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Structure, function and evolution of stomata from a bryological perspective

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Abstract

Stomata are key innovations for the diversification of land plants. They consist of two differentiated epidermal cells or guard cells and a pore between that leads to an internal cavity. Mosses and hornworts are the earliest among extant land plants to have stomata, but unlike those in all other plants, bryophyte stomata are located exclusively on the sporangium of the sporophyte. Liverworts are the only group of plants that are entirely devoid of stomata. Stomata on leaves and stems of tracheophytes are involved in gas exchange and water transport. The function of stomata in bryophytes is highly debated and differs from that in tracheophytes in that they have been implicated in drying and dehiscence of the sporangium. Over the past decade, anatomical, physiological, developmental, and molecular studies have provided new insights on the function of stomata in bryophytes. In this review, we synthesize the contributions of these studies and provide new data on bryophyte stomata. We evaluate the potential role of stomata in moss and hornwort life histories and we identify areas that will provide valuable data in ascertaining the evolutionary history and function of stomata across land plants.

Introduction

Although the existence of stomata in the earliest fossil plants from the upper Silurian and the ubiquitous occurrence across tracheophytes identify stomata as important innovations in plant evolution, the paucity of studies focusing on bryophytes has limited our understanding of stomatal biology and evolution. Studies over the past decade have begun to address important questions from morphology, development and physiology, but the central questions on the evolution and function of stomata in early land plants remain elusive. The absence of stomata in liverworts and in several lineages of mosses and hornworts has confounded interpretations of evolutionary change in stomata based on phylogenetic analyses. As inferred by the Qiu *et al.* (2006) hypothesis that liverworts are sister to the remaining land plants, stomata originated following the liverwort divergence with a number of subsequent losses in mosses and hornworts. The evolution of bryophyte stomata is more complicated if hornworts are basal or if bryophytes are monophyletic as hypothesized more recently by Wickett *et al.* (2014) and Cox *et al.* (2014). Clearly, resolving the phylogenetic position of the mosses, hornworts and liverworts and whether they are monophyletic or paraphyletic, is key to establishing the origin and modifications of stomata through evolutionary history.

This paper explores current findings related to the unique stomata of bryophyte and the contributions of studying bryophytes to the general knowledge of the structure, development and function of stomata in plants. We identify the specific challenges of studying stomata in bryophytes and point to areas for future study that are critical in understanding the evolution of stomata with or without a resolved phylogeny.

Stomata occurrence in bryophytes

Stomata in bryophytes are located exclusively on the sporophyte. Typically, in mosses they are found at or near the base of the sporangium (capsule) and in hornworts along the length of the sporophyte, which is a growing sporangium anchored by a foot. Although the early fossil record demonstrates the occurrence of stomata on sporangia in polysporangiates (Edwards *et al.* 1998), bryophytes are the only living embryophytes with this condition. Losses of stomata are readily interpreted from current phylogenetic hypotheses in both hornworts and mosses (Renzaglia *et al.* 2017). The occurrence of stomata in *Leiosporoceros*, the sister taxon to other hornworts, supports plesiomorphy in hornworts, with two losses in the highly reduced genus *Notothylas* and the more derived *Megaceros*/ *Nothoceros*/ *Dendroceros* lineage (Fig. 1) (Renzaglia *et al.* 2008; 2017).

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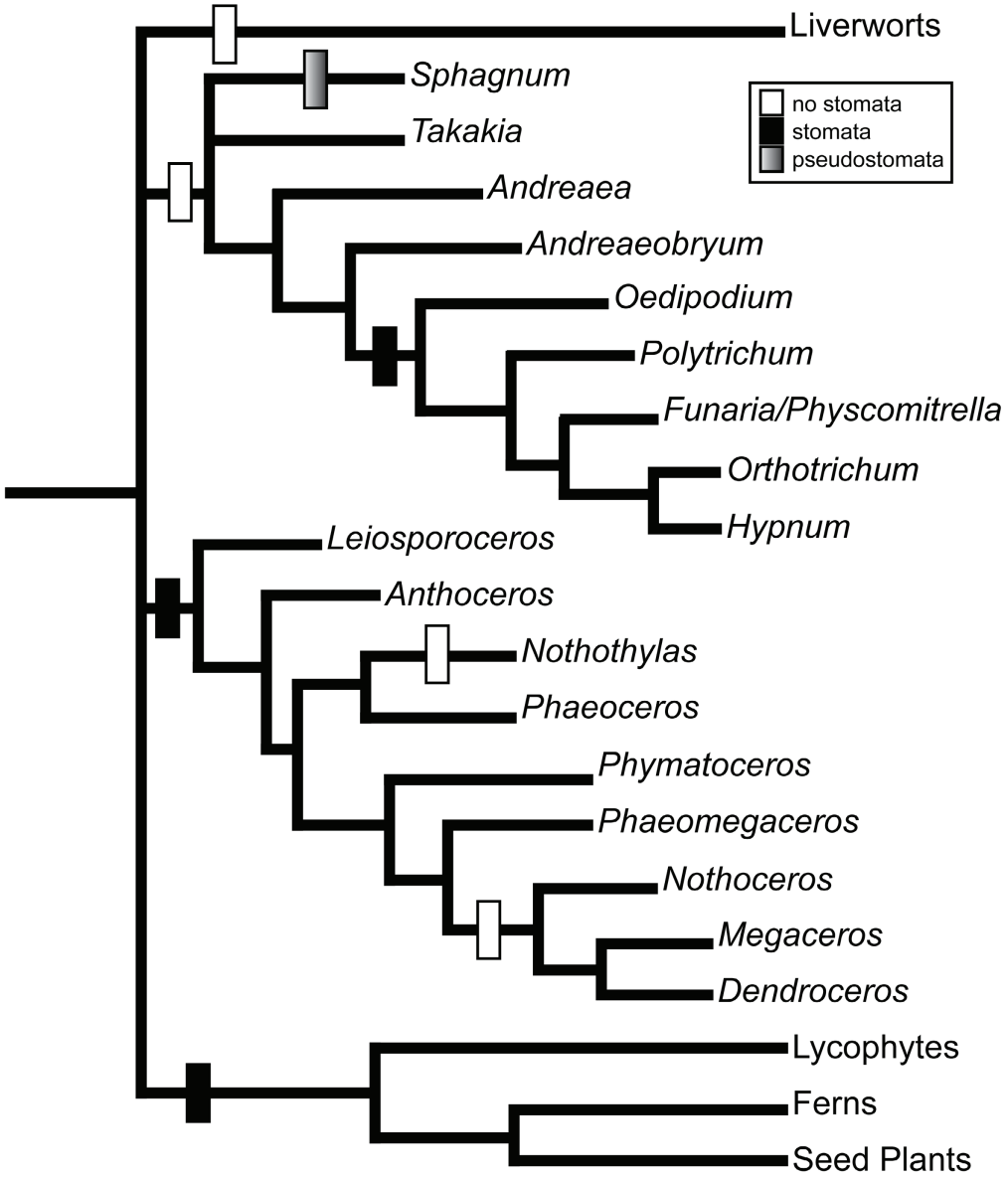

FIGURE 1. Phylogenetic tree of stomata evolution in land plants.

