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Anatomy, ultrastructure and physiology of hornwort stomata

Jessica Regan Lucas

Introduction:

Hornworts, phylum anthocerotophyta, constitute a small group of plants which range from tropical to temperate climates. As in all bryophytes, the gametophyte is the dominant life stage of hornworts. The gametophyte is a simple thallus, superficially resembling that of simple thalliod liverworts. The sporophyte consists of a foot embedded in the gametophyte and a long cylindrical sporangium. A basal meristem indeterminately produces new cells throughout the growing season. Due to the basal meristem, all stages of cellular development can be found in the sporophyte. In the center of the sporangium is a sterile columella, often just rows of 16 cells. Surrounding the columella are sporogenous cells which give rise to alternating tiers of pseudoeleaters and spores. Generally containing two chloroplasts with pyrenoids, assimilative cells surround the sporogenous cells and make up most of the sporophyte. A single layer of epidermal cells with cuticle form the boundary between the atmosphere and the assimilative cells. Longitudinally oriented, stomata are scattered amongst nondifferentiated epidermal cells (Bold 276-279).

The term stoma (plural=stomata) refers to two specialized epidermal cells on a plant body and the aperture which forms between them. In tracheophytes, stomata are integral to water transport in plants because evaporation of water through the stomatal pore draws water through the water conducting tissue. Diurnal rhythms of stomatal movement allow maximum levels of carbon assimilation to occur while minimizing water loss. Though the exact rhythm varies for each plant, it is strongly correlated to the plant's carbon dioxide concentration mechanism, i.e. C3, C4, or CAM (Taiz and Zeiger 1998). Since a water transport system is crucial to success on land, the development of stomata was a landmark in plant evolution. It is generally assumed that stomata are homologous among plant groups, but a growing amount of evidence implies otherwise, especially among the basal phyla (Renzaglia et al. 2000).

Although diverse in microanatomy, all stomata are composed of two adjacent guard cells that function in response to changes in turgor pressure. In face view, two basic guard cell morphologies are found: reniform and graminicous, the latter predominated in grasses and other monocots. For most plants, specialized epidermal cells originate either perigenously or mesogenously, which serve as companions to guard cells. These subsidiary cells act as ion reservoirs necessary to ionic fluxes accompanying stomatal movement. An opening forms when a turgid pair of adjacent guard cells separate from each other; no pore is present in a flaccid set of guard cells. Special wall

thickenings and microfibril arrangements facilitate the differential swelling of the guard cells.

Light is the most important environmental factor that causes the characteristic opening and closing of stomata, but humidity and carbon dioxide concentration also influence guard cell movement (Taiz and Zeiger 522). Such triggers, through internal signals, cause solutes to accumulate in the guard cells and water to consequently diffuse into the cells. Sucrose, potassium, chloride, and malate ions are the principal solutes regulating the osmotic potential in guard cells (Outlaw 1983).

Virtually all tracheophytes develop stomata but the presence of these structures in bryophytes is not universal. Of moss lineages, only the more advanced true mosses possess functional stomata, the Takakiales and Andreaeales lack stomata completely and rudimentary stomata are found on *Sphagnum* moss capsules. Stomata of true mosses are found only on the base of the capsule, a region known as the apophysis. In hornworts, stomata are restricted to only three of the six genera (Renzaglia et al 2000). *Anthoceros*, *Folioceros* and *Phaeoceros* form stomata while *Dendroceros*, *Notothylas*, and *Megaceros* lack them. The fact that stomata occur in all groups of embryophytes except liverworts supports the hypothesis that the liverworts are the earliest divergent extant group of land plants (Kenrick and Crane 1997). Until recently, the speculation that liverworts are the basal-most embryophyte group was widely accepted. Accumulating molecular (Hedderon et al. 1998, Nishiyama and Kato 1999) and morphological data (Garbary and Renzaglia 1998, Renzaglia et al. 2000) suggest that hornworts, rather than liverworts, are the oldest living group of land organisms. In such analyses, liverworts form a monophyletic clade with mosses. Along with the presumption that the different bryophyte groups diverged from one another before the sporophyte had evolved much complexity, these phylogenies suggest that a reevaluation of the evolution of stomata is in order (Renzaglia et al 2000).

