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Mechanism of plasmodesmata formation in characean algae in relation to evolution of intercellular communication in higher plants

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Abstract. It is generally accepted that higher plants evolved from ancestral forms of the modern charophytes. For this reason, we chose the characean alga, *Chara corallina* Klein ex Willd., em. R.D.W. (*C. australis* R. Br.), to determine whether this transition species produces plasmodesmata in a manner analogous to higher plants. As with higher plants and unlike most green algae, *Chara* utilizes a phragmoplast for cell division; however, in contrast with the situation in both lower and higher vascular plants, the developing cell plate and newly formed cell wall were found to be completely free of plasmodesmata. Only when the daughter cells had separated completely were plasmodesmata formed across the division wall. Presumably, highly localized activity of wall-degrading (or loosening) enzymes inserted into the plasma membrane play a central role in this process. In general appearance characean plasmodesmata are similar to those of higher plants with the notable exception that they lack an appressed endoplasmic reticulum. Further secondary modifications in plasmodesmal structure were found to occur as a function of cell development, giving rise to highly branched plasmodesmata in mature cell walls. These findings are discussed in terms of the evolution of the mechanism for plasmodesmata formation in algae and higher plants.

Key words: Cell wall – *Chara* – Phragmoplast – Plasma membrane – Plasmodesmata

Introduction

The ability to establish and control cell-to-cell communication played an obvious important role in organismal evolution. In this regard it has long been recognized that plants evolved a special mechanism to produce a cell wall, during cytokinesis, that does not completely separate the daughter cells. This incomplete separation is achieved by the formation of special plasma-membrane-lined cytoplasmic bridges, termed plasmodesmata, that create an integrated cytoplasmic continuum referred to as the symplasm. In higher plants, a continuum of the endoplasmic reticulum (ER) is also maintained from cell-to-cell, as the ER forms the central region of the plasmodesmata (Robards and Lucas 1990; Lucas et al. 1993). High-resolution electron-microscopic examination of plasmodesmata has revealed that globular proteins (3 nm diameter) are embedded in the inner and outer leaflets of the plasma membrane and ER, respectively (Ding et al. 1992a; see also Tilney et al. 1991). These plasmodesmal proteins divide the cytoplasmic sleeve into micro-channels that have diameters of about 2.5 nm (Ding et al. 1992a) and establish a size exclusion limit of approximately 800–1000 Da (see Robards and Lucas 1990 and references cited therein). Small molecules involved in intercellular communication can diffuse through these micro-channels, whereas selective macromolecular trafficking appears to occur through plasmodesmata by a process analogous to nucleocytoplasmic transport (Lucas et al. 1993).

Ultrastructural studies on higher-plant plasmodesmata have established that there are two distinctly different mechanisms by which these special cytoplasmic bridges can be formed across the wall. Primary plasmodesmal formation has been demonstrated to be related to the mechanism of cell division, which occurs by cell plate formation (Robards and Lucas 1990). During cytokinesis, a new cell wall is produced by coalescence of vesicles at the cell plate. A structure called the phragmoplast, which contains microtubules oriented perpendicular to the developing cell plate, appears to direct cell plate formation. The incomplete separation of the two daughter cells is accomplished by the controlled positioning of ER within and perpendicular to the forming cell plate (Porter and Machado 1960; Hepler 1982). As the new wall develops, this ER becomes appressed and, together with the plasma membrane surrounding it, forms the primary plasmodesma.

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Secondary plasmodesmata represent new cytoplasmic bridges that are formed, completely or partially, post-cytokinetically across the existing cell wall. The importance of secondary plasmodesmal formation to normal plant development and function has only recently been recognized (Lucas et al. 1993). Developmental and ultrastructural studies performed on a range of plant species and tissues indicate that secondary plasmodesmata can be formed de novo, or by the modification of existing primary plasmodesmata (Ding et al. 1992b, 1993; Lucas et al. 1993). The steps involved in the de-novo formation of secondary plasmodesma have been very elegantly illustrated for situations where cells are brought into contact as a consequence of callus formation, such as occurs during grafting or wound healing (Kollmann et al. 1985; Kollmann and Glockmann 1985, 1991). At present it is not known whether such secondary plasmodesmata are formed by the same mechanism that generates secondary plasmodesmata during the normal process of cell and tissue maturation.

Although the appearance of plasmodesmata is generally considered to be a seminal event in the evolution of structurally complex higher plants (see Gunning and Robards 1976), little is known about the events that gave rise to the evolution of this intercellular organelle. Specifically, there is little information on when and where the mechanisms for primary and secondary formation of plasmodesmata evolved. Knowledge relating to these events is critical to our understanding of the evolution of complex plant form and function (Lucas et al. 1993).

One approach to elucidating the origin(s) of plasmodesmata is to examine the most 'primitive' extant members of the green plant family, the Chlorophyta (green algae) from which higher plants are thought to have evolved (Graham et al. 1991; Graham and Kaneko 1991). The Chlorophyta is comprised of single-celled and multicellular species of varying degrees of structural complexity. Species that have evolved bodies (thalli) with functionally differentiated regions, complex cellular relationships or localized growth patterns generally have some form of intercellular cytoplasmic continuity. This continuity ranges from pits and cytoplasmic strands to structures recognizable as plasmodesmata. Members of the Charales, in particular, merit close examination, since they have been considered by some evolutionary biologists to be living representatives of the end of the algal line of descent that led to the evolution of land plants (Pickett-Heaps 1975; Graham et al. 1991; Graham and Kaneko 1991). In this regard it is important to note that members of the Charales are morphologically quite advanced, having a thallus which consists of elongate internodal cells separated by a multicellular nodal complex of smaller cells (Pickett-Heaps 1975). Growth is by the activity of an apical initial, a characteristic also found in many lower vascular plants. The reproductive structures are also complex, consisting of protective sterile cells which surround the gametes. Significantly, cytokinesis in *Chara* has been reported to occur by cell plate formation with the involvement of a phragmoplast (Pickett-Heaps 1967b; Turner 1968).

In view of their proposed evolutionary position, we undertook an ultrastructural examination of the structure and development of plasmodesmata in some typical members of the Charales with the prospect that such a study would provide critical information on the evolution and biology of plasmodesmata in higher plants. In particular, we addressed the following questions. First, is plasmodesmal formation in *Chara* directly associated with cytokinesis, as is primary plasmodesmal formation in higher plants? Second, will the mechanism of plasmodesmal formation in *Chara* support the hypothesis for co-evolution of a specific cytokinetic mechanism and the ability to produce primary plasmodesmata? Third, is there a close evolutionary relationship between algal and higher-plant plasmodesmata?

