Protoplasma (2002) 220: 39–49
DOI 10.1007/s00709-002-0033-8

PROTOPLASMA
Printed in Austria

Ultrastructure of vascular cambial cell cytokinesis in pine seedlings preserved by cryofixation and substitution

K. H. Rensing[1,*], **A. L. Samuels**[1], and **R. A. Savidge**[2]

[1] Department of Botany, University of British Columbia, Vancouver, British Columbia
[2] Faculty of Forestry and Environmental Management, University of New Brunswick, Fredericton, New Brunswick

Received February 11, 2002; accepted May 31, 2002; published online October 31, 2002

Summary. Trees depend on the secondary vascular cambium to produce cells for new xylem and phloem. The fusiform cells of this lateral meristem are long and narrow, presenting special challenges for arranging the mitotic spindle and phragmoplast. Fusiform cambial cells of *Pinus ponderosa* and *Pinus contorta* were studied by cryofixation and cryosubstitution which preserved ultrastructure and phases of cytokinesis with a resolution not previously attained. Membranous structures including the plasma membrane, tonoplast, and those of other organelles were smooth and unbroken, indicating that they were preserved while the protoplasm was in a fully turgid state. Mitotic spindles separated daughter chromosomes diagonally across the radial width of the cells. The cell plate was initiated at an angle to the cell axis between the anaphase chromosomes by a microtubule array which organized vesicles at the phragmoplast midline. Within the phragmoplast, vesicles initially joined across thin tubular projections and then amalgamated into a tubulo-vesicular network. Axial expansion of the cell plate generated two opposing phragmoplasts connected by a thin, extended bridge of cell plate and cytoplasm that was oriented along the cell axis. In the cytoplasmic bridge trailing each phragmoplast, the callose-rich tubular network gradually consolidated into a fenestrated plate and then a complete cell wall. Where new membrane merged with old, the parent plasmalemma appeared to be loosened from the cell wall and the membranes joined via a short tubulo-vesicular network. These results have not been previously reported in cambial tissue, but the same phases of cytokinesis have been observed in cryofixed root tips and suspension-cultured cells of tobacco.

Keywords: Secondary vascular cambium; *Pinus* spp.; High-pressure freezing; Freeze substitution; Cell plate; Phragmoplast.

Abbreviations: CFS cryofixation and cryosubstitution; ER endoplasmic reticulum; HPF high-pressure freezing; PPB preprophase band.

* Correspondence and reprints: Department of Botany, University of British Columbia, 6270 University Boulevard, Vancouver, BC V6T 1Z4, Canada.

Introduction

The meristematic secondary vascular cambium produces radial files of fusiform cells destined for either xylem or phloem. Although it may be the most frequently occurring type of meristematic cell division in terrestrial plants, scant attention has been paid to the remarkable nature of this periclinal division in recent decades (Evert and Deshpande 1970, Goosen-de Roo et al. 1984, Farrar and Evert 1997). Observations made by light microscopy early in the twentieth century, detailed the mechanics of mitosis and cell plate formation in the highly elongated cells (Bailey 1920a, b). Since the cells can be more than 500 times longer than they are wide, special adaptations to divide along the axial length of the cell include an angled mitotic spindle, elongate phragmoplast, and finally a new cell wall growing between daughter cells by the action of two phragmoplasts moving in opposite directions. In the ensuing years, the nature of the phragmoplast, a cytoskeletal complex directing cell plate formation, has been revealed by electron microscopy and immunofluorescence (Farrar and Evert 1997, Chaffey and Barlow 2002). The nature of the membrane structures producing the new cell plate has remained enigmatic because the cambial cells are notoriously difficult to preserve by conventional techniques for electron microscopy.

Conventional chemical fixatives disrupt the cell membranes by their slow penetration, induction of osmotic stress and fragmentation of delicate membrane networks (Rensing 2002). The volumes of the elongated, fusiform cambial cells are dominated by the

large central vacuole, with only a thin layer of parietal cytoplasm pressed against the cell wall capable of being cross-linked by fixatives. The gradual penetration of chemical fixatives into the cell means that it may take several minutes for cytoplasmic streaming to halt and cellular structures to be stabilized (Mersey and McCully 1978). When cambial cells undergoing cytokinesis were prepared by chemical fixation, the cell plate consisted of lines of vesicles and mixtures of sizes of vesicles (Farrar and Evert 1997). Similar results have been reported for chemical fixation of the forming cell plate in primary meristems and cell cultures (reviewed in Samuels et al. 1995, Staehelin and Hepler 1996).

The development of techniques for cryofixation and cryosubstitution (CFS) has provided a new means for studying plant cells by transmission electron microscopy. Cryofixation freezes cells within milliseconds, capturing dynamic cellular events without placing the cells under osmotic or pH stress. During cryosubstitution, water is extracted from the tissues directly from the frozen state and fixative is allowed to penetrate the sample, uniformly, at low temperature. CFS is thus very good for studying transient membrane configurations such as in Golgi (Staehelin et al. 1990) and during endocytosis (Galway et al. 1993). When cytokinesis in tobacco primary growth was studied with CFS, the forming cell plate consisted of a series of delicate reticular membrane networks suspended in the phragmoplast (Samuels et al. 1995). The goal of this research was to investigate the cryofixed ultrastructural features of cytokinesis in conifer secondary vascular cambium, with emphasis on the unique features imposed by the highly elongate nature of the cambial cells and the features of membrane structures shared by primary and secondary growth. The results give a detailed view of the low-resolution, but extremely accurate, work done by Bailey (1920a, b).

Material and methods

Two species of pines, *Pinus ponderosa* and *Pinus contorta*, were utilized for these studies. No ultrastructural differences were noted between the two.

Plunge freezing

Seedlings were grown outdoors in planters (15 by 60 cm) and destructively sampled at 2-week intervals through two consecutive springs. Detailed tissue preparation techniques were described previously (Rensing 2002). In summary, thin radial slices of the cambium and an adjacent portion of the xylem and phloem were hand-cut from radially bisected 2.5 cm stem lengths and then placed in unbuffered 0.2 M sucrose as a cryoprotectant for a minimum of 30 min. Tissue slices from the cryoprotectant were placed on formvar-coated wire support loops, the excess fluid was wicked away, and then they were immediately plunged into liquid propane at –186 °C using a Reichert KF80 immersion cryofixation system (Leica Microsystems Heidelberg GmbH, Heidelberg, Federal Republic of Germany). Frozen slices still on the loops were transferred in liquid nitrogen to specimen baskets in a prechilled Reichert CSauto cryosubstitution apparatus. Oven-dried molecular sieve was added to provide water scavenging, and then the machine was closed and the substitution medium of 1.5% osmium tetroxide in fresh dry acetone was slowly injected. Substitution proceeded for 90 h at –90 °C, and then the tissues were warmed to 15 °C at a rate of 5 °C per hour. The slices were then moved to fresh acetone at 15 °C, separated from the plunging loops, and placed in 1 ml of acetone in capped vials on a rotating mixer. One drop of Spurr's resin (Spurr 1969) was added to each vial every 5 min until the mixture was approximately 25% resin. After 2 h in 50% resin, the tissue slices were left in open vials containing 75% resin for 12 h, then 100% resin with twice daily changes for 3 days. The infiltrated samples were polymerized in fresh Spurr's resin at 60 °C.