Unlike mosses and tracheophytes that have well-defined water conducting strands, hornworts lack such tissue. Because of this anatomical peculiarity, the water transport function typically attributed to stomata is highly questionable in these plants. Intercellular spaces form interior to anthocerote stomata therefore gas exchange is a possible function of these stomata. Unlike other archegoniates, diurnal movements appear to be absent in bryophytes. Although true moss stomata have been reported to function as do those of tracheophytes (Garner and Paolillo 1973), Paton and Pierce's (1954) comprehensive study of bryophyte stomata calls into question physiological similarities between vascular and nonvascular plant stomata.

In the present study it was hypothesized that stomata in hornworts were independently derived from those of other embryophytes. This hypothesis was tested by observing the development, ultrastructure and physiology of hornwort guard cells. First, the cells were examined with the light and fluorescent microscopes to determine general cellular features and stomatal distribution. Light and transmission electron microscopy were utilized to explore development and ultrastructure of the cells. General patterns of development were visualized using scanning electron microscopy. To determine whether the cells function in response to ionic changes as in other plants, stomata were stained with cobaltinitrite and fast violet B to localize potassium cations and organic acids (malate) respectively. By observing stomata at various times during day and night, the presence of circadian rhythms was evaluated. Effects of the phytohormone, abscisic acid (ABA), which is responsible for the closing of stomata during water stress, was explored in hornworts. Stomatal features of hornworts will be evaluated in comparison with those of mosses and tracheophytes.

Materials and Methods:

Live plants were collected in southern Illinois by KSR and JRL. In late June 2000, *Anthoceros agrestis* and *Phaeoceros leavis* were collected from a recently tilled cornfield in Jackson county in Southern Illinois. *P. leavis* was collected in Cove Hollow (Jackson County) in October 1999. Many collections were received by mail. *A. punctatus* was collected in Oregon and provided by Dr. David Wagner. *A. caucasicus* and *P. carolineaus* from the Iberian peninsula were contributed by Cecilia Sergio. Dr. Martha Cook contributed *Phaeoceros carolineaus* that was collected from a Illinois State University greenhouse in Normal, Illinois. A California collection of *P. leavis* and *P. mohrii* were acquired from Dr. Terry O'Brien. *Anthoceros* sp. was collected in Idaho by Jim Thompson. The plants were kept under continuous light in covered dishes and moistened as needed with distilled water until utilized. Voucher specimens were deposited in the personal herbarium of KSR and JRL.

Light microscopy:

Tissue embedded in a 1:1 Spurr's/Polybed resin (see TEM) was cut into thick sections with a diamond knife and adhered with heat to a glass slide. The sections were then stained with 1.5% (w/v) aqueous toluidine blue containing sodium borate, allowed to cool and gently rinsed with water. Sectioned specimens were monitored to evaluate the stage of development, cell and tissue organization. Optimal material was further examined in the TEM (see below).

Fresh sporophyte tissue was prepared by longitudinally cutting the diploid phase in half and scraping the epidermis clean of cells. The epidermis was mounted in distilled water and viewed on an Olympus BX40 light microscope. Aqueous ruthenium red (1:5000) was used to visualize pectin in epidermal peels. To evaluate possible diurnal cycles in stomatal movement, *A. punctatus* collections were exposed to 12 hours of light and 12 hours of dark and observed for stomatal closure. Plants were observed at four different times during the day and night to observe the aperture and scored either as open, closed, or occluded, which was determined by focusing through the pore. Around 150 stomata were observed. Epidermal peels of moss capsules and hornwort sporophytes were dehydrated on glass slides with glycerin and 80% ETOH. Four sporophytes were dehydrated, each with about ten stomata.

All light and fluorescence micrographs were taken using either 50 ASA Ilford black and white or 400 ASA Provia color slide film on an Olympus PM-30 camera attached to a Leitz Orthoplan Microscope.

Fluorescence Microscopy:

Epidermal peels were prepared and mounted in a 0.4M sorbitol fluoromount solution and autofluorescence was recorded. The addition of sorbitol was necessary to prevent plasmolysis. A 1:500 mixture of DAPI in 0.4M sorbitol fluoromount solution was applied to other epidermal peels to determine the location of nuclei in guard cells. Aniline blue stain (0.005% w/v) was applied for twelve hours to detect callose in epidermal peels.

