American Journal of Botany 84(9): 1169–1178. 1997.

COMPARATIVE ULTRASTRUCTURE OF PLASMODESMATA OF *CHARA* AND SELECTED BRYOPHYTES: TOWARD AN ELUCIDATION OF THE EVOLUTIONARY ORIGIN OF PLANT PLASMODESMATA[1]

MARTHA E. COOK,[2,5] LINDA E. GRAHAM,[2] C. E. J. BOTHA,[3] AND COLLEEN A. LAVIN[4]

[2] Department of Botany and [4] Intergrated Microscopy Resource, University of Wisconsin-Madison, Madison Wisconsin 53706; and [3] Department of Botany, Schönland Botanical Laboratories, Rhodes University, P. O. Box 94, Grahamstown, 6140, South Africa

We have used transmission electron microscopy to examine plasmodesmata of the charophycean green alga *Chara zeylanica,* and of the putatively early divergent bryophytes *Monoclea gottschei* (liverwort), *Notothylas orbicularis* (hornwort), and *Sphagnum fimbriatum* (moss), in an attempt to learn when seed plant plasmodesmata may have originated. The three bryophytes examined have desmotubules. In addition, *Monoclea* was found to have branched plasmodesmata, and plasmodesmata of *Sphagnum* displayed densely staining regions around the neck region, as well as ring-like wall specializations. In *Chara,* longitudinal sections revealed endoplasmic reticulum (ER) that sometimes appeared to be associated with plasmodesmata, but this was rare, despite abundant ER at the cell periphery. Across all three fixation methods, cross-sectional views showed an internal central structure, which in some cases appeared to be connected to the plasma membrane via spoke-like structures. Plasmodesmata were present even in the incompletely formed reticulum of forming cell plates, from which we conclude that primary plasmodesmata are formed at cytokinesis in *Chara zeylanica.* Based on these results it appears that plasmodesmata of *Chara* may be less specialized than those of seed plants, and that complex plasmodesmata probably evolved in the ancestor of land plants before extant lineages of bryophytes diverged.

Key words: bryophytes; *Chara*; charophycean algae; *Monoclea*; *Notothylas*; plant evolution; plasmodesmata; *Sphagnum.*

Plants are divided into cells that remain cytoplasmically connected via plasmodesmata. Several workers have emphasized the unity of the plant body, and have stressed the importance of plasmodesmata in maintaining cell-to-cell communication within the plant as an integrated organism (Niklas, 1989; Kaplan and Hagemann, 1991; Lucas and Wolf, 1993). It has been suggested that plasmodesmata may serve as channels for simple diffusion and complex molecular trafficking, and that plant development at the cell, tissue, and organ levels may be regulated by means of plasmodesmata in the apical meristem (Lucas, Ding, and Van der Schoot, 1993; Duckett et al., 1994; Epel, 1994; Lucas, 1995; Lucas et al., 1995).

Many researchers have examined the ultrastructure of primary plasmodesmata in seed plants (López-Sáez, Giménez-Martin, and Risueño, 1966; Hepler, 1982; Gunning and Overall, 1983; Robards and Lucas, 1990; Ding, Turgeon, and Parthasarathy, 1992; Botha, Hartley, and Cross, 1993 and references cited therein), which are formed at cytokinesis when endoplasmic reticulum (ER) is incorporated into the forming cell plate (Hepler, 1982). The ER that passes through the plasmodesma forms what is commonly accepted as the desmotubule. The model for plasmodesmatal ultrastructure proposed by Ding, Turgeon, and Parthasarathy (1992), based on tobacco tissue preserved by high-pressure freezing and freeze substitution, suggests that particles are associated with the inner leaflet of the plasma membrane and the outer leaflet of the desmotubule, and that spoke-like extensions connect the plasma membrane and the desmotubule in the expanded central regions of mature plasmodesmata. This model is supported by more recent work using chemically preserved grass tissue (Botha, Hartley, and Cross, 1993). Other features of seed plant plasmodesmata include narrow neck regions that may be associated with sphincters (Oleson and Robards, 1990), and ring-like wall specializations (Badelt et al., 1994).

The present study focuses on the ultrastructure of plasmodesmata of charophycean algae and putatively basal bryophytes in an attempt to further elucidate the evolutionary origin of seed plant plasmodesmata. Land plants (embryophytes), including the bryophytes, appear to be a monophyletic group that arose from an ancestor held in common with certain modern charophycean green algae (Graham, Delwiche, and Mishler, 1991; Mishler et al., 1994; McCourt et al., 1996). Common ancestry suggests that modern seedless plants and charophytes can be

[1] Manuscript received 26 April 1996; revision accepted 5 January 1997.

The authors thank Lee Wilcox, M.V. Parthasarathy, Robyn Overall, and Ned Friedman for helpful discussion; Ray Evert for the use of TEM facilities; Yasuko Kaneko for her bryophyte fixation protocol; Scott Kroken for isolation of *Notothylas* and for collection of *Sphagnum*; Gary Breckon and Karen Renzaglia for collection, respectively, of *Monoclea* and *Notothylas*; and George Headley for assistance with sending computer images between Madison and Grahamstown via the internet. High pressure freezing was done at the Integrated Microscopy Resource, which is supported by NIH Biomedical Research Technology Grant RR00570.

[5] Author for correspondence, current address: Department of Evolutionary, Population, and Organismic Biology, University of Colorado at Boulder, Campus Box 334, Boulder, Colorado 80309 (mcook@colorado.edu).

used in tracing the evolution of more complex features of seed plants. Therefore, we used a comparative approach to ascertain when the complex features of higher plant plasmodesmata may have arisen.

Charophycean algae include organisms with a wide range of body types, from unicells (desmids) and unbranched filaments (*Spirogyra, Mougeotia*), to the more complex branched filamentous or parenchymatous bodies of *Coleochaete* and Charales (Graham, 1993). Based on molecular, biochemical, and cellular evidence, *Coleochaete* and the Charales are thought to be the closest extant algal relatives to land plants (Graham, Delwiche, and Mishler, 1991; Manhart, 1994; McCourt et al., 1996), and it is in these more complex charophytes that plasmodesmata are known to occur (Graham, 1993). Graham and Kaneko (1991) pointed out that cell division mechanisms of charophycean algae exhibit an evolutionary progression from furrowing with a rudimentary phragmoplast in Zygnemataceae (unbranched filaments), to centrifugal cell plate formation with a plant-like phragmoplast in the complex forms that possess plasmodesmata. Since cytokinesis in *Chara* is known to involve a plant-like phragmoplast and entrapment of ER in the forming cell plate (Pickett-Heaps, 1967b), it seems reasonable to expect that the internal structure of its plasmodesmata might be similar to that of seed plants, hence we chose to examine this member of the Charales.

Pickett-Heaps (1967b, fig. 27; 1968, figs. 23, 24) and Kwiatkowska and Maszewski (1986, fig. 17) have demonstrated ER traversing plasmodesmata in *Chara*. However, Pickett-Heaps (1967a, b) also reported that in older walls plasmodesmata contain various substructures that do not appear to be directly connected with the ER. Furthermore, Spanswick and Costerton (1967) found internal structure in only a few *Nitella translucens* plasmodesmata, and Fischer and MacAlister (1975) and Franceschi, Ding, and Lucas (1994) did not observe ER in plasmodesmata of *Chara corallina*. In addition, Franceschi, Ding, and Lucas (1994) found no plasmodesmata in young cell plates of *Chara corallina*, concluding that all plasmodesmata in that species are formed secondarily. The internal structure and formation of plasmodesmata in the Charales are thus controversial.

