibitors may instead interfere with the membrane-trafficking system responsible for inserting the carriers into the plasma membrane (Geldner et al., 2001). Nevertheless, these inhibitors at low concentrations have their more pronounced effects on those developmental processes that depend on auxin transport.

The overall objective of this chapter is to characterize the role that auxin plays in the axial elongation of the sporophytes of common plants representing the three divisions of extant bryophytes. Our aim is to use the information gathered to aid our consideration of the evolutionary origins of these structures in the early land plants. Intact sporophytes of the hornwort *Phaeoceros pearsonii* (Howe) Prosk., the liverwort *Pellia epiphylla*

(L.) Corda, and the moss *Polytrichum ohioense* Renauld & Cardot were exposed to exogenous auxin as well as to an auxin antagonist to evaluate whether auxin acts to regulate axial elongation in these sporophytes. Conventional agar-block techniques for measuring polar transport of radio labelled auxin were modified to accommodate the small cross-sectional areas of bryophyte sporophytes. Those techniques were then applied to measure auxin transport in axial sections in the absence or presence of inhibitors that affect various transport steps. These experiments demonstrated that auxin plays distinctive roles in the regulation of axial growth of the sporophytes from different bryophyte divisions. This knowledge has significant implications for our considerations about the origins of axial sporophytes in the early land plants.

## MATERIALS AND METHODS

*Plant material—Phaeoceros pearsonii* gametophytes bearing young sporophytes were collected on Thurber Road in Santa Cruz, California, USA, by Drs. Daniel Norris and William Doyle. *Pellia epiphylla* gametophytes with preemergent sporophytes were collected on the campus of the University of Pittsburgh at Bradford in Bradford, Pennsylvania, USA, with the assistance of Drs. Francis Mulcahy and Mary Puterbaugh. *Polytrichum ohioense* gametophytes with dormant sporophytes were collected on the campus of the University of Maryland in College Park, Maryland, USA. All three species were grown on soil from the original habitat at room temperature under ca. 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of fluorescent light until the young sporophytes reached the desired length of 1 cm. Older moss sporophytes 2–3 cm in length were also used in certain experiments. Coleoptiles of *Zea mays* cv. Jubilee (R H Shumways, Graniteville, South Carolina, USA)

were grown in the dark for 4 d after which they received 8–10 h of red light at 2.46 mmol $\cdot m^{-2} \cdot s^{-1}$ before being returned to darkness until used. The coleoptiles were cut just above the soil line and then scored to remove the primary leaf from the center of the coleoptile so that only coleoptile tissues were used.

*Growth response assays*—Stock solutions ($10^{-2}$ mol/L) of p-chlorophenoxyisobutyric acid (PCIB) (Aldrich Chemical Company, Milwaukee, Wisconsin, USA) or indole-3-acetic acid (IAA) (Sigma, St. Louis, Missouri, USA) were prepared in 95% ethanol and then were added to 0.7% cool molten phytoagar (Sigma) to obtain final concentrations of $10^{-5}$ mol/L in 100 x 15-mm polystyrene petri dishes. An equivalent amount of 95% ethanol was added to the control plates. Intact sporophytes of *Phaeoceros pearsonii*, *Pellia epiphyll*a, and *Polytrichum ohioense* were carefully dissected from surrounding gametophytic tissue under a dissecting microscope. *Polytrichum* sporophytes were completely removed from the gametophyte, while the sporophyte of *Pellia epiphylla* and *Phaeoceros pearsonii* were dissected with minimal gametophytic tissue remaining near the foot that could not be removed because of the delicate nature of the structures. Then 25–30 sporophytes for each treatment were placed horizontally on the agar surface of the petri plates, their original lengths were measured with the ocular micrometer of a dissecting microscope at 75x magnification. The dishes were placed in the constant darkness except for brief intervals needed to measure their lengths under the dissecting microscope every 24 h. The data from each treatment of each species were presented as the mean net growth of the sporophytes at each 24-h interval ± the standard error among replicate sporophytes.

*Auxin transport assays*—The experiments designed to measure auxin transport in bryophyte sporophytes were carried out using conventional agar-block methods (McCready and Jacobs, 1963; Mitchell and Livingston, 1968), modified to accommodate the small cross-sectional areas of bryophyte sporophytes. All donor blocks contained $10^{-6}$ mol/L 5-[$^{3}$H]-IAA (specific activity of 25 Ci/mmol, American Radiolabeled Chemicals, St. Louis, Missouri, USA), and receiver blocks were composed either of water agar or $10^{-5}$ mol/L N-(1-naphthyl)phthalamic acid (NPA) (Pfaltz and Bauer, Stamford, Connecticut, USA). NPA stock ($10^{-3}$ mol/L) in 95% ethanol was added to molten Bactoagar (Difco Laboratories, Detroit, Michigan, USA) and allowed to cool in a 3-mm-diameter glass tube to create receiver blocks with a final concentration of $10^{-5}$ mol/L NPA. Sporophyte sections 5 mm in length were cut with a miniature scalpel (Roboz Surgical Instruments, Rockville, Maryland, USA) from the midregion of the setae of *Polytrichum ohioense* and *Pellia epiphyll*a. Sections (5 mm) were similarly cut from the immature capsule located just above the intercalary meristem of *Phaeoceros pearsonii*. These sections were placed in a horizontal orientation between the 3-mm-diameter cylindrical donor and receiver blocks of 1.8% Bactoagar mounted on microscope slides under a glass chamber designed to maintain high humidity at room temperature. A physical gap separated the lanolin-mounted slides to ensure that no capillary movement of water occurred between the agar blocks. Single time point experiments for characterizing the transport polarity and inhibitor effects in each species utilized 5–15 sporophyte sections, which were placed in the transport chamber for 3 h. Time course experiments for measuring the accumulated amount of basipetal transport in each species used five different sections for each time point taken every hour for 5 h. In

either case, the receiver block from each section was placed in 5 mL of Biosafe II scintillation fluid overnight and then counted for 5 min in an LKB Wallac 1219 Rack Beta liquid scintillation counter (95% counting efficiency, LKB Instruments, Gaithersburg, Maryland, USA). Identical methods were used for measuring auxin transport in 5-mm sections obtained 1.0 cm below the apical region of *Zea mays* coleoptiles.

Additional experiments were run to evaluate auxin transport in the setae of older sporophytes (2–3 cm in length) of *Polytrichum ohioens*e. Experiments were carried out in the same manner as the assay described earlier with the variation that $10^{-5}$ mol/L NPA or $10^{-5}$ mol/L NOA (Aldrich Chemical Company) was added to both the donor and the receiver blocks. The NOA was dissolved in 95% ethanol at a stock concentration of $10^{-2}$ mol/L. The control donor and receiver blocks had 1% (v/v) 95% ethanol added to simulate the amount of added ethanol in the inhibitor solutions of the other trials. The transport assay was run on 10 sporophytes for each treatment for 3 h. Identical methods were used for measuring auxin transport in *Zea mays* coleoptiles obtained as described earlier.

*Data analysis for transport experiments*—All counts per minute (cpm) from the liquid scintillation counter were divided by the counting efficiency to yield the corresponding disintegrations per minute (dpm), which were converted into curies ($2.2 \times 10^{6}$ dpm = 1 Ci) and then into moles by dividing by the specific activity of [$^{3}$H]-IAA (25 Ci/mmol) reported by the manufacturer. Time course data for basipetal transport were directly plotted as amount (fmol) vs. time (h). The slope of each best-fitted line represented the

intensity (amount per unit time) of basipetal transport. Basipetal transport velocity (distance per unit time), which corresponded to the transport rate of the first molecules to reach the receiver block, was calculated as the length of the section divided by the x-intercept of the line. Transport flux (amount per unit area per unit time) was calculated by dividing the transport intensity by the cross-sectional area of each section, as determined from measuring the dimensions of the seta, capsule, and coleoptile section with an ocular micrometer in a dissecting microscope. Cross-sectional areas for solid moss sections were calculated by the formula $\pi r^2$, where r is the radius, and for hollow liverwort and hornwort sections by the formula $\pi(r_o^2 - r_i^2)$, where $r_o$ is the outer radius and $r_i$ is the inner radius. Cross-sectional areas for hollow elliptical maize coleoptiles were calculated by the formula $\pi(R_a R_b - r^2)$, where $R_a$ is the minor outer radius of the ellipse, $R_b$ is the major outer radius of the ellipse, and $r$ is the radius of the hollow inner circle. Transport polarity was calculated as the ratio of basipetal transport intensity to acropetal transport. Percentage inhibition was calculated as the ratio of transport intensity in inhibited structures vs. the transport intensity in control structures times 100%. The data are presented as the mean ± the standard error among replicate sections.

## RESULTS

*General sporophyte morphology*—In general, almost all sporophytes of the three lineages of extant bryophytes mature as tripartite linear structures, but the developmental processes responsible for generating these sporophytes are strikingly different in the three lineages (Smith, 1955; Bold et al., 1987; Renzaglia et al., 2000; Crum, 2001). The mature sporophyte of the representative liverwort *Pellia epiphylla* develops an apical

capsule, a basal foot, and an intermediate seta (Fig. 3-1 A-C). The liverwort embryo differentiates into three tiers destined to develop into those three structures in the mature sporophyte. Subsequently, the unexpanded sporophyte remains protected by the enlarged calyptra and the adjacent involucre while the capsule undergoes precocious differentiation to produce mature spores and narrow elators (Fig. 3-1B). The unexpanded seta is uniformly composed of unspecialized cells lacking any differentiation (Thomas, 1980). When the environmental conditions are conducive to spore dispersal, the seta cells start rapidly absorbing water resulting in diffuse cell elongation in the absence of compensatory cell division or substantial wall biosynthesis (Thomas and Doyle, 1976). This simple turgor-driven mechanism mediates seta elongation at a rate approaching 1 mm/h (Watson, 1971). Therefore, it appears that the sole purpose of the ephemeral liverwort seta is to suddenly elevate the mature capsule above the gametophyte to effect the almost simultaneous dispersal of its spores.

The mature sporophyte of a typical hornwort like *Phaeoceros pearsonii* consists of a linear sporangium and a basal foot with an intervening intercalary meristem that divides to generate new sporangial cells throughout sporophytic growth (Fig. 3-1 D-F). The hornwort embryo also exhibits a basal tier destined to become the foot and an apical tier representing the future tip of the capsule (Campbell, 1918; Smith, 1955; Crum, 2001). The intermediate tier develops into a narrow band of dividing cells called an intercalary meristem that undergoes unifacial divisions on its capsule side, with the result that the new cells compose almost the entire capsule (Fig. 3-1F). The persistent activity of the intercalary meristem generates an indeterminate capsule, in which spore development is a sequential process with new sporogenous cells originating near the

meristem and mature spores being released from distal regions over several months. Thus, the axis of the hornwort sporophyte is largely composed of the linear capsule itself (Fig. 3-1F), in marked contrast to the elongated setae of the other bryophytes.

The mature sporophyte of the mosses, such as *Polytrichum ohioens*e, can also be divided into a foot, seta, and capsule (Fig. 3-1 G–I). The young *Polytrichum* sporophyte grows as a bipolar structure with transient apical cells at opposite poles. The activity of these apical cells and their derivatives results in the javelin-shaped structure illustrated in Fig. 3-1H (Smith, 1955; Lal and Bhandari, 1968; Bold et al., 1987; Crum, 2001). Subsequently, an intercalary meristem arises near the base of the future capsule, and the unifacial activity of this meristem is responsible for generating all the remaining seta cells (French and Paolillo, 1975b). Because seta elongation in the mosses depends in part on repeated cell divisions, it is a gradual process, as opposed to the rapid growth of liverwort setae by simple cell elongation. The *Polytrichum* seta has considerable cellular differentiation with an outer epidermis, an underlying cortex, and a central strand composed of water-conducting cells called hydroids, sugar-conductive cells called leptoids, and supportive stereids (Hébant, 1977). Although spore maturation is a simultaneous process within the late-maturing capsule, the spores are gradually disseminated in most mosses due to the activity of the peristome.

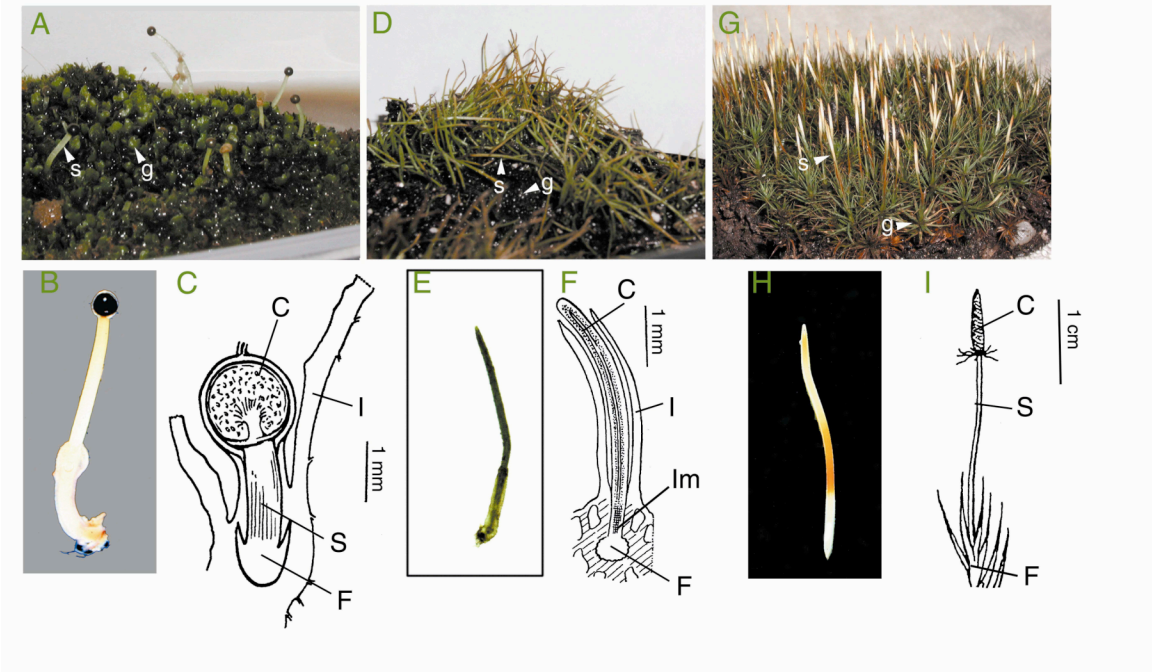



**Fig. 3-1**: A-C. The sporophytes of *Pellia epiphylla*. A.Gametophytes bearing sporophytes. B. Detached sporophyte. C. Drawing of longitudinal section of sporophyte. Reprinted with permission fromWatson (1971). D-F. The sporophytes of *Phaeoceros pearsonii*. D. Gametophytes bearing sporophytes. E. Detached sporophyte. F. Drawing of longitudinal section of sporophyte. Reprinted with permission from Watson (1971). G-I. The sporophytes of *Polytrichum ohioense*. G. Gametophytes bearing sporophytes. H. Detached sporophyte. I. Drawing of longitudinal section of sporophyte. Reprinted with permission from Bold (1973). Abbreviations: C, capsule; S, seta; F, foot; Im, intercalary meristem; I, involucre; s, sporophyte; g, gametophyte.

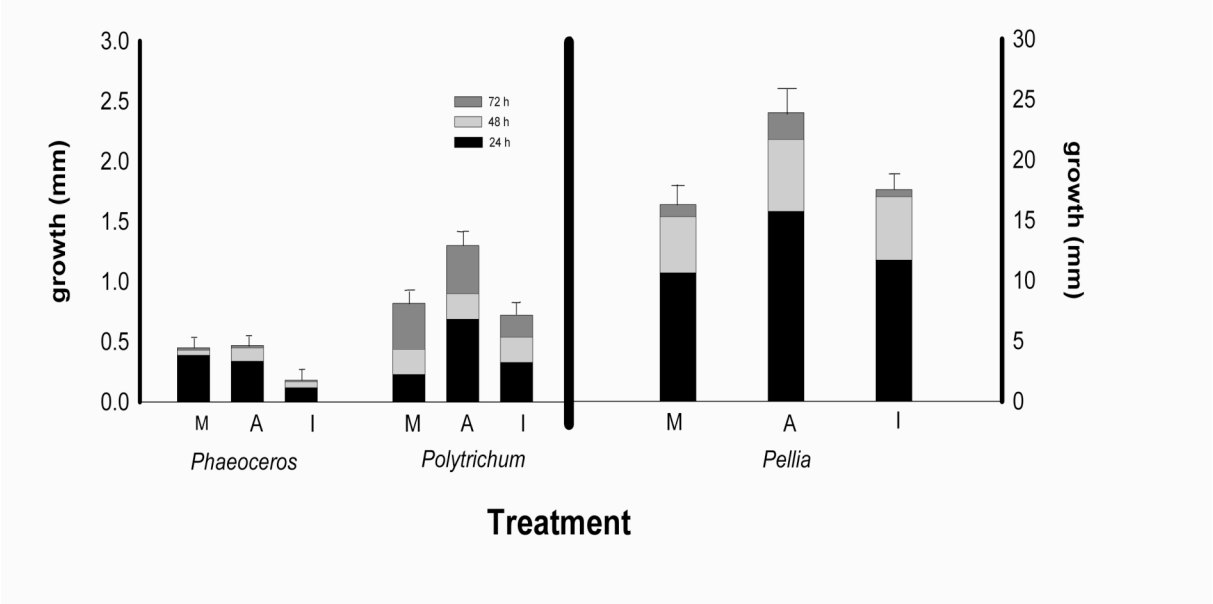



**Fig. 3-2**: *In vitro* growth of isolated sporophytes of *Phaeoceros pearsonii*, *Pellia epiphylla*, and *Polytrichum ohioense* measured every 24 h for 72 h in response to control medium (M), indole-3-acetic acid (A), or *p*-chlorophenoxyisobutyric acid (I). Data are presented as means + 1 SE among 25-30 replicate sporophytes.

*Auxin effects on axial elongation*—Young sporophytes from *Phaeoceros pearsoni*i, *Pellia epiphyl*l, and *Polytrichum ohioensis* were isolated from their respective gametophytes and exposed to an auxin (IAA) or an auxin antagonist (PCIB) at a concentration of $10^{-5}$ mol/L to determine if auxin acts to regulate axis elongation in bryophyte sporophytes. The elongation responses of 25 sporophytes exposed to each treatment were measured every 24 for 72 h (Fig. 3-2).

Immature sporophytes of *Phaeoceros pearsonii* ranged in length from 4 to 14 mm at the start of the experiment. Control sporophytes exhibited a mean increase of 0.39 mm in the first day and 0.06 mm over the next 2 d for a total mean increase of 0.45 mm. The growth response of these hornwort sporophytes was almost identical to that of the IAA treatment, with a mean increase of 0.34 mm in the first 24 h and a total mean increase of 0.47 mm for the entire experiment. However, hornwort sporophytes subjected to the PCIB treatment grew only 0.18 mm over 3 d, which represented a 60% reduction in total growth relative to the control sporophytes.