More than 500 plunge-frozen tissue slices were sectioned at 0.6 µm with a Reichert Ultracut E microtome. The sections were mounted on glass slides and stained with toluidine blue O at pH 11 (O'Brien and McCully 1981) for observation by conventional light microscopy. Approximately 10% of the slices showed signs of good preservation (intact protoplasm); relevant sections were photographed on Kodak Tmax film (Eastman Kodak Company) with a Wild MPS46/52 camera system on a Leica Laborlux compound microscope.

High-pressure freezing

Dormant container-grown seedlings were planted in planters (15 by 60 cm) and placed in a growth chamber set at 24 °C and constant light. The seedlings were destructively sampled over a three-week period. Cambial slices were hand-cut as above in 0.2 M sucrose. The tissues were high-pressure frozen in a Balzers HPM 010 (Balzers Instruments, Balzers, Liechtenstein). Frozen samples were freeze substituted for 120 h in 2% osmium tetroxide and 8% dimethoxypropane in acetone (Kaeser 1989), using a dry ice–acetone bath. The tissues were then warmed to –20 °C in a freezer for 4 h and then to 4 °C in a refrigerator for 4 h, after which they were brought to room temperature. The tissues were transferred to fresh dry acetone, then gradually embedded and sectioned for light microscopy as above.

Well-preserved sections were further sectioned at 70 nm for transmission electron microscopy. Thin sections were mounted on formvar-coated and uncoated copper 75-mesh hexagonal grids, stained with 1% uranyl acetate in 50% ethanol for 10 min and Sato's lead (Sato 1967) for 5 min, and then photographed on sheet film with a Hitachi 7000 (Nissei Sangyo America Ltd., Pleasanton, Calif., U.S.A.) or a Zeiss (Carl Zeiss, Jena, Federal Republic of Germany) EM10C transmission electron microscope.

Immuno-gold labeling

Callose immunolabeling using a mouse monoclonal antibody to β-1,3-glucan (Biosupplies Australia, Pty. Ltd., Parkville, Victoria, Australia) was performed on Spurr-embedded, osmicated sections mounted on formvar-coated nickel grids. Following a 10 min etching with saturated aqueous sodium metaperiodate, nonspecific protein binding was blocked with 5% Carnation nonfat dry milk in 20 mM phosphate buffer (pH 7.0) with 0.25 M sodium chloride and 0.1%

Tween 20 (PBST). Sections were incubated for 1 h in primary (anti-callose) antibody diluted 1 : 10 in PBST with 1% nonfat dried milk, rinsed for 20 s in a stream of PBST, then incubated in 10 nm diameter gold-labeled polyclonal goat anti-mouse antibody (Ted Pella Inc., Redding, Calif., U.S.A.) in PBST for 1 h. The sections were then rinsed for 20 s each in a stream of PBST followed by distilled water and dried. The grids were stained with 2% aqueous uranyl acetate for 30 min and Reynolds' lead citrate (1963) for 10 min and then observed and photographed by electron microscopy. Controls included omission of the primary antibody and preincubation of the primary antibody with 1 mg of laminarin per ml to block the epitope binding site.

Results

Evidence of small ice crystal growth during cryofixation was apparent at high magnification only in plunge-frozen material. This was at the useful limit of resolution for this material and thus did not greatly affect the ultrastructure. The prime advantage of HPF was a success rate for well fixed tissue of over 90% compared to about 10% for plunge freezing. By both methods of CFS, ultrastructural features of the phases of mitosis and cytokinesis in fusiform cambial cells were preserved with a resolution not previously reported. Membranous structures including plasmalemma, tonoplast, and those of other organelles were all smooth and unbroken and appeared to be fixed in a fully turgid state.

Despite the observation of numerous fusiform cells in all stages of the cell cycle, no evidence of preprophase bands (PPB) of microtubules was found. Following the breakdown of the nuclear envelope in early mitotic cells, spindle microtubules were laid out at an angle to the cell axis, with the spindle poles associated with opposite periclinal walls (Fig. 1 A). Endoplasmic reticulum (ER) was present between the microtubules, but organelles such as mitochondria and plastids were excluded from the spindle. The chromosomes were thereby displaced axially as well as laterally so that nuclei re-formed adjacent to opposite periclinal walls, with one slightly above the other (Fig. 2). Nonkinetochore microtubules extended across the metaphase plate as mitosis progressed to anaphase.

The phragmoplast formed among the nonkinetochore microtubules at telophase, prior to the reformation of the nuclear membrane (Fig. 1 B). The initial orientation of the cell plate between the nuclei (Figs. 1 C and 2 A) reflected the angled orientation of the mitotic spindles (Fig. 1 A), but once the cell plate extended beyond the daughter nuclei, it became realigned parallel to the long axis (Fig. 2 B, C). Decondensation of the chromatin and re-formation of the nuclei took place after cell plate formation was initiated (Fig. 1 C) and well before the cell plate was extended through the length of the cell (Fig. 2 C). The cell plate fused with the parent plasmalemma along the sides of the cell while the cell plate continued to extend axially, producing two opposing phragmoplasts each of which formed a rounded plug (bolus) within the vacuole (Fig. 2 B, C). Cytokinesis in the fusiform cell continued as the two boluses migrated in opposite directions along the extended axis of the cell (Fig. 2 C). The bolus maintained a fairly constant size as it built the cell plate through the length of the cell.

Each bolus of the migrating phragmoplast (Fig. 3) was dominated by the microtubule array and the forming cell plate with an outer shell of cytoplasm and organelles. Microtubules were arranged radially about the developing cell plate throughout most of the bolus. The trailing part of the microtubule array was sometimes angled from the cell plate toward the direction of growth (Fig. 3 A), while the leading portion often curved back so that the microtubules converged at the lateral border of the phragmoplast, making the array heart or umbrella shaped. Mitochondria were restricted primarily to the periphery of the phragmoplast, although some were present toward its leading edge. Smooth and ribosomal ER were present mostly in the lateral regions of the phragmoplast, but smooth ER was occasionally interspersed within the microtubule array. Golgi with associated vesicles also were present in the peripheral area, but in median sections, they were more frequently observed at the trailing part of the phragmoplast.

Cell plate assembly could be roughly separated into 4 phases within the structures of the phragmoplast and trailing cytoplasmic bridge. First, the most distal region of the phragmoplast at the leading edge resembled the earliest phases of cell plate formation in late anaphase (Fig. 1 C). This region was characterized by abundant phragmoplast microtubules and clusters of vesicles axially aligned at the leading border of the cell plate (Figs. 4 and 5). Many vesicles were joined either through narrower projections, making dumbbell shapes, or by direct contact, making irregular elongated shapes. Thin membranous bridges, seen in cross section in oblique sections, were visible between some of the vesicles (Figs. 4 and 5 A). Most vesicles had a distinct darkly stained coating with a somewhat lighter-stained interior. Clathrin-coated vesicles were not present.