Little previous ultrastructural or physiological work has been done on bryophyte plasmodesmata. Two studies of internal transport in bryophytes support a concept of complex plasmodesmatal structure compatible with the function of molecular trafficking (Rydin and Clymo, 1989; Trebacz and Fensom, 1989). The latter authors showed that internal transport in the liverwort *Conocephalum conicum* is similar to that in cells of *Nitella* with respect to rate of transport and reaction to pressure changes, and concluded that transport was similar in a filament and in a tissue. Wall pits with perforations that are thought to be plasmodesmatal channels have been described in various liverworts (Hébant, 1978; Giordano et al., 1989; Ligrone and Duckett, 1994). Similar plasmodesmatal connections occur in the leaflets (Schnepf and Sych, 1983), stem, and fascicles (Baker, 1988) of the *Sphagnum* gametophore. We have found only two reports documenting the occurrence of seed-plant-like plasmodesmata with desmotubules in bryophytes; that of Schnepf and Sawidis (1991) on protonemal filaments of the moss *Funaria hygrometrica*, and that of Ligrone and Duckett (1994) on the thallus of the liverwort *Asterella wilmsii*.

Phylogenetic relationships among the seedless plants are incompletely resolved, but it appears that bryophytes are basal to the tracheophytes, and that the liverworts may be the earliest divergent bryophytes (Mishler and Churchill, 1984, 1985; Waters et al., 1992; Mishler et al., 1994). To increase the chances of finding features reflecting those of the common ancestor of both bryophytes and vascular plants, rather than derived features of bryophytes, we chose taxa for which there is evidence of relatively early divergence within each of the three bryophyte divisions. *Monoclea* is considered by Schuster (1984) to be a basal liverwort because it exhibits a synthesis of features found in other liverworts. In addition, archesporial cells of *Monoclea* are monoplastidic (Brown and Lemmon, 1992), and monoplastidy is a pleisiomorphic plant characteristic thought to have been inherited from the charophycean green algal ancestors of land plants. Characteristics that suggest *Notothylas* is an early divergent hornwort include a thallus that stops apical growth after fertilization, synchronous spore production, and poorly developed pseudoelaters (Hyvönen and Piippo, 1993; Hasegawa, 1994). *Sphagnum* is considered to be a basal moss taxon based on analysis of chloroplast ribosomal RNA genes (Mishler et al., 1992) and on various sperm characteristics, including presence of a single starch grain in a solitary plastid, a character shared with hornworts (Garbary, Renzaglia, and Duckett, 1993). Therefore, we examined the ultrastructure of plasmodesmata of *Monoclea*, *Notothylas*, and *Sphagnum*.

MATERIALS AND METHODS

Specimen collection and culture—A culture of unknown provenance, which we identified as *Chara zeylanica* (based on Wood, 1967), was obtained from a soil-water tank that has been maintained in our laboratory for >20 yr. An unidentified charalean protonema was collected from Spring Lake in Green Lake County, Wisconsin. Specimens of *Monoclea gottschei* were collected from municipality Orocovis, barrio Ala de la Piedra, Toro Negro Commonwealth Forest, Puerto Rico in late February of 1994, and kept alive on wetland soil in a terrarium. *Notothylas orbicularis* was collected from Carter County, Tennessee. Capsules were surface sterilized in 20% Clorox for 30 min, with sonication for the first 5 min, rinsed and sliced open, and spores sown on agarized Guillard's medium (Sigma). A single thallus was eventually subcultured and propagated vegetatively on agarized half-strength Knop's medium (Schofield, 1985). *Sphagnum fimbriatum* was collected from Jyme Bog in Oneida County, Wisconsin in July of 1993. Capsules were surface sterilized for 5 min in a solution of 5% Clorox in water, rinsed, and left to dry in a closed but not sealed petri dish. When the capsules dried they released spores into the dish. These were retrieved by adding a few drops of water to the dish with a Pasteur pipette, then pipetting out the spore-laden drops onto a plate of agarized half-strength Knop's medium (Schofield, 1985). We examined the resulting protonemal plates rather than the apices of mature shoots, since this juvenile stage might be more likely to retain ancestral features than would the adult.

Preservation and electron microscopy—Shoot tips of *Chara zeylanica* were fixed in three different ways. (1) Stepwise chemical fixation (SCF), which we designed especially for charalean algae, was based on Taylor's (1988) work on the osmotic effects of buffers and fixatives on *Nitella flexilis*. Progressively higher concentrations of glutaraldehyde in

25 mmol/L phosphate buffer (pH 7) were introduced as follows: 0.5, 1.0, 1.5, and 2.0%, with shoot tips immersed in each solution for 30 min. Following the primary fixation, tissue was rinsed in buffer three times for 10 min each, postfixed for 1 h in 1% osmium tetroxide, rinsed three times for 10 min each in water, and stained for 30 min in a 0.5% aqueous solution of uranyl acetate (UA). Specimens were then dehydrated in a graded ethanol series, infiltrated in two steps with Spurr's resin, embedded, and polymerized in a 70°C oven. (2) Potassium ferricyanide (KFe) postfixation to enhance ER and other membranes was adapted from Hepler (1982). This differed from SCF in that 0.8% potassium ferricyanide [$K_3Fe(CN)_6$] was included along with osmium in the postfixation, no UA was used, dehydration was with acetone, and infiltration took place in three steps. (3) High-pressure freezing (HPF) took place in a buffered sucrose solution modified from Ding, Turgeon, and Parthasarathy (1992). Shoot tips were immersed in a 20 mmol/L HEPES buffer solution (pH 8.5) containing 2 mmol/L $CaCl_2$, 2 mmol/L KCl, and 0.2 mol/L sucrose for 30–60 min and frozen with a Balzers HPM010 high-pressure freezer (Balzers Union, Liechtenstein), the buffered sucrose solution also being used to fill in space not taken up by the specimens in the planchettes. Specimens were freeze substituted with 1% osmium in acetone at −80°C for 3 d, −20°C overnight, brought slowly to room temperature over a period of 2 h, rinsed with pure acetone, and infiltrated, embedded, and polymerized as for SCF above. The unidentified charalean protonema was preserved via SCF.

Specimens of *Monoclea* and *Notothylas* were sliced in fixative into pieces of <2 mm^2. Three-week-old *Sphagnum* protonemal plates were fixed whole, on a chunk of agar on which they had been growing. Fixation was with 2% glutaraldehyde in 0.05 mol/L phosphate buffer, pH 7, for 4.5 h, with occasional agitation, followed by six 10-min rinses in the phosphate buffer. Specimens were postfixed for 2 h in 2% osmium tetroxide, dehydrated in a graded acetone series (10 min each solution, 100% three times), infiltrated with Spurr's resin in three steps (50%—1 h, 75%—1 h, and 100%—overnight, with a fresh change of 100% the next day), embedded, and polymerized overnight in fresh 100% resin in a 70°C oven.

Thin sections (silver-gold) were cut using a Sorvall MT2-B Ultra Microtome with a Dupont diamond knife, stained for 5–10 min with aqueous Reynold's lead citrate, and viewed using a JEOL JEM-1200 EX electron microscope at either 60 or 80 kV. For *Chara zeylanica*, nodes of the main axis as well as branch nodes were examined. Sections of *Monoclea* and *Notothylas* were cut tangential to the edge of the thallus on a lobe near a meristematic notch, while those of *Sphagnum* were cut parallel to the protonemal surface. Using the terminology for dorsiventral thalli employed by Renzaglia (1978), the sections cut were transverse and horizontal longitudinal.

RESULTS

Mature plasmodesmata of *Chara zeylanica* have neck constrictions that are discernible in longitudinal section (Figs. 1, 2) as well as in transverse section (Fig. 3). Neck constrictions are observed in transverse section by comparing diameters of plasmodesmata in the median region of the wall, where sections are cut through the larger central cavities (Fig. 3, right), with diameters of the narrower neck regions near the edge of the wall, next to the cytoplasm (Fig. 3, left). Internal ultrastructure of the plasmodesmata seen in longitudinal view (Figs. 1, 2, 8, 9, 10) is difficult to interpret in all three treatments. In the plasmodesma in Fig. 1 there is a linear internal structure (arrow), but despite the presence of abundant ER at the cell periphery (Fig. 8, arrowhead), plasmodesmata were observed only occasionally to have ER closely associated with them (Figs. 1, 2, 9, 10, arrowheads). Such associated ER was seen more often in the HPF treatment (Figs. 9, 10), even though ER did not stain well in this treatment. In transverse view, on the other hand, internal structure is frequently evident (Figs. 3, 4, 7, 11), even at low magnification (Fig. 3, arrowheads). Some plasmodesmata also display short linear extensions that radiate out from the plasma membrane (Fig. 3, arrow). Figs. 4, 7, and 11 demonstrate, in all three treatments of *Chara zeylanica*, presence of a central structure (arrows) that appears to be connected to the plasma membrane via spoke-like structures (aligned with arrowheads). In addition, a central structure was found in plasmodesmata of the protonema of an unidentified charalean species (Fig. 6, arrow). The apparent central structure in longitudinal view of a protonemal plasmodesma (Fig. 5, arrowhead) may be a glancing section of the plasma membrane in the foreground.

