The 2D to 3D growth transition in the moss *Physcomitrella patens*

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Abstract

The colonization of land by plants coincided with and was most likely facilitated by the evolution of 3-dimensional (3D) growth. 3D growth is a pivotal feature of all land plants, but most develop in a way that precludes genetic investigation. In the moss *Physcomitrella patens*, 3D growth (gametophores) is preceded by an extended 2-dimensional (2D) growth phase (protonemata) that can be propagated indefinitely. Studies using *P. patens* have thus elucidated some of the molecular mechanisms underlying 3D growth regulation. This review summarizes the known molecular mechanisms underlying both the formation of gametophore initial cells and the development of the 3D growth in gametophores.

Introduction

A crucial episode in the history of life on earth was the colonization of land by plants approximately 470 million years ago [1,2]. Movement to the terrestrial environment exposed plants to harsh conditions such as drought, UV irradiation, high temperatures and gravity without the support of water. Transitioning from water to land therefore demanded a significant change in growth habit. One of the key evolutionary innovations that accompanied the colonization of land was the evolution of three-dimensional (3D) growth. In charophyte algae, the sister group to land plants, growth occurs from apical initial cells that can only divide in one or two planes to produce filaments, mats or branches [3,4]. By acquiring apical initial cells that could cleave in three planes, land plants were able to develop the morphological 'toolkit' (e.g. vasculature, roots, stomata, flowers, seeds) required to survive and thrive on land (Figure 1).

3D growth is a pivotal feature of all land plants, but angiosperms develop in a way that renders genetic investigation of the two-dimensional (2D) to 3D growth transition difficult. This is largely because the onset of 3D growth occurs during early embryogenesis and disrupting 3D growth can lead to lethality. By contrast, in the moss *Physcomitrella patens*, a representative of the earliest land plant lineage, 3D growth is preceded by an extended 2D filamentous growth phase that can be propagated indefinitely. *P. patens* is therefore an ideal model organism in which to genetically dissect 3D growth. This review summarizes the known molecular mechanisms underlying 3D growth in *P. patens*.

The 2D to 3D growth transition in *Physcomitrella*

The *P. patens* life cycle begins with haploid spore germination and the formation of a chloronemal initial cell, which self-renews and divides to produce filaments of chloronemal cells. A chloronemal initial cell will eventually convert into a caulonemal initial cell, which self-renews and divides to produce filaments of caulonemal cells. Chloronemata and caulonemata are collectively known as protonemata [5,6,7]. Caulonemal cells can divide in two planes to form side branch initials. Most of these develop into 2D secondary chloronemata or caulonemata (~95%) but some develop into gametophore initial cells (~5%) to begin the 3D growth trajectory [7,8**,9**]. There are important morphological distinctions between cells that are fated to become '2D' and those that are fated to become '3D'. 2D protonemal initial cells extend by tip growth and the division plane orientation in these cells is roughly perpendicular to the caulonemal cell from which they are derived [10]. Conversely, 3D gametophore initial cells swell by probable diffuse growth, and divide in an oblique manner to form the apical and basal cells of the bud (Figure 2). Subsequent divisions of the apical and basal cells occur obliquely and perpendicular to the first division plane. Two successive rotating cell divisions establish a tetrahedral apical cell, which divides in spiral-like manner to self-renew and produce a mature 3D gametophore bearing rhizoids, antheridia and archegonia [8**].

Cytokinin

It has been known for many years that cytokinin induces gametophore bud formation, and that once 3D form is established, cytokinin signalling is rapidly downregulated [11-15]. Auxin operates antagonistically to cytokinin during this process, and exogenous treatment with auxin can reverse the effects of cytokinin treatment [12]. All groups of the isoprenoid cytokinins, the most common class of cytokinins, are represented in *P. patens* [16], where they bind to CHASE domain-containing histidine kinases that function as *bona fide*

cytokinin receptors. Notably, disruption of receptor function reduces gametophore initial cell formation and perturbs gametophore development [17]. Unlike angiosperms, in which cytokinin affects the patterning of groups of stem cells, cytokinin affects development at the single-cell level in *P. patens*; firstly, to promote the formation of a gametophore initial cell from a caulonemal cell, and secondly to promote differentiation of this initial into a multicellular 3D gametophore [14,17].