The initial lengths of immature setae of *Pellia epiphylla* ranged from 8 to 24 mm at the beginning of the experiment. Liverwort sporophytes in the controls elongated an average of 16.29 mm over 72 h, while the IAA-treated sporophytes grew 25.90 mm over the same interval, which means that IAA promoted the elongation of liverwort setae by 58%. The liverwort sporophytes grown in the PCIB treatment had a mean total increase of 17.53 mm, which was not significantly different from the control response.

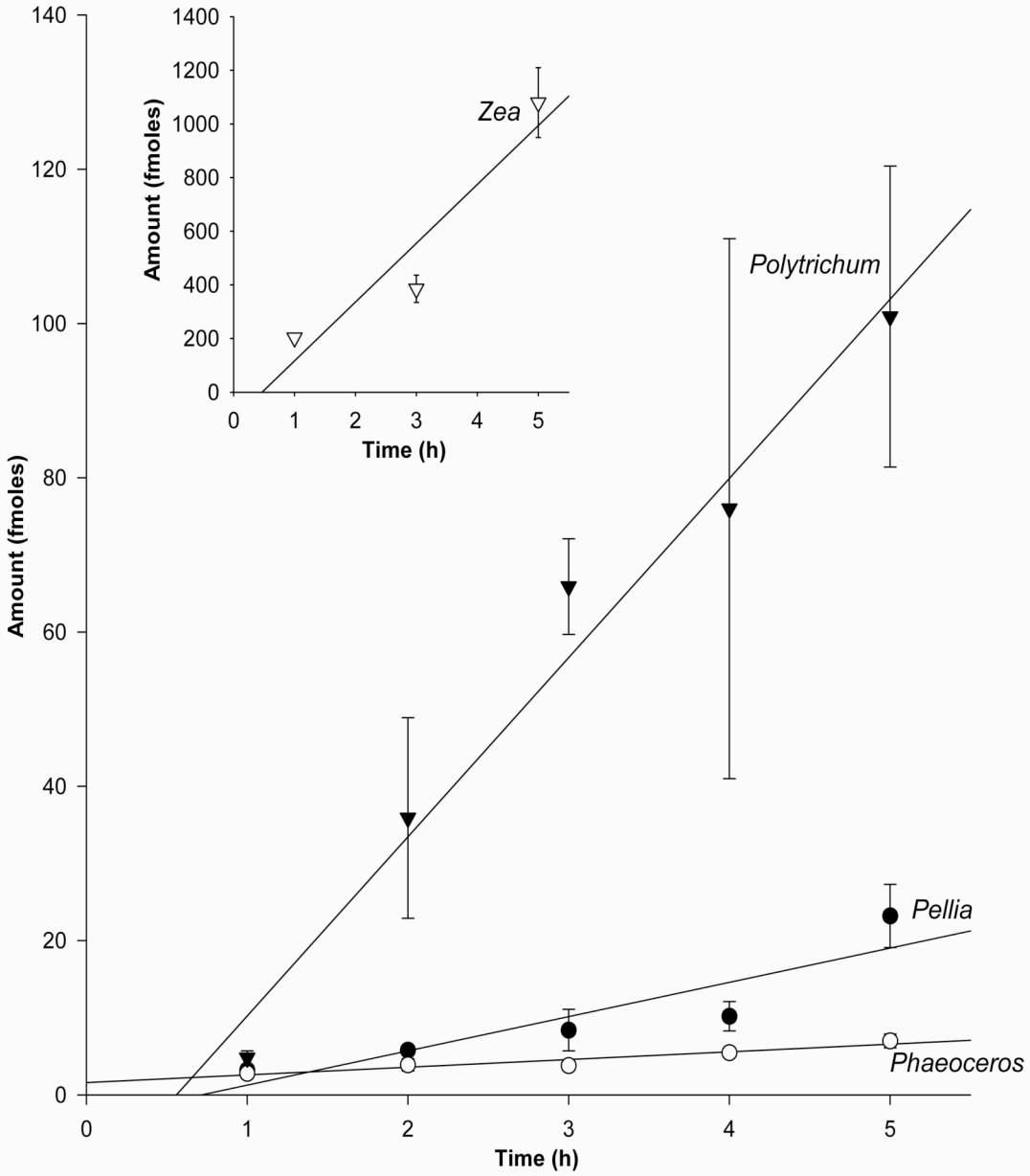
The young sporophytes of *Polytrichum ohioens*e, which had initial lengths of 12 to 21 mm, grew rather consistently within each treatment during the entire experiment. Moss sporophytes displayed a total mean increase of 0.82 mm in the control treatment vs. 1.30 mm and 0.72 mm in the IAA and PCIB treatments, respectively. Thus, IAA caused an increase in total elongation approaching 60% in these sporophytes.

In conclusion, overall growth rates of young *Pellia* and *Polytrichum* sporophytes significantly increased in response to exogenous IAA, but they did not respond to the anti-auxin treatment. By contrast, young *Phaeoceros* sporophytes reacted with the opposite sensitivity to these experimental treatments. The experiment thus suggested that endogenous IAA acts to regulate axis elongation in all three bryophytes.

*Time course of basipetal auxin movement*—Axial sections from the sporophytes of *Phaeoceros pearsoni*i, *Pellia epiphyll*a, and *Polytrichum ohioense* and from the coleoptiles sections of *Zea mays* were placed into a conventional agar-block apparatus with $^{3}$ H-IAA in the donor blocks to determine the time course of basipetal movement. The amount of $^{3}$ H-IAA was measured in the receiver blocks every hour for 5 h to construct the curves depicted in Fig. 3-3 and characterized in Table 3-1.

Time course data from immature sporophytes of *Phaeoceros pearsonii* were represented by the line $y = 1.0x + 1.6$ (see Table 3-1, Fig. 3-3) whose slope corresponded to 1.0 fmol auxin moved per hour. The velocity (distance per unit time) of auxin transport could not be resolved for *Phaeoceros* sporophytes because the limited amount of auxin transported resulted in a best-fit line that did not cross the x-axis. Hornwort

**Fig. 3-3**: Basipetal auxin movement in isolated sporophytes of *Phaeoceros pearsonii* (open circles), *Pellia epiphylla* (closed circles), and *Polytrichum ohioense* (closed triangles) and in isolated coleoptiles of *Zea mays* (open triangles) in an agar-block apparatus over 5 h. Data are presented as means ± 1 SE among five to 15 replicate structures for every collection of each species at each hour.


Amount (fmoles)
Time (h)
Zea
Polytrichum
Pellia
Phaeoceros

| Organism | Structure | Best-fit line | Velocity (mm/h) | Mean Cross Sectional Area ($mm^2$) | Flux ($fmoles/mm^2 \bullet s$) |
|---|---|---|---|---|---|
| **Phaeoceros pearsonii** | Immature capsule | y = 1.0x + 1.6 | n/d | 0.17 | $1.6 \times 10^{-3}$ |
| **Pellia epiphylla** | Young seta | y = 4.4x – 3.2 | 6.9 | 0.21 | $5.9 \times 10^{-3}$ |
| **Polytrichum ohioense** | Young seta | y = 23.2x – 13.0 | 8.9 | 0.14 | $4.6 \times 10^{-2}$ |
| **Zea mays** | Coleoptile | y = 219.3x – 101.8 | 11.0 | 1.99 | $3.1 \times 10^{-2}$ |

**Table 3-1**: Key parameters characterizing basipetal auxin transport in three bryophyte sporophytes and in maize coleoptile. The equations correspond to the best-fit lines calculated on the basis of the amount of radioactivity in the receiver blocks at each hour starting at 1 h, as depicted in Fig. 3-3.

sporophytes had a mean cross-sectional area of 0.17 $mm^2$, which meant that their basipetal flux of auxin movement was equal to 1.6 x $10^{-3}$ fmol $mm^{-2} \cdot s^{-1}$

Instead of using the conventional term of auxin *amounts* to compare auxin movement among the different structures, auxin *fluxes* were utilized here to normalize the profound size differences among these structures. Auxin movement in young setae of *Pellia epiphylla* was characterized by the line $y = 4.4x - 3.2$. Extrapolating this line to the x-axis resulted in the calculated auxin movement velocity of 6.9 mm/h. These setae had an average cross-sectional area of 0.21 $mm^2$, with a calculated flux of 5.9 x $10^{-3}$ fmol $\cdot$ $mm^{-2} \cdot s^{-1}$. Thus, the auxin flux in *Pellia* setae was over 3.5-fold higher than the flux in *Phaeoceros* sporophytes. Time course data for the young setae of *Polytrichum ohioense* were best represented by the line $y = 23.2x - 13.0$, which led to a predicted velocity for auxin movement of 8.9 mm/h. Moss setae displayed a mean cross-sectional area of 0.14 $mm^2$, which was somewhat less than that of the other two bryophyte sporophytes. However, their basipetal flux of 4.6 x $10^{-2}$ fmol $\cdot$ $mm^{-2} \cdot s^{-1}$ was almost 30 times higher than the flux in *Phaeoceros* sporophytes and almost eight times higher than the flux in *Pellia* sporophytes.

Even though the maize coleoptile is a hollow structure, it offers a much greater cross-sectional area for auxin movement (1.99 $mm^2$) than do the bryophyte sporophytes. The line $y = 219.3x - 101.8$ best represented the time course data for maize coleoptiles, which had a transport velocity of 11 mm/h. Their slope of 219.3 fmol auxin transported per hour is approximately 10-fold higher than the slope of the movement in moss sporophytes. However, the auxin flux in maize coleoptiles was 3.1 x $10^{-2}$ fmol $\cdot$ $mm^{-2}$ $\cdot$

$s^{-1}$ , which is 67% of the flux recorded in *Polytrichum* sporophytes. Thus, the moss sporophyte appears to have evolved a mechanism for moving auxin that is comparable to those acting in flowering plant structures.

*Polarity and inhibitor sensitivity of auxin movement*—Polar auxin transport in flowering plants is typically sensitive to certain inhibitors (see Introduction). Thus, to compare this process in bryophyte sporophytes vs. maize coleoptiles, auxin movement was characterized in both acropetal and basipetal directions in the presence or absence of these inhibitors.

Control sections from immature capsules of hornwort sporophytes transported 5.9 ± 0.7 fmol (326 ± 39 dpm) 3 H-IAA in the basipetal direction and 6.1 ± 0.3 fmol (337 ± 14 dpm) in the acropetal direction during 3-h experiments, which resulted in a basipetal to acropetal (B/A) polarity ratio of 1.0 (Table 3-2). The absence of evident auxin polar transport, along with the very low levels of auxin movement measured in Fig. 3-2, suggests that auxin movement may result from simple diffusion in hornwort sporophytes. The addition of the inhibitor NPA to the receiver block had virtually no effect, which is consistent with an apparent lack of an auxin transport apparatus (Table 3-2). Thus, this experiment suggested that auxin efflux carriers are absent from hornwort sporophytes and/or they are almost completely insensitive to transport inhibitors.

Seta sections from *Pellia epiphylla* sporophytes were similarly measured to have a B/A ratio of 1.1 (Table 3-2), suggesting that auxin movement also lacks polarity in these sporophytes. However, $10^{-5}$ mol/L NPA in the receiver blocks reduced basipetal movement from 8.4 ± 1.8 fmol to 3.7 ± 0.3 fmol auxin (56% inhibition) and acropetal

movement from 7.6 ± 0.9 fmol to 5.0 ± 0.8 fmol (33% inhibition). This sensitivity to NPA suggests that auxin efflux carriers are present in liverwort sporophytes.
The somewhat higher NPA-mediated inhibition of basipetal transport implies that efflux carriers may be some-what more likely to reside near the basal ends of seta cells.

The B/A ratio of 9.3 of the control setae of *Polytrichum ohioense* sporophytes confirmed basipetal auxin transport (Table 3-2). However, the addition of NPA in the receiver blocks effected only slight, but equivalent inhibitions of basipetal transport (17%) and acropetal transport (14%). These results could be attributed to preferential but not exclusive distribution of auxin efflux carriers to basal locations, even though the carriers and/or their intracellular transport system were apparently insensitive to NPA. Because of the structural complexity of the *Polytrichum* seta, a second possibility was that basipetal and acropetal transport might occur in the peripheral cortex and central vascular strand, respectively, within the *Polytrichum* seta.

By contrast, maize coleoptiles exhibited a B/A ratio of 674, which means that auxin transport is almost exclusively basipetal in these structures. NPA caused a 99% inhibition of basipetal transport, but it did not affect the negligible amount of acropetal transport.

The possibility that *Polytrichum* setae might have two opposing pathways for auxin transport was evaluated by examining the transport capabilities of older moss sporophytes, which would presumably contain more mature vascular tissue. Transport assays were performed in the agar-block apparatus as before in the presence or absence of the inhibitors of the auxin influx carrier (NOA) and the auxin efflux carrier (NPA). In

these experiments, $10^{-5}$ mol/L NPA or $10^{-5}$ mol/L NOA were incorporated into both the donor and receiver blocks to ensure effective inhibition.

In the control experiments, older moss sporophytes transported 54.6 ± 8.6 fmol of auxin in the basipetal direction over 3 h (Table 3-3), which is roughly similar to the basipetal transport in younger moss sporophytes (Table 3-2). However, acropetal auxin transport in older sporophytes was 61.7 ± 14.7 fmol (Table 3-3), which is almost nine times higher than the acropetal transport in young sporophytes (Table 3-2). Auxin transport in older sporophytes was strongly affected by NPA: basipetal transport was reduced to 31.9 ± 4.5 fmol (41.4% inhibition) while acropetal transport was decreased to 24.8 ± 4.5 fmol auxin (59.9% inhibition). NOA application resulted in only 4% inhibition of basipetal transport but 64.7% inhibition of acropetal transport. The enhanced levels of acropetal transport and its pronounced sensitivity to NOA suggest that the bidirectional auxin transport occurs in *Polytrichum* sporophytes via two different pathways. On the other hand, the control coleoptile sections of *Zea mays* transported 266.6 ± 717.8 fmol auxin in the basipetal direction. NPA greatly decreased this transport to 54.3 ± 6.9 fmol auxin (99.6% inhibition). Acropetal transport in coleoptile sections was less affected by NPA because 47.3 ± 2.5 fmol auxin was reduced to 39.8 ± 4.0 fmol (15.7% inhibition). With the addition of NOA to the receiver blocks, both basipetal and acropetal auxin transport decreased substantially in maize coleoptiles by 52.6% and 34.5%, respectively.

**Table 3-3**: Transport polarity and inhibitor sensitivity of auxin transport in older moss sporophytes and maize coleoptiles placed in the agar-block apparatus for 3 h. Data are presented as the mean ± the standard error among 10 replicate structures. NPA, N-(1-naphthyl)phthalamic acid; NOA, (1-napthoxy)acetic acid.

| Organism | Structure | Treatment | Basipetal transport | | Acropetal transport | | Polarity |
|---|---|---|---|---|---|---|---|
| | | | dpm | fmoles | dpm | fmoles | B/A |
| ***Phaeoceros pearsonii*** | Immature capsule | Control | 326 ± 39 | 5.9 ± 0.7 | 337 ± 14 | 6.1 ± 0.3 | 1.0 |
| | | $10^{-5}$ M NPA | 365 ± 29 | 6.6 ± 0.5 | 320 ± 29 | 5.8 ± 0.5 | --- |
| | | % Inhibition | | 0 | | 6 | --- |
| ***Pellia epiphylla*** | Young seta | Control | 461 ± 97 | 8.4 ± 1.8 | 416 ± 49 | 7.6 ± 0.9 | 1.1 |
| | | $10^{-5}$ M NPA | 205 ± 16 | 3.7 ± 0.3 | 277 ± 45 | 5.0 ± 0.8 | --- |
| | | % Inhibition | | 56 | | 33 | --- |
| ***Polytrichum ohioense*** | Young seta | Control | 3793 ± 444 | 69.0 ± 8.1 | 409 ± 36 | 7.4 ± 0.7 | 9.3 |
| | | $10^{-5}$ M NPA | 3165 ± 556 | 57.5 ± 10.1 | 352 ± 16 | 6.4 ± 0.3 | --- |
| | | % Inhibition | | 17 | | 14 | --- |
| ***Zea mays*** | Coleoptle | Control | 233972 ± 5088 | 4254 ± 92.5 | 347 ± 52 | 6.3 ± 0.9 | 674 |
| | | $10^{-5}$ M NPA | 2433 ± 388 | 44.2 ± 7.1 | 339 ± 52 | 6.2 ± 1.0 | --- |
| | | % Inhibition | | 99 | | 2 | --- |

**Table 3-2**: Transport polarity and NPA sensitivity of auxin transport in three bryophyte sporophytes and a maize coleoptile placed in the agar-block apparatus for 3 h. Data are presented as the mean ± the standard error among five to 15 replicated structures. NPA, N-(1-naphthyl)phthalamic acid.

| Organism | Structure | Treatment | Basipetal transport | | | Acropetal transport | | |
|---|---|---|---|---|---|---|---|---|
| | | | dpm | fmoles | % Inhibition | dpm | fmoles | % Inhibition |
| **Polytrichum ohioense** | Older seta | Control | 3001 ± 474 | 54.6 ± 8.6 | --- | 3394 ± 810 | 61.7 ± 14.7 | --- |
| | | $10^{-5}$ M NPA | 1757 ± 250 | 31.9 ± 4.5 | 41.4 | 1362 ± 247 | 24.8 ± 4.5 | 59.9 |
| | | $10^{-5}$ M NOA | 2881 ± 506 | 52.4 ± 9.2 | 4.0 | 1199 ± 150 | 21.8 ± 2.7 | 64.7 |
| **Zea mays** | Coleoptile | Control | 674662 ± 39480 | 12266.6 ± 717.8 | --- | 2599 ± 140 | 47.3 ± 2.5 | --- |
| | | $10^{-5}$ M NPA | 2986 ± 378 | 54.3 ± 6.9 | 99.6 | 2190 ± 221 | 39.8 ± 4.0 | 15.7 |
| | | $10^{-5}$ M NOA | 31952 ± 8054 | 5809.6 ± 146.4 | 52.6 | 1703 ± 139 | 31.0 ± 2.5 | 34.5 |

## DISCUSSION

*Auxin regulation of axis elongation*—The sporophytes of three different bryophytes in this paper exhibit distinctive processes of axial elongation, which correlate with the profound differences in their auxin responses. The linear sporophytes of the hornwort *Phaeoceros pearsonii* do not develop specialized setae for axis elongation, but instead they have a persistent intercalary meristem that generates an elongated capsule. Although the auxin-antagonist experiment suggests that endogenous auxin regulates the activity of this meristem, this auxin appears to move by simple diffusion in the apoplast of the elongating capsule without any detectable polarity or carrier activity. The sporophytes of the liverwort *Pellia epiphylla* and of the moss *Polytrichum ohioense* have both evolved intervening setae that are specialized for axis elongation. Liverwort setae are uniformly composed of parenchymatous cells, which elongate by means of diffuse growth (Thomas, 1980). Our results confirm that this elongation process is sensitive to auxin levels, as was reported in earlier work (Schnepf et al., 1979; Thomas, 1980). Even though auxin does not exhibit polar movement within *Pellia* setae, the experiments with transport inhibitors reveals that its movement does involve the activity of transmembrane auxin carriers in seta cells. Thus, this type of auxin movement can be classified as apolar facilitated diffusion.