Transmission Electron Microscopy:

Sporophytes were dissected from the gametophyte and cut into small pieces while in 4% glutaraldehyde in 0.05 M cacodylate buffer. At room temperature for 4 hours, the tissue was fixed in 4% glutaraldehyde in 0.05 M cacodylate buffer and then overnight at 4°C. The tissue is washed three times, over two hours, in cacodylate buffer (0.05 M, pH 7.2), postfixed in 2% OsO₄ in same buffer, rinsed in water, and *en bloc* stained in 2% aqueous uranyl acetate (UA) for 16 hours at 4°C. After dehydration in a graded acetone series, the material was infiltrated slowly over six days with a 1:1 mix of Spurr's/Polybed resin and cured at 65°C for 16 hours. Specimens were thick sectioned and stained with 1.5% toluidine blue with sodium borate and monitored for the presence of stomata. Promising blocks were thin sectioned and poststained with ethanolic UA and basic lead citrate for 5 minutes each. Observations were made on a Hitachi H500 TEM.

Scanning Electron Microscopy:

The tissue was fixed as in the TEM fixation then dehydrated in a graded ethanol series. After dehydration, the tissue was critically pointed dried, placed on stubs, and coated with gold and palladium. Specimens were viewed on a Hitachi S570 SEM.

Histochemical stains:

Potassium localization: Sporophytic epidermal peels were stained in Macallum's reagent prepared as follows (Raschke and Fellows 1971). Four grams cobaltous nitrate and seven grams sodium nitrate were dissolved in 13ml of distilled water after which 2ml glacial acetic acid was added. This solution was stirred for one hour to finish preparation. The stain was centrifuged for three minutes and chilled to 4°C before use. All cells were removed from the epidermis as described above while in the stain and on a freezing Petri dish, a dish filled with ice that was necessary to keep the stain and tissue at a low temperature. At least three minutes passed before the tissue was rinsed with cold distilled water. Two minutes of staining with cold 3% (v/v) aqueous ammonium sulfide solution on a freezing Petri dish followed. Before the tissue was mounted in water and viewed, it was rinsed again in cold water. Black crystals are indicative of the potassium ion. Since this is a ubiquitous ion in cells it is common to find some staining all over the peel and heavy staining in the active assimilative cells. In control plants (*Zea mays*), guard and subsidiary cells stand out from the rest of the epidermis due to their high activity, in comparison with the rest of the epidermal cells.

This stain was performed on 11 different sporophytes from five species and approximately 195 stomata were observed.

Organic ions: A 0.5% (w/v) Fast Violet B solution in 0.1M Tris buffer (pH 8.0) was used to visualize organic ions (Palevitz et al. 1981). The solution deteriorates quickly so must be mixed immediately before each use. Keeping the stain free from light and on ice slows the deterioration process which is visualized by a color change in the solution from clear, bright yellow to opaque red-orange. Epidermal tissue was stained for three minutes while covered then rinsed in buffer and mounted in water. The tissue must be examined quickly for the stain dissipates with time. Vacuoles in positive cells stain pink.

Five species of hornworts were sampled, 14 sporophytes with about 235 total stomata.

Movement:

Hornworts were generally kept under continuous light but the *A. punctatus* collection was transferred into darkness for 12 hours each night. After three days of this, plants were immediately sliced longitudinally, mounted in water, and viewed on an Olympus microscope at various times during the day and night. No more than two minutes were needed to prepare the slides. Plants were observed directly after removing them from the dark, midday, before placing them in the dark, and while they were in the dark.

Sporophytes were cut longitudinally and floated on a drop of aqueous ABA solution. The hormone concentrations were 1×10^{-3} , 6×10^{-5} , and 6×10^{-6} . The tissue was checked about every half hour for two hours to determine if the stomata had reacted to the hormone. For each concentration two sporophytes with about ten stomata were used. Two capsules of *Campylium hispidulum* were prepared as hornwort sporophytes and floated in the 1×10^{-3} ABA solution. Eight to fifteen stomata were present on each apophysis.

Results:

To coherently describe stomata and their walls, a set of terms is commonly used (Fig. 1A, B). The area where the two cells separate from each other is the pore or aperture. The cell wall that surrounds the stomatal pore and where the two guard cells meet is designated the ventral wall, the opposite anticlinal wall is termed the dorsal wall. The tangential wall that faces the atmosphere is the outer wall and the opposite paradermal wall is the inner wall. Often at the junction of the ventral wall surrounding the pore with either the inner or outer wall, wall materials accumulate forming inner and outer ledges respectively.