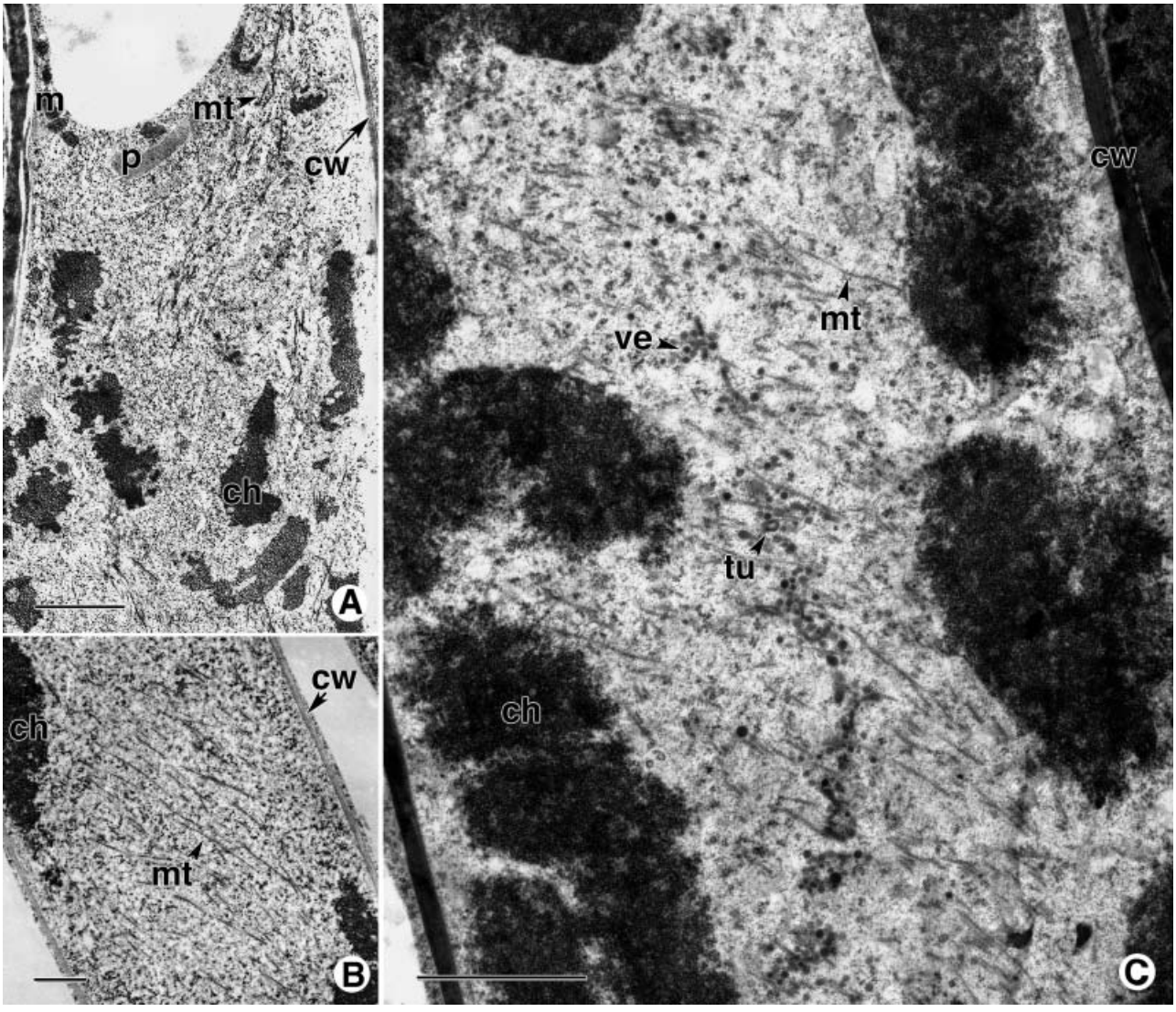

Fig. 1 A–C. Mitosis and initiation of cytokinesis. **A** Plunge freezing. During anaphase, the spindle microtubules (*mt*) are oriented at an angle to the cell axis and nonkinetochore microtubules span the area between the two groups of separating chromosomes (*ch*). Mitochondria (*m*) and plastids (*p*) are excluded from the nuclear region. **B** HPF. By telophase, the space vacated by the separating chromosomes is populated by early phragmoplast microtubules (*mt*) oriented perpendicular to the spindle. **C** HPF. The cell plate is initiated by fusion of vesicles (*ve*) that appear within the microtubule arrays emanating from the chromosomes. The early cell plate is oriented at an angle to the cell axis. *cw* Cell wall, *tu* tubule. Bars: 1 μm

Adjacent to this region of the cell plate, but still within the microtubule-rich phragmoplast, was a second area dominated by a network of membranous tubules formed from the fusion of many vesicles (Fig. 5B). Vesicles, often associated with phragmoplast microtubules, were fused with other vesicles and to the membranous networks. The vesicles and the membranous network still had a darkly stained coating and contents. Individual vesicles and vesicles "budding" from the tubules were also present and exhibited a coating characteristic of clathrin. Within the phragmoplast, the cell plate was often wavy or contorted (Fig. 2B, C) but became axially aligned outside the phragmoplast such that the new walls radially bisected the cells.

Outside the phragmoplast, the cytoplasm on either side of the cell plate became thinner with fewer organelles. This transition zone was the third characteristic phase of cell plate development (Fig. 5C). In general, the darkly stained coating and contents in the forming membrane decreased through this zone (lower portions of Fig. 5C, D). Microtubules were no longer present, while mitochondria, Golgi and ER were very close to the forming membrane. The mem-

branous network became tighter packed, consisting of numerous interwoven tubular structures. Fewer individual vesicles were present, although some clathrin-coated vesicles were seen along the membrane.

In the fourth phase of cell plate development, the membranes appeared to be in long axially oriented tubules with occasional gaps which, in oblique sections (not shown), indicated a fenestrated plate structure. The darkly stained coating was no longer present. Further towards the center portion of the cytoplasmic bridge, consolidation of the membrane was complete.

Callose was localized in the forming cell plate, starting at the transition between the tubulo-vesicular network and the tubular network where the phragmoplast microtubules were no longer present (i.e., where the phragmoplast microtubules ended) (Fig. 5D). Immunolabeling showed that callose remained in the cell plate over an extended distance from the phragmoplast. Towards the oldest portion of the complete plate, labeling gradually decreased.

Fusion of the cell plate with the parent plasmalemma at the cell tips was not captured because of the low probability of sectioning and identifying such areas. Rather, membrane fusion was observed from oblique sections of the lateral walls (Fig. 6). The cell plate joined the parent plasmalemma across a short tubulo-vesicular network. The vesicles and microtubules (Fig. 6B) represented the remnants of the phragmoplast. Many clathrin-coated vesicles adjacent to both the parent and cell plate membranes indicated a high rate of membrane turnover. Recycling of membrane material was implied by vesicles budded from the more mature part of the cell plate via tubelike protrusions (Fig. 6A). The parent membrane protruded from the wall; the gap was occupied by a fibrillar material that was distinct from the parent wall. Callose was present in the tubular network immediately adjacent to the parent membrane as well as in the more mature region of the cell plate (Fig. 6B).

