ANALYSES OF STRUCTURE-FUNCTION RELATIONSHIPS IN THE EVOLUTION OF EMBRYOPHYTE SHOOT MERISTEMS: A TRANSCRIPTOMIC SURVEY

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Cornell University 2014

Abstract

Shoot apical meristems (SAMs) have increased in complexity over the course of embryophyte evolution from single apical initials that generate the gametophytic body plans in bryophytes, to the histologically stratified, multicellular SAMs that give rise to the entire sporophytic shoot in angiosperms. Despite this diversity in shoot apical structure, our understanding of the genes involved in SAM function is largely limited to the angiosperms. In this thesis, I use laser microdissection coupled with RNA-sequencing (LM-RNAseq) to generate domain specific gene expression profiles for meristem cells as well as sporophytic bryophyte embryos. These data were used to address major questions pertaining to transitions in embryophyte evolution. In chapter two I explore the developmental genetic programs that control multidimensionally dividing bud cells versus unidimensionally dividing tip cells in the moss *Physcomitrella patens.* I identify over 4,000 transcript profiles distinguishing the two stem cell types. Moreover the bud cell transcriptomes have significantly up-regulated programs involving meristem development and asymmetric cell division. From this work I propose a model wherein the merger of these two programs allows the unicellular moss meristem to balance its essential functions of self-maintenance with organogenesis. In chapter three I look into the molecular basis of sporophyte

shoot meristem evolution. I ask if angiosperm meristem patterning genes expressed in the sporophytic SAM of *Zea mays* are expressed in the gametophytic SAM, or in the non-meristematic sporophyte, of the model bryophytes *Marchantia polymorpha* and *Physcomitrella patens*. I identify an abundance of up-regulated genes involved in stem cell maintenance and organogenesis in the maize SAM and in both the gametophytic meristem and sporophyte of moss, but not in *Marchantia*. I use these findings to build a framework for sporophytic meristem evolution involving the concerted selection of ancestral meristem gene programs from gametophyte-dominant lineages. In chapter four I investigate the functional relationships amongst the AC-type meristem structures found in *Selaginella* and *Equisetum* and the angiosperm meristem structure found in maize. The analyses indicate that pluripotent cell functions reside within the prominent AC. I also identify homologs for angiosperm SAM maintenance genes across multiple domains in the *Equisetum* and *Sellaginella* SAMs, implying that meristem maintenance is not restricted to the prominent AC that defines these SAMs. Moreover, the transcriptional profiles for the two AC-type SAMs are definitively distinct from one another, providing the first molecular support for the convergent evolution of AC-type SAM structures within these vascular plant lineages. The data presented here bring a new awareness to the developmental genetic processes that may have contributed towards pivotal innovations in land plant evolution.

BIOGRAPHICAL SKETCH

Margaret was born and raised in Portland, Oregon, where she developed a passion for running and the outdoors. When she moved to New York City to attend Barnard College in 2003, she was planning on a career in sports medicine. Her future directions quickly shifted halfway through college, while she was taking a Plant Physiology course from Professor Kristen Shepard. Professor Shepard's emphasis on the clever experimental designs that underlie our basic understanding of plant development sparked Margaret's interest in lab research. Margaret started her first project in the Shepard lab the following summer, investigating *CLAVATA* gene function in the *Arabidopsis* shoot apical meristem. After graduating from Barnard in 2007, she took a position as a research assistant in Dr. Jianhua Li's lab at the Arnold Arboretum. While there, she contributed to studies of the molecular phylogenies for the maples and the gymnosperms. She moved to Ithaca, NY in August 2008 to start as a graduate student in the Cornell University Plant Biology PhD program. Margaret quickly joined the Scanlon lab after Professor Mike Scanlon proposed a thesis project investigating the evolution of shoot apical meristem development in ancient land plant lineages.

This thesis is dedicated to Danny Frank

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To Dezi Elzinga, who has made Cornell awesome from the first semester of classes in the Fall of 2008 to this last semester of finishing my thesis. She is a true best friend with whom I can share my struggles and celebrate my successes. To my parents, Joe and Haven Frank, who have surrounded me with love and support in all of my directions in life. Finally, to David Goldberg, who taught me that each day is an opportunity to pursue what you love. I look forward to *many* more days together.

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CHAPTER 1

INTRODUCTION

1.0.1 Overview

The discovery that the entire above ground portion of plants can be traced back to the activity of a small population of cells referred to as the shoot apical meristem (SAM), was first published in a PhD thesis from 1759 (Wolff). Since that time, tremendous advancements in our understanding of the structure, function, and molecular patterning of the SAM have been made. The eighteenth and nineteenth centuries are marked by extensive investigations into the histological organization of diverse SAMs. These studies revealed that while the principal functions of self-maintenance and organogenesis are conserved across land plants, SAM anatomical organization is diverse. In the twentieth century, chimeric analyses and microsurgical studies revealed histogenic relationships within SAM structures and their corresponding contributions to lateral organs. More recently, the advent of model plants with sequenced genomes and tools for tracking gene function have enabled the discovery of molecular programs that are essential for SAM development. However, these recent findings are largely limited to the angiosperms. While there are extensive data from historical experiments concerning SAM structure and function in ancient land plant lineages, very little is known about the molecular genetic toolkit that patterns development in these lineages. The work presented in this thesis comprises an attempt to address this deficiency. It extends the predicted functions of known SAM programs to anciently-evolved lineages of land plants, and uncover novel molecular programs within each of these newly-sequenced model plants.

1.0.2 The ancestors to the land plants

The first apical meristems for the streptophyte (green plant) clade likely evolved within the Charales, an algal order that is sister to all land plants (Graham and Wilcox, 2000; Karol et al., 2001). This algal SAM is restricted to the gametophytic generation (the only multicellular generation in charophycean green algae) and is composed of an individual cell with one or two cutting faces in *Chara* and *Nitella* (respectively) that form filamentous or planar arrays of daughter cells to create simple, multicellular body plans (Bold, 1973; Graham and Wilcox, 2000; Niklas, 1997). Anciently-evolved extant embryophyte lineages contain (1) apical meristems with three or more cutting faces, and (2) multicellular sporophytes. These novel innovations are believed to be correlated with the movement of plants onto land (Graham and Wilcox, 2000). An increase in the number of cutting faces on the apical initial allowed plants to build cellular sectorial arrays in three dimensions and the expansion of the sporophyte into a multicellular generation provided the foundation for the development of complex, diploid body plans.

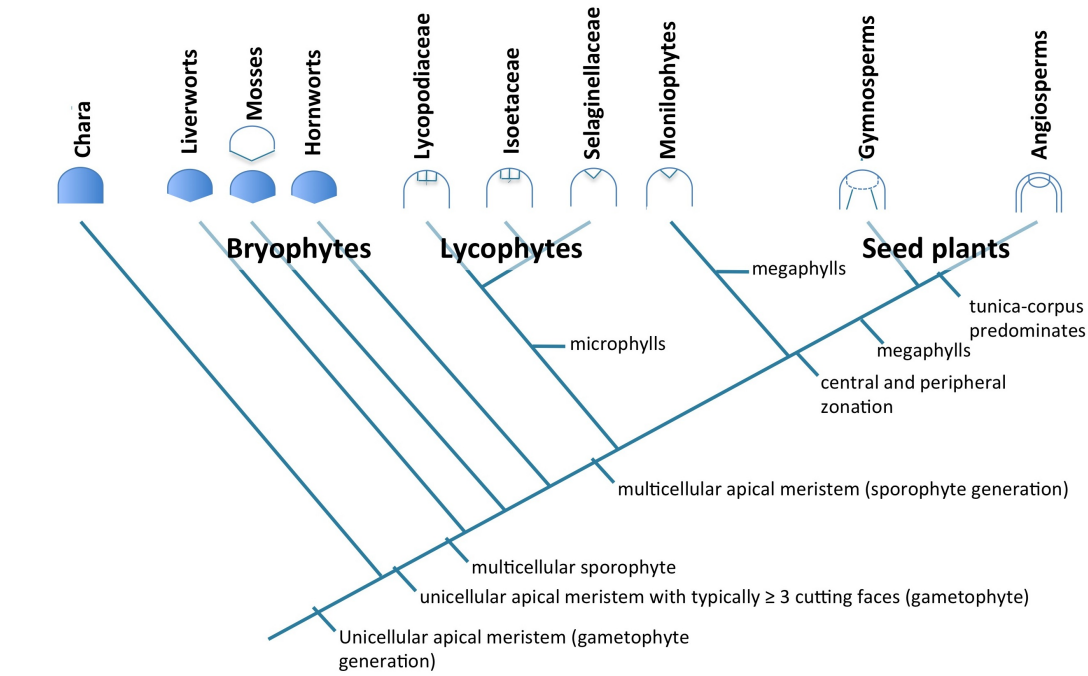
The bryophytes, a paraphyletic group that contains the liverworts, mosses, and hornworts, are the most anciently-evolved extant embryophytes (Qiu et al., 2006). Given the sparse fossil record for the pre-vascular land plants, the bryophyte lineages serve as a proxy through which we can explore the early history of the embryophytes. These lineages are characterized by having a haploid-dominant life cycle in which the gametophytic generation is nutritionally independent and almost always larger than the sporophytic generation.

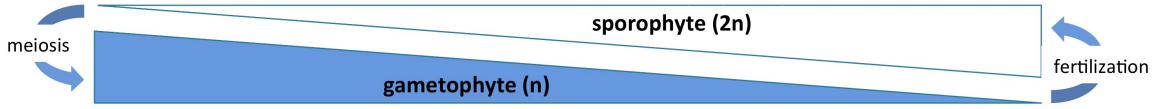
Mosses are the first lineage to evolve axial growth during the gametophyte generation. Although these axes (gametophores) are photosynthetic, produce

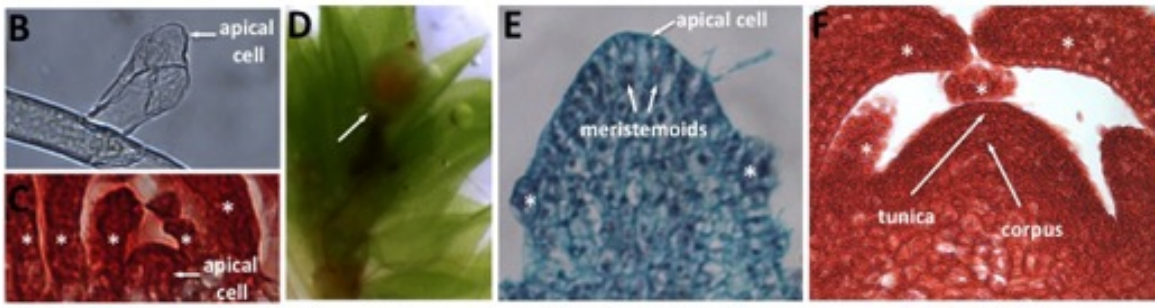
lateral leaf-like organs (phyllids), and have water-conducting cells that are functionally similar to vascular plant sporophytes, this body plan is analogous rather than homologous to those of vascular plant sporophytes (reviewed in Graham and Wilcox, 2000). Mosses are also the earliest living plant lineage to grow from an apical initial (Mishler and Churchill, 1984), and exhibit polar auxin transport during the sporophyte generation (Fujita et al., 2008; Poli et al., 2003). Apical initial cells of the moss sporophyte give rise to the sprophytic capsule, although these determinate cell division patterns are generally considered to be non-meristematic. In spite of their non-meristematic function, recent studies suggest that the sporophytic apical cells of moss are homologous to the indeterminate SAMs of more recently derived vascular land plants (Friedman and Moore, 2004).

Figure 1.1: **Evolution of shoot apical structures in the embryophytes**

Filled shapes represent gametophytic apices and empty shapes represent sporophytic shoot apices. Boxes denote plural initials and triangles denote apical-cell type meristems. The dashed line (Gymnosperms) represents a zonation pattern found in most but not all gymnosperm taxa, and solid lines represent zonations and histological stratification that are found in all taxa. (modified from Imaichi 2008). (B) Tetrad stage of an initiating *Physcomitrella* gametophore with an apical initial cell and two lateral bud cells. (C) Apical section of a mature *Physcomitrella* gametophore, showing a single apical cell with its prominent nucleus (arrow) surrounded by leaf-like phyllid primordia (asterisks) that are formed via division of the apical cell. (D) Mature *Physcomitrella* sporophyte (arrow) attached to a gametophore. (E) Shoot apical meristem (SAM) of *Selaginella* showing a large triangular apical cell surrounded by meristemoids cells formed by division of the apical cell. Microphyll leaf primordia (asterisks) form at the SAM periphery. (F) The *Arabidopsis* SAM contains a two-layered tunica, maintained as a clonal layer via exclusive anticlinal divisions, surrounding an internal corpus wherein cell divisions occur in all planes. Megaphyllous leaf primordia (asterisks) initiate at the SAM periphery.


Chara
Liverworts
Mosses
Hornworts
Lycopodiaceae
Isoetaceae
Selaginellaceae
Monilophytes
Gymnosperms
Angiosperms
Bryophytes
Lycophytes
Seed plants
megaphylls
tunica-corpus predominates
megaphylls
microphylls
central and peripheral zonation
multicellular apical meristem (sporophyte generation)
multicellular sporophyte
unicellular apical meristem with typically ≥ 3 cutting faces (gametophyte)
Unicellular apical meristem (gametophyte generation)


meiosis
sporophyte (2n)
gametophyte (n)
fertilization


B
apical cell
C
apical cell
D
E
apical cell
meristemoids
F
tunica
corpus
Physcomitrella patens gametophore
Physcomitrella patens sporophyte
Selaginella moellendorffii
Arabidopsis thaliana

1.0.3 Evolution of the vascular plants

Developmental innovations that characterize the bryophyte-to-vascular plant transition include the rise of sporophytic dominance, and indeterminate multicellular SAMs that are capable of branching. Ancient polysporangiophytes (defined as having branched sporophytes) arose during this transition from gametophyte-dominant to sporophyte-dominant lineages. The discovery of fossil polysporangiophytes has provided invaluable clues toward understanding the chronology and ontogeny of these key innovations (Stewart and Rothwell, 1993). In particular, *Aglaophyton major* (formerly *Rhynia major*) (Kidston and Lang, 1917) combines morphological characters that group it with both the bryophyte and early vascular plant lineages. For example, the *Aglaophyton* sporophyte is dichotomously branched, but is of diminutive stature and lacks lignified conducting cells (Edwards, 1986). These and other paleobotanical studies suggest a gradual transition to sporophyte-dominant life cycles during embryophyte evolution.

Ancient polysporangiophytes are hypothesized to be the last common ancestor to the two surviving lineages of vascular plants (the lycophytes and the euphyllophytes), and are thus indispensable for discerning homology versus homoplasy in extant lineages (Banks, 1968; Gensel, 2008). These plants grew as bare axes, lacking roots, shoots, and leaves (Gensel, 2008); implying that these structures evolved separately in the lycophytes and euphyllophytes. Comparative developmental genetic studies in the shoot apices of lycophytes, monilophytes, and seed plants have demonstrated that both conserved (Harrison et al., 2005) and divergent (Floyd and Bowman, 2006; Prigge and Clark, 2006) molecular mechanisms regulate leaf initiation and growth.

Lycophyte meristems comprise two structural categories: (1) apical cell (AC) type meristems in *Selaginella* and (2) plural initial cell meristems in *Lycopodium* and *Isoetes* (Campbell, 1895; Stevenson, 1976). AC-type meristems have one prominent central cell in the shape of an inverted pyramid that functions as an apical initial (Clowes, 1961). These initials have 2 to 5 cutting faces that give rise to daughter meristemoid cells, which then divide both anticlinally and periclinally to generate the corpus of the shoot apex (Harrison et al., 2007; Popham, 1951) analyzed genetic chimeras in *Selaginella kraussiana* and concluded that AC-type meristems actually contain *two* initial cells, indicating that the single pyramidal apical cell in the shoot apex is not the sole contributor to initial cell divisions. However, the identity of this second meristematic initial remains enigmatic (Jones and Drinnan, 2009).

SAMs within the monilophyte lineages (horsetails and ferns) have meristems that are superficially very similar to those of the *Selaginella*, however, the apical cells of these meristems are broader, and exhibit less variability in the number of cutting faces (Dengler, 1983; Hagemann, 1980; Imaichi and Kato, 1989; Imaichi and Kato, 1991; Popham, 1951; Siegert, 1974). Previous models suggested that sporophytic AC-type meristems evolved in the ancient polysporangiophytes, rendering the AC-type of *Selaginella* and monilophytes as homologous structures. However, phylogenetic and paleobotanical data refute this hypothesis. Anatomically preserved SAMs from the early polysporangiophytes lack AC-type meristem structures (Edwards, 1993; Hueber, 1992; Kidston and Lang, 1920; Wolff, 1759). Furthermore, considering the phylogenetic placement of *Selaginella* as sister to *Isoetes*, and in a derived position relative to *Lycopodium*, parisomony suggests an ancestral SAM with plural initial cells. Taken together, this evidence suggests that AC-type structures evolved independently in the

lycophytes and the euphyllophytes; however, this question is far from being answered. Most of the early vascular plant lineages are extinct, leaving large gaps in our understanding of SAM structural evolution in early vascular plants. Chapter four of this thesis utilizes next generation sequencing of SAM subdomains to address questions concerning the independent origins of AC-type meristems.

1.0.4 The seed plant shoot apex

SAM structural evolution in the seed plants, the last major group to evolve, trends toward increased zonation and histological stratification. The gymnosperms are the first extant lineage to exhibit distinct central (CZ) and peripheral zones (PZ) that perform the well-defined roles of meristem cell proliferation and lateral organ initiation (respectively). Tunica-corpus histology, in which meristems are stratified into clonally-related outer and inner cell files, first arose within the gymnosperms and became predominant within the angiosperms (Graham and Wilcox, 2000; Karol et al., 2001; Schmidt, 1924). While tunica-corpus organization is essential for angiosperm SAM development (Bold, 1973; Graham and Wilcox, 2000; Niklas, 1997; Reinhardt, 2003; Reinhardt et al., 2005), the absence of this feature in pre-angiosperm lineages marks it as a structural requirement that is only relevant to more recently evolved plants.

1.0.5 Functional investigations into SAM organization in ferns and angiosperms

The significance of structurally diversity on SAM function has been examined through the use of elegant genetic and surgical studies (Graham and Wilcox, 2000; Szymkowiak and Sussex, 1996). Cytochimeras obtained through colchicine-induced polyploidy were utilized to demonstrate that the histological layers of the angiosperm SAM are clonally distinct (Qiu et al., 2006; Satina et al., 1940). These experiments also provided evidence for position-dependent cell fate acquisition in plants, as opposed to the lineage-dependent paradigms that characterize animal development (Clowes, 1961; reviewed in Graham and Wilcox, 2000). Time-lapse observations of *Selaginella* meristems suggest that this position-dependent model for cell fate acquisition can be extended to AC-type meristems (Jernstedt et al., 1994; Mishler and Churchill, 1984). Studies using radiation-induced somatic clonal sectors have provided insights into the number and persistence of initial cells within distinct meristematic tissues (Dawe and Freeling, 1991; Fujita et al., 2008; Poli et al., 2003). In angiosperms, these studies revealed that initial cell populations within the SAM are continuously diverted towards the flanks of the meristem, and are replaced by daughter cells of stem cell initials in the SAM core (Friedman and Moore, 2004; Irish and Sussex, 1992; McDaniel and Poethig, 1988; Poethig, 1987). The recent extension of these experimental strategies to *Selaginella* demonstrates that the AC-type SAM also harbors transient, apical initial cells (Harrison et al., 2007; Stewart and Rothwell, 1993).

Finally, microsurgical experiments have provided key insights into the domain-specific functions and developmental fates of initials cells within an-

giosperm and AC-type SAMs(Kidston and Lang, 1917; Steeves, 1989). For example, surgical analyses illustrated that a region of cells surrounding the AC in ferns is sufficient for whole plant regeneration on minimal media, whereas seed plants require more extensive nutritional supplementation or the inclusion of young leaf primordia to survive (Edwards, 1986; Steeves, 1989). These dramatic differences in regeneration potential between ferns and seed plants may reflect differences in SAM autonomy between these two lineages.

Damage to the CZ of the angiosperm SAM, either through surgical incision, micropuncture, or laser ablation can lead to either SAM abortion or regeneration. Meristem arrest occurs when all SAM initials are destroyed, while re-specification of SAM organization and shoot regeneration occurs when a small reservoir of SAM initials survive (Ball, 1952; Ball, 1955; Ball, 1980; Banks, 1968; Gensel, 2008; Pilkington, 1929; Reinhardt, 2003; Steeves and Sussex, 1989). Reorganization of the SAM center following injury is correlated with molecular re-patterning of the SAM (Gensel, 2008; Reinhardt, 2003). Similar experiments in AC-type fern meristems reveal that SAM regenerating is also possible following micropuncture of the prominent AC (Harrison et al., 2005; Steeves, 1989). Whereas the AC is thought to be the sole initial of the fern SAM, these findings suggest that a complete re-specification of the stem cell initial ensues following AC ablation, similar to what was found following laser ablation of the root quiescent cell in *Arabiodopsis* (Scheres et al., 1995). As yet, analogous SAM wounding experiments in *Selaginella* are lacking.

1.0.6 Genetic regulation of the angiosperm SAM

Genetic regulation of SAM function has been extensively explored in angiosperms, revealing parallel pathways that interact to specify networks of SAM activity. These include the *CLAVATA-WUSCHEL* loop that is responsible for maintaining SAM size, the *KNOX-ARP* pathway that antagonistically balances pluripotency and organogenesis within the SAM, the *Class III HD-Zip* pathway(s) that specifies lateral organ polarity and SAM maintenance, and several chromatin remodeling complexes that mediate between pluripotent and differentiated chromatin states. Each of these pathways have been studied in great detail; here, I provide a summary of the main findings, a more extensive coverage of SAM maintenance pathways can be found in the following excellent reviews (Barton, 2010; Byrne, 2006; Carles and Fletcher, 2003; Clark, 2001; Floyd and Bowman, 2006; Hake et al., 2004; Hay and Tsiantis, 2010; Perales and Reddy, 2012; Prigge and Clark, 2006; Shen and Xu, 2009).

Fasciated (enlarged) meristems that develop flowers with supernumerary organs and oversized fruits have been observed in agricultural fields for centuries (Campbell, 1895; Stevenson, 1976; White, 1948). Genetic screens for fasciated SAM phenotypes in Arabidopsis led to the discovery of the *CLAVATA1/2/3* (*CLV1/2/3*) genes, the first known regulators of SAM size (Clark et al., 1993; Clark et al., 1995; Clowes, 1961; Galun, 2007; Kayes and Clark, 1998; Leyser and Furner, 1992). An independent screen for the opposing phenotype uncovered the *wuschel* (*wus*) mutation, which fails to maintain the SAM stem cell population (Harrison et al., 2007; Laux et al., 1996; Popham, 1951). The antagonistic phenotypes of *clv1/2/3* and *wus* mutants, the expression patterns of the *CLV* and *WUS* genes, and genetic analyses of *clv* and *wus* mutants contribute to a

model for a negative-feedback signaling loop that maintains SAM size (Carles and Fletcher, 2003; Jones and Drinnan, 2009). *CLV3* encodes a small peptide that accumulates in the upper layers of the SAM and moves apoplastically to lower layers (Dengler, 1983; Fletcher et al., 1999; Hagemann, 1980; Imaichi and Kato, 1989; Imaichi and Kato, 1991; Popham, 1951; Siegert, 1974). In the substrata of the SAM, CLV3 binds receptor-like kinases including the CLV1-CLV2 heterodimer and mediates a signaling pathway that maintains SAM size by restricting *WUS* expression to a small region of cells in the organizing center (OC) of the SAM (Jeong et al., 1999; Kinoshita et al., 2010; Miwa et al., 2009; Ogawa et al., 2008; Zhu et al., 2010). WUS, in turn, up-regulates *CLV3* expression in a non-cell autonomous manner, leading to its own transcriptional inhibition (Brand et al., 2000; reviewed in Carles and Fletcher, 2003; Schoof et al., 2000).

A parallel pathway involving antagonistic interactions between *KNOTTED1-like Homeobox* (*KNOX*) regulators of cell indeterminacy and *ASYMMETRIC LEAVES1/ROUGH SHEATH2/PHANTASTICA* (*ARP*) lateral organ patterning genes was initially discovered through independent mutant screens in maize and *Antirrhinum* (Byrne et al., 2000; Clark et al., 1996; Schneeberger et al., 1998; Timmermans et al., 1999; Vollbrecht et al., 1991; Waites and Hudson, 1995). The founding member of this pathway, *Knotted-1*, was identified as a dominant mutation that causes ectopic outgrowths along vascular bundles in the maize leaf (Freeling and Hake, 1985). Further investigation of angiosperm *KNOX* genes revealed that *KNOX* expression marks indeterminate cell fate in the SAM, and is essential for maintaining the SAM stem cell population (Endrizzi et al., 1996; Jackson et al., 1994; Kerstetter et al., 1997; Smith et al., 1992; Vollbrecht et al., 2000). Furthermore, mutants that fail to repress *knox* expression in lateral organ founder cells develop leaves that are defective in axial patterning (Henderson et

al., 2005; Scanlon et al., 1996; Schneeberger et al., 1998). The connection between *Kn-1* specification of SAM indeterminacy and *ARP*-mediated lateral organ patterning was made when the maize mutant *rough sheath2*, which phenocopies *kn-1D* overexpression alleles, was cloned and identified as an ortholog of the *KNOX* regulator *PHANTASTICA* (Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999).

Analyses in Arabidopsis show that *KNOX* expression is downregulated in later stages of primordia development by a heterodimeric complex between the ARP protein ASYMMETRIC LEAVES 1 (AS1) and the LATERAL ORGAN BOUNDARIES domain containing protein, ASYMMETRIC LEAVES 2 (AS2). Interestingly, ectopic KNOX expression in *as1/as2* double mutants is not observed during the earliest stages of leaf development, instead, local auxin maxima generated by polar auxin transport turns *KNOX* expression off (Hay et al., 2006; Reinhardt et al., 2003). At later stages, AS1-AS2 interact with a chromatin remodeling complex (HIRA) and directly bind to *KNOX* promoters to keep *KNOX* expression off during leaf development (Guo et al., 2008; reviewed in Hay and Tsiantis, 2010; Phelps-Durr et al., 2005). Intriguingly, this antagonistic interaction is not found in ferns, where *KNOX* proteins accumulate in initiating leaf primordia, however remnants of the pathway have been identified in *Selaginella* SAMs, suggesting a complicated but ancient history for this pathway (Harrison et al., 2005; Sano et al., 2005).

Beyond controlling leaf initiation, the founding *ARP* gene *PHAN* was originally isolated for its role in specifying adaxial-abaxial (ad-ab; i.e. - top-bottom/dorsi-ventral) polarity (Waites and Hudson, 1995). Analysis of *phan* phenotypes inspired a model for leaf development, in which the juxtaposition

of upper and lower leaf patterning pathways is essential for proper blade outgrowth (reviewed in Eckardt, 2004). In line with such a model, the identification of a slue of genes controlling ad-ab patterning has shown that a loss of adaxial or abaxial specification is sufficient to disrupt proper blade outgrowth (reviewed in Byrne, 2012).