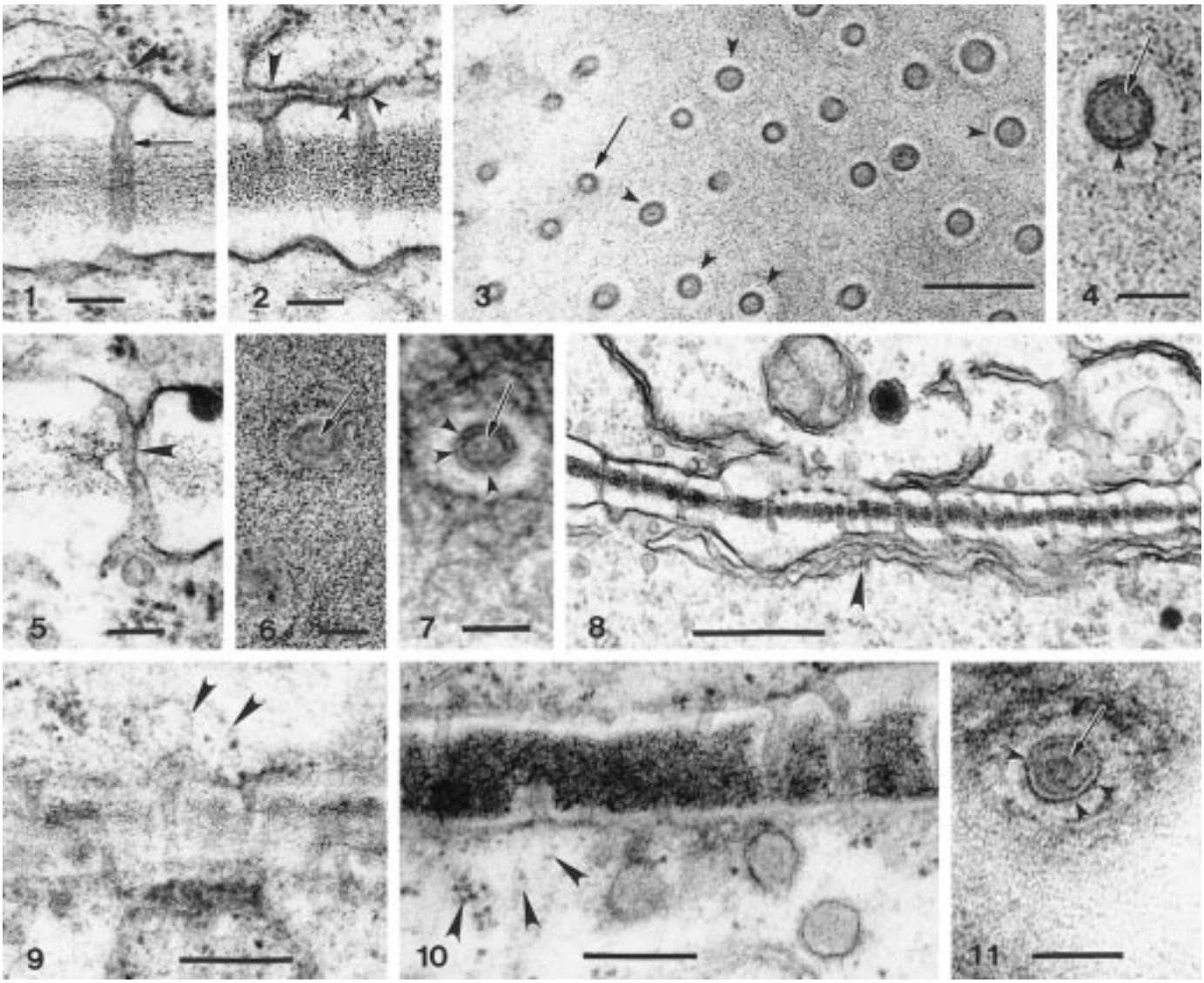
During cytokinesis in *Chara zeylanica*, ER is incorporated in the forming cell plate (Fig. 12, large arrowhead). Associated with the plate are coated vesicles (Fig. 13, arrow) that are much larger than the diameters of nearby plasmodesmata (Fig. 13, arrowheads). Plasmodesmata are found in very young plates that are from 25 to 50 nm thick (Figs. 12–14, small arrowheads), and in reticulate, incompletely formed edges of cell plate where they connect with the mother cell wall (Fig. 15, arrowheads). Cell plate formation occurs simultaneously across the cell, except for the edges, which lagged behind (Fig. 15).

In *Monoclea*, *Notothylas*, and *Sphagnum* longitudinal views of plasmodesmata show a desmotubule that connects to ER in the cell proper (Figs. 16–19, 20, 24, 29, large arrowheads). Transverse views also show a desmotubule in plasmodesmata of all three bryophytes. In the only transverse view we have of a plasmodesma in *Monoclea* (Fig. 19, small arrowhead), the internal structure observable is the desmotubule. Transverse views of plasmodesmata of *Notothylas* and *Sphagnum* appear to display, in addition to the desmotubule (Figs. 21–23, 25–27, arrows), spoke-like structures that connect the desmotubule to the plasma membrane (Figs. 23, 26, 27, aligned with small arrowheads).

Found in *Sphagnum*, but not in the other genera, are ring-like wall specializations (Fig. 28, arrowheads) and connected short, linear structures that appear to extend to the plasma membrane (Figs. 26, 27, aligned with big arrowheads). Fixation with potassium ferricyanide reveals densely staining regions that are associated with necks of plasmodesmata in *Sphagnum* (Fig. 29, arrows). Branched plasmodesmata (Figs. 16, 17) were found in *Monoclea*. No evidence for neck constrictions near the plasma membrane–cell wall interface is evident in any of the three bryophytes.