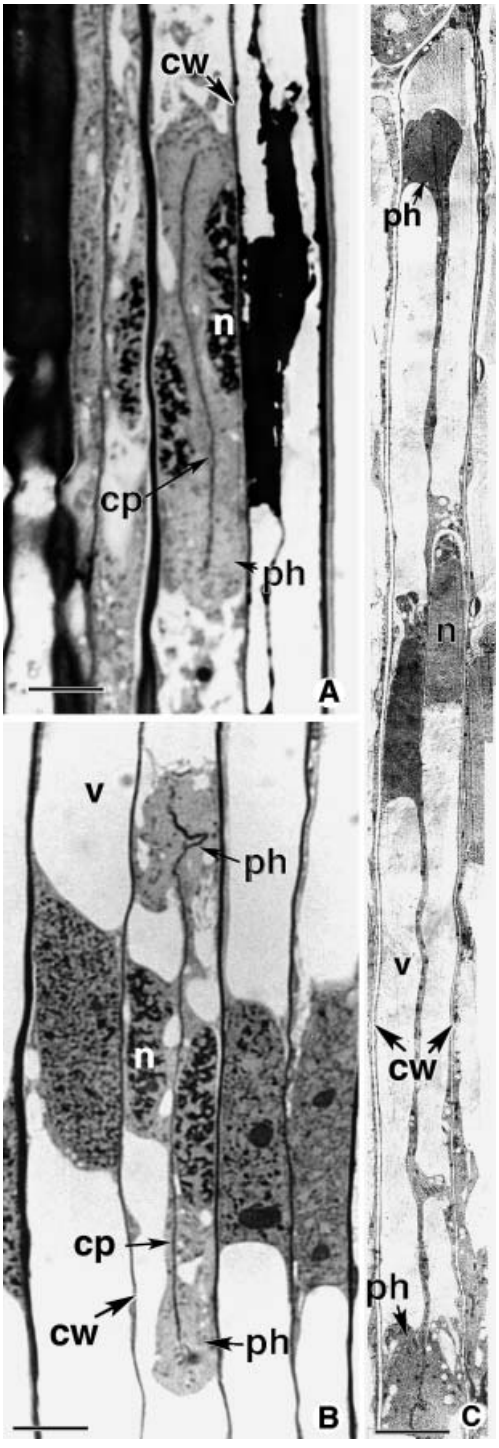

Fig. 2A–C. Progression of the phragmoplast and reassembly of the nuclei during cytokinesis. **A** Light micrograph. As the phragmoplast (*ph*) extends the cell plate (*cp*) beyond the re-forming nuclei (*n*), it curves toward the cell midline. **B** Light micrograph. With greater extension of the cell plate and further re-formation of the nuclei, the cell plate (*cp*) between the nuclei realigns with the cell axis and the phragmoplast (*ph*) separates into two. Wavy cell plate formation as in the upper phragmoplast is common but not universal. *v* Vacuole. **C** Electron micrograph. The phragmoplasts (*ph*) have extended the cell plate through the vacuole (*v*), well beyond the fully re-formed nuclei (*n*). The cell plate remains aligned midway between the parent cell walls. The phragmoplasts do not make contact with both periclinal walls through the periclinal width of the cell. *cw* Cell wall. Bars: 5 μm

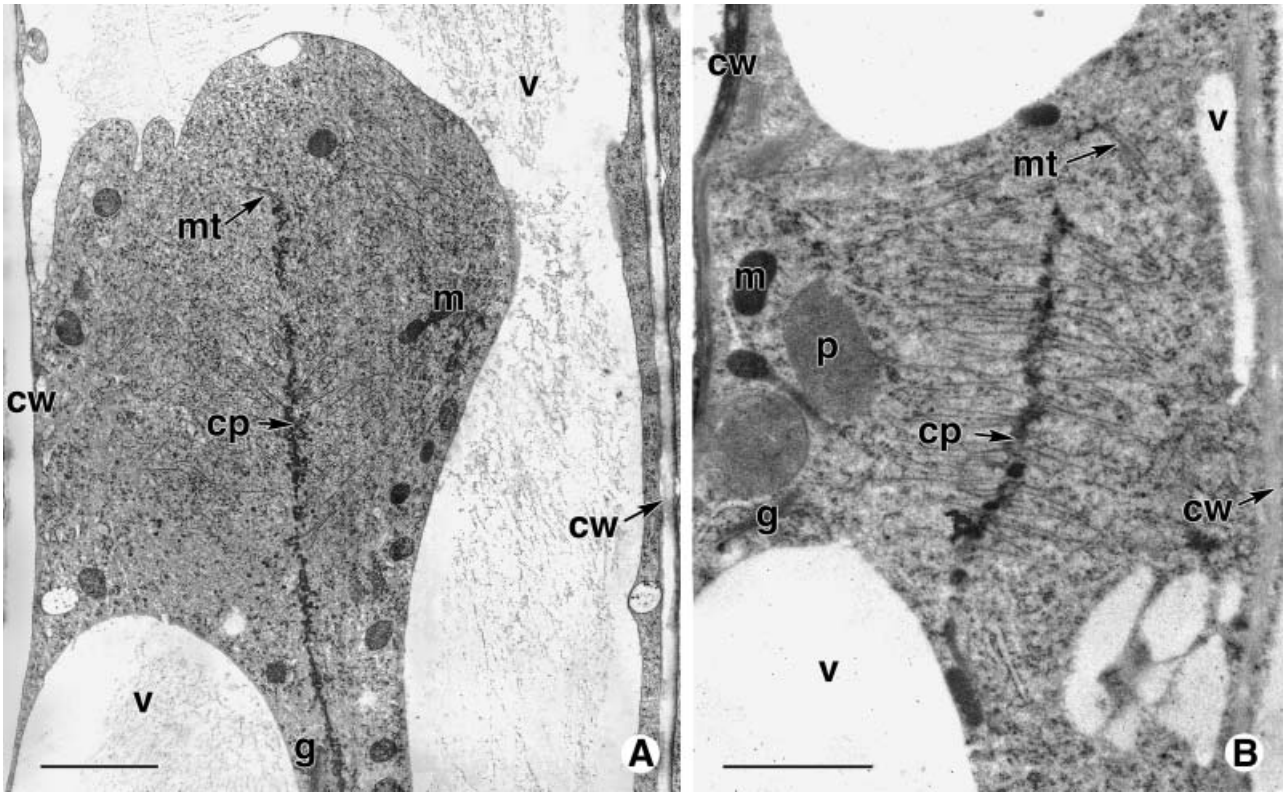

Fig. 3 A, B. Phragmoplast and phragmosome. There is no significant cytoplasmic bridge extending in advance of the expanding cell plate (see also Fig. 2). Microtubules (*mt*) at the leading edge of the array angle toward the direction of cell plate growth. **A** Plunge freezing. The bolus, made up of the phragmoplast and phragmosome (see text), moves through the vacuole (*v*) as the cell plate (*cp*) is extended. Microtubules radiate from the widest part of the bolus to the midline where the vesicles are assembled into a cell plate. **B** HPF. Golgi (*g*), mitochondria (*m*), and plastids (*p*) were excluded from the phragmoplast microtubule array. Although the cytoplasm spans the width of the cell in this section, portions of the continuous vacuole (*v*) are still present on the right. *cw* Cell wall. Bars: 1 µm