From an evolutionary perspective, the most well-studied members of this pathway are the *Class III HOMEODOMAIN LEUCINE-ZIPPER* (*HD-ZIP III*) genes, which regulate adaxial cell patterning and SAM maintenance in a redundant fashion (Prigge et al., 2005). Fitting with their proposed function, *HD-ZIP III* genes are expressed in adaxial domains of leaves, and also in the SAM (reviewed in Byrne, 2006; Emery et al., 2003; McConnell et al., 2001). Opposing *HD-ZIP III* function are the abaxial patterning genes, including the *KANADI* (*KAN*) gene family, *AUXIN RESPONSE FACTOR3* (*ARF3*), and the *YABBYs* (*YABs*) (Bowman, 2000; Eshed, 2004; Kerstetter et al., 2001; Siegfried et al., 1999). An additional layer of dorsiventral regulation involves trans-acting small interfering RNA (ta-siRNA) signaling across ad-ab boundary (reviewed in Chitwood et al., 2007; Douglas et al., 2010; Garcia et al., 2006; reviewed in Husbands et al., 2009; Juarez et al., 2004; Kidner and Martienssen, 2004; reviewed in Kidner and Timmermans, 2007; Nagasaki et al., 2007; Nogueira et al., 2007; Timmermans et al., 2004). The combined functions of these genes yield a network of redundant and mutually antagonistic interactions between the ad-ab surfaces of the developing leaf primordium, ultimately creating a polarized lateral organ.

Chromatin remodeling plays an essential role in directing cell fate transitions in the SAM. *Fasciata1* (*fas1*) and *fasciata2* (*fas2*) were first identified along with clv1 in a screen for enlarged SAMs (Leyser and Furner, 1992). Later cloning

of these genes revealed homology to proteins in the human chromatin assembly factor-1 (CAF-1) complex, which is involved in loading newly replicated DNA onto histones (Kaya et al., 2001b; Smith and Stillman, 1989). FAS1, FAS2, and a third protein MULTICOPY SUPPRESSOR 1 (MSI1) form a multimeric complex that is essential for regulating the size of the SAM stem cell niche (Kaya et al., 2001b; Kaya et al., 2001a). MSI1 also associates with the polycomb group complex2 (PRC2) to direct specific changes in the reproductive state in the meristem (Hennig et al., 2003; Khler et al., 2003). PRC complexes regulate several processes in plant development (reviewed in Hennig and Derkacheva, 2009); in the vegetative SAM, a PRC2 complex containing CURLY LEAF (CLF) regulates SAM proliferation by catalyzing repressive H3K27 trimethylation marks at the *KNOX* locus (Xu and Shen, 2008). The PRC1 complex interprets this repressive mark by mediating chromatin compression at these sites, ensuring the down-regulation of *KNOX* expression in initiating lateral organs (Xu and Shen, 2008). The SWI/SNF ATPases SPLAYED (SYD) and BRAHMA (BRM) also contribute to maintenance of the stem cell niche by restricting *WUS* expression within the SAM (Farrona et al., 2004; Kwon et al., 2005; Wagner and Meyerowitz, 2002). Several chromatin remodelers have been shown to mediate lateral organ-specific changes in chromatin state. For example, the HIRA complex associates with AS1 and AS2 to repress *KNOX* expression in lateral organs (mentioned above) (Phelps-Durr et al., 2005) and the CHD3 chromatin remodeler, *PICKLE*, mediates lateral organ initiation presumably by controlling GA responsiveness (Henderson et al., 2004; Ogas et al., 1999; Ori et al., 2000).

Interwoven throughout these intersecting genetic pathways are networks of phytohormone signalling. Cytokinin (CK) has well-described roles in cell proliferation and shoot development (reviewed in Werner and Schmulling, 2009),

whereas gibberellic acid (GA) and auxin promote cell differentiation and lateral organ patterning (Reinhardt et al., 2003; reviewed in Shani et al., 2006; Zhao, 2010). KNOX proteins directly repress GA production and promote biosynthesis of CK, thereby promoting stem cell indeterminacy in the SAM (Bolduc and Hake, 2009; Jasinski et al., 2005; Sakamoto et al., 2001; Yanai et al., 2005). This up-regulated cell proliferation is likely mediated by CK activation of the CK receptor WOODEN LEG1 which causes the direct up-regulation of *WUS* expression (Gordon et al., 2009; Leibfried et al., 2005; reviewed in Sablowski, 2009).

Polar auxin transport is required for lateral organ initiation (Reinhardt et al., 2000). The auxin efflux carrier PIN-FORMED1 generates auxin maxima that function to repress *KNOX* expression and promote lateral organ initiation (Hay et al., 2006; Reinhardt et al., 2000; Reinhardt et al., 2003; Scanlon, 2003) A role for auxin during leaf polarity is also implied. Auxin induces expression of *ARF3* in leaf abaxial domains; in addition to promoting abaxial specification, ARF3 directly represses cytokinin biosynthesis enzymes to maintain lateral organ identity in the initiating leaf (Cheng et al., 2013). These examples provide a conceptual summary of the complex shoot patterning networks operating in the SAM. A more thorough treatment of this subject can be found in recent reviews (e.g. Shani et al., 2006).

1.0.7 Genetic regulation in anciently evolved embryophyte lineages

A great deal of progress in our understanding of the developmental genetic programs that operate in anciently derived land plant lineages has been made since

the release of the *Phycomitrella* and *Selaginella* genomes (Banks, 2009; Dolan, 2009; reviewed in Langdale, 2008; Niklas and Kutschera, 2009; Niklas and Kutschera, 2010; Nishiyama et al., 2011; Prigge and Bezanilla, 2010; Rensing et al., 2008). Here, I will focus on work that has contributed towards answering three major questions concerning early land plant history:

(1) How is the transition from filamentous to multidimensional growth regulated in the model bryophyte *Physcomitrella patens*?

(2) How did an indeterminate shoot apical meristem evolve, and become dominant, in the sporophytic generation?

(3) What is the functional relationship between the apical cell-type meristems found in seedless vascular plant lineages and plural initial-type meristems found in the lycophytes and seed plants?

1.0.8 Transition from unidimensional to multidimensional growth

The transition from unidimensional to multidimensional stem cell growth in the green plant lineage allowed for the construction of parenchymatous body plans capable of colonizing the vertical landscape (Niklas, 1997; Niklas and Kutschera, 2009). This transition in growth habit occurs in the algal sister lineage to the embryophytes, and is reflected in the life cycles of extant bryophytes (Harrison et al., 2009; Niklas and Kutschera, 2009; Steemans et al., 2009). Auxin and cytokinin play pivotal roles during multidimensional growth regulation in mosses(Ashton and Cove, 1977; Ashton et al., 1979; Grimsley et al., 1977; Johri

and Desai, 1973; Schumaker and Dietrich, 1998). Auxin promotes the transition from feeding (chloronema) to foraging (caulonema) during the filamentous stage of growth and cytokinin triggers axial bud initiation on foraging cells (Schumaker and Dietrich, 1998).

Recently, genetic interactions with cytokinin and auxin signalling pathways have contributed to models for how these hormone-mediated transitions occur. For example, paralogs of the bHLH transcription factor *RHD SIX-LIKE1*, are up-regulated in the presence of auxin and are necessary and sufficient to direct the chloronema to caulonema transition (Jang and Dolan, 2011; Pires et al., 2013). On the other hand, *AP2-class* transcription factors from the *AINTEGUMENTA/PLETHORA/BABY BOOM* (*APB*) gene family are up-regulated in the presence of cytokinin, where they function redundantly to induce bud initiation (Aoyama et al., 2012). In addition to transcription factors, multiple small RNA pathways are identified as key players in determining filamentous versus axial growth(Axtell, 2009; Cho et al., 2012; reviewed in Prigge and Bezanilla, 2010; Saleh et al., 2011). Questions concerning the innovation of a multidimensionally dividing gametophore bud cell that coordinates the meristematic function of organogenesis and self-maintenance are addressed in chapter two. Bioinformatic comparisons of cell-enriched protonemal tip versus bud transcriptomes were used to identify the molecular features that distinguish filamentous from multidimensional stem cell growth. These data inspire a new model for how the unicellular moss SAM balances organogenesis and stem cell-maintenance.

1.0.9 Evolution of the sporophyte shoot meristem

Evolution of the sporophyte SAM represents a landmark event that enabled the development of elaborate sporophyte body plans (Ligrone et al., 2012a; Ligrone et al., 2012b; Niklas, 1997; Niklas and Kutschera, 2009). However, developmental genetic shifts that allowed for indeterminate growth in the sporophytic generation are still poorly understood. Two models for this evolutionary innovation include the neo-functionalization of sporophytic gene programs to function in SAM organization, or the transfer of gametophytic SAM programs into the sporophyte generation. Recent work examined the sporophytic transcriptomes of two different moss species demonstrated that many of the key genes involved in angiosperm sporophyte development are also expressed in the moss sporophyte. This study concluded that programs regulating complex development in angiosperm bodies have ancient origins in simple sporophyte body plans (O'Donoghue et al., 2013; Szvnyi et al., 2011). Further support for this model is derived from functional studies of *Physcomitrella* homologs for *KNOTTED 1-like HOMEOBOX* (*KNOX*) genes, which are known to mark indeterminate shoot meristem cells in angiosperms (Vollbrecht et al., 1991). Two independent groups demonstrated that loss of *Class I KNOX* function in *Physcomitrella* conditions mutant phenotypes that are restricted to the sporophyte generation (Sakakibara et al., 2008; Singer and Ashton, 2007). A recent study of the *Class II KNOX* genes in *Physcomitrella*, demonstrated that these genes also specify sporophyte development; *class II knox* deletion mutants develop protonemal outgrowths from the sporophytic embryo, exhibiting a phenomenon called apospory (Bower, 1890; Haig, 2008; Niklas and Kutschera, 2009; Sakakibara et al., 2013). The presence of *KNOX* function in the determinate sporophyte generation of moss and not in the gametophyte indicates that other factors were essential for the evolution

of sporophyte dominance. One potential mechanism that has gained clarity in recent years involves epigenetic changes during the alternation of generations. Deletion mutations of *clf* or *fertilization independent endosperm* (*fie*) PRC2 complex members results in the apogamic initiation of sporophyte-like structures in the gametophytic generation (Mosquna et al., 2009; Okano et al., 2009). Moreover, these sporophytes are able to branch, suggesting that chromatin remodeling may be a central role in the evolution of sporophyte indeterminacy.

Whether the sporophytic meristem evolved through neo-functionalization of sporophytic gene networks or via recruitment of gametophytic programs is an unanswered question(Dolan, 2009; Langdale, 2008; Niklas and Kutschera, 2009). These two hypotheses are not mutually exclusive; the data gathered thus far suggest that some combination of neo-functionalization and trans-generational recruitment may have transpired during embryophyte evolution. However, the picture is far from being resolved. As yet, all comparisons between bryophyte gametophyte and vascular plant sporophyte generations are based on whole plant gene expression profiles, which fail to resolve the molecular genetic relationships of the sporophyte and gametophytic initial cells. Furthermore, comprehensive transcriptional analyses of bryophyte sporophytes have been restricted to the moss lineage, rendering it impossible to examine broad trends in the early evolution of the sporophyte generation. In chapter three, we utilized laser microdissection and next generation RNA sequencing to address whether angiosperm meristem patterning genes expressed in the sporophytic shoot apical meristem (SAM) of *Zea mays* are expressed in the gametophytic SAMs or in the non-meristematic sporophytes of the model bryophytes *Marchantia polymorpha* and *Physcomitrella patens*. These data provide strong transcriptomic support for a model wherein sporophyte meristem evolution in-

volved the concerted selection of ancestral meristem gene programs in a pre-sporophyte dominant lineage.

1.0.10 Evolutionary relationships of AC-type meristems

Despite a rich history of anatomical and morphological experiments in the seedless vascular plant lineages, relatively little is known about the molecular patterning of these early vascular plant meristems (reviewed in Ambrose and Purugganan, 2012). In the fern *Ceratopteris richardii*, homologs of both *CLASS I KNOTTED1-LIKE HOMEOBOX* (*KNOX*) and *CLASS III HOMEODOMAIN LEUCINE ZIPPER* (*HD-Zip III*) genes are transcriptionally restricted to the sporophyte generation, suggesting that these genes do not function in the fern gametophyte (Sano et al., 2005). Likewise, *Selaginella kraussiana* homologs for *CLASS I KNOX*, *ASYMMETRIC LEAVES1/ROUGH SHEATH2/PHANTASTICA* (*ARP*), *HD-ZIP III* genes show transcript accumulations in distinctive subdomains within the shoot apex ((Harrison et al., 2005); (Prigge and Clark, 2006); (Floyd and Bowman, 2006)). These studies indicate that a set of genes with well-described functions in angiosperm SAM development may be present in the anciently evolved AC-type SAMs of seedless vascular plants. However, beyond this very short list, there is little information concerning the genetics of these uniquely structured SAMs. In chapter four, we establish the first molecular markers for AC identity, and connect gene expression patterns with SAM subdomain-specific function for the monilophyte *Equisetum arvense* and the lycophyte *Selaginella moellendorfii*. This study represents the first comprehensive look into SAM-enriched transcriptomics of seedless vascular plants. The data presented in this chapter implies that the AC-type shoot meristem structure

evolved convergently in the lineages that gave rise to *Selaginella* and *Equisetum*.

Claude Wardlaw, a botanist who made significant contributions towards our understanding of AC function during the mid twentieth century, wrote the following statement in the abstract of his 1957 paper concerning the use of new cytological techniques on fern meristems (reviewed in Gifford, 1983; Steeves, 1989; Wardlaw, 1957a; Wardlaw, 1957b):

> *"After one hundred years of research we still do not know why leaf primordia are formed at the apical meristem, or why, indeed, apical meristems exist at all...With this in mind, any new theory or technique that affords a fresh approach to the manifold problems of the apex is likely to be welcomed"*
> -W. C. Wardlaw, 1957

While this statement no longer holds true for the angiosperms, it encapsulates the relative dearth of molecular genetic information available for pre-angiosperm plant lineages. This thesis is largely motivated by the desire to employ new technologies to address long-standing questions in plant evolution.

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CHAPTER 2

TRANSCRIPTOMICS OF MULTIDEMENSIONAL CELL GROWTH IN THE MOSS *PHYSCOMITRELLA PATENS*

2.1 ABSTRACT

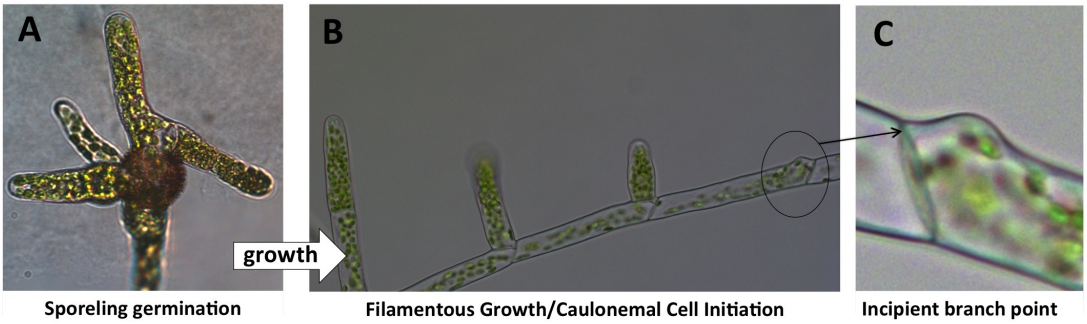
Haploid moss gametophytes harbor distinct stem cells types, which include tip cells that divide in single planes (1D tip cells) to generate filamentous protonemata, and those dividing multidimensionally (3D bud cells) to yield three-dimensional gametophores. Transition from 1D to 3D growth occurs progressively during the moss life cycle, and is thought to mirror the evolution of the first terrestrial plants from Charophycean green-algal ancestors (Graham and Wilcox, 2000; Karol et al., 2001; Raven et al., 2005). The innovation of 3D plant body plans facilitated colonization of the vertical landscape, and enabled development of complex vegetative and reproductive plant morphologies. Despite its profound evolutionary significance, the molecular programs regulating this transition from 1D to 3D meristematic plant growth are poorly understood. In this study we used cell-enriched transcriptome sequencing to uncover more than 4,000 transcript profiles distinguishing 1D protonematal tip cells from 3D gametophore bud cells in the moss *Physcomitrella patens*. While the transcriptomes of both tip and bud cells harbor molecular signatures of actively dividing meristematic cells, the majority of differentially expressed (DE) genes were identified in 3D bud cells. Our transcriptomic data suggest that the combined differential accumulation of shoot patterning transcripts and genes promoting asymmetric cell division accompanied the transition from unidimensional filamentous growth to development of a multidimensional body plan in moss.

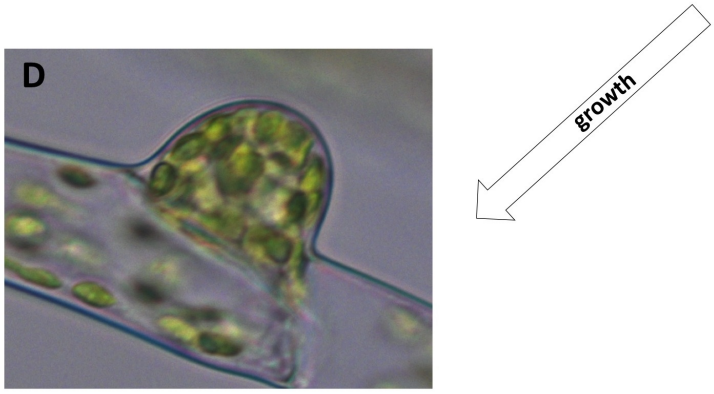
2.2 INTRODUCTION

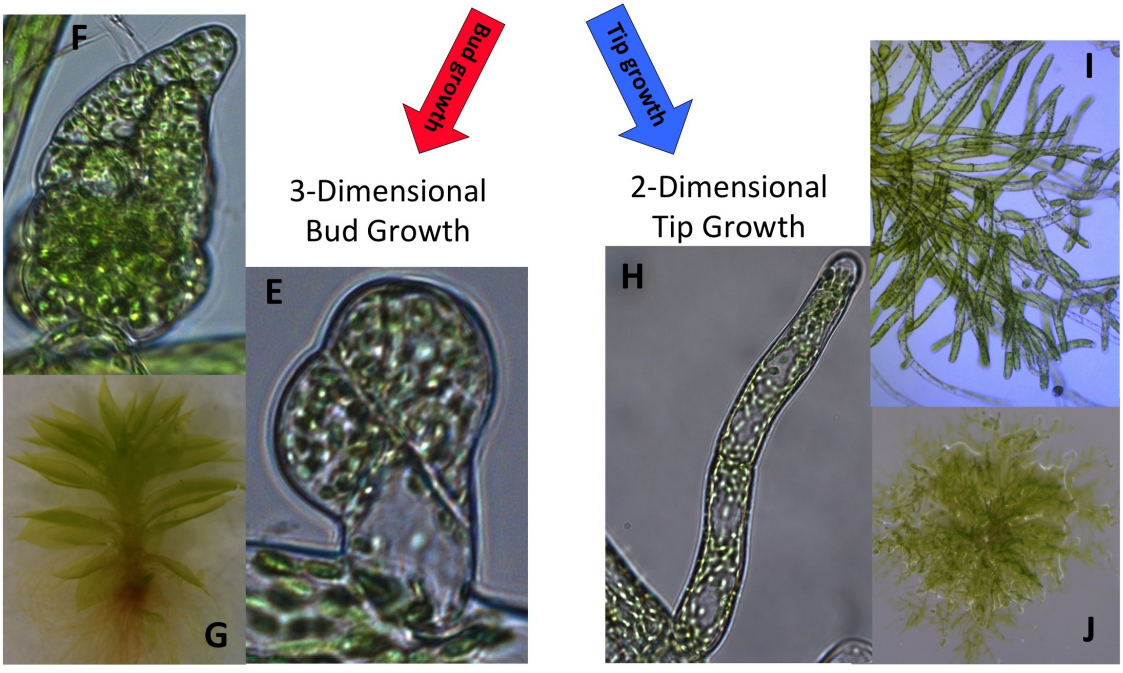
Vascular plants develop from multidimensionally-dividing (3D) cells, whereas unidimensional (1D) cell divisions that generate filamentous growth are generally restricted to two specialized cell types (pollen tubes and root hairs). Development in Charophycean green algae, the closest living relatives of land plants, is wholly composed of 1D cell divisions (reviewed in McCourt et al., 2004). The transition to 3D meristematic cells capable of generating the morphologically complex, parenchymatous body plans of land plants probably occurred in an aquatic ancestor to the land plants (Graham and Wilcox, 2000; Niklas, 1997; Niklas and Kutschera, 2010). Remnants of this critical developmental innovation can be seen in the bryophytes, comprising the oldest extant lineages of land plants. Unlike vascular plants, germinating spores of the seedless, non-vascular bryophytes divide unidimensionally to form filamentous, chloroplast-rich cells called chloronema (Figure 2.1A). Under environmental stimuli and in the presence of auxin, chloronema transition into a second filamentous cell type (caulonema) with few chloroplasts and oblique cell walls (Figure 2.1B). Caulonema (Figure 2.1 C) usually continue filamentous growth as protonemal tip cells (Figure 2.1H-J), although approximately 5% of caulonemata (Cove and Knight, 1993) transition into 3D stem cells called gametophore buds (Figure 2.1D). Bud cells are meristematic, and thus form shoot axes and photosynthetic lateral organs called phyllids (Figure 2.1 E-F).

Figure 2.1: **Tip versus bud developmental decision occurs at incipient caulonemal branch points**

Moss sporeling initiates development with chloroplast-rich chloronemal protonemata or tip cells (A). Caulonemal protonemata develop from chloronema (B), these cells with relatively few chloroplasts and oblique cell walls initiate branch cells. An incipient branch cell initiating as a bulge is circled in black and enlarged in (C). As branch cells initiate they face a decision of developmental fate (D), they can either continue growth as filamentous protonemata (H) or cleave in multiple planes to commence three-dimensional bud growth (E). Buds continue to cleave three-dimensionally from the meristem cell to give rise to young gametophores (F) that continue to develop into shoot-like mature gametophores (G). Tip cells (H) divide in one plane giving rise to filamentous growth (I) that comprises a two-dimensional moss colony (J).


A
B
C
growth
Sporeling germination
Filamentous Growth/Caulonemal Cell Initiation
Incipient branch point


D
growth
Tip versus Bud Developmental Decision


F
G
E
Bud growth
Tip growth
3-Dimensional
Bud Growth
2-Dimensional
Tip Growth
H
I
J

Gametophore buds comprise a single meristematic cell that simultaneously performs indeterminate and organogenic functions (Harrison et al., 2009). In seed plants the shoot meristem is separated into functionally distinct multicellular zones, wherein the meristem center (CZ) divides infrequently and maintains the indeterminate stem cell niche, and the meristem periphery (PZ) divides rapidly and promotes organ initiation (reviewed in Steeves and Sussex, 1989). A fundamental question in plant development asks how a unicellular shoot meristem assimilates the combined functions of stem cell indeterminacy and organogenesis, functions that are typically performed by a multicellular shoot meristem structures in vascular plants.

Key genetic factors known to regulate moss stem cell identity include parallel small RNA pathways, chromatin remodelers, transcription factors and cell cycle regulators (update all required refs: (Aoyama et al., 2012; Arif et al., 2012; Axtell, 2009; Axtell and Bartel, 2005; Axtell and Bowman, 2008; Axtell et al., 2007; Cho et al., 2012; Ishikawa et al., 2011; Jang and Dolan, 2011; Khraiwesh et al., 2010; Mosquna et al., 2009; Nishiyama et al., 2012; Okano et al., 2009; Pires et al., 2013; Sakakibara et al., 2013; Saleh et al., 2011; Talmor-Neiman et al., 2006). Conspicuously absent from the list of known stem cell regulators are moss homologs of the *Class I KNOTTED 1-like Homeobox* (*Class I KNOX*) genes, which play indispensable roles in specifying shoot meristem indeterminacy in angiosperms (Hay and Tsiantis, 2010; reviewed in Vollbrecht et al., 1991). Targeted knockouts of moss *KNOX* genes yield no gametophytic phenotypes, suggesting that distinct stem-cell maintenance pathways function in the shoot meristems of moss and angiosperms (Sakakibara et al., 2013; Sakakibara et al., 2008; Singer and Ashton, 2007).

The combined use of laser microdissection and next generation sequencing technologies (LM-RNAseq) enables specific cell/tissueenriched gene expression analyses. Here we generated Illumina-based transcriptomes from microdissected 1D protonemal tip cells and 3D bud cells from *Physcomitrella patens*, a model moss with a fully sequenced genome (Rensing et al., 2008; Zimmer et al., 2013). Our goals were three-fold: (1) to explore the extent to which genetic programs of angiosperm shoot meristems operate in the unicellular meristems of moss; (2) to discover molecular mechanisms that distinguish 1D and 3D moss stem cells; and (3) to identify transcriptional profiles that are shared between bud and tip cells of the moss gametophyte.

Our transcriptomic comparisons revealed thousands of gene transcripts that distinguish 1D tip cells from 3D bud cells in moss. While both stem cell transcriptomes are enriched for genes predicted to function during cell cycle regulation and pluripotency, the bud cell transcriptomes show differential accumulation of shoot patterning genes and regulators of asymmetric cell division. This data suggest a model for the evolution of multidimensional growth in land plants, via the combined acquisition of shoot meristematic functions and capacity for asymmetric cell divisions.

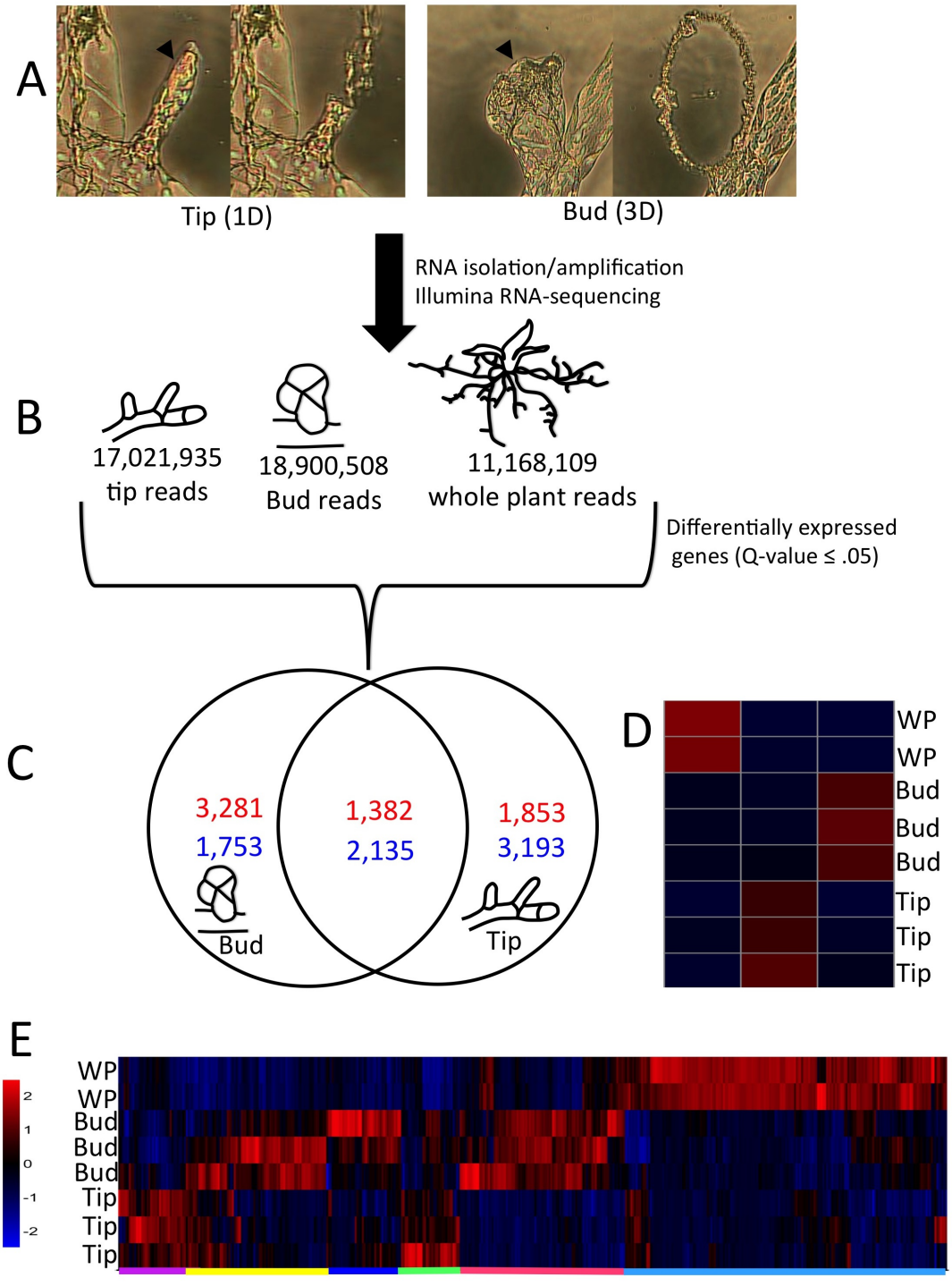
2.3 RESULTS AND DISCUSSION

2.3.1 Thousands of gene transcripts distinguish 1D from 3D stem cells

Development of the moss *Physcomitrella patens* ensues with filamentous tip growth to form protonemata, and ultimately transitions to 3D growth from bud cells. Since both 1D-filamentous and 3D growth patterns arise from single meristematic cells, laser microdissection and transcriptomic profiling of these two, distinct, stem cell types is efficiently straightforward (Figure 2.2B). In total, over 70 million 100 base pair reads were generated and aligned to the *Physcomitrella* reference genome. Applying an adjusted P value (Q value) of $<$.05, 6,957 significantly differentially expressed genes (DEGs) were identified between the tip and bud cell populations (Supplemental Table 2.1). Among these were 4,472 DEGs that exhibited $>$2-fold differential expression, As shown in Figure 2.2C, 1,043 DEGs were up-regulated in tip cells and 3,429 of were up-regulated in bud cells.

