Cytoskeletal discoveries in the plant lineage using the moss *Physcomitrella patens*

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Abstract

Advances in cell biology have been largely driven by pioneering work in model systems, the majority of which are from one major eukaryotic lineage, the opisthokonts. However, with the explosion of genomic information in many lineages, it has become clear that eukaryotes have incredible diversity in many cellular systems, including the cytoskeleton. By identifying model systems in diverse lineages, it may be possible to begin to understand the evolutionary origins of the eukaryotic cytoskeleton. Within the plant lineage, cell biological studies in the model moss, *Physcomitrella patens*, have over the past decade provided key insights into how the cytoskeleton drives cell and tissue morphology. Here, we review *P. patens* attributes that make it such a rich resource for cytoskeletal cell biological inquiry and highlight recent key findings with regard to intracellular transport, microtubule-actin interactions, and gene discovery that promises for many years to provide new cytoskeletal players.

Keywords Actin · Microtubules · Myosin · Kinesin · Organelle transport · Tip growth · Phragmoplast

Introduction

The eukaryotic cytoskeleton is a complex dynamic array of proteins that drive fundamental cellular processes including membrane trafficking, motility, morphology, and division. At the tissue level, the cytoskeleton is central in directing spatial organization across macroscopic length scales. Stemming mainly from studies in opisthokonts, an in-depth understanding, ranging from in vitro reconstitutions of cellular processes, such as actin-based bacterial propulsion (Loisel et al. 1999) to counting the number of molecules required for events such as cytokinesis (Wu and Pollard 2005) has painted a complex picture of the cytoskeleton. However, there are major eukaryotic lineages where our understanding of how the cytoskeleton drives cellular processes remains largely unexplored. This untapped biodiversity may hold unifying evolutionary insights for cell biology, or it may uncover unique

molecular interactions which if deployed strategically could provide future therapeutic solutions for diseases where cell biology has failed.

With respect to cell biology, plants represent one of these understudied lineages. Like the budding and fission yeasts, plants have cell walls and one of the major functions of the cytoskeleton is to pattern that wall to direct cell growth and division (Bashline et al. 2014; Szymanski and Staiger 2018). However, unlike yeasts, multi-cellular plants in addition to building individual cells, also must organize these cells into tissues and ultimately the plant body without the benefit of cell motility. How plants pattern the extracellular matrix over macroscopic length scales from within individual cells is an open and fascinating question.

To address this question, it is useful to identify plant species amenable to molecular genetic and cell biological inquiry. Among plants, the moss *P. patens* has a number of attributes ranging from cytological to genetic that make it a powerful model system to study plant cell biology, and the cytoskeleton in particular. As a bryophyte, the macroscopic *P. patens* plant body is quite small, reaching a maximum height on the order of a couple centimeters. Because of this small size, all tissues are amenable to microscopic observation. Two key genetic attributes, namely the fact that a majority of the plant body is haploid and the ability to integrate DNA via homologous recombination (Schaefer and Zryd 1997; Strepp et al. 1998),

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have further promoted the use of *P. patens* for gene-based discovery investigation, or reverse genetic studies.

P. patens develops from a single-celled haploid spore, which germinates into an essentially two-dimensional array of filaments, known as protonemata (Fig. 1). As the plant matures, adult shooting structures, known as gametophores, develop from the protonemata (Fig. 1). The emergence of the bud, the predecessor to the gametophore, represents a fundamental developmental switch from two- to three-dimensional tissue patterning. At the apices of the gametophores, both male and female organs develop (Fig. 1). A flagellate sperm is released from male organs (antheridia) and swims through the thin film of water present on the plant to the female organs (archegonia), ultimately fertilizing the egg. The fertilized egg develops into the diploid phase of the life cycle known as the sporophyte (Fig. 1). Within the sporophyte, meiosis occurs generating haploid spores in the spore capsule, which are then subsequently released into the environment to produce another generation of plants (Fig. 1).