Cytokinin negatively regulates *PpMiR534a*, which targets transcripts of *P. patens BLADE-ON-PETIOLE 1/2* (*PpBOP1*/*2*) for cleavage, a positive regulator of gametophore initial cell formation. Deletion mutants of *PpMIR534a* undergo premature bud formation, while gametophore development itself is unaffected [15]. Another gene believed to be positively regulated by cytokinin is *PpCESA5*, a member of the *CELLULOSE SYNTHASE* (*CESA*) gene family that is indispensable for cell expansion and cytokinesis in *P. patens*. *Ppcesa5* mutants are indistinguishable from wild-type plants at the 1-cell, 2-cell and 4-cell stages of bud development. However, during later stages, enlarged and irregular initial cells are formed, resulting in callus-like buds that fail to accumulate crystalline cellulose in their cell walls [18,19] (Figure 3). Cytokinin-responsive gene expression is thus an important mechanism for delivering cytokinin signalling outputs.

Division plane orientation

In Arabidopsis, the microtubule-associated protein TONNEAU1 is required for preprophase band (PPB) formation and for the regulation of oriented cell divisions throughout development [20, 21]. There are two reports that describe how the mitotic spindle is orientated in *P. patens* buds to specify division planes. Both reports corroborate the role of TONNEAU1 in cellular patterning within developing gametophores, since two independent *tonneau1* deletion mutants both exhibit short and radially compressed gametophores [22,23]. In the first study, *tonneau1* deletion mutants fail to correctly orient cell division planes from the onset of bud initiation. Mutants did not form a PPB and thus it was concluded that cell division plane orientation in developing gametophores occurs in a TON1/PPB-dependent manner [22]. In the second study, *tonneau1* deletion mutants did not exhibit defective cell division plane orientation from the onset of bud initiation, but during later stages of gametophore development. The authors suggested that a cytoplasmic microtubule organizing centre known as the ‘gametosome’ is instead responsible for orienting the mitotic spindle during the transition to 3D growth and thus early divisions occur in a TON1/PPB-independent manner [23].

The subcellular localization of the exocyst subunit EXO70.3d alters when a cell decides that it is time to divide; EXO70.3d localizes to cell plates during cell division but otherwise resides in the cytosol. *Ppexo70.3d* mutants are unable to stably maintain stem cell identity and a variety of phenotypes have been observed. *Ppexo70.3d* mutants exhibit delayed or defective cytokinesis in protonemata, and gametophores can develop from chloronemata. In addition, *Ppexo70.3d* mutants exhibit cell division plane defects, which are evident from the second or third division of the gametophore initial. Most of the buds proceed to form a stunted gametophore, but mutants are reproductively sterile. Exceptionally, some mutant buds initiate 3D growth but then undergo developmental arrest or, in other cases, gametophore initials exhibit the capacity to revert to a protonemal cell fate [24].

AP2-like transcription factors

Genes encoding four AP2-like transcription factors are necessary for gametophore induction in *P. patens*. These genes are orthologous to the Arabidopsis genes *AINTEGUMENTA*, *PLETHORA* and *BABY-BOOM* (*APB*), all of which are involved in aspects of angiosperm development. Single disruption mutants of *PpAPB1*, *PpAPB2* and *PpAPB3* are indistinguishable from wild type. However, fewer gametophores are formed in *Ppapb4* disruption mutants and gametophore formation is completely abolished in quadruple mutants of *PpAPB1-4*, even in the presence of cytokinin. Gametophore initial cells are replaced by protonemal initial cells in these lines and hence stem cell identity is perturbed. Interestingly, ectopic expression of *PpAPB4* promotes the formation of supernumerary buds, which implies that *PpAPB4* (at least) is sufficient to drive gametophore development. *PpAPB* gene expression is positively regulated by auxin and distinguishes side branch initial cells with 3D gametophore fate from those with 2D secondary protonemal fate (Figure 2). The expression of all four PpAPB proteins persists in side branch initials that become gametophore initial cells, but expression fades in those initials that acquire a protonemal identity. *PpAPB* genes function redundantly to promote gametophore initial cell formation, with *PpAPB4* as the major contributor. The PpAPBs are considered to be the master regulators of the 2D-3D growth transition [9**].

