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Ultrastructural study on mitosis and cytokinesis in *Scytosiphon lomentaria* zygotes (Scytosiphonales, Phaeophyceae) by freeze-substitution

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Summary. The ultrastructure of mitosis and cytokinesis in *Scytosiphon lomentaria* (Lyngbye) Link zygotes was studied by freeze fixation and substitution. During mitosis, the nuclear envelope remained mostly intact. Spindle microtubules (MTs) from the centrosome passed through the gaps of the nuclear envelope and entered the nucleoplasm. In anaphase and telophase, two daughter chromosome masses were partially surrounded with endoplasmic reticulum. After telophase, the nuclear envelope was reconstructed and two daughter nuclei formed. Then, several large vacuoles occupied the space between the daughter nuclei. MTs from the centrosomes extended toward the mid-plane between two daughter nuclei, among the vacuoles. At that time, Golgi bodies near the centrosome actively produced many vesicles. Midway between the daughter nuclei, small globular vesicles and tubular cisternae accumulated. These vesicles derived from Golgi bodies were transported from the centrosome to the future division plane. Cytokinesis then proceeded by fusion of these vesicles, but not by a furrowing of the plasma membrane. After completion of the continuity with the plasma membrane, cell wall material was deposited between the plasma membranes. The tubular cisternae were still observed at the periphery of the newly formed septum. Microfilaments could not be observed by this procedure. We conclude that cytokinesis in the brown algae proceeds by fusion of Golgi vesicles and tubular cisternae, not by a furrowing of the plasma membrane.

Keywords: Brown alga; Centrosome; Cytokinesis; Freeze-substitution; Microtubule; *Scytosiphon lomentaria*.

Introduction

Ultrastructural observations on cytokinesis of brown algae have been reported in *Ascophyllum nodosum* (L.) Le Jolis (Rawlence 1973), *Pylaiella littoralis* (L.) Kjellm. (Markey and Wilce 1975), *Fucus vesiculosus* L. (Brawley et al. 1977), *Cutleria cylindrica* Okamura (La Claire 1981), and *Sphacelaria tribuloides* Meneghini (Katsaros et al. 1983). There is still much controversy regarding the mechanism of cytokinesis in brown algae. In *Ascophyllum nodosum*, cytokinesis is accomplished by the outgrowing of a partition membrane, not a furrowing of the plasma membrane, while in *Pylaiella littoralis*, *Fucus vesiculosus*, *Cutleria cylindrica*, and *Sphacerlaria tribuloides* cytokinesis is carried out by the centripetal development of a plasma membrane furrow in which Golgi vesicles may fuse. However, it has been commonly observed that cytokinesis begins after the completion of mitosis. In land plants, a phragmoplast appears at anaphase, and in some members of green algae (Pickett-Heaps 1975, Woods and Triemer 1981, Lokhorst et al. 1988) and red algae (Broadwater et al. 1995, Klepacki et al. 1995) cytokinesis is accomplished by the furrowing of the plasma membrane, which starts either prior to or during mitosis.

Characteristic microtubule (MT) systems such as phycoplast and phragmoplast in green algae and land plants are not recognizable in brown algae. In ultrastructural observations of brown-algal cells, MT arrays become gradually obscure during telophase. From immunofluorescence microscopy, it is clear that MTs from the centrosomes elongate toward the mid-plane between two daughter nuclei. The distribution of MTs was observed as a "cage", instead of a phycoplast or a phragmoplast (Kropf et al. 1990; Allen and Kropf 1992; Katsaros and Galatis 1992; Bisgrove et al. 1997; Nagasato et al. 1998, 2000). MT-depolymerizing and -stabilizing agents, such as nocodazole and taxol, inhibit cytokinesis (Kropf et al. 1990, Allen and Kropf 1992, Karyophyllis et al. 1997, Dimitriadis et al. 2001).

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Also, in land plants, the phragmoplast which contains not only MTs but also microfilaments (MFs) (Zhang et al. 1993, Staehelin and Hepler 1996) appears between two separating daughter chromosome masses during anaphase. In brown algae, it is suggested that MFs are involved in cytokinesis on the basis of either direct staining of MFs (Karyophyllis et al. 2000) or experiments using cytochalasin B and D (Brawley and Robinson 1985, Kropf et al. 1990). However, how the MTs and MFs participate in cytokinesis has remained unclear, because detailed ultrastructural observations using an adequate preparation were few.