Materials and methods

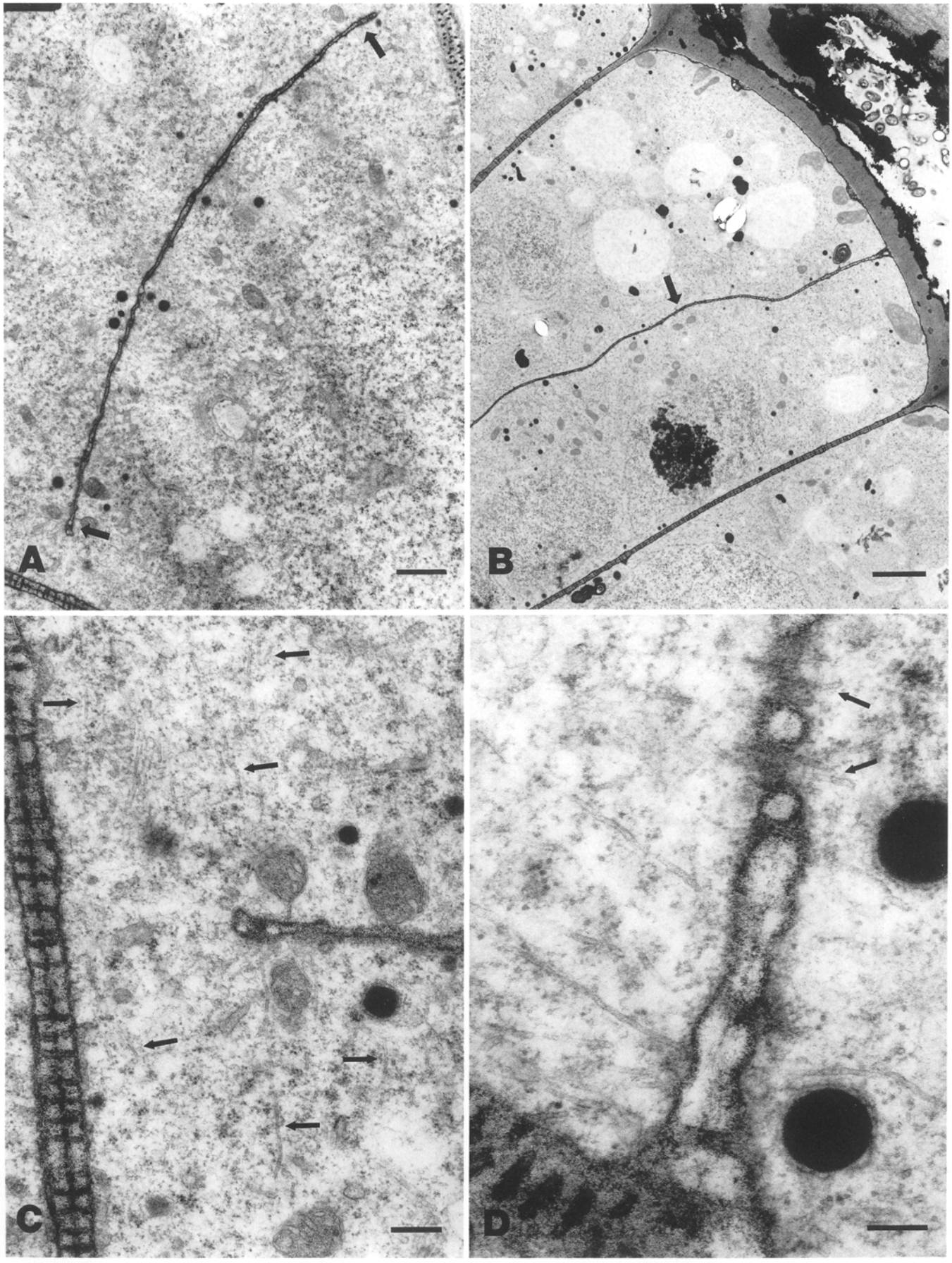
Plant material. The present study focussed on plasmodesmal formation in male plants of *Chara corallina*. Additional observations were also made on *Chara braunii*, and *Nitella translucens*. Details relating to laboratory culture conditions and tissue manipulation have been previously described (Lucas 1975; Franceschi and Lucas 1980). Tobacco plants were grown under controlled environmental conditions as previously described (Ding et al. 1992b). Mature leaf tissue was used to obtain images of secondary plasmodesmata in higher plants.

Transmission electron microscopy. As the giant cells of *Chara* are particularly sensitive to plasmolysis, during tissue preparation for electron microscopy, a protocol was developed which specifically targeted preservation of the cell wall-plasma membrane interface. Unless otherwise noted, plant material excised from the vegetative apex was fixed in a combination of 2.5% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde, 1% (w/v) tannic acid, 1 mM $CaCl_2$, in 25 mM sodium cacodylate buffer (pH 7.2) for 16 h at 4°C. Samples were postfixed in a combination of 1% (w/v) osmium tetroxide and 0.5% (w/v) tannic acid in 25 mM sodium cacodylate buffer (pH 7.2) for 2 h at room temperature. After washing with cacodylate buffer, the samples were dehydrated with an acetone series (10 steps) and then slowly infiltrated (9 steps) with epoxy resin (Spurr 1969). All fixatives were prepared just prior to use.

Mature tobacco leaves were prepared for electron microscopy as previously described (Ding et al. 1992b).

Thin sections were cut with a diamond knife and picked up onto nickel grids. The sections were stained with a 1:3 mix of aqueous 2% (w/v) uranyl acetate: 1% (w/v) potassium permanganate that was prepared and filtered just before use. This protocol was found to give optimal staining of the plasma membrane, plasmodesmata and cell walls. Sections were examined with a Hitachi (Hitachi Scientific Instruments, Mountain View, Calif., USA) 600 transmission electron microscope (TEM; operated at an accelerating potential of 100 kV) or a Philips (Eindhoven, The Netherlands) EM410LS TEM operated at 80 kV.

Fig. 1A–D. Cell division and cell wall formation in *Chara corallina*. **A** Cytokinesis occurs by cell plate formation. The ends of the developing plate are indicated by *arrows*. Bar = 1 μm; × 9600 **B** The new cell wall (*arrow*) is very thin and has a wavy appearance. Bar = 3 μm; × 3300 **C** Edge of a developing cell plate showing the perpendicular microtubules of the phragmoplast (*arrows*) and lack of ER association. Bar = 0.1 μm; × 31500 **D** Junction of newly formed cell wall with wall of mother cell. Note microtubules of the phragmoplast, some of which have been trapped within the region of the forming wall (*arrows*). Bar = 0.2 μm; × 58200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Results

Mechanism of cell division. We confirmed that *Chara* cells divide by cell plate formation (Fig. 1A). The newly formed wall was very thin relative to adjacent walls of the daughter cells and usually had a somewhat wavy appearance (Fig. 1B) which straightened as the daughter cells expanded. A phragmoplast, with microtubules arranged perpendicular to the cell plate, was found to be involved with cell plate formation. However, unlike the situation in higher plants, the ER was never found to be positioned between the fusing vesicles during cell plate development and, surprisingly, the cell plate was found to be free of plasmodesmata (Fig. 1C). Occasionally, microtubules of the phragmoplast appeared to become trapped between the fusing vesicles of the cell plate. This phenomenon was most often observed in the situation where the plate approached the lateral walls of the mother cell, and plasmodesma-like structures were produced (Fig. 1D). However, these structures were highly variable in size, were not very frequent, and did not persist after the wall was completed.