Figure 2.2: **Laser microdissection enables the identification of Tip and Bud molecular fingerprints**

(A) Laser microdissection enables specific isolation of tip and bud cells. (B) Between 10 and 20 million sequence reads were generated for each sample type, giving rise to over ten thousand DEGs (C) in pairwise comparisons between each SC type and the whole plant (WP) transcriptomes. (D) K-means clustering of all differentially expressed genes between SC and WP transcriptomes shows discrete independent groupings amongst the three sample types. (E) Heatmap of differentially expressed genes between whole plant (WP), bud, and tip transcriptomes in reads per million scaled by row. Major clusters formed in row hierarchical clustering are indicated with purple, yellow, blue, orange, and green color blocks. Value bar of scaled read counts is shown to the left of the heatmap with red and blue indicating up-regulated and down-regulated transcript accumulation (respectively). Genes that are up-regulated and down-regulated in SC versus WP transcriptomes in C are shown in red and blue (respectively).


A
Tip (1D)
Bud (3D)
RNA isolation/amplification
Illumina RNA-sequencing
B
17,021,935
tip reads
18,900,508
Bud reads
11,168,109
whole plant reads
Differentially expressed
genes (Q-value ≤ .05)
C
3,281
1,753
1,382
2,135
1,853
3,193
Bud
Tip
D
WP
WP
Bud
Bud
Bud
Tip
Tip
Tip
E
2
1
0
-1
-2
WP
WP
Bud
Bud
Bud
Tip
Tip
Tip

Comparisons of the bud transcriptome and the whole plant transcriptome reveals that 65% of all DEGs were up-regulated in bud cells, whereas just 37% were upregulated in the protonemal tips (Figure 2.2C). Overall, the bud transcriptome has more than 1,500 more up-regulated DEGs than the tip transcriptome. Previous transcriptomic comparisons of maize shoot meristems and whole seedlings reported an equivalent number of SAM up-regulated genes (Brooks et al., 2009; Ohtsu et al., 2007). Our data suggest that the moss tip cell is a transcriptionally repressive fate, whereas bud cells are transcriptionally active relative to whole moss plants. Taken together, these results predict that moss tip cells and bud cells harbor dramatic differences in chromatin state.

2.3.2 Gene ontology enriched in 3D bud cells (developmental patterning) and 1D tip cells (photosynthesis)

Our analyses of transcript accumulation in tip and bud cells identified thousands of up-regulated (1,382 transcripts) and down-regulated (2,135 transcripts) DEGs in comparisons to whole plants. Utilizing the R-program topGO and a P-value cutoff of <0.05, gene ontology (GO) enrichment analyses were employed to identify predicted gene functions that are significantly enriched in these datasets. 3D stem cells are replete for genes predicted to be involved in molecular patterning and development, including transcription factors, protein kinanses, genes involved in cell fate determination and morphogenesis, and transmembrane efflux-transport (Supplemental Table 2.2). Other GO categories enriched in bud cells include cell wall orientation, and cellular component localization of the preprophase band and phragmoplast. Surprisingly, some GO cat-

egories implicated during tip growth are also enriched in gametophores, such as tip growth, pollen tube growth, and other microtubule-based processes. Traditionally, protonema are acknowledged to be the only tip growing cells of moss, however, our data suggest that gametophores also display some components of tip-like development.

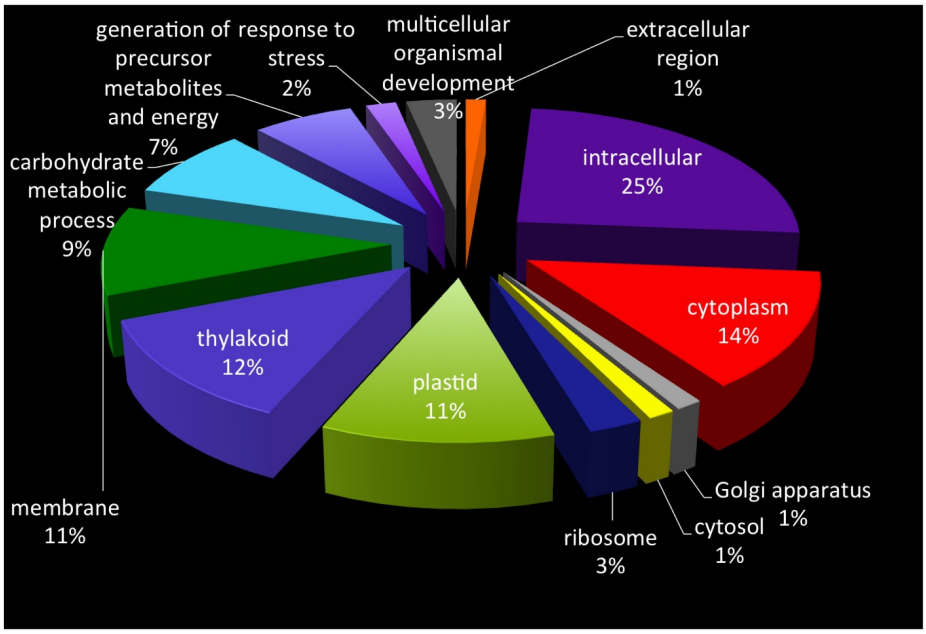
In contrast to the abundance of developmental patterning GO categories identified in the bud transcriptome, the tip transcriptome is predominately enriched for photosynthetic functions (Supplemental Table 2.2). Over 70% of the cellular component GO annotations identified in the tip cell transcriptome are functions targeted to the chloroplast (Figure 2.3A), which likely reflects the fact that the protonemal tip cell produces all the organelles that are inherited by subapical, daughter cells of the protonema. Root hair development GO categories are also enriched in tip cells, which reflects the shared tip-growth strategies found in moss protonema and angiosperm root hairs (Rounds and Bezanilla, 2013). Intriguingly, GO analyses indicate that bud cells utilize a distinct, molecular mechanism of tip growth that is homologous to angiosperm pollen-tube cell division, and unlike the root-hair homologous mechanisms found in protonemal tip cells.

Figure 2.3: **GO Slim enrichment categories functionally separate tip and bud cells**

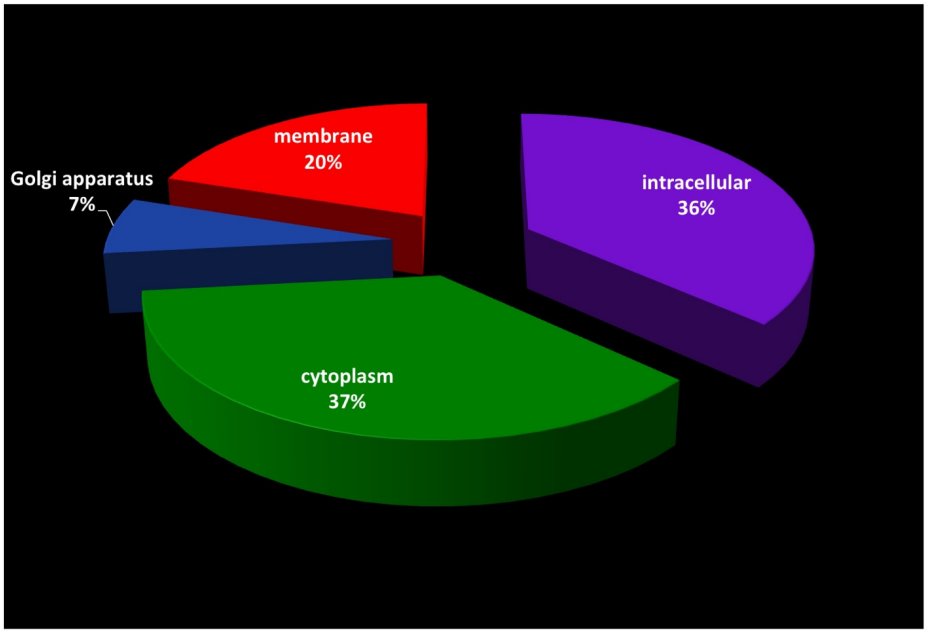
(A) Pie chart representations of Cellular Component GO Slim categories shows strong enrichment for plastid targeted DEGs in tip cells and increased cytoplasm and membrane targeting of bud cells. (B) Pie charts of GO slim molecular function categories for tip and bud cells show that more than 50% of tip cell DEGs have predicted catalytic activities likely related to photosynthetic process, whereas bud DEGs are predominately involved in transporter, hydrolase, and transerase activities.

A

Tip

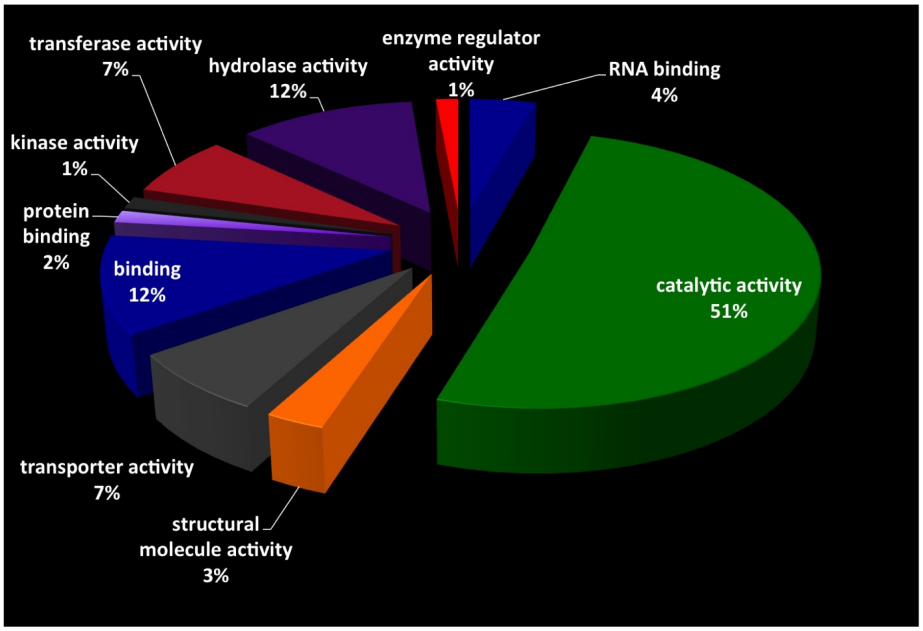

generation of precursor metabolites and energy 7%
response to stress 2%
multicellular organismal development 3%
extracellular region 1%
intracellular 25%
carbohydrate metabolic process 9%
cytoplasm 14%
thylakoid 12%
plastid 11%
membrane 11%
Golgi apparatus 1%
cytosol 1%
ribosome 3%

Bud

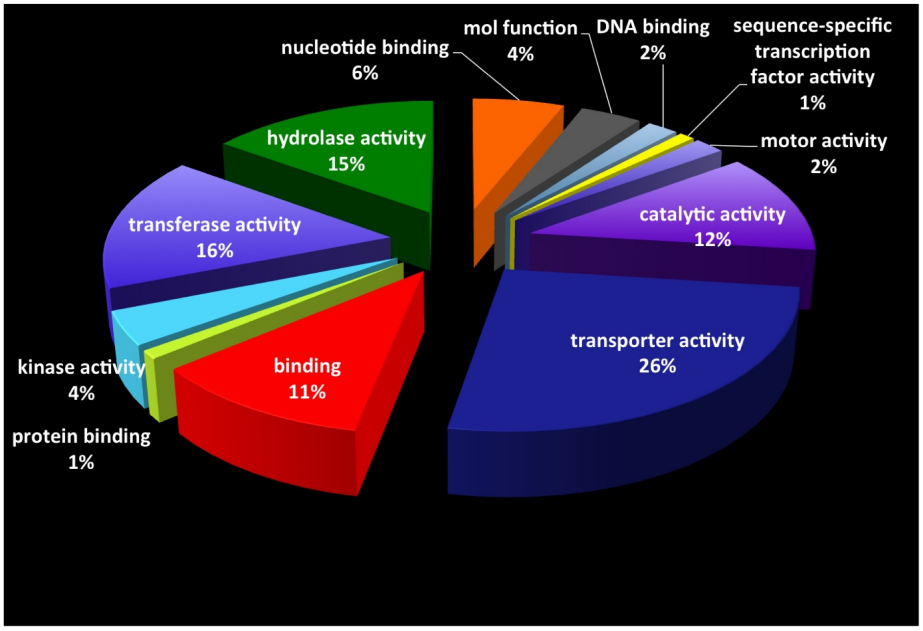

membrane 20%
Golgi apparatus 7%
intracellular 36%
cytoplasm 37%

B

Tip


transferase activity
7%
hydrolase activity
12%
enzyme regulator
activity
1%
RNA binding
4%
kinase activity
1%
protein
binding
2%
binding
12%
catalytic activity
51%
transporter activity
7%
structural
molecule activity
3%

Bud


nucleotide binding
6%
mol function
4%
DNA binding
2%
sequence-specific
transcription
factor activity
1%
motor activity
2%
hydrolase activity
15%
catalytic activity
12%
transferase activity
16%
transporter activity
26%
kinase activity
4%
binding
11%
protein binding
1%

Surprisingly, none of the GO categories enriched in protonema involve transcriptional regulation, hormone responses, or epigenetic modifications. Previous transcriptomic investigations of protonemal cell reprogramming revealed a similar dearth of developmental genes (Busch et al., 2013). As the primordial cell type following the germination of haploid spores (Figure 2.1A) and the initial stage of moss regeneration in response to wounding, the protonemal tip cell may comprise the "default" developmental state for moss and is transcriptionally repressed relative to gametophore bud cells.

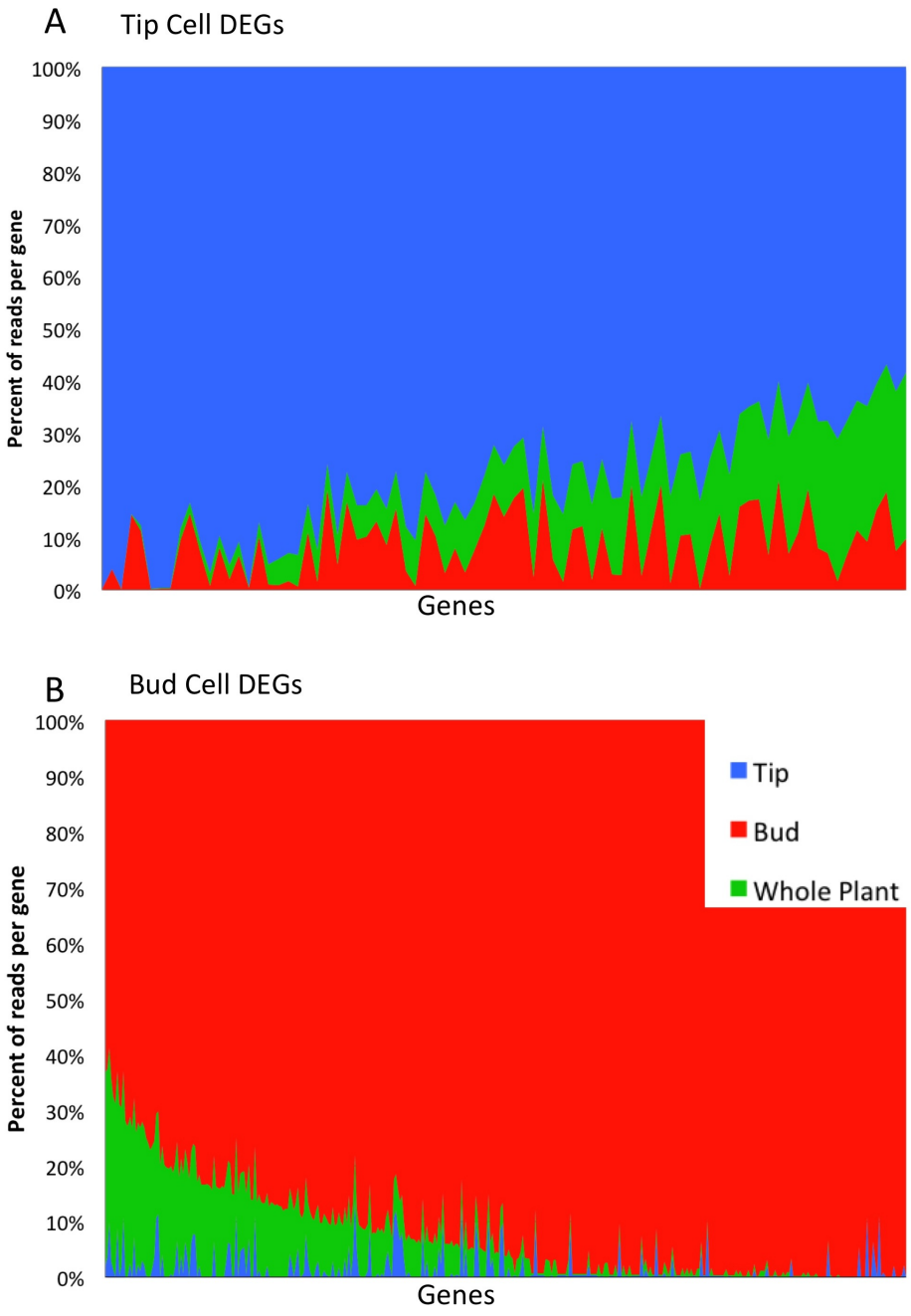
2.3.3 Shoot meristem patterning and asymmetric cell division genes functions are up-regulated in bud cells

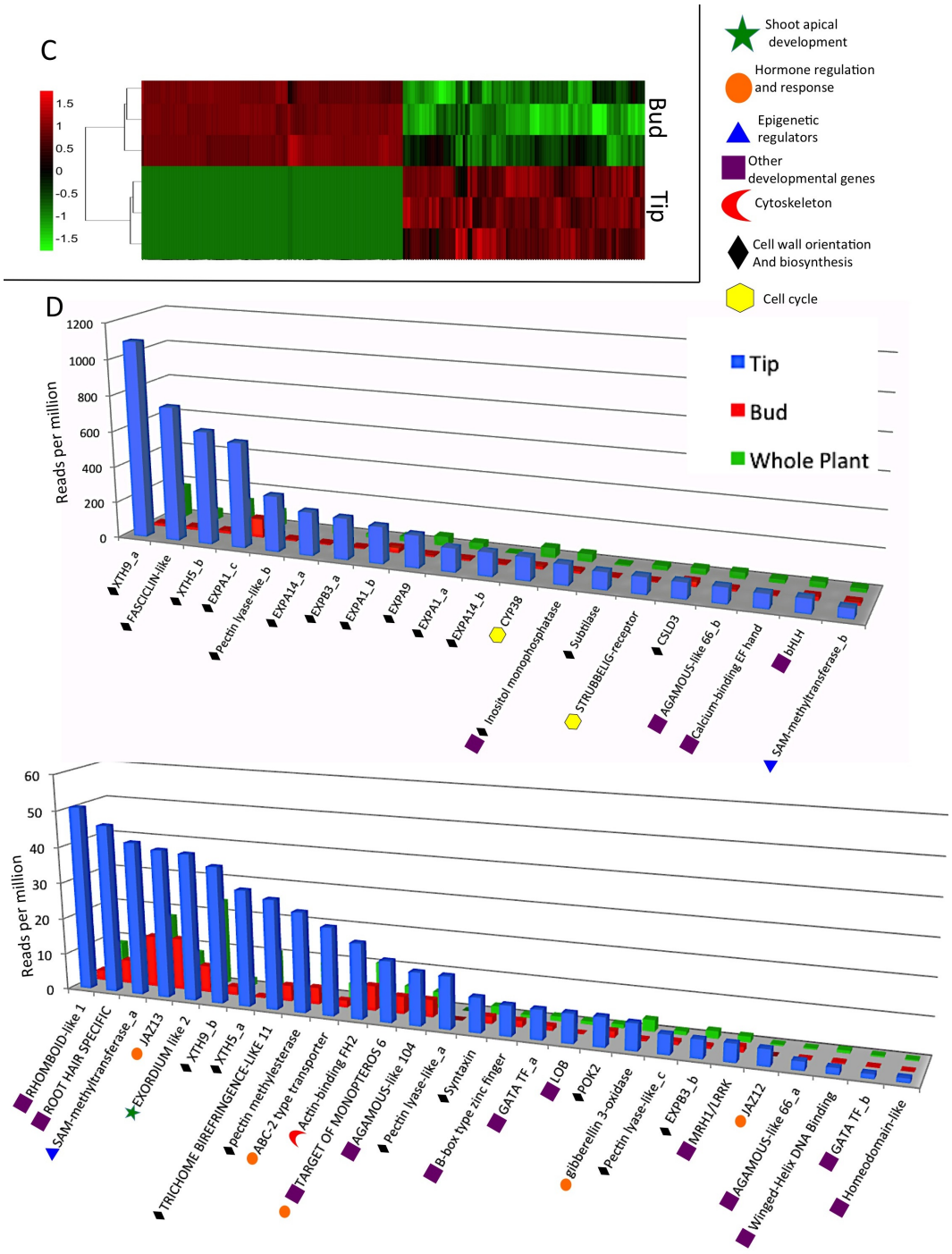
Toward the identification of DE candidate genes that may specify tip and bud cell identity and function, our cell-specific transcriptomes were mined for homologs of angiosperm genes with described developmental functions. Transcripts were selected from seven functional categories: shoot apical development; transcription factors; hormone biology; epigenetic regulation; cytoskeleton dynamics; cell wall biogenesis and orientation; and cell cycle regulation. Pairwise comparisons of specific gene transcripts that are upregulated in tip (75 genes) or bud cell transcriptomes (100) relative to whole moss plants are depicted in Figures 2.5-2.6. Notably, hundreds of DEGs with unknown functions were identified as specifically up-regulated in bud or tip cell transcriptomes (Figure 2.4). This is not surprising, considering that 48% of the genes in the *P. patens* genome cluster independently from all other sequenced plant genes (Zimmer et al., 2013).

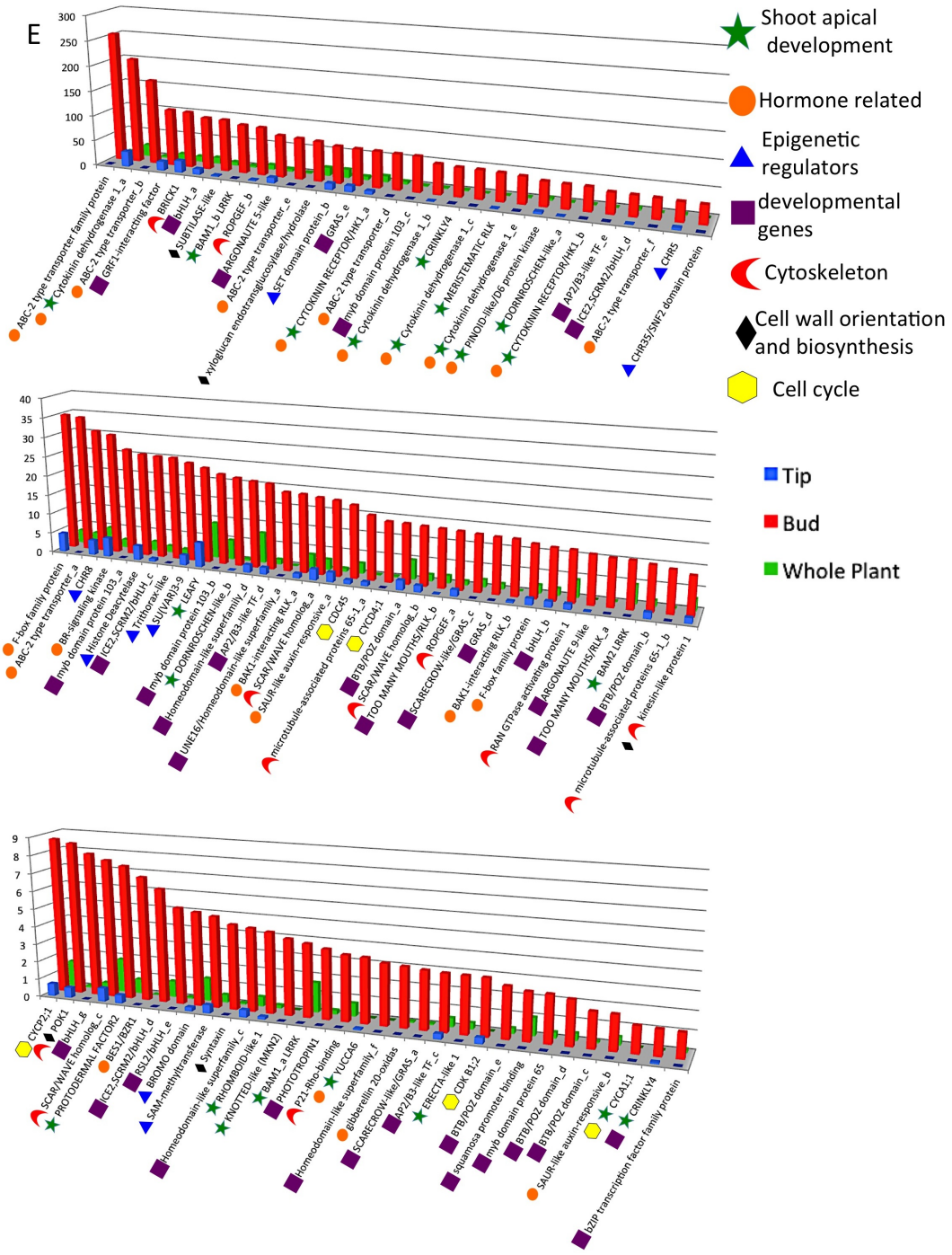
An obvious trend observed in our pairwise comparisons is the up-regulated expression in bud cells of transcripts predicted to direct shoot meristem patterning and asymmetric cell division. Examples include moss homologs of *Arabidopsis* receptor-signaling genes that regulate shoot meristem size and patterning, including: *BARELY ANY MERISTEM 1* (*BAM1*) and *BAM2*; *MERISTEMATIC RECEPTOR LIKE KINASE* (*MRLK*); the *MAPKK-kinase YODA* (*YDA*); *ERECTA-LIKE*, *CRINKLY4* (*CR4*) and the calpain encoding signalling gene *DEFECTIVE KERNEL1* (*DEK1*) (Becraft et al., 2001; Becraft et al., 2002; DeYoung et al., 2006; Hord et al., 2006; Lukowitz et al., 2004; Mandel et al., 2014).