In the vicinity of a new cell partition, it has been reported that mitochondria (Rawlence 1973, Markey and Wilce 1975, Brawley et al. 1977), endoplasmic reticulum (ER) (Markey and Wilce 1975, La Claire 1981), chloroplasts (Brawley et al. 1977), physodes (Schoenwaelder and Clayton 1998, 2000), and Golgi bodies (Brawley et al. 1977, Katsaros et al. 1983) are present. Cells of *Sphacelaria tribuloides* and *Fucus vesiculosus* have many Golgi bodies in the cytoplasm including the perinuclear region and they aggregate at the future division plane (Brawley et al. 1977, Katsaros et al. 1983). The Golgi bodies approach the cell division plane apparently to supply vesicles related to the progression of cytokinesis. However, there has been no direct evidence indicating the involvement of Golgi bodies in cytokinesis.

Conventional chemical fixation and preparation of brown algae for electron microscopy is often inadequate, and many investigators have faced several problems, e.g., preservation of membrane structures including Golgi bodies, ER, etc. and the cytoskeletal elements like MTs and MFs. Several serious problems arise by these conventional procedures. First, the cells contain physode vesicles with abundant phenolic compounds, observed as osmiophilic, membrane-enclosed structures in electron microscopy (McCully 1968, Pellegrini 1980, Ragan 1981, Clayton and Beakes 1983). The contents of the vesicles are highly reactive and hard to stabilize. Clayton and Beakes (1983) reported that preservation of phenolics varied depending to the fixation methods. Second, the cell wall of brown algae is composed of cellulose, alginate, and fucans (McCully 1966a, b; Evans and Holligan 1972; Chi et al. 1999), which can be extracted during fixation and dehydration, and as a result, the cell wall expands a little and the plasma membrane detaches from the cell wall. Moreover, mucilage materials such as alginic acid interrupt infiltration of fixatives into the cytosol.

In brown algae, the process of cytokinesis still remains obscure. This probably is caused by the difficulty of fixation for transmission electron microscopy (TEM) mentioned above. Therefore, it has not been possible to link the behavior of MTs and MFs during cytokinesis observed by fluorescence microscopy and Golgi-derived vesicles relating to cell division by TEM. In the present study, *Scytosiphon lomentaria* zygotes were used for the study of cytokinesis in brown-algal cells for the following reasons: only one or two Golgi bodies exist near the centrosome in the cell (Clayton and Beakes 1983); each centrosome remains at the opposite poles of two nuclei after mitosis. Thus, Golgi bodies are located away from the future division plane. These characters are quite useful for analyzing the relationship between MTs and Golgi-derived vesicles in cytokinesis. Furthermore, the small Scytosiphon zygotes and germlings (ca. 5 µm diameter) are easy to prepare for freeze substitution.