In the laboratory, the life cycle is reliably completed within 3 months. However, it is also possible to short-circuit the life

cycle and instead vegetatively propagate the plant, which is primarily accomplished by either damaging tissue by homogenization or by generating a suspension of single cells. Shredded moss tissue from any developmental stage rapidly repairs itself by de-differentiating into protonemata. To generate a suspension of single cells, known as protoplasts, the cell walls of protonemata are enzymatically digested. Given proper osmotic pressure, protoplasts are viable, regenerate their cell walls, and eventually polarize producing protonemata leading to the development of an entire plant. Since protoplasts can be easily transformed with exogenous DNA (Schaefer et al. 1991), plants regenerated from transformed protoplasts represent individual transformation events.

Moss, like all land plants, has a cytoskeleton, comprised of actin filaments and microtubules. In many plants, the actin cytoskeleton powers intracellular motility, characterized by vigorous cytoplasmic streaming. Interestingly in moss, organelles move at very reduced velocities compared to plant cells that exhibit cytoplasmic streaming (Furt et al. 2012). Whether cytoplasmic streaming was lost in moss or gained in other lineages is unclear. Nevertheless, the absence of streaming

Fig. 1 *P. patens* life cycle. A haploid spore produces a polarized extension that continues to grow by tip growth to establish the juvenile stage of the plant, known as protonemata. Protonemata produce side branches, or as the plant matures, transition from two- to three-dimensional growth by producing a bud. The bud contains a single apical stem cell. Mature gametophores produce male (antheridia) and female (archegonia) organs at their apex. Flagellate sperm are released from antheridia and swim through a thin layer of water that coats the plant to

an archegonium where it ultimately fertilizes the egg. Fertilization can occur on the same gametophore, or the sperm can swim to a neighboring gametophore. Thus, while *P. patens* primarily self-fertilizes, it can out-cross, which is essential for producing mapping populations. Upon fertilization, the egg develops into the sporophyte, which remains attached to the gametophore. Meiosis occurs in the sporophyte producing spores, which are then released into the environment

represents an opportunity to investigate modes of intracellular transport that would otherwise be masked in cells with cytoplasmic streaming. Because of the simple tissues that are easy to alter genetically, it is possible to query intracellular transport under various conditions including the pharmacological ablation of the cytoskeleton or in plants harboring specific mutations.

Here, we highlight recent key plant cytoskeleton discoveries made in moss. These studies have provided fundamental insights into processes such as intracellular transport, actin-microtubule cross talk, and the identification of novel cytoskeletal-associated proteins. Finally, we suggest several areas where moss promises to continue to make impacts in our understanding of the cytoskeleton.

Kinesin-mediated organelle transport in plant cells

In many plants, actomyosin motility has been considered the dominant force for long-range intracellular transport of vesicles and organelles. Plants have two classes of myosins: myosin VIII and myosin XI. Myosin XI most closely resembles myosin V and is widely acknowledged to power cytoplasmic streaming (Ryan and Nebenführ 2018). Seed plant genomes tend to have at least 10 myosin XI genes. In contrast, moss, which lacks cytoplasmic streaming, only has three myosin XI genes (Peremyslov et al. 2011). Two are functionally redundant and required for polarized growth (Vidali et al. 2010), and the third is significantly more divergent (Peremyslov et al. 2011). Thus, the expansion of myosin XI genes in seed plants may have occurred as a result of tissue complexity and the need to drive streaming in these diverse tissues.

Interestingly, genome sequencing has revealed that plants possess relatively large numbers of kinesin genes in comparison to myosin genes. For example, *Arabidopsis* has 61 kinesins and 17 myosins (Reddy and Day 2001a; Reddy and Day 2001b; Lee and Liu 2004) while *P. patens* has 78 kinesins and only 8 myosins (Lee and Liu 2004; Vidali et al. 2010; Wu et al. 2011; Shen et al. 2012). The retention of so many kinesins in plants and the similar numbers of kinesins between seed plants and moss prompts us to consider the possibility that microtubule-dependent mechanisms may contribute to processes such as intracellular transport (Cai and Cresti 2012). But evidence for kinesin-driven microtubule-based transport in plant cells has been limited by the confounding activity of cytoplasmic streaming and the difficulty of performing reverse genetics with large gene families anticipated to exhibit functional overlap among family members.