Formation of plasmodesmata. Examination of developing cells in the vegetative apex of *Chara* allowed for the reconstruction of the mechanism by which plasmodesmata are formed in this species. As described above, the forming cell plate was free of plasmodesmata and, unlike the situation in higher plants, the ER was rarely observed in the vicinity of the forming wall (Fig. 2A). The youngest complete walls that were observed had a low density of plasmodesmata but did have numerous indentations or evaginations of the plasma membrane (Fig. 2B), and in these regions small particles were positioned between the membrane and the wall material. Slightly older walls had funnel-shaped plasmodesma-like structures (Fig. 2C), which apparently resulted from the fusion of membrane evaginations originating from the neighboring daughter cells. As the young wall became thicker, by addition of wall materials, the funnel-shaped plasmodesmata took on a typical tubular conformation (Fig. 2D).

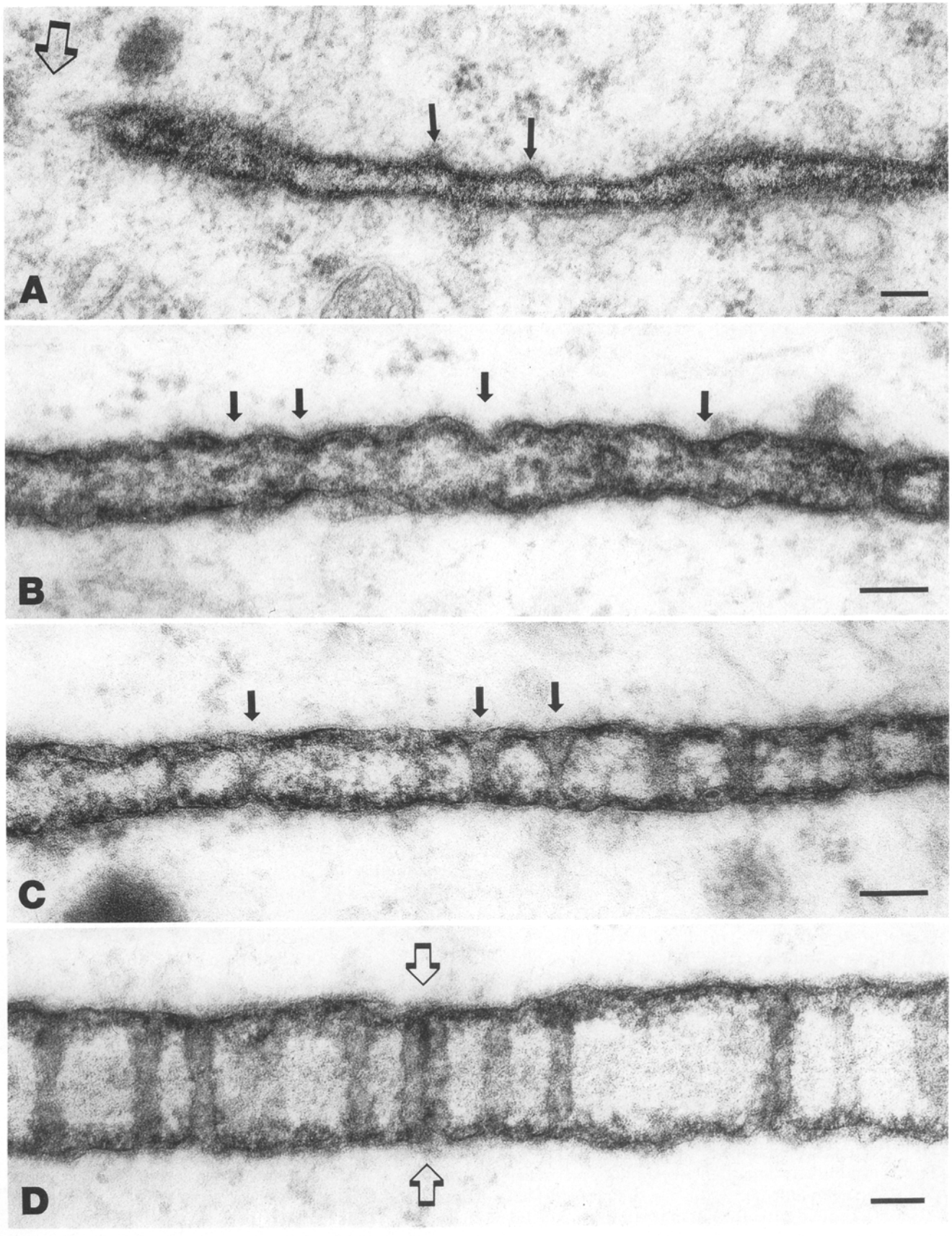
Our interpretation of these results is that *Chara* plasmodesmata are formed by evagination of the plasma membrane out into the cell wall in concert with hydrolysis (or loosening) of the wall matrix. Internal hydrostatic pressure would help drive this process, and this probably explains the funnel shape of the early plasmodesmata, where less wall material has been hydrolyzed at the point of initial fusion with the opposite plasma membrane. Eventually, a complete cytoplasmic bridge is produced that reconnects the two daughter cells. These results indicate that most, if not all, plasmodesmata are formed secondarily in this species. Quite frequently the plasmodesmata were observed to form so close together that they appeared to fuse along one surface (Fig. 2D): further evidence for the formation of secondary plasmodesmata. Again, it is important to note that the ER was never found to be associated with plasmodesmata at any stage of either cell plate or plasmodesmal development.

It is important to emphasize that most of the figures shown here have been printed to optimize visualization of the plasma membrane and plasmodesmata. Because of the fixation and contrasting techniques used, the cytoplasmic constituents are faintly stained and do not show up clearly in many of the micrographs. Figure 3 consists of overexposed prints which demonstrate that the preparation techniques used do in fact give excellent overall preservation of cell components, including the ER. Our observations of a lack of ER association with developing cell walls and plasmodesmata are based on such images.

Plasmodesmal structure in young and mature cell walls. Plasmodesmata were very abundant within the walls of the *Chara* thallus (Fig. 4A). Their structure was variable and dependent upon both the age of the cross-wall as well as the cell type being connected by the plasmodesmata. The simplest type of plasmodesma consisted of a cylindrical plasma-membrane-lined pore with an inner diameter of 20–25 nm. At this stage of plasmodesmal development particles could be detected along the outer surface of the plasmodesma (Fig. 4B, C) and the ER was still absent from within the pore (Fig. 4C). These simple unbranched plasmodesmata were observed in recently formed cell walls of the thallus and antheridia (male reproductive structures) as well as in some radial walls between the smaller cells of the mature nodal complex.