Material and methods

Culture

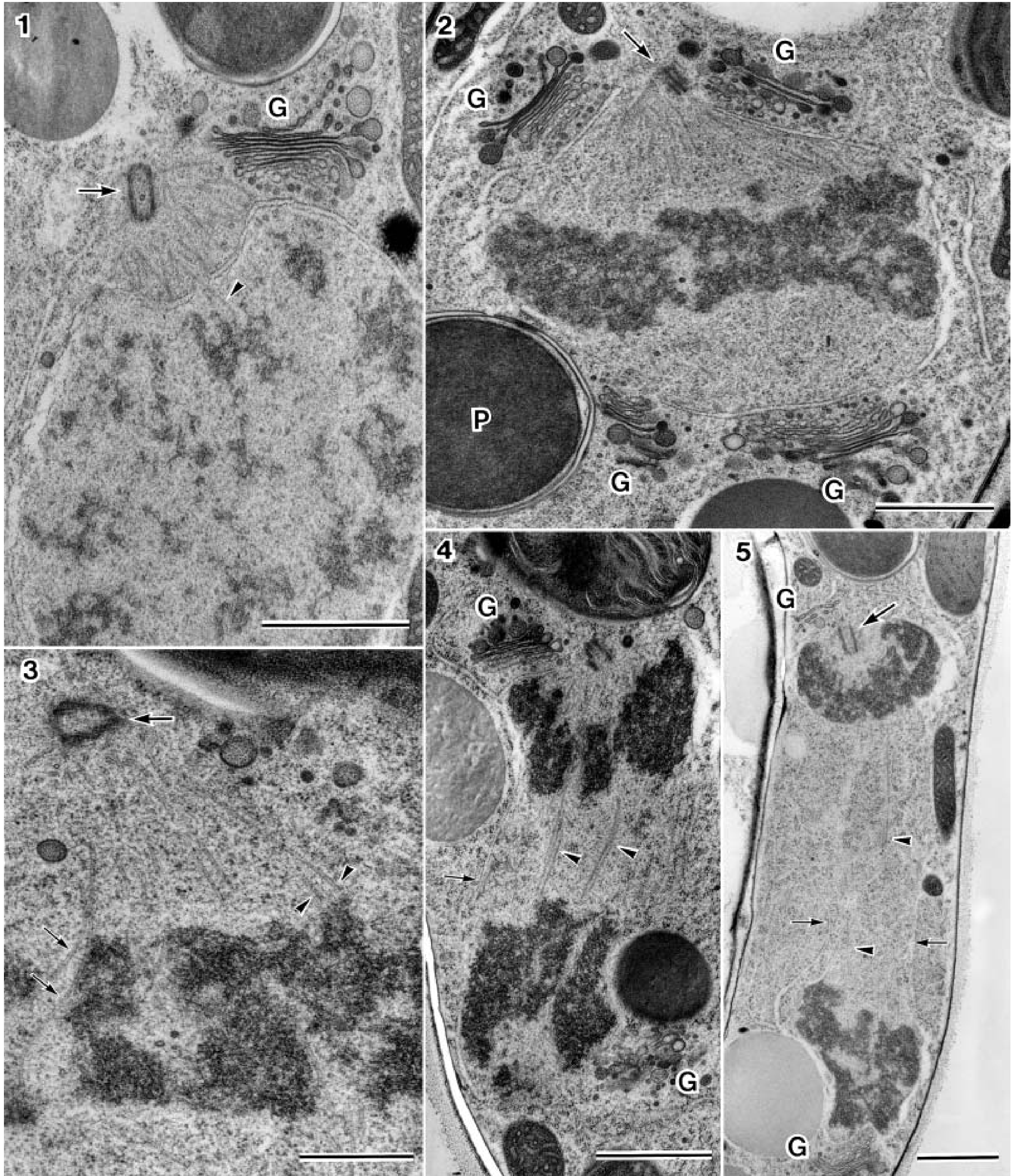
Mature male and female gametophytes of *Scytosiphon lomentaria* (Lyngbye) Link were collected from March to May in 2000 and 2001, at Charatsunai, Muroran, Hokkaido (42 °19′N, 140 °59′E). Liberation of male and female gametes and induction of plasmogamy have been described previously (Nagasato et al. 1999, 2000). Female gametes were first inoculated into a drop of PESI medium (Tatewaki 1966) on gel support films (ATTO Co., Tokyo, Japan), which were previously washed with distilled water, cut into a less than 1 cm side length triangle, and attached on the petri dishes by adhesive tape. After female gametes had settled, the male gametes were added. Then swimming male gametes were washed out with sterilized seawater and the zygotes on the films were incubated with PESI medium under fluorescent lamps (30–40 µmol/m^2 · s photon flux density) at 14 °C long-day condition (14 h light, 10 h dark).

Electron microscopy

Zygotes at 32–34 h after fertilization on the gel support films were rapidly frozen by putting the films with samples into liquid propane previously cooled to −180 °C by liquid nitrogen and immediately transferred into liquid nitrogen. They were then transferred into cooled acetone (−85 °C) containing 2% osmium tetroxide and were stored at −85 °C for about 2 days. After that, samples in this fixative were put at −20 °C for 2 h and 4 °C for 2 h, respectively. Finally the temperature of the fixative was gradually allowed to rise to room temperature. The samples were then washed with acetone at room temperature several times and embedded in Spurr epoxy resin on aluminum foil dishes. When the samples were embedded in the resin, the surface of the gel support films with the samples was placed upside-down on the upper surface of the resin. Serial sections were cut with a diamond knife on Porter-Blum MT-1 ultramicrotome and mounted on formvar-coated slot grids. Sections were stained with uranyl acetate and lead citrate and observed with a Hitachi H-300 electron microscope. In this experiment, we examined nearly complete serial sections of each zygote.