However, recent studies in moss have demonstrated that both chloroplasts and the nucleus use kinesin-driven microtubule-based transport (Vidali et al. 2007; Suetsugu et al. 2010; Suetsugu et al. 2012; Miki et al. 2015; MacVeigh-Fierro et al.

2017; Yamada et al. 2017; Yamada and Goshima 2018). Driving forces that move the nucleus are varied and for example depend on the tissue type and developmental stage in animals (Gundersen and Worman 2013). In *Arabidopsis*, myosin XI-i was shown to bind to the nuclear envelope via the SUN/WIP/WIT complex and translocate the nucleus in an actin-dependent manner in root cells (Zhou et al. 2012; Tamura et al. 2013). However, Tamura et al. demonstrated that nuclear movement in response to light, known as photorelocation was unimpaired in mutants lacking myosin XI-i function, suggesting that other mechanisms may also contribute to nuclear movement (Tamura et al. 2013).

In moss protonemal cells, the nucleus exhibits a variety of different movements (Miki et al. 2015). During interphase, the nucleus in the apical cell moves towards the cell tip at the same rate as cell growth maintaining a centralized position within the cell (Fig. 2a). After chromosome segregation, the two daughter nuclei move rapidly towards the cell center of each daughter cell (Fig. 2b), then briefly back towards the newly formed cell plate (Fig. 2c), and finally reverse direction again to the cell center but at a slower speed than the initial movement (Fig. 2d). In a branching cell, shortly before nuclear envelope breakdown, the nucleus migrates a long distance from the center of the subapical cell to the branch site where mitosis and cell division occur.

Using reverse genetics and live cell imaging, recent studies have identified three kinesins that mediate nuclear movement in protonemal cells (Miki et al. 2015; Yamada et al. 2017; Yamada and Goshima 2018). Depletion of any of these three kinesins leads to abnormal nuclear movement or positioning. Because the cytoplasmic microtubule organization has a well-defined orientation, with microtubules plus-ends pointing towards the cell tip in interphase (Fig. 2a), it is easy to identify the direction of cargo transport along microtubules (Hiwatashi et al. 2014). In the absence of the armadillo repeat-containing kinesin (ARK), a plant-specific plus-end directed motor, the nucleus resides near the cell plate (Miki et al. 2015). On the other hand, when the minus-end directed class II kinesin-14, KCH (kinesin with calponin homology domain) is depleted, the nucleus moves towards the tip of the cell (Yamada and Goshima 2018) (Fig. 2a). These data indicate that a balance of plus- and minus-end directed motility functions to properly position the nucleus (Fig. 2a).

Interestingly, during cell plate expansion, while the cytoplasmic microtubules are reforming, the polarity of the microtubules in the apical cell is inverted with respect to an interphase cell (Fig. 2b), with the plus ends near the growing cell plate and the minus ends pointing towards the cell center (Yamada et al. 2017). At this stage just after chromosome segregation, the class VI kinesin-14, kinesin-like calmodulin-binding protein (KCBP), a minus-end directed kinesin, localizes to the nuclear surface and helps the nucleus move rapidly towards the cell center. However, as interphase

Fig. 2 Nuclear movements in *P. patens* protonemata. **a** Kinesin-dependent microtubule-based motility positions the nucleus during interphase. Two representative fluorescence images of wild type and Δkch apical protonemal cells expressing histone-mRFP labeling the nucleus (magenta) demonstrate that in the absence of KCH, the nucleus resides at the tip of the cell. Autofluorescence was used to image the chloroplasts (cyan). Scale bars, 20 μm . The diagram on the right illustrates that interphase cytoplasmic microtubules have their plus ends pointing towards the tip. Antagonistic plus- and minus-end directed kinesins help to keep the nucleus at the cell center. **b** Diagram illustrates that just after chromosome

segregation, as the cell plate is being built in the middle of the cell, the cytoplasmic microtubule organization in the apical cell is reversed from what is observed in interphase. At this stage, the minus-end directed kinesin, KCBP associates with the nuclear envelope and moves the nucleus rapidly to the middle of the cell. **c** During the final stages of cell plate maturation, the cytoplasmic microtubules in the apical cell re-orient and the nucleus moves slightly backwards towards the cell plate. **d** After re-establishment of the interphase cytoplasmic array in the apical cell, ARK transports the nucleus to the center of the apical cell