DISCUSSION

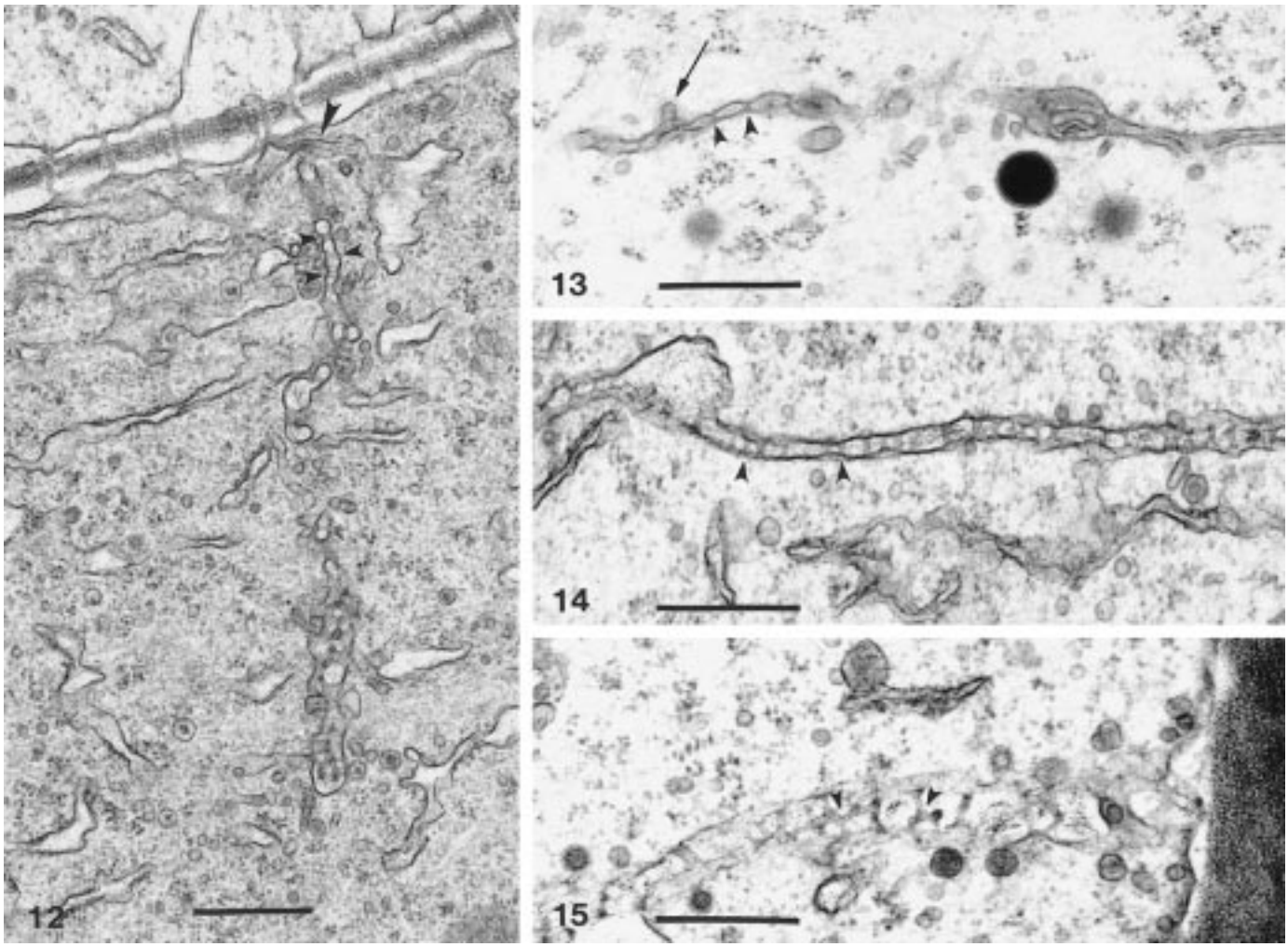
This study provides support for the reports of Pickett-Heaps (1968) and Kwiatkowska and Maszewski (1986) that ER traverses plasmodesmata in *Chara* at some locations. In addition, it presents detailed information about the ultrastructure of plasmodesmata in *Chara zeylanica*. Important features shared by plasmodesmata of plants and *Chara zeylanica* are neck constrictions and a central structure from which spoke-like extensions appear to



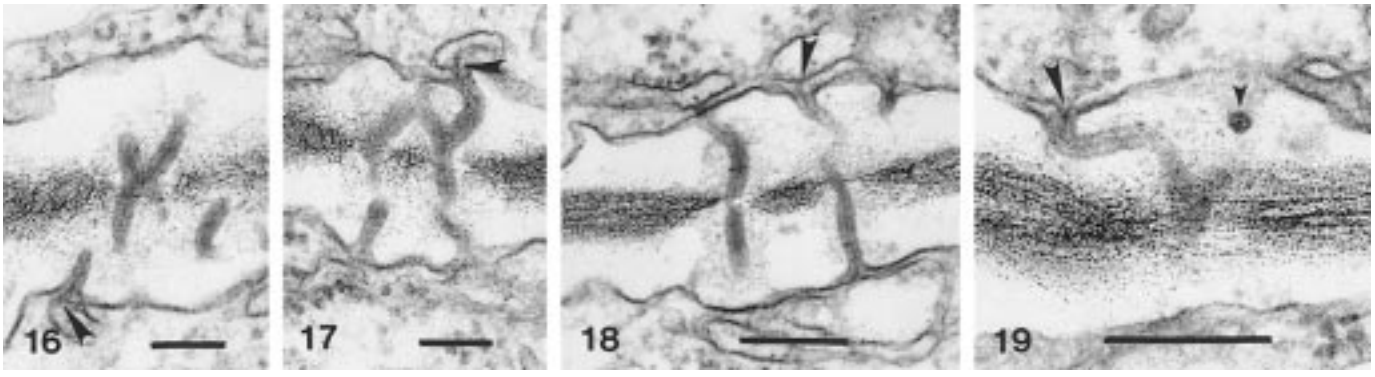
Figs. 1–11. Charalean plasmodesmata preserved via SCF (Figs. 1–6), KFe (Figs. 7–8), and HPF (Figs. 9–11). All are of *Chara zeylanica*, except Figs. 5 and 6, which are of an unidentified charalean protonema. **1, 2.** Longitudinal views in branch node. ER (arrowheads) appears to be closely associated with these plasmodesmata. Also note neck constrictions. A central linear structure (arrow) can be seen in the plasmodesmata in Fig. 1. Bars = 100 nm. **3.** Transverse views in branch node, a number of which (arrowheads) have internal structure, and one of which (arrow) has small linear extensions radiating from the plasma membrane. Smaller profiles near neck regions near the cytoplasm are at left, while wider profiles deeper in the wall are at right. Bar = 200 nm. **4.** Transverse view in branch node. Note central structure (arrow), and spoke-like structures that appear to connect the central structure to the plasma membrane (aligned with small arrowheads). Bar = 50 nm. Figs. 5, 6. Unidentified charalean protonema. **5.** Longitudinal view of plasmodesma from branch axis with apparent central structure (arrowhead) that may be a glancing section of plasma membrane. Bar = 100 nm. **6.** Transverse view from main axis with central structure (arrow). Bar = 50 nm. **7.** Transverse view of plasmodesma in main axis. Note central structure (arrow), and spoke-like structures that appear to connect the central structure to the plasma membrane (aligned with small arrowheads). Bar = 40 nm. **8.** ER (arrowhead) along new wall of peripheral cell, transverse view of main axis node. Bar = 500 nm. **9.** Central cell wall, transverse view of main axis node. Longitudinal view of plasmodesmata that appear to have ER (arrowheads) closely associated with them. Bar = 200 nm. **10.** Longitudinal view of branch axis. ER (arrowheads) closely associated with plasmodesmata. Bar = 200 nm. **11.** Transverse view of plasmodesma in main axis with central structure (arrow), and spoke-like structures that appear to connect the central structure to the plasma membrane (aligned with small arrowheads). Bar = 50 nm.

connect directly to the plasma membrane. While these features are not as prominent or as common in *Chara zeylanica* as they are in higher plants, it is possible that numerous modifications in plasmodesmatal ultrastructure may well have occurred during the several hundred million years since charophycean algae and plants diverged from a common ancestor. The electron-opaque structures that appear to radiate with the outer edge of the plasma membrane are in agreement with those described by Franceschi, Ding, and Lucas (1994) in *Chara corallina*. Similar structures have been reported in ferns and in barley (Badelt et al., 1994).