The picture is less clear in mosses where early divergent taxa such as *Takakia*, *Andreaea* and *Andreaeobryum* are entirely devoid of stomata, and the homology of pseudostomata in Sphagnopsida and stomata of true mosses (Bryophytina) is controversial (Fig. 1) (Merced 2015b). Stomata are widespread from *Oedipodium* throughout peristomate mosses but are absent in scattered taxa. Within mosses, stomata-less genera or species are interspersed among stomata-bearing taxa. For example, species of *Dicranella, Dicranodontium, Grimmia, Cryphaea,* and *Leucodon* have stomata, while others lack them. Stomata are absent in *Atrichum* and *Pogonatum* while other genera of the Polytrichaceae have them. There seems to be no relation between the absence of stomata and the size of the capsule (Merced & Renzaglia 2013); habitat conditions may influence the number, position and distribution of stomata in bryophytes but do not appear to be responsible for the absence of stomata (Paton & Pearce 1957).

In surface view stomata of bryophytes are similar to those in tracheophytes and consist of specialized guard cells with a pore between that leads to a substomatal cavity. Guard-cell number is normally two (Fig. 2A, B, C), but stomata of 3–5 cells are frequently found in unrelated mosses (Fig. 2 E, F) and are probably related to abnormal development (Paton & Pearce 1957, Hedenäs 2007, Field *et al.* 2015). The Funariaceae are characterized by a single binucleate guard cell (Fig. 2D, G), also documented in species of *Polytrichum, Polytrichastrum* and *Buxbaumia* (Paton & Pearce 1957). This type of stoma results from an incomplete cytokinesis, since the cell walls do not reach the ends of the cells (Sack & Paolillo 1985). Stomata can be at the same level as epidermal cells (Fig. 2B, C, E), slightly raised (Fig. 2A), raised significantly (Fig. 2H), slightly sunken (Fig. 2K) or sunken (Fig. 2I, J). Pores in some mosses are round (Fig.

2D) or elongated (Fig. 2G). The distinctive pseudostomata of *Sphagnum* consist of two cells that partially separate but do not form a pore or substomatal cavity (Fig. 2K–M). In hornworts guard cell are always two, with an elongated pore aligned with the length of the sporophyte and stomata are raised slightly above the epidermis when first formed (Fig. 2N) (Pressel *et al.* 2009). Asymmetrical stomata with oblique pores occur in proximity to the suture line in hornworts and may contribute to dehiscence of the cylindrical sporophyte (Villarreal & Renzaglia 2015).