progresses, cytoplasmic microtubules switch polarity generating the interphase microtubule array (Fig. 2c, d), which has microtubules with their minus ends pointing towards the cell center. During this reorientation, the nucleus briefly moves back towards the cell plate (Fig. 2c). With a fully established cytoplasmic microtubule array, the nucleus then moves to the cell center in an ARK kinesin-dependent manner (Fig. 2d). Taken together, these studies provide excellent examples of how the moss model system has begun to and will continue to untangle the contribution of individual kinesins within the large kinesin superfamily. Future studies will provide insights into how these kinesins are regulated throughout the cell cycle, and how the nucleus is guided in other tissue types as well as during branching in protonemata.

Studies in moss are also contributing to our understanding of chloroplast positioning and motility (Suetsugu et al. 2012; MacVeigh-Fierro et al. 2017; Yamada et al. 2017). Chloroplasts are essential organelles required for photosynthesis. As such, chloroplast positioning is tightly regulated to optimize photosynthetic efficiency. In weak light, chloroplasts accumulate to capture light efficiently. In contrast, strong light induces chloroplast avoidance. Recent studies have identified kinesins that drive microtubule-dependent chloroplast transport in moss. Depletion of a class II kinesin-4, Kinesin4II, which does not associate with mitotic structures and is predicted to be a plus-end directed motor (Shen et al. 2012; Miki et al. 2014), leads to the inability of chloroplasts to avoid high light (MacVeigh-Fierro et al. 2017) (Fig. 3a). During interphase, KCBP transports chloroplasts (Fig. 3b) as well as the nucleus to microtubule minus-ends (Fig. 2b). Independent of light, depletion of KCBP causes abnormal accumulation of

chloroplasts at the cell tip (Yamada et al. 2017) (Fig. 3b) where microtubules plus-ends are coalesced. This accumulation may result from unbalanced antagonistic forces leading to excessive plus-end directed transport, which suggests the presence of an as of yet unidentified plus-end directed motor antagonizing KCBP resulting in uniform chloroplast distribution along the length of the cell (Fig. 3b). These studies point to two systems controlling chloroplast movement/positioning: (1) a light-dependent chloroplast repositioning, and (2) a light-independent mechanism that maintains chloroplasts evenly distributed along the length of the cell.

Interestingly in basal land plants, such as mosses and ferns, chloroplasts photorelocate in response to both red and blue light (Kadota et al. 2000; Sato et al. 2001; Kawai et al. 2003). Using actin and microtubule depolymerizing drugs, Sato et al. showed that actin mediates the blue light response, while microtubules mediate both red and blue light responses (Fig. 3c). In seed plants, however, chloroplasts only respond to blue light (Zurzycki 1980). In Arabidopsis, actin reorganizes around chloroplasts upon light irradiation and this chloroplast-associated actin network is thought to generate the driving force for chloroplast movement (Kadota et al. 2009; Kong et al. 2013). Similar results have also been described in moss (Yamashita et al. 2011) (Fig. 3c), indicating that actin-dependent motility appears to be conserved from moss to seed plants.

An ancient connection, however, between microtubule and actin-mediated chloroplast movement may have recently been uncovered. The class V kinesin-14, also known as kinesin-like protein for actin-based chloroplast movement (KAC), was identified in a screen for Arabidopsis mutants exhibiting