DEFECTIVE KERNEL 1

DEFECTIVE KERNEL 1 (*DEK1*) encodes a membrane-bound protease, characterized by the presence of 21 transmembrane domains, an internal loop and a highly conserved calpain domain [25]. Cytosolic calpains were lost when plants made the transition to land, leaving DEK1 as the single calpain of land plants. The calpain domain is regarded as the biologically active form of DEK1 [26**,27]. In angiosperms, DEK1 is required for cell wall orientation in the developing embryo [28]. In *P. patens*, *dek1* deletion mutants produce an increased number of gametophore initial cells (~4 times more than wild type), which correlates with upregulation of *PpAPB* gene expression. Furthermore, the first oblique cell division of the gametophore initial cell is unaffected, but cell plate formation during the second division is defective and occurs randomly in *Ppdek1* deletion mutants compared to wild type (Figure 3). Consequently, gametophore development is arrested. Deletion of the loop or linker regions of *PpDEK1* results in the production of intermediate numbers of gametophore initial cells (~2 times more than wild type) and the second division of the apical cell is not usually as severely affected [27,29]. *PpDEK1* is thus a negative regulator of gametophore initial cell formation [26**,27]. The calpain domain of PpDEK1 can complement the *Ppdek1* deletion mutant, and also the Arabidopsis *dek1-3* mutant phenotype. This suggests that, concomitant with the colonization of land, an ancient DEK1 protein was recruited to orient cell division planes in plants, and this role has been functionally conserved across the span of land plant evolution [26**,30].

NO GAMETOPHORES 1

The *NO GAMETOPHORES 1* (*NOG1*) gene was identified using a forward genetics approach and encodes a ubiquitin-associated protein. *NOG1* is absent from algal genomes but is represented by a single copy gene in land plant genomes, with some exceptions. In *P. patens*, *nog1* deletion mutants produce fewer gametophore initial cells than wild type, even in the presence of cytokinin. This correlates with downregulation of *PpAPB* gene expression. Although buds can initiate in these mutants, development usually arrests by the

five-cell stage of gametophore development (Figure 3). Moreover, misoriented cell division planes are observed in these buds as early as the first oblique division of the gametophore initial cell. *PpNOG1* is thus a positive regulator of gametophore initial cell formation [31**]. A homozygous mutation in the rice *NOG1* homologue HEADING AND GRAIN WEIGHT (*HGW*) causes embryo lethality, which implies that the initiation of 3D growth in early embryo development may be affected. Furthermore, *HGW* is co-expressed with genes associated with ubiquitination and proteolysis, suggesting that NOG1 in angiosperms functions as a bona fide ubiquitin-associated protein [32]. Thus, it is plausible that the role of NOG1 in 3D growth regulation has been retained since plants first appeared on land.

CLAVATA

In angiosperms, the CLAVATA (CLV) signalling pathway coordinates stem cell proliferation with differentiation within the shoot apical meristem in a non-cell-autonomous manner [32-36]. No CLV signalling pathway components exist in the chlorophyte or charophyte algae. However, core components of the CLV pathway arose in the earliest land plants, coincident with the evolution of 3D growth [36,37**]. In *P. patens*, there are no CLV2 or CORYNE (CRN) homologues. The core CLV signalling module of land plants thus comprises differentiation-promoting CLV3-like (CLE) peptides, which bind to membrane-localised CLV1/BARELY ANY MERISTEM (BAM) or RPK2 kinases, to activate an intracellular signal transduction cascade. In *P. patens*, there are seven *CLE* genes (*PpCLE1-7*), two *CLV1/BAM*-like genes (*PpCLV1a* and *PpCLV1b*) and a single *RPK2* homologue (*PpRPK2*). Disruption of these genes perturbs the 2D to 3D growth transition. The first oblique division of the gametophore initial cell is misoriented in *PpcleAmiR1-3* and *PpcleAmiR4-7* mutants. The first oblique division of the gametophore initial cell is correct in *Ppclv1a/1b* disruption mutants, but division planes are misplaced thereafter. *Pprpk2* disruption mutants exhibit cell division defects at a later stage of bud development, and these are less severe than those of *PpcleAmiR1-3*, *PpcleAmiR4-7* and *Ppclv1a/1b* mutants. Interestingly, all of these mutant classes produce supernumerary buds, and gametophore development is defective (Figure 3). Hence, CLAVATA signalling in *P. patens* negatively regulates gametophore initial cell formation. Application of *P. patens* CLE peptides prevent stem cell proliferation within the Arabidopsis root apical meristem, indicating that the role for CLAVATA in regulating division plane orientation has been retained throughout the course of land plant evolution [38**].

OTHER PUTATIVE REGULATORS

Compared to protonemal initials, the gametophore initial cell transcriptome is enriched in genes required for regulating biosynthesis and signalling of auxin and cytokinin, which are known to act antagonistically during the 3D growth transition. There is also an enrichment of genes necessary for cytoskeletal control and asymmetric cell division, which include those related to *YODA*, *TOO MANY MOUTHS*, *SHORTROOT* and *SCARECROW*, all of which have well characterized roles in angiosperms. Also, in abundance are genes that contribute towards shoot apical meristem development, such as CLAVATA signalling components [39].