Results

In *S. lomentaria* cells (Clayton and Beakes 1983), one, or occasionally two, Golgi bodies were near each centrosome near the nuclear envelope (Figs. 1, 2, 4, and 5). At 32–34 h after fertilization, zygotes entered first mitosis. Just before mitosis, a pair of centrioles derived from a male gamete duplicated and moved to the future mitotic spindle poles (Motomura 1992; Nagasato et al. 1998, 2000). In prophase, electron-dense



chromosomes were conspicuous and MTs extending from the centrosome entered the nucleoplasm through nuclear-envelope gaps at the poles (Fig. 1). In metaphase, chromosomes became arranged at the equator, and numerous MTs elongated toward chromosomes from near the centrosome at both poles (Fig. 2). From observations of consecutive serial sections, it became clear that several MTs emanating from the mitotic poles terminated on chromosomes. However, a distinctive kinetochore structure could not be found at the MT terminal site on the chromosomes (Figs. 2 and 3). Other MTs from the poles directed through the sides of each chromosome and elongated toward the other pole (Fig. 3). In anaphase, bundles composed of two or three MTs were observed between two daughter chromosomes (Fig. 4). In telophase, MTs were less well developed compared to other stages of mitosis. However, MTs were observed between the two daughter chromosome masses (Fig. 5).

After telophase, the nuclear envelope was reformed around two daughter nuclei (Fig. 6), condensed chromosomes gradually diffused and the nucleolus reappeared. By the completion of mitosis, some large vacuoles moved to occupy the space between the daughter nuclei (Fig. 6). Each pair of centrioles remained at the former poles of the spindle (Fig. 6). Golgi bodies near centrosomes produced a lot of small globular vesicles containing dense material (Figs. 7 and 8). MTs extended from the centrosome (Fig. 7) toward the mid-part of the cell (Fig. 8). The Golgi-derived vesicles associated with these MT bundles apparently migrated toward the future division plane (Fig. 8). Figure 9 shows two kinds of vesicles, i.e., Golgi-derived globular vesicles and tubular cisternae, starting to aggregate midway between daughter nuclei. The electron density of membranes of Golgi-derived globular vesicles and tubular cisternae was high and similar to that of the plasma membrane (Fig. 9).

The globular vesicles and tubular cisternae lined up horizontally at the future division plane. Figures 10–12 show a selection from consecutive serial sections of a zygote. Many vesicles and cisternae accumulated at the periphery of the cell (Figs. 10–12). They apparently fused successively and formed flat membranous sacs with lightly stained contents (Fig. 13). We never observed a furrowing of the plasma membrane.

Figures 14 and 15 show two of consecutive serial sections of a zygote. Flat membranous sacs formed by the fusion of vesicles and cisternae appeared to expand along the future division plane (Fig. 14). These sacs were connected (Fig. 16) and reached the plasma membrane (Figs. 15 and 16). After that, the deposition of cell wall material could be observed (Fig. 17). The number of globular vesicles gradually decreased, while tubular cisternae remained near the new cell wall. These tubular cisternae were frequently found just beneath the plasma membrane during other developmental stages in zygotes and were not restricted to cytokinesis (data not shown). Plasmodesmata were not detected in the nascent cell partition. MFs could not be definitely found through mitosis and cytokinesis, even though the freeze substitution technique was used.

Figure 18 shows a diagrammatical summary of cytokinesis of *S. lomentaria* zygotes.

Discussions

This is the first observation of spindle formation and cytokinesis in brown-algal cells by freeze fixation and substitution, which gave us very good images of mem-