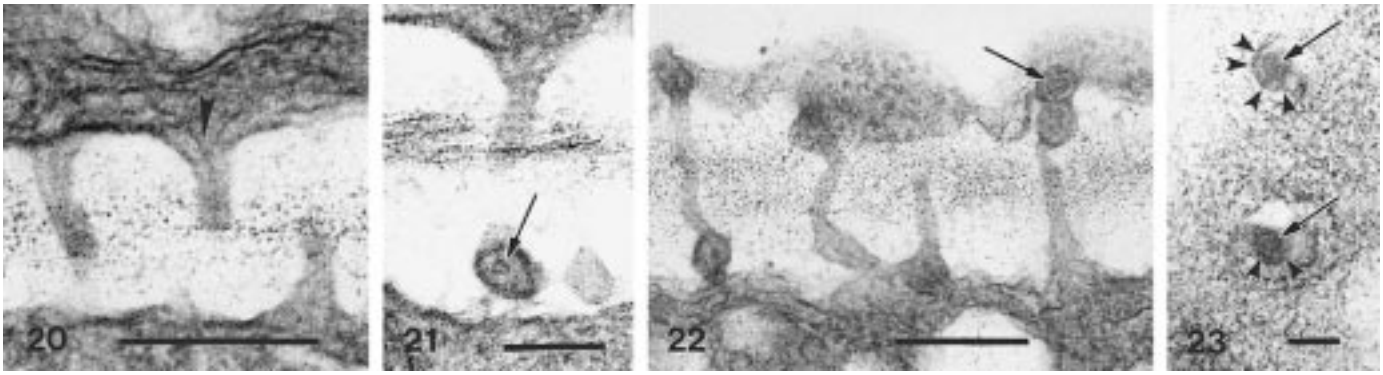
Our results for *Chara* are consistent across three fixation treatments, providing some assurance that the internal structures that are reported here are not artifacts. In the HPF treatment there is a difference in appearance



Figs. 12–15. Cytokinesis in *Chara zeylanica.* Preservation via KFe (Figs. 12, 14, 15) and HPF (Fig. 13). Bars = 500 nm. **12.** Longitudinal view of branch node in which peripheral cell is being cut off. ER (large arrowhead) is trapped in the forming cell plate, and plasmodesmata (small arrowheads) are already present when the plate is only 36 nm thick. Figs. 13–15. Longitudinal views of plasmodesmata in branch axis. **13.** Plate ranges in width from 25 nm where plasmodesmata (small arrowheads) traverse it, to 50 nm elsewhere. Note coated vesicle (arrow) has a greater diameter than do nearby plasmodesmata. **14.** Plasmodesmata (small arrowheads) traverse the plate along its length. Plate is 50 nm thick. **15.** Reticulate edge of plate as it fuses with mother cell wall (right). Plasmodesmata (small arrowheads) are present in the reticulum.

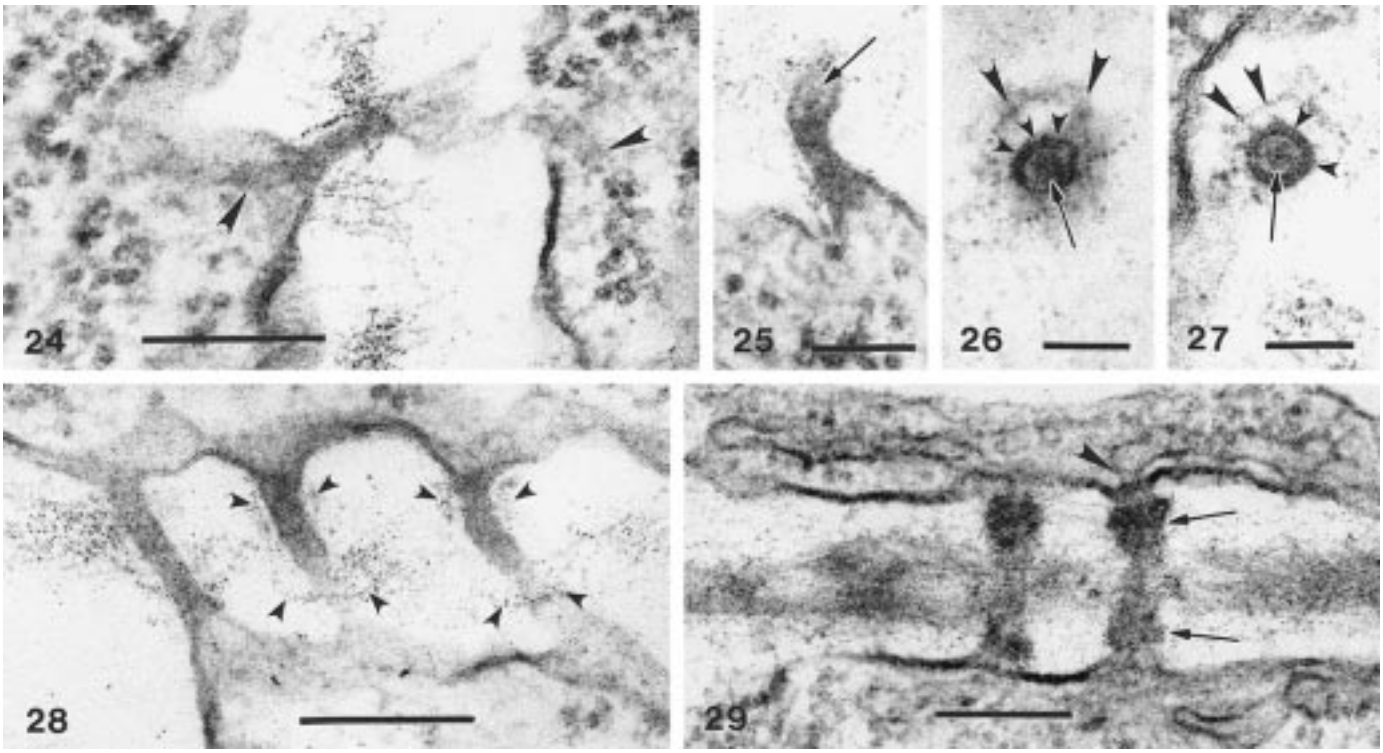


Figs. 16–19. *Monoclea.* **16, 17.** Longitudinal views of branched plasmodesmata with internal ER (arrowheads). Bars = 125 nm. **18, 19.** Longitudinal views of plasmodesmata showing that ER traversing the plasmodesma is continuous with ER in the cell proper (large arrowheads). Fig. 19 contains a transverse view of a plasmodesma (small arrowhead), with desmotubule visible in center. Bars = 200 nm.



Figs. 20–23. *Notothylas.* **20.** Longitudinal view of plasmodesma in which plasmodesmatal ER is seen to connect to ER in cell proper (arrowhead). Bar = 200 nm. **21, 22.** Plasmodesmata curve in the wall so that both longitudinal and transverse views are seen. Desmotubules are indicated (arrows) in transverse view. Bars = 100 nm. **23.** Transverse views showing desmotubules (arrows) and spoke-like structures that appear to connect the desmotubule to the plasma membrane (aligned with small arrowheads). Bar = 50 nm.

between the central structure, which is well stained, and intracellular ER, which is difficult to see. Such differences are also apparent in HPF tobacco plasmodesmata (Ding, Turgeon, and Parthasarathy, 1992). Generally, in plant tissues fixed by different methods, membranes associated with plasmodesmata appear modified in comparison with those that are not associated with plasmodesmata. For example, there appear to be no H^+-ATPases in plasma membranes associated with plasmodesmata, while they are associated with intracellular plasma membrane (Fleurat-Lessard, 1995), and differences have been reported in the preservation of desmotubules and intracellular ER (Hepler, 1982; Oleson and Robards, 1990) and between plasma membrane inside and outside plasmodesmata (Ding, Turgeon, and Parthasarathy, 1992). In addition, various degradative chemical treatments have been found to affect intercellular membranes differently from those in the cell proper, possibly indicating that connections between the desmotubule and intracellular ER are not as strong as are connections between desmotubule



Figs. 24–29. *Sphagnum.* **24.** Longitudinal view showing ER (arrowheads) traversing plasmodesma. Bar = 200 nm. **25–27.** Transverse views showing desmotubules (arrows). Bars = 100 nm. **26, 27.** Spoke-like structures appear to connect the desmotubule to the plasma membrane (aligned with small arrowheads), and linear extensions appear to connect the plasma membrane to ring-like wall specializations (aligned with large arrowheads that touch ring-like wall specializations). Bars = 50 nm. **28.** Longitudinal views of plasmodesmata displaying apparent ring-like wall specializations (small arrowheads). Bar = 200 nm. **29.** KFe, longitudinal view. ER is seen entering plasmodesma (arrowhead). Neck regions (arrows) stain densely. Bar = 200 nm.

and intercellular plasma membrane (Tilney et al., 1991; Oparka, 1994; Turner, Wells, and Roberts, 1994).