Fig. 3 Chloroplast movements in *P. patens* protonemata. **a** High light induces an avoidance response, which in *P. patens* depends on Kinesin4II (Kin4II)-mediated microtubule-based transport. **b** Independent of light, chloroplasts are evenly distributed along the length of the protonemal cell. Autofluorescence images of chloroplasts in wild type and $\Delta kcbp$ apical protonemal cells show that in the absence of KCBP, chloroplasts accumulate in the center of the cell. Scale bars, 20 μ m. The diagram at the bottom illustrates that chloroplast positioning likely relies on antagonistic plus- and minus-end directed kinesins as well as on KAC-dependent formation of actin filaments around the chloroplast. KAC appears to have lost microtubule-binding and gliding

activities, but the existence of a connection between the actin- and microtubule-based transport mechanisms is currently unknown. **c** Chloroplast photorelocation in response to blue light is mediated by both actin and microtubules. In response to blue light, KAC-dependent actin formation results in chloroplast motility. The molecular basis of the microtubule-based response is currently unknown. In response to red light, chloroplasts are transported on microtubules. Since Kin4II is the motor responsible for high fluency light avoidance, Kin4II presumably mediates this in response to red light. However, the molecular basis for the accumulation response is an open question

defects in chloroplast motility (Suetsugu et al. 2010). In the absence of KAC, chloroplasts were detached from the plasma membrane and unable to move in response to light (Suetsugu et al. 2010). Live imaging with the talin live-cell actin marker (Kost et al. 1998) revealed that chloroplast-associated actin did not form in the *kac1kac2* mutant, suggesting that KAC is a key regulator of chloroplast-associated actin formation. Interestingly, KAC was found to not bind microtubules and one of the KAC homologs in moss did not exhibit any microtubule gliding activity (Suetsugu et al. 2010; Jonsson et al. 2015). Even though KAC is classified as a member of the kinesin-14 subfamily, KAC instead binds actin filaments in vitro (Suetsugu et al. 2010). Given its biochemical activity, it is likely that KAC plays a role in chloroplast motility via the actin cytoskeleton rather than the microtubule cytoskeleton. In moss, depletion of the two KAC homologs resulted in abnormal chloroplast accumulation around the nucleus (Suetsugu et al. 2012). The accumulated chloroplasts were unable to photorelocate, and actin dynamics was altered in lines silencing both KAC genes (Shen et al. 2015). These data further support that KAC activity functions through the actin cytoskeleton (Fig. 3a). However, the striking similarity to kinesins suggests that at some point in evolution, this molecule may have been coordinating both actin- and microtubule-dependent chloroplast movement.

In moss, microtubule-dependent transport underlies both nuclear and chloroplast motility. In contrast in seed plants, the

nucleus and chloroplasts are primarily thought to move along the actin cytoskeleton (Shimmen and Yokota 2004; Shimmen 2007; Tamura et al. 2013). However, recent studies in tobacco identified light-independent microtubule-mediated chloroplast dynamics (Kumar et al. 2018; Erickson et al. 2018). These studies found that stromules, tubular extensions from the chloroplast surface, extend along microtubules but not along actin filaments. In fact, actin filaments are dispensable for stromule extension. Instead, actin was found to provide stromule anchor points (Kumar et al. 2018). The direction of stromule extension correlated strongly with the direction of chloroplast movement. However, it is still unclear whether stromules guide chloroplasts or generate the driving force for motility. Nevertheless, these studies demonstrate that microtubule-mediated chloroplast movement is present in seed plants and is thus not limited to only basal lineages. In addition, the strong conservation of kinesins from mosses to seed plants and the ability of KCBP to bind and transport phospholipid vesicles (Yamada et al. 2017) suggest that general transport may be mediated by these conserved kinesins across land plants.

Actin and microtubule interactions during cell division and polarized growth

Cytokinesis is a complex and consequential biological process that has captured the fascination of cell biologists for decades.

Because plant cells are encased in cell walls, cell shape and subsequent tissue development are greatly influenced by where cell division planes are positioned (Facette et al. 2018). In most plant cells, the division plane is marked before nuclear envelope breakdown by a microtubule-based structure called the preprophase band. The preprophase band microtubules disassemble during prophase but several preprophase band proteins are left behind at the cell cortex marking the site where the new cell wall will be inserted during cytokinesis. Unlike animal and fungal cells, which rely on an actomyosin contractile ring constricting the plasma membrane to form a cleavage furrow, plant cytokinesis involves the *de novo* synthesis of membrane and cell wall material to build a new partition between the two daughter cells. After anaphase, a structure composed of two antiparallel microtubule arrays called the phragmoplast originates in the center of the cell. Golgi-derived vesicles are delivered along the microtubule tracks accumulating in the center of the microtubule array in a disk-shaped structure called the cell plate, which expands outward. Eventually, the cell plate is connected to the parental wall and completes the separation of the two daughter cells (Müller and Jürgens 2016; Rasmussen and Bellinger 2018).