Discussion

Phragmoplasts in fusiform cells

The diaphragm of cytoplasm in which the nucleus is suspended just prior to mitosis and which delimits the plane of cytokinesis has been termed the phragmosome (Sinnot and Bloch 1941). Phragmosomes were found to lie between the developing phragmoplasts and the lateral walls of dividing cambial cells in *Ulmus americana* and *Tilia americana* (Evert and Deshpande 1970). However, longitudinal sections demonstrated that extended phragmosomes did not traverse the axial length of fusiform cells ahead of the phragmoplasts in *Robinia pseudoacacia* (Farrar and Evert 1997). This was also the case in pine fusiform cambial cells, where phragmoplast microtubules were very close to the leading edge of the axially advancing bolus. The lack of a complete phragmosome between all edges of the phragmoplast and the future site of cell plate fusion is not unique to cambial cells (Palevitz 1986, Esau and Gill 1991). Therefore, it may be helpful to use Esau's (1965) description of the phragmosome as the living medium in which the phragmoplast and the cell plate develop. It can include the cellular constituents adjacent to the phragmoplast that directly support cell plate production, such as cytoplasm, mitochondria, Golgi, ER, and ribosomes. Then, in fusiform cambial cells, the phragmosome surrounds the phragmoplast; together they make up the bolus that extends the cell plate axially through the large central vacuole.

The different microtubule arrays that bring about mitosis and cytokinesis form an intergrading continuum (Brown and Lemmon 1997). Phragmoplast microtubules, which direct vesicles to the forming cell plate (Esau 1977), are thought to be organized from spindle-interzone microtubules (Staehelin and Hepler 1996, Valster and Hepler 1997). In embryogenic culture cells of *Picea glauca*, spindle-interzone microtubules were infrequent during metaphase, but by anaphase the more numerous microtubules gave rise

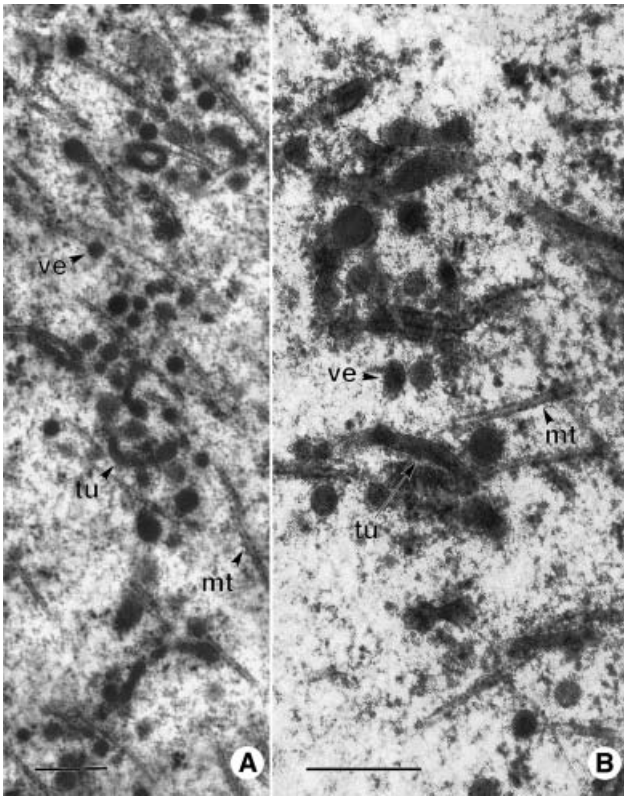

Fig. 4 A, B. Vesicle fusion; HPF. **A** Vesicles (*ve*) have a darkly staining coating that was distinct from clathrin. Individual vesicles were often joined to others via short membranous tubules (*tu*) producing dumbbell shapes but also via longer tubules in curved or horseshoe-shaped configurations. **B** Membrane tubules (*tu*) are smaller in diameter than vesicles (*ve*) but notably wider than microtubules (*mt*). Bars: 0.1 μm

to the phragmoplast (Fowke et al. 1990). Similarly, this was found to be the case in fusiform cells of *Pinus* spp., where few interzone microtubules were present while spindles were present, whereas many microtubules occupied the space between the separated chromosomes at the initiation of the cell plate during telophase. Once formed, the phragmoplast serves as a stabilizing center for the continued production of microtubules (Brown and Lemmon 1997).

In many plant cells, assembly of the cell plate occurs within a centrifugally expanding phragmoplast ring that eventually reaches the lateral cell wall, where the cell plate fuses with the existing cell membrane (Smith 2001). In fusiform cells, cytokinesis becomes quite different from this basic model. As in isodiametric cells, the cell plate is initiated and expands centrifugally from between the separated chromosomes following anaphase. However, while its lateral borders quickly reach and fuse with the plasmalemma, its upper and lower borders must extend much further before a fusiform cell is fully bisected. Therefore, the initial single ring-shaped phragmoplast splits into two phragmoplasts that assemble the cell plate in opposite directions (Bailey 1920a, b).

In tobacco BY-2 culture cells, phragmoplast microtubules were perpendicular to the cell plate (Samuels et al. 1995) with their "plus" (fast-growing) ends within the forming cell plate (Shibaoka 1993), but in *Pinus* spp., the phragmoplast microtubules were sometimes angled from the cell plate towards the direction of growth. The microtubules at the leading border of the phragmoplast also often curved forward toward the direction of cell plate growth. As the cell plate starts to mature in the trailing region of the phragmoplast, the microtubules are broken down and the tubulin is recycled to the regions of active vesicle assembly (Yasuhara et al. 1993). When microtubule depolymerization is prevented by taxol (Shibaoka 1993), or when relocation of tubulin is inhibited by caffeine (Valster and Hepler 1997), centrifugal growth of the phragmoplast is inhibited. Nishihama and Machida (2001) propose that neither the pushing force of the cell plate nor the pulling force of the parental walls is necessary for the "enlargement" of the phragmoplast; assembly of new microtubules at the leading edge of the phragmoplast forces the expansion of the phragmoplast. The forwardly oriented arrangement of phragmoplast microtubules and the lack of an extended phragmosome in cambial cells is consistent with assembly of new microtubules driving cell plate expansion.

Control of cell plate orientation

It is not known what directs the lateral borders of the cell plate to fuse to parent plasmalemma in the correct position. The path of phragmoplast development and thus the new cell wall position are not controlled by the orientation of the mitotic spindles (Palevitz 1986, Pickett-Heaps et al. 1999). In *Pinus* spp., the spindles were depolymerized and the daughter nuclei fully formed while the phragmoplasts were still extending the cell plate. This contrasts with primary meristems, in which cell plate formation began at late anaphase and was complete by late telophase. Also, in fusiform cells, the spindles were semitransversely oriented, whereas the cell plate was produced parallel to the long axis. Following the oblique separation of daughter chromosomes in guard mother cells of *Allium cepa*, the expanding edges of the phragmoplast reorient to find the fusion site on the parent wall, following which