While we cannot rule out the possibility that cross-sectional thickness is such that neck constriction profiles have been interpreted as central plasmodesmatal structures, and that the spoke-like structures are linear extensions radiating out from the plasma membrane of a neck constriction, the plasma membrane thickness in the figures presented in this paper is much greater than it would be if the central structure was a neck constriction. Furthermore, the central structure does not exhibit typical unit membrane structure, while the plasma membrane does. It is curious that longitudinal views do not clearly demonstrate ER traversing plasmodesmata, since we observed ER being trapped in a forming cell plate. However, it may be difficult to routinely demonstrate plasmodesmatal ultrastructure via electron microscopy, because ER may be out of the plane of section (Esau and Thorsch, 1985), or because the cross-sectional size of the plasmodesma is small in relation to the thickness of the section (Robards and Lucas, 1990). In addition, it may be that the desmotubule is less stable than the plasma membrane inside the plasmodesma, as was demonstrated in protonemata of the moss *Funaria* (Schnepf and Sawidis, 1991).

We present evidence that primary plasmodesmata are formed during cytokinesis in *Chara zeylanica.* Primary formation of plasmodesmata is demonstrated by entrapment of ER in forming cell plate, and by presence of plasmodesmata in very young walls (as indicated by wall thickness), including the reticulate, forming edge of a new cell plate as it attaches to the mother cell wall.

Our results with respect to plasmodesmatal ultrastructure and formation of plasmodesmata in *Chara zeylanica* differ from those of workers studying other charalean taxa (Spanswick and Costerton, 1967; Fischer and MacAlister, 1975; Franceschi, Ding, and Lucas, 1994) in that we found internal plasmodesmatal structure, as well as primary plasmodesmata formed during cytokinesis. We believe that these differences may be attributable to species or to fixation method. A brief survey of fixation methods reveals that details of charalean plasmodesmatal ultrastructure are more often observed when low concentrations of fixative and low osmolarity buffers are used (Pickett-Heaps, 1968; Kwiatkowska and Maszewski, 1986; the present study) than with a harsher fixation protocol (Spanswick and Costerton, 1967; Fischer and MacAlister, 1975; Franceschi, Ding, and Lucas, 1994). It is unclear whether plasmolysis, which might be associated with fixation, would substantially alter plasmodesmatal substructure (Oparka, 1994; Oparka, Prior, and Crawford, 1994; Bewley, 1995), but changes in osmolarity have been found to affect neck constrictions (Schulz, 1995). Schulz (1995) suggested that HPF may be problematic for preservation of plasmodesmata because cryoprotectants may affect osmolarity and because freeze substitution requires an extended fixation period.

Potassium permanganate fixation, which highlights membranes (Hayat, 1970; Bozzola and Russell, 1992), resulted in spectacular views at lower magnification of ER traversing plasmodesmata in *Zea mays* root tips (Lucas, Ding, and Van der Schoot, 1993), though no fine structural details of plasmodesmata at higher magnification were presented. We tried this method (3% $KMnO_4$ in 0.05 mol/L pH 7 phosphate buffer for 2 h at 4°C) on *Chara zeylanica,* but found that it caused much artifactual damage, producing an amorphous cytoplasm and indistinct plasma membrane. Furthermore, potassium permanganate is thought to denature proteins (Hayat, 1970), so even with modification of this technique, we would not expect to see fine details of plasmodesmata if there were proteinaceous particles associated with the desmotubule wall and the inner plasma membrane leaflet, as suggested for plants by Ding, Turgeon, and Parthasarathy (1992), Botha, Hartley, and Cross (1993), and Lucas, Ding, and Van der Schoot (1993).

Additionally, different developmental stages could also explain differences between the results presented here and those of other authors. There is a great range in the sizes of charalean plasmodesmata, and there seems to be some correlation between the size of an individual plasmodesma and its location in the thallus (Spanswick and Costerton, 1967; Fischer, Dainty, and Tyree, 1974). A general size range of 25–50 nm was found in *Chara zeylanica,* but this does not include branched plasmodesmata, in which the central cavity may be 400 nm or more (Spanswick and Costerton, 1967; Franceschi, Ding, and Lucas, 1994), or apparent coalescence of (for example) four, 80-nm plasmodesmata into one plasmodesma of 200 nm in diameter (M. E. Cook, personal observation, unpublished data).

Given this great variety in diameter, it seems reasonable that plasmodesmata in different parts of the thallus may be specialized for different purposes. The 45-kDa size exclusion limit (SEL) reported for *Nitella* and obtained from internodal cells in the main axis (Kikuyama et al., 1992) suggests that these cells could be modified for axial macro molecular transport by developmental loss of any internal ER substructure at the plasmodesmatal connections with nodal cells, or by initial development of plasmodesmata devoid of internal ER. The former idea is supported by Pickett-Heaps's (1967b) observations that ER is trapped in the cell plate during cell division in *Chara,* but that central structures of plasmodesmata in older cells lack connections to the ER. Alternatively, the degree of evolutionary loss of internal plasmodesmatal structure may vary with location in the charalean thallus or among taxa.

Based on previously published data, *Chara* plasmodesmata exhibit a range in diameter of 25–100 nm, including the plasma membrane (Pickett-Heaps, 1967a, 1968; Kwiatkowska and Maszewski, 1986; Franceschi, Ding, and Lucas, 1994). Plasmodesmata described in this paper have diameters ranging from 12 to 27 nm at the neck constrictions, and from 25 to 50 nm in the center. These measurements are generally in keeping with those in the model of Ding, Turgeon, and Parthasarathy (1992) of higher plant plasmodesmata, in which the neck averages 24 nm and the central cavity 35 nm. Given the similarities between size and structure of charalean plasmodesmata and those of higher plants, it is curious that their SELs of 45 kDa and 1 kDa (Lucas and Wolf, 1993 and references therein), respectively, are so different. Citovsky (1993) identified a relationship between the SEL and the channel size of higher plant plasmodesmata, with the channel size (2.5 nm, according to Ding, Turgeon, and

Parthasarathy, 1992) being a little larger than the globular particle of 2 nm, which corresponds to the 1-kDa SEL (Robards and Lucas, 1990 and references therein). Lucas and Wolf (1993) suggested that since a 9-nm globular particle corresponding to the 45-kDa charalean exclusion limit is much smaller than the inner diameter of a plasmodesma, globular proteins may be associated with the plasma membrane, preventing larger particles from passing through. This suggested feature in charalean algae is the same as in higher plants, except that no internal ER was described in Charales by these workers. But Kikuyama et al. (1992) stated that since a 45-kDa SEL was obtained after 24 h following injection of fluorescein-conjugated protein in *Nitella,* then an accurate comparison with higher plants would be to wait 24 h in those as well, rather than just the customary few minutes, if plant cells could be stabilized that long. It would also be useful to conduct an SEL experiment on *Chara zeylanica* to test the consistency of the results of Kikuyama et al. (1992) among charalean species.

Our results differ from those of Franceschi, Ding, and Lucas (1994) in that we found primary plasmodesmata formed during cytokinesis in *Chara zeylanica,* while they reported that in *Chara corallina* plasmodesmata are formed secondarily by vesicles fusing with the plasma membrane and releasing enzymes that hollow out the wall, first into a funnel shape, and finally into a straight tube 35–50 nm in diameter. We found similar vesicles fusing with the plasma membrane in young walls of *Chara zeylanica,* but plasmodesmata were already present, and no funnel-shaped plasmodesmata were observed. Furthermore, the size of the vesicles was much larger than the plasmodesmata, so it seems unlikely that a vesicle containing wall-degrading enzymes could act precisely enough to create these plasmodesmata.