Branched plasmodesmata have previously been reported in characean algae (Pickett-Heaps 1967a; Spanswick and Costerton 1967; Kwiatkowska and Maszewski 1976, 1986; Franceschi and Lucas 1982; Lucas et al. 1993), and in fact are the major form in walls of mature cells. These plasmodesmata are complex structures having many branches emanating from a large sinus located in the middle lamella region of the cell wall (Fig. 5A). The number of branches present appeared to depend upon the size of the cross-wall, with the greatest number of branches occurring in plasmodesmata between the internodal and nodal cells. When viewed in longitudinal section, the smaller branched plasmodesmata of *Chara* had a similar appearance to secondary plasmodesmata of higher plants (Fig. 5B), except that in *Chara* the central region (sinus) was more extensive. Tangential sections through the mature *Chara* wall (Fig. 5C) provide a more accurate indication of the degree of the branching, as well as the extent of intercellular connection afforded by these branched plasmodesmata. The pattern produced in this

Fig. 2A–D. A reconstruction of the process of plasmodesmal formation across walls of *Chara corallina.* **A** Part of a developing cell plate (one end at *open arrow*) with a general absence of plasmodesmata. Note presence of some membrane invaginations (*solid arrows*). Some microtubules, perpendicular to the cell plate, can be seen. Bar = 3 μm; ×87300 **B** New cell wall with evagination of the plasma membrane into the cell wall matrix (*arrows*). Bar = 0.1 μm; ×127000 **C** Slightly older wall with funnel-shaped plasma-membrane-lined cytoplasmic channels (*arrows*). Bar = 0.1 μm; ×126000 **D** Cell wall with newly formed plasmodesmata. Some plasmodesmata have formed so close together that they appear to be fused along one surface (*open arrows*). Bar = 0.1 μm; ×99000 The events shown here indicate that plasmodesmata are formed secondarily across cell walls by activities associated with the plasma membrane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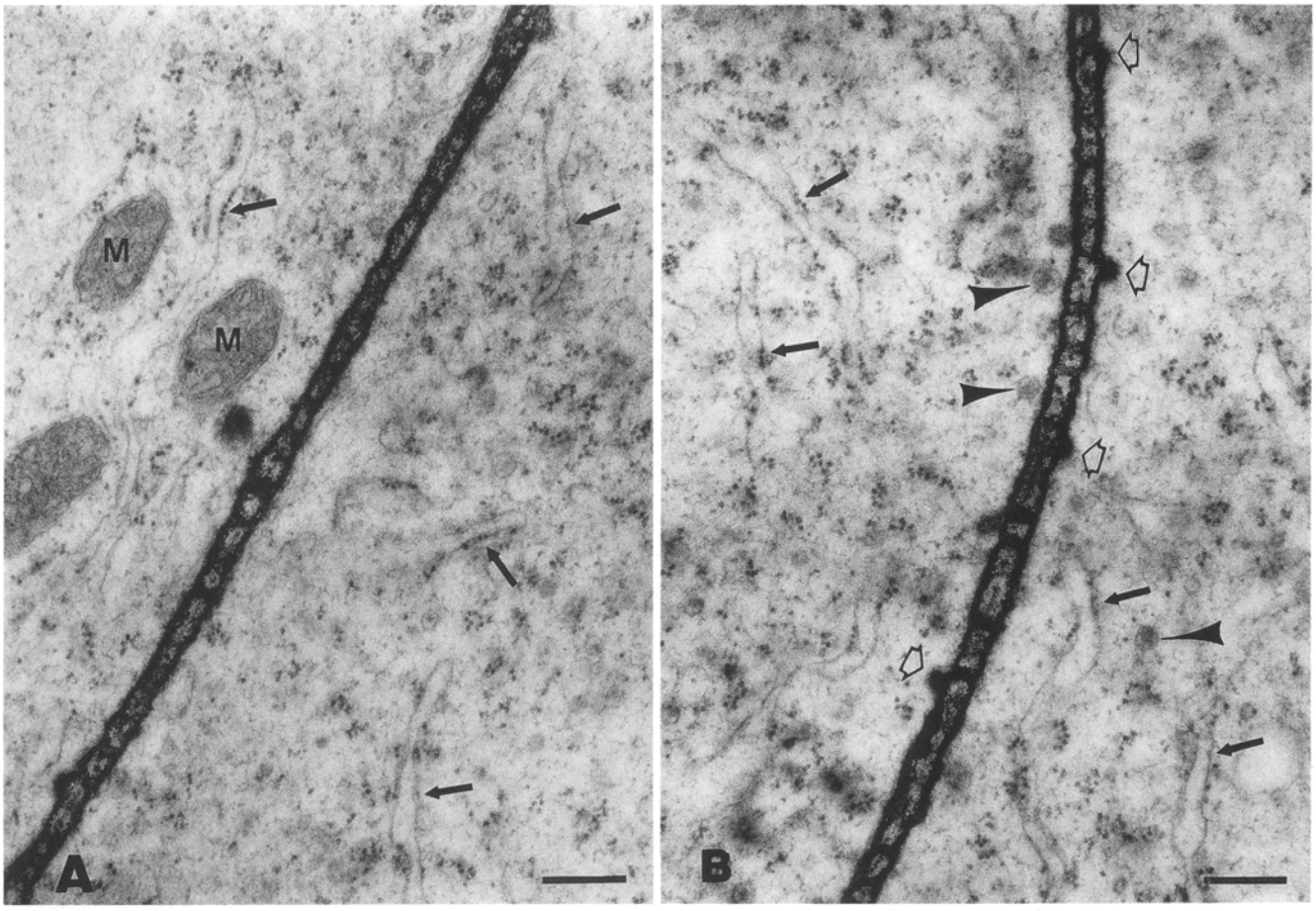


Fig. 3A,B. New cell walls with developing plasmodesmata (similar stage to Fig. 2C). Micrographs have been printed to show cytoplasmic components and thus walls/plasmodesmata are overexposed. **A** Mitochondria (*M*) are well preserved as is the ER (*arrows*). **B** Endoplasmic reticulum (*arrows*) is abundant but is not specifically associated with the cell wall or plasmodesmata. Note coated plasmamembrane invaginations (*open arrows*) and smooth vesicles (*arrowheads*) which are common at this stage of development. Bars = 0.3 μm; × 36000

type of section is reminiscent of the pattern of plasmodesmata in pit fields of higher plants.

Formation of branched plasmodesmata. A careful examination of a range of cell walls that reflected a developmental gradient within the *Chara* thallus provided important details on the process by which further cytoplasmic bridges (branches) are added to the simple secondary plasmodesma. The relative age of a cell wall could easily be determined by position and thickness, as seen in thick sections prepared for light-microscopic examination (see also Fig. 1B). Cells at the desired developmental stage were identified by light microscopy and then prepared for examination by transmission electron microscopy. The following mechanism for branch formation of plasmodesmata was established. The simple, secondary plasmodesmata that were first formed, following cell plate formation, began to expand radially within the middle lamella portion of the cell wall (Fig. 6A). At this stage, small particles could be seen between the wall and plasmodesmal surface and an electron-dense material developed in the middle portion of the plasmodesma (Fig. 6A, B). Tangential sections showed that this electron-dense material was actually in the plasmodesma, rather than being associated with the underlying wall material (Fig. 6B).