Figure 2.4: **Key developmental and cellular regulators uniquely define uni-dimensionally and multi-dimensionally dividing SCs**

Hundreds of genes with unknown functions are significantly enriched in tip and bud transcriptomes (A-B). Heatmap shows massive up and down regulation of the top 1,000 differentially expressed genes between bud and tip transcriptomes (C). Digital in situs for cell function and development genes that are significantly up-regulated in protonema tio cells relative to bud cells and whole plant transcriptomes (D) or are up-regulated in bud cells relative to tip and whole plant transcriptomes (E). Expression data is shown as the percent of total reads contributed by each sample. Individual genes are plotted along the Y-axis. Red, green, and blue indicate protonema, shoot, and whole plant transcript levels (respectively).


A
Tip Cell DEGs
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percent of reads per gene
Genes
B
Bud Cell DEGs
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percent of reads per gene
Genes
Tip
Bud
Whole Plant


C
1.5
1
0.5
0
-0.5
-1
-1.5
Bud
Tip
Shoot apical development
Hormone regulation and response
Epigenetic regulators
Other developmental genes
Cytoskeleton
Cell wall orientation And biosynthesis
Cell cycle
D
Reads per million
1200
1000
800
600
400
200
0
Tip
Bud
Whole Plant
XTH9_a
FASCICLIN-like
XTH5_b
EXPA1_c
Pectin lyase-like_b
EXPA14_a
EXPB3_a
EXPA1_b
EXPA9
EXPA1_a
EXPA14_b
CYP38
Inositol monophosphatase
Subtilase
STRUBBELIG-receptor
CSLD3
AGAMOUS-like 66_b
Calcium-binding EF hand
bHLH
SAM-methyltransferase_b
Reads per million
60
50
40
30
20
10
0
RHOMBOID-like 1
ROOT HAIR SPECIFIC
SAM-methyltransferase_a
JAZ13
EXORDIUM like 2
XTH9_b
XTH5_a
TRICHOME BIREFRINGENCE-LIKE 11
pectin methylesterase
ABC-2 type transporter
Actin-binding FH2
TARGET OF MONOPTEROS 6
AGAMOUS-like 104
Pectin lyase-like_a
Syntaxin
B-box type zinc finger
GATA TF_a
LOB
POK2
gibberellin 3-oxidase
Pectin lyase-like_c
EXPB3_b
MRH1/LRRK
JAZ12
AGAMOUS-like 66_a
Winged-Helix DNA Binding
GATA TF_b
Homeodomain-like


E
Shoot apical development
Hormone related
Epigenetic regulators
developmental genes
Cytoskeleton
Cell wall orientation and biosynthesis
Cell cycle
Tip
Bud
Whole Plant

A variety of chromatin-remodeling complexes regulate the developmental switch from indeterminate to determinate cell fates within the CZ and PZ of angiosperm shoot meristems (reviewed in Guyomarc'h et al., 2005; Kwon et al., 2005). Multiple transcripts predicted to function in chromatin-remodeling are upregulated in the meristematic bud cells of *Physcomitrella*, but not in protonemal tip cells. These include homologs of *Arabidopsis* SWI/SNF complex components (*SET1*, *BROMO1*, and *Trithorax-like* genes) (*Trx*). In addition, cell cycle homologs that have described interactions with chromatin-remodeling complexes are specifically up-regulated in the bud cell transcriptome, such as the direct SWI/SNF interactor *E2F*, as well as *CYCLINs* (*CDC/CYCs*), and *CYCLIN-DEPENDENT KINASES* (*CDKs*) (Wildwater et al., 2005).

More significantly, bud cells are enriched for specific cell cycle regulators implicated in to control asymmetric cell division, including homologs of *CYCB1;1*, *CDKA1;1*, and *E2F* (Heidstra, 2007). A critical innovation in the evolution of multicellularity, the acquisition of asymmetric cell divisions is essential for the development of differentiated tissues and complex plant architectures (Inagaki and UMEDA, 2011; Maughan and Menges, 2007; Menges et al., 2005). One predicted function that is enabled by expression of genes promoting asymmetric cell division is the formation of lateral organ (phyllid) precursor cells from unequal cell division in the gametophore bud cell (Figure 2.1 F-G). In marked contrast, just two cell cycle regulators are identified as differentially expressed in the tip transcriptomes (the putative LRRK cell cycle regulator *STRUBBELIG* and Rhodanase cell cycle regulator *CYCLOPHILIN 38* (Chevalier et al., 2005; Eyuboglu et al., 2007).

Additional shoot patterning genes specifically upregulated in the bud cell

include 25 genes predicted to control biosynthesis and signalling of the phytohormones auxin, cytokinin and brassinosteroid. Most intriguingly, two moss homologs of the *PIN-FORMED* (*PIN*) polar auxin efflux genes (PAT) are identified in the bud cells. Moreover, a putative homolog for *PINOID*, a protein kinase involved in the polar localization of PIN proteins is also up-regulated in the bud cells (Benjamins et al., 2001). Current paradigms preclude a role for PIN-mediated PAT during moss gametophore development (Fujita et al., 2008; Poli et al., 2003; Viaene et al., 2013). As yet, no PAT mutants have been analyzed in *Physcomitrella*, although time-lapse studies of moss development indicate that organization and determination of gametophytic cell fate is coordinated through local signaling (Harrison et al., 2009). Taken together, these data suggest that a re-examination of PAT function in the haploid generation of moss is warranted.

A plethora of transcription factor networks are described in the control of cell-specific fates and developmental patterning in angiosperms. Strikingly, predicted transcription factor transcripts upregulated in the moss protonemal tip cells included no known homologs to angiosperm patterning genes (Figures 2.5-2.6). In contrast, transcripts upregulated in bud cells but not tip cells include moss homologs of several angiosperm transcription implicated in shoot patterning, such as *DORNROSCHEN* (*DRN*) and *LEAFY* (*LFY*), the epidermal cell-fate genes *PROTODERMAL FACTOR2* (*PDF2*) and *SCREAM2* (*SCRM*), and the *Class I KNOX MOSS-KNOTTED-RELATED HOMEOBOX 2* (*MKN2*) (Abe et al., 2003; Cole et al., 2013; Kanaoka et al., 2008; Vollbrecht et al., 1991; Weigel et al., 1992). Our discovery of up-regulated expression of *MKN2* in the gametophore bud cell was unexpected; previous genetic analyses indicated that *KNOX* gene function is restricted to the sporophyte generation (Sakakibara et al., 2013; Sakakibara et

al., 2008; Singer and Ashton, 2007). In support of our findings however, RT-PCR analyses demonstrated *MKN2* transcript accumulation in moss gametophores and sporophytes, and *MKN2-GUS* fusion reporter lines revealed *KNOX* promoter activity in the gametophytic egg cell, which are mitotic products of the gametophore bud cell (Sakakibara et al., 2008). Taken together, these data predict that future investigations may uncover previously not described, perhaps redundant, *KNOX* gene function in the *Physcomitrella* gametophore.

The concerted up-regulation of homologs for angiosperm shoot patterning genes and regulators of asymmetric cell division inspires a model wherein synergy of these genetic pathways contributes to novel, developmental innovations found in gametophores but not protonema. In this view, emergent properties conferred by these combined developmental functions may enable the unicellular moss bud cell to balance stem cell-maintenance functions conferred by meristem patterning genes, and organogenesis conferred by asymmetric division programs that overlay programs of lateral organ initiation. This model for the innovation of meristematic functions in gametophore bud cells draws analogies to the role of meristemoid mother cells (MMCs) during stomatal development. Asymmetric division of MMCs generates a variety of cell types comprising the stomatal complex, however this pluripotency of MMCs is short-lived (reviewed in Dong and Bergmann, 2010). The earliest paleobotanical evidence for stomata coincides with the appearance of the bryophyte mosses (Ruszala et al., 2011; Vaten and Bergmann, 2012). It is alluring to speculate that the emergence of developmental pathways enabling meristematic function in the moss gametophore may have played a similar role during stomatal evolution.

2.4 MATERIALS AND METHODS

2.4.1 Plant culture

Physcomitrella patens ssp. *Patens* (Hedwig) ecotype Gransden 2004 spores were germinated and grown on .8% agar BCDAT plates overlayed with cellophane disks, as previously described (Nishiyama et al., 2000). For protonema tip cell isolation, sporelings were harvested after ten days and prepared for laser microdissection as described (Scanlon et al., 2009). To isolate gametophore buds, ten-day-old plantlets were transferred to BCD media supplemented with 1 μM naphthalenacetic acid (NAA) for 5 days, then transferred to BCD media supplemented with 1 μM Kinetin for 24 hours (as described in Johri and Desai, 1973). All plants were grown at 25 deg C with 16 hour day length cycles.

2.4.2 Plant harvest and laser microdissection

For protonema tip cell collection, 10-day-old sporelings were harvested from cellophane disks and fixed in ice-cold acetone overnight. Bud cell collection involved similar procedures, except bud-producing 18-day-old sporelings treated with NAA and BAP were harvested. After 24 hours fixation, fixed sporelings were adhered directly onto charged HistoBond slides (VWR, cat number UNIB75251) and left to dry at 37 deg C for six hours. The slides were then directly used for P.A.L.M. laser microdissection following the Zeiss manufacturing specifications. Selected protonema tip cells and tetrad stage gametophore bud cells were microdissected; at least 100,000 sq microns of plant tissue were collected for each cell type. Whole plant samples of 10-day-old sporelings, and

16-day-old NAA-treated/BAP-treated sporelings were prepared by grinding entire shoots in liquid nitrogen. RNA was extracted from the collected tissues using the PicoPure RNA Isolation Kit (Life Technologies) and in vitro amplified using a TargetAmp 2-round aRNA Amplification Kit 2.0 (Epicentre). Three biological replicates were prepared for the tip and bud cell samples; two whole plant replicates were prepared from the 10-day-old and 16-day-old plantlets.

2.4.3 Illumina library construction and sequencing

Each amplified RNA sample was prepared for sequencing following the protocols (Kumar et al., 2012) with a modified procedure for single sample processing. The libraries were ligated to adapters with barcodes 3-nucleotides in length and pooled for 8-plex sequencing on an Illumina HiSeq 2000 at the Cornell Genomic Facility.

2.4.4 Sequence processing and differential gene expression analysis

Barcoded sequences were sorted, and then clipped. Bases with a PHRED quality score <15 were trimmed from the reads using the software package Lucy (Chou and Holmes, 2001; Li and Chou, 2004). Trimmed reads of each species were aligned to their corresponding reference genomes using GSNAP (Wu and Nacu, 2010) and uniquely-mapped reads were filtered. Uniquely mapped reads were defined as reads that aligned to the reference genome with ≤ 2 mismatches per 36 bp, and less than 5 mismatches per 75 bp. Genes that had at least one mapped

read in two different samples were used for differential expression test. The R package QuasiSeq (http://cran.rproject.org/web/packages/QuasiSeq) was used to test the null hypothesis that expression of a given gene is not different between every pair of tissues. The generalized linear model Quasi-likelihood spline method assuming negative binomial distribution of read counts was used to test the null hypothesis. The 75% quantile of reads from each sample was used as the normalization factor (Bullard et al., 2010). P-values of all the statistical tests were converted to adjusted p-values (q-values) (Nettleton et al., 2006). A false discovery rate (FDR) of 5% (q-value) was used to account for multiple testing.

Venn diagrams were constructed using the R package VennDiagram (http://cran.r-project.org/web /packages/VennDiagram/VennDiagram.pdf). Heatmaps were produced using the R package pheatmap (http://cran.r-project.org/web/ packages/pheatmap/index.html). Values were scaled by row and rows were clustered using the default k-means clustering parameters provided in the software. The R package colorRamp (http://stat.ethz.ch/R-manual/R -devel/library/grDevices/html/colorRamp.html) was used to produce a gradient of color values corresponding to gene fold change values.

2.4.5 GO Enrichment tests

Gene Ontology (GO) enrichment tests were performed using the R package TopGO (http://www.bioconductor.org/packages/2.13/bioc/html/topGO.html). The background gene set consisted of genes with ≥ 1 mapped read per million across all samples. Significantly upregulated transcripts with a q-value of

≤ .05 were tested for enriched GO terms. *Physcomitrella* gene ID to GO mappings from Zimmer et al. (2013) were used to join genes to GO categories. The Fisher T-test was used to test for significant enrichment of GO categories in protonema vs gametophore bud transcriptomes and differentially expressed bud and protonema versus whole plant transcriptomes. GO categories with a P-value ≤ .05 were considered significant. GO slim categories were identified from the enriched GO categories using the AgBase GoSlimViewer tool (http://www.agbase.msstate.edu/cgi-bin/tools/goslimviewer.pl)

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CHAPTER 3

TRANSCRIPTOMIC EVIDENCE FOR THE EVOLUTION OF SHOOT MERISTEM FUNCTION IN SPOROPHYTE-DOMINANT LAND PLANTS VIA CONCERTED SELECTION OF ANCESTRAL GAMETOPHYTIC AND SPOROPHYTIC GENETIC PROGRAMS

3.1 ABSTRACT

Alternation of generations, in which the haploid and diploid stages of the life cycle are each represented by multicellular forms that differ in their morphology, is a defining feature of the land plants (embryophytes). Anciently derived lineages of embryophytes grow predominately in the haploid gametophytic generation from shoot apical meristems (SAMs) that give rise to photosynthetic structures with either leaf-like organs or a ribbon-like thallus. More recently evolved plant lineages have multicellular SAMs, and photosynthetic shoot development is restricted to the sporophyte generation. The molecular genetic basis for this evolutionary shift from gametophyte dominant to sporophyte dominant life cycles remains a major question in the study of land plant evolution. We used laser microdissection and next generation RNA sequencing to address whether angiosperm meristem patterning genes expressed in the sporophytic SAM of *Zea mays* are expressed in the gametophytic SAMs, or in the non-meristematic sporophytes, of the model bryophytes *Marchantia polymorpha* and *Physcomitrella patens*. A wealth of up-regulated genes involved in stem cell maintenance and organogenesis are identified in the maize SAM and in both the gametophytic meristem and sporophyte of moss, but not in *Marchantia*. Significantly, meiosis-specific genetic programs are expressed in bryophyte

sporophytes, long before the onset of sporogenesis. Our data suggest that this upregulated accumulation of meiotic gene transcripts suppresses indeterminate cell fate in the *Physcomitrella* sporophyte, and overrides the observed accumulation of SAM patterning genes. A model for sporophytic meristem evolution via the concerted selection of ancestral meristem gene programs from gametophyte-dominant lineages is discussed.

3.2 INTRODUCTION

The alternation of generations is a fundamental feature of land plants (embryophytes), and is defined as a life cycle whereby organisms produce morphologically dissimilar offspring that in turn give rise to progeny resembling the parents (Haig, 2008). All embryophytes display this diplobiontic form of alternation of generations, in which the haploid and diploid stages of the life cycle are each represented by multicellular forms. In this strategy the haploid generation initiates as meiotic spores that divide mitotically to form the multicellular gametophyte, which ultimately gives rise to gametes (Figure 3.1 A-D). Fusion of the haploid gametes during fertilization initiates the diploid stage, which undergoes mitosis to form the multicellular sporophyte (Figure 3.1 E). Meiosis within specialized germinal cells of the sporophyte will regenerate the haploid spores to complete the diplobiontic life cycle (Figure 3.1 F).

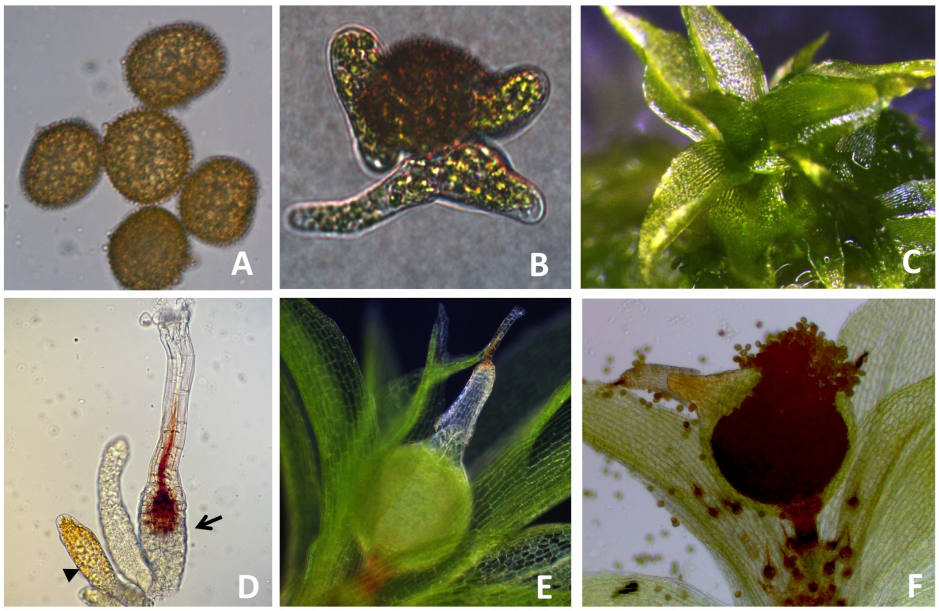


Figure 3.1: **Developmental stages in the gametophyte-dominant life cycle of the bryophyte *Physcomitrella patens***

Haploid spores (A) germinate and grow as filamentous, photosynthetic protonema (B). Three dimensional gametophore growth is initiated from protonemal filaments (C), and archegonia (arrow) and antheridia (arrow head) reproductive structures are formed at the apex of the gametophore (D). Fertilization of the archegonial egg cell by sperm initiates the sporophyte generation (E). Meiosis within the sporophyte capsule produces haploid spores that are released to start a new cycle (F).

Embryophytes evolved approximately 500 million years ago from haploid dominant Charophycean green alga (Graham and Wilcox, 2000; Haig, 2008; Karol et al., 2001; Steenstrup, 1845). In the Charophyta, multicellular growth is restricted to the gametophytic generation; the unicellular sporophyte (zygote) in these species undergo meiosis following fertilization without any intervening mitotic cell divisions (Graham and Wilcox, 2000; Niklas, 1997; Niklas

and Kutschera, 2009). While the first embryophytes are long extinct, insight into their developmental morphology may be gleaned from analyses of extant members of anciently derived embryophyte lineages. The bryophytes, comprising the liverworts, mosses, and hornworts, are the oldest, extant land plant lineages (Gensel, 2008; Wellman, 2003; Wellman and Gray, 2000). Despite their extreme morphological diversity, all bryophytes share a gametophyte-dominant life cycle, and lack apical meristematic growth during the sporophyte generation (Shaw and Goffinet, 2000). Whereas photosynthetic organs such as phyllids or thalli are produced by the indeterminate gametophytes of moss and liverworts respectively, bryophytic sporophytes are determinate structures that do not produce lateral organs (Goffinet and Buck, 2012; Haig, 2008; Ligrone et al., 2012a; Ligrone et al., 2012b).

The rise of sporophyte dominance and the reciprocal reduction of the gametophyte generation in later plant lineages is a defining trend in embryophyte evolution. The evolution of a multicellular sporophyte generation preceded the appearance of the oldest known embryophytes identified in the fossil record (Kenrick and Crane, 1997), and the origin of the embryophyte life cycle remains a topic of spirited debate (Bower, 1890; Haig, 2008; Niklas and Kutschera, 2010). This innovation of the multicellular sporophyte within the embryophytes involved: (1) a delay in the onset of meiosis following formation of the zygote; (2) retention of the zygote in maternal tissues; (3) nutrient transfer from the gametophyte to the zygote, and (4) the interpolation of mitotic cell divisions into the diploid sporophyte (Graham and Wilcox, 2000; Niklas, 1997; Niklas and Kutschera, 2010). The molecular genetic basis for the shift from gametophytic to sporophytic dominance is unclear, however logic suggests that this new developmental paradigm required the innovation of indeterminate, meris-

tematic growth during the sporophyte generation (Ligrone et al., 2012b). Thus, deciphering the origins and molecular fingerprints of meristematic genetic programs in the plant sporophyte is a major task in the study of land plant evolution.

Recent transcriptomic analyses of two model moss sporophytes (*Funaria hygrometrica* and *Physcomitrella patens*) demonstrated that many key genes involved in angiosperm sporophyte development are also expressed in the moss sporophyte, suggesting that the genetic programs regulating the complex morphological development of angiosperms may have ancient origins in the simple body plans of determinate, bryophyte sporophytes (O'Donoghue et al., 2013; Szovenyi et al., 2011). Further support for this model comes from analyses of *Class I KNOTTED 1-like HOMEOBOX* ((*KNOX*) genes, well-described markers of indeterminate, shoot-meristematic cell-fate (Vollbrecht et al., 1991). Genetic studies in *Physcomitrella patens* have reported that *KNOX* gene function is restricted to the sporophyte (Sakakibara et al., 2008; Singer and Ashton, 2007). In contrast to this sporophyte-centric view of SAM evolution, moss homologs of the *AINTEGUMENTA/PLETHORA/BABY BOOM* (*APB*) family of angiosperm stem cell-niche regulators are shown to function in the moss gametophytic meristem (Aoyama et al., 2012).

As yet, transcriptomic comparisons between bryophyte gametophyte and vascular plant sporophyte generations have relied on whole-plant comparisons, which lack the fine resolution required to interrogate meristem-specific patterns of gene expression. In addition, comprehensive transcriptional analyses of bryophyte sporophytes are heretofore restricted to the moss lineage, excluding the more ancient embryophytic sporophytes. In this study, laser-

microdissection transcriptomics (LM-RNAseq) was utilized to compare the transcriptional profiles of the sporophytic SAM of the angiosperm *Zea mays* to those of the gametophyte SAMs and non-meristematic sporophytes of the bryophytes *Physcomitrella patens* and Marcantia polymorpha. These transcriptomic profiles were used to address three fundamental questions regarding the evolution of the sporophyte meristem:

(1) What is the molecular basis for the shift from a gametophyte-dominant to a sporophyte-dominant life cycle?

(2) What are the shared molecular genetic networks that describe a functional SAM in both the gametophytic generation of model bryophytes and in the sporophytic generation of a model vascular plant?

(3) What are the unique developmental genetic pathways that have evolved separately in ancient gametophyte-dominant lineages and in a more recent sporophyte-dominant plant?

Our transcriptomic data identified expression of SAM genetic programs in both the gametophytic SAM and the non-meristematic sporophyte in moss, but not in *Marchantia*. The data lead to a model for the evolution of sporophyte-dominant SAM function in vascular plant lineages via concerted selection of meristematic gene programs from both the gametophyte and sporophyte stages in an ancestral bryophyte. Furthermore, our data suggests that SAM developmental programs identified in angiosperms evolved *after* the appearance of the liverworts. In addition, transcripts predicted to promote meiotic function were identified in bryophyte sporophytes, which were harvested long before the onset of sporogenesis. Taken together with the absence of these meiotic transcripts

in the sporophytic maize SAM, our data suggest a model for the early termination of meristematic function in the moss sporophyte via the early onset of meiotic programs.

3.3 RESULTS AND DISCUSSION

3.3.1 Laser microdissection and RNA-sequencing (LM-RNAseq) of meristems and bryophyte sporophytes enables the construction of cell-type molecular signatures

A long-standing question in embryophyte evolution has asked whether gene programs essential for directing indeterminate functions in the sporophytic SAM arose through the neo functionalization of sporophyte-specific transcripts, or via recruitment of meristem programs from the gametophytic generation? (Langdale, 2008; Niklas and Kutschera, 2009; Niklas and Kutschera, 2010; O'Donoghue et al., 2013; Sakakibara et al., 2008; Szovenyi et al., 2011). To test these hypotheses, we performed cell-enriched transcriptomics in the gametophyte-dominant model bryophytes *Physcomitrella patens* (a moss) and *Marchantia polymorpha* (a liverwort), and in the sporophyte-dominant angiosperm *Zea mays* (maize). Specifically, Illumina-based RNA-seq profiles were generated for laser microdissected gametophytic SAMs and young non-meristematic sporophytes from *Physcomitrella* and *Marchantia* (Figure 3.2 A-J). These were compared with transcriptomes isolated from the sporophytic SAM of maize (Figure 3.2 K-M). Each of these cell-enriched samples was compared

with whole plant transcriptomes, to identify up-regulated and down-regulated genes with significant differential expression (false discovery rate FDR $>.05$). Thousands of significantly differentially expressed genes (DEGs) were identified for the SAM-enriched and sporophyte-enriched samples. These robust differential gene expression patterns ultimately enabled the construction of complex molecular signatures for each cell type (Figure 3.3).

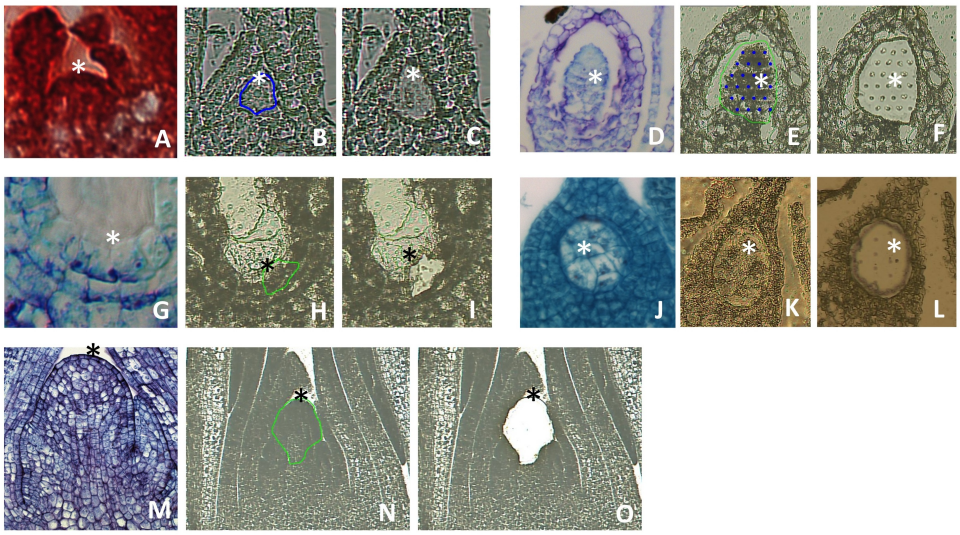
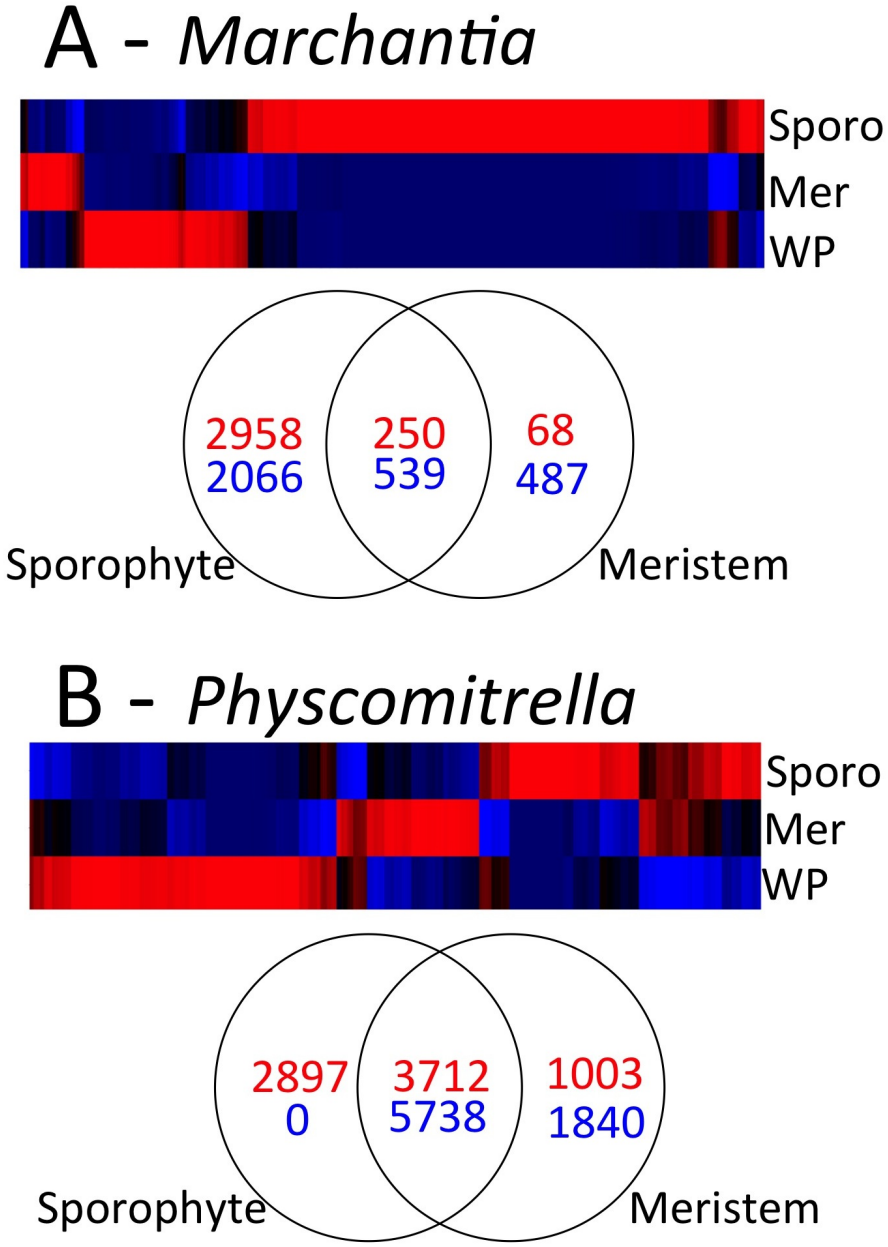


Figure 3.2: **Laser microdissection of SAMs and bryophyte sporophytes**

Gametophytic SAMs and sporophytic embryos were isolated from the haploid-dominant moss *Physcomitrella* (A-C, D-F) and liverwort *Marchantia polymorpha* (G-I, J-L) and compared with cells from the sporophytic maize SAM (M-O). Isolated structures are marked with an asterisk.