Although we were unable to resolve internal structure other than the desmotubule in *Monoclea,* we observed spoke-like structures connecting the desmotubule with the plasma membrane in *Notothylas* and *Sphagnum,* and smaller linear structures radiating out from the plasma membrane in *Sphagnum.* Examination with a magnifying glass of micrographs in Schnepf and Sawidis (1991, fig. 3) and Ligrone and Duckett (1994, fig. 5E) reveals that both of these features are present in another moss and in a liverwort, respectively. We did not observe neck constrictions at the cell wall–plasma membrane interface in our micrographs or in those of Schnepf and Sawidis or Ligrone and Duckett. This characteristic of plasmodesmata in vascular plants (Oleson and Robards, 1990) and in mature plasmodesmata of *Chara zeylanica* does not, therefore, appear to be correlated closely with internal structure. In addition, the apparent ring-like wall specializations observed around plasmodesmata in *Sphagnum,* while reported in ferns and in barley (Badelt et al., 1994), were not observed in *Monoclea, Notothylas,* or *Chara zeylanica,* and are therefore likely to be evolutionarily independent of internal plasmodesmatal structure. Cytochalasin has been shown to affect the size and structure of neck regions of plasmodesmata, perhaps indicating that actin is responsible for stabilizing plasmodesmata or even regulating intercellular transport via plasmodesmata (White et al., 1994), hence the quality of actin preservation could affect the appearance of plasmodesmata in preserved tissue. Given the presence of neck constrictions in *Chara zeylanica* and densely staining regions and ring-like wall specializations in *Sphagnum,* it would seem that a mechanism for regulation of intercellular transport via plasmodesmata evolved early.

We found bryophyte plasmodesmata to be similar to those of seed plants and charalean algae. Spoke-like structures connecting the desmotubule and the plasma membrane have been reported in plasmodesmata in *Nicotiana tabacum* (Ding, Turgeon, and Parthasarathy, 1992), and in the grass *Themeda triandra* (Botha, Hartley, and Cross, 1993). Short, linear structures that radiate from the plasma membrane have been reported in *Chara corallina* (Franceschi, Ding, and Lucas, 1994) and *Chara zeylanica* (this paper), in the fern *Nephrolepis exaltata* (Badelt et al., 1994), and in barley, *Hordeum vulgare* (Badelt et al., 1994). Spoke-like structures connecting the desmotubule and the plasma membrane and the smaller, linear structures that radiate out from the plasma membrane are evident in plasmodesmata of lettuce, *Lactuca sativa* (Hepler, 1982, fig. 14), in the water fern *Azolla pinnata* (Overall, Wolfe, and Gunning, 1982, fig. 13), and in sugarcane, *Saccharum* (Robinson-Beers and Evert, 1991, fig. 7).

Our consistent finding of a central structure in cross-sectional views of some *Chara* plasmodesmata under three different methods of preservation does not completely resolve the controversy surrounding the possibility that there are desmotubules in *Chara,* but it does indicate that there is some internal structure present in plasmodesmata of *Chara zeylanica.* Furthermore, our results strongly indicate that there are primary plasmodesmata formed at cytokinesis in *Chara zeylanica.* We conclude that plasmodesmata of *Chara* may be less specialized than those of seed plants, either because the complex plasmodesmata of seed plants had not yet evolved at the time *Chara* branched from a common ancestor with plants, or because of loss of complex plasmodesmata that are not as important in an organism such as *Chara,* with its particular morphology and life style. The plasmodesmata of the bryophytes we examined possessed features similar to those of seed plants. Our survey of a putatively basal liverwort, hornwort, and moss suggests that the complex ultrastructure of modern seed plant plasmodesmata probably arose before bryophytes diverged from the seed plant lineage. Our hypotheses that (1) central structures in *Chara* plasmodesmata are homologous to desmotubules and (2) that land plant plasmodesmata are monophyletic could be tested if proteins unique to desmotubules are eventually identified.

LITERATURE CITED

BADELT, K., R. G. WHITE, R. L. OVERALL, AND M. VESK. 1994. Ultrastructural specializations of the cell wall sleeve around plasmodesmata. *American Journal of Botany* 81: 1422–1427.

BAKER, R. G. E. 1988. The morphology and distribution of pits in the cell walls of *Sphagnum. Journal of the Hattori Botanical Laboratory* 64: 359–365.

BEWLEY, J. D. 1995. Physiological aspects of desiccation tolerance—a retrospect. *International Journal of Plant Sciences* 156: 393–403.

BOTHA, C. E. J., B. J. HARTLEY, AND R. H. M. CROSS. 1993. The ultrastructure and computer-enhanced digital image analysis of plasmodesmata at the Kranz mesophyll-bundle sheath interface of

Themeda triandra var. *imberbis* (Retz) A. Camus in conventionally fixed leaf blades. *Annals of Botany* 72: 255–261.

BOZZOLA, J. J., AND L. D. RUSSELL. 1992. Electron microscopy. Jones and Bartlett, Boston, MA.

BROWN, R. C., AND B. E. LEMMON. 1992. Polar organizers in monoplastidic mitosis of hepatics (Bryophyta). *Cell Motility and the Cytoskeleton* 22: 72–77.

CITOVSKY, V. 1993. Probing plasmodesmatal transport with plant viruses. *Plant Physiology* 102: 1071–1076.

DING, B., R. TURGEON, AND M. V. PARTHASARATHY. 1992. Substructure of freeze-substituted plasmodesmata. *Protoplasma* 169: 28–41.

DUCKETT, C. M., K. J. OPARKA, D. A. M. PRIOR, L. DOLAN, AND K. ROBERTS. 1994. Dye-coupling in the root epidermis of *Arabidopsis* is progressively reduced during development. *Development* 120: 3247–3255.

EPEL, B. L. 1994. Plasmodesmata: composition, structure and trafficking. *Plant Molecular Biology* 26: 1343–1356.

ESAU, K., AND J. THORSCH. 1985. Sieve plate pores and plasmodesmata, the communication channels of the symplast: ultrastructural aspects and developmental relations. *American Journal of Botany* 72: 1641–1653.

FISCHER, R. A., J. DAINTY, AND M. T. TYREE. 1974. A quantitative investigation of symplasmic transport in *Chara corallina.* I. Ultrastructure of the nodal complex cell walls. *Canadian Journal of Botany* 52: 1209–1214.

———, AND T. J. MACALISTER. 1975. A quantitative investigation of symplasmic transport in *Chara corallina.* III. An evaluation of chemical and freeze-substituting techniques in determining the in vivo condition of the plasmodesmata. *Canadian Journal of Botany* 53: 1988–1993.

FLEURAT-LESSARD, P., S. BOUCHÉ-PILLON, C. LELOUP, W. J. LUCAS, R. SERRANO, and J.-L. BONNEMAIN. 1995. Absence of plasma membrane H^+-ATPase in plasmodesmata located in pit-fields of the young reactive pulvinus of *Mimosa pudica* L. *Protoplasma* 188: 180–185.

FRANCESCHI, V. R., B. DING, AND W. J. LUCAS. 1994. Mechanism of plasmodesmata formation in characean algae in relation to evolution of intercellular communication in higher plants. *Planta* 192: 347–358.

GARBARY, D. J., K. S. RENZAGLIA, AND J. G. DUCKETT. 1993. The phylogeny of land plants: a cladistic analysis based on male gametogenesis. *Plant Systematics and Evolution* 188: 237–269.

GIORDANO, S., R. C. COBIANCHI, A. BASILE, AND V. SPAGNUOLO. 1989. The structure and role of hyaline parenchyma in the liverwort *Lunularia cruciata* (L.) Dum.. *Giornale Botanico Italiano* 123: 169–176.

GRAHAM, L. E. 1993. Origin of land plants. John Wiley & Sons, New York, NY.

———, C. F. DELWICHE, AND B. D. MISHLER. 1991. Phylogenetic connections between the 'green algae' and the 'bryophytes'. *Advances in Bryology* 4: 213–244.

———, AND Y. KANEKO. 1991. Subcellular structures of relevance to the origin of land plants (embryophytes) from green algae. *Critical Reviews in Plant Sciences* 10: 323–342.

GUNNING, B. E. S., AND R. L. OVERALL. 1983. Plasmodesmata and cell-to-cell transport in plants. *BioScience* 33: 260–265.

HASEGAWA, J. 1994. New classification of Anthocerotae. *Journal of the Hattori Botanical Laboratories* 76: 21–34.

HAYAT, M. A. 1970. Principles and techniques of electron microscopy. Van Nostrand Reinhold, New York, NY.

HÉBANT, Ch. 1978. Development of pores in water-conducting cells of the liverwort *Hymenophyton flabellatum* (Metzgeriales, Bryophytes). *Protoplasma* 96: 205–208.