Each plasmodesma continued to expand radially in the central region of the wall and developed discrete evaginations into the wall matrix (Fig. 6C). These evaginations appeared to grow towards the wall surfaces to eventually fuse with the plasma membrane to produce new cytoplasmic bridges. The end result of this activity was an expanded region of the plasmodesma, in the middle lamella region of the wall which formed a sinus, or median cavity, from which numerous cytoplasmic branches radiated to form a complex multi-branched plasmodesma in the mature cross-wall (Fig. 5A, C). Again, the ER was never directly associated with any aspect of this developmental sequence. These observations demonstrate that the complex plasmodesmata of *Chara* are produced through processes that take place as post-cytokinetic events.

Plasmodesmata in other species. The structure of the plasmodesmata in other *Chara* and *Nitella* species examined

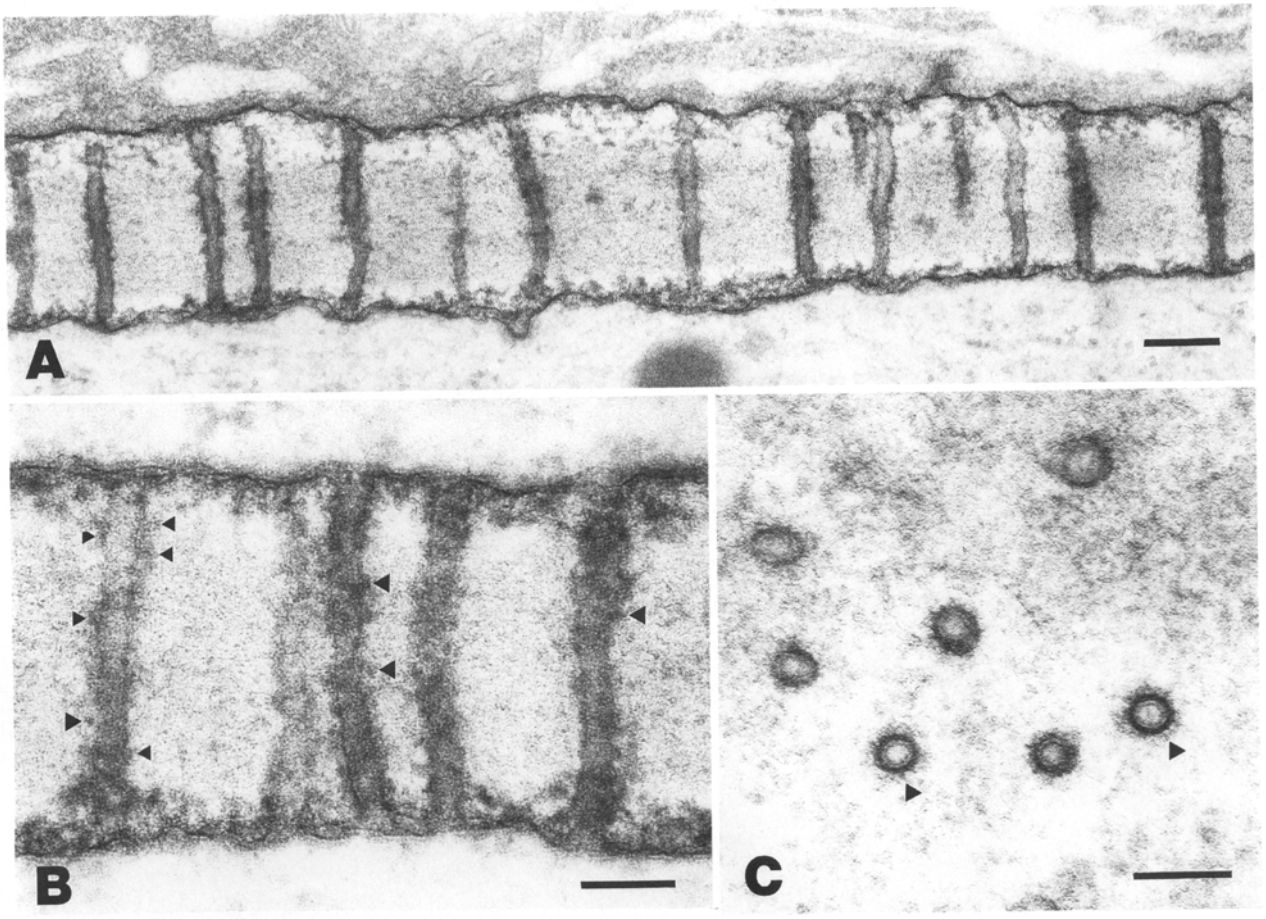


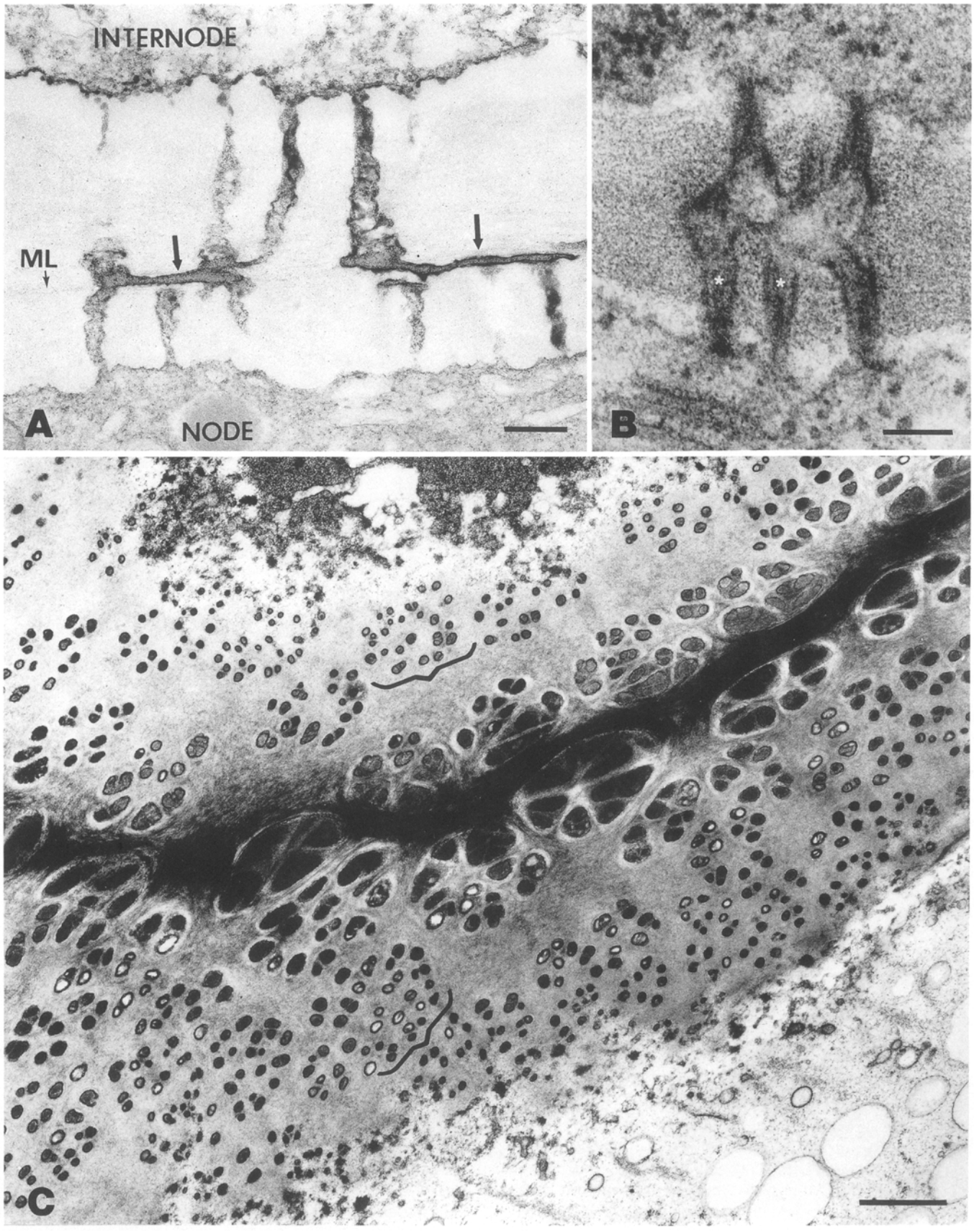
Fig. 4A–C. General structure of mature, simple plasmodesmata in young walls of *Chara corallina*. **A** Newly formed cross-walls between cells of developing shoot tip are traversed by numerous plasmodesmata. Bar = 0.1 μm; × 50000 **B** Fixation protocol employed resulted in plasmodesmal images having a uniform diameter along their length, and an absence of any constriction in the vicinity of the orifice. Note small particles along the outer surface (*arrowheads*) of plasmodesmata and lack of appressed ER. Bar = 0.1 μm; × 123000 **C** Cross-section through plasmodesmata demonstrating diameter of about 20–25 nm (inner), particles radiating from outer plasmamembrane surface (*arrowheads*) and a lumen free of ER. Bar = 0.1 μm; × 129000