Although the cell plate is built from the center and expands outward, where it will be inserted into the parental wall is actually determined by molecular landmarks on the cell cortex left behind by the preprophase band. How a plant cell determines the future division plane and how this information is transmitted from the cell cortex to the phragmoplast are fascinating questions (Rasmussen and Bellinger 2018). The cellular machinery involved in this well-orchestrated process has been steadily uncovered over the years (Smertenko et al. 2017). Not surprisingly, microtubules and many microtubule-binding proteins play important roles in these processes. In addition to microtubules, actin is also present in the preprophase band and phragmoplast. However, since cytokinesis can proceed in the absence of actin, it has been less clear how actin contributes to cell division.

Using moss protonemal filaments as a model, Wu and Bezanilla probed the function of actin in cytokinesis and discovered that actin nucleators localize to the phragmoplast midzone, where actin filaments are generated (Wu and Bezanilla 2014). Live cell imaging revealed that cell plate expansion occurs within a dynamic meshwork of actin filaments linking the phragmoplast midzone to the cell cortex (Fig. 4). This study also found that class VIII myosins together with actin guide phragmoplast expansion. Plants lacking all five class VIII myosins have inappropriately positioned cell plates, particularly in branching cells. Furthermore, myosin VIII was found to localize to microtubule plus ends in the phragmoplast midzone. In the absence of either actin or myosin VIII, peripheral phragmoplast microtubules were detached from the expanding cell plate resulting in aberrant cell plate deposition, indicating that myosin VIII acts to link

microtubules to actin filaments thereby guiding cell plate expansion (Fig. 4) (Wu and Bezanilla 2014).

Expansion of the cell plate is an example of polarized secretion in plant cells. As described in moss, microtubules and actin work together to coordinate the direction of expansion (Wu and Bezanilla 2014). Plant cell growth, in general, relies on secretion of cell wall material, and subsequent cell patterning depends on spatial regulation of the deposition of cell wall carbohydrates and cell wall modifying enzymes (Eng and Sampathkumar 2018). How spatial regulation of secretion is achieved is an active area of investigation. A subset of plant cells, such as root hairs, pollen tubes, and the protonemal cells of moss, have a very simple elongated geometry. These cells grow by polarized secretion of flexible cell wall at the apex of the cell, where local cell expansion occurs due to high internal turgor pressure (Rounds and Bezanilla 2013). Actin is absolutely essential for tip growth and studies from a variety of tip growing systems, including in the last decade from moss protonemata, have provided mechanistic insights into the regulation of actin dynamics required for polarized growth (Vidali and Bezanilla 2012; Bascom et al. 2018). In contrast to actin, it is less clear how microtubules impact polarized growth, which may be a result of the fact that microtubule depolymerizing drugs have a varied effect on tip growth in different cell types. For example, loss of microtubules in angiosperm pollen tubes has no effect on growth (HESLOP-HARRISON et al. 1988; Åström et al. 1995). Other cell types such as root hairs and moss protonemata continue to grow in the absence of microtubules. However, without microtubules, root hairs and protonemata often produce multiple tips or change their growth direction frequently (Doonan et al. 1988; Bibikova et al. 1999).

Recent findings in moss have revealed the importance of actin-microtubule coordination for maintaining persistent polarized growth (Wu and Bezanilla 2018). As described, moss protonemal cells have cytoplasmic microtubules oriented along the long axis of the cell with their plus ends pointed towards the cell apex (Fig. 2a). The activities of plant-specific kinesins, KCH, and a class II kinesin orphan known as kinesin for interdigitated microtubules (KINID) are required to coalesce the microtubule plus ends at the cell tip (Fig. 4) (Hiwatashi et al. 2014; Yamada and Goshima 2018). In an actively growing cell, a cluster of actin filaments is also found near the cell apex (Vidali et al. 2009; Furt et al. 2013), overlapping with the coalescing microtubules (Wu and Bezanilla 2018). This actin cluster is composed of dynamic actin filaments, but is stably present over long periods of times during growth. The position of the actin cluster closely correlates with the site of cell expansion (Wu and Bezanilla 2018). Wu and Bezanilla found that myosin VIII localizes to the clustered microtubule plus ends and links the two cytoskeletons (Fig. 4). Coalescing microtubule plus ends intersecting with the actin cluster ensure the delivery of the actin