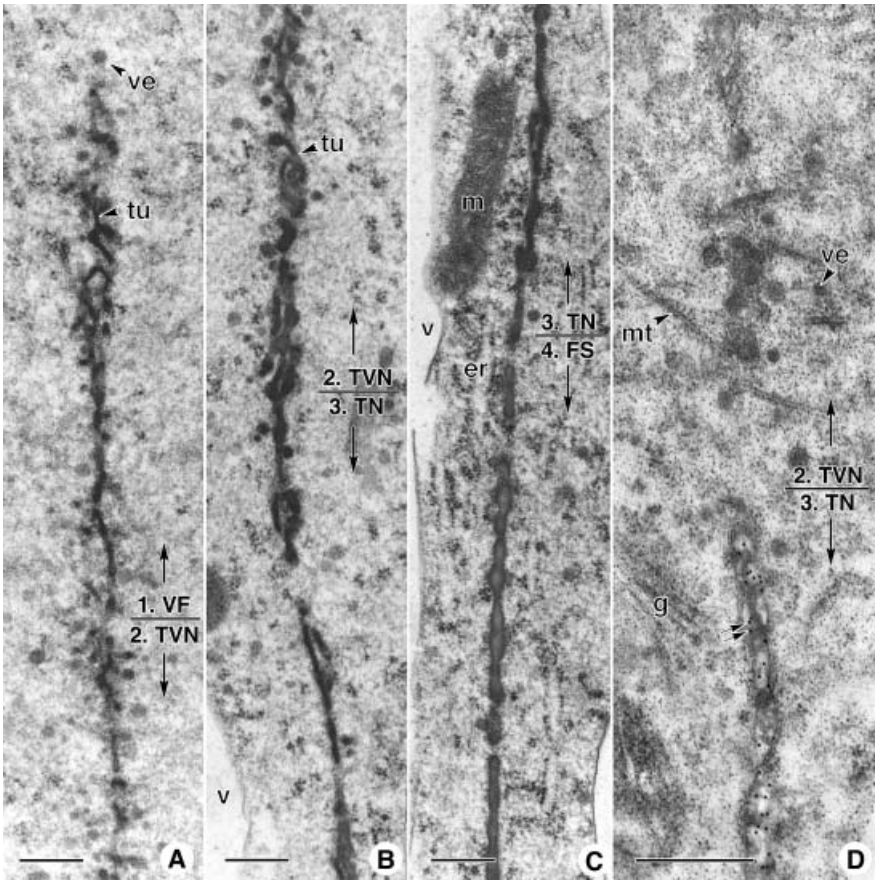

Fig. 5 A–D. Maturation of the cell plate; HPF. A–C Sequential (end-to-end) images from a single section. Separation of the cell plate into four phases is only approximate, as each phase grades into the next. **A** The first phase of cell plate formation, vesicle fusion (1. VF), occurs as Golgi-derived vesicles (*ve*) are joined via membranous tubules (*tu*) (see also Fig. 4). The second phase was the amalgamation of the fusion tubules into a more reticular, tubulo-vesicular network (2. TVN). Individual vesicles continued to join the network during this phase. The network and vesicles retained a dark coating. **B** Where the tubulo-vesicular network graded into the tubular network (3. TN), phragmoplast microtubules were no longer present and the cytoplasm narrowed to a thin layer confined by the vacuole (*v*). Although some vesicles are present in the younger portion, the cell plate in this third phase of development is dominated by branched tubular membranes. Organelles such as endoplasmic reticulum (*er*) and mitochondria (*m*) can be found immediately adjacent to the cell plate. **C** In the fourth phase of maturation, the cell plate consolidated into a fenestrated sheet (4. FS) with only intermittent gaps. **D** Anticallose labeling (double arrows) demonstrates that callose deposition increases dramatically between the tubulo-vesicular network and the tubular network where the microtubule (*mt*) array is no longer present and organelles such as Golgi (*g*) are immediately adjacent to the new membrane. Bars: 0.1 μm

the cell plate straightens and rotates into the final plane of division (Palevitz 1986). Realignment of the cell plate in pine fusiform cells occurred when it had expanded axially past the nuclei, presumably when its lateral borders had fused to the parent plasmalemma.

When some plant cells prepare to divide during the G_2 phase, the cortical microtubule cytoskeleton is redeployed to a narrow PPB girdling the cell (Simmonds 1986, Fowke et al. 1990, Mews et al. 1997, Pickett-Heaps et al. 1999). The location of the PPB normally predicts, but does not determine, the site of cell plate fusion with the parent wall and thus the plane of cytokinesis (Manandhar et al. 1996; Gimenez Abian et al. 1997, 1998; Murata and Wada 1997). In cambial cells, a putative PPB would form an axially oriented band on the radial walls. However, PPBs were not observed in this study of pine cambial cells or in dividing fusiform cells of *Robinia pseudoacacia* (Farrar and Evert 1997), *Ulmus americana*, or *Tilia americana* (Evert and Deshpande 1970). Also, using antitubulin immunolocalization with confocal microscopy, PPBs were not located in dividing fusiform cambial cells of *Populus tremula*, *Aesculus hippocastanum* (Chaffey and Barlow 2002), *Pinus pinea*, or

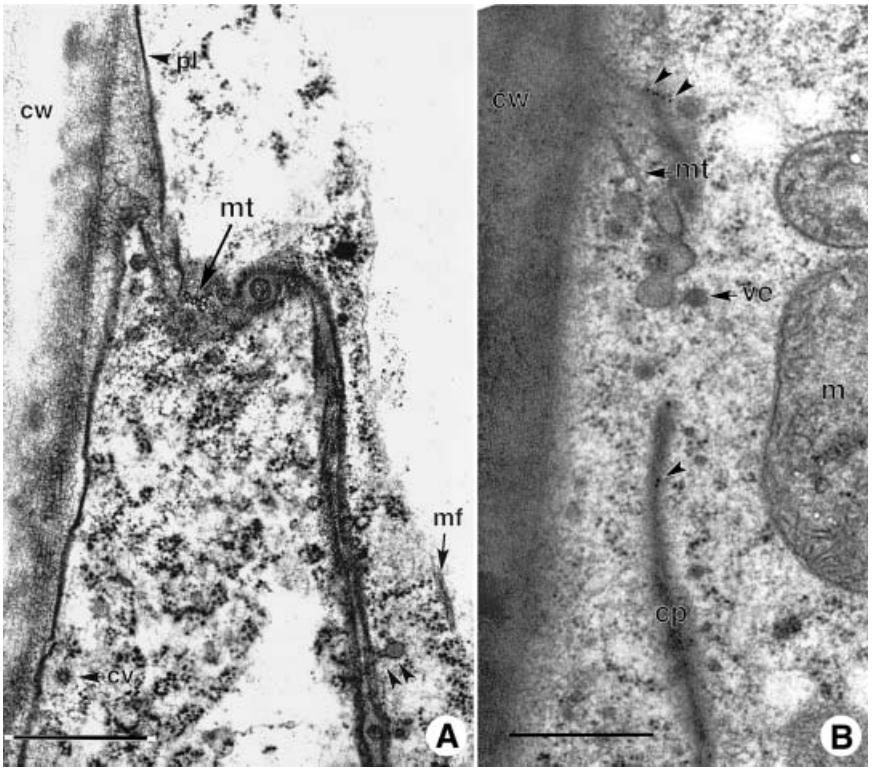

Fig. 6 A, B. Fusion of the cell plate to the parent membrane. These sections were cut at an angle to the cell axis. **A** Plunge freezing. At the line of fusion, the cell plate membrane separates the parent plasmalemma (*pl*) which protrudes from the cell wall (*cw*). A fibrillar material apparently loosened from the parent wall occupies the gap between the two. Clathrin-coated vesicles (*cv*) are present singly and attached to the cell plate membrane across thin stalks (double arrowheads). Microfilament bundles (*mf*) are seen close to the new membrane. **B** HPF. A short tubulo-vesicular network containing callose (arrowheads) separates the cell plate (*cp*) and the parent cell wall (*cw*). Few microtubules (*mt*) remain from the phragmoplast. *m* Mitochondrion. Bars: 0.25 μm

Abies sachalinensis (Oribe et al. 2001). Although not conclusive, evidence thus far suggests that fusiform cambial cells do not form PPBs.