By contrast, young moss setae develop a subapical meristem beneath the apical region destined to become the capsule (Wenderoth, 1931; French and Paolillo, 1975b). In addition, the older setae of many mosses, including *Polytrichum ohioens*e, develop a central strand of vascular tissue (Hébant, 1977). Therefore, the cell types and

meristematic activity in *Polytrichum* setae are somewhat similar to those features in vascular plant axes. This paper has presented considerable evidence that auxin acts to regulate the elongation of *Polytrichum* setae. In young setae, exogenous auxin mediates a 50% increase in axis elongation, and it undergoes polar transport in the basipetal direction at a flux (amount per cross-sectional area per unit time) greater than the flux measured in maize coleoptiles. In older setae, auxin is transported at high rates in both directions. In light of the differing inhibitor sensitivities of acropetal and basipetal transport, they may occur in separate cellular pathways within moss setae. Because acropetal transport becomes more pronounced near the time of vascular tissue differentiation in the setae, it seems reasonable to speculate that acropetal transport occurs in the vascular tissue to supply the auxin that is presumably required for the delayed process of capsule differentiation. Lastly, polar auxin transport in moss setae bears some remarkable similarities to this process in vascular plant axes. Bidirectional polarized transport is also observed in certain vascular plant organs; for instance, auxin transport in angiosperm roots is basipetal in the peripheral cortex, but it is acropetal in the central stele (Rashotte et al., 2000; Swarup et al., 2001). This bidirectional auxin movement appears to play an essential role in pattern formation and cellular differentiation in roots (Sabatini et al., 1999; Friml et al., 2002; Grebe et al., 2002).

In summary, among the three bryophyte lineages, hornwort sporophytes appear to exhibit the simplest structural features and hormonal regulation for generating an elongated axis. Almost all liverwort sporophytes develop elongated setae for elevating their capsules, but liverwort setae have growth mechanisms and auxin movements that are quite different from those operating in moss setae. Moss setae and vascular plant

organs have similar structural features and hormonal regulation, which may be indicative of common developmental mechanisms operating in both types of plant axes.

*Evolutionary implications*—As is clear from emerging perspectives from the field of evolutionary developmental biology (Raff, 1996; Knoll and Carroll, 1999; Peterson and Davidson, 2000; Cronk, 2001), the regulatory mechanisms operating in embryos and young organisms are generally conserved within particular lineages over great evolutionary time scales. The evidence is consistent with the notion that auxin has played a critical role in the regulation of plant developmental processes ever since the origin of the land plant lineage. First of all, auxin serves as the principal hormone for regulating embryo development, at least in vascular plants (Cooke et al., 2003). Bryophyte gametophytes tend to have auxin biosynthetic pathways, auxin movement characteristics, and auxin-mediated responses that are rather similar to those features in vascular plant sporophytes (Cooke et al., 2002). This leads to the plausible interpretation that the vascular plants are not likely to have evolved *de novo* mechanisms governing auxin regulation of developmental processes, but rather they modified preexisting mechanisms already operating in the early land plants. Lastly, given that the bryophytes seem to represent the earliest divergent lineages of land plants (Kenrick and Crane, 1997; Qiu et al., 1998; Nickrent et al., 2000; Renzaglia et al., 2000; Karol et al., 2001; Delwiche et al., 2004), the present report concerning auxin effects on the axial growth of bryophyte sporophytes may provide significant insights into the early evolution of land plant sporophytes.

The unique structural events and hormonal regulation in young hornwort and liverwort sporophytes make it impossible to link the process of axial elongation in either group to the comparable process in vascular plants. By contrast, the remarkable structural and physiological similarities in the axial elongation of moss setae and vascular plant axes supports the plausible interpretation that mosses may be the sister group to vascular plants. In particular, it appears reasonable to speculate that this elongation mechanism, which is based on persistent apical or subapical meristems, early axis differentiation, and bidirectional polarized auxin transport, evolved in their common ancestor before the divergence into separate lineages.

From these considerations, the following scenario for the evolution of the sporophytic axes of early land plants may be plausible. Microfossil evidence from the Middle Ordovician Period has indicated that the earliest land plants were likely to have a bryophyte-grade of structural organization, at least with respect to spore morphology (Gray, 1985; Edwards and Wellman, 2001; Graham and Gray, 2001). These first plants gave rise to different lineages, including those that would ultimately evolve into the hornwort, liverwort, and moss-vascular plant lineages. No paleobotanical evidence exists to resolve the issue of whether these lineages diverged before or after they evolved the ability to generate axial sporophytes. Nevertheless, insofar as extant bryophytes possess very different mechanisms for elevating their sporangia, it seems reasonable to propose that the diversification of bryophyte lineages did precede the independent origins of axial sporophytes. Of the earliest lineages of land plants, only the putative moss–vascular plant lineage appears to have evolved an elongation mechanism preadapted for

generating the large multiaxial sporophytes that have been the most prominent members of the terrestrial flora ever since 400 my BP.

Our enthusiasm for this scenario is dampened by the realization that the question of bryophyte evolution can be viewed as yet another ‘‘abominable mystery’’ plaguing plant evolutionary biology (Kenrick and Crane, 1997; Niklas, 1997; Bateman et al., 1998; Goffinet, 2000). Molecular phylogenetic studies have unequivocally established the three bryophyte lineages as being the earliest divergent lineages of extant plants, although the specific order of their divergence remains unresolved to date (Qiu et al., 1998; Goffinet, 2000; Nickrent et al., 2000; Karol et al., 2001; Delwiche et al., in press). The earliest mesofossils with possible bryophyte affinities have been identified as miniature branching axes in Lower Devonian rocks (Edwards et al., 1995; Edwards, 2000; Edwards and Axe, 2000). The meager macrofossil record for putative bryophytes, viz. thalloid organisms bearing monosporangiate axes, consists of a few compression fossils, such as *Sporogonites* (Lower Devonian), perhaps representing an early hornwort or thalloid moss, and *Pallaviciniites* (Upper Devonian), closely resembling certain modern liverworts (Taylor and Taylor, 1993; Goffinet, 2000). By contrast, the macrofossil record of the early land plants appears to emphasize the rapid diversification of numerous multiaxial protracheophytes and vascular plants starting in Upper Silurian and Lower Devonian strata (Taylor and Taylor, 1993; Kenrick and Crane, 1997; Bateman et al., 1998). The evidence available from well-preserved fossils in the Rhynie Chert (Lower Devonian) indicates that these plants are likely to have undergone isomorphic alternation of generations, as opposed to the heteromorphic life cycles of extant bryophytes (Kenrick and Crane, 1997). Therefore, the central dilemma in bryophyte evolution is how to

resolve the apparent conflict between the early divergence of bryophyte lineages, as predicted by molecular analyses, vs. the late appearance of recognizable bryophytes in the fossil record. Kenrick and Crane (1997) proposed that stem-group bryophytes have gone unrecognized because they may lack the most distinctive characteristics of extant crown groups. It is therefore conceivable that the monosporangiate axes of extant bryophytes do not represent the ancestral condition, but instead these axes were evolutionarily derived from reduced polysporangiate structures. This perspective must necessarily confound any facile interpretation that the auxin regulation of axial elongation in extant bryophyte sporophytes reflects the developmental mechanism involved in the evolution of the sporophytic axes of the earliest bryophytes.

A second, related problem arises in our interpretation that the underlying mechanism of axial elongation arose in the common ancestor of the mosses and vascular plants, which implies that the development of the sporophytic axis is a homologous process in these two groups. According to the most recent phylogenetic analysis of the mosses (Newton et al., 2000), the earliest divergent moss order is the problematic Sphagnales, which elevate their short sporophytes via extended gametophores. This mechanism of capsule elevation in Sphagnales may represent the basal state in the mosses, or it may be a derived adaptation in response to their aquatic habit (L. E. Graham, University of Wisconsin, Wisconsin USA, personal communication). In the former case then, the appearance of polar auxin transport in the sporophytic axes of later-divergent Polytrichales would be attributable to independent recruitment as opposed to a single origin in the common ancestor of these two groups.

In conclusion, the observations made in this chapter indicate that the three divisions of extant bryophytes utilize different developmental mechanisms for regulating axial elongation of their sporophytes. The auxin regulation of axial elongation in moss sporophytes is quite reminiscent of the same process in certain vascular plant organs, which supports the intriguing interpretation that it originated in a common ancestor of the moss and vascular plant lineages. Of course, the prediction that these two lineages are indeed sister to each other remains to be validated by further phylogenetic analyses.

# CHAPTER 4

# POLAR AUXIN TRANSPORT IN THE MOSS *Polytrichum ohioense* : DEVELOPMENTAL REGULATION, ENVIRONMENTAL SENSITIVITY, AND EVOLUTIONARY IMPLICATIONS

## INTRODUCTION

The plant hormone indole-3-acidic acid (IAA, auxin) plays an important role in angiosperm development with direct involvement in embryogenesis, tropisms, vascular tissue formation, and root formation. This perspective has recently been greatly strengthened by molecular investigations on the model system *Arabidopsis thaliana*. For example, auxin binding protein 1 (ABP1) has been found to be required for organized cell elongation and division in *Arabidopsis* embryogenesis (Chen et al., 2001). During embryogenesis, the basal localization of the auxin efflux carrier PIN1 has been shown to be critical for the formation of the shoot-root axis (Steinmann et al., 1999) and vascular tissue formation (Gailweiler et al., 1998). The auxin influx carrier AUX1 has been observed to promote lateral root formation by facilitating auxin distribution between the sink and source tissues in *Arabidopsis* seedlings (Marchant et al., 2002). However, little attention has been granted to the metabolism, transport, and action of auxin in the bryophytes and pteridophytes.

Current knowledge about the regulation of auxin metabolism in the entire land plant lineage was summarized by Sztein et al. (2000). It appears that the tryptophan–

independent pathway is the predominant auxin biosynthetic pathway in the charophytes and land plants. Together the liverworts and charophytes have a higher percentage of free IAA, slow conjugation rates, and low levels of total IAA metabolites suggesting that free IAA levels are regulated by a biosynthesis-degradation strategy (Sztein et al., 2000). In direct comparison, other bryophytes and vascular plants appear to regulate lower free IAA levels using a conjugation-hydrolysis strategy. Conjugation rates become greater for mosses/hornworts, pteridophytes, and seed plants respectively. Another trend is the switch from an amide conjugation strategy in the liverworts, hornworts, and mosses to a combined amide- and ester-conjugation strategy in the vascular plants (Sztein et al., 2000).

By examining the effects of auxin-regulatory compounds on the sporophytes of the three divisions of extant bryophytes, I (Chapter 3) identified developmental mechanisms that may have been involved in the evolution of axial sporophytes in early land plants. The altered growth of isolated young sporophytes exposed to applied auxin (IAA) or an auxin antagonist (*p*-chlorophenoxyisobutyric acid) suggested that endogenous auxin acts to regulate the rates of axial growth in the hornworts, liverworts, and mosses. The hornwort sporophyte exhibited a low auxin flux, was insensitive to transport inhibitors, and did not express transport polarity; these results suggested that the hornwort sporophyte moved auxin by simple diffusion. Liverwort sporophytes had higher auxin fluxes and were sensitive to the transport inhibitors but the transport still lacked polarity, thereby suggesting apolar facilitated diffusion as the auxin transport mechanism. In young *Polytrichum* sporophytes, auxin movement was predominantly basipetal and occurred at high fluxes exceeding those measured in maize coleoptiles. In

older *Polytrichum* sporophytes, acropetal auxin flux had increased beyond the level measured for basipetal flux. Differing inhibitor sensitivities for acropetal and basipetal fluxes suggested that moss sporophytes might carry out bi-directional polar transport in different cellular pathways, which resembles the transport in certain angiosperm structures (Aloni et al., 2003; Berleth et al., 2000; Friml et al., 2003)

The overall objective of this paper was to characterize the bi-directional auxin transport in older moss sporophytes that was observed in Chapter 3. Modified agar-block techniques for measuring polar transport of radiolabelled auxin were utilized to accommodate the small cross-sectional area of the moss sporophyte and its subsequent dissected tissue types of auxin (Chapter 3). To my surprise, total acropetal transport in sporophytes collected for this research was significantly reduced compared to the levels measured in previous collections. These results prompted further investigations into: 1) the fluxes and polarities of auxin transport occurring in different tissues within moss sporophytes at different developmental stages, and 2) the annual weather patterns correlating with different levels of acropetal auxin fluxes. The results obtained from the experiments suggested that polar auxin transport played important roles in moss sporophyte development; however, auxin transport in moss sporophytes appeared to manifest greater variability in response to environmental conditions than did the same process in angiosperm sporophytes.

## MATERIALS AND METHODS

*Plant material— Polytrichum ohioense* gametophytes with dormant sporophytes were collected on the campus of the University of Maryland in College Park, Maryland, USA

in winter and stored in the refrigerator at 4 °C to maintain dormancy until needed for experimental manipulations. Then the plants were transferred to growth conditions at room temperature under ca. 100 μmol $m^{-2}$ •$s^{-1}$ of fluorescent light until the proper developmental stage was reached. Young, pre-capsule, and early capsule sporophytes were utilized in the following experiments. The young sporophytes, allowed to grow for 3-5 days, were equivalent to those utilized in Chapter 3 at 1.0 - 1.5 cm in length. Pre-capsule sporophyte setae were somewhat longer than, but roughly equivalent to, the older sporophyte setae observed in Chapter 3. Pre-capsule sporophytes were grown for 3-5 weeks and the setae reached the length of 4-6 cm. Young sporophytes had a green seta while the pre-capsule sporophyte seta was green-brown. Seta width and capsule area shape and size were similar between the young and pre-capsule sporophytes. Early-capsule sporophytes grown for 6 – 8 weeks had the longest seta, 6-9 cm, of the three stages used. Morphologically the early-capsule sporophyte produced a small swollen capsular on top of the brown seta, but the seta width was equivalent to its width at the other two stages.

*Auxin transport assays*—The experiments designed to measure auxin transport in bryophyte sporophytes were carried out using conventional agar-block methods (McCready and Jacobs, 1963; Mitchell and Livingston, 1968), modified to accommodate the small cross-sectional areas of moss sporophytes (Chapter 3). Experiments were run to evaluate auxin transport polarity in the setae of young, pre-capsule, and early-capsule sporophytes. All donor blocks contained $10^{-6}$ mol/L 5-[$^{3}$ H]-IAA (specific activity of 20 Ci/mmol, American Radiolabeled Chemicals, St. Louis, Missouri, USA), and receiver

blocks were composed of water agar made with Bactoagar (Difco Laboratories, Detroit, Michigan, USA). The transport chambers were constructed as described in Chapter 3. Single time point experiments for characterizing the transport polarity utilized 5–20 sporophyte sections, which were placed in the transport chamber for 3 h. Time course experiments for measuring the accumulated amount of basipetal or acropetal transport used 5-30 different sections for each time point taken at 1 h, 3 h, and 5 h.

Additional experiments were run to examine auxin transport in the cortex and vascular tissue of the seta. A glass capillary tube was pulled into a thin point and then the very edge of the point was broken off to create a boring apparatus that was slid between the cortical and vascular regions of each seta section described above. The boring apparatus was slowly pushed into the seta resulting in the vascular tissue being pushed out of the seta section. Transport assays were run exactly as described above on both the cortex and vascular cylinder.

The receiver block from each section was placed in 5 mL of Biosafe II scintillation fluid overnight and then counted for 5 min in an LKB Wallac 1219 Rack Beta liquid scintillation counter (95% counting efficiency, LKB Instruments, Gaithersburg, Maryland, USA). All calculations of transport parameters were done exactly as described in Chapter 3.

*Temperature and precipitation data* – All temperature and precipitation data were collected by the Maryland State Climatologist Office (2004) from the Beltsville, MD weather station 7 miles from the collection site. Since original data were presented in

degrees Fahrenheit and inches, they were converted into degrees Celsius and cm, respectively, before being presented in Fig. 4-2.

## RESULTS

*Sporophyte development*–The young sporophyte of *Polytrichum ohioense* grows as a bipolar structure with transient apical cells at opposite poles resulting in a javelin-shaped structure (Smith 1955; Lal and Bhandari, 1968; Bold et al. 1987; Crum 2001). An intercalary meristem, which arises at the base of the future capsule, generates the remaining seta cells (French and Paolillo, 1975), which undergo cell elongation to form the mature seta. The seta differentiates to form an outer epidermis, an underlying cortex, and a central strand composed of water-conducting cells called hydroids, sugar-conducting cells called leptoids, and supportive stereids (Hébant, 1977). At the end of sporophyte development, the capsule region swells, and the spores mature simultaneously and are disseminated by the activity of the peristome.

All *Polytrichum ohioense* gametophytes with attached sporophytes were collected from their habitat in the winter. The moss was grown on soil from the original habitat at room temperature until the young stage sporophytes reached the desired length of 1.5 cm. Pre-capsule stage sporophytes 4 – 6 cm in length and early-capsule stage sporophytes 6 – 8 cm in length were also used in certain experiments.

*Developmental analysis of polar auxin transport* –Transverse sections from the setae of the three developmental stages were placed into a conventional agar-block apparatus with

$^{3}$H-IAA in the donor blocks in order to characterize the time course of auxin movement in both basipetal and acropetal directions. The amount of $^{3}$H-IAA was measured in the receiver blocks at 1, 3, and 5 h to construct the curves depicted in Fig. 4-1 and characterized in Table 4-1.

Young sporophytes carried out basipetal auxin transport at a velocity of 6 mm/h and a flux of 0.404 fmoles $mm^{-2}$ $s^{-1}$ as ascertained from the equation $y = 213.9x - 179.9$ (Fig 4-1, Table 4-1). Basipetal transport in the pre-capsule sporophytes showed almost a two-fold decrease in velocity to 3.6 mm/h but a decrease of almost four-fold to 0.146 fmoles $mm^{-2}$ $s^{-1}$ in flux as calculated from the equation $y = 78.7x - 110.4$. In contrast, the young-capsule sporophyte had a velocity of 7.2 mm/h in the basipetal direction that was greater than the other stages observed. Utilizing the line $y = 31.4x - 21.7$ to calculate the basipetal flux, a further decrease to a flux of 0.059 fmoles $mm^{-2}$ $s^{-1}$ in the young-capsule stage.