Figure 3.3: **Unique molecular signatures define the meristem and sporophyte cells from *Marchantia* and *Physcomitrella***

Comparisons between the LM cell-enriched and whole plant transcriptomes in *Marchantia* (A) and *Physcomitrella* (B) enabled the identification of thousands of DEGs that define the meristem and sporophyte cell types. Up-regulated DEGs are indicated in red and down-regulated DEGs in blue.


A - Marchantia
Sporo
Mer
WP
2958
2066
250
539
68
487
Sporophyte
Meristem
B - Physcomitrella
Sporo
Mer
WP
2897
0
3712
5738
1003
1840
Sporophyte
Meristem

3.3.2 Bryophyte sporophytes are transcriptionally similar to the maize angiosperm SAM

Homologous gene family clusters were constructed to test for shared differential gene expression patterns across *Marchantia*, *Physcomitrella*, and maize. A total of 8,156 homologous gene families were constructed to test for differential gene expression patterns across *Marchantia*,*Physcomitrella*, and maize. A comparison of the sporophytic maize SAM and the gametophytic bryophyte SAMs demonstrates that less than 3% (248) of the gene families share differential expression patterns across the three meristems (Figure 3.4 A). . This set of shared SAM gene families includes epigenetic regulators such as the Polycomb Group Complex 2 (PRC2) gene *CURLY LEAF* (*CLF*), *CHROMATIN REMODELING 4*, and the *HISTONE ACETYL TRANSFERASEs* (*HATs*). Conspicuously absent are key gene families involved in meristem establishment and maintenance, such as the *Class I KNOTTED 1-like HOMEOBOX* (*KNOX*) genes, the *CLASS III HOMEODOMAIN LEUCINE-ZIPEERs* (*HD-ZIP IIIs*), and the *CLAVATA-WUSCHEL* signaling pathway members. (Emery et al., 2003; Hay and Tsiantis, 2010; Schoof et al., 2000; Vollbrecht et al., 1991).

In contrast to the cross-species SAM comparisons, more than 8% (720) of the gene families share differential expression patterns across the sporophyte samples (Figure 3.4 B). Our data are in agreement with previously-reported generation-biased transcriptome patterns found between the moss sporophyte and angiosperm SAM transcriptomes (O'Donoghue et al., 2013; Szovenyi et al., 2011). Importantly, the *Class I KNOX* gene family, a marker of indeterminate cell populations in angiosperm shoot meristems (Vollbrecht et al., 1991), is up-regulated in moss and liverwort sporophytes, and in the maize sporophytic

SAM as compared to whole plants. The presence of *KNOX* genes in these sporophyte cross-species comparison but not in *both* the moss and liverwort gametophyte SAMs is compatible with previous studies demonstrating that KNOX genes are involved in specifying sporophyte-specific development in diverse green plant lineages (Lee et al., 2008; Sakakibara et al., 2008; Sano et al., 2005; Singer and Ashton, 2007). In moss sporophytes *Class I KNOX* expression is associated with transient meristematic activity within the sporophytic apical cell and intercalary meristem, suggesting that these transcripts are indeed marking *short-lived* indeterminate functions (Sakakibara et al., 2008). Insights into the function of *Class I KNOX* genes in the *Marchantia* sporophyte, which exhibits *no* localized meristematic activity, may uncover the ancestral role of this gene family in this most ancient, extant land plant lineage.

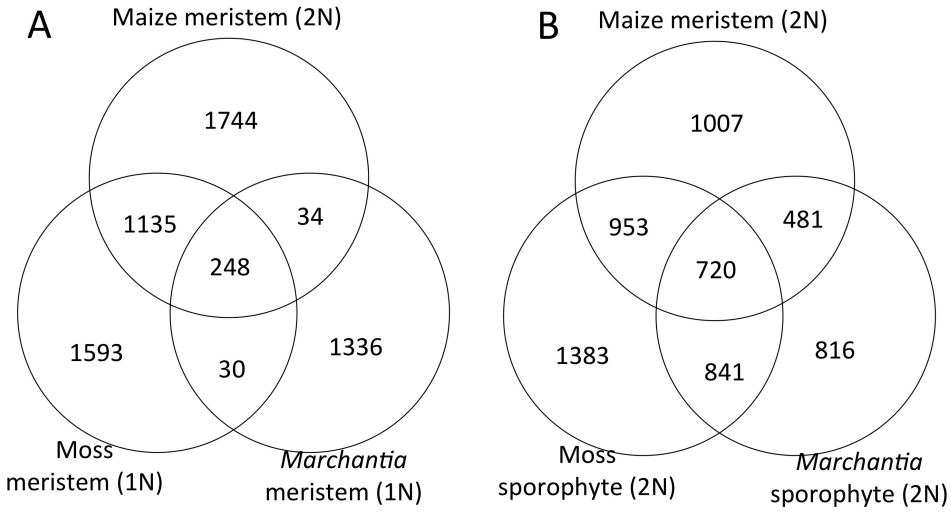


Figure 3.4: **Non-meristematic sporophytes are transcriptionally closer to the sporophytic maize meristem than are their gametophytic meristem counterparts**

Cross species comparisons of DE gene families in the gametophytic SAMs of *Marchantia* and *Physcomitrella* and the sporophytic SAM of maize identifies 248 shared gene families (A). In contrast, 720 gene families share DE patterns across the gametophytic bryophyte SAMs and the sporophytic maize SAM.

Additional DE gene families found specifically in the sporophyte cross-species comparison include the *ARGONAUTE* (*AGO*) gene family and several predicted chromatin-remodeling genes, in agreement with previous reports that small RNA biogenesis and the regulation of epigenetic patterning are ancient developmental features of sporophytes from basally-derived lineages (Axtell and Bartel, 2005; Axtell et al., 2007). Conspicuously absent from these cross-species sporophytic comparisons are members of phytohormone-related gene families, receptor-signaling gene families, and additional transcription factor families known to be crucial to angiosperm meristem function (Brooks et al., 2009; Canales et al., 2005; Dodsworth, 2009; Golz, 2006; Wolters and Juergens, 2009; Yadav et al., 2009)

Principle component analyses (PCAs) were performed to sort the cell-enriched and whole plant transcriptomes from *Physcomitrella* and *Marchantia*, based upon their statistical variance (Figure 3.5) (Ringner, 2008). In PC1 and PC2 the *Marchantia* biological replicates clustered into distinctly separate groups (Figure 3.5 A), whereas the *Physcomitrella* sporophyte and meristem replicates clustered relatively close to one another and distant from whole plant samples (Figure 3.5 B). Moreover, over 60% of the shared moss DEGs also show similar patterns of up-regulation and down-regulation relative to the whole plant (Figure 3.4 D). Unlike moss however, comparable cross-correlations are not identified between the sporophyte and the gametophytic SAM transcriptomes of *Marchantia*.

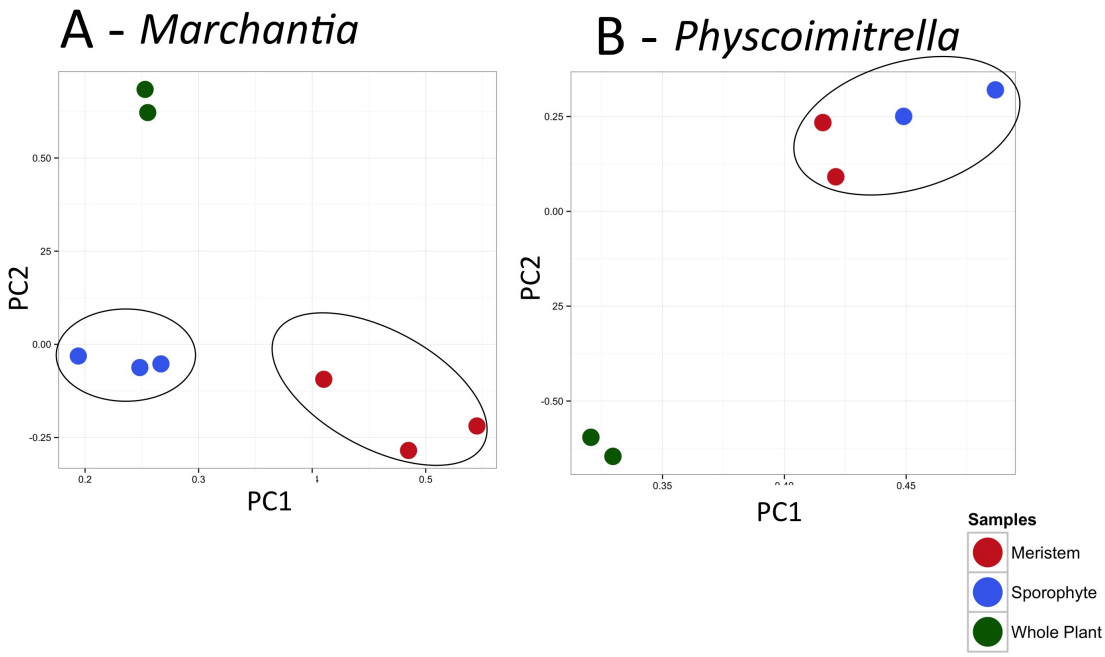


Figure 3.5: **The gametophytic meristem and sporophyte transcriptomes show pleiotropic expression patterns in *Physcomitrella* but not in *Marchantia***

Principle Component Analyses with entire transcripomes for bryophyte meristem (red), sporophyte (blue), and whole plant (green) samples in *Marchantia* (A) and *Physcomitrella* (B) separate individual biological replicates by their statistical variance. *Marchantia* transcriptomes segregate into separate cell-type groupings on the PC1 and PC2 axes (A), whereas sporophyte and meristem transcriptomes from *Physcomitrella* co-segregegate (B).

3.3.3 Homologs of angiosperm SAM patterning transcripts identified in moss sporophytes and gametophytes

Systematic searches of the DEGs within both the determinate sporophyte and indeterminate gametophyte SAM of moss uncovered a plenitude of transcripts with homology to known angiosperm shoot patterning genes, such as regulators of transcription, signaling, epigenetic patterning, phytohormone biosynthesis and response, and cell-cycle control genes (Figure 3.6; Supplemental Table 3.1). Notable DEGs included moss homologs of genes implicated in SAM maintenance and function, such as a the *Class I KNOX* gene *MKN2*, a *WUSCHEL-like HOMEOBOX* (*WOX*) transcription factor, homologs of *DORNROSCHEN-like* (*DRN-like*) and *TERMINAL EAR-like 1* (*TEL1*), as well as *LATERAL SUPPRESSOR* (*LAS*) (Kirch et al., 2003; Laux et al., 1996; Veit et al., 1998; Vollbrecht et al., 1991)

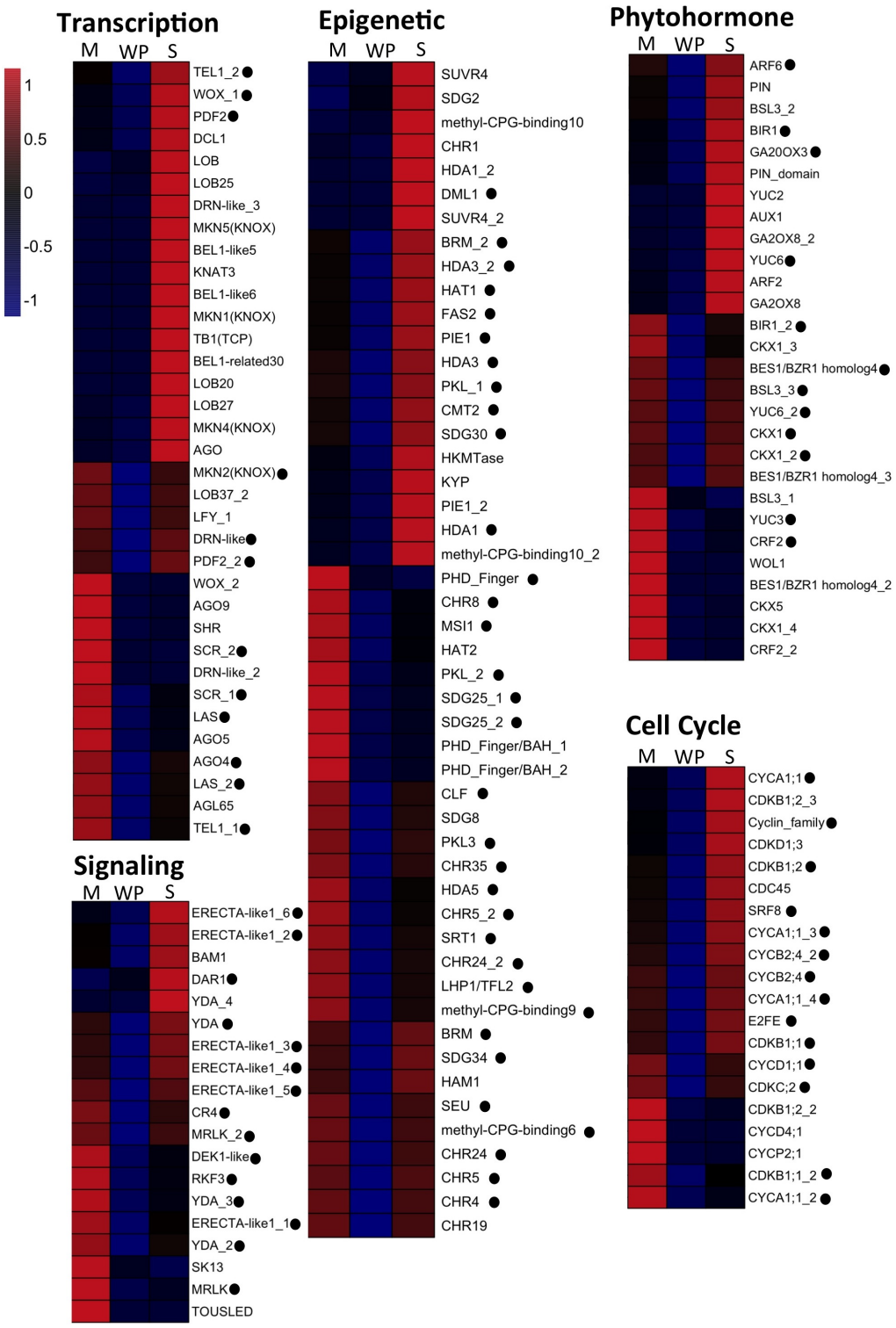
Conspicuously up-regulated in the moss sporophyte are several additional *KNOX* gene family members and homologs of the related family of *BELLRINGER1-like HOMEOBOX* (*BEL1*) genes; KNOX and BEL-like proteins heterodimerize and thereby down-regulate meristem-maintenance functions of KNOX transcription factors in angiosperms (Bellaoui et al., 2001; Bhatt et al., 2004; Cole et al., 2006; Smith et al., 2002). The combined up-regulation of *KNOX* and *BEL1-like* transcripts in the moss sporophyte suggests a possible model for acquisition of determinate cell fate in the moss sporophyte, wherein KNOX-BEL heterodimerization contributes to repression of KNOX-induced pluripotency. Genetic knockouts of *BEL1-like* function will test the efficacy of this model. In keeping with this theme, several moss homologs of the *KNOX*-repressive *LATERAL ORGAN BOUNDARY* (*LOB*) genes and a homolog of the dosage-

dependent, growth-repressive angiosperm gene *Teosinte Branched1* (*TB1*) are also upregulated in the moss sporophyte and may likewise contribute to suppression of indeterminacy (Doebley et al., 1995; Shuai et al., 2002).

Pluripotent cell activity is established and maintained through the restructuring of chromatin states (Shen and Xu, 2009; Ho and Crabtree, 2010). In the angiosperm SAM, chromatin remodelers coordinate the balance between indeterminacy in the CZ and lateral organ initiation in the PZ (reviewed in Shen and Xu, 2009). For example, *KNOX* gene expression is restricted to the CZ by members of the PRC1 and PRC2 ((Barrero et al., 2007)), while *WUS* expression is restricted to the center of the SAM by *FASCIATA1/FASCIATA2* (*FAS1/FAS2*) (Kaya et al., 2001; Shen and Xu, 2009). In moss, PRC2 genes were shown to maintain developmental programs for gametophytic growth by repressing sporophyte differentiation (Mosquna et al., 2009; Okano et al., 2009). We uncovered 79 up-regulated DEGs encoding epigenetic regulators in our laser-microdissected moss transcriptomic samples, with over half of these genes showing up-regulated expression patterns in both the moss sporophyte and gametophyte samples (Figure 3.6; Supplemental Table 3.1). Most notably, homologs for the PRC1 gene *TERMINAL FLOWER 2* (*TFL2*), the PRC2 genes *CLF* and *MULTICOPY SUPPRESSOR OF IRA1* (*MSI1*), as well as *FASCIATA2* (*FAS2*) are all up-regulated in the moss meristem and sporophyte. Furthermore, three paralogs of *PICKLE*, a SWI/SNF-type chromatin remodeler known to mediate lateral organ initiation, are also up-regulated in the meristem and sporophyte samples (Ori et al., 2000). These findings suggest that in the bryophytic ancestor(s) of sporophytedominant plants, ancient homologs of extant angiosperm SAM-chromatin remodelers functioned pleiotropically in both the indeterminate gametophyte *and* in the indeterminate sporophyte.

Figure 3.6: **Homologs of angiosperm SAM patterning transcripts are identified in moss sporophytes and gametophytes**

Angiosperm SAM patterning genes that control transcription, signaling, epigenetic patterning, phytohormone biology, and cell cycle were identified as up-regulated in the *Physcomitrella* meristem and sporophyte samples relative to the whole plant. Genes that are significantly up-regulated in both meristem and sporophyte samples are indicated with a black circle. M = SAM, WP = Whole Plant, and S = Sporophyte. Up-regulated expression is shown in red and down-regulated expression in blue (as indicated in the color key).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MKN5(KNOX)
BEL1-like5
KNAT3
BEL1-like6
MKN1(KNOX)
TB1(TCP)
BEL1-related30
LOB20
LOB27
MKN4(KNOX)
AGO
MKN2(KNOX)
LOB37_2
LFY_1
DRN-like
PDF2_2
WOX_2
AGO9
SHR
SCR_2
DRN-like_2
SCR_1
LAS
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AGO4
LAS_2
AGL65
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SDG2
methyl-CPG-binding10
CHR1
HDA1_2
DML1
SUVR4_2
BRM_2
HDA3_2
HAT1
FAS2
PIE1
HDA3
PKL_1
CMT2
SDG30
HKMTase
KYP
PIE1_2
HDA1
methyl-CPG-binding10_2
PHD_Finger
CHR8
MSI1
HAT2
PKL_2
SDG25_1
SDG25_2
PHD_Finger/BAH_1
PHD_Finger/BAH_2
CLF
SDG8
PKL3
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SDG34
HAM1
SEU
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CHR19
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YUC2
AUX1
GA2OX8_2
YUC6
ARF2
GA2OX8
BIR1_2
CKX1_3
BES1/BZR1 homolog4
BSL3_3
YUC6_2
CKX1
CKX1_2
BES1/BZR1 homolog4_3
BSL3_1
YUC3
CRF2
WOL1
BES1/BZR1 homolog4_2
CKX5
CKX1_4
CRF2_2
Cell Cycle
M WP S
CYCA1;1
CDKB1;2_3
Cyclin_family
CDKD1;3
CDKB1;2
CDC45
SRF8
CYCA1;1_3
CYCB2;4_2
CYCB2;4
CYCA1;1_4
E2FE
CDKB1;1
CYCD1;1
CDKC;2
CDKB1;2_2
CYCD4;1
CYCP2;1
CDKB1;1_2
CYCA1;1_2
Signaling
M WP S
ERECTA-like1_6
ERECTA-like1_2
BAM1
DAR1
YDA_4
YDA
ERECTA-like1_3
ERECTA-like1_4
ERECTA-like1_5
CR4
MRLK_2
DEK1-like
RKF3
YDA_3
ERECTA-like1_1
YDA_2
SK13
MRLK
TOUSLED

Ultimately, all of the developmental programs discussed above are coordinated through the activities of phytohormone regulatory networks. Twenty-eight DEGs with predicted phytohormone function are identified in our laser microdissected moss samples. Both meristem and sporophyte transcriptomes showed up-regulated expression of *CYTOKININ OXIDASEs* (*CKXs*), *CYTOKININ RESPONSE FACTORs* (*CRFs*), auxin biosynthesis genes (*YUCCAs*), and *AUXIN RESPONSE FACTORs* (*ARFs*) (El-Showk et al., 2013; Zhao, 2010). This data suggests that a dynamic balance of cytokinin and auxin mediated signaling, similar to what is found in angiosperm SAMs, coordinates the development of both indeterminate meristem and determinate sporophyte structures in moss (Su et al., 2011). Notably, a homolog of the cytokinin receptor *WOODENLEG1* (*WOL1*) is only up-regulated in the gametophytic meristem (Inoue et al., 2001). Cytokinins mediate pluripotent cell functions in both angiosperms and moss (Kurakawa et al., 2007; Schumaker and Dietrich, 1998; Su et al., 2011), and gametophyte-specific expression *WOL1* may contribute to indeterminate cell-fate acquisition in the moss SAM. Furthermore, the significant up-regulated expression of genes involved in polar auxin transport (PAT) in the moss sporophyte but not the gametophyte, including homologs of *PINNOID* (*PIN*) and the auxin influx carrier *AUXIN RESISTANT 1* (*AUX1*), is in keeping with previous findings that auxin-mediated cell differentiation predominately functions in the determinate sporophyte (Fujita et al., 2008; Poli et al., 2003).

In this study, we generally assumed that homologous gene sequences encode broadly similar functions in bryophytes and angiosperms. Although we expect that exceptions to these assumptions undoubtedly exist, a number of moss developmental genetic analyses support our presumptions. For example, the calpain protease encoded by the moss *DEK1* complements the *Arabidopsis*

dek1 mutant, and both maize and moss *dek1* mutants are shootless gametophytes (R. Quatrano, personal communication of submitted data). Notable exceptions include moss *LFY* homologs, which have evolved a divergent function in moss, and fail to complement Arabidopsis *lfy* mutants (Maizel et al., 2005; Sayou et al., 2014). This study provides a data enriched foundation for future functional analyses of moss development.

3.3.4 *Marchantia* meristem and sporophyte transcriptomes contain a lack of known developmental regulators

The majority of DEGs up-regulated in the moss sporophyte are not DE in the liverwort counterpart; however, exciting exceptions exist. For example: the chromatin remodelers *CLF* and *FAS2*; a *HD-ZIP III* transcription factor; and a *Class I KNOX* gene are all up-regulated in the *Marchantia* sporophyte transcriptome (Table 3.2). Class *I KNOX* expression in the *Marchantia* sporophyte suggests that ancestral *KNOX* gene function may not involve shoot meristem maintenance, since *Marchantia* sporophytes lack localized regions of meristematic growth (Coulter, 1914; Goffinet and Buck, 2012; Leitgeb, 1877). Understanding the developmental evolution of *Class I KNOX* function during sporophytic, shoot meristem specification will be a key piece of the puzzle to understand the rise of sporophyte dominance in the embryophytes. One possible explanation for the paucity of developmental programs identified in the *Marchantia* sporophyte transcriptomes may be its relatively simple ontogeny. Sporophyte development in liverwort involves uniform cell divisions, whereas in mosses transient meristems function to produce morphologically more elaborate sporo-

phytic structures (Coulter, 1914; Goffinet and Buck, 2012; Leitgeb, 1877). Our *Marchantia* transcriptomic data suggests that this most ancient lineage of embryophytes utilizes quite distinct mechanisms for of developmental patterning.

Unlike the pleiotropic expression patterns observed for DEGs in the moss meristem and sporophyte, very few developmental regulators show significant up-regulated expression in both the *Marchantia* meristem and sporophyte samples (Supplemental Table 3.2). Putative angiosperm SAM maintenance homologs that are found in both of the *Marchantia* cell-enriched transcriptomes include SET-domain containing genes involved in chromatin remodeling, a GRAS transcription factor, the mRNA processing gene *MAGO NASHI* (Park et al., 2009; Shen and Xu, 2009; Stuurman et al., 2002) (Supplemental Table 3.1). These data raises the possibility that gene programs evolved to serve pleiotropic functions in the gametophytic SAM and determinate sporophyte only *after* the evolution of the liverworts.

These data demonstrate that the pleiotropic gene expression patterns observed in the *Physcomitrella* meristem and sporophyte transcriptomes are not present in *Marchantia*. Assuming that these trends hold true for the liverwort and moss clades in general, this data raises a possible scenario in which gene networks that are essential for modern-day sporophytic SAM development originally served pleiotropic functions in both the non-meristematic sporophyte and the gametophytic meristem in an ancient land plant ancestor. This ancestor likely existed after the divergence of the liverwort lineage and before the evolution of the mosses. Selection pressures on these genes to serve pleiotropic functions in the sporophyte and gametophytic meristem may have created a rewiring of sporophyte programs, enabling the evolution of sporophytic meris-

tem patterning.

3.3.5 Meiotic gene transcripts are abundant in the determinate bryophyte sporophytes

Although the liverwort and moss sporophytes used in this study were microdissected early in development (Figure 3.2 D & J), both their transcriptomes are replete for meiotic gene programs (Supplemental Tables 3.1 and 3.2). Upregulation of meiosis-specific genes such as *DISRUPTED MEIOTIC CDNA 1* (*DMC1*), *ASY1*, and *DYAD* (a homolog of *AMEIOTIC 1*) in the moss sporophytes, and *DMC1* as well as *HOMOLOGOUS-PAIRING PROTEIN 2* (*HOP2*) in the *Marchantia* sporophytes indicates that these young sporophytes are molecularly programmed for meiosis long before there any histological evidence of sporogenesis (Armstrong et al., 2002; Bishop et al., 1992; Leu et al., 1998; Ross et al., 1997). This very early onset of meiotic programs may explain why moss sporophytes are unable to achieve indeterminate growth, in spite of their transient meristematic activity and their abundance of meristem-patterning genes. Meiotic delay enabled the evolution of a multicellular diploid generation in early embryophytes (Graham and Wilcox, 2000; Niklas, 1997; Niklas and Kutschera, 2010); an extension of this process into later embryophyte lineages may explain the progressive expansion of the sporophyte generation in plant lineages that evolved after the bryophytes. In line with this view, ectopically initiated sporophyte-like bodies in the gametophyte generation of moss are able to develop branched architectures reflecting semi-indeterminate growth habits (Okano et al., 2009). The absence of meiosis in the gametophyte provides one

explanation for this result.