Cell plate assembly

Gu and Verma (1997) aptly described membrane fusion in the phragmoplast as the creation of an "extracellular compartment inside the cell". The series of delicate networks making up the cell plate in fusiform cells of *Pinus* spp. parallels the processes outlined by Samuels et al. (1995). The region occupying the leading half of the phragmoplast was characterized by the presence of microtubules and the start of fusion of the Golgi-derived vesicles. Individual vesicles were joined across narrow membranous tubes and across short, wider membranous projections producing dumbbell-shaped structures. Phragmoplastin, a cell plate membrane protein concentrated at the growing margins between the phragmoplast microtubules, is thought to induce fusion tubules in the vesicles of the phragmoplast (Gu and Verma 1997). This would produce reticulate or stellate bodies in the plane of the cell plate (Verma 2001) that would become the tubulo-vesicular network of Samuels et al. (1995).

Within the phragmoplast, the cell plate was often highly contorted (wavy), indicating that the assembly of vesicles into a tubulo-vesicular network is rather loosely controlled within the microtubule array. However, the cell plate is guided to the cell midline when the phragmoplast is depolymerized and callose deposition begins. As in BY-2 cells (Samuels et al. 1995, Samuels and Staehelin 1996), callose in *Pinus* spp. was faintly detected in the microtubule-associated tubulo-vesicular network, and then its amount increased rapidly in the tubular network. Callose is believed to be a critical stabilizing component of the cell plate following disassembly of the phragmoplast microtubules. When callose deposition is prevented by caffeine treatment, the fusion network of vesicles rapidly degrades into individual vesicles that are subsequently broken down (Samuels and Staehelin 1996). Radio-labeling studies have shown that β-1,3-glucan (callose) synthesis occurs at the cell plate rather than in the ER or Golgi (Kakimoto and Shibaoka 1988) and cell-plate-specific callose synthase has been characterized (Hong et al. 2001).

The phragmoplast initiates the assembly of the cell plate (Shibaoka 1993, Samuels et al. 1995), but the consolidation of the cell plate, from the tubular network through production of the fenestrated plate (Samuels et al. 1995), takes place outside (or trailing) the microtubule array. In fusiform cells, these phases occurred within a relatively thin cytoplasmic layer containing ER, Golgi, mitochondria, and occasional plastids. Medially, the closing of the fenestrae completed the cell plate formation, while the phragmoplast continued its extension distally. This contrasts with tobacco cells, where the fusion of the cell plate to the parent membrane coincides with the closing of the fenestrated plate (Samuels et al. 1995).

The surface area of the vesicles delivered by the phragmoplast is greater than the surface area of the new plasmalemma (Otegui et al. 2001), so a mechanism for membrane recycling must exist. Clathrin-coated vesicles are involved in selectively concentrating types of molecules (Hawes et al. 1999) and could be involved in the removal of membrane components no longer needed. As in tobacco cells (Samuels et al. 1995), clathrin-coated vesicles and buds were present in the tubulo-vesicular network and the tubular network of the cell plate in fusiform cells of *Pinus* spp. Galway et al. (1993) charted the course of labeled plasmalemma from coated pits and vesicles to uncoated vesicles, Golgi and trans-Golgi membranes and then to multivesicular bodies, suggesting that multivesicular bodies may be part of the path for membrane degradation.

The cell plate initially joined to the parent wall via numerous fingerlike projections in a heterotypic fusion that appeared to be similar to the homotypic fusion of Golgi-derived vesicles in the early phragmoplast. At these sites, the parent membrane protruded from the parent wall with the space occupied by a loose fibrillar substance. Since such fibrillar material was not seen elsewhere, it most likely arose from the parent wall, although it had a distinctly different appearance from the underlying wall. It may also be that the fibrillar material was created by substances associated with the phragmoplast. Intercellular spaces were not present in the cambial zone, and where walls in different planes met, the junctions were rounded rather than angular. Strong and rounded cell wall junctions would be produced by "weaving" newly created cell wall polysaccharides into a network that was part of the parent wall rather than by merely laying new wall fibers onto the existing ones.

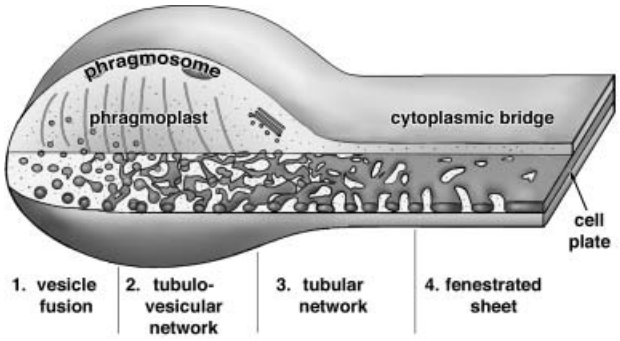

Fig. 7. Model of cell plate production in fusiform cambial cells. Cytokinesis divides the parent cells across their long axis. As the cell plate expands, the initial, single phragmoplast splits into two that push axially through the vacuole in opposite directions. As represented here, each phragmoplast and surrounding phragmosome constitute the bolus in which the vesicle fusion and tubulovesicular network phases of cell plate assembly take place. At the leading edge of the phragmoplast, microtubules angle forward and vesicle fusion is initiated across narrow membranous tubules. This fusion network amalgamates into a highly branched tubulovesicular network in the trailing region of the phragmoplast. The bolus tapers to a thin cytoplasmic bridge soon after depolymerization of the phragmoplast microtubules. This coincides with reduced vesicle fusion and increased callose deposition in the tubular network. Maturation of the callose-stabilized tubular network into a fenestrated sheet and then a complete cell plate occurs over an extended distance in the cytoplasmic bridge trailing the bolus

Conclusions

Periclinal cytokinesis in fusiform cambial cells exhibits remarkable cell plate production due to the challenge of partitioning these long and highly vacuolate cells. After the initial phases of cell plate development, the phragmoplast is no longer circular but splits into two as the cell plate is extended across the long axis of the cell (Fig. 7). Rather than the phragmoplast and phragmosome building the cell plate through cytoplasm, they extend it through a great length of vacuole. The cell plate often undulates within the phragmoplast but is directed to the cell midline at its trailing edge. Callose is deposited in the cell plate at the trailing border of the phragmoplast and remains in the plate over an extended distance. Completion of the cell plate into a continuous, closed membrane also takes place over an extended length outside the phragmoplast well before the completion of cell plate extension. Cell plate fusion to the lateral parent walls occurs well before the upper and lower borders are fully extended.

Acknowledgments

Funding was provided by the Canadian Forestry Service, Maritimes Division, and the Natural Science and Engineering Research

Council of Canada. We are indebted to Dr. J. N. Owens and the Department of Biology, University of Victoria for use of the plunge cryofixation equipment.