Acropetal transport velocity could not be resolved for any stage because the limited amount of auxin transported resulted in best-fit lines that did not cross the x-axis (Fig. 4-1). However, acropetal fluxes in the acropetal direction could be determined for all three stages utilizing their best-fit lines (Table 4-1). The young sporophytes exhibited a time course of acropetal transport which was represented by the line $y = 0.3x + 1.9$ that translated into a flux of $5.5 \times 10^{-4}$ fmoles $mm^{-2}$ $s^{-1}$. In contrast to the young sporophytes, the time courses of pre-capsule sporophytes and young-capsule sporophytes was characterized by similar lines, $y = 2.4x + 0.9$ and $y = 3.1x + 0.4$, respectively, which corresponded to similar acropetal fluxes at $4.4 \times 10^{-3}$ and $5.9 \times 10^{-3}$ fmoles $mm^{-2}$ $s^{-1}$, respectively.

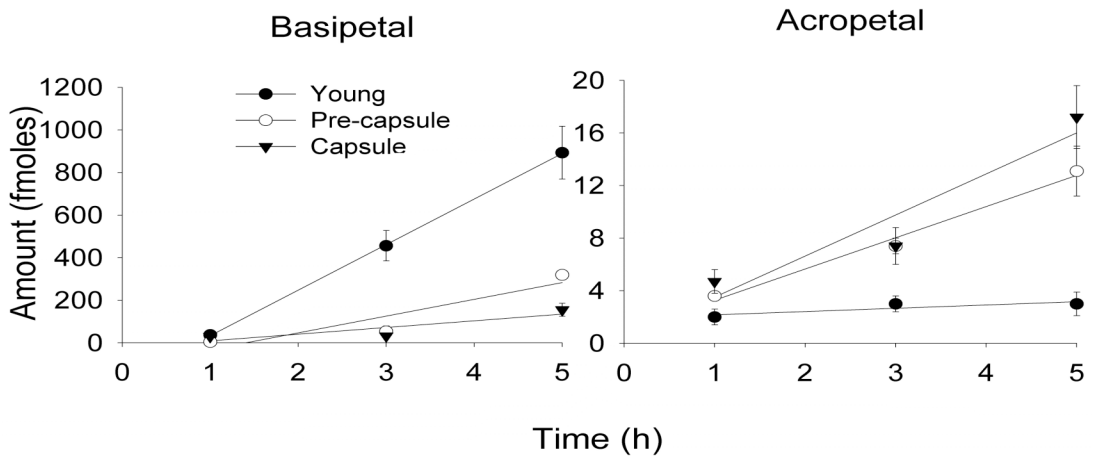



**Fig 4-1**. Basipetal and acropetal auxin transport in isolated seta sections of young (closed circles), pre-capsule (open circles), and early-capsule (closed triangles) sporophytes from an agar-block apparatus over 5 h. Data are presented as means ± 1 SE among five to 15 replicate sporophytes for every collection of each stage at 1, 3, and 5 hours. The term replicate refers to individual sporophytes. The data from the replicates presented in the figure were obtained in a representative experiment that was repeated 8 times.

| Stage | Orientation | Best-fit line | B/A ratio | Velocity (mm/h) | Flux (fmoles/mm$^2$ s) |
|---|---|---|---|---|---|
| Young | Basipetal | y = 213.9x – 179.9 | 152 | 6.0 | 0.404 |
| | Acropetal | y = 0.3x + 1.9 | | -- | 5.5 x $10^{-4}$ |
| Pre-capsule | Basipetal | y = 78.7x – 110.4 | 7.3 | 3.6 | 0.146 |
| | Acropetal | y = 2.4x + 0.9 | | -- | 4.4 x $10^{-3}$ |
| Young capsule | Basipetal | y = 31.4x – 21.7 | 4.3 | 7.2 | 0.059 |
| | Acropetal | y = 3.1x + 0.4 | | -- | 5.9 x $10^{-3}$ |

**Table 4-1**: Key parameters characterizing basipetal and acropetal auxin transport in three sporophyte stages of *Polytrichum ohioense*. The equations correspond to the best-fit lines calculated on the basis of the amount of radioactivity in the receiver blocks at 1, 3, and 5 h time points, as depicted in Fig. 4-1.

Basipetal auxin transport was considerably higher in all three developmental stages when compared to acropetal transport (see Table 4-1, Fig. 4-1). The 3 h basipetal/acropetal (B/A) ratio for young sporophytes was 152, and this ratio dropped considerably to 7.3 and 4.3 in the pre-capsule and early-capsule sporophytes, respectively. Even though it appears that the velocity of basipetal transport was not greatly affected by sporophyte maturation, the amount of auxin transported per cross-sectional area per second (flux) decreased with sporophyte maturation (Table 4-1).

The sporophytes used in this experiment were collected from the same population as those collected in the years 2000-2001 and 2001-2002 for the experiments described in Chapter 3. Table 4-2 represents the total basipetal and acropetal transport over 3 h in whole sections of young and pre-capsule setae from the three different years. As the immature sporophytes from the 2000-2001 and 2001-2002 collections were released from dormancy and reinitiated their development auxin transport decreased in the basipetal direction but increased in the acropetal direction, and this trend culminated into basipetal and acropetal transport becoming essentially equal or a B/A ratio of 1 (Table 4-2). Young and pre-capsule sporophytes from the 2003-2004 season were examined for 3 hours utilizing the same agar-block assay and at equivalent developmental stages so direct comparisons to the young and older sporophytes from the 2000-2001 and 2001-2002 seasons could be made in both the acropetal and basipetal orientations. Basipetal auxin transport was 456.0 ± 71.3 fmoles in the young sporophyte setae from the 2003-2004 season which is 5.5 times higher than the 2000-2001 and 2001-2002 seasons. Similarly, acropetal auxin transport for the 2003-2004 season decreased to 2.8 ± 0.5

| Stage | Transport Direction | 2003 – 2004 Transport (fmoles) | 2003 – 2004 Replicate number | 2000 – 2001 Transport (fmoles) | 2000 – 2001 Replicate number | 2001 – 2002 Transport (fmoles) | 2001 – 2002 Replicate number |
|---|---|---|---|---|---|---|---|
| **Young** | Basipetal | 456.0 ± 71.3 | 9 | 85.4 ± 8.1 | 15 | 54.4 ± 7.3 | 5 |
| | Acropetal | 2.8 ± 0.5 | 12 | 9.2 ± 0.8 | 5 | 9.2 ± 1.3 | 5 |
| **Pre-Capsule** | Basipetal | 54.0 ± 25.6 | 10 | 44.7 ± 0.4 | 6 | 68.2 ± 10.8 | 10 |
| | Acropetal | 7.0 ± 1.4 | 11 | 37.1 ± 0.3 | 6 | 77.1 ± 18.4 | 10 |

**Table 4-2**: Total basipetal and acropetal transport over 3 hours in whole sections of young and pre-capsule setae taken from the same population of *Polytrichum ohioense* sampled in three different years. Data are presented as the mean ± standard error among replicate. The term replicate refers to individual sporophytes. The data from the replicates presented in the table were obtained in a representative experiment that was repeated 6 times.

fmoles, 1/3 of the amount determined in the 2000-2001 and 2001-2002 seasons. In the pre-capsule setae, basipetal auxin transport in the 2003-2004 season was roughly comparable as that transport in the other years, but acropetal transport was 7.0 ± 1.4 fmoles which was 5-11 times less than the previous years (Table 4-2). Several sub-populations as well as all stages of sporophyte development from early pre-capsule to mature capsule from the 2003-2004 season were sampled with similar observations (data not shown). Therefore, I was completely surprised by these observations that the sporophytes from the 2003-2004 season did not possess obvious bi-directional transport as observed in the past.

*Structural analysis* – In an effort to determine whether or not sporophytes from 2003-2004 might be carrying out cryptic acropetal transport, whole sections, cortical regions, and vascular cylinders of young and early-capsule setae were placed into the agar-block apparatus with $^{3}$H-IAA in the donor blocks to determine the transport activities of the different seta tissues (Table 4-3). The early-capsule sporophytes were utilized instead of the pre-capsule sporophytes for this experiment because they exhibited slightly more low levels of acropetal transport than pre-capsule sporophytes. The values for transport intensity in whole sections and cortical regions were determined from time course experiments in the basipetal and acropetal directions. Preliminary experiments on vascular cylinders resulted in almost instantaneous auxin accumulation in the receiver block suggesting that the auxin label was wicking across the vascular cylinder into the receiver block. Lanolin paste was also tried as an alternative method for delivering the auxin from the donor block but the transport intensities in all these experiments were

| Stage | Transport Direction | Whole sections | | Cortical Regions | | Vascular Regions | |
|---|---|---|---|---|---|---|---|
| | | Intensity (fmoles/h) | Flux (fmoles/cm$^2$ s) | Intensity (fmoles/h) | Flux (fmoles/cm$^2$ s) | Intensity (fmoles/h) | Flux (fmoles/cm$^2$ s) |
| **Young** | Basipetal | 213.98 | 40.43 | 119.33 | 32.82 | 94.65 | 58.43 |
| | Acropetal | 0.13 | 0.03 | 1.23 | 0.34 | -1.10 | -0.68 |
| **Early-Capsule** | Basipetal | 31.43 | 5.94 | 105.43 | 29.00 | -74.01 | -45.68 |
| | Acropetal | 3.13 | 0.59 | 1.98 | 0.54 | 1.15 | 0.71 |

**Table 4-3**: Polar transport in whole sections, cortical regions, and vascular regions of the setae of *Polytrichum ohioense* at young and early-capsule stages. Intensity data for whole sections and cortical regions were determined from time course experiments as described in the Materials and Methods. The intensity data for vascular regions were calculated as the difference between the data for whole sections and cortical regions. Flux data were calculated as described in the Materials and Methods. The term replicate refers to individual sporophytes. The data from the replicates presented in the table were obtained in a representative experiment that was repeated 8 times.

much lower than those observed in agar-block experiments. Because I was never successful in acquiring reliable time course data from vascular regions, the transport intensity in vascular regions was calculated as the difference between the values measured in whole sections and cortical regions.

Both the young and early-capsule sporophytes had a whole and cortical region cross-sectional area of 0.15 $mm^2$ and 0.10 $mm^2$, respectively, so that the transport parameters from the two stages can be compared without normalizing the data (Table 4-3). In this experiment, whole sections of young sporophytes showed basipetal auxin transport at an intensity of 213.98 fmoles/h while the cortical region obtained from whole sections moved 119.33 fmoles/h of auxin basipetally. Therefore the difference in the amount of auxin transported in the basipetal direction between the whole sections vs the cortical regions must have been supplemented by 94.65 fmoles/h of auxin through the vascular region. Basipetally, whole sections of young sporophytes exhibited a flux of 40.43 fmoles $cm^{-2}$ $s^{-1}$, cortical regions exhibited 32.82 fmoles $cm^{-2}$ $s^{-1}$ and the vascular region exhibited 58.43 fmoles $cm^{-2}$ $s^{-1.}$ The basipetal flux in vascular tissue was actually higher than the cortex due to the smaller cross-sectional area of the vascular tissue. The intensity and flux of acropetal transport were negligible in both regions of these young sporophytes (Table 4-3). In the early-capsule sporophytes, basipetal transport in whole sporophytes occurred an intensity of 31.43 fmoles/h, which represents seven-fold decrease when compared to the young sporophytes. By contrast, 105.43 fmoles/h of auxin were basipetally transported through the cortical region of early capsule sporophytes, which is very similar to the measurement of cortical transport in young sporophytes. Thus, a basipetal intensity of –74.01 fmoles/h was calculated for the

vascular region, which means that the presence of the vascular tissue caused an apparent reversal in the direction of auxin transport. However, when the section orientation was reversed for measuring direct acropetal transport whole sections of early-capsule sporophyte transported 3.13 fmoles/h in the acropetal direction, with 1.98 fmoles/h measured in the cortex and 1.15 fmoles/h calculated in the vascular tissue. Possible causes of the apparent reversal of auxin transport in the vascular tissue of early-capsule sporophytes are evaluated in the Discussion.

In summary, young sporophytes carry out basipetal auxin transport in both the cortex and the vascular tissue. In contrast, the early-capsule sporophyte exhibits a significant decrease in basipetal transport that appears to be attributable to reversed flow through the vascular region.

*Seasonal differences* – Several workers have reported that internal structure of moss sporophytes is less developed under the influence of high humidity, diminished light intensity, and/or burial in soil (Davy de Virville, 1927; Leach, 1930). Based on these findings, plus his own Hebant (1977) concluded that the development of vascular tissue in moss sporophytes is highly dependent on environmental conditions. Therefore I decided to examine the precipitation and temperature data obtained from a weather monitoring station within 7 miles of the collection site for the years 2000-2004 (Fig. 4-2), in order to examine possible correlations between the weather condition and the relative intensity of bi-directional transport observed in different seasons.

I observed the following seasonal events in moss sporophyte development in the population used for my studies. Persistent gametophytes bear antheridia and archegonia

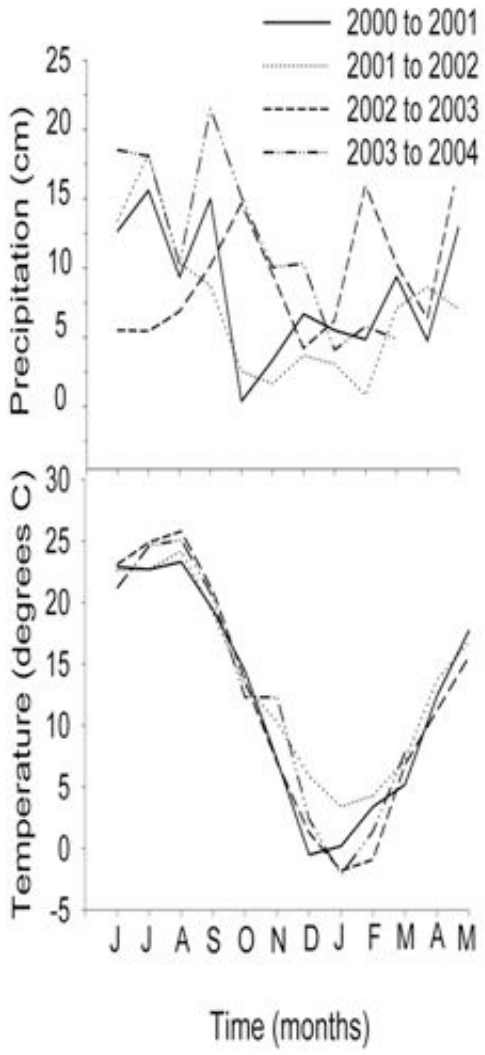
in July, after which fertilization and the initial stages of sporophyte development proceed until the young sporophyte become dormant in late October or early November. From November through March or April sporophytes remain dormant, as measured by elongation of the seta. Once the temperatures begin to warm up, the sporophyte completes seta elongation and capsule maturation, with spore release occurring in May and June.

The pattern of sporophyte development is obviously related to the four-season climate in Maryland. Winter, occurring from late-November through mid-March, has an average freeze period of 185 days with January being the coldest month. Spring lasts from March to May with average temperatures around 10 °C (Maryland State Climatologist Office, 2004). May has the highest average monthly rainfall at about 11.5 cm (Maryland State Climatologist Office, 2004). Summers are hot and humid with July having highest temperatures ranging in the mid to upper 20's°C. Finally, Fall generally lasts from September through early-November with mild temperatures and moderate rainfall.

The moss population at the collection site experienced some climatic extremes during the years that I studied these plants. During the critical period of initial sporophyte development, the 2000-2001 collection experienced the coldest July in the last 50 years, which was followed by a drought lasting from September through November. The plants collected for the 2001-2002 experiments were subjected to a more severe, and possibly a more influential drought than occurred in the 2001 Fall. Therefore, the exposure to drought conditions may have pre-disposed the sporophytes

collected during the 2000 and 2001 winters to have developed more vascular tissue, which may in turn have affected acropetal auxin transport.

**Fig. 4-2** Monthly temperature and precipitation data for the College Park/Beltsville MD area from 2000-2004. All data were originally reported in degrees Fahrenheit and inches, respectively(Maryland State Climatologist Office) but were converted into degrees Celsius and cm.

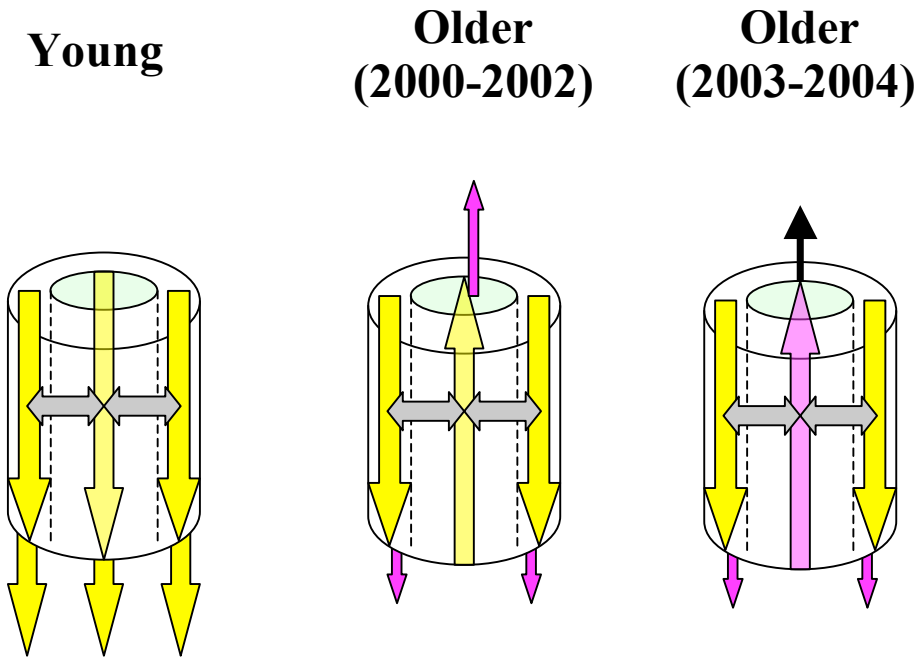

2000 to 2001
2001 to 2002
2002 to 2003
2003 to 2004
Precipitation (cm)
25
20
15
10
5
0
Temperature (degrees C)
30
25
20
15
10
5
0
-5
J J A S O N D J F M A M
Time (months)

## DISCUSSION

The evidence presented in Chapter 3 and this chapter suggests the polar auxin transport in moss sporophytes is dependent on developmental stage, transporting tissue, and environmental conditions. In young sporophytes, auxin transport occurs in the basipetal direction, at higher fluxes than those even occurring in maize coleoptiles, and in both the cortex and vascular cylinders. In older sporophytes, lower fluxes of basipetal transport do persist in the cortex. However, the interpretation of my results on acropetal transport in older sporophytes is less straightforward. Previous descriptions of bi-directional auxin transport in angiosperm roots have generally not even mentioned the critical role that the endodermis must play in the transport process. Since the mechanism of auxin transport involves auxin secretion from the basal ends of the transporting cells, which is coupled to the apoplastic diffusion between those cells, then the complete separation of two opposing pathways requires the presence of an apoplastic barrier, such as the Casparian bands in angiosperm roots, to prevent lateral diffusion between two pathways.