HEPLER, P. K. 1982. Endoplasmic reticulum in the formation of the cell plate and plasmodesmata. *Protoplasma* 111: 121–133.

HYVÖNEN, J., AND S. PIIPPO. 1993. Cladistic analysis of the hornworts (Anthocerotophyta). *Journal of the Hattori Botanical Laboratory* 74: 105–119.

KAPLAN, D. R., AND W. HAGEMANN. 1991. The relationship of cell and organism in vascular plants. *BioScience* 41: 693–703.

KIKUYAMA, M., Y. HARA, K. SHIMADA, K. YAMAMOTO, AND Y. HIRAMOTO. 1992. Intercellular transport of macromolecules in *Nitella.* *Plant Cell Physiology* 33: 413–417.

KWIATKOWSKA, M., AND J. MASZEWSKI. 1986. Changes in the occurrence and ultrastructure of plasmodesmata in antheridia of *Chara vulgaris* L. during different stages of spermatogenesis. *Protoplasma* 132: 179–188.

LIGRONE, R., AND J. G. DUCKETT. 1994. Thallus differentiation in the marchantialian liverwort *Asterella wilmsii* (Steph.) with particular reference to longitudinal arrays of endoplasmic microtubules in the inner cells. *Annals of Botany* 73: 577–586.

LÓPEZ-SÁEZ, J. F., G. GIMÉNEZ-MARTIN, AND M. C. RISUEÑO. 1966. Fine structure of the plasmodesm. *Protoplasma* 61: 81–84.

LUCAS, W. J. 1995. Plasmodesmata: intercellular channels for macromolecular transport in plants. *Current Opinion in Cell Biology* 7: 673–680.

———, B. DING, AND C. VAN DER SCHOOT. 1993. Plasmodesmata and the supracellular nature of plants. *New Phytologist* 125: 435–476.

———, AND S. WOLF. 1993. Plasmodesmata: the intercellular organelles of green plants. *Trends in Cell Biology* 3: 308–315.

———, S. BOUCHÉ-PILLON, D. P. JACKSON, L. NGUYEN, L. BAKER, B. DING, AND S. HAKE. 1995. Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. *Science* 270: 1980–1983.

MANHART, J. R. 1994. Phylogenetic analysis of green plant rbcL sequences. *Molecular Phylogenetics and Evolution* 3: 114–127.

MCCOURT, R. M., K. G. KAROL, M. GUERLESQUIN, AND M. FEIST. 1996. Phylogeny of extant genera in the family Characeae (Charales, Charophyceae) based on rbcL sequences and morphology. *American Journal of Botany* 83: 125–131.

MISHLER, B. D., AND S. P. CHURCHILL. 1984. A cladistic approach to the phylogeny of the "bryophytes." *Brittonia* 36: 406–434.

———, AND ———. 1985. Transition to a land flora: Phylogenetic relationships of the green algae and bryophytes. *Cladistics* 1: 305–328.

———, L. A. LEWIS, M. A. BUCHHEIM, K. S. RENZAGLIA, D. J. GARBARY, C. F. DELWICHE, F. W. ZECHMAN, T. S. KANTZ, AND R. L. CHAPMAN. 1994. Phylogenetic relationships of the "green algae" and "Bryophytes." *Annals of the Missouri Botanical Garden* 81: 451–483.

———, P. H. THRALL, J. S. HOPPLE, JR., E. DE LUNA, AND R. VILGALYS. 1992. A molecular approach to the phylogeny of bryophytes: cladistic analysis of chloroplast-encoded 16S and 23S ribosomal RNA genes. *Bryologist* 95: 172–180.

NIKLAS, K. J. 1989. The cellular mechanics of plants. *American Scientist* 77: 344–349.

OLESON, P., AND A. W. ROBARDS. 1990. The neck region of plasmodesmata: general architecture and some functional aspects. *In* A. W. Robards et al. [eds.], Parallels in cell to cell junctions in plants and animals, 145–170. Springer-Verlag, Berlin.

OPARKA, K. J. 1994. Plasmolysis: new insights into an old process. *New Phytologist* 126: 571–591.

———, D. A. M. PRIOR, AND J. W. CRAWFORD. 1994. Behavior of plasma membrane, cortical ER and plasmodesmata during plasmolysis of onion epidermal cells. *Plant, Cell and Environment* 17: 163–171.

OVERALL, R. L., J. WOLFE, AND B. E. S. GUNNING. 1982. Intercellular communication in *Azolla* roots. I. Ultrastructure of plasmodesmata. *Protoplasma* 111: 134–150.

PICKETT-HEAPS, J. D. 1967a. Ultrastructure and differentiation in *Chara* sp. I. Vegetative cells. *Australian Journal of Biological Sciences* 20: 539–551.

———. 1967b. Ultrastructure and differentiation in *Chara* sp. II. Mitosis. *Australian Journal of Biological Sciences* 20: 883–894.

———. 1968. Ultrastructure and differentiation in *Chara* (*fibrosa*). IV. Spermatogenesis. *Australian Journal of Biological Science* 21: 655–690.

RENZAGLIA, K. S. 1978. A comparative morphology and developmental anatomy of the Anthocerotophyta. *Journal of the Hattori Botanical Laboratory* 44: 31–90.

ROBARDS, A. W., AND W. J. LUCAS. 1990. Plasmodesmata. *Annual Review of Plant Physiology and Plant Molecular Biology* 41: 369–419.

ROBINSON-BEERS, K., AND R. F. EVERT. 1991. Fine structure of plasmodesmata in mature leaves of sugarcane. *Planta* 184: 307–318.

RYDIN, H., AND R. S. CLYMO. 1989. Transport of carbon and phospho-

rous compounds about *Sphagnum*. *Proceedings of the Royal Society of London B* 237: 63–84.

SCHNEPF, E., AND Th. SAWIDIS. 1991. Filament disruption in *Funaria* protonemata: occlusion of plasmodesmata. *Botanica Acta* 104: 98–102.

———, AND A. SYCH. 1983. Distribution of plasmodesmata in developing *Sphagnum* leaflets. *Protoplasma* 116: 51–56.

SCHOFIELD, W. B. 1985. Introduction to bryology. Macmillan, New York, NY.

SCHULZ, A. 1995. Plasmodesmal widening accompanies the short-term increase in symplasmic phloem unloading in pea root tips under osmotic stress. *Protoplasma* 188: 22–37.

SCHUSTER, R. M. 1984. New manual of bryology, vol. 2. The Hattori Botanical Laboratory, Japan, and The Cryptogamic Laboratory, Hadley, MA.

SPANSWICK, R. M., AND J. W. F. COSTERTON. 1967. Plasmodesmata in *Nitella translucens*: structure and electrical resistance. *Journal of Cell Science* 2: 451–464.

TAYLOR, D. P. 1988. Direct measurement of the osmotic effects of buffers and fixatives in *Nitella flexilis*. *Journal of Microscopy* 150 (part 1): 71–80.

TILNEY, L. G., T. J. COOKE, P. S. CONNELLY, AND M. S. TILNEY. 1991. The structure of plasmodesmata as revealed by plasmolysis, detergent extraction, and protease digestion. *Journal of Cell Biology* 112: 739–747.

TREBACZ, K., AND D. FENSOM. 1989. The uptake and transport of ^{14}C in cells of *Conocephalum conicum* L. in light. *Journal of Experimental Botany* 40: 1089–1092.

TURNER, A., B. WELLS, AND K. ROBERTS. 1994. Plasmodesmata of maize root tips: structure and composition. *Journal of Cell Science* 107: 3351–3361.

WATERS, D. A., M. A. BUCHHEIM, R. A. DEWEY, AND R. L. CHAPMAN. 1992. Preliminary inferences of the phylogeny of bryophytes from nuclear-encoded ribosomal RNA sequences. *American Journal of Botany* 79: 459–466.

WHITE, R. G., K. BADELT, R. L. OVERALL, AND M. VESK. 1994. Actin associated with plasmodesmata. *Protoplasma* 180: 169–184.

WOOD, R. D. 1967. Charophytes of North America. Stella's Printing, West Kingston, RI.