References

Bailey IW (1920a) The cambium and its derivative tissues III: a reconnaissance of cytological phenomena in the cambium. Am J Bot 7: 417–434

– (1920b) The formation of the cell plate in the cambium of the higher plants. Proc Natl Acad Sci USA 6: 197–200

Brown RC, Lemmon BE (1997) Transition from mitotic apparatus to cytokinetic apparatus in pollen mitosis of the slipper orchid. Protoplasma 198**:** 43–52

Chaffey N, Barlow P (2002) Myosin, microtubules, and microfilaments: co-operation between cytoskeletal components during cambial cell division and secondary vascular differentiation in trees. Planta 214: 526–536

Esau K (1965) Plant anatomy. Wiley, New York

– (1977) Anatomy of seed plants. Wiley, New York

– Gill RH (1991) Distribution of vacuoles and some other organelles in dividing cells. Bot Gaz 152: 397–407

Evert R, Deshpande B (1970) An ultrastructural study of cell division in the cambium. Am J Bot 57: 942–961

Farrar JJ, Evert RF (1997) Ultrastructure of cell division in the fusiform cells of the vascular cambium of *Robinia pseudoacacia*. Trees 11: 203–215

Fowke LC, Attree SM, Wang H, Dunstan DI (1990) Microtubule organization and cell division in embryogenic protoplast cultures of white spruce (*Picea glauca*). Protoplasma 158: 86–94

Galway ME, Rennie PJ, Fowke LC (1993) Ultrastructure of the endocytotic pathway in glutaraldehyde-fixed and high-pressure frozen/freeze-substituted protoplasts of white spruce (*Picea glauca*). J Cell Sci 106: 847–858

Gimenez-Abian MI, Gimenez-Martin G, Navarrete MH, De La Torre C (1997) Microtubular structures developed in response to a carbamate herbicide in plant mitosis. Protoplasma 200: 65–70

– Utrilla L, Canovas JL, Gimenez-Martin G, Navarrete MH, De La Torre C (1998) The positional control of mitosis and cytokinesis in higher-plant cells. Planta 204: 37–43

Goosen-de Roo L, Bakhuisen T, van Spronsen P, Libbenga K (1984) The presence of extended phragmosomes containing cytoskeletal elements in fusiform cambial cells of *Fraxinus excelsior* L. Protoplasma 122: 145–152

Gu X, Verma DPS (1997) Dynamics of phragmoplastin in living cells during cell plate formation and uncoupling of cell elongation from the plane of cell division. Plant Cell 9: 157–169

Hawes CR, Brandizzi F, Andreeva AV (1999) Endomembranes and vesicle trafficking. Curr Opin Plant Biol 2: 454–461

Hong Z, Delauney AJ, Verma DPS (2001) A cell plate-specific callose synthase and its interaction with phragmoplastin. Plant Cell 13: 755–768

Kaeser W (1989) Freeze-substitution of plant tissues with a new medium containing dimethoxypropane. J Microsc 154: 273–288

Kakimoto T, Shibaoka H (1988) Cytoskeletal ultrastructure of phragmoplast-nuclei complexes isolated from cultured tobacco cells. Protoplasma Suppl 2: 95–103

Manandhar G, Apostolakos P, Galatis B (1996) Cell division of binuclear cells induced by caffeine: spindle organization and determination of division plane. J Plant Res 109: 265–275

Mersey B, McCully ME (1978) Monitoring the course of fixation of plant cells. J Microsc 114: 49–76

Mews M, Sek FJ, Moore R, Volkmann D, Gunning BES, John PCL (1997) Mitotic cyclin distribution during maize cell division: implications for the sequence diversity and function of cyclins in plants. Protoplasma 200: 128–145

Murata T, Wada M (1997) Formation of a phragmosome-like structure in centrifuged protonemal cells of *Adiantum capillus-veneris* L. Planta 201: 273–280

Nishihama R, Machida Y (2001) Expansion of the phragmoplast during plant cytokinesis: a MAPK pathway may MAP it out. Curr Opin Plant Biol 4: 507–512

O'Brien TP, and McCully ME (1981) The study of plant structure: principles and selected methods. Termarcarphi Pty, Melbourne

Oribe Y, Funada R, Shibagaki M, Kubo T (2001) Cambial reactivation in locally heated stems of the evergreen conifer *Abies sachalinensis* (Schmidt) Masters. Planta 212: 684–691

Otegui MS, Mastronarde DN, Kang B-H, Bednarek SY, Staehelin LA (2001) Three-dimensional analysis of syncytial-type cell plates during endosperm cellularization visualized by high resolution electron tomography. Plant Cell 13: 2033–2051

Palevitz BA (1986) Division plane determination in guard mother cells of *Allium*: video time-lapse analysis of nuclear movements and phragmoplast rotation in the cortex. Dev Biol 117: 644–654

Pickett-Heaps JD, Gunning BES, Brown RC, Lemmon BE, Cleary AL (1999) The cytoplast concept in dividing plant cells: cytoplasmic domains and the evolution of spatially organized cell division. Am J Bot 86: 153–172

Rensing KH (2002) Chemical and cryo-fixation for transmission electron microscopy of gymnosperm cambial cells. In: Chaffey NJ (ed) Wood formation in trees: cell and molecular biology techniques. Taylor and Francis, New York, pp 65–81

Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17: 208–212

Samuels AL, Staehelin LA (1996) Caffeine inhibits cell plate formation by disrupting membrane reorganization just after the vesicle fusion step. Protoplasma 195: 144–155

– Giddings TH, Staehelin LA (1995) Cytokinesis in tobacco BY-2 and root tip cells: a new model of cell plate formation in higher plants. J Cell Biol 130: 1345–1357

Sato T (1968) A modified method for lead staining of thin sections. J Electron Microsc 17: 158–159

Simmonds DH (1986) Prophase bands of microtubules occur in protoplast cultures of *Vicia hajastana* Grossh. Planta 167: 469–472

Sinnott EW, Bloch R (1941) Division in vacuolate plant cells. Am J Bot 28: 225–232

Shibaoka H (1993) The use of tobacco BY-2 cells for the studies of the plant cytoskeleton. J Plant Res (Special Issue) 3: 3–15

Smith LG (2001) Plant cell division: building walls in the right places. Nat Rev Mol Cell Biol 2: 33–39

Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26: 31–43

Staehelin LA, Hepler PK (1996) Cytokinesis in higher plants. Cell 84: 812–824

– Giddings TH, Kiss JZ, Sack FD (1990) Macromolecular differentiation of Golgi stacks in root tips of *Arabidopsis* and *Nicotiana* seedlings as visualized by high pressure frozen and freeze-substituted samples. Protoplasma 157: 75–91

Valster AH, Hepler PK (1997) Caffeine inhibition of cytokinesis: effect on the phragmoplast cytoskeleton in living *Tradescantia* stamen hair cells. Protoplasma 196: 155–166

Verma DPS (2001) Cytokinesis and building of the cell plate in plants. Annu Rev Plant Physiol Plant Mol Biol 52: 751–784

Yasuhara H, Sonobe S, Shibaoka H (1993) Effects of taxol on the development of the cell plate and of the phragmoplast in tobacco BY-2 cells. Plant Cell Physiol 34: 21–29