By contrast, moss sporophytes lack an apoplastic barrier between the cortex and the vascular cylinder, which has the following consequences for interpreting the data presented in this chapter (Fig.4-3). In young sporophytes, both the cortical and vascular pathways are carrying out basipetal auxin transport. Thus the fluxes measured at the cut surfaces of the seta of young sporophyte sections do probably correspond to the actual fluxes moving through the two tissues. However, it appears that the vascular tissues in older sporophytes are mediating acropetal transport, with the result that the fluxes of this

**Fig. 4-3**: A model of auxin transport through the cortical (white cylinder) and vascular regions (green cylinder, dotted lines) of the *Polytrichum ohioense* seta in young and older sporophytes. Direction and quantity of auxin is represented by the arrows.



cross-current transport occurring in older sporophytes should be significantly higher inside the sections than those measured at their cut surfaces. In those older sporophytes collected in the 2000-2001 and 2001-2002 seasons, acropetal transport is directly visualized in whole sections because the acropetal transport flux is appreciably higher than the lateral diffusion. However, the acropetal transport flux in older sporophytes collected in the 2003-2004 season is apparently similar to lateral diffusion; consequently, this acropetal transport is only visualized via its ability to reduce the basipetal flux measured from whole sections relative to the much higher level measured from isolated cortical tissues. If one considers the observations of older sporophytes from the 2003-2004 season, it is also conceivable that the vascular tissue is secreting endogenous transport inhibitors, such as flavonoids, into the cortex, thereby causing the observed reduction in basipetal transport observed in whole sections. However, this alternative explanation fails to account for the significant fluxes of acropetal transport observed in the older sporophytes from earlier seasons.

*Compare and contrast to angiosperm work*—Molecular genetic evidence suggest that bi-directional auxin circulation is established in young *Arabidopsis* embryos (Steinmann et al., 1999). During the earliest stages of *Arabidopsis* embryogenesis, the auxin efflux protein PIN1 is randomly distributed in relation to the longitudinal axis. However, from the mid-globular stage onwards, PIN1 is restricted to the basal side of the central procambial cells and to the apical side of the outer epidermal and cortical cells. The coordination of this localization of this protein is dependent upon GNOM-dependent vesicle trafficking (Steinmann et al., 1999). *gnom* embryos distribute their PIN1 proteins

in a random fashion with the result that *gnom* and *pin1* embryos fail to develop a central axis. Therefore it appears that the establishment of cell polarity results in the localization of polar auxin transport proteins, which appears to circulate auxin in a basipetal direction down the vascular cylinder and in an acropetal direction up the outermost cell layers. This auxin circulation appears to play a critical role in establishing the root-shoot axis of the mature embryo.

Bi-directional auxin flow is also known to occur in various organs of the angiosperms. For example, auxin is directionally transported from young apical regions in developing organs such as leaves (Berleth et al., 2000; Aloni et al., 2003), and lateral roots (Laskowski et al., 1995). During the development of *Arabidopsis* leaves, for example, the site of highest free-auxin concentration production starts at the tip of the primordium, progresses to the leaf margins, and finally moves to the central regions of the leaf establishing a basipetal pattern leaf maturation and vascular differentiation (Aloni et al., 2003; Berleth et al., 2000). However, in roots, auxin is transported in the phloem to the root tip and then it circulates basipetally via polar auxin transport (*Coleus*: Goldsmith et al., 1973; *Phaseolus*: Davies and Mitchell, 1972; Mitchell and Davies, 1975; *Pisum*: Cambridge and Morris, 1996; *Zea*: Shaw and Wilkins, 1974; *Arabidopsis*: Rashotte et al., 2003). Auxin transport in roots has been even further described by Friml et al.(2003). Auxin is supplied to a root cap by PIN1- and PIN4-dependent acropetal transport in the vascular tissue. PIN3 is thought to move auxin laterally from the collumella cells after which auxin is basipetally transported through outer root cap and epidermis cells by an AUX1-dependent influx and a PIN2-dependent efflux (Friml et al., 2003) Benkova et al. (2003) visualized the dynamic gradients of auxin at the tips of

organ primordia for cotyledons, shoots, and roots in *Arabidopsis.* PIN proteins mediated these gradients regardless of morphology or developmental origin (Benkova et al., 2003). Thus, PAT acts as a general mechanism for the axis formation and vascular tissue differentiation in angiosperm organogenesis.

Even though PIN had not been characterized in the bryophytes, Chapter 3 established that auxin regulates axial growth in all bryophyte divisions with all groups exhibiting different mechanisms for moving auxin. In young *Polytrichum* sporophytes, basipetal auxin movement at high fluxes exceeded those measured in maize coleoptiles while older sporophytes exhibited equitable basipetal and acropetal transport with different inhibitor sensitivities. These results suggested a bi-directional polar transport existed in different cellular pathways that resembled the transport witnessed in angiosperm structures.

Therefore, the mosses contain a nascent auxin transport system that is influenced by the environment and developmental timing, and in the vascular plants this system is more highly regulated. Finally, further studies are necessary to test the depth of influence the environment has on PAT in mosses. However, some features of bi-directional auxin transport in the mosses are simpler than those in the angiosperms. In particular, the absence of apoplastic barriers decreases the relative efficiency of bi-directional flow. A second difference is the greater variability of acropetal transport in moss vascular tissue in response to environmental conditions, as opposed to the tight regulation observed in seed plants (Schrader et al., 2003).

Finally, it appears that the mechanism of polar auxin transport in the mosses is homologous to the transport mechanism operating in the angiosperms, at least as judged

by similar inhibitor sensitivities. This evidence supports the interpretation that polar auxin transport arose in the common ancestor of the moss and angiosperm lineages before their divergence into separate lineages. However, angiosperm embryos circulate auxin down the vascular tissue and up through the outer cell layers, whereas older moss sporophytes circulate auxin in the opposite direction. It seems reasonable to hypothesize from this evidence that polar auxin transport was independently recruited in these two lineages as the hormonal mechanism for establishing their sporophytic axes.

# CHAPTER 5

# THE EFFECTS OF AUXIN REGULATORS AND POLAR TRANSPORT INHIBITORS ON THE EMBRYOGENESIS OF THE FERN *Marsilea vestita*

## INTRODUCTION

In comparative morphological studies, embryos and larvae of extant animals were exhaustively examined as the first step in the characterization of different metazoan body plans (Raff, 1996; Gilbert, 2000). More recently, molecular investigations demonstrated that the regulated expression of homeobox (Hox) genes is responsible for establishing the anterior-posterior axis in the body plans of all bilateral animals (Knoll and Carroll, 1999; Pederson and Davidson, 2000; Carroll et al., 2001). By contrast, extant plants exhibit three distinctive embryonic body plans (Cooke et al., 2004). Linear tripartite embryos, which are subdivided into three regions destined to develop into absorptive foot, seta or intercalary meristem, and spore-producing capsule, are observed in the liverworts, hornworts, and mosses, even though these organisms utilize different cellular mechanisms to accomplish axial growth (Crum, 2001; Chapter 3). Pteridophytes develop

unipolar embryos, which are subdivided into shoot apex, leaf, root, and foot quadrants; subsequently, the shoot apex of each unipolar embryo continues to generate all the leaves and subtending roots of the developing sporophyte (Troll, 1943; Goff and Kaplan, 1988). In gymnosperms and angioserms, bipolar embryos produce shoot and root apical meristems at opposite poles, with the result that plant bodies exhibit central axes with opposing shoot and root systems (Troll, 1943; Groff and Kaplan, 1988). Although it is clear that plants and animals must use different developmental mechanisms for establishing their respective body plans (Meyerowitz, 2002), much less is known about the specific mechanisms operating in plants.

The hormone auxin (indole-3-acetic acid, IAA) appears to plays a central role in the embryogenesis of several flowering plants. For example, both somatic and zygotic embryos of *Daucus carota* exhibit a greater surge in free IAA levels following callus initiation and fertilization, respectively (Michalczuk et al., 1992a; Ribnicky et al., 2002; Ljung et al., 2002). The initial surge has been attributed to the activation of a tryptophan-dependent pathway for IAA biosynthesis, at least in the case of somatic embryogenesis (Michalczuk et al., 1992b). It seems reasonable to conclude that high levels of free IAA are required to mediate cell proliferation in the early globular embryo. Subsequently, lower free IAA levels, which are maintained by a tryptophan-independent biosynthetic pathway, are measured in the later stages of carrot embryogenesis, as the embryo starts to initiate the elongation of its bipolar axis. (Michalczuk et al., 1992a,b; Ribnicky et al., 2002). This interpretation is supported by inhibitor studies on carrot embryos and by molecular studies on Arabidopsis mutants. Fujimura and Komamine (1979) reported that IAA, 2,4,6-trichlorophenoxyacetic acid (2,4,6-T), and $\alpha$-(p-chlorophenoxy) isobutyric

acid (PCIB) were all effective inhibitiors of total embryogenesis in carrot cultures. However, treating these cultures with IAA and 2,4-dichlorophenoxyacetic acid (2,4-D) did not disrupt the sequence nor the polarity of individual stages in embryo development; instead, these treatments caused early embryos to revert to undifferentiated callus (Schiavone and Cooke, 1987). Similar experiments were done with *Brassica juncea*, which confirmed that the auxin analogs and inhibitors have comparable effects on another angiosperm system (Hadfi et al., 1998). In Arabidopsis, it has been shown that a homologous null mutation in auxin binding protein 1 (ABP1) results in embryo lethality (Chen et al., 2001). These embryos develop normally until the early globular stage where they become unable to produce a bilateral structure because cells fail to elongate. Therefore, it can be concluded that ABP1 is required for organized cell elongation and division during embryogenesis (Chen et al., 2001).

The evidence available to date suggests that polar auxin transport (PAT), or the polar movement of auxin through the activities of influx and efflux carriers (Muday et al., 2003), is the fundamental mechanism for initiating and maintaining the central axis in developing embryos. Using PAT inhibitors 1-N-naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA)*(*Lomax et al., 1995*),* it is possible to show the causal relationship between PAT and embryo development. In *Daucus carota,* the bipolar growth of advanced embryonic stages is blocked by PAT inhibitors, which often mediate the formation of additional lateral growth axes (Schiavone and Cooke, 1987). In comparison, PAT inhibitors induced the formation of fused cotyledons in globular embryos but not in heart-shaped embryos when used on *in vitro* cultures of *B. juncea* embryos (Liu et al., 1993). Hadfi et al. (1998) also observed collar-cotyledons forming

when NPA was added to developing embryos of *B. juncea*. But they also observed a small percent of very early globular embryos treated with NPA developing into twins and ball-shaped embryos suggesting that auxin transport is necessary to establish a proper apical-basal axis. Finally, several Arabidopsis mutants, such as theauxin efflux carrier pin1 and gnom, exhibit severe phenotypes that are blocked at the late globular stage. The molecular basis of these phenotypes is directly tied to PAT. PIN1 is involved in auxin efflux from a cell (Galweiler et al., 1998) and PIN positioning is thought to occur via GNOM-dependent vesicle trafficking (Steinmann et al., 1999, Muday et al., 2003). Interestingly, certain bryophytes, such as mosses, are also observed to carry out PAT in their sporophytic axes (Chapter 3). However, it is unknown whether auxin biosynthesis and polar transport play analogous roles during the development of the unipolar embryos of the vascular non-seed plants called pteridophytes.

The fern *Marsilea vestita* is an ideal system for studying pteridophyte embryogenesis for several reasons: 1) various treatments can be directly applied to the growth medium (Klink and Wolniak, 2001), 2) gamete development and activation occurs within 12-15 hours of hydration, 3) embryo development is observable after 24 hours of hydration (Laetsch, 1967; Rice and Laetsch, 1967; Machlis and Rawitscher-Kunkel, 1967), and 4) gross morphology of early development can be easily observed with a common dissecting microscope. The overall goal of this paper is to characterize the auxin regulation of embryo development in *M. vestita*. The experimental strategy used was to expose developing embryos to various regulators of auxin action, including auxin analogues, biosynthesis inhibitors, and polar transport inhibitors, known to affect angiosperm embryos. The strong auxin NAA and the anti-auxin PCIB were utilized to

examine the direct effects of auxin action on fern development while the biosynthetic inhibitors 6-fluoroindole (6FI) and 2-mercaptobenzimidazole (MBI) were applied to characterize the role of auxin metabolism in fern embryogenesis. Lastly, the polar transport inhibitors TIBA and NPA were used to examine the role of auxin transport during the development of the unipolar embryonic axis.

## MATERIALS AND METHODS

*Plant material*—Marsilea sporocarps were originally obtained from Dr. Peter Hepler (University of Massachusetts, Amherst, MA) as a gift to Dr. Stephen Wolniak (University of Maryland, College Park, MD). The source of the sporocarps was a natural population of ferns growing at Lake Lagunita, at Stanford CA. In the greenhouses of the University of Maryland, seven 500-gallon ponds were constructed to the dimensions of 3.72 m x 1.5 m x 31 cm. Plantlets were grown in 8-inch pots within the ponds, and sporocarps were induced by allowing the ponds to dry down. Sporocarps for this work were a gift from Dr. Stephen Wolniak.

*Treatment of embryos* --For each experimental treatment, eight fully expanded and regularly shaped sporocarps were cut at one end by a razor blade and were germinated in a 500 ml Erlenmeyer flask containing 500 ml of sterile 10% Murashige and Skoog (MS) Basal Salt Mixture medium (Sigma, St. Louis, MO, USA) supplemented with MS

Modified Vitamin Powder (Sigma, St. Louis, MO, USA) at 22-24°C. The cut sporocarps remained in this germination pretreatment for 14 h during which the sporocarps released the microspores and megaspores, the microgametophytes and megagametophytes completed their development, and the sperm fertilized the egg cells in the archegonia. Although various media, such as Laetsch's medium, are reported as being suitable for supporting subsequent embryonic development (Laetsch, 1967; Rice and Laetsch, 1967), preliminary experiments indicated that this modified MS medium was the only medium other than autoclaved tap water that was able to support synchronous embryonic growth with no apparent inhibition of initial organ development. All compounds, including TIBA (Sigma T-7267, sodium salt), NPA (Pfaltz & Bauer, Inc. 5G-NO3165), NAA (Sigma N-0640), PCIB (Aldrich Chemical Co. 19,777-7), MBI (Aldrich Chemical Co. M320-5), and 6FI (Sigma F-1876), were initially dissolved in 95% ethanol at a stock concentration of $3.3 \times 10^{-2}$ M. Various auxin-regulatory compounds were added to a final concentration of $3.3 \times 10^{-5}$ M to the flasks at 14 h in order to determine their effects on embryonic development. The control treatment consisted of a comparable 1000x dilution of 95% ethanol.

Ten hours after the start of the experimental treatment (24 h total time), the embryo-bearing megagametophytes floating on the solution surface were pipetted into 10 ml of fresh medium including the experimental treatment in a 15 ml polypropylene conical tube (FALCON Blue Max Jr. 17 x 120 mm style 35-2097). This transfer was accomplished by using modified Pasteur pipettes whose tips had been broken off and then flamed to provide a larger opening for drawing up the embryos. After embryos re-floated to the solution surface, the embryos were transferred to another conical tube again

with fresh medium. The embryos were thus washed five times for each treatment. Then the embryos were transferred to fresh experimental media in 35 x 10 mm polystyrene Petri dish (FALCON 35-1008). This transfer was repeated 24 h later. Morphological observations concerning various archegonial and embryonic features were made with the dissecting microscope at 48 and 72 h after cutting the sporocarp, which corresponded to 34 and 58 h exposure to the experimental treatments. Leaf angle was measured using a *camera lucida* attached to a compound microscope, and all other measurements were taken *via* an ocular micrometer under a dissecting microscope.

*Microtechnique* --In brief, embryos with attached nutritive cell following either 24 or 48 h after sporocarp incision were pipetted into glass vials, and the excess medium was removed before adding the formalin-acetic acid- 70% alcohol (1:1:18) fixative. The vials were then vacuum-infiltrated for 30 m, after which they were placed in 4° C for 36 h. A sharp glass needle drawn from a 1.0 mm diameter glass capillary tube was then used to puncture the attached nutritive cell, and the embryos were exposed to the fixative for an additional 36 h at 4° C. The embryos were dehydrated with successive 2-hour ethanol treatments of 70% (3 times), 80%, 90%, 100% (3 times) at 4° C. The embryos were embedded in methacrylate according to standard procedures (Ruzin, 1999) except that each lasted 12 h. Embryos were sectioned to 2-5 microns using glass knives with a Reichert model ultramicrotome. The methacrylate was etched by a 15 m acetone wash, and then the slides were stained with 1% toludine blue O for 20 s. Wet mount slides were examined with DIC microscopy, and the images were stored as tiff files.

## RESULTS

*Initial gametophyte and embryo development* --Fully expanded and regularly shaped sporocarps were more likely to produce viable spores. During imbibition, these sporocarps swelled apart and the sorophores (gelatinous rings) emerged bearing the sori. Once megaspores (0.7 –0.75 mm long and egg-shaped) and microspores (round and 0.075 mm in diameter) were released from the sori, they initially sank to the bottom of the flask but then rose to the surface due to the hydration of their outermost walls.

Simultaneously, each microspore matured into a microgametophyte with two antheridia while each megaspore developed into a megagametophyte with a single apical archegonium. In *M. vestita*, sperm populations were active for 3-3.5 h at the ambient temperature of 22-25°C (Rice and Laetsch, 1967). The sperm are attracted chemotactically to the archegonium, and fertilization occurred soon after sperm discharge approximately 10 hours following sporocarp incision at room temperature (for further details, see Laetsch, 1967; Rice and Laetsch, 1967; Machlis and Rawitscher-Kunkel, 1967).

Following fertilization, fern zygotes undergo an initial stage of rapid cell proliferation that leads to a four-part embryo including root, leaf, foot, and shoot apex quadrants. The activity of the shoot apex will ultimately generate all additional organs, with the roots rising at the bases of the leaves (Gifford and Foster, 1989). In more detail, the zygote secretes a basal cell wall in the plane of the archegonium axis, which is then followed by two more divisions that divide the original zygote into octants. Four octants constitute an epibasal hemisphere which gives rise to the leaf and shoot apical meristem and the other four constitute a hypobasal tier that gives rise to the root and foot (Bower, 1963). The organ primordia start to exhibit their characteristic structures as they burst through the venter of the archegonium (Bower, 1963). The general features of *Marsilea* embryo development are summarized in Figure 5-1.