Figs. 1–5. Mitosis of *S. lomentaria* zygotes

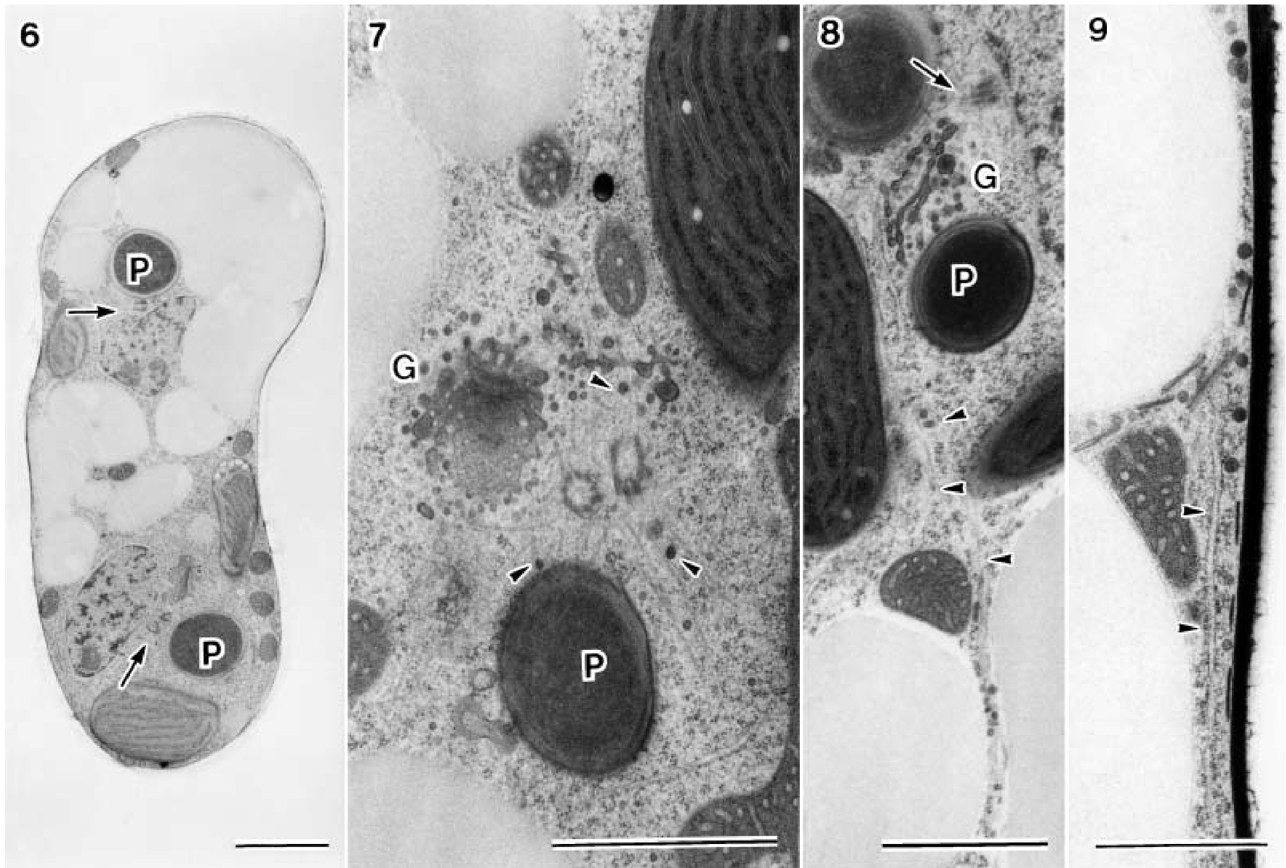
Fig. 1. Polar region of prophase nucleus. Numerous MTs develop from around centriole (arrow) toward nucleus. Nuclear envelope near the centriole becomes invaginated. Some MTs enter the nucleoplasm passing through a gap of the nuclear envelope (arrowhead). Golgi body (*G*) always lies close to centrioles. Bar: 1 µm

Fig. 2. Metaphase. Chromosomes are arranged at the equator of spindle. Nuclear membrane is almost intact except at the polar region (arrow). Golgi bodies (*G*) are near polar regions. A pyrenoid (*P*) exists near a Golgi body. Bar: 1 µm

Fig. 3. Polar region of metaphase nucleus. This is another section of the cell shown in Fig. 2. MTs elongate from around the centriole (arrow) to chromosomes. Distinctive kinetochores on the chromosomes cannot be observed. Some MTs terminate on the chromosome (arrowheads). Other MTs run at the sides of chromosome (small arrows). Bar: 0.5 µm

Fig. 4. Anaphase. Remnant of nuclear envelope (arrow) and bundles of MTs (arrowheads) are observed between two daughter chromosome masses. Bar: 1 µm

Fig. 5. Telophase. Bundles of MTs (arrowheads) disappear gradually. Two daughter chromosome masses are partially connected by ER (small arrows). Arrow shows centriole. Bar: 1 µm



Figs. 6–9. Ultrastructure of *S. lomentaria* zygotes after completion of mitosis

Fig. 6. Each of two pairs of centrioles (arrows) exists at the opposite poles. Several vacuoles occupy the space between daughter nuclei. Each pyrenoid (*P*) is located near centrioles. Bar: 2 µm