Development:

The first visible stomatal precursor is a rounded epidermal cell with a prominent starch filled chloroplast (Fig. 2A). This guard mother cell elongates and its anticlinal walls bulge into neighboring epidermal cells (Fig. 2B). Typically, one equal longitudinal, anticlinal division of this cell forms the two guard cells (Fig. 2C). Occasionally a slightly oblique division of the rounded guard mother cell will yield two guard cells. No subsidiary cells are formed in the development of the guard cells or surrounding epidermal cells. Wall thickenings begin to form in the midregion of the outer ventral wall before the middle lamella breaks down (Fig. 2D). During the degradation of the middle lamella, intercellular spaces form schizogenously internal to the stoma within the assimilative layer. Sheets and strings of remnant middle lamella material can be seen

between guard cells, surrounding epidermal cells, and subtending assimilative cells (Fig 3A). Concomitantly wall material is laid down primarily to form highly thickened inner and outer walls (Fig 3A). Upon initial separation of the two guard cells, the ventral thickened inner and outer wall regions are pulled apart and act as ledges (Fig. 3D). A mature stoma averages 70 μm long, 30 μm wide, and 20 μm deep; the aperture in face view is approximately a third of the length and a fifth of the width of the complex (Fig. 3A).

A regimented pattern for stomatal placement seems to be lacking in hornworts, for their appearance on the epidermis is not evenly distributed or in distinct arrangements. Rarely stomata are found that are directly adjacent to one another. More stomata are seen on older sections of sporophyte and no mature stomata are found on the sporangium still within the involucre. Rounded guard mother cells are found on all ages of sporophyte epidermis, from areas which have no mature stomata to the tip of the dehiscing sporangium. Often the older stomata are occluded with perhaps a waxy or mucilaginous material so that even if the pore is open little gas exchange could occur. Bacteria have been found living in the pore amongst the occlusion.

Organelles:

Despite their unique shape, the one to two large chloroplasts of guard cells make stomata easy to identify (Fig. 1B). Although all epidermal cells have starch filled chloroplasts while still in the involucre (Fig. 4A), throughout development the chloroplasts of nonspecialized epidermal cells contract, degenerate (Fig. 4B) and lose their ability for electron transport (Fig. 4D). All epidermal chloroplasts are adjacent to the inner tangential wall. In guard cells, the plastids are generally found in the ends of the guard cells. Like other actively photosynthetic plastids of hornworts, guard cell plastids have a pyrenoid (Fig. 4E). The nucleus is generally associated with the chloroplast. DAPI staining has shown the placement of the guard cell nucleus to be mainly in the center of the cells, yet this is somewhat variable. Characteristic of plant cells are vacuoles and guard cells are no exception. The one large vacuole occupies the central area of guard cells, next to the pore. Membrane bound multivesicular bodies are found in guard cells. Chloroplasts of assimilative cells which border the intercellular spaces are positioned adjacent to the space (Fig. 4C).

Wall Structure:

In tangential section, the guard cells are reniform in shape and an irregularly jagged projection partially covers the aperture on the outer surface. The two guard cells'

lumen are oval in cross section towards the transverse walls and appear circular at the pore. In longitudinal anticlinal section from dorsal side approaching the pore is a transition from a long oval cell to a centrally constricted, dumbbell shaped cell (Fig. 5A). Although the guard cell is elongate, it is still much shorter than other epidermal cells. However in cross section, guard cells are larger than other epidermal cells.

The guard cell walls are stratified into three distinct layers of parallel microfibrils, oriented differently from one another (Figs. 5A, B). These layers are difficult to see in the ventral wall where the two guard cells meet because the wall is compressed (Fig. 5D). Moreover, this wall does not autofluoresce as all other epidermal walls do. The microfibrils of the oldest wall layer are longitudinally oriented. Net radial micellation was not found.

At the junction of the ventral wall with either the inner or outer wall, ledges are present. The outer ledge extends into a thick outer wall that spans half way across the width of the cells. Continuous with epidermal cells, cuticle covers the outer wall. The inner wall ledge is even more pronounced; the greatest accumulation of wall materials occurs in the center of this wall. Radially aligned microfibrils are found in the inner ledge close to the lumen (Fig. 5E). Covering the entire inner wall and ventral wall surrounding the pore are middle lamella remnants (Fig. 6C). Near the transverse walls, neither the outer or inner wall is very broad. TEM observations have shown the outer ledge to be bordered on the outermost side by a thin layer of wall material oriented parallel to the outer wall (Fig. 6B). Beneath this layer is an area of branched fibrillar wall material. Ruthenium red staining has shown that pectin is the principle constituent of the outer wall outgrowth (Fig. 6A).