When control embryos were sectioned at 24 h, they exhibited a four-quadrant spherical appearance with dense cells (Fig. 5-2 a). Approximately 26% of the control embryos appeared to exhibit an unorganized pattern of cell division, which is typically associated with embryo abortion. This effect may have been caused by the presence of

ethanol in the control medium necessary to dissolve the compounds. The embryos were surrounded by the venter of an archegonium that was two cell layers thick (Fig. 5-2 a). In control experiments, rhizoids developed in a cluster from the outer layer of the archegonium, in the general location of the first root, prior to the appearance of the organs (see Fig. 5-1). The first root developed to an average length of 54 ± 2 μm by 48 h, and to 1097 ± 212 μm by 72 h. Next, the first leaf developed to 440 ± 72 μm in length in 48 h, 2980 ± 281 μm in length after 72 h. This leaf was positioned at a leaf angle of approximately 80 degrees from the longitudinal axis of the original megaspore (Table 5-1).

**Fig. 5-1**: Montage of Marsilea embryo development. A, longitudinal section of a macrospore X 120 (Smith, 1955). B, stages in development of sterile tissue and archegonium of a megagametophyte X 215 (Smith, 1955). C, surface view of a megagametophyte after fertilization X 80 (Smith, 1955). D, vertical section through a young sporophyte in the natural position X 325 (Smith, 1955). E, surface view of a megagametophyte with a young sporophyte X book +200%(Smith, 1955). F, surface view of a megagametophyte with an older sporophyte X book +200% (Smith, 1955). (C., calyptra; L., leaf; F., foot; Rh., rhizoids; Rt., root; SA., shoot apex)

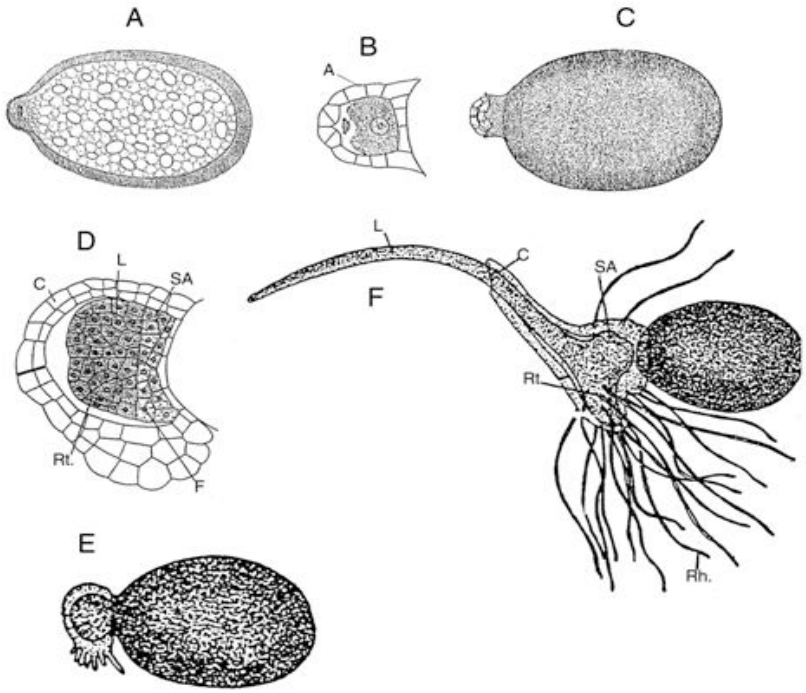
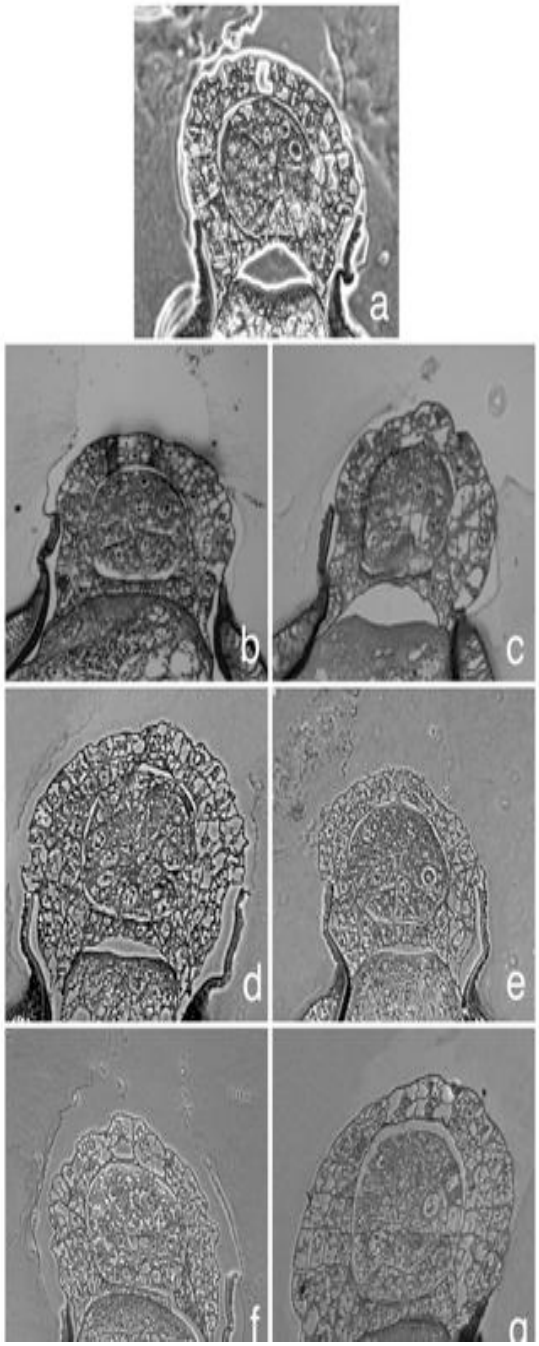

A
B
A
C
D
L
C
SA
Rt.
F
L
C
SA
F
Rt.
Rh.
E

**Fig 5-2**: 24 h embryos treated with different auxin biosynthesis, action, and transport inhibitors. A. Control, B. MBI, C. 6FI, D. NAA, E. PCIB, F. TIBA, G. NPA



| Treatment | Average Rhizoid Length (microns) | | Unorganized Embryos (%) | Organized Embryos | | | |
|---|---|---|---|---|---|---|---|
| | | | | First Leaf Length (microns) | | First Root Length (microns) | |
| | 48 h | 72 h | | 48 h | 72 h | 48 h | 72 h |
| **EtOH** | 595 ± 56 | 1974 ± 128 | 26.0 | 440 ± 72 | 2980 ± 281 | 54 ± 2 | 1097 ± 212 |
| **MBI** | 515 ± 57 | 1859 ± 184 | 33.6 | 360 ± 41 | 2144 ±193 | 55 ± 7 | 432 ± 92 |
| **6FI** | 122 ± 17 | 426 ± 75 | 95.5 | 137 ± 18 | 268 ± 32 | 0 ± 0 | 0 ± 0 |
| **NAA** | 450 ± 70 | 1264 ± 221 | 57.5 | 150 ± 51 | 816 ± 92 | 0 ± 0 | 32 ± 10 |
| **PCIB** | 403 ± 83 | 1564 ± 116 | 30.5 | 250 ± 51 | 1797 ± 336 | 43 ± 9 | 189 ± 31 |
| **TIBA** | 350 ± 28 | 1611 ± 292 | 33.6 | 277 ± 30 | 1712 ± 387 | 59 ±14 | 230 ± 52 |
| **NPA** | 206 ± 47 | 1786 ± 194 | 29.0 | 221 ± 37 | 865 ± 134 | 15 ± 5 | 233 ± 72 |

**Table 5-1** :Structural changes in embryos treated with biosynthesis, analogue, and transport inhibitors as measured under a dissection microscope.

*Auxin biosynthesis inhibitors* --MBI and 6FI are reported to inhibit the *Zea mays* tryptophan-dependent pathway, at least in in vitro experiments with *Zea mays* endosperm (Ilic et al., 1999). In these experiments, MBI appeared to mediate a pronounced inhibition of IAA biosynthesis while 6FI was a somewhat weaker inhibitor, but the mechanisms of action for both inhibitors are unknown (Ilic et al., 1999). Therefore, MBI and 6FI were utilized to evaluate the role that auxin biosynthesis might play in fern embryogenesis.

More than 95% of the 6FI-treated embryos exhibited unorganized divisions producing small cells that were more densely cytoplasmic than the control embryos. The 6FI-treated embryos that managed to develop vegetative organs showed a dramatic phenotype for each organ: 1) a 5-fold decrease in rhizoid length in both the 48 h and 72 h embryos, 2) a 3- and11- fold decrease in leaf length for the 48 h and 72 h embryos, respectively and 3) almost complete inhibition of root elongation (Table 5-1). In direct comparison, 33.6% of the MBI-treated embryos were unorganized. By 24 h, MBI-treated embryos were also composed of much smaller densely cytoplasmic cells as compared to the control embryos (Fig. 5-2 b). The embryos able to develop beyond the initial stage of cell proliferation appeared similar to control embryos, except that their roots were shorter at 72 h (Table 5-1). It is intriguing that fern embryos show the opposite sensitivity to these two biosynthesis inhibitors, when compared to their affects on maize endosperm.

*Endogenous auxins* --Synthetic auxins such as 2,4-D and NAA tend to undergo less turnover and less conjugation than exogenous IAA (see Ribnicky et al., 1996).

Therefore, these chemicals are often used in somatic embryogenesis research because they can induce persistent auxin-mediated responses. By definition, a strong auxin (e.g. NAA) enhances an auxin-mediated response because it competes and binds to the receptor better than IAA. Auxin analogues (or weak auxins), like PCIB, have little activity by themselves but they do inhibit the effects of endogenous IAA. Since PCIB affects can be overcome by the simultaneous application of exogenous IAA, it appears that PCIB is also acting as a competitive inhibitor of endogenous IAA (Lomax et al., 1995).

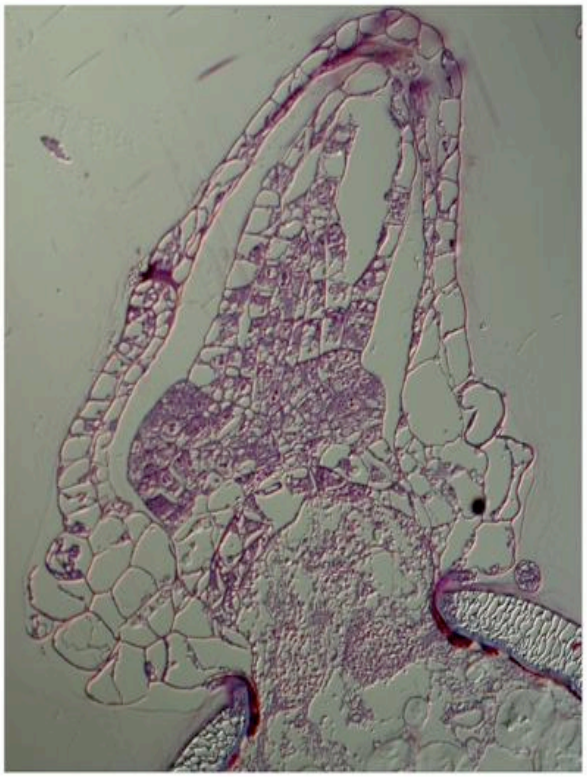
When the archegonia and the embryos were exposed to NAA, they exhibited an enlarged rounded appearance caused by an increase in cell volume without an obvious increase in cell number (Fig. 5-2 d). In this treatment, 57.5% of the embryos had unorganized cell divisions and the surrounding archegonium had an appearance of callus growth. Rhizoids emerged from many cells all over the surface of the NAA treated archegonia by 24 h in contrast to the localized rhizoids on control archegonia (Fig. 5-3). If the embryos survived past the initial spherical stage, they developed a relatively normal leaf and a stunted root (Table 5-1). For example, 48 h embryos had no visible root, and 72 h embryos had short roots averaging 32 μm ± 10 μm in length. The predicted high levels of persistent NAA within the embryo are the most likely explanation for the failure of root elongation as well as the circumferential rhizoid distribution. However, metabolic measurements of endogenous auxin and NAA concentrations within *Marsilea* embryos are needed to confirm these predictions.

When PCIB-treated embryos were compared to the controls, it was found that exposure to PCIB results in normal embryo formation, with only 30.5% of the 24 h

treated embryos appearing unorganized, which is equivalent to the control embryos (Fig. 5-2 e). This embryo formation in this treatment differed only in the delay in developmental timing as compared to the control (Table 5-1). This delayed development continued as long as fresh PCIB solution was added to the embryos every 24 hours. Otherwise, the organs of the embryos grew to the same lengths as those of the control (data not shown).

*Polar auxin transport inhibitors* --The transport inhibitors NPA and TIBA prevent polar auxin transport by blocking auxin efflux; however, these two inhibitors have different effects on auxin transport. The phytotropin NPA can inhibit both polar auxin transport and the lateral transport associated with tropisms but the non-phytotropin TIBA affects only polar transport (Lomax et al., 1995). The site of NPA action is controversial. NPA may bind to a protein that interacts with another protein at the efflux carrier. However, recent molecular evidence suggests that NPA may have indirect effects on polar transport by interfering with the secretion process responsible for delivering the efflux carrier to a basal position in the transporting cells (Muday et al., 2003). TIBA appears to bind to the auxin-binding site on the efflux carrier, which explains why it shows weak polar transport (Lomax et al., 1995).

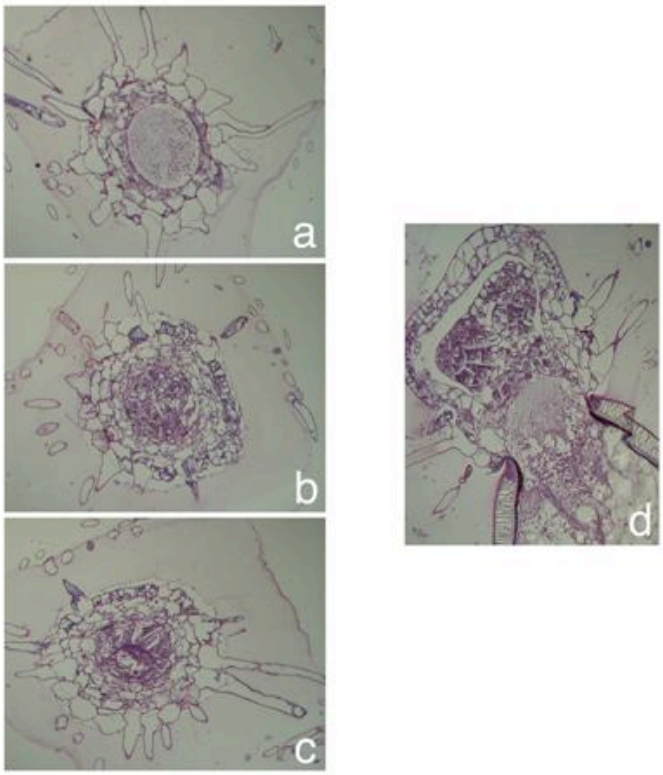
**Fig 5-3**: 48 h NPA-treated embryo within an archegonium showing the 0-20º leaf angle in relation to the original longitudinal axis of the megagametophyte.



Even though NPA did not cause significantly more embryo abortion then the control treatment, the first leaves of NPA-treated embryos emerged through the apex of the archegonium at small angles between 0 to 20° as opposed to the 70 to 80° angles of control embryos (Fig. 5-4). Thus, it appears that NPA also altered the initial axis position of leaf development in the young embryo. The leaves in NPA-treated embryos at 48 h were curly and approximately 50% and 75% shorter than the control leaf measurements at 48 h and 72 h, respectively. If the embryos were given fresh NPA solution after the initial 24 and 48 hours, then the curly leaf effect became even more severe (data not shown).

Finally, TIBA-treated embryos have similar morphologies and slightly shorter length for all measured organs when compared to the controls; therefore it appears that TIBA just delayed growth (Table 5-1). However, interestingly, a small percent of embryos in each experiment exhibit a shift in the axis of root development (data not shown). Further experimentation must be done to understand how auxin transport regulates leaf position; nevertheless, it is worth noting that the effects of transport inhibitors are much less severe on fern embryos than angiosperm embryos.

**Fig 5-4**: 48 h NAA-treated embryos showing the circumferential rhizoid pattern. a-c. Serial cross-sections of the megagametophyte. d. Longitudinal section of the megagametophyte.


a
b
c
d

## DISCUSSION

Studying the effects of auxin analogues and inhibitors on fern embryogenesis allows us to connect what we understand about cell proliferation and cell elongation in angiosperms with what might be happening in the pteridophytes. The biosynthesis inhibitors, 6FI and MBI, affect the initial stages of cell proliferation resulting in the formation of embryos, most of which abort in the presence of 6FI. The weak auxin, PCIB, delays growth and development in all stages of fern embryogenesis, which suggests a general requirement of auxin action through this process. On the other hand, the strong auxin NAA mediates rapid cell proliferation that causes enlarged, often disorganized embryos. The high levels of auxin activity appear to disrupt endogenous gradients, as judged by the uniform distribution of archegonial rhizoids and high rate of aborted embryos failing to proceed to organ formation. Finally, polar auxin transport inhibitors changed leaf angle and had other minor effects like leaf curling. Therefore, since TIBA caused no significant developmental abnormalities, polar auxin transport probably plays a limited role in axis establishment in fern embryos.

The major groups of land plants have evolved three different body plans (Cooke et al., 2004). All bryophyte divisions show a tripartite body plan that is first expressed in the young embryo (see Chapter 3). Pteridophyte embryos undergo a series of segmentation divisions resulting in the formation of a globular-shaped embryo composed of four quadrants: shoot apex, first leaf, first root, and foot (Campbell, 1918; Smith, 1955; Wardlaw, 1955; Gifford and Foster, 1989). This grade of plant organization is said to be unipolar since the shoot apex is solely responsible for generating the growth axis of the postembryonic plant (Groff and Kaplan, 1988). By contrast, the seed plants

exhibit bipolar organization with the embryonic shoot and root apices arising at opposite poles during the globular stage of embryonic development. These apical meristems are responsible for perpetuating the bipolar organization (Groff and Kaplan, 1988; Kaplan and Cooke, 1997).

The evidence available to date suggests that auxin is ultimately involved in the establishment of these different body plans. In seed plant body plans, auxin biosynthesis appears necessary to mediate the rapid cell division for forming the globular embryo and its polar transport helps to establish the bipolar axis (Michalczuk et al., 1992a, 1992b; Ribnicky et al., 1996, 2002). Both synthetic auxin and polar auxin transport inhibitor experiments in several different species have supported this concept (Schiavone and Cooke, 1987; Liu et al., 1993; Fischer et al., 1997; Hadfi et al., 1998). Certain bryophytes, such as mosses, share with the seed plants the ability to carry out polar auxin transport in their sporophytic axes (Chapters 3, 4). This study suggests that auxin biosynthesis and perhaps polar transport play analogous roles during pteridophyte embryogenesis.