Fig. 4 Similarities between actin-microtubule interactions in tip growth and cytokinesis. Left, confocal images of lifeact-mEGFP labeling actin (green) and mEGFP-tubulin labeling microtubules (magenta) in the phragmoplast midzone (top) and near the cell apex (bottom). Scale bars, 5 μ m. Right, diagrams illustrating the actin and microtubule interactions occurring in the boxed region of the phragmoplast and at the cell apex in a tip-growing cell. In both cases, microtubule plus-ends are linked to a highly dynamic actin structure. During cell division, cell plate expansion occurs along the plane dictated by the actin filaments and myosin VIII

coordinates the interaction between microtubule plus-ends and actin. KINID is indispensable for the formation of interdigitated antiparallel microtubules in the phragmoplast midzone, which are required for cell plate expansion (Hiwatashi et al. 2008). At the apex of a growing cell, KINID and KCH focus the plus ends of the microtubules, while the dynamic actin cluster predicts the site of cell expansion. Without focused microtubules, or without the link to microtubules mediated by myosin VIII, actin cluster formation and cell expansion become irregular

nucleating factor class II formins to the established site of actin polymerization, therefore maintaining persistent directional growth (Wu and Bezanilla 2018).

Forward genetics, a tool to identify new cytoskeletal associated proteins

Highly efficient gene targeting in *P. patens* coupled with its predominant haploid lifestyle has driven reverse genetic approaches, rather than phenotype-based discovery, or forward genetics. Paradoxically, generating mutants in *P. patens* is rather straight-forward and was first accomplished several decades ago (Ashton et al. 1979). However, identifying the causal lesions responsible for the mutant phenotypes proved to be challenging. A candidate gene-based complementation approach was the first successful method used to identify one of the original mutations generated in *P. patens* 30 years after

isolating the mutant (Prigge et al. 2010). However, now with the ease of genome sequencing, it has become possible to identify the causal lesions underlying mutants of interest (Stevenson et al. 2016; Moody et al. 2018).

In one particularly elegant study, Ding et al. carried out a conditional genetic screen for mutants that no longer grew at 32 °C, a temperature that supports wild-type *P. patens* growth (Ding et al. 2018). Isolating conditional mutants was a key advantage because mutant plants grew relatively normally at 25 °C, the permissive temperature, enabling completion of their life cycle, which was required to generate a mapping population. Using whole genome re-sequencing, Ding et al. discovered that *CONDITIONAL LOSS of GROWTH 1* (*GLoG1*) encodes an ancient protein conserved in amoeba and across the entire land plant clade. While live cell imaging demonstrated that CLoG1 associates with microtubules during interphase and cell division, loss of function studies demonstrated that *clog1* mutants divide normally. These data

suggest that CLOG1 plays an essential role during cell growth. CLOG1 associates preferentially with depolymerizing microtubule ends and silencing CLOG1 affects microtubule dynamics, suggesting that CLOG1 plays a critical role in regulating dynamics of the interphase microtubule array (Ding et al. 2018). This study is an excellent example of the power of moss genetics and cell biology for identifying novel cytoskeletal regulators.

Future directions

Genetic manipulation in *P. patens* using homologous recombination to integrate transgenes into the genome has been very successful. However, there have been a number of limitations, including the fact that there are few selectable markers available for transformation. To generate deletions in multiple genomic loci, the selectable markers need to be removed so that they can be used again for subsequent transformations. While removal has been straight-forward using CRE-lox technology (Sauer 1998), it is nevertheless time-consuming. Recently, CRISPR-mediated genome editing has been used to generate knock outs, including deletions in multiple loci from a single transformation (Lopez-Obando et al. 2016; Collonnier et al. 2017; Leong et al. 2018). Because the Cas9 and single guide RNAs need only be expressed transiently, the selection cassette never integrates into the genome and it is possible to re-transform with the same selectable marker. Thus, CRISPR-mediated genome editing promises to be a particularly powerful approach in moss and will make it feasible to rapidly accomplish many of the proposed future directions.