was found to be the same as for *Chara corallina* (see also Pickett-Heaps 1967a; Spanswick and Costerton 1967; Kwiatkowska and Maszewski 1976, 1986; Lucas et al. 1993). Complete developmental studies will be conducted on these and other algal species (to be published elsewhere), but preliminary results indicate that the mechanism of secondary plasmodesmal formation is essentially the same as described for *Chara corallina* (data not shown).

Discussion

The present results unequivocally establish that plasmodesmal formation in *Chara corallina* and, presumably in the Charales, occurs after cell walls have been completely formed (Figs. 1, 2). An important corollary, therefore, is that plasmodesmata can be formed completely independent of the mechanics involved in cytokinesis. Hence, co-evolution of a particular cell-division mechanism, or structure, such as the phragmoplast, and plasmodesmal formation is not supported. Obviously, the mechanism of plasmodesmal formation in *Chara* is fundamentally different from the processes that give rise to the formation of the higher-plant primary plasmodesma. Because they are produced post-cytokinetically, the *Chara* plasmodesmata, simple or branched, are truly secondary plasmodesmata (see Ding et al. 1992b; Lucas et al. 1993).

Interestingly, a similar situation appears to have evolved in the brown algae, where the septal cross-walls are produced by annular infurrowing. Here the cells of the thallus are often interconnected by specialized "pits" containing orderly arrays of many simple plasmodesmata (Bisalputra 1966). Numerous ultrastructural studies performed on evolutionarily primitive to advanced members of the Phaeophyta indicate the presence of simple, plasma-membrane-lined cytoplasmic bridges (Oliveira and Bisalputra 1973; Schmitz and Srivastava 1974, 1975; Schmitz and Kühn 1982) that interconnect the cells of the meristoderm, cortex and medulla (Steinbiß and Schmitz 1973; Schmitz and Kühn 1982). The presence of these simple plasmodesmata in the brown algae suggests that these species also evolved a mechanism that enabled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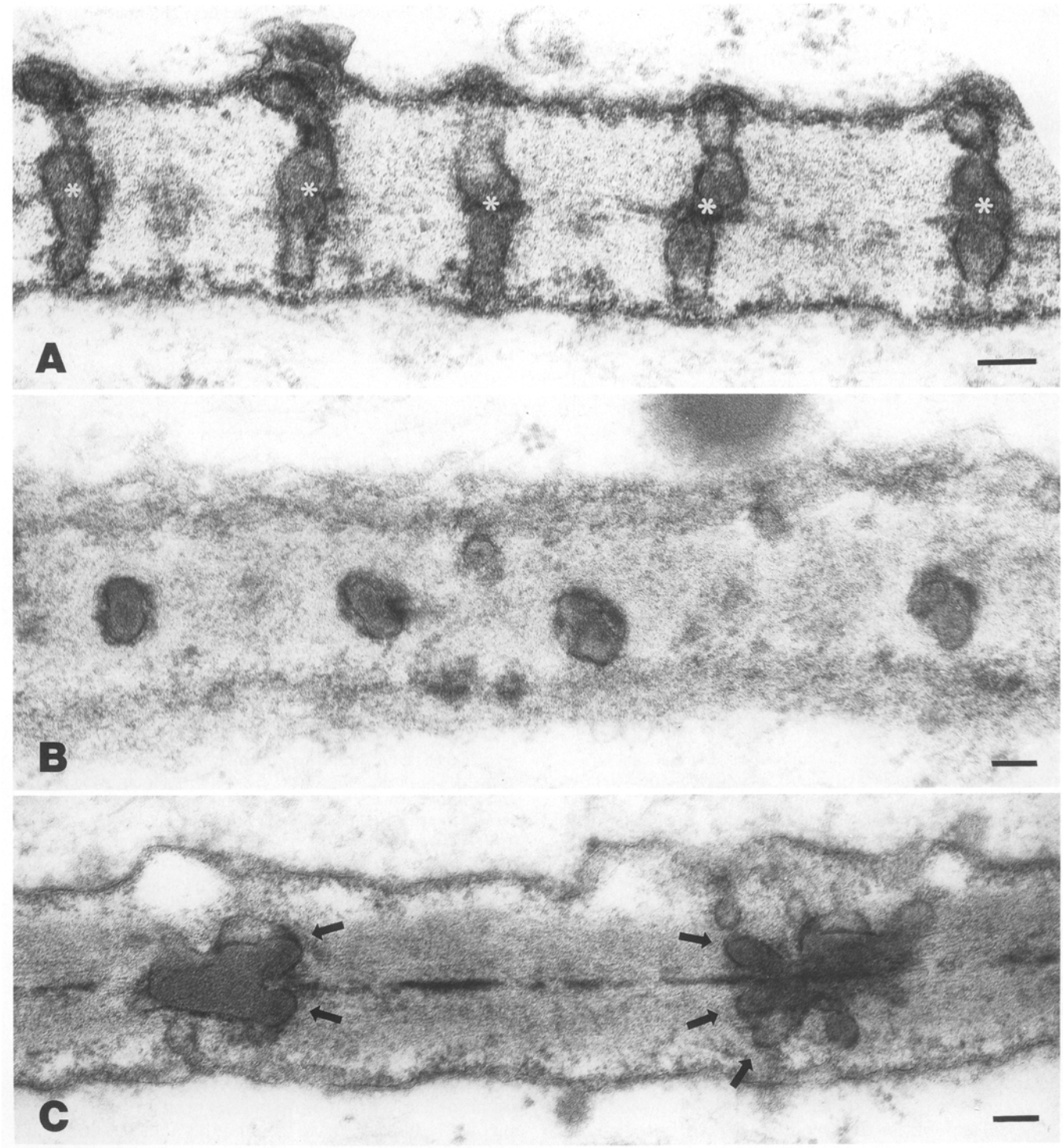