As the initial effort to study the role of auxin in fern embryogenesis, this work utilized the same inhibitors of auxin biosynthesis, action, and transport that have traditionally been used in angiosperm systems. It is clear that I have only begun this research effort. Because the spores of this fern can readily be transformed with inhibiting dsRNAs corresponding to various developmental genes which can knock-out specific gene expression (Klink and Wolniak, 2000), this technology should allow us to further evaluate the role of the auxin transport carriers and other auxin regulating proteins in fern embryogenesis. It should be interesting to run RNAi experiments with the genes that

encode such proteins as PIN, AUX1/IAA, and ABP. If the genes targeted are not required for gametogenesis, then the corresponding RNAis may interfere with embryo development that would then provide further insight into the development and evolutionary roles of auxin in land plant embryogenesis.

# CHAPTER 6

## CONCLUSIONS: JUST THE BEGINNING

Today, IAA action research is mainly focused on the seed plants but there are a few older reviews devoted to other specific green plant groups, such as the algae (Bradley, 1991), liverworts (Maravolo, 1980), and mosses (Christianson, 1999). This dissertation takes a comparative approach to characterizing IAA action in all plants, with emphasis on the non-seed plants, specifically the bryophytes and pteridophytes. This approach allows flexibility in uncovering important answers about phenomena that are not easily assessable in other organisms or specific groups. For example, Bennett et al. (2002) suggested that in relation to hormones, complex signal transduction networks composed of multiple, interacting, and redundant pathways are regulating anigosperm development but Sztein et al. (2000) showed the lower plants utilized simpler mechanisms of IAA regulation meaning that they may more readily reveal the fundamental mechanisms of auxin regulation than the seed plants.

**Evolutionary implications of auxin action and polar auxin transport in land plants**

This dissertation begins to outline a picture of IAA regulation on development, with respect to auxin transport, in several basal groups in the land plant lineages. My observations indicate that the three divisions of extant bryophytes utilize different developmental mechanisms for regulating axial elongation of their sporophytes. The auxin regulation of axial elongation in moss sporophytes is quite reminiscent of the same process in certain vascular plant organs, which supports the interpretation that it originated in a common ancestor of the moss and vascular plant lineages. This work may not be complete, but it does provide several surprising perspectives about the evolutionary patterns in auxin action in green plants. Auxin transport exists in the entire land plant lineage and the only significant differences appear to involve the relative sophistication of the transport across the species (e.g., inhibitor sensitivity, transport mechanism). Also auxin responses, like tropisms and apical dominance, play a critical role in the development of bryophytes, pteridophytes, and seed plants. Therefore, the seed plants may not have evolved *de novo* mechanisms for mediating IAA responses, but have modified pre-existing mechanisms already present in the early land plants. These auxin-driven mechanisms may provide significant insights into the early evolution of land plant sporophytes, specifically the origins of axial sporophytes.

**Future experimentation in polar auxin transport**

Since this dissertation was just the beginning of examining polar auxin tranport in the lower plants from a physiological and evolutionary perspective, polar auxin transport assays can continue to be applied to answer questions that remain in the bryophyte

gametophytes and sporophytes. In Chapter 4, I began to observe the effects of different environmental conditions on the polar auxin transport in moss sporophtyes. These questions can begin to be answered by growing mosses under specific conditions that mimic different environmental characteristics, like humidity and temperature ranges, and then examining polar auxin transport in the resulting gametophytes and sporophytes. Comparable experiments could also be run in the newly developed fern system, *Marsilea*, for a more complete comparative study.

The development of model systems in lower land plants opens the way for experimental observations to be approached in an evolutionary perspective. For example, *Physcomitrella*, which is reportedly capable of homologous recombination (Schaefer and Zryd, 2001), and *Marsilea* which can be studied with RNAi (Klink and Wolniak, 2001), provide us with the opportunity to look at polar auxin transport from a mechanistic approach with the added benefit of genetic techniques to further classify the mechanism. However, in order to complete the most comprehensive examination of polar auxin transport evolution, so-called exemplar lower plant species in key evolutionary lineages need to be examined (e.g., *Sphagnum*, the most basal moss lineage according to Newton et al., 2000).

Understanding the significance of polar auxin transport in the sporophytes of the lower land plants is important but polar auxin transport must be re-examined in the corresponding gametophytes in order to achieve a true evolutionary perspective. Previous researchers explored polar auxin transport in several species of basal plants (e. g., Wochok and Sussex, 1973; Maravolo 1976; Gaal et al., 1982) but direct comparisons between these species was difficult because each researcher utilized a different, often

idiosyncratic, experimental design due to varying gametophyte structure across the species tested. However, the situation has changed for the better now: other researchers have a more sophisticated understanding of the cellular mechanism for polar auxin transport, including the auxin transport carriers and the inhibitors that affect them, and I have contributed to the development of several basal plant model systems for studying auxin regulation. I have also successfully modified the assay for small sporophyte samples. Therefore, a more uniform and complete study of gametophyte structures should occur.

Once the basic mechanism of polar auxin transport in basal land plants has been characterized by the above experiments, the next logical step is to start studying the molecular aspects of this process in these plants. For example, the auxin transport carriers AUX/IAA and PIN should be characterized by an approach used to study those carriers in the angiosperms. In principle, one could approach this research by identifying the homologs from the cDNA library for the species being studied, and sequencing those genes for sequence comparisons with the angiosperm homologs. The next step would exploit the unique ability of the moss *Physcomitrella pattens* to carry out homologous recombination (i. e., the insertion of a gene in the site already occupied by an allele of the same gene). You could replace the wild-type gene with various alleles to test for functional activity and/or genetic redundancy. Another approach could involve the use of double stranded RNA mediated interference (RNAi) in *Marsilea vestita* in order to evaluate the role of auxin transport carriers and other auxin regulating proteins in fern embryogenesis. The spores of this plant can readily take up small dsRNAs corresponding to various developmental genes (Klink and Wolniak, 2000). Thus, if these genes are not

required during gametogenesis, then it may be possible for persistent RNA to interfere with the early events in embryogenesis. I would start by evaluating the effects of RNAi knock-outs of such proteins as PIN, AUX1/IAA, and ABP.

In order to get a complete picture of the evolution of auxin transport and its role on axial initiation, it is important to visualize auxin gradients in the lower land plants. For example, one method would be to analyze the expression of the β-glucuronidase gene (GUS) fused to the highly active synthetic auxin response element referred to as DR5 (Ulmasov et al., 1997), which can be used to detect elevated free-auxin concentrations (Aloni et al., 2003). It might also be informative to use immunocytochemistry to try to localize the transport proteins; basically the antibodies available from angiosperm homologs might react with the basal plant proteins.

**In Conclusion…**

This dissertation contributes significantly to the knowledge of auxin action in land plants and the role of auxin during axial initiation. It was the first attempt to examine all available literature on specific auxin actions in lower plants and to organize it into a cohesive scheme. This was the first work that studied polar auxin transport in the sporophytes of the three extant bryophyte lineages. Because of identical experimental techniques and normalized data, the evolutionary predictions and observations about the evolution of polar auxin transport and its role on axial initiation are strong. Examining the novel fern *Marsilea*, allowed it to be developed into a model system for future embryogenesis and auxin transport work. In conclusion, this work begins to open many avenues for future research in development, polar auxin transport, and auxin action

among the basal plants. My future research in evolutionary physiology of the lower land plants, as a new approach to complement bioinformatics and phylogenetics, is wide open.

# APPENDIX I.

## THE DEVELOPMENT OF A PROTOCOL FOR DETERMINING THE AUXIN BIOSYNTHETIC PATHWAY AT DIFFERENT STAGES OF ZYGOTIC EMBRYOGENESIS IN *Daucus Carota*

### INTRODUCTION

It is well established that the hormone auxin (indole-3-acetic acid, IAA) plays a central role in the regulation of carrot embryogenesis. In particular, Michalczuk et al. (1992a) measured 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA) and their conjugate levels in *Daucus carota* callus suspension cells and developing somatic embryos. When the embryos were exposed to 2,4-D, the researchers found total endogenous IAA levels increased to levels greater than 600 ng/g FW in both the embryonic and non-embryonic lines. Once removed from 2,4-D containing medium, the embryonic line showed a decline in free and conjugated metabolites (ca. 20 ng/g FW) approximately 10 times lower than the non-embryonic line. IAA levels were highest in preglobular embryos and lowered as the developmental stages proceeded. Michalczuk et al. (1992a) suggested that high IAA levels may be necessary but not sufficient for initial embryogenic events, while low levels are associated with later stages of embryo

development. Ribnicky et al. (2002) showed in zygotic carrot embryos that there was a 80-fold increase in free IAA levels following fertilization from basal levels of 25 ng/g FW in unfertilized ovules to over 2,000 ng/g FW in late globular and early heart stages. One can then say that similar changes in auxin levels occur during both somatic and zygotic embryogenesis.

Michalczuk et al. (1992b) examined the regulation of IAA biosynthetic pathways in carrot cell cultures. Isotopic enrichment of IAA by different stable isotope-labeled precursors (deuterium oxide, [$^{15}$N]indole, and $^{2}H_5$-L-tryptophan) was used to characterize the different points of the proposed contributors of different IAA biosynthetic pathways to the auxin levels at different embryonic stages. It was determined that somatic embryos (heart and torpedo) grown in medium without 2,4-D showed a 10-fold decrease in the level of IAA (30 ng/g FW) from the levels observed in the initial cell clusters (497 ng/g FW). When cell clusters were grown in the presence of 2,4-D there was no sign of embryo formation and IAA levels were high (548 ng/g FW). Label experiments with either deuterium oxide ($^{2}H_2O$) or [$^{15}$N]indole showed similar IAA enrichment in callus proliferating with 2,4-D and embryos developing in the absence of 2,4-D. However, when fed $^{2}H_5$-tryptophan, the IAA enrichment was at least 7 times higher in carrot callus cultures proliferating in the presence of 2,4-D than in the embryos developing in the absence of 2,4-D. The $^{2}H_20$ incorporation showed that any difference in labeling was not due to the absence of IAA production. Therefore, Michalczuk et al. (1992b) suggested that a tryptophan-independent pathway for IAA synthesis was activated during somatic embryogenesis.

Ribnicky et al. (1996) examined the effects of exogenous auxins on the metabolism of excised hypocotyl cultures of carrot. They measured concentrations of free and conjugated IAA by mass spectrometry technology after feeding stable isotope-labeled internal standards of [$^2H_4$]-IAA, 2,4-D, and α-naphthaleneacetic acid (NAA). When 2,4-D was applied, callus proliferation occurred on the excised hypocotyl. The 2,4-D fed hypocotyls accumulated large quantities of free 2,4-D but it did not greatly affect endogenous IAA levels. In contrast, NAA accumulated high free concentrations in organogenic callus and then as conjugated forms during embryo development. Again, NAA did not greatly affect endogenous IAA levels. [$^2H_4$]-IAA was rapidly conjugated, did not cause noticeable growth pattern, and seemed to have a possible feedback inhibition on IAA biosynthesis. From these experiments it is thought that exogenous auxins do not alter the IAA biosynthetic pathway because endogenous tryptophan or IAA pools were not affected in most cases.

A few generalizations can be drawn from the above papers. In both somatic and zygotic embryos, a great surge of free auxin is apparently required to form the globular embryo, and then this level decreases as the embryo matures (Michalczuk et al., 1992a; Ribnicky et al., 2002). High initial concentrations of free auxin seem to signal callus proliferation while lower concentrations allow for organized development of a bipolar somatic embryo (Ribnicky et al., 1996). It also appears that during somatic embryogenesis the tryptophan-dependent pathway is the utilized pathway for callus proliferation, but the tryptophan-independent pathway is active in post-globular embryos (Michalczuk et al., 1992b). Therefore, one might hypothesize that a similar switch in auxin biosynthetic pathway might also occur during zygotic embryogenesis. This

hypothesis can be tested by feeding labeled anthranilate to developing zygotic embryos, and then measuring the relative incorporation of the label into the IAA and tryptophan pools. We would expect to observe a primarily labeled tryptophan pool, or the tryptophan-dependent pathway, during the initial proliferation of globular embryo formation. If anthranilate labels the IAA pool, the tryptophan-independent pathway would be functioning and we expect to observe this during the post-globular embryo stages of development.

This appendix represents the progress of the protocol development necessary to run the biosynthesis experiments suggested by the above research. I needed to devise developmental influx/uptake procedures, labeling experiments, and analytical microtechnique to make this research possible.

## MATERIALS AND METHODS

*Developmental index for determining embryonic stage* -- Four to eight seeds of *Daucus carota* var. Danville (Meyer Seed Co., Baltimore MD) were sown in long tuber pots in the University of Maryland greenhouses. The seedlings were not supplemented additional light and therefore grew through the year's day length and intensity for the region. Once plants produced roots of 6 - 8 inches long and 2 – 3 inches thick, the roots were collected and vernalized for 8 - 10 weeks. After vernalization the roots were replanted and floral development progressed normally with natural habit insects aiding pollination. Floral development was followed daily for 8 months. Fifty (50) individual flowers were examined under a dissection microscope for each developmental stage and notes were taken of the following characteristics: the color, shape, length, and stripping

pattern of the ovules; the presence, length and color of trichomes; and the presence, color and size of the nectaries. Ovule dissection was performed in order to relate these external characteristics to the specific embryonic developmental stages occurring inside the ovule. A developmental index was generated for relating external features to embryonic development.

*An experimental system for performing in vivo labeling studies* --Umbels at varying developmental stages were cut from plants grown at the University of Maryland greenhouses and immediately placed in water. Once the cut end of the stem was completely covered by lanolin, umbel stems were surface sterilized by soaking in 1% Triton X-450 (Sigma) solution for 60 s, 10% commercial bleach for 90 s, and sterile water for 30 s repeated three times. After the last water soaking, the lanolin-covered portion of the stem was cut off, and the umbel was immediately placed into the sterile growth apparatus containing the growth medium. This apparatus was a 25-ml glass graduated cylinder modified to hold a total volume of 15 ml. A polypropylene S. T. 19/38 joint (Aldrich) was used to cap the graduate cylinder. A hole was drilled into the cap in order to fit a 1 inch section of a glass pasteur pipet. Several media were explored for *in vivo* development of the embryos (refer to Results for more information). A sterile 1% Murashige and Skoog medium (MS) (M-8900 Sigma) with 0.5% sucrose (Fisher) solution was made in distilled water and adjusted to pH 6.0.

*Preliminary uptake experiments* -- Initial eosine uptake studies were carried out to visually determine if carrot umbels would incorporate label into particular organs,

specifically the ovule and embryos. A 100-ml beaker with 30 ml of 0.5% eosin solution was set into a growth chamber at the above conditions. Umbels of different developmental stages were cut from the plant, placed in water, and re-cut under the eosin solution. The umbel was held to the side of the beaker by stopcock grease. The plants were observed under a dissection microscope at 75 X at the established time points at various times in order to develop a time course for dye movement into the ovules. A similar experiment was run using [$^{14}$C]-anthraniliate in the above growth apparatus and the umbels were then placed into a tissue culture chamber set at a 24 h light cycle at 100 µmole/m2/s, 27 °C and 60% humidity. Twenty-five ml (25 ml) of 500 µM anthranilate (Sigma) solution was prepared in 1 % MS supplemented with 0.5% sucrose at pH 6 and it was spiked with 3 µCi or 15 µCi of $^{14}$C-anthranilate (56 mCi/mmol, 0.1 mCi/ml) for each umbel tested. At zero and 24 h, ovules and embryos were collected and counted via a scintillation counter to determine the amount of anthranilate/ mg FW.

*Labeling studies:* In order to run the tryptophan microtechnique (see below), one must label umbels utilizing the growth apparatus outlined above. To the sterile1% MS media supplemented with 0.5% sucrose, a final concentration of 500 µM $^{15}$N-anthranilic acid was obtained after stock anthranilic acid solution was sterilized by suction filtration. The umbels were then placed into a tissue culture chamber set at a 24 h light cycle at 100 µmole/m$^2$/s, 27 °C and 60% humidity. Embryos were collected under a dissection microscope and immediately placed into a microcentrifuge tube sitting on dry ice and then they were stored at -80°C.

*Tryptophan Analysis Microtechnique* -- The tryptophan analysis technique presented in Michalezuk et al. (1992) was modified in order to quantify the auxin pool levels in zygotic embryos at the fmole range.

*Tissue Preparation* -- Corn (*Zea mays*) endosperm or watercress leaf, from 1 mg to 10 mg, was placed into microcentrifuge tube and frozen at –80 °C. A sample was frozen in liquid $N_2$ and was homogenized in 40 µl isopropanol-water (70:30) by a pellet pestle (No. 749520 Knotes Glass Co., Vineland N. J., USA) modified to fit inside the closed microcentrifuge tube (Ribnickey et al. 1998). The sample, with the pestle inside of the microcentrifuge tube to reduce loss, was placed on ice for 1 hour for isotope equilibration, after which time, the pestle was rinsed with 20 µl isopropanol (70:30) and this rinse was added to the rest of the sample. The sample was then centrifuged for 5 min at 10,000 x g, the supernatant was kept, and the pellet was washed with 20 µl isopropanol-water (70:30), re-spun for 2 min at 10,000 x g. Both supernatants were combined for the remainder of experiment. Then approximately 50,000 – 70,000 dpm of [5-$^3$H]Trp (0.5 KbQ; Amersham, 1 TBq/mmol) tracer was added to the supernatants.

*Sample purification, methylation, and HPLC* -- Dowex 50W-X2, 200-400 mesh, was equilibrated with 1 M HCl and washed with deionized water. One hundred µl (100 µl) of freshly prepared Dowex was added to the supernatants, vortexed for 30 s and set on ice for 30 min. The sample was then centrifuged at 3000 x g for 2 min, the supernatant was removed and to the pellet 100 µl of 2N $NH_4OH$ was added to the Dowex to elute the tryptophan. Again the pellet was centrifuged at 3000 x g for 2 min. This supernatant was collected with the first elutant and placed into a 300 µl conical glass reaction minivial.

An azeotropic distillation *in vacuo* was performed, first with 80 µl absolute ethanol, then 40 µl dicloromethane, then 40 µl absolute ethanol, and finally by 40 µl of dicloromethane. Immediately after the last dichloromethane distillation, 100 µl of anhydrous methanol (dehydrated by distillation from magnesium activated with iodine) and 50 µl of acetic anhydride (Supelco) were added to the sample vial. The flask was tightly capped, vortexed for 1 min and put into a 55° C heating block for 1.5 h. The methanol and acetic anhydride was then evaporated *in vacuo*, the residue was dissolved in 50 µl of methanol-water (50:50) and injected into a Waters NovaPak C18 column (15 cm x 3.9 mm) attached to a Waters 600MS HPLC pumping system. The column was eluted with methanol-water (30:70) and all fractions with radioactivity presence were collected, evaporated to dryness and dissolved in a total of 10 µl of ethyl acetate for GC-MS analysis.

*GC-MS Analysis* -- GC-MS analysis was performed on a Hewlett-Packard 6890 gas chromatograph connected to a 5973 mass selective detector. The GC was equipped with a 15 m x 0.237 mm Varion 5000 fused silica capillary column (J & W Scientific). Helium was used as a carrier gas and analysis was followed as done by Michalezuk et al. (1992). Retention time was approximately 11 minutes depending on the column length. Ions at m/z 130 and 131 (unlabeled and labeled quinolinium ions) and at 260 (unlabeled molecular ions) were monitored for quantitative analysis of tryptophan.