SUMMARY

The 2D to 3D growth transition in *P. patens* is induced by cytokinin and is currently known to be regulated at many levels by microRNAs, transcription factors, cell wall biogenesis genes, microtubule-associated proteins

and cell signalling mechanisms. With mutants in both proteasome (*PpMCB1*, [40]) and N-end rule (*PpATE,* [41,42]) degradation pathways perturbing (but not abolishing) gametophore development, and with roles for *PpDEK1* and *PpNOG1* during this process, it has become increasingly apparent that this growth transition is also regulated by post-translational modifications.

Disruption mutants of *P. patens DEK1*, *NOG1* and *CLAVATA* are affected in gametophore initial cell formation as well as division plane orientation in developing gametophores, processes that are both linked to cytokinin [14,17,26**,27,31**,38**]. It remains unclear how these genes are connected to the cytokinin pathway. However, it seems likely that a cytokinin feedback loop operates within side branch initials, which is initiated by *APB* gene targets and then maintained by DEK1, NOG1 and CLAVATA (Figure 4). The emergence of all of these genes coincided with the evolution of 3D growth and likely facilitated the water to land transition.

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FIGURE LEGENDS

Figure 1: Morphological innovations that emerged during land plant (embryophyte) evolution

Land plants (embryophytes) emerged from the charophyte algae approximately 470 million years ago, coincident with the evolution of 3D growth. All land plants also produce a multicellular sporophyte and undergo an alternation of generations. Other morphological innovations include vasculature, roots (evolved at least twice in land plants), leaves (evolved at least twice in land plants), seeds and flowers. The lycophytes, Monilophytes, Gymnosperms and Angiosperms are all represented as monophyletic clades. For simplicity, the Bryophytes are represented as a paraphyletic group.

Figure 2: 3D gametophore initials are morphologically distinguishable from 2D protonemal initials

Schematic diagram showing the morphological distinction between 3D gametophore (left) and 2D protonemal (right) initials. 2D protonemal initials extend by tip growth and divide parallel to the caulonemal cell from which they are derived (denoted by 'C'). 3D gametophore initials swell by diffuse growth and then divide obliquely to the parental caulonemal cell. Arrows indicate the direction of growth. The gametophore initial is indicated by a red dot (adapted from [8**]).

Figure 3: Gametophore phenotypes of 3D-defective mutants

Schematic diagrams of bud formation in mutants that fail to make the transition from 2D to 3D growth. Representative images at the 2-cell, 4-cell and 5-10 cell stage are shown. The first division of each bud is indicated by a red arrow. Wild type buds produce normal numbers of buds and undergo a normal developmental program. The first oblique division of the gametophore initial cell is misoriented in *PpcleAmiR1-3*, *PpcleAmiR4-7* and *Ppnog1-R* mutants. The first oblique division of the gametophore initial cell is correct in *Ppclv1a/1b* and *Δdek1* mutants, but division planes are misplaced thereafter. Cell division defects are not observed in *Pprpk2* and *Ppcesa5* mutants until later stages of development. *Ppcesa5* mutants produce the same number of buds as wild type. *PpcleAmiR1-3*, *PpcleAmiR4-7*, *Ppclv1a/1b* and *Δdek1* mutants all produce supernumerary buds whereas *Ppnog1-R* mutants produce fewer buds than wild type. All of these mutants undergo premature developmental arrest and fail to produce 3D gametophores.

Figure 4: Speculative model for 3D growth regulation in *P. patens*

(a) Negative regulation of gametophore initial cell formation. CLE peptides bind to membrane-localized CLV1 or RPK2 receptor kinases, to activate a MAPK signalling cascade. This results in the phosphorylation and subsequent activation of the DEK1 calpain domain, the biologically active form of DEK1. The activated DEK1 calpain facilitates the cleavage of an activator of APB gene transcription; APB genes are thus transcriptionally repressed. PM denotes plasma membrane. **(b)** Positive regulation of gametophore initial cell formation. NOG1 binds to a ubiquitinated APB repressor and targets it for proteasome-mediated degradation. APB genes are thus transcriptionally activated, which in turns activates the transcription of cytokinin response regulators. DEK1, NOG1 and CLAVATA then cooperate to coordinate an independent feedback loop for correct division plane orientation in gametophores.