Fig. 5A–C. Branched plasmodesmata in mature cross-walls between a nodal and an internodal cell. **A** Longitudinal section through plasmodesmata illustrating sinus (*arrow*) in the center of the wall which interconnects branches from the plasma membrane of adjacent cells (fixed without tannic acid; *ML*, middle lamella). Bar = 0.3 μm; × 38200 **B** Secondary plasmodesmata in cross-wall of *Nicotiana tabacum*. Note presence of appressed ER (*stars*). Bar = 0.1 μm; × 130000 **C** Tangential section through a mature *Chara* wall shows that the branches are arranged in circular or elliptical patterns (*brackets*), and the appressed ER is absent from these branches. Bar = 1 μm; × 16000

Fig. 6A–C. Reconstruction of the mechanism of secondary modification of plasmodesmata. **A** As the cell wall grows in thickness and length, simple plasmodesmata develop an electron-dense central region (*asterisks*) and begin to expand at this region. Bar = 0.1 μm; × 102000 **B** Cross-section through this central region clearly demonstrates that the electron-dense material is located inside the plasmodesmata. Bar = 0.1 μm; × 76000 **C** Tangential section through a maturing cell wall shows the numerous evaginations of the plasmodesmata surface into the cell wall matrix (*arrows*). These evaginations will grow towards one or the other cell surface until they fuse with the plasma membrane to form complete branches. The central region of the plasmodesmata in this section can be seen to be more sinus-like in nature. Bar = 0.1 μm; × 76000

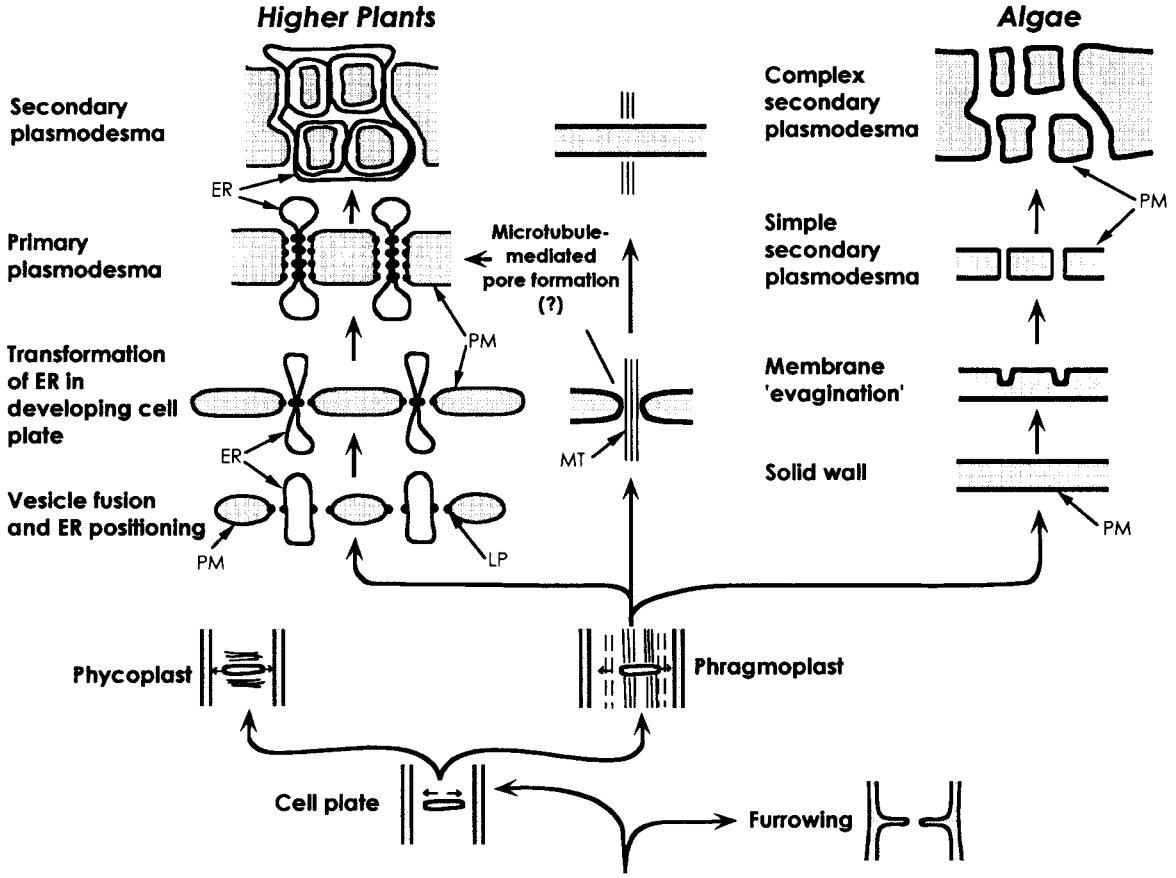

Fig. 7. Comparison between mechanisms of plasmodesmal formation in characean algae and higher plants. Plasmodesmata in characean algae are formed only secondarily across the newly formed wall. During cell plate development, microtubules (*MT*) can become entrapped between fusing vesicles, forming unstable "pores". However, the MT depolimerize and these pores then disappear giving rise to the establishment of a solid cell wall. Evagination of the plasma membrane (*PM*) into the new wall then leads to the formation of simple secondary plasmodesmata. These simple secondary plasmodesmata can undergo further modification to become highly branched complex secondary plasmodesmata. In contrast, higher plants form plasmodesmata during cell plate development by the positioning of the ER between fusing vesicles. This process may involve interactions between specific protein molecules associated with the fusion vesicles and the ER (linking proteins [*LP*]; *solid circles*). Transformation of ER into an appressed form occurs as the wall increases in thickness. Further modification of primary plasmodesmata through the addition of new cytoplasmic branches can give rise to secondary plasmodesmata. The important question that remains to be resolved is where in the course of terrestrial plant evolution did the mechanism of primary plasmodesmal formation first appear

them to form secondary plasmodesmata through septal walls as well as across sites where cells make contact. The elegant ultrastructural studies performed on *Laminaria groenlandica* (Schmitz and Srivastava 1974) provide unequivocal support for this hypothesis. Thus, based on our present studies on *Chara* and the earlier studies on the brown algae, it would appear that secondary plasmodesmal formation is an evolutionarily primitive, rather than a modern characteristic (Fig. 7).