Cell division in moss protonemata occurs without a preprophase band in both apical cells, which divide symmetrically, and branching cells, which divide asymmetrically (Doonan et al. 1985). Interestingly, a mutant in *Arabidopsis* was recently reported to undergo normal cell division even in the absence of microtubules in the preprophase band (Schaefer et al. 2017). Together these data suggest that microtubules might not be the essential element in the preprophase band required to mark the future division plane. In fact, moss protonemata may represent an evolutionarily conserved system containing a minimal set of components required for marking the future site of cell division, providing an ideal genetic and cell biological system to identify division site markers in the preprophase band independent of microtubules.

Towards this end, a study demonstrated that one of the *P. patens* class VIII myosins localizes to the preprophase band and cortical division zone when expressed in tobacco BY-2 cells (Wu and Bezanilla 2014). In moss, myosin VIII localized to a band in the cortical region of the side branch where the future cell wall is inserted (Wu and Bezanilla 2014). While there is no known marker for either the preprophase band or the cortical division site in moss, the localization of myosin

VIII is reminiscent to the preprophase band and cortical division zone and suggests that myosin VIII may mark these structures. Future studies aimed at identifying landmark proteins conserved from moss to seed plants will help to establish the minimal components comprising the preprophase band and cortical division zone as well as the possible role of myosin VIII and actin in these structures.

Work in moss has also revealed interesting similarities between cytokinesis and tip growth. In both cases, microtubule plus ends are embedded in a meshwork of actin filaments (Wu and Bezanilla 2014; Wu and Bezanilla 2018). Both places are sites of heavy membrane trafficking and carry high volumes of vesicles containing cell wall material. These Golgi-derived vesicles are transported on microtubule tracks during cytokinesis, raising the possibility that microtubule-dependent transport may occur during tip growth as well as more generally in plant cells. In the future, drawing comparisons between tip growth and cytokinesis while investigating the functions of new proteins might accelerate our understanding of both processes.

The simple moss body plan may also help to unlock a number of fundamental unanswered questions. For example, to build a new plant body, regenerating protoplasts must first re-build their cell wall. Armed with a new cell wall, a protrusion emerges from the protoplast generating the highly elongated protonemal cells. Thus, protoplast regeneration provides a system to study both de novo cell wall synthesis as well as establishment of cell polarity. In addition to protoplast regeneration, polarity establishment occurs in the germinating spore as well as in side branch formation in the filamentous protonemata (Fig. 1). While polarity establishment has been well studied in yeast and animals, plants and other eukaryotes do not necessarily have identifiable homologues of yeast and animal polarity proteins. Thus, deciphering the molecular basis of the establishment of polarity during protoplast regeneration as well as branch formation in moss will help identify components found in plant systems and may provide a framework to identify commonalities across many diverse eukaryotic systems.

The transition from juvenile filamentous protonemata to adult shooting structures requires a switch from two- to three-dimensional tissue morphogenesis. Strikingly, this switch occurs in a single stem cell in the bud (Fig. 1), which gives rise to all the cells in the gametophore, representing an extremely simplified developmental system (Harrison et al. 2009; Kofuji and Hasebe 2014). Cell expansion within the gametophore occurs in a developing tissue that is only a single-cell layer thick. Coupled with recent advances using microfluidic chambers that have successfully imaged the entire process of gametophore formation (Bascom et al. 2016) and forward genetics for gene discovery (Moody et al. 2018), gametophore development promises to be an excellent model system for analyzing tissue morphogenesis in plants.

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Compliance with ethical standards

Conflict of interest Shu-Zon Wu declares that she has no conflict of interest. Moe Yamada declares that she has no conflict of interest. Darren R. Mallett declares that he has no conflict of interest. Magdalena Bezanilla declares that she has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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