Histochemical stains:

Results for the cobaltinitrate stain varies with age of the sporophyte. Positive staining is seen in all epidermal cells from the foot up to a region with mature stomata (Fig. 7A). Localization of stain precipitate within the guard cells occurs above this region (Fig. 7B). A few scattered epidermal cells also stained in this area, but their occurrence was not patterned. In the oldest region of the sporophyte, no staining occurs in any cells. This section of the sporangium has dehisced and the assimilative cells appear functionless. Mostly the stain is seen in the lumen of the cells; positive reactions occur on walls also. Often the stomatal pore heavily stains, even in dehisced regions (Fig. 7B).

As for the potassium stain, a gradient of staining for malate emerges on the sporophyte. In the youngest pieces of sporophyte all cells stain positively (Fig. 7C). A

localization is seen as guard cell precursors develop (Fig. 7D). Epidermal cells level with spores that have been dispersed do not stain. Seemingly random epidermal cells also stain positive for organic ions.

Movement:

Guard cells are generally always open, although a few closed stomata usually are found on a sporophyte. Dark adapted plants mainly have open stomata as do plants observed during the nighttime. Attempting to force the stomata closed by using glycerine results in roughly a third of stomata closing, while the use of alcohol does not close the stomata. In both treatments, all epidermal cells lose water, seen by the constriction of the plasmalemma and cellular contents. Unfortunately the actual movement of stomata that did close was not observed. Neither a 6×10^{-5} or 1×10^{-4} solution of ABA caused the closure of stomata.

Moss stomata:

Only a few capsules were tested for potassium, organic ions, ABA and dehydration. Preliminary results showed that all epidermal apophysis cells stain positively for potassium and malate while the operculum is still retained on the capsule. After releasal of the operculum, no cells on the epidermis stain. At either development stage, the 1×10^{-4} solution of ABA did not close the stomata. Adding glycerine to capsule peels quickly closed stomata. Some stomata on the capsule were obvious because of bright red plastids in the guard cells.

Discussion:

Development: Due to the basal meristem in hornwort sporophytes all stages of stomatal development are observed on one mature sporophyte. At the rounded guard mother cell stage can be arrested from developing into a mature stoma. Since stomata are not produced in equal numbers at all ages or are evenly distributed on the sporophyte, it is likely there is little regulation of the development of the precursors. Epidermal cells are generally larger than guard cells but this is not true of hornworts. Mucilage is readily produced by hornworts, the cavities on the ventral thallus of the gametophyte are filled with mucilage (Renzaglia 1978); the occlusions, generally of mature stomata, appear to be composed of similar material. Many plugged stomata are found on dehiscd sporophyte tissue so water loss would not be a concern, but such considerations would affect living regions of the sporophyte. Even so no water transport system is present in hornworts (columella), therefore the water that is lost only affects the apoplast and

surrounding cells. Certainly this loss is minimal in comparison to the combined effects of water conducting tissue and evaporative loss via stomata that occurs in tracheophytes and mosses. The fact that substomatal chambers form and the placement of the assimilative cells' chloroplasts next to the chamber leaves little doubt that gas exchange.

Organelles: The complement of organelles within guard cells of anthocerotes is similar to those of other plants. Moderate grana stacks, starch granules occur in the chloroplasts, and a large vacuole occupies in the central cell region. The central placement of the vacuole differs from many plants that have vacuoles situated near the endwalls (Zeiger et al. 44). Angiosperm guard cell chloroplasts are thought to function slightly differently from actively photosynthetic cells (Shimazaki et al. 1989). The chloroplast is thought to assist turgor operated movement by shuttling Calvin-Benson intermediates between the stroma and cytosol. The localization of Rubisco in the pyrenoid may have profound effects on this process (Vaughn et al. 1992).