We propose a model in which the combined influence of meiotic delay and meristem program evolution in the sporophyte opened the door for indeterminate sporophytic growth, giving way to the sporophyte dominant lineages.

3.4 CONCLUSIONS

Our data suggests that angiosperm SAM patterning genes have ancient origins in the sporophyte generation and the gametophytic meristem of early land plants. We propose a mechanism in which concerted selection for these pleiotropic developmental programs to function in both indeterminate gametophytic growth and transient sporophytic growth laid the foundations for sporophytic SAM patterning. Furthermore, we find evidence for the early upregulation of meiotic programs in both bryophyte sporophytes but not in the maize sporophyte SAM.

Whether these genes originally functioned in just one generation and were recruited to function in another is possible. The lack of developmental programs in our *Marchantia* transcriptomes makes it difficult to unequivocally answer such a question, however, further exploration within the bryophytes should clarify whether shoot meristem programs simultaneously evolved for function in both generations or had ancestral functions in one and were transferred into the other.

3.5 MATERIALS AND METHODS

3.5.1 Plant culture

Wild-type *Physcomitrella patens* spores from the Gransden isolate (Ashton and Cove, 1977) were germinated and grown on .8% agar BCD plates overlaid with cellophane disks, as previously described (Nishiyama et al., 2000). Subcultures were maintained by homogenizing protonemal cells in sterile water using a Power Gen 125 homogenizer (Fischer Scientific). Reproductive development in *Physcomitrella* was induced under short-day, cold conditions (as described in Cove et al., 2009).

Marchantia polymorpha male and female Takaragaike-1 and Takaragaike-2 accessions, respectively, were obtained from the Kohchi lab (Kyoto University; (Chiyoda et al., 2008)) and assexually grown from gemmae on .8% agar half-strength Gamborgs B5 plates as outlined in (Chiyoda et al., 2008; Gamborg et al., 1968). *Marchantia* sexual reproduction was induced under long day conditions and sporophytes were obtained by manually crossing the male and female accessions (as described in (Chiyoda et al., 2008).

Zea mays seedlings from the B73 background were grown in the Cornell University greenhouse under a 16-hour light cycle at 28 deg C.

3.5.2 Plant harvest and laser microdissection

For meristem isolation, *Physcomitrella* gametophores were collected from BCD plates 21 days after subculture; *Marchantia* gametophytes were harvested 9 days

after plating; and *Z. mays* seedlings were isolated 16 days after planting. For sporophyte isolation, *Physcomitrella* sporophytes were collected ten days after crossing and *Marchantia* sporophytes were collected thirteen days after crossing. All meristem and sporophyte samples were fixed in ice-cold acetone overnight and processed for laser microdissection as outlined in (Scanlon et al., 2009). Approximately 100,000 sq microns of cells were harvested for each meristem and sporophyte biological replicate (Figure 3.2). Two-three biological replicates were prepared per sample-type. RNA was extracted from all collected tissues with the PicoPure RNA Isolation Kit (Life Technologies, cat number KIT0204) and in vitro amplified using a TargetAmp 2-round aRNA Amplification Kit 2.0 (Epicentre, cat number TAU2R51224).

Whole plant samples were collected in liquid nitrogen in coordination with meristem harvesting for each of the three species. Specifically: *P. patens* plants were harvested from BCD plates 21 days after subculture; *M. polymorpha* plants were harvested from .5X Gamborgs plates 9 days after plating gemmae; and *Z. mays* whole seedlings were collected 16 days after planting. RNA isolation and amplification of the freshly ground tissue followed the same procedures as outlined for the laser microdissected samples (above).

3.5.3 Illumina library construction and sequencing

Each amplified RNA sample was prepared for sequencing following the protocols of (Kumar et al., 2012) with a modified procedure for single sample processing. The libraries were ligated to barcoded adapters and pooled for 8-plex sequencing on an Illumina HiSeq 2000 at the Cornell University CLC DNA se-

quencing facility (http://cores.lifesciences.cornell.edu/brcinfo/?f=1).

3.5.4 Sequence processing and differential gene expression analysis

Barcoded sequences were sorted, and then clipped. Bases with a PHRED quality score <15 were trimmed from the 3 prime end using the software package Lucy (Chou and Holmes, 2001; Li and Chou, 2004). Trimmed reads of each species were aligned to their corresponding reference genomes using GSNAP (Wu and Nacu, 2010) and uniquely-mapped reads were filtered. Uniquely mapped reads were defined as reads that aligned to the reference genome with <2 mismatches per 36 bp, and less than 5 mismatches per 75 bp. Genes that had at least one mapped read in two different samples were used for differential expression test. The R package QuasiSeq (http://cran.rproject.org/web/packages/QuasiSeq) was used to test the null hypothesis that expression of a given gene is not different between every pair of cell samples. The generalized linear model Quasi-likelihood spline method assuming negative binomial distribution of read counts was used to test the null hypothesis. The 75% quantile of reads from each sample was used as the normalization factor (Bullard et al., 2010). P-values of all the statistical tests were converted to adjusted p-values (q-values) (Nettleton et al., 2006). A false discovery rate (FDR) of 5% (q-value) was used to account for multiple testing.

3.5.5 Orthologous gene family identification

Conserved Gene Families were identified using OrthoMCL 2.0 (Chen et al., 2007). BlastP searches were used to identify clusters of reciprocal best blast hits among all six species. For species without an existing set of protein annotations, amino acid sequence of the longest Open Read Frame (ORF) present within an assembled transcript was used (minimum length 80 amino acids). Annotations for the function of a conserved orthologous group were pulled first from *Arabidopsis* TAIR 10 annotations, then from Phytozome's annotations of the *Physcomitrella* genome and finally from the B73 RefGen v2 functional annotations (Goodstein et al., 2012).

3.5.6 Data visualization with Venn diagrams and heatmaps

Venn diagrams were constructed using the R package VennDiagram. Heatmaps were produced using the R package pheatmap. Values were scaled by row and rows were clustered using the default K-means clustering parameters provided in the software. The R package colorRamp was used to produce a gradient of color values corresponding to gene fold change values.

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CHAPTER 4

CONVERGENT EVOLUTION OF APICAL-CELL SHOOT MERISTEMS IN TWO ANCIENT PLANT LINEAGES

4.1 INTRODUCTION

Unlike animals, plants develop throughout their entire life cycle due to the activities of pluripotent populations of cells called meristems. The shoot apical meristem (SAM) gives rise to the entire above ground portion of the plant. Despite its conserved functions during organ initiation and stem cell maintenance, SAM anatomy, histology, and architecture varies markedly across the land plant lineages (Clowes, 1961; Evert, 2006; Gifford and Foster, 1989; Popham, 1951; Steeves, 1989). Seed plant SAMs comprise dozens to hundreds of cells that are organized into distinct histological layers and zones corresponding to function, whereas seedless plant SAMs typically house a single, prominent, inverted-pyramidal, meristematic initial called the *apical cell* (AC) (Clowes, 1961; Evert, 2006; Popham, 1951; Schmidt, 1924). Initially described as critical to all shoot meristematic activities, a variety of alternative functional models arose after it was discovered that AC-type meristems are restricted to seedless plant lineages (Hansteins, 1868; Hofmeister, 1851; Nägeli, 1845; Steeves, 1989). For a time, it was even believed that the AC was functionally inactive (D'amato, 1975); however, extensive prodding, poking, and poisoning of AC-type meristems has re-established the AC as a mitotically active, initial cell of the meristem (reviewed in Gifford, 1983; Steeves, 1989). As yet, the molecular genetic toolkit that confers pluripotent properties within the AC remains a mystery. Most seed plant SAMs are stratified into functional zones that correlate with the expression of shoot

developmental markers (Mayer et al., 1998; Schneeberger et al., 1998; Timmermans et al., 1999; Yadav et al., 2009). Pluripotent cells occupy the central zone (CZ), while organogenesis is relegated to the flanks of the meristem, called the peripheral zone (PZ). Resolving whether or not the AC of monilophyte and *Selaginella* meristems is the functional equivalent of the angiosperm CZ remains a major question in ancient land plant biology (Friedman and Moore, 2004).

The molecular framework for our current understanding of shoot meristem development in AC-type meristems rests on a small number of comparative studies investigating the functions of angiosperm meristem gene homologs in seedless vascular plants. In the fern *Ceratopteris richardii*, homologs of both *CLASS I KNOTTED1-LIKE HOMEOBOX* (*KNOX*) and *CLASS III HOMEODOMAIN LEUCINE ZIPPER* (*HD-Zip III*) genes are transcriptionally restricted to the sporophyte generation, suggesting that these genes function in sporophytic but not gametophytic meristem development in ferns (Sano et al., 2005). Likewise, *Selaginella kraussiana* homologs for *CLASS I KNOX*, *ASYMMETRIC LEAVES1/ROUGH SHEATH2/PHANTASTICA* (*ARP*), *HD-ZIP III* genes show transcript accumulations in distinctive subdomains within the shoot apex (Floyd and Bowman, 2006; Harrison et al., 2005; Prigge and Clark, 2006).

Equally intriguing as the enigmatic function(s) of the AC is the evolutionary history of plants that display AC-type SAM structure. All living vascular plants comprise two distinct evolutionary lineages that diverged over 420 million years ago during the Devonian: the lycophytes (*Lycopodium, Isotes,* and *Selaginella*) and the euphyllophytes (ferns, horsetails, and seed plants) (Clowes, 1961; Evert, 2006; Gifford and Foster, 1989; Kenrick and Crane, 1997; Popham,

1951; Steeves, 1989). The lycophytes are organized into three phylla, including the basal genus, *Lycopodium*, and two derived sister genera, *Isotes* and *Selaginella* (Clowes, 1961; Evert, 2006; Popham, 1951; Schmidt, 1924; Wikstrom, 2001). The euphyllophytes, on the other hand, comprise all remaining vascular plants including the seedless vascular monilophytes (ferns and horsetails) and all seed plants (Hofmeister, 1851; Nägeli, 1845; Pryer et al., 2001; Steeves, 1989). AC-type meristems are found both in *Selaginella* species and in the monilophytes, suggesting two possible scenarios for the evolution of this meristem structure (D'amato, 1975; Imaichi, 2008; Kato and Imaichi, 1997). Either AC-type SAM structures were present in the last common ancestor to the lycophytes and euphyllophytes and were independently lost from *Lycopodium* and *Isotes*, or this structure evolved convergently in the separate ancestors of *Selaginella* and the monilophytes. While *Selaginella* and monilophyte SAM structures are superficially similar, *Selaginella* ACs are generally more narrow and have a variable number of cutting faces when compared with those from monilophyte SAMs (Dengler, 1983; reviewed in Gifford, 1983; Hagemann, 1980; Imaichi and Kato, 1989; Imaichi and Kato, 1991; Siegert, 1974; Steeves, 1989). Paleobotanical evidence also supports a model for convergent evolution of AC-type SAM structure (Hueber, 1992; Mayer et al., 1998; Schneeberger et al., 1998; Timmermans et al., 1999; Yadav et al., 2009). The only taxa with an anatomically preserved SAMs that preceded the lycophyte-euphyllophyte split lacked AC-type meristem structures (Edwards, 1993; Friedman and Moore, 2004; Hueber, 1992; Kidston and Lang, 1920). Intriguingly, AC meristems from both lineages have plasmodesmatal densities that are approximately three-fold higher than in the representative SAMs from all other vascular plant lineages (Imaichi and Hiratsuka, 2007; Sano et al., 2005). However, this shared character likely reflects a func-

tional constraint of AC-type SAM structures, and is thus more likely the result of homoplasy rather than homology (Floyd and Bowman, 2006; Harrison et al., 2005; Imaichi, 2008; Prigge and Clark, 2006).

The emergence of new transcriptomic tools and the release of the *Selaginella* sequenced genome enables a more intensive strategy toward uncovering the molecular functions that operate in structurally distinct, AC-type meristem subdomains (Banks, 2009; Nishiyama et al., 2011). In this study we employ laser-microdissection (LM) and Illumina-based RNA-sequencing (RNAseq) to investigate the transcriptomic profiles that mark SAM subdomains for two, anciently-derived vascular plants that diverged more than 400 million years ago(Banks, 1968; Kenrick and Crane, 1997; Niklas and Banks, 1990). We demonstrate the first-described molecular markers for AC identity, and connect gene expression patterns with SAM subdomain-specific function for the monilophyte *Equisetum arvense*, and the lycophyte *Selaginella* moellendorfii. This study represents the first comprehensive, SAM-enriched, transcriptomic analysis of seedless vascular plants. Our data suggest that independent developmental programs are at work within these two AC-type meristems, supporting the view that AC-type meristems convergently evolved in the separate lineages leading to *Selaginella* and *Equisetum*. The data are discussed in light of previous models for the phylogenetic relationship of these ancient SAM structures.

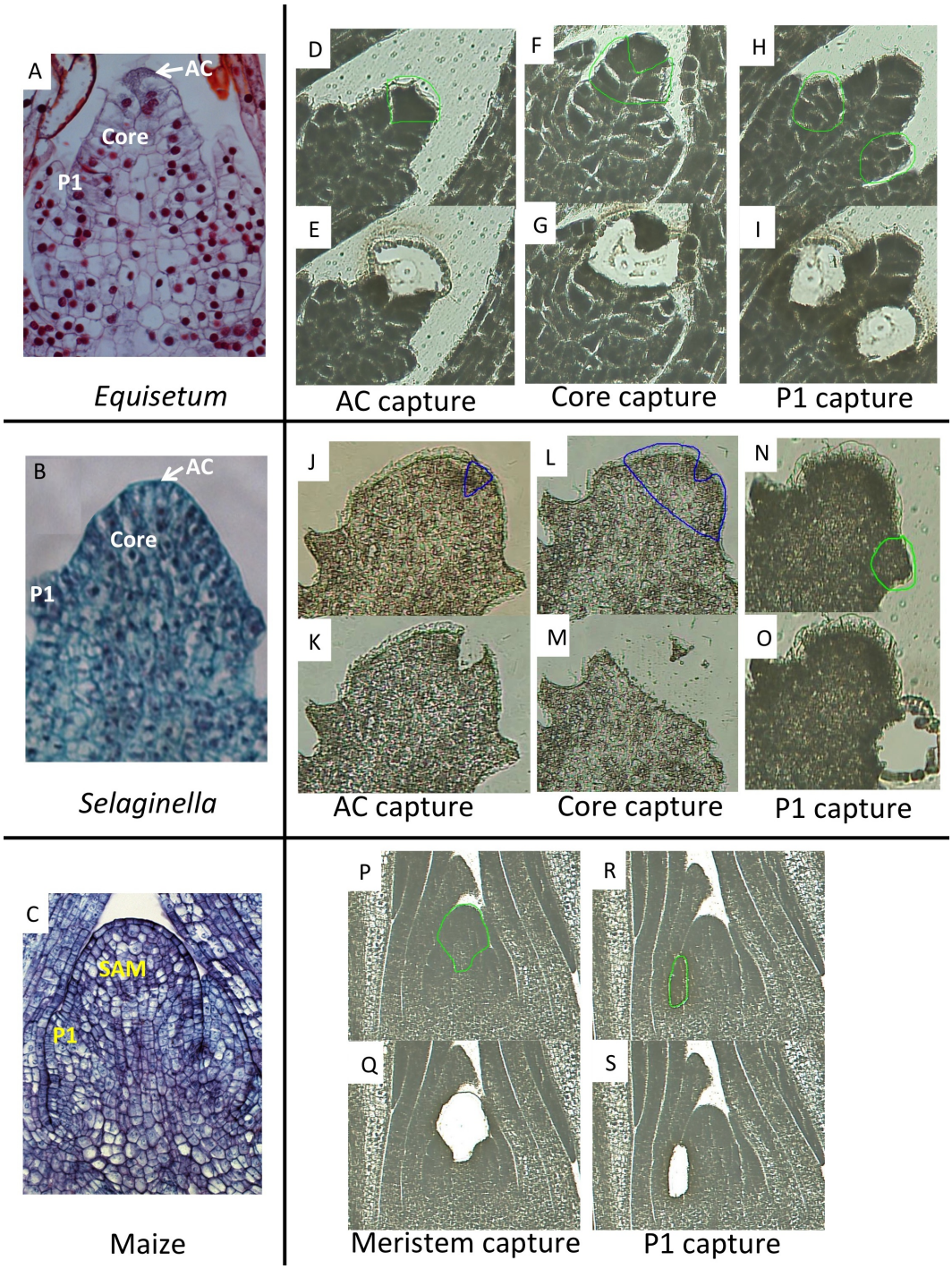
4.2 RESULTS AND DISCUSSION

Laser microdissection of shoot apical transcriptomes

Comparative transcriptomics of functional cell types from diverse lineages is a robust method for examining the molecular history of cell and organ evolution (Arendt, 2005; Arendt, 2008). We used LM-RNAseq to compare the molecular fingerprints of cells enriched for subdomains within *Selaginella*, *Equisetum*, and maize shoot apices. Three apical domains were isolated from the *Selaginella* and *Equisetum* SAMs: the AC domain, comprising the lone AC (Figure 4.1 D-E and J-K); the core domain, comprising the cells below the AC and above the first initiating lateral organ primordium (Figure 4.1 F-G and L-M); and the youngest initiating leaf primordium (P1) (Figure 4.1 H-I and N-O). Whole SAM and P1 domains were also isolated from maize, and used for comparisons of transcriptomic dynamics in angiosperm SAMs (Figure 4.1). LM-RNAseq of these SAM subdomains generated hundreds of significantly [false discovery rate (FDR) less than .05] differentially expressed genes (DEGs) for each cell type relative to whole plant transcriptomes (Figure 4.2). Ultimately, the transcriptomes for the SAM subdomains described here were used to test for the presence of homologous developmental programs across these three species, and to identify unique developmental programs operating within each species.

Figure 4.1: **Laser microdissection enables the collection of specific subdomain-enriched samples of apical cell-type and angiosperm meristems**

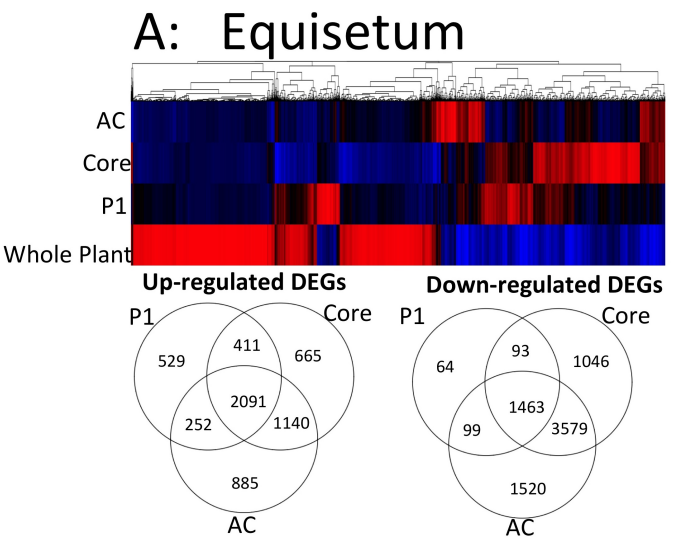
Equisetum (A) and *Selaginella* (B) have meristem structures with prominent ACs, and angiosperms have tunica-corpus structure (C). Two separate meristem subdomains were collected from the AC-type meristems and compared with the multicellular meristem of maize. AC and core domains were isolated from *Equisetum* (D-E and F-G) and *Selaginella* (J-K and L-M) (respectively) and compared with the shoot meristem cells captured from maize (P-Q). Initiating leaf primordia (P1) were isolated from *Equisetum* (H-I), *Selaginella* (N-O), and maize (R-S).

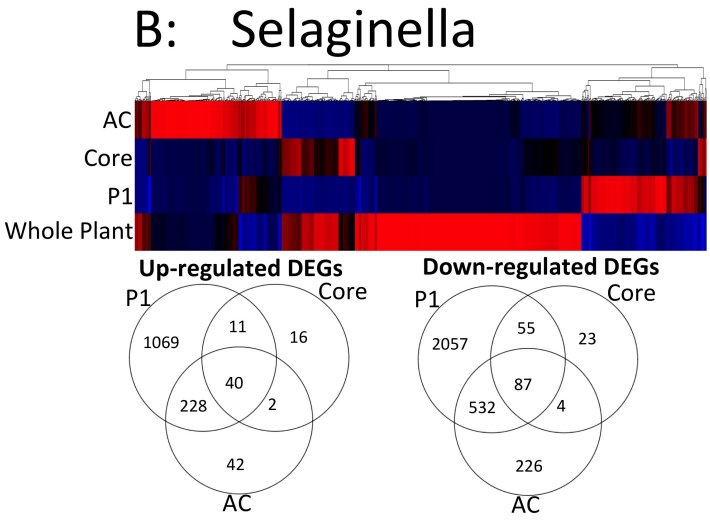

A
AC
Core
P1
Equisetum
D
E
AC capture
F
G
Core capture
H
I
P1 capture
B
AC
Core
P1
Selaginella
J
K
AC capture
L
M
Core capture
N
O
P1 capture
C
SAM
P1
Maize
P
Q
Meristem capture
R
S
P1 capture

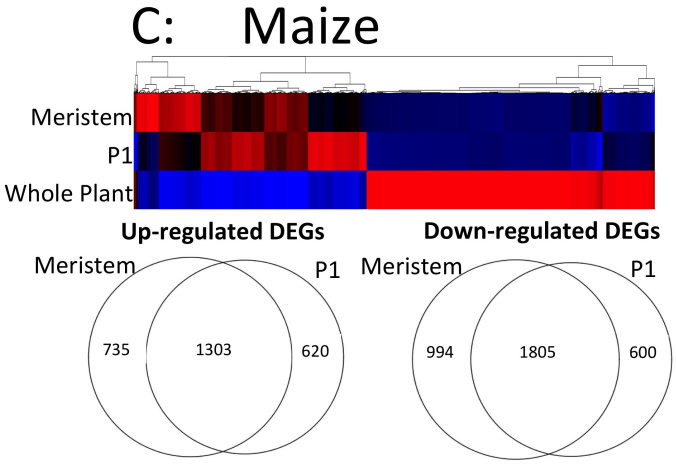
An inter-species heatmap was assembled in order to visualize relative transcript accumulation of genes with well-described roles in SAM initiation, maintenance, and function across the SAM subdomains. This gene list included transcriptional regulators, hormone biosynthesis/response genes, DNA repair and chromosomal maintenance factors, and components of small RNA biogenesis (Brooks et al., 2009; Canales et al., 2005; Dodsworth, 2009; Golz, 2006; Husbands et al., 2009; Shen and Xu, 2009; Wolters and Juergens, 2009; Yadav et al., 2009). Phytozome and OrthoMCL were used to identify gene homologs across the three species (Chen et al., 2007; Goodstein et al., 2012). Significant differential transcript accumulation (FDR $>$.05) within each of the meristem subdomains was determined using EdgeR (Robinson and Oshlack, 2010) by performing pairwise comparisons between of each subdomain and whole plant transcriptomes for each respective species.

Figure 4.2: **Laser microdissection enables the generation of SAM subdomain molecular fingerprints**

Equisetum (A), *Selaginella* (B) and maize (C) SAMs each contain thousands of significantly differentially expressed genes


A: Equisetum
AC
Core
P1
Whole Plant
Up-regulated DEGs
P1
Core
AC
529
411
665
2091
252
1140
885
Down-regulated DEGs
P1
Core
AC
64
93
1046
1463
99
3579
1520

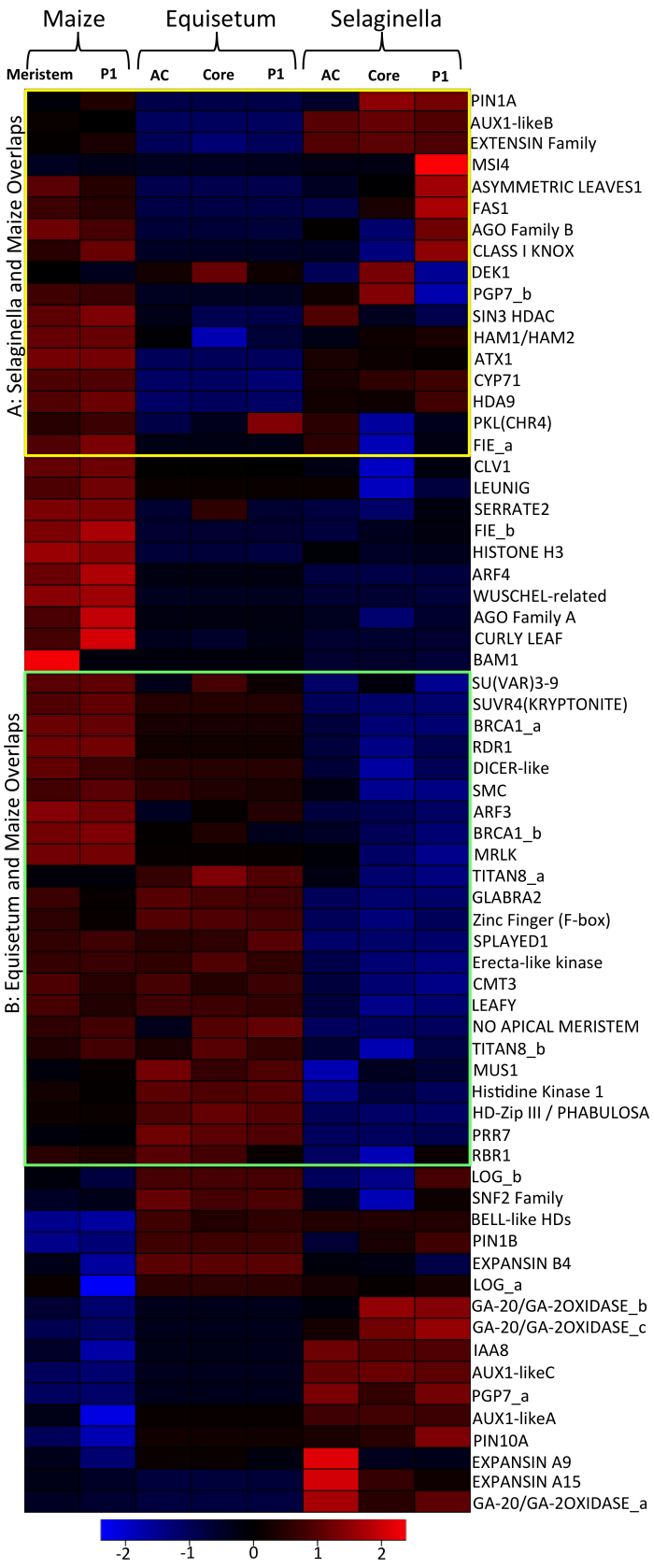

B: Selaginella
AC
Core
P1
Whole Plant
Up-regulated DEGs
P1
Core
AC
1069
11
16
40
228
2
42
Down-regulated DEGs
P1
Core
AC
2057
55
23
87
532
4
226


C: Maize
Meristem
P1
Whole Plant
Up-regulated DEGs
Meristem
P1
735
1303
620
Down-regulated DEGs
Meristem
P1
994
1805
600

Gene homologs that were significantly up-regulated between *Equisetum* and maize shoot apical domains relative to whole plant samples form a block of eighteen gene groups (outlined in green; Figure 4.3, overlap B). These genes include the chromatin maintenance gene groups such as *CHROMOMETHYLASE 3* (*CMT3*), and *SPLAYED* (*SYD*), as well as key transcriptional regulators: *GLABRA2* (*GL2*), *LEAFY* (*LFY*), and *NO APICAL MERISTEM* (NAM). *Selaginella* and maize shoot apical domains also share a block of gene homologs with up-regulated transcript accumulation (outlined in yellow; Figure 4.3, overlap A) including the auxin transporters *PIN* and *AUX1-like* (Friml and Palme, 2002). Several genes were up-regulated in individual subdomains of the *Selaginella* shoot apex relative to the whole plant samples, for example the *Selaginella ARP* homolog is solely upregulated in P1 samples whereas the homolog of a described regulator of *PIN* auxin efflux, *P-GLYCOPROTEIN7* (*PGP7*), is only up-regulated in the *Selaginella* core domain (Geisler and Murphy, 2006). Both *Equisetum* and *Selaginella* shoot apices show increased accumulation of the putative shoot patterning gene *DEFFECTIVE KERNEL 1* (*DEK1*) in their core domains, whereas accumulation of the cytokinin-activating gene *LONELY GUY 1* (*LOG1*) (Kurakawa et al., 2007)) and the auxin efflux carrier *PIN* are both up-regulated in the P1 samples for these two species (Becraft et al., 2002; Kurakawa et al., 2007). Remarkably, aside from these *relatively few* shared patterns of transcript accumulation, *Selaginella* and *Equisetum* show distinct transcriptional profiles. These data suggest that SAM function proceeds via mostly independent developmental pathways in the AC-type meristems of *Selaginella* and *Equisetum*.