Examination of plasmodesmal formation in *Chara* suggests that secondary modification of plasmodesmata, as is also seen in higher plants, may represent a primitive characteristic. It is interesting to note that the end result of secondary modification of plasmodesmata in *Chara* is similar in appearance to secondary plasmodesmata and pit fields in higher plants (cf. Fig. 5A and B). Branching of the *Chara* plasmodesmata most probably evolved in response to the extensive radial expansion and thickening of the wall during cell growth (from approximately 40 μm, as in figure 1B, to 0.5–1 mm in mature branches). Rather than forming more new plasmodesmata through an increasingly longer path, the existing plasmodesmata are modified to produce numerous branches, each covering only half the distance through the wall. The end result is the maintenance of an adequate intercellular pathway for the support of normal transport function. It is also possible that, as in higher plants, these branched secondary plasmodesmata evolved to potentiate the execution of specialized physiological functions (see Ding et al. 1993).

Our results also establish that the evolution of the phragmoplast preceded the acquisition of the mechanism that ultimately resulted in the formation of the primary plasmodesma. This conclusion is consistent with the fact that, in the green algae, simple plasmodesmata have been reported within species that use either the phycoplast (microtubules arranged parallel to the plane of the forming cell plate; see Fig. 7) or the phragmoplast to orches-

trate the processes of cell plate formation (Stewart et al. 1973). The cytoskeleton must in some way direct the trafficking of Golgi-derived vesicles to the margin of the forming cell plate. The molecular events underlying these trafficking processes must have evolved independently of the mechanism(s) that potentiated the ability to produce a membrane-generated pore (Fig. 7).

Interestingly, in contrast to the brown algae, plasmodesmata have yet to be identified in green algae that undergo cell division by annular furrowing. This suggests that the ability to produce secondary plasmodesmata may have evolved, independently, in the brown and green algae. However, the test for this hypothesis must await the identification of the genes responsible for secondary plasmodesmal formation in the Phaeophyta and Chlorophyta. Given the general structural similarity between the *Chara* and higher-plant secondary plasmodesmata, it will be of considerable interest to ascertain whether the higher-plant genes that encode for this process are homologous with those of the green algae.

The actual process of forming the simple cytoplasmic bridge across the *Chara* cell wall must involve a mechanism that imparts spatial control over the site where the secondary plasmodesma will form. Our observations suggest that proteins are inserted into the plasma membrane to form a restricted, 'dome-shaped' *in*vagination (Fig. 2A). These proteins must be held in place by some form of cytoskeletal elements (see Franceschi and Lucas 1980) and result in highly localized hydrolytic removal of the cell wall matrix. Turgor pressure would then force the initial *in*vagination to extend out into the region of the wall, where the matrix material had already been digested, resulting in the *e*vagination of the plasma membrane. At present we cannot determine whether this process occurs asymmetrically from one daughter cell, or simultaneously from both plasma-membrane surfaces. However, the events that give rise to the formation of additional cytoplasmic branches across the maturing cell wall implicate a unidirectional fusion process in which the evaginating membrane crosses the wall to fuse with the opposite plasma membrane (Fig. 7).

These simple, 'open' plasmodesmata should allow rapid transport of nutrients and medium-sized macromolecules, while restricting movement of large proteins and structural units like ribosomes. Kikuyama et al. (1992) used microinjection techniques to establish that molecules (proteins) of up to 45 kDa could move from cell-to-cell in *Nitella*. A globular molecule of this mass should have a diameter of approximately 9 nm (Lucas and Wolf 1993), which is considerably smaller than the measured inner diameter of these secondary plasmodesmata (20–25 nm). This restriction in plasmodesmal size exclusion limit may reflect the presence of proteins that are either embedded in or anchored to the inner leaflet of the plasma membrane. A close inspection of the transverse sections presented in Fig. 4C reveals electron-dense structures that might be proteins. The existence of such regulatory proteins would be consistent with an earlier model of plasmodesmata in the filamentous green alga, *Bulbochaete hiloensis* (Fraser and Gunning 1969).

Functional specializations, as seen in the reproductive structures of *Chara*, appear to be accomplished by merely restricting the formation, or temporary plugging, of plasmodesmata (Kwiatkowska and Maszawski 1976, 1985, 1986; Kwiatkowska 1988, 1991). During growth and development in higher plants, cells become specialized and more complex symplasmic domains are established (Robards and Lucas 1990). Therefore, a mechanism for selective trafficking of informational macromolecules in general is necessary. The inclusion of the appressed ER, as in the plasmodesmata of higher plants, reduced the plasmodesmal size exclusion limit from 45 kDa in characean cells to 800 to 1000 kDa for passive diffusion of small molecules while also potentiating the ability to engage in selective macromolecular trafficking (Fisher et al. 1992; Lucas et al. 1993). This potential to engage in selective intercellular trafficking of informational molecules may well have greatly refined the plant's ability to regulate differential gene expression during development. In an evolutionary sense, such selective trafficking of molecules and information may have played key roles in allowing the development of the complexity of cellular structure and physiology seen in vascular plants.

The mechanism for primary formation of plasmodesmata must have evolved as a separate event from that of secondary plasmodesmata formation as seen in *Chara*. This presumably occurred at some point higher in the evolutionary tree. At least two possible scenarios can be envisaged for this development (Fig. 7). In one, during the course of evolution, the phragmoplast of the ancestral forms of the Charales that gave rise to the land plants gained the ability to position the ER within the forming cell plate, thus producing the primary plasmodesmata. Here the microtubules of the phragmoplast are envisaged to have evolved a central role in the "entrapment" of the ER. It is interesting to note that in *Chara*, microtubules can be "trapped" in the developing cell plate, although this does not result in stable primary plasmodesmata formation. In the second scenario, a phragmoplast with the ability to "anchor" rather than "entrap" the ER to form primary plasmodesmata evolved independently of the phragmoplast of the Charales, perhaps in the ancestors of certain unknown or uncharacterized species. The membrane-membrane interactions associated with the anchoring of the ER to the developing plasma membrane may have been the key point leading to the formation of a stable pore that, over evolutionary time, gave rise to the formation of the primary plasmodesma.

With the exception of plastids and mitochondria, little is known about the evolution of organelles in eukaryotic organisms. The DNA content of the above organelles has been a useful tool for such studies, but is lacking in other important organelles and cellular structures. Reconstruction of the events or pressures leading to organelle features of modern organisms is thus very difficult and often restricted to the theoretical. Careful examination of ultrastructural development in extant organisms which are considered primitive or representative of ancestral lines may provide a means of exploring the evolution of eukaryotic cellular structure. The results presented here not only give us new insight into the development of the intercellular communication channels of vascular plants,

but also indicate that expanded studies of selected green algae and bryophyte species may hold great potential for a reconstruction of the evolution of plasmodesmata, a critical step in the pathway leading to complex plant forms.

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