Wall Structure: Pronounced walls are found on the inner and outer surface of stomata, especially at the junction with the ventral wall. The outer ledge of pectin appears to be a middle lamella relic from the separation of the guard cells more than an active accumulation of wall material. Pectin is been thought to constitute a large part of guard cell walls but only the middle lamella of several plants stains positively with ruthenium red (Zeiger et al. 75). The sculpted inner ledge must be planned.

Wall layering in guard cells is reported for few species (Sack and Paolillo 1983), but it is distinct in hornworts. The apparent lack of radial micellation could severely affect stomata's ability to form an aperture. Thin dorsal and ventral walls, found in hornworts and most plant stomata, could allow the distortion of cell shape during movement (Paton and Pierce 1957). No exceptionally thin walls areas are observed which would function as a hinge.

Histochemical stains: Stomata are only histochemically discernible from other epidermal cells at one maturation stage, the final stages of spore maturation. The gradient of staining results correlates to the maturation of the sporophyte. After the spores have been dispersed there is no need for the sporophyte of equal age to continue functioning, therefore no staining is seen. In young sporophytic cells, all epidermal cells including guard cells and their precursors stain positively for potassium and malate. This is due to the near equal health of these young cells, although the chloroplasts of cells in the guard cell lineage are always conspicuous. Because of these chloroplast being more developed than surrounding epidermal cells, malate localizations are seen on younger sporophyte zones than potassium localizations. The heavy potassium localizations in the pore are likely due either to occlusions or organisms living in the aperture.

The results of maturation are also seen in moss stomata which do not show stain localizations in the young stages of capsule development and do not stain after the operculum was lost. More intensive studies need to be pursued to determine at which stage moss guard cells do exhibit ion localization and how that relates to spore development. In addition, the stomatal precursors of model plants such as *Zea mays* or *Vicia faba* should be explored histochemically.

Histochemically and morphologically no subsidiary cells are present on hornwort or moss sporophytes. Anomomytic stomata are found in all bryophytes and other nonrelated vascular plants. Such guard cells apparently do not need subsidiary cells to function, but vascular plants which have no subsidiary cells probably lost the need to form such cells unlike bryophytes which appear to be incapable of designing subsidiary cells.

Movement. Stomatal movement, diurnal or otherwise, does not seem to occur in hornworts. The only environmental factor which closes hornwort stomata is desiccation (Paton and Pierce 1953). Desiccation reduces the size of all vacuoles so certainly the turgor operated stomata should shut during water stress. Glycerine effectively dehydrates the epidermal peel, but not all stomata closed. The protoplasts were seen to plasmolyse so inadequate infiltration of the glycerine is not the explanation for inconsistency of stomatal closure. Possibly the stomata are unable to close at certain stages of development. Completely inflexible walls would prevent the movement of stomata, but as of yet no information has been collected regarding this. Moss stomata do close when dehydrated with glycerine.

ABA is found in all vascular plants and has been detected in mosses; liverworts have a physiologically similar hormone, lunularic acid (Tiaz and Zeiger 1972). Its presence in hornworts is undetermined though. Epidermal peels of hornwort sporophyte did not respond to ABA treatment, neither did the moss capsule epidermis. These results are quite preliminary, for a wide range of ABA concentrations was not studied and previous studies have shown *Funaria hygrometrica* to close when exposed to ABA (Garner and Paolillo 1973).

Conclusions:

The development of hornwort stomata is very simple. This is indicated by the single longitudinal division of the guard cell precursor, pectinous ledges, lack of subsidiary cells, and lack of radial micellation. Gas exchange seems to be a likely function of hornwort stomata, but the absence of vascular tissue makes water transport improbable. Histochemical stains for malate and potassium indicate that guard cells

localize ions for a short time- after the differentiation of the epidermis and before spore dispersal. Diurnal guard cell movements do not occur in hornworts. Neither dehydration or ABA treatment effects the guard cells in respect to movement.

It is still unclear whether or not hornwort stomata are homologous to stomata of vascular plants. The prominent chloroplast, the localization of ions, and the role of stomata in gas exchange suggest that anthocerotale stomata are related to those of other embryophytes. However, the lack of vascular tissue and stomatal movement counter the homologous theory. Also in opposition to this paradigm is the distinct wall structure of hornwort guard cells. A multilayered wall and ledges of pectin have only been reported for a few other plants. In the future to elucidate the homology of these structures, the effect of ABA should further be studied. Also the guard cells' ability to transport ions, which is essential for movement, should be determined.

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