Figure 4.3: **Heatmap shows shared transcript accumulation patterns amongst SAM subdomains**

SAM transcripts shows blocks of shared transcript accumulation patterns between *Selaginella* and maize (overlap A) and *Equisetum* and maize (overlap B). Scaled transcript values are shown in the color key with blue indicating down-regulated expression and red indicating up-regulated expression.


Maize
Equisetum
Selaginella
Meristem
P1
AC
Core
P1
AC
Core
P1
A: Selaginella and Maize Overlaps
B: Equisetum and Maize Overlaps
PIN1A
AUX1-likeB
EXTENSIN Family
MSI4
ASYMMETRIC LEAVES1
FAS1
AGO Family B
CLASS I KNOX
DEK1
PGP7_b
SIN3 HDAC
HAM1/HAM2
ATX1
CYP71
HDA9
PKL(CHR4)
FIE_a
CLV1
LEUNIG
SERRATE2
FIE_b
HISTONE H3
ARF4
WUSCHEL-related
AGO Family A
CURLY LEAF
BAM1
SU(VAR)3-9
SUVR4(KRYPTONITE)
BRCA1_a
RDR1
DICER-like
SMC
ARF3
BRCA1_b
MRLK
TITAN8_a
GLABRA2
Zinc Finger (F-box)
SPLAYED1
Erecta-like kinase
CMT3
LEAFY
NO APICAL MERISTEM
TITAN8_b
MUS1
Histidine Kinase 1
HD-Zip III / PHABULOSA
PRR7
RBR1
LOG_b
SNF2 Family
BELL-like HDs
PIN1B
EXPANSIN B4
LOG_a
GA-20/GA-2OXIDASE_b
GA-20/GA-2OXIDASE_c
IAA8
AUX1-likeC
PGP7_a
AUX1-likeA
PIN10A
EXPANSIN A9
EXPANSIN A15
GA-20/GA-2OXIDASE_a
-2
-1
0
1
2

To quantitatively test our hypothesis that AC-type SAM structure evolved convergently in *Selaginella* and *Equisetum*, the transcriptional abundance of gene-families was examined. Gene family trees were constructed for maize, *Selaginella*, and *Equisetum* using OrthoMCL and Phytozome (Chen et al., 2007; Goodstein et al., 2012). A total of 8,156 gene families were identified, of which 3,143 contain homologs across all three species. Shared gene family expression patterns were determined by testing whether or not members of each gene family were significantly up-regulated or down-regulated within each species. Few gene families were up-regulated across the SAM domains of all three species: 21 gene families have shared upregulation amongst the AC domains and the maize meristem; two gene families have shared up-regulation amongst the core domains and the maize meristem; and three gene families have shared up-regulation amongst the P1 domains (Figure 4.4). With a few notable exceptions, these shared transcriptional families are predominately annotated with housekeeping functions such as ribosomal biogenesis, histone family proteins, and mitochondrial maintenance, suggesting that there are very few developmental parallels amongst the shoot apices of maize, *Selaginella* and *Equisetum*.

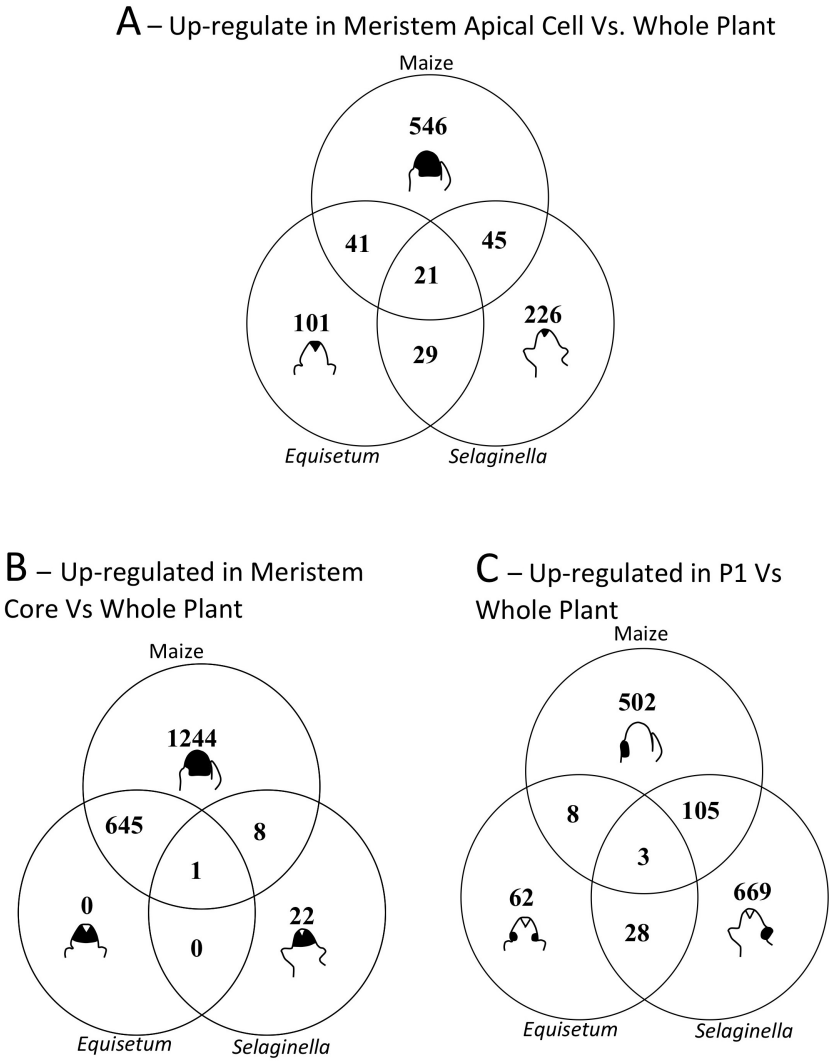


Figure 4.4: ***Equisetum* and *Selaginella* shoot apices express independent developmental programs**

Venn diagrams of up-regulated DEG families across shoot apical domain transcriptomes from *Equisetum*, *Selaginella*, and maize. Number of up-regulated gene families in subdomains of the *Selaginella*, *Equisetum*, and maize AC (A), Core (B), and P1 (C).

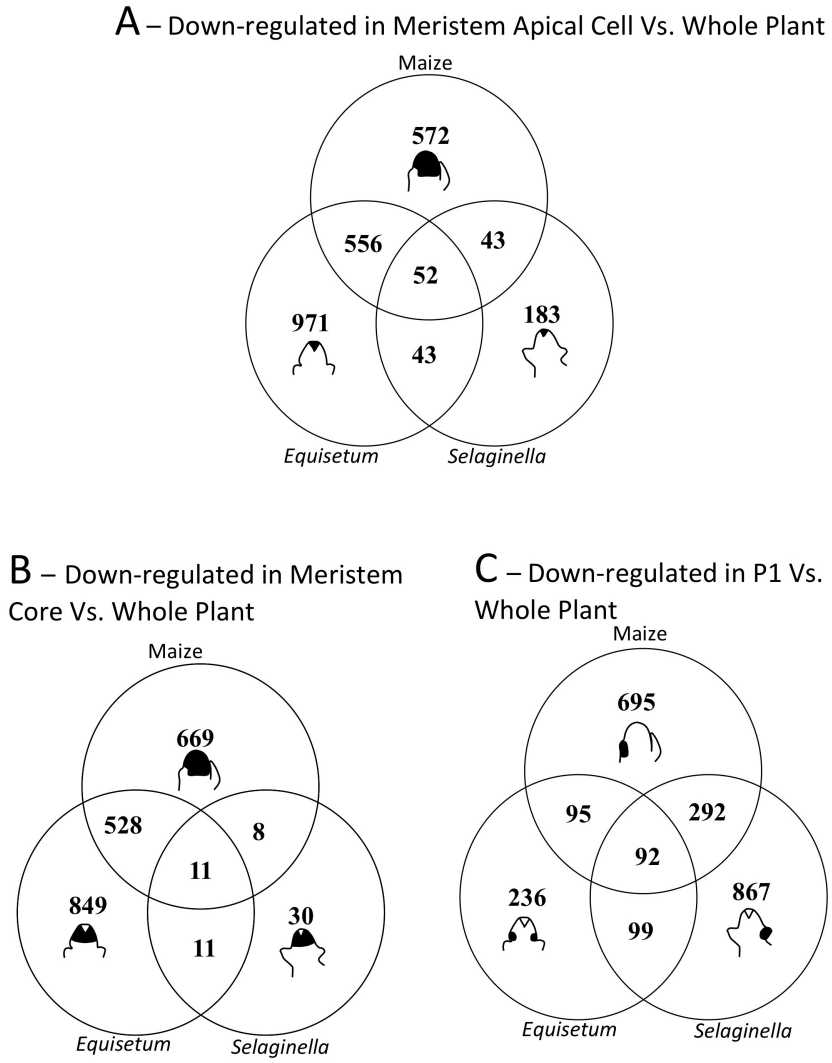


Figure 4.5: ***Equisetum* and *Selaginella* shoot apices express independent developmental programs**

Venn diagrams of down-regulated DEG families across shoot apical domain transcriptomes from *Equisetum*, *Selaginella*, and maize. Number of down-regulated gene families in subdomains of the *Selaginella*, *Equisetum*, and maize AC (A), Core (B), and P1 (C).

Perhaps the more interesting exception includes the shared up-regulation of the *PHABULOSA-LIKE HD-ZIP III* (*PHB*) family within the leaf primordia of maize, *Equisetum*, and *Selaginella* (Table 4.3). The *HD-ZIP III* family of transcription factors has well-described functions during leaf patterning, meristem maintenance, and vasculature development in angiosperm lineages (reviewed in Galun, 2007). *In situ* hybridization of *HD-ZIP III* homologs in a related *Selaginella* species (*S. kraussiana*) demonstrated that these genes are expressed in the shoot meristem and in young initiating leaves. However, this *HD-ZIP III* transcript accumulation connects back to the central vascular strand of the stem, suggesting that these transcription factors function during vascular patterning rather than in leaf development within the lycophyte lineage (Floyd and Bowman, 2006; Prigge and Clark, 2006). Genetic analyses are required to determine whether or not shared expression of *HD ZIP III* transcripts in the *Selaginella*, *Equisetum*, and maize P1 transcriptomes reflect shared programs for leaf development, or vasculature patterning, or both processes.

Direct comparison between the AC domains of *Selaginella* and *Equisetum* reveals shared up-regulation of 50 gene families, which are also replete for predicted housekeeping functions (Figure 4.4 A; Table 4.1). Within the meristem core domains of *Selaginella* and *Equisetum*, a lone *HISTONE SUPERFAMILY PROTEIN* is the only gene family with up-regulated transcript accumulation in both species (Figure 4.4 B; Table 4.2). These distinct molecular profiles between the functionally-related meristem structures of *Selaginella* and *Equisetum* comprise strong evidence for the convergent evolution of AC-type meristem organization in these two species.

Table 4.1: Up-regulated Gene Families in the Meristem AC Domain

Gene	***Selaginella***	***Equisetum***	**Maize**
Ribosomal biogenesis	Y	Y	Y
Histone Superfamily	Y	Y	Y
PROHIBITIN 4	Y	Y	Y
4 Unknown proteins	Y	Y	Y
PHABULOSA		Y	Y
LEAFY		Y	Y
PRR7		Y	Y
AUXIN RESPONSE FACTOR 3	Y		Y
BARELY ANY MERISTEM 1	Y		Y
REPRESSOR OF GA1-3	Y		Y
ERECTA-like 2	Y		Y

Table 4.2: Up-regulated Gene Families in the Meristem Core Domain

Gene	***Selaginella***	***Equisetum***	**Maize**
PHABULOSA		Y	Y
LEAFY		Y	Y
CHR11		Y	Y
BARELY ANY MERISTEM 1	Y		Y
ROP-interactive CRIB (RIC)	Y		Y
REPRESSOR OF GA1-3	Y		Y

Table 4.3: Up-regulated Gene Families in P1 Domain Across Species

Gene	***Selaginella***	***Equisetum***	**Maize**
PHABULOSA	Y	Y	Y
LEAFY		Y	Y
SPLAYED 1		Y	Y
CYCLIN-DEPENDENT KINASE1		Y	Y
BARELY ANY MERISTEM 1	Y		Y
AUXIN RESPONSE FACTOR 3	Y		Y
AUXIN RESPONSE FACTOR 12	Y		Y
TOPLESS-related	Y		Y
MERISTEMATIC RECEPTOR-like KINASE	Y		Y
AP2/B3 TF	Y		Y
SET-domain containing	Y		Y

4.2.1 Transcriptomic comparisons support shared shoot meristem developmental programs in pairwise comparisons between *Equisetum* or *Selaginella* and maize

Unlike in the pairwise comparison between the AC-type meristems, a number of developmental gene regulators with up-regulated expression were identified in comparisons of *Equisetum* and maize transcriptomes. All *Equisetum* and maize meristem domains share significantly increased transcript accumulation for homologs of *PHB* and *LEAFY* (*LFY*), two gene families that evolutionarily-

conserved functions in angiosperm SAM development (Bowman, 2000; Floyd and Bowman, 2006; Floyd and Bowman, 2010; Maizel et al., 2005; Prigge and Clark, 2006; Sayou et al., 2014; Schultz and Haughn, 1991; Weigel et al., 1992). *Selaginella* and maize shoot apices also share significantly increased transcript accumulation for homologs of *BARELY ANY MERISTEM1* (*BAM1*), *AUXIN RESPONSE FACTOR3* (*ARF3*), *REPRESSOR OF GA1* (*RGA1*), all of which show disrupted SAM development when mutated in *Arabidopsis* (Cheng et al., 2013; DeYoung et al., 2006; Peng et al., 1997). Moreover, independent pairwise comparisons of the P1 transcriptomes of *Equisetum* and maize, and of *Selaginella* and maize each revealed shared up-regulation of transcriptional programming genes, epigenetic patterning genes, and biochemical markers for differentiating cells, including *BAM1*, *ARF3*, *TOPLESS-related 2* (*TPL2*), and *AUXIN RESPONSE FACTOR 12* (*ARF12*) (Table 4.3).

The independent recruitment of homologous developmental pathways to function in convergent structures is amply demonstrated in both plants and animals (Gehring, 2005; Harrison et al., 2005; Menand et al., 2007). Our transcriptomic comparisons demonstrate that more than 100 gene families are shared between the transcriptomes of the microphyllous leaves of *Selaginella* and the megaphyllous leaves of maize (Andrews and Forbes, 1975; Banks, 1968; Gensel, 1992; Tomescu, 2009). Although it is possible these leaf developmental networks were recruited independently during convergent evolution of *Selaginella* and maize leaves, it is more parsimonious to suggest that a relatively small number of transactivating switch genes were convergently recruited, and their multiple, conserved, downstream targets simply *followed* recruitment of these master regulators. In line with this view, *ARP* genes involved in angiosperm leaf patterning are also expressed in initiating microphyllous leaves in *Selaginella* (Harrison

et al., 2005; Waites and Hudson, 1995).

In contrast to the strong signal for shared gene programs between *Selaginella* and maize P1 samples, only eleven gene family transcripts are up-regulated in both *Equisetum* and maize leaf primordia. These include key regulators of early stages in lateral organ development, like *PHB* as well as the interacting factors *LFY* and *SYD1* (Emery et al., 2003; Wagner and Meyerowitz, 2002). Also found, were gene families without described roles in leaf development such as a *Eukaryotic Translation Initiation Factor* family and an *ADP-RIBOSYLATION POLYMERASE* family. This marked incongruity in the shared homologies of the leaf transcriptomes between *Equisetum* and maize may be explained by the arrested development of *Equisetum* leaves, which terminate as tiny non-photosynthetic vestiges surrounding the node (Golub and Wetmore, 1948). Alternatively, the disparate expression patterns between *Equisetum* and maize leaves could be the result of convergent evolution of the two leaf types, a scenario that has been suggested previously (Boyce and Knoll, 2002).

4.2.2 Both the AC and core domains of *Selaginella* and *Equisetum* SAMs house distinct patterning genes

In order to further our understanding of the distinct developmental pathways and novel structure-function organization within the AC-type SAMs of *Selaginella* and *Equisetum*, we performed intra-species transcriptomic comparisons amongst the distinct AC, core, and P1 subdomains of these SAMs (Figure 4.2). Hundreds to thousands of DEGs were identified within most of the SAM subdomains, constituting distinct molecular fingerprints for each collected cell types.

Notably, detection of statistically significant, DEGs in the *Selaginella* meristem core transcriptomes was difficult. We suggest that these issues reflect the developmental activity of the *Selaginella* apical core and its function during shoot branching. The branched shoot architecture of *Selaginella* is accomplished via continuous bifurcation of the SAM, such that any sampling of *Selaginella* meristems will be uncoordinated regarding developmental time and proclivity to branching (Hagemann, 1980). Thus, any microdissected sample of core domains microdissected from a pool of *Selaginella* SAMs comprises a heterogeneous mixture of different developmental time points with regard to shoot branching, which generates variable quantification of transcriptomic reads, relatively high P-values, and statistically unacceptable false discovery rates. To overcome the noise created by this heterochronic sampling, we identified transcripts that exhibited consistently distinct transcript reads in pairwise comparisons between biological replicates (explained in Materials and Methods). Genes with previously-describe expression patterns were identified in the *Selaginella* core domain using this strategy, including the *Class I KNOX* homolog g135843 (Harrison et al., 2005). The verification of RNAseq-identified, domain-enriched, gene candidates via *in situ* hybridization provides further validation of this strategy (discussed below; Figure 4.8).

To gain insight into the functional domains within *Selaginella* and *Equisetum* AC-type SAMs we clustered the implicated genes into a Self-Organizing Map (SOM) based on SAM subdomain expression patterns. We used the R som package (http://cran.r-project.org/web/packages/som/som.pdf) to deconstruct our complex RNAseq datasets into a network of co-expresssion clusters, in which similar clusters group closer together on the map. The SOMs generated for the *Equisetum* and *Selaginella* SAM transcriptomes delineated tran-

scriptionally distinct subdomains within the shoot apices for each of these species. The molecular separation of these subdomains was further confirmed with in situ hybridization analyses in *Selaginella*. Unfortunately, a similar attempt at in situ hybridization in *Equisetum* failed, likely as a result of the silica-rich cell walls in this species (Holzhüter et al., 2003).

4.2.3 The Apical Cell is a Molecularly Distinct Domain Which Contains Homologous Programs for Angiosperm SAM Maintenance

The meristematic function of the AC during seedless vascular plant is long standing question in experimental botany (Gifford, 1983; Steeves, 1989). Here, we identify hundreds of genes that are distinctly expressed in the AC compared to the neighboring core domain of *Equisetum* SAMs. Most notably, *SYD1* and *LFY*, proteins that physically interact to regulate meristem maintenance in angiosperms, were identified together in an AC enriched *Equisetum* cluster (cluster 3,4; Table 4.4) (Kwon et al., 2005; Wagner and Meyerowitz, 2002). Several additional homologs essential for angiosperm SAM development were also identified, such as *CYTOKININ RESPONSE 1* (*CRE1*), *MADS HOMEOBOX* (*MADS*) transcription factors, *BELLRINGER1-like HOMEODOMAIN* (*BEL1*) genes (Table 4.4) (Byrne et al., 2003; Inoue et al., 2001; Rosin et al., 2003). Taken together, these patterns suggest that the *Equisetum* AC houses an abundance of developmental regulators that are central to angiosperm SAM maintenance.

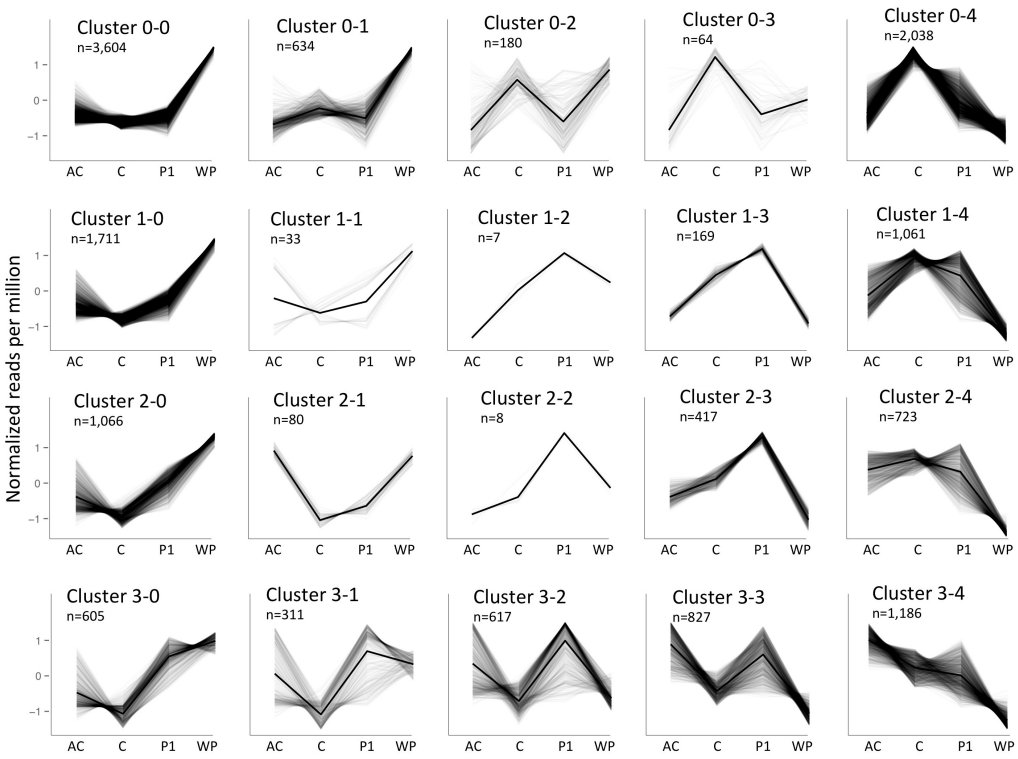


Figure 4.6: **Self Organizing Map of *Equisetum* shoot apical transcriptomes reveals large clusters of developmental regulators that are up-regulated in shoot apical domains.**

Cluster coordinates and the number of genes grouped within the cluster is indicated above each transcriptional pattern. AC = Apical Cell, C = Core, P1 = Leaf Primordium, and WP = Whole Plant.

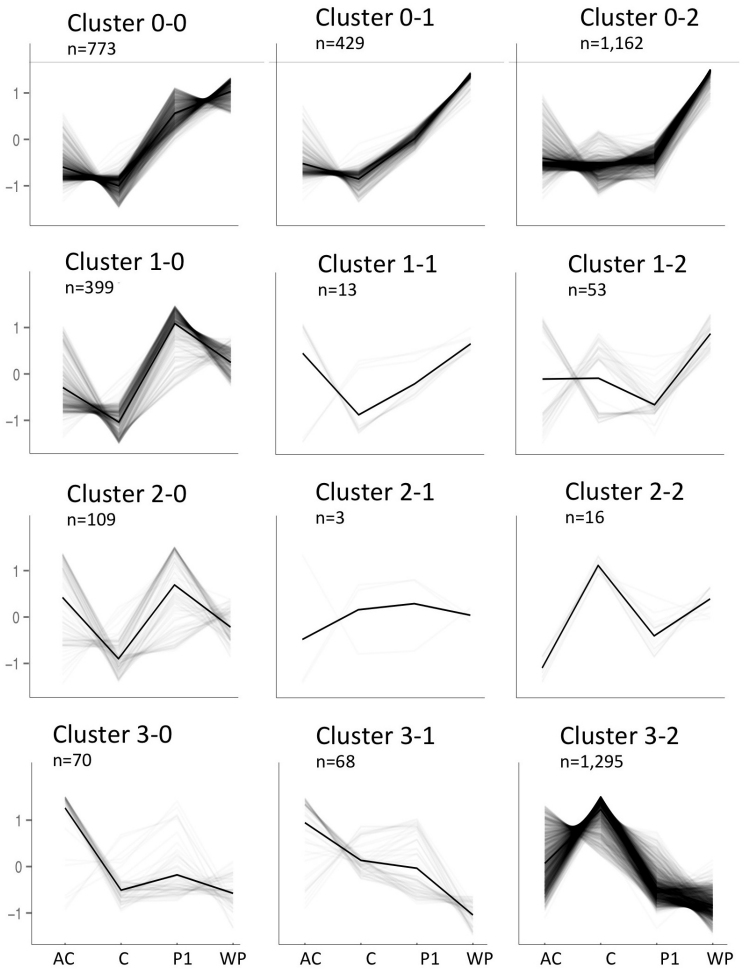


Figure 4.7: **Self Organizing Map of *Selaginella* shoot apical transcriptomes reveals large clusters of developmental regulators that are up-regulated in shoot apical domains**

Cluster coordinates and the number of genes grouped within the cluster is indicated above each transcriptional pattern. AC = Apical Cell, C = Core, P1 = Leaf Primordium, and WP = Whole Plant.

In line with our findings in *Equisetum*, the *Selaginella* SOM shows molecularly distinguishable patterns between the AC and core domains (Figure 4.7). The *Class I KNOX* gene g159366 was identified in the AC-enriched cluster (0,4), suggesting that the AC domain of *Selaginella*, like that of *Equisetum*, shares

Table 4.4: *Equisetum* SOM Membership

Gene Annotation	**Cluster ID**
CUP-SHAPED COTYLEDON3	1-3
bHLH transcription factor-like protein	1-4
TIR1/AFB auxin receptor	2-3
MADS-box protein	2-4
KNAT3-like	3-0
bZIP	3-0
HDZip I	3-0
FAMA	3-2
auxin influx carrier	3-2
CHROMATIN REMODELING 4	3-2
MIKC-type MADS-box	3-2
SIN3 histone deacetylase complex	3-3
MADS-box protein	3-3
HDZip IV	3-3
BELL1-like	3-3
SPLAYED	3-4
cytokinin receptor 1	3-4
LEAFY	3-4
CHROMOMETHYLASE 3	3-4

Table 4.5: *Selaginella* SOM Gene Membership

Gene Annotation	**Cluster ID**
Auxin Efflux Carrier	0-2
AUXIN RESPONSE FACTOR	0-2
HOMEODOMAIN-like 0-2 X	
SAUR-like auxin responsive	0-2
MYB-like	0-3
F-BOX	0-4
KNOX/KNAT6-like	0-4
CLAVATA1a	1-4
HERCULES2	3-2
KANADI	3-3
AP2/B3-like transcription factor	3-4
TASSELSEED2	3-4
ETHYLENE RESPONSE FACTOR	3-4
WUSCHEL-related Homeobox	3-4
SMC	3-4
E2F transcription factor	3-4

molecular similarities with angiosperm SAMs. Notably, the *Class I KNOX* identified here is a distinct paralog of a previously-described *Selaginella Class I KNOX* expressed in the core domain (Harrison et al., 2005). These data suggest that multiple *Class I KNOX* genes may function in the *Selaginella* shoot apex, similar to what is found in the angiosperm SAM (Vollbrecht et al., 1991).