## RESULTS

*A developmental index for carrot zygotic embryogenesis* -- *Daucus carota* is the primary *in vitro* embryogenesis system in large part because it is easy to collect somatic embryos at different stages. Thus, in order to compare somatic vs. zygotic embryogenesis, one must be able to predict and dissect out zygotic embryos at specific developmental stages from the plant. A developmental index was created that utilizes specific external characteristics of the changing ovary to determine a particular embryonic stage (Table A-1). In order to determine the developmental stage of the *in vivo* plant umbels and its ovules, the developmental index was utilized. It is important to note that one ovule and not the other on an individual flower can be fertilized and therefore, fertilization was determined by the increase in trichome length of approximately 700-1000 μm on individual ovules.

*Labeling an in vivo system* -- Umbels, 5 – 15 cm in diameter, were cut off of the plant and grown in a tissue culture chamber under different environmental conditions. Umbels 5-9 cm in diameter reliably produced viable seed in 7-12 d under 27°C, 60% humidity, 24-h light at 100 μmole/$m^2$/s when grown in the presence of 1% MS supplemented with 0.5% sucrose. Distilled water alone and a 1% MS solution produced embryos to the torpedo stage but did not produce viable seed. The 10% MS supplemented with 0.5% sucrose did not produce viable seed either even though the rest of the embryo morphology appeared mature (data not shown). Umbels larger than 5-9 cm in diameter tend to have higher transpiration rates and take much longer to develop (data not shown). This is a serious concern due to the cost of stable label and the amounts needed during development.

| Embryo stage | Embryo length (μm) | Trichome length (μm) | Ovary length (μm) | Morphological variation |
|---|---|---|---|---|
| Globular | 133 | 1300-1600 | 2700 | Bright green ovary (lime) with dark green stripes |
| Early heart | 266 | 1600-2000 | 3300-4000 | Green ovary with green stripes and 1-2 browning stripes, no pleats |
| Late heart | 399-665 | 2000 | 3300 | Green ovary, no pleats to slight pleats visible |
| Early torpedo | 931-1064 | 2000-2600 | 2600-3300 | Brown stripes down ovary especially where the ovaries separate from each other; most of the ovary is green; pleats |
| Late torpedo | 1064 | 2000 | 2600-4000 | Brown ovary with brown pleats ~665-931 um wide, some green may be visible, trichomes are still full |
| Mature | 1330 | 1300 | 2600 | Ovary is brown with raised pleats which are brown ~<665 um wide; hard to touch; trichomes appear thin |

**Table A-1:** A developmental index for zygotic embryogenesis in *Daucus carota*. Data are presented as the mean of 200 flowers observed daily, over 8 months.

Once the *in vivo* development was understood through the use of a developmental index, it became necessary to establish a method for successfully labeling developing embryos with a "heavy" anthranilate ($^{15}N$), a biochemical predecessor, in order to quantify the relative enrichmentnt of the endogenous auxin pool via GC-MS. Initial time course studies with eosin dye were carried out to visually ensure that a labeled compound would reach the desired plant tissue, in this case the developing embryo (Table A-2). Pre-fertilized ovules exhibited the fastest labeling with the entire umbel being pink in 6 h; in contrast, young (globular/heart shaped embryos) and older (heart/torpedo embryos) ovules only showed red color in the funiculus at 6 h. Once the dye study verified uptake, the same experiment was completed using a radioisotope to better quantify labeling levels in individual ovules to ensure levels measurable by the GC-MS. Both 3 μCi and 15 μCi of $^{14}C$-anthranilate added to umbel-growth- media resulted in approximately 2500 pg anthranilate/mg FW labeling of the heart shaped embryo (Table A-3). Even though individual ovules exhibited more label once the amount of $^{14}C$-anthranilate was increased, the embryo's anthranilate pool appears to saturate. Therefore in order to ensure accurate conclusions to be made, sample size must be large enough during the anthranilate studies.

| **Time** (m) | **Pre-fertilized ovules** | **Young fertilized ovules** (globular/heart shaped embryos) | **Old fertilized ovules** (heart/torpedo embryos) |
|---|---|---|---|
| 0 | All green | All green | All green |
| 10 | See pink to the start of the umbel | Stem is pink | n/c |
| 35 | Veins of ovary are pink | Individual umbel shoots are pink | Stem is pink |
| 60 | Ovule tip is pink, nectaries and style are pink | Veins of ovary are pink | Some pink in individual umbel shoots |
| 90 | Funiculus is pink | n/c | n/c |
| 120 | n/c | n/c | n/c |
| 180 | n/c | n/c | n/c |
| 240 | Petals are red, ovule tip is darkening | Slightly pink at tip of ovule | n/c |
| 300 | Ovary is dark pink | n/c | n/c |
| 360 | Entire plant pink/red | Funiculus is pink | Funiculus is pink |
| 480 | n/c | n/c | n/c |

**Table A-2**: Observations of developing umbels exposed to 0.5% Eosin water solution for various times to determine where fed label would accumulate. Abbreviation n/c, no change.

| **Amount of $^{14}$C-anthranilate** (uCi) | **Ovule** (pg anthranilate/mg FW) | **Embryo** (pg anthranilate/mg FW) |
|---|---|---|
| 3 | 7300 | 2436 |
| 3 | 7000 | 2373 |
| 15 | 13680 | 2239 |

**Table A-3**: $^{14}$C-anthranilate embryo label accumulation calculations after two different anthranilate concentration studies.

In conclusion, a modified glass graduated cylinder served as an excellent delivery system for feeding and rapid development of the umbel. This apparatus in conjunction with the above mentioned growth medium, when sterilely assembled, would reliably feed label, stable or radioactive, to the *Daucus carota* umbel with a radioactivity overall loss of only 7.2% (data not shown). Several $^{15}$N-anthranilate labeled samples have been collected to date (Table A-4).

*Tryptophan analysis microtechnique development* -- David Ribnicky had successfully scaled down the traditional auxin biochemical analysis for determination of free, conjugated, and total auxin levels in plants. However, in order to determine if the tryptophan-dependent or –independent pathway predominates during a particular developmental stage, one must also look at the relative enrichment of tryptophan pool levels following precursor labeling. Thus, I needed to scale down that procedure for my experiments.

When one is performing biochemistry on small sample volumes, product loss is the number one concern. Two critical areas of traditional sample loss in the scaled-down protocol occur at the pellet washing stage and the Dowex exchange stage. Table A-5 shows that a pellet must be washed in order to maintain sample, but 4% loss is minimum with upper limits as much as 32%.

Table A-6 examines Dowex exchange recovery. From the results, there is a direct correlation between the amount of Dowex used and the amount of sample recovered with approximately 80-100 ul Dowex being optimal.

| Embryo stage | Vial code | Number in vial | Total tissue weight (mg) |
|---|---|---|---|
| Globular | G1 | 20 | 2.0 |
| | G2 | 11 | 1.1 |
| Heart | H1 | 24 | 4.8 |
| | H2 | 9 | 1.8 |
| Torpedo | T1 | 21 | 6.3 |
| | T3 | 30 | 9.0 |
| | T4 | 30 | 9.0 |
| | T5 | 35 | 10.5 |
| | T6 | 35 | 10.5 |
| | T7 | 30 | 9.0 |

**Table A-4**: $^{15}$N-Anthranilate labeling experiments done to date

| | Trial 1 | Trial 2 |
|---|---|---|
| Total Supernatant counts | 43,034.31 dpm | 38,575.12 dpm |
| Homogenization pellet – pre wash | 27,452.49 dpm (64%) | 33,344.88 dpm (86%) |
| Homogenization pellet - post wash | 1,735.56 dpm (68% of total) | 3,512.44 dpm (96% of total) |

**Table A-5**: Recovery increase due to pellet wash. Abbreviation dpm, disintigrations per minute

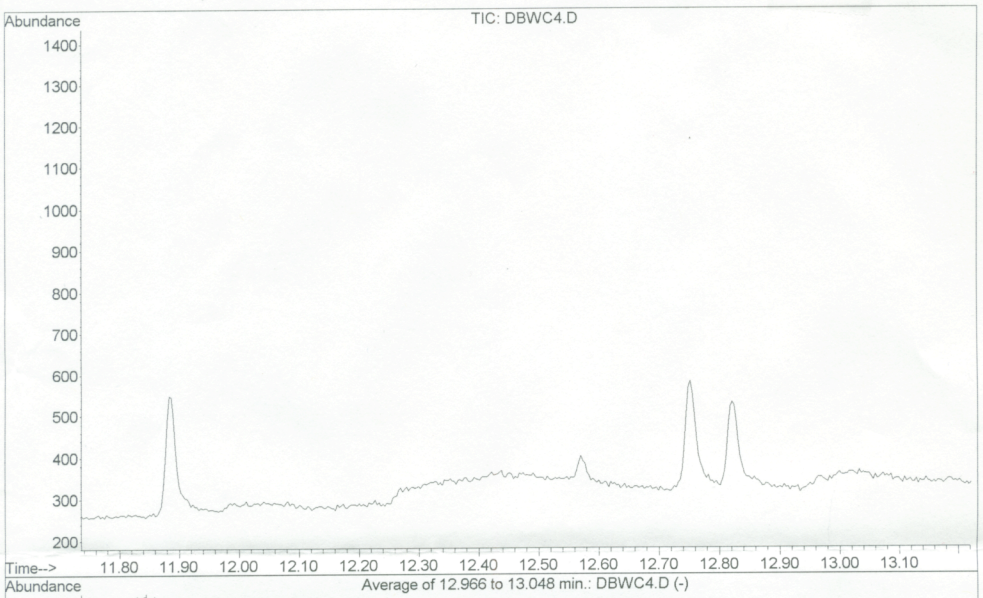
| Dowex (ul) | Supernatant – pre $NH_4OH$ (dpm) | Supernatant – post $NH_4OH$ (dpm) |
|---|---|---|
| 20 | 42,749.26 | 24,861.77 |
| 40 | 14,770.84 | 54,716.31 |
| 60 | 23,702.51 | 33,388.48 |
| 80 | 15,586.64 | 54,472.37 |
| 100 | 18,417.62 | 50,295.68 |

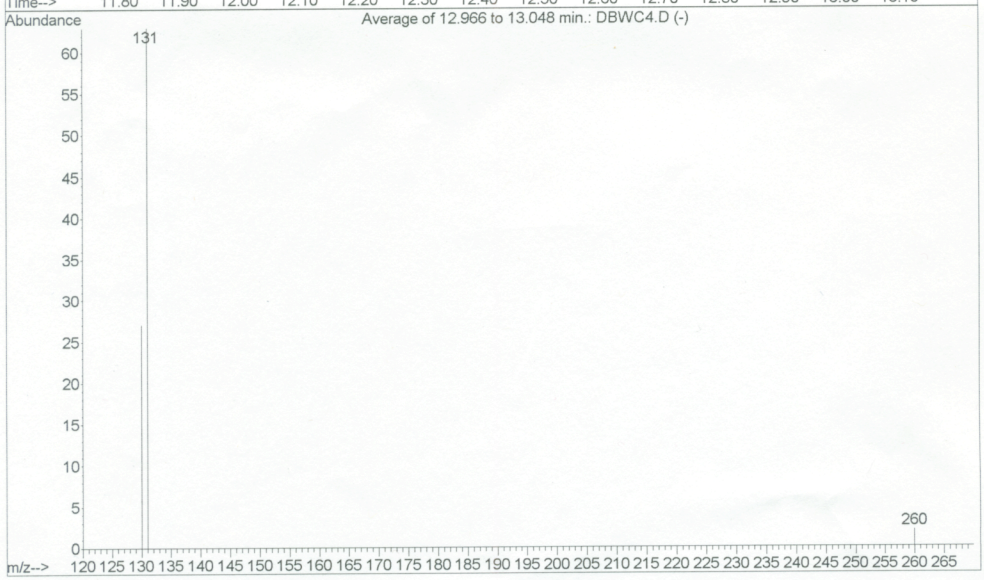
**Table A-6**: Dowex direct exchange recovery. Abbreviation dpm, disintegrations per minute

**Fig. A-1**: GC-MS analysis for tryptophan in 4mg of watercress leaf tissue. The top graph is the ion scan from the GC-MS. The bottom graph is the ion quantification for endogenous tryptophan and the $^{3}$H-tryptophan tracer. Ions at m/z 130 and 131 (unlabeled and labeled quinolinium ions) and at 260 (unlabeled molecular ions).

```
File       : C:\MSDCHEM\1\DATA\TEST\DBWC4.D
Operator   : db
Acquired   : 15 Jan 2002  17:22     using AcqMethod DBTRPSIM
Instrument :    Instrumen
Sample Name: DB watercress #4, 4mg tissue, trp
Misc Info  :
Vial Number: 1
```

| | |
|---|---|
| 130.00 | 27.0 |
| 131.00 | 63.0 |
| 260.00 | 2.0 |



As far as the results are concerned, this is the progress of the protocol development necessary to run the remaining analysis of zygotic embryos.

## CONCLUSION

Through this protocol, which was developed over several years, one is now able to examine the role of auxin biosynthesis and metabolism within a zygotic embryo system and compare them to the trends seen in the somatic callus studies (Michalczuk et al. 1992; Ribnicky et al. 1996). Examining the relative incorporation of anthranilate label into the IAA and tryptophan pools during the different embryogenesis stages; globular, heart, and torpedo, one can establish the activity of the tryptophan-independent and tryptophan-dependent pathways at different stages of embryo formation. It is predicted that the tryptophan-dependent pathway that is utilized for callus proliferation in somatic studies is also responsible for the initial cell proliferation seen in globular embryos. Switching to a trypophan-independent pathway for long term growth and development would be expected to occur in post-globular embryos.

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## Education

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|---|---|---|
| 1993 -1997 | University of Pittsburgh, Bradford | B. S. Biology |
| 1997– 2004 | University of Maryland, College Park | Ph. D. Plant Biology |

## Employment

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|---|---|
| 1996 – 1997 | Tutor at University of Pittsburgh, Bradford PA |
| 1997 – 2004 | Various instructional positions at University of Maryland, College Park MD |

### Professional Honors and Awards

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|---|---|
| 2004 | Center of Teaching Excellence Distinguished Teaching Assistant Award |
| 2003 | A.J. Sharp Award For Best Student Paper (American Bryological and Lichenological Society and the Bryological and Lichenological Section of the BSA) |
| 1998 | Center of Teaching Excellence Distinguished Teaching Assistant Award |
| 1997 | Graduated from University of Pittsburgh Summa Cum Laude |
| 1997 | University of Pittsburgh Student Life Award |
| 1994-1997 | Beta Beta Beta Biological Honor Society |
| 1993-1997 | National Dean's List |
| 1993-1997 | University of Pittsburgh Dean's List |
| 1993-1997 | University of Pittsburgh Presidential Scholar |
| 1993 | Alpha Lambda Delta Freshman Honor Society |

## Grants and Fellowships

| | |
|---|---|
| 2000 | McDonald Service Award ($2000) |
| 2001 | Moyer Summer Research Fellowship ($700) |
| 2002 | Moyer/Cox Summer Research Fellowship ($2000) |
| 2003 | Jacob K. Goldhaber Travel Grant ($225) |
| 2003 | College of Life Science Travel Award ($300) |
| 2003 | Moyer Summer Research Fellowship ($500) |
| 2003 | Molecular Systematics of Bryophytes Symposium and Moss 2003 Travel Grant Award (supported by Deep Gene) ($500) |
| 2004 | Cox Award for Research Excellence in Plant Biology in Cell Biology and Molecular Genetics ($1000) |

## Teaching Experience

*Laboratory Instruction:*

BSCI 211 Cell Biology and Physiology– Fall/Spring 1997-1998 UMD
BSCI 411 Plant Physioloy – Fall 1998, 1999, 2001, 2004 UMD
BSCI 225 Plant Diversity – Fall 2001 UMD
BSCI 421 Eukaryotic Cell Biology – Fall/Spring 2002-2003, Spring 2004 UMD

*Laboratory Coordinator:*

BSCI 421 Eukaryotic Cell Biology – Fall 2003 UMD

*Course coordinator and classroom lecturer:*

BSCI 411 Plant Physiology – Fall 2000 UMD
BSCI 125 Plant Biology for Non-Majors – Spring 2002 UMD

*Laboratory supervision of undergraduate students:*

Angeline David at UMD from 1998-1999
Franklin Johnson at UMD, summer 2003

**Publications:**

1. Cooke, T. J., Poli, DB., Sztein, A. E., and Cohen, J. D. 2002. Evolutionary patterns in auxin action. Plant Mol Bio 49: 319-338.

2. Poli, DB., Jacobs, M., and Cooke, T. J. 2003. Auxin regulation of axial growth in bryophyte sporophytes: its potential significance for the evolution of early land plants. Am J Bot 90: 1405-1415.

3. Cooke, T. J., Poli, DB., and Cohen, J. D. 2004. Did auxin play a crucial role in the evolution of novel body plans during the late Silurian – early Devonian radiation of vascular plants? In A. R. Hemsley and I. Poole (eds.), Evolution of plant physiology, pp. 85-107. Elsevier Academic Press: Amsterdam.

4. Poli, DB., Klink, V., and Cooke, T. J. (in prep) The role of auxin on *Marsilea vestita* embryogenesis.

5. Poli, DB. and Cooke, T. J. (in prep) Polar auxin transport in the moss Polytrichum ohioense: developmental regulation, environmental sensitivity, and evolutionary implications.

**Scientific Meeting Representations**

Cooke, T. J., Sztein, A. E., Poli, DB., and Cohen, J. D. Evolutionary trends in auxin regulation and their significance for plant embryogenesis. EMBO Workshop on Auxin: The first international meeting devoted to the mechanisms of auxin action. Calcatoggio, France, May 13-19, 2000.

Poli, DB., Jacobs, M., and Cooke, T. J. Auxin regulation of axial growth in bryophyte sporophytes: its potential significance for the evolution of early land plants. Botany 2003 (Botanical Society of America Annual meeting). Mobile, Alabama, USA, July 24-29, 2003.

Poli, DB., Jacobs, M., Sztein, A. E., Cohen, J. D., and Cooke, T. J. The role of auxin in plant evolution. Moss 2003. St. Louis, Missouri, USA, September 5-11, 2003

## Professional Service

| | |
|---|---|
| 1997 – 2005 | Lab Safety Officer (UMD) |
| 1999 – 2002 | Graduate Student Office Coordinator for H. J. Patterson Hall (UMD) |
| 1998 – 1999 | CBMG Graduate Student President (UMD) |
| 1997 – 1998 | Programs, Curriculum, and Courses Committee (UMD) |

### Society Memberships

| | |
|---|---|
| 1997 – current | American Society of Plant Biologists |
| 1997 – current | Botanical Society of America |
| 2003 – current | American Bryological and Lichenological Society |