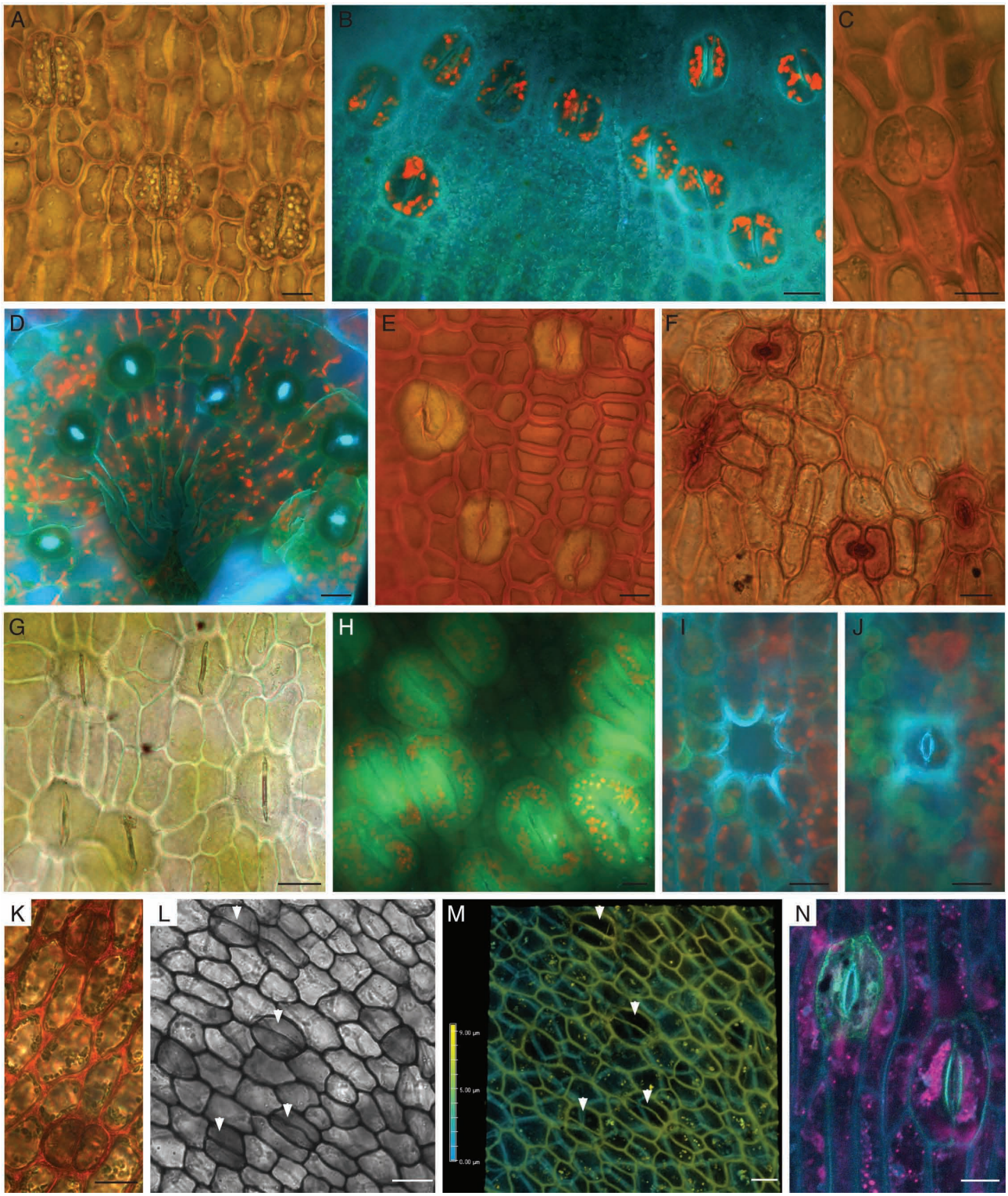

FIGURE 2. Stomata diversity in bryophytes (bright field, fluorescence and confocal microscopy). A. *Pohlia*. B. *Bartramia* guard cells with chloroplasts (orange) in fluorescence microscopy. C. *Pleurozium*. D. Fluorescence image of *Physcomitrella* sporophyte with stomata. E. *Hypnum*. F. *Fissidens*. G. *Funaria*. H. *Polytrichum* stomata in fluorescence microscopy. H–I. Fluorescence images of sunken stomata of *Orthotrichum* at the epidermal level (H) and at pore (I). K–L. Pseudostomata of *Sphagnum*. M. Depth coded 3D reconstruction of epidermis and cortex of *Sphagnum* capsule, color represents cells at the same level (same as L). N. *Phaeoceros* confocal image of guard cells with chloroplasts (purple). Scale bars = 20µm.

Stomata size, number and distribution

Guard cells of many bryophytes are the same size or larger than epidermal cells (Fig. 2E, H, N) in contrast to smaller guard cells typical of tracheophytes (Fig. 3). In cross section, stomata of bryophytes vary in size, with hornwort guard cells considerably larger than epidermal cells (Fig. 3A) and those of mosses smaller than surrounding cells (Fig. 3B). Variability in cross section of stomata across model species is evident in the hornwort *Anthoceros* (Fig. 3A), moss *Physcomitrella* (Fig. 3B), lycophyte *Selaginella* (Fig. 3C) and flowering plant *Arabidopsis* (Fig. 3D). Stomata in *Arabidopsis* are remarkably small in comparison to surrounding cells (Fig. 3D). Stomata size is positively correlated with genome size in angiosperms and negatively correlated with stomatal density, however adaptations to habitat can affect guard cell size (Beaulieu *et al.* 2008, Jordan *et al.* 2015). The relationship between guard cell size (including pore size) and stomata density in tracheophytes is important because it determines the diffusive conductance of CO_2 and the efficiency of guard cell function (Franks & Beerling 2009). No correlation between guard cell and genome size exists in hornworts (Renzaglia *et al.* 2017) but this relationship is yet to be tested in mosses. Bryophyte stomata density, aperture and size do not respond to differences in environmental CO_2 concentrations (Baars & Edwards 2008, Field *et al.* 2015). Measurements of stomatal density are rarely used in bryophytes because guard cells are not uniformly distributed and the size of the area where stomata are located varies greatly. Differences in the number of stomata within species probably result from changes in growing conditions (Paton & Pearce 1957, Erzberger 2003).

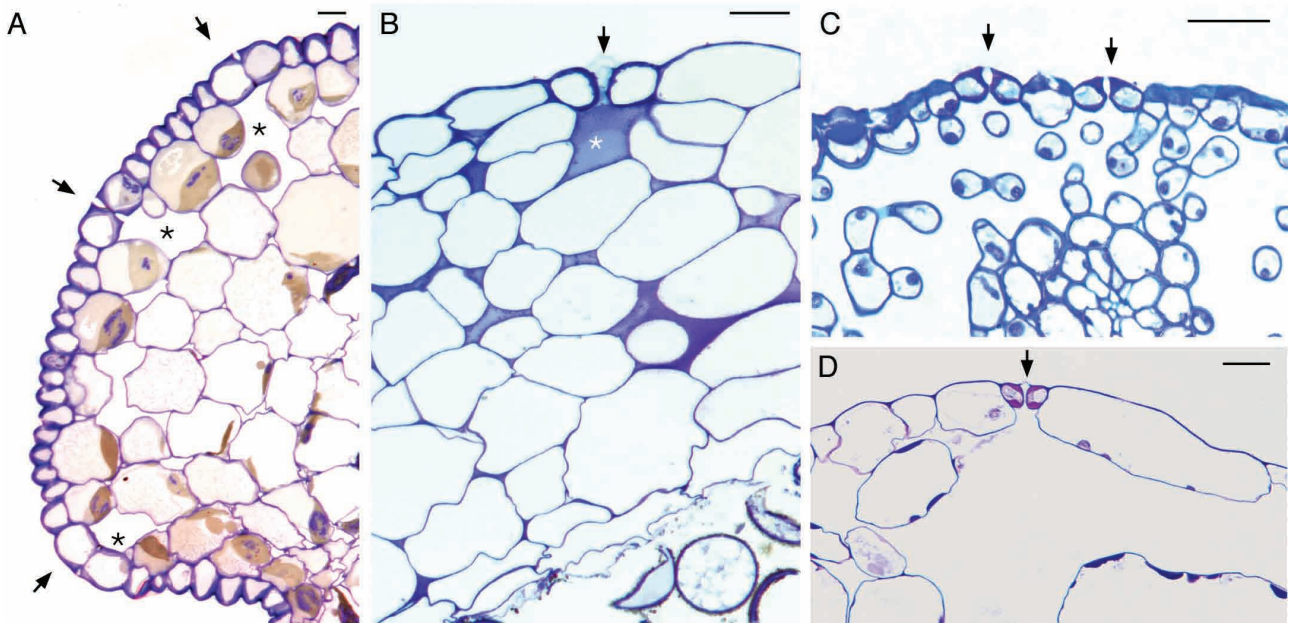

FIGURE 3. Stomata across model species. A. hornwort *Anthoceros*, B. moss *Physcomitrella*, C. Lycophyte *Selaginella* and D. flowering plant *Arabidopsis*. Scale bars = 20μm.

Across the phylogeny of bryophytes, the number of stomata is more likely related to the size, shape and elaboration of the sporophyte (Merced & Renzaglia 2013). The experiments of Baars & Edwards (2008) and Field *et al.* (2015) suggest that bryophyte stomata number and size are not determined by environmental factors and hence do not reflect the plasticity evident in tracheophytes to compensate for changes in ambient CO_2 over time (Casson & Grey 2008, Franks & Carsson 2014, Dow *et al.* 2014). Nevertheless, spacing of stomata is regular in mosses and hornworts (Fig. 2, 4A) in that it typically follows the 'one-cell spacing' rule. In *Funaria* less than 4% of stomata touch each other (Merced & Renzaglia 2016). Development of bryophyte stomata is regulated by genes homologous to those of angiosperms. Key regulatory elements SCREAM and SMF (SPEACHLESS, MUTE, FAMA), signaling peptides and membrane receptors (EPF, TMM, ERECTA) are known to be present and involved in stomatal development in the moss *Physcomitrella* (MacAlister & Bergmann 2011, Cain *et al.* 2016, Chater *et al.* 2016, Chater *et al.* 2017). Othologs of these genes have been recently identified in the genome of *Anthoceros* (Chater *et al.* 2017)

Microscopic observations of other mosses and hornworts also point to regular separation of stomata (Fig. 2), but some exceptions are perplexing such as *Polytrichum* where stomata are clustered in the apophysis and so close to each other that opening of the pore seems unlikely (Fig. 2H, 5I). Distribution and patterning of stomata is genetically controlled and necessary for proper function and optimal gas exchange in tracheophytes (Dow *et al.* 2014). We hypothesize that spacing stomata in bryophytes is not as critical and tightly regulated as in angiosperms as evidenced by clusters and

pairs of stomata in many species. Instead separating stomata from each other may be required for pore and substomatal cavity formation in bryophytes (Merced & Renzaglia 2016). We postulate that the developmental mechanism to distribute stomata in response to the environment was fine-tuning in tracheophytes with the advent of leaves.

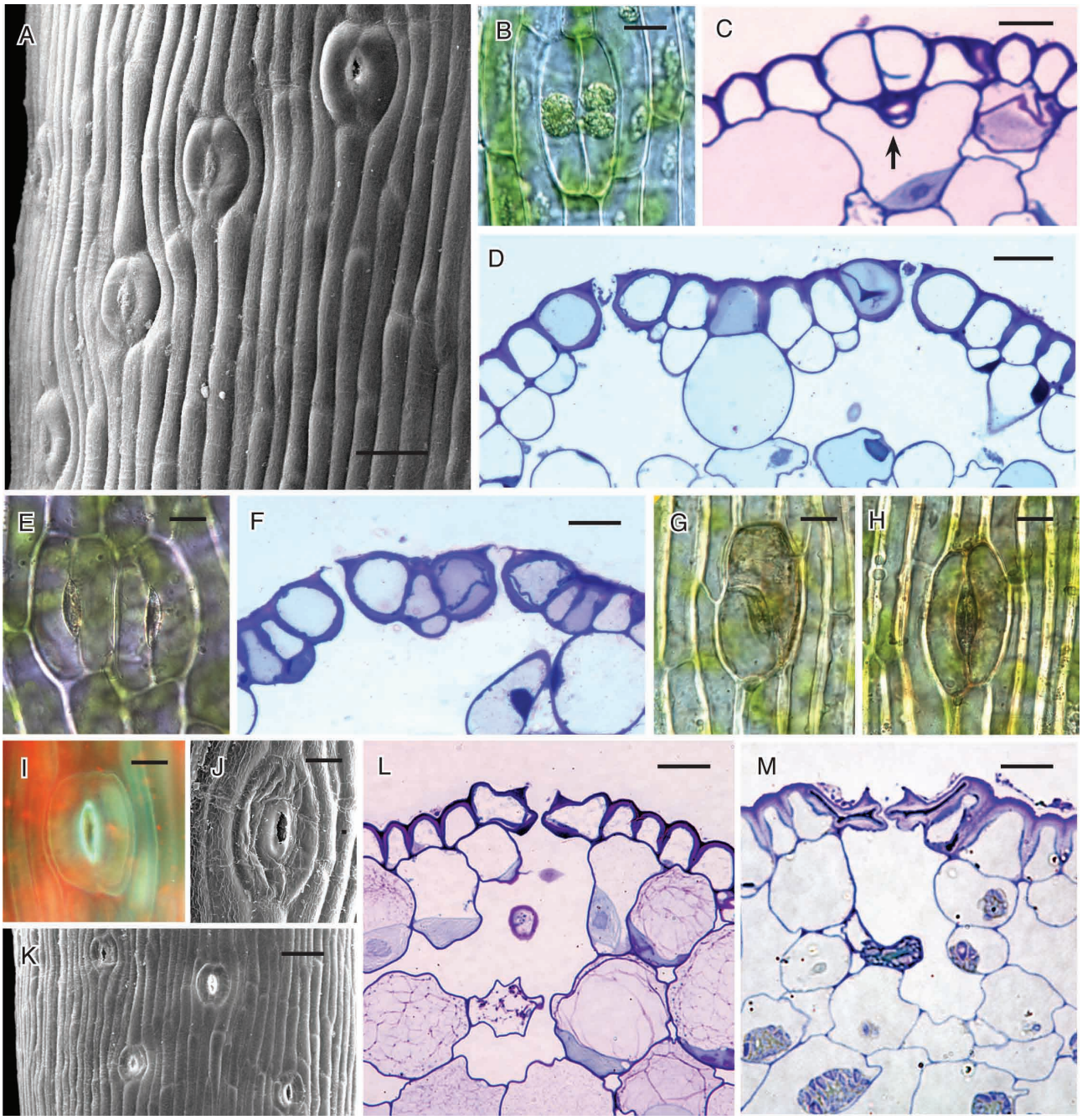

FIGURE 4. Stomata of hornworts. A, J, K. *Anthoceros adscendens*. B, C, E, G, H, I, L. *Phaeoceros carolinianus*. D,F,M. *Leiosporoceros dussii*. Arrow in C indicates the initiation of substomatal cavity beneath guard cells before pore formation. J–K. Collapsing or collapsed stomata. Scale bars=20 µm except A, K = 50 µm

Sporophyte anatomy and stomatal development

As in tracheophytes, stomata in bryophytes develop from a symmetric division of a guard mother cell (GMC). Guard mother cells in mosses form at the spear stage before capsule expansion (Garner & Paolillo 1973a,b, Paton & Pearce 1957) and GMCs are arranged in files (Merced & Renzaglia 2016). The division of GMCs to form guard cells and separation of ventral walls to open the pore occurs during capsule expansion as the sporangium begins to differentiate. In hornworts GMCs are found below the involucre and divide to form the two guard cells before emerging from this protective tissue; above the involucre guard cells separate to open the pore (Pressel *et al.* 2014, Renzaglia *et al.* 2017). Guard mother cells can be distinguished by being larger, rounded, slightly raised over the epidermis and with more chloroplasts or larger chloroplasts in hornworts compared to surrounding epidermal cells.

Stomata fully develop before spores in both mosses and hornworts (Fig. 4, 5A) (Merced & Renzaglia 2016, Renzaglia *et al.* 2017). Substomatal cavities are always associated with stomata in hornworts and mosses; the pore opens into a network of spaces in the underlying photosynthetic tissue (Figs. 3A–B, 4D, L, M, 5A, I, J) (Paton & Pearce 1957, Merced and Renzaglia 2013, 2016, Pressel *et al.* 2014). The schizogenous internal spaces are filled with fluid when first formed and they dry with opening of stomatal pores; the space may increase by breakdown of cells within the air-filled chambers (Merced & Renzaglia 2016, Renzaglia *et al*. 2017). The fluid may fill the cavities throughout development of spores (Fig 3B). Capsules of bryophytes lacking stomata are devoid of spongy tissue and have a reduced to absent apophysis in mosses (Merced 2015a, Chater *et al.* 2016).

The peculiar stomata of hornworts

Given they occur on an elongating sporangium, it is not surprising that stomata of hornworts exhibit a suite of features not before reported in other extant plants (Renzaglia *et al.* 2017). Stomata are large and irregularly scattered along the length of the sporophyte (Fig. 4A, K). Stomata originate within the confines of the involucre through a symmetrical division in an epidermal initial (Fig. 4B, C). Young guard cells are surrounded by thin walls and are wider than surrounding epidermal cells (Fig. 4A, C). Formation of the substomatal cavity begins at the inner-most region where guard cells meet and it progressively expands in a schizogenous manner (Fig. 4C, D). Typically, stomata are solitary and evenly distributed along the sporophyte, but occasionally side-by-side stomata are produced (Fig. 4E, F). A diagonal division produces asymmetrical guard cells in stomata near the sporophyte suture (Fig. 4G).

As the substomatal cavity expands, guard cells develop differential wall thickening, forming a prominent outer ledge that surrounds the pore, a thickened inner wall and thin ventral, outer and dorsal walls (Fig. 4D, F, I). Guard cells overlie internal cavities that lead to a system of spaces in the assimilative tissue that is initially filled with fluid and dries where stomatal pores open (Pressel *et al.* 2014, Renzaglia *et al.* 2017). This fluid does not label with pectin epitopes and therefore cannot be identified as mucilage. The most peculiar feature of hornwort stomata is that after they develop wall thickenings, they die and collapse (Fig. 4H–M). This occurs in regions of the sporophyte where spores are differentiating, but not mature. Following guard cell collapse, the spongy assimilate tissue facilitates CO_2 acquisition as evidenced by prominent chloroplasts adjacent to intercellular spaces. The sporogenous tissue is surrounded by pectin-containing mucilage that persists along the length of the sporophyte up to the dehiscence region. Mucilage surrounding spores progressively dries down following stomata collapse. This process precedes sporophyte dehiscence and is necessary for spores to separate and disperse. Guard cell wall thickenings ensure that stomata maintain an enlarged external surface area as well as remain perched over the substomatal space (Fig. 4L, M).

Sunken stomata of *Orthotrichum*

The position, shape and number of stomata have been used as taxonomic characteres in mosses (Erzberger 2003, Hedenas 1989). In *Orthotrichum,* species with immersed (sunken) stomata and superficial stomata were traditionally place in two different subgenera. However, the most recent molecular classification suggests that the six subgenera with sunken stomata evolved independently three times (Sawicki *et al.* 2012). The position of stomata in Holartic species of *Orthotrichum* correlates to karyotype in that species with a chromosome number of 6 have superficial stomata and almost all species with 11 chromosomes have sunken stomata (Vitt 1971). Factors that influence stomatal position remain to be investigated; it is uncertain if the level of stomata in the epidermis correlates with environmental conditions during development or if chromosome number or genome size are contributing factors.

Expanded green capsules of *Orthotrichum pusillum* are surrounded by a plicate calyptra, and stomata develop and fully mature before sporogenesis while the sporophyte is still covered by the calyptra (Fig. 5A). Sporophyte anatomy and stomata ultrastructure in developed capsules of *Orthotricum* are similar to other mosses with open pores that connect the external environment to the inside network of air spaces adjacent to cells with large and abundant chloroplast, suggesting a role in CO_2 acquisition and water transpiration (Merced & Renzaglia, 2013) (Fig. 5A). The sunken stomata of *Orthotrichum* are positioned below the epidermis of the capsules (Fig. 5A, B), with surrounding epidermal cells that protrude over guard cells (Fig. 2I, J, 5A–G). These 'subsidiary cells' are of different shape and size from other epidermal cells (Fig. 5B, C, G), and a thick electron-lucent material fills the cell wall where they protrude above guard cells (Fig. 5G). Guard cell walls are thicker where outer and inner walls meet ventral walls (Fig. 5E), and outer walls are thicker at their polar end (Fig. 5D). A large vacuole occupies the majority of the cytoplasm displacing the chloroplast to the edge of the guard cell (Fig. 5D, E, F). Numerous small oil droplets line the inside of the walls (Fig. 5 F, H) and are more abundant at the polar end compared to the pore region (Fig. 5D, E). The association of guard cells, surrounding epidermal cells and spongy cortex in this tiny moss parallels the stomatal complex of tracheophytes.

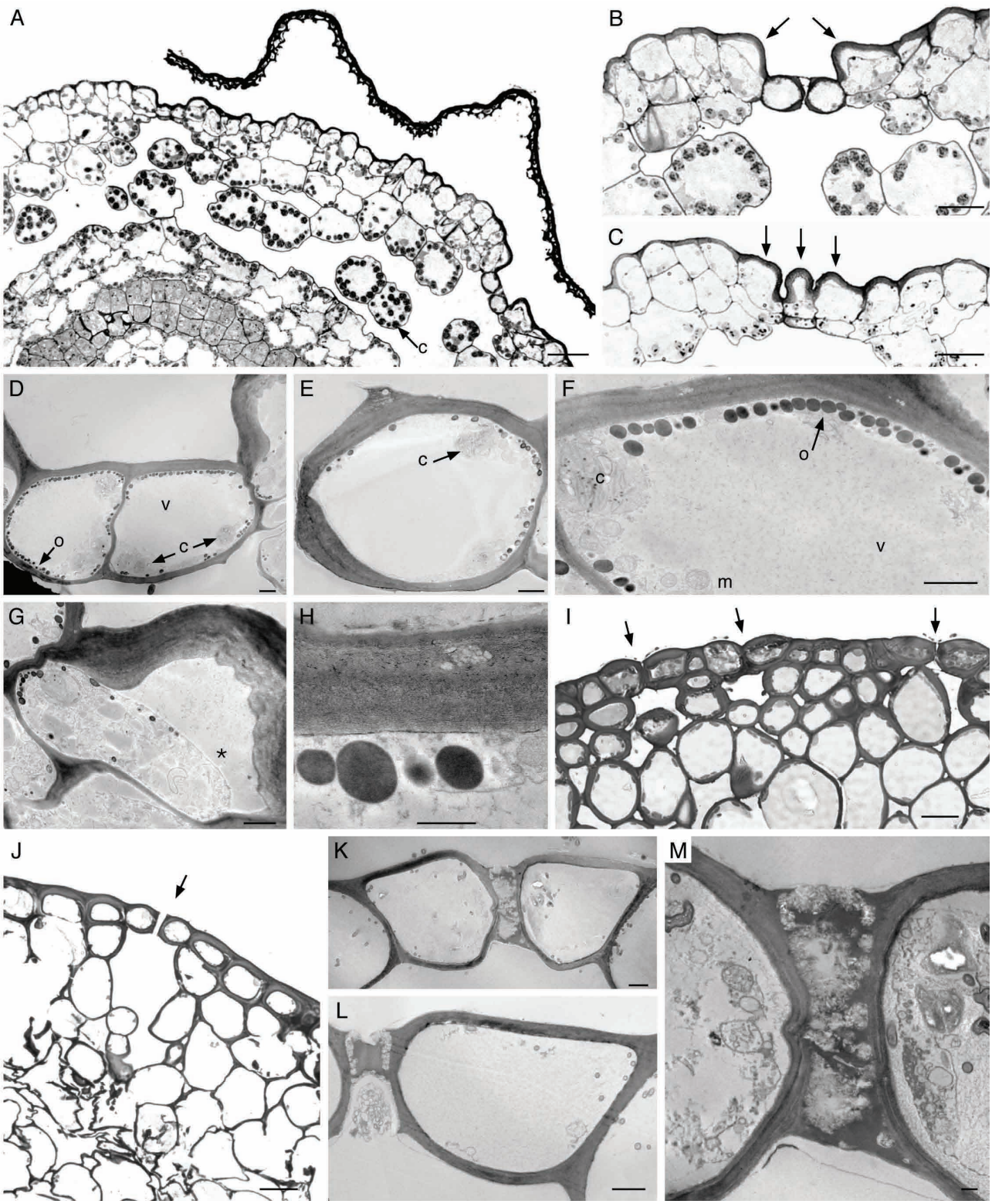

FIGURE 5. Anatomy of moss capsules and stomata structure. A–H *Orthotrichum pusillum*. I. *Polytrichum ohioense*. J. *Hypnum curvifolium*. K–M Stoma in brown open capsules of *Funaria flavicans*. A = 35 µm; B–C, I–J = 20 µm; D–G = 2 µm; H, M, N = 500nm; K, L=2µm.

Pore obstruction and fluid in spaces

A peculiar condition of aging bryophyte stomata is that the pores are often occluded with wax-like material (Fig. 5K, L, M) (Paton & Pearce 1957, Merced & Renzaglia 2013). Obstructed pores can be found near open pores in hornworts (Fig. 4A) and in mature capsules of mosses (Fig. 2C, F,). Liquid often fills substomatal cavities and intercellular spaces when pores are clogged, as is seen in Figure 3B in a *Physcomitrella* capsule with nearly mature spores. Obstructed pores are common in conifers and are sometimes found in angiosperms, although wax plugs reduce stomatal maximum

conductance they function in reducing transpiration and entrance of pathogens (Brodribb & Hill 1997). Involvement of stomata in gas exchange under this condition is highly suspect because the capsule lacks air-filled intercellular spaces well after spore differentiation. It is possible that liquid in intercellular spaces within a species such as *Physcomitrella* is variable, or even environmentally induced, as it is not always evident in sections of capsules. Alternatively, lack of fluid may be an artifact of fixation. The occurrence and role of fluid in spaces as related to pore obstruction remain to be examined.

Function and physiology of bryophyte stomata

Many aspects of the anatomy and ultrastructure of bryophyte stomata support their role in gas exchange (CO_2 acquisition, O_2 release and water loss), but few studies have looked at opening and closing of stomata in mosses and hornworts. Paton and Pearce (1957) performed experiments with several mosses and *Anthoceros* in various environmental conditions (darkness/light, CO_2, humidity) and concluded that bryophyte stomata did not respond to external stimuli but instead closed when the water content of the capsule dropped. Garner and Paolillo (1973a) showed that stomata in *Funaria hygrometrica* closed in response to darkness and reopened when exposed to white light and that open stomata treated with abscisic acid (ABA) closed. This response was limited to only a few days after capsule expansion when the sporophyte is nearly self-sufficient in terms of carbon fixation (Paolillo & Bazzaz 1968, Proctor 1977). More recently, Chater *et al.* (2011) reported a closing response of moss stomata to ABA, elevated CO_2 concentration and darkness in expanding sporophytes of *Physcomitrella* and *Funaria*.

Stomata movement in hornwort has been less studied. Stomata closure as a result of application of ABA was reported once in the literature (Hartung *et al.* 1987). However, Lucas & Renzaglia (2002) found that stomata in hornworts are unresponsive to ABA and do not have diurnal cycles, instead stomata are closed when immature and remain open when mature (Paton & Pearce 1957, Pressel *et al.* 2014). Most recently, Pressel, Renzaglia and Duckett (unpublished) examined potassium ion concentrations in guard cells and adjacent cells of hornwort using x-ray microanalysis and found that potassium mass-proportion is lower in guard cells of newly opened stomata than in the adjacent epidermal cells. They also reported that only a slight reduction in aperture dimensions occurs after desiccation and plasmolysis, and no changes in aperture dimension follow ABA treatments and darkness.

So few bryophytes have been studied that it is hard to extrapolate any physiological studies to other taxa with stomata. Moreover, it is difficult to determine if the responses noted are due to passive movement of water or active hormonal control. Several studies point to passive closing of stomata in response to low water status in lycophytes and ferns without ABA involvement (Brodribb & McAdam 2011, McAdam *et al.* 2016). Higher concentrations of ABA in liverworts and hornworts are associated with dry conditions suggesting that stomatal closure may be an overall stress response (Hartung *et al.* 1987). Regulatory elements of ABA response in stomata of angiosperms are expressed and upregulated in expanding green sporophytes of *Physcomitrella* (O'Donoghue *et al.* 2013). Cross complementation assay of the *Physcomitrella* OPEN STOMATA 1 (PpOST1) in *Arabidopsis ost1* mutants recovered stomata response to ABA (Chater *et al.* 2011). Closing of stomata in response to ABA involves activation of SLAC1 anion channel by OST1 and although genes that encode these elements are present in the green alga *Klebsormidium*, the liverwort *Marchantia* and the moss *Physcomitrella*, only in *Physcomitrella* do both elements, PpOST1 and PpSLAC1, form a functional complex that activates the anion channel similar to angiosperms (Lind *et al.* 2015). These components of the ABA dependent drought stress response originated before the diversification of embryophytes but it is still controversial when the use of this gene regulatory network was employed for stomata function (Chater *et al.* 2013, Lind *et al.* 2015, McAdam *et al.* 2016).

Current understanding of the physiology of stomata is based on tracheophytes, making it problematic to extrapolate these concepts and processes to explain bryophyte physiology. The effects of the environment (e.g., gravity, water surface tension, air currents) on bryophyte and tracheophyte physiology could be fundamentally different due to scale related differences (Proctor 2009). In particular, active closing and opening of stomata can be difficult to understand since stomata develop and usually open in mosses under the cover of a calyptra that protects against desiccation (Budke *et al.* 2013, Budke & Goffinet 2016). In hornworts stomata develop inside the involucre, open above it and then collapse (Renzaglia *et al.* 2017). This development pattern leaves a small window for the possibility of active movement of guard cells; if that is the case, it will happen at the youngest region near the base of the sporophyte that is not exposed to severe drying. The absence of responses to environmental cues and ABA coupled with the unique architecture and fate of stomata in hornworts is consistent with the inability of these stomata to open and close. The lack of arabinan-containing pectins in guard cell walls of hornworts supports this conclusion as this type of pectin is present in young guard cells of mosses that are reported to be capable of moving and is essential for active movements of flowering plant stomata (Merced & Renzaglia 2014, Renzaglia *et al.* 2017).

Alternative functions of stomata in bryophytes

Similar anatomical features between most bryophytes and tracheophytes support a gas exchange role for stomata in both groups. Stomata of mosses and hornworts lead to cavities that connect to aerenchymatous tissue with chloroplasts much like leaf mesophyll (Merced & Renzaglia 2013, 2016, Renzaglia *et al.* 2017). Water-conducting tissue is present in the seta of most mosses (Hébant 1977), supporting a possible role in transpiration, capsule hydration, and even in restoring fluid in intercellular spaces (Haig 2013). However, it is not clear what the contribution of stomata is to CO_2 acquisition for photosynthesis of the sporophyte and it has been argued that gas exchange is not the primary role of stomata in bryophytes (Pressel *et al.* 2014, Field *et al.* 2016). Cuticular plugs that obstruct pores of mature stomata may be a means to close pores in lieu of guard cell movement (Fig. 5K, L). The existence of fluid in intercellular spaces and occluded pores are counterintuitive to a role in gas exchange but may indicate stomata are involved in water movement, transpiration and drying of the sporophyte.

The hypothesis that stomata evolved to facilitate sporophyte drying, and active movement through hormonal and osmotic triggers evolved later with leaves, assumes that stomata of bryophytes have retained the ancestral function (Ligrone et al 2012a, Haig 2013). Guard cell wall architecture facilitates dehydration, shape changes, and dehiscence of the capsule, supporting a common function of moss and hornwort stomata (Ligrone *et al.* 2012b, Merced & Renzaglia 2013; Chater *et al.* 2016, Renzaglia *et al*. 2017). Chater *et al.* (2016) demonstrated that losing stomata in mosses can be accomplished in one step, by eliminating either one of transcription factors that initiate stomata differentiation (SMF or SRCM) and without major consequences to sporophyte development and anatomy, except that capsule dehiscence is delayed. These results support a role of moss stomata in drying and dehiscence of the capsule.

Pseudostomata of *Sphagnum*

The absence of true stomata in early divergent mosses complicates the interpretation of a single origin of stomata in land plants (Fig. 1). Pseudostomata in Sphagnopsida may provide some clues to this evolutionary problem but do not explain the absence of stomata in *Takakia*, *Andreaeobryum* and *Andreaea*. It was known since Schimper's study in 1858 that *Sphagnum* had stomata-like structures that do not form an open pore. *Sphagnum* pseudostomata development and ultrastructure was described by Bouldier (1988) who considered them as reduced epidermal cells without any of the structural characteristics of guard cells. Duckett *et al.* (2009) described that during desiccation of the capsule, pseudostomata are the first cells to dry until they collapse and partially separate but never open internally; they proposed that pseudostomata are involved in changing the shape of the capsule and explosive discharge of spores. Ultrastructure of pseudostomata shows similarities with young stomata of other bryophytes, including separation of pseudostomatal cells by cuticle deposition, layering of walls after "pore" initiation and walls rich in pectins (Merced 2015b). Merced (2015b) proposed that pseudostomata are modified stomata that suppressed substomatal cavity formation, which in turn interrupted pore development. Capsules of Sphagnopsida are operculate but lack peristomes. Most have pseudostomata scattered across the middle of the capsule but *Ambuchanania*, the sister taxon to *Sphagnum*, has numerous pseudostomata on the lower half of the capsule (Yamaguchi *et al.* 1990). Reminiscent of stomata in true mosses that are also operculate, this restriction to the lower capsule region could be interpreted as plesiomorphic in mosses. A search of the draft genome of *Sphagnum* could not clearly identify stomata related genes (Chater *et al.* 2017). Clarification of the homology of pseudostomata and bryophyte stomata may require transcriptomic studies integrated with physiological and structural data. Similar developmental fates of early death and collapse in hornwort stomata and pseudostomata of Sphagnopsida are intriguing similarities that must be considered when evaluating homology with guard cells in other bryophytes.

Evolution of Stomata

With a new phylogenetic hypothesis that places hornworts sister to other land plants (Wickett *et al.* 2014), a reinterpretation of the evolution of stomata is warranted. The absence of stomata in liverworts is consistent with the complete maturation of liverwort sporophytes and spores inside of protective structures (Crandall-Stotler *et al.* 2008). If liverworts are sister to mosses, either the absence of stomata in liverworts and valvate mosses is plesiomorphic and stomata first appeared in true mosses, or stomata were lost independently in early lineages (Fig. 1). Loss of stomata is common in bryophytes and thus the latter interpretation is plausible. The absence of stomata in the valvate mosses *Takakia*, *Andreaea* and *Andreaeobryum* is curious and suggests that stomata evolved in mosses with operculate capsules. Similar to *Sphagnum*, *Andreaea* does not have a seta and capsules develop before being lifted by a pseudopodium, however the relationship between these conditions and stomata is not clear.

In order to assess the evolution of stomata we must take a step back in time and consider the evolution of the organography of the first land plant. The first fossils with stomata were from rocks just over 400 million years old.

These plants consisted of branching axes with terminal sporangia. Planar leaves did not exist and did not evolve until CO_2 levels fell some 30 million years later (Beerling *et al.* 2001). In their morphology, these earliest plants resembled bryophyte sporophytes that also lack leaves and have terminal sporangia. Remarkably, the stomata in these late Silurian/early Devonian fossils possess collapsed guard cells and elongated epidermal cells similar to those of hornworts (Renzaglia *et al.* 2017). We hypothesize that stomata on leafless axes are subjected to different developmental and physiological constraints from those on planar leaves.

Two competing models for stomata evolution are still under debate, 1) the single-step model proposes that stomata in early land plants evolved with an ABA response and active movement, and 2) the gradualistic model that proposes stomata movement originated as a simple passive mechanism in response to water content (Sussmilch *et al.* 2017). The bryophytes as sister to the rest of land plants are key in elucidating this problem but more studies are needed because of conflicting evidence supporting both hypothesis. It seems unlikely that stomata on sporangia such as those in bryophytes would be responsive to environmental cues in the same way that is documented in vegetative leaves of angiosperms. Bryophyte sporangia are never very elongated nor do they persist beyond one growing season. Passive mechanisms for closure would likely evolve first as sporophytes increased in stature through embryo differentiation and the evolution of apical meristems (Ligrone *et al.* 2012a, b). Complex physiological mechanisms that regulate a suite of responses to seasonal and diurnal environmental stimuli would not be associated with the sporophyte of bryophytes but are critical to tracheophyte evolution.

The genes involved in the acquisition of stomatal physiology can be expected to undergo progressive changes with co-option of early genes for more complex regulatory roles as leaves diversified. Stomatal spacing genes and those that regulate ABA are found across plants and are more critical in spacing and regulating stomata in angiosperms where transpiration must be precisely coordinated with carbon acquisition. The tendency of small guard cells of angiosperms to respond quickly to the environment does not pertain to hornworts were genome sizes are not correlated with guard cell sizes, this has yet to be tested in mosses (Renzaglia *et al.* 2017). Guard cell differentiation in bryophytes involves the production of differentially thickened walls after pore opening. Changes in guard cell wall ontogeny were necessary for stomatal movement as modifications of pectinaceous wall constituents were integrated for wall flexibility and resilience (Merced & Renzaglia 2014).

We must consider that it is possible that stomata in mosses, hornworts and tracheophytes are homoplasious. Independent evolution or re-evolution of a structure has been documented in other organisms (Collin & Miglietta 2008). For stomata, this scenario seems unlikely due to similarities in development, guard cell architecture, anatomy of surrounding tissue, and shared stomata-related genes (Chater *et al.* 2017). If stomata are homologous, there is also a possibility that those in bryophytes are highly derived and have lost the physiological functions attributed to tracheophyte stomata. Similar changes have been demonstrated in stomata on floral organs that are highly modified and unresponsive to environmental cues. This scenario also seems untenable when the condition of the earliest fossil plants is considered, i.e., sporangia and lack of leaves in these fossils are ancestral traits that are shared with bryophytes. At present, we see the preponderance of data supporting a single origin of stomata in the first terrestrial plants with more sophisticated physiological biochemistry evolving as tracheophytes diversified.

Conclusions

The widespread occurrence of stomata across all plant groups except liverworts and their occurrence in the earliest fossil plants identify stomata as a key innovation that supported the early diversification of plants on land. Nevertheless, our knowledge of stomatal development and physiology in seed free plants remains scanty. Even with the pivotal role bryophytes assume in stomatal evolution, the structure, development and function of stomata within mosses and hornworts have only been scrutinized in very recent years. Studies using the model moss *Physcomitrella* are challenging and might not reflect the reality of the great majority of mosses because of its reduced cleistocarpic capsule with few stomata, small apertures and poorly developed intercellular spaces. Physiological and genetic studies need to be replicated in additional mosses and hornworts that represent the entire range of diversity in sporangial and stomatal complexity. Important questions remain to be answers such as: 1) To what extend are stomata essential to the development of the sporophyte (in species that have them) and how do stomata enhance fitness of sporophytes?; 2) Do environmental pressures have an effect on stomata position and density in bryophytes?; 3) What accounts for the absence of stomata in several lineages, i.e., are the same genes involved with stomata loss across taxa?; 4) Are there anatomical features across bryophytes with stomata that are absent in taxa without stomata?; 5) Are there

characteristics related to sporophyte development that are unique to mosses with stomata? and 6) Are guard cell size and genome size correlated in mosses? In regards to anatomy and morphology, there is little information on capsule, seta and placental anatomy in mosses, features that may show correlations with stomatal number, position and function. Similarly, the complexity of conducting tissue and the association of this tissue with spongy tissue and stomata are not well characterized. It is unclear if there is a relationship between number of stomata, rate of capsule development and longevity of spores. If stomata function in capsule drying, it follows that capsules without stomata will open more slowly and will be longer lived than those with stomata. Excellent models to address morphological features *vis a vis* stomata location, structure and number are *Orthotrichum,* with diverse positions and conditions of stomata, and genera in the Polytrichales with and without stomata.

Although much remains to investigate, recent studies of bryophyte stomata have provided a more comprehensive perspective on stomata. Minimally, these studies have raised concerns about generalizing on stomatal function across plants, and have pointed to the importance of bryophytes in understanding stomatal biology and evolution. With the imminent availability of genomes and transcriptomes of additional mosses and hornworts, the genetic control of development and the evolution of stomatal genes may be examined more thoroughly. For the present, studies of bryophyte stomata are changing our perspective on the evolution of these minute cellular entities that were so critical to land plant diversification and survival.

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