In situ hybridization analyses of AC-upregulated genes strongly suggest that

the AC comprises a distinct molecular domain. Two gene candidates, a homeodomain transcription factor and a *PIN1* auxin efflux carrier, were selected as markers for AC function based on their distinct expression values within the AC transcriptomes, and their putative roles in development. Remarkably, the *Homeodomain TF* gene specifically marks a single AC and serves as the first described marker for AC identity (Figure 4.8 C). *PIN1* transcripts, on the other hand, form a cloud of expression surrounding the two apical cells in the bifurcating meristem (Figure 4.8 B). Notably, this transcript accumulation pattern is reminiscent of auxin efflux carrier gene expression in the angiosperm root, where PIN1 functions as an auxin-funnel feeding the root stem-cell niche (i.e. the quiescent center - QC) (Blilou et al., 2005). Furthermore, the presence of this predicted, PIN-generated auxin pool within the *Selaginella* AC correlates with significant up-regulation of an *AUXIN RESPONSE FACTOR* (*ARF*) (gene g115320) in the up-regulated AC SOM cluster (0,2), suggesting that high auxin levels are accumulating in the AC. Intriguingly, this predicted auxin maximum in the *Selaginella* SAM apex is not yet described in angiosperm shoot apices, but is reminiscent of auxin dynamics in the *Arabidopsis* root. Specifically, a network of PIN auxin efflux carriers coordinates with the ARF-regulated AP2-type transcription factor PLETHORA to specify an auxin maximum in the QC (Blilou et al., 2005).

The fact that both QC and AC cells are specified at presumptive auxin maxima and are centrally located within the meristems within which they function, suggests similarities between the *Selaginella* SAM and the *Arabidopsis* root apical meristem. Evidence for analogous genetic functions in root/shoot apical development is described previously (reviewed in Benfey, 1999; Sarkar et al., 2007). Furthermore, initiating *Selaginella* rhizophores (analogous to root struc-

tures) can switch identities and become shoots if the neighboring SAM is ablated (reviewed in Halperin, 1978), emphasizing the blurred line between root and shoot identity in *Selaginella*. Moreover, the expression of *Class I KNOX* genes in both the rhizophore and SAM of *Selaginella* demonstrates the shared use of molecular machinery in these two opposing meristems (Kawai et al., 2010). Short of proposing that the *Selaginella* shoot meristem is simply an angiosperm root meristem, the data presented here raises the possibility that the *Selaginella* SAM shares aspects of root developmental organization. This mosaic of root and shoot-like patterning may explain the developmental flexibility between rhizophore and shoot specification in *Selaginella* (Webster, 1969).

4.2.4 The Core Domain Contains Gene Expression Patterns Indicative of PZ Function and is Transcriptionally Distinct from the AC

The core domain comprises a multicellular region of leaf-generating cells that subtends the prominent AC; in *Selaginella*, dichotomous branching is also coordinated in the SAM core (Hagemann, 1980). Reflecting the dynamic growth processes within the core domain, clusters for up-regulated core gene expression in both *Equisetum* and *Selaginella* SOMs contain a profusion of SAM developmental regulators. Most intriguing is the accumulation of transcripts homologous to angiosperm dorsiventral patterning genes. For example, *Equisetum* homologs for *PHB* and *SERRATE* (*SE*), which specify adaxial-abaxial leaf patterning, are up-regulated within the core clusters (Table 4.4) (Emery et al., 2003; Grigg et al., 2005). Likewise, the *Selaginella* core clusters include a *KANADI* (*KAN*) gene

family homolog, whose members play well-described roles in abaxial lateral organ specification in angiosperms (Kerstetter et al., 2001). Transcript accumulation of these leaf polarity genes in the core domains of *Equisetum* and *Selaginella* suggests that homologous pathways functioned in the SAM of ancient vascular plants. Moreover, the expression of genes putatively involved in leaf patterning provides molecular support to the conclusions drawn from previous anatomical studies, which suggested that the core domain functions as a SAM peripheral zone (PZ) (Dengler, 1983; Hagemann, 1980; Wardlaw, 1957).

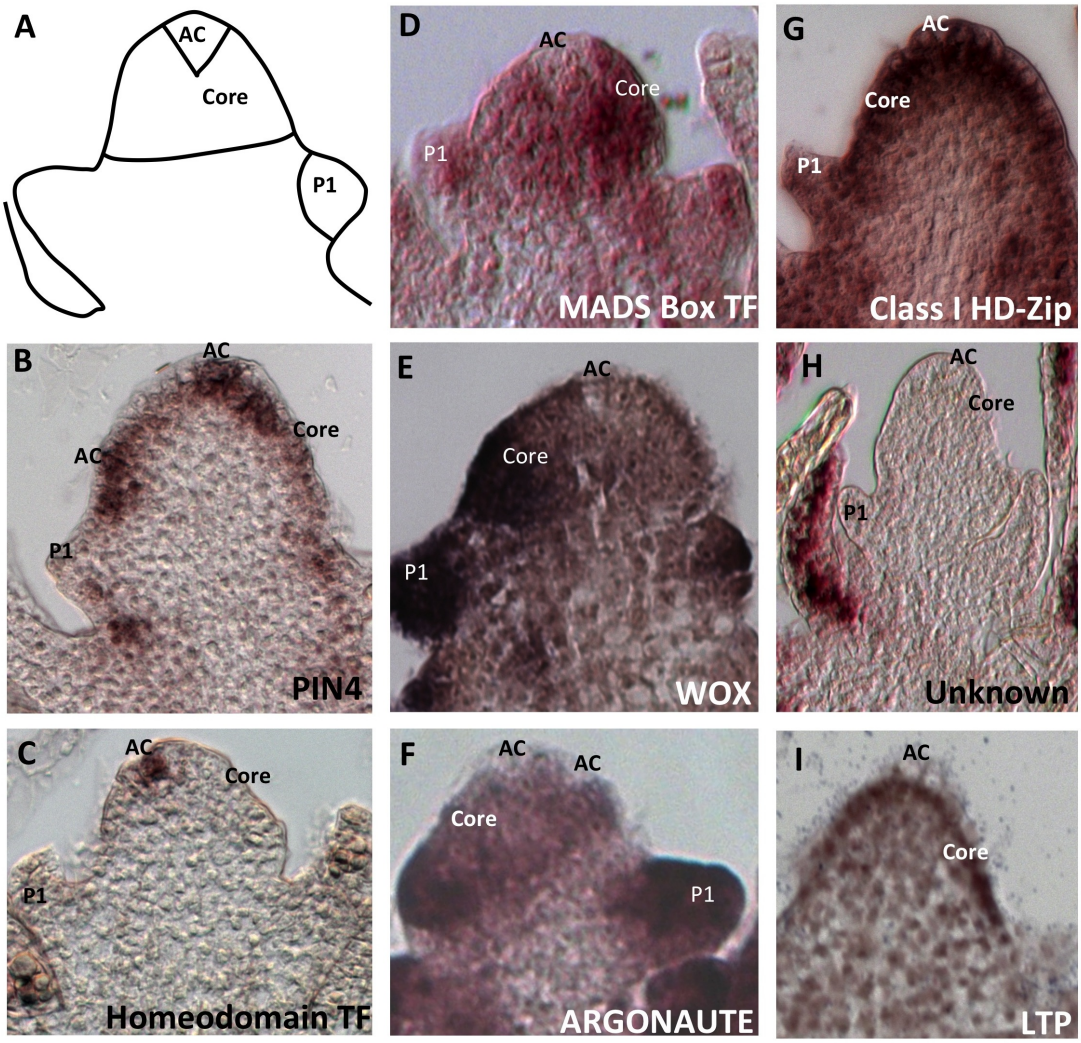
We performed in situ hybridizations for several core-enriched genes from *Selaginella*, in order to identity the specific, SAM cell-tissue accumulation patterns of these core genes. Three developmental markers for the *Selaginella* core (a *MADS-Box* transcription factor (Figure 4.8 - D), a *WUSCHEL-like Homeobox* gene (*WOX*) (Figure 4.8 E), and a putative *ARGONAUTE* (*AGO*) (Figure 4.8 F)] were thus selected, based on their high expression levels within the core domain and their homology to known developmental regulators in angiosperms. All three transcripts exhibit expression in both the SAM core and in leaf primordia, supporting a developmental model in which lateral organ patterning initiates in apical regions of the SAM core, close to the AC. Significantly, all three core transcripts are not detected in the AC domain, emphasizing the distinct separation between the AC and this presumed patterning of lateral organ initiation. These discrete SAM expression domains are reminiscent of CZ versus PZ patterning in angiosperms, where genes involved in lateral organ initiation are repressed in the CZ (Yadav et al., 2013).

Moreover, the striking expression pattern of *AGO* in incipient leaf primordia suggests that, as in angiosperms, small RNAs function in patterning the

Selaginella leaf. Further investigation into which microRNAs interact with this AGO will determine whether or not these small RNA pathways are homologous to leaf polarity programs described in angiosperms (Emery et al., 2003; Reinhart et al., 2002) (Juarez et al., 2004) (Mallory and Reinhart, 2004) (Kidner and Martienssen, 2004) (Nagasaki et al., 2007) (Nogueira et al., 2007) (Douglas et al., 2010). Our *in situ* hybridizations provide key insights into the organization of the *Selaginella* SAM; the AC and core domains define discrete boundaries of transcript accumulation and presumed function, whereas similar, PZ-type functions are suggested for P1 and core domains.

Figure 4.8: ***In situ* hybridization reveals key regulators of *Selaginella* meristem domain function**

(A) Diagram of the three microdissected meristem domains: AC = Apical Cell domain, Core = surrounding cells below the AC and above the first initiating leaf, and Leaf = youngest histologically visible lateral organ. *PIN4* (B) and *Selaginella Homeodomain* transcription factor (C) serve as apical cell developmental markers. D-F: *MADS Box*, *WOX*, and *AGO* genes mark lateral organ initiation; these genes have distinct expression patterns in the meristem core and leaf domains but are absent from the apical cell domain. G and I *Class I HD-Zip* and *Lipid Transfer Protein* genes are predominately expressed in the outer cell layer throughout the meristem. H Selaginella unknown protein only accumulates in more mature leaves.


A
AC
Core
P1
D
AC
Core
P1
MADS Box TF
G
AC
Core
P1
Class I HD-Zip
B
AC
Core
AC
P1
PIN4
E
AC
Core
P1
WOX
H
AC
Core
P1
Unknown
C
AC
Core
P1
Homeodomain TF
F
AC
AC
Core
P1
ARGONAUTE
I
AC
Core
LTP

The lack of transcriptional changes noted in our LM-RNAseq analyses of the *Equisetum* core and P1 domains may reflect the vestigial state of leaves in this lineage. Gene pathways that regulate the development of organs that are no longer under functional selection frequently accumulate mutations. For example, eye degeneration in cavefish is associated with diminished *Pax6* function and dramatically reduced levels of gene expression relative to surface dwelling fish (Meng et al., 2013; Strickler et al., 2001). Alternatively, the *Equisetum* P1 expression patterns may reflect differences in the pathways controlling leaf development in the monilophytes versus the lycophytes and seed plants (reviewed in Vasco et al., 2013).

4.2.5 Putative Markers for Epidermal Cell Function are Expressed in the Outer Cell Layers of ALL SAM Domains

Several crucial genes for angiosperm SAM patterning are expressed across multiple domains of the meristem (McConnell et al., 2001). Toward the identification of similar phenomena within the AC-type SAMs, we found that SOM clusters of *Equisetum* and *Selaginella* transcripts up-regulated across all meristem domains contain a relatively small number of essential SAM patterning genes (Figure 4.6, Clusters (2,3),(2,4); Fig 4.7, Cluster (1,4); Table 4.4; Table 4.5). Notable members of this list include homologs of the SAM-size regulator *CLAVATA2* and the auxin receptor *TRANSPORT INHIBITOR RESPONSE 1* (*TIR1*), which are identified in the *Selaginella* and *Equisetum* up-regulated SAM clusters, respectively (Dharmasiri et al., 2005; Kayes and Clark, 1998). *In situ* hybridization patterns of selected genes upregulated in all three SAM domains further sup-

port our SOM cluster identifications. Transcripts of a *Class I HD-ZIP* transcription factor (Figure 4.8 G) and a *LIPID TRANSFER PROTEIN* (*LTP*) (Figure 4.8 I) accumulate throughout the outer cell layer of the apex. Notably, this pattern is similar to that observed in markers of the angiosperm SAM L1, despite the absence of genetically distinct layering in AC-type SAMs. Localization of this *Selaginella LTP* in the outer cell layer suggests that this protein may be involved in epidermal cuticle development, similar to the putative role of family 1 LTPs in angiosperms (reviewed in Yeats and Rose, 2008). As yet, there are no known angiosperm Class I HD-ZIP genes with analogous expression patterns to the one identified in *Selaginella* (reviewed in Ariel et al., 2007). One possible role for this *Class I HD-ZIP* protein would be in epidermal patterning, a function typically reserved for the *Class IV HD-ZIPs* in angiosperms. Further functional studies of these genes, and many of the other genes identified in this transcriptomic study will deepen our understanding of the developmental processes at work within these AC-type meristems.

4.3 CONCLUSIONS

In agreement with an abundance of paleobotanical, phylogenetic, and comparative anatomical studies of the evolution AC-type SAM structure, our comparative transcriptomic analyses of *Selaginella* and *Equisetum* SAM subdomains provide overwhelming molecular support for the convergent evolution of AC-type SAM structures in these two vascular plant lineages. Questions concerning the location of the stem cell niche in the AC-type SAM, a subject of intense experimental creativity over the past century, are also addressed herein. Our analyses demonstrate that the AC and the core domains are transcriptionally distinct,

similar to the functionally distinct gene expression patterns observed in the CZ and PZ of the angiosperm SAM. Lastly, we identified transcriptional evidence for auxin maxima within the AC of *Selaginella*, suggesting that a developmental mechanism similar to the QC of angiosperm roots may support AC function in the *Selaginella* SAM. The depth and specificity of transcriptomic sequencing generated in this study provides a solid foundation for future investigations into the molecular mechanisms of AC-type SAM function.

4.4 MATERIALS AND METHODS

4.4.1 Plant Culture

14-day-old Maize B73 seedlings were grown under greenhouse conditions. *Selaginella moellendorfii* plants were ordered from Plant Delights Nursery (Clayton, NC) and maintained under a humidity dome at 25 deg C with natural light conditions. *Equisetum arvense* shoot apices were collected from a wild population in Ithaca, NY (GPS coordinates Lattitude: 42 deg 28 ft 28.5846 inch and Longitude: -76 deg 27 ft 1.7058 inch). Total above ground plant samples were harvested for RNA at the same time that shoot apices were collected for laser microdissection.

4.4.2 Laser microdissection and RNA amplificaiton

Laser microdissections and RNA isolations from microdissected samples followed the procedures outlined in (Scanlon et al., 2009). At least 100,000 sq microns were isolated for each biological replicate. Maize apices were microdis-

sected into meristem and P1 domains (Figure 4.1). *Selaginella* and *Equisetum* apices were microdissected into meristem Apical Cell, meristem core, and initiating P1 domains (Figure 4.1). Whole plant samples were prepared by grinding up the entire shoot for each species in liquid nitrogen. RNA was extracted from all collected tissues using a PicoPure RNA Isolation Kit (Life Technologies, cat number KIT0204) and in vitro amplified using a TargetAmp 2-round aRNA Amplification Kit 2.0 (Epicentre, cat number TAU2R51224). Three biological replicates were prepared for maize meristem and leaf samples; two replicates were prepared for all other samples.

4.4.3 Illumina Library Construction and sequencing

Following linear RNA amplification (Epicentre, cat number TAU2R51224) RNA was prepared for Illumina sequencing following the protocols of (Kumar et al., 2012), with modifications for single sample processing. The libraries were ligated to adapters with barcodes 3-nucleotides in length and pooled for 8-plex sequencing on an Illumina HiSeq 2000 at the Cornell University CLC DNA sequencing facility (http://cores.lifesciences.cornell.edu/brcinfo/?f=1).

4.4.4 Sequence processing and differential gene expression analysis

The three base barcode at the 5 prime-end of each read was used to sort sequenced reads into sample types. The barcode sequence was then clipped. Bases with a PHRED quality score ¡ 15 were trimmed from the reads (Ew-

ing et al., 1998a; Ewing et al., 1998b) using the software package Lucy (Li and Chou, 2004). Trimmed reads of each species were aligned to their corresponding reference genomes using GSNAP (Wu and Nacu, 2010) and confidently mapped reads were filtered if it mapped uniquely (less than 2 mismatches every 36 bp and less than 5 bases for every 75 bp as tails) and used for subsequent analyses. Genes having an average of at least one mapped read per million sequence reads in at least two samples were used for the differential expression test. The R package edgeR (http://www.bioconductor.org/packages/2.13/bioc/html/edgeR.html) was used to find differentially expressed genes between samples (Robinson and Oshlack, 2010; Robinson and Smyth, 2007; Robinson and Smyth, 2008). A False discovery rate (FDR) of 5 percent (q-value) was used to account for multiple testing.

4.4.5 Orthologous Gene Family Identification and Differential Expression

Conserved Gene Families were identified using OrthoMCL 2.0 (Chen et al., 2007). BlastP searches were used to identify clusters of reciprocal best blast hits among all six species. For species without an existing set of protein annotations, amino acid sequence of the longest Open Read Frame (ORF) present within an assembled transcript was used (minimum length 80 amino acids). Annotations for the function of a conserved orthologous group were pulled first from *Arabidopsis* TAIR 10 annotations, then from Phytozome's annotations of the *Physcomitrella* genome and finally from the B73 RefGen v2 functional annotations (Goodstein et al., 2012).

The Gene Families were identified as differentially expressed within each species if at least one member of the gene family was significantly differentially expressed according to the EdgeR analysis. Gene Families were determined as having shared differential expression across species if the same family was differentially expressed in more than one species.

4.4.6 Self Organizing Maps and Data Diagrams

Self Organizing Maps (SOMs) were constructed using the R package som (http://cran.r-project.org/web/packages/som/). Average reads per million for genes that were differentially expressed in one or more apical domain to whole plant pairwise comparison were added to a data matrix. The read counts were scaled by row using the normalize function detailed in the som package. SOM parameters for both *Equisetum* and *Selaginella* were as follows: 4 X 5 and 3 X 4 (respectively), and topology = hexagonal.

Triple and Quintupple overlapping Venn diagrams were constructed using the R package VennDiagram. The heatmap of meristem gene expression patterns across species was produced using the R Heatmap function and the color key was made using the Heatmap.2 function in ggplot2 (Wickham, 2009). Meristem genes for each species were identified using Phytozome gene family searches and BLAST annotations for the *Equisetum* transcriptome. Genes that were not expressed or did not have homologs in at least two species were excluded from the heatmap. In many cases there were multiple gene family members for each species, we gave preference to the genes that were expressed in our samples. A data matrix of fold change values between apical domain

and whole plant was used as input for the R heatmap function. Values were scaled by row and the R package colorRamp (http://stat.ethz.ch/R-manual/R-devel/library/grDevices/html/colorRamp.html) was used to produce a gradient of color values corresponding to gene fold change values.

4.4.7 *In situ* hybridizations

The procedures outlined in (Jackson et al., 1991) were followed with slight modifications. *Selaginella* apices were sectioned into slices 8 microns thick. Either full length or partial length cDNA fragments were cloned with gene specific primers. Digoxigenin labeled probes were in vitro transcribed from the cDNA clones following the manufacturers specifications (Roche). All hybridizations were carried out at 50 deg C and SSC wash steps at 55 deg C. Slides were imaged using a Zeiss AxioCam MRc5 camera with a brightfield or DIC lens.

4.4.8 Preparation and assembly of the *Equisetum arvense* transcriptome

Young stems were collected and frozen in liquid nitrogen from a wild population of *Equisetum arvense* in Ithaca, NY. RNA was extracted (Wan and Wilkins, 1994) and 500 ng of total RNA was selected and amplified using the TargetAmp aRNA amplification kit (Epicentre Biotechnologies). The product was purified using the RNeasy Mini Kit (Qiagen) and cDNA libraries were made using the SuperScript Choice System (Invitrogen), with a mix of both polyT and random hexamer DNA primers for first strand synthesis and only random hexamers for

the second strand. cDNAs were purified using the PureLinkPCR Purification Kit (Invitrogen) and libraries were prepared for 454 pyrosequencing using a 454 Genome Sequencer FLX system with titanium chemistry, according to manufacturers instructions (Roche) and then sequenced at the Cornell University CLC DNA sequencing facility (http://cores.lifesciences.cornell.edu/brcinfo/?f=1).

The raw sequence files in SFF format were base called using the Pyrobayes base caller (Quinlan et al., 2008). The sequences were then processed to remove low quality regions and adaptor sequences using programs LUCY (Chou and Holmes, 2001) and SeqClean (http://compbio.dfci.harvard.edu/tgi/software). The resulting high quality sequences were then screened against the NCBI UniVec database and *E. coli* genome sequences to remove possible contamination. Sequences shorter than 30 base pairs were discarded. The processed high-quality sequences were assembled de novo using iAssembler (Zheng et al., 2011). After assembly, the unigenes were annotated by BLAST searches against GenBank (http://www.ncbi.nlm.nih.gov/genbank) non-redundant protein (nr) with a cut off e value of 1e-5.

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CHAPTER 5

CONCLUSION

In this thesis, we analyze meristem cell-enriched transcriptomes to address long-standing questions concerning land plant evolution. In chapter two we explore the developmental genetic programs that control multidimensional versus unidimensional growth in the moss *Physcomitrella patens*. We identify over 4,000 transcript profiles distinguishing 1D protonematal tip cells from 3D gametophore bud cells in the moss *Physcomitrella patens*. While both cell types harbor molecular signatures that are indicative of active cell division, we find that the bud cells are greatly enriched for programs involving meristem development and asymmetric cell division. From this data we propose a model wherein this novel combination of meristem and asymmetric cell division programs allow the unicellular moss meristem to balance its essential functions of self-maintenance and organogenesis.

In chapter three we examined the molecular basis for sporophyte shoot meristem evolution. We ask whether angiosperm meristem patterning genes expressed in the sporophytic SAM of *Zea mays* are expressed in the gametophytic SAMs, and/or in the non-meristematic sporophytes, of the model bryophytes *Marchantia polymorpha* and *Physcomitrella patens*. We identify an abundance of up-regulated genes involved in stem cell maintenance and organogenesis in the maize SAM and in both the gametophytic meristem and sporophyte of moss, but not in *Marchantia*. In addition, we find meiosis-specific genetic programs are expressed in bryophyte sporophytes, long before the onset of sporogenesis. From this data, we suggest that this upregulated accumulation of meiotic gene transcripts suppresses indeterminate cell fate in the *Physcomitrella* sporo-

phyte, and overrides the observed accumulation of SAM patterning genes. We use these findings to build a framework for sporophytic meristem evolution involving the concerted selection of ancestral meristem gene programs from gametophyte-dominant lineages.

In chapter four we investigate the functional relationships amongst the AC-type meristem structures found in *Selaginella* and *Equisetum* and the angiosperm meristem structure found in maize. Our analyses provide transcriptional evidence indicating that pluripotent cell functions reside within the prominent AC. Putative genes for SAM maintenance were found across multiple domains in the *Equisetum* and *Sellaginella* SAMs, implying that multiple domains act in a concerted fashion to promote meristem maintenance in the AC-type SAM. Moreover, the transcriptional profiles for the two AC-type SAMs are decidedly distinct from one another, providing the first molecular support for the convergent evolution of AC-type SAM structures within these vascular plant lineages.

Previous molecular genetic analyses probing these fundamental questions in embryophyte evolution have predominately relied on comparative analyses of angiosperm candidate genes. The data presented in this thesis represents a comprehensive view of meristem transcriptomics in these basal land plant lineages. The depth and specificity of transcriptomic sequencing generated in these studies provides a solid foundation from which future investigations into the molecular mechanisms of AC-type SAM function can be launched. There are however, several notable limitations of this technology. RNA sequencing is a reflection of transcript accumulation patterns, and while it does offer a data rich strategy for selecting candidate genes for future studies, it does not prescribe function. Furthermore, the model species that were analyzed in this dissertation

diverged from the angiosperm lineage over 400 million years ago. In our analyses, we generally assumed that homologous gene sequences encode broadly similar functions in bryophytes, pteriodophytes, and angiosperms. Although we expect that exceptions to these assumptions undoubtedly exist, a number of moss developmental genetic analyses support our presumptions. For example, the calpain protease encoded by the moss *DEK1* complements the Arabidopsis *dek1* mutant, and both maize and moss *dek1* mutants are shootless gametophytes (R. Quatrano, personal communication of submitted data). Notable exceptions include moss *LFY* homologs, which have evolved a divergent function in moss, and fail to complement *Arabidopsis lfy* mutants (Maizel et al., 2005; Sayou et al., 2014).

In order to test the functional significance of interesting genes identified in chapters two and three, we have started generating knockdown, knockout, and protein fusion lines in collaboration with the Bezanilla lab (University of Massachusetts, Amherst). For these functional analyses we selected moss homologs for *BARELY ANY MERISTEM 1*, *SCREAM2*, and a *Class I KNOTTED1-like* HOMEOBOX gene, all of which were up-regulated in the gamtophore bud and gametophore meristem transcriptomes analyzed in chapters two and three. These functional tests will hopefully help demonstrate the efficacy of the LM-RNAseq strategy used in this thesis. Any results from these experiments will be included in the publications that come out of chapters two and three of this thesis.

Ontological staging is another limitation inherent to cross species analyses. The species that were harvested in this study employ different developmental strategies, making it very difficult to compare similar stages of growth. For ex-

ample, *Selaginella* grows through dichotomous branching, while maize and *Equisetum* form axillary branches. Every effort was made to compare samples that were at similar developmental stages. All meristems were harvested while the plants were in a vegetative growth stage, and the bryophyte sporophytes were isolated at early stages of development (approximately one week after fertilization). In spite of this effort, we did run into at least one instance in which different developmental strategies made it difficult to perform cross species comparisons. Namely, the dichotomous branching habit of *Selaginella* made it difficult to detect significantly differentially expressed genes in the meristem core domain, confounding our quantitative comparisons between the two pteridophyte core samples. While differences in ontological staging present challenges to molecular genetic analyses, they are an innate property of any experiment in comparative development.

Perhaps the most conspicuous drawback of this study stems from limited taxon sampling. We focused this project on species with available reference genomes and/or transcriptomes. Thus, each major plant lineage compared in this thesis was represented by a single species. Moreover, the established model organisms for these lineages are not necessarily the most appropriate species for answering our questions. *Marchantia polymorpha* and *Physcomitrella patens* are both situated in more derived positions within the liverworts and mosses, respectively, and thus do not necessarily represent the ancestral habits of these lineages. While it would be desirable to extensively sample across the bryophyte and pteridophyte lineages, such work is beyond the scope of this project. One extension of this work would be to survey these lineages in more depth, and address whether or not our conclusions of the data hold true in a more general sense.

Despite these limitations, our findings demonstrate that LM-RNAseq is an effective tool for rapidly expanding our knowledge of the developmental genetic programs that operate in understudied plant lineages. Furthermore, the comprehensive nature of the data produced through this method offers a new type of information for testing old hypotheses concerning plant evolution. We believe that the methodology employed in these projects will prove invaluable as the rate of genome sequencing far outpaces the speed at which a candidate gene approach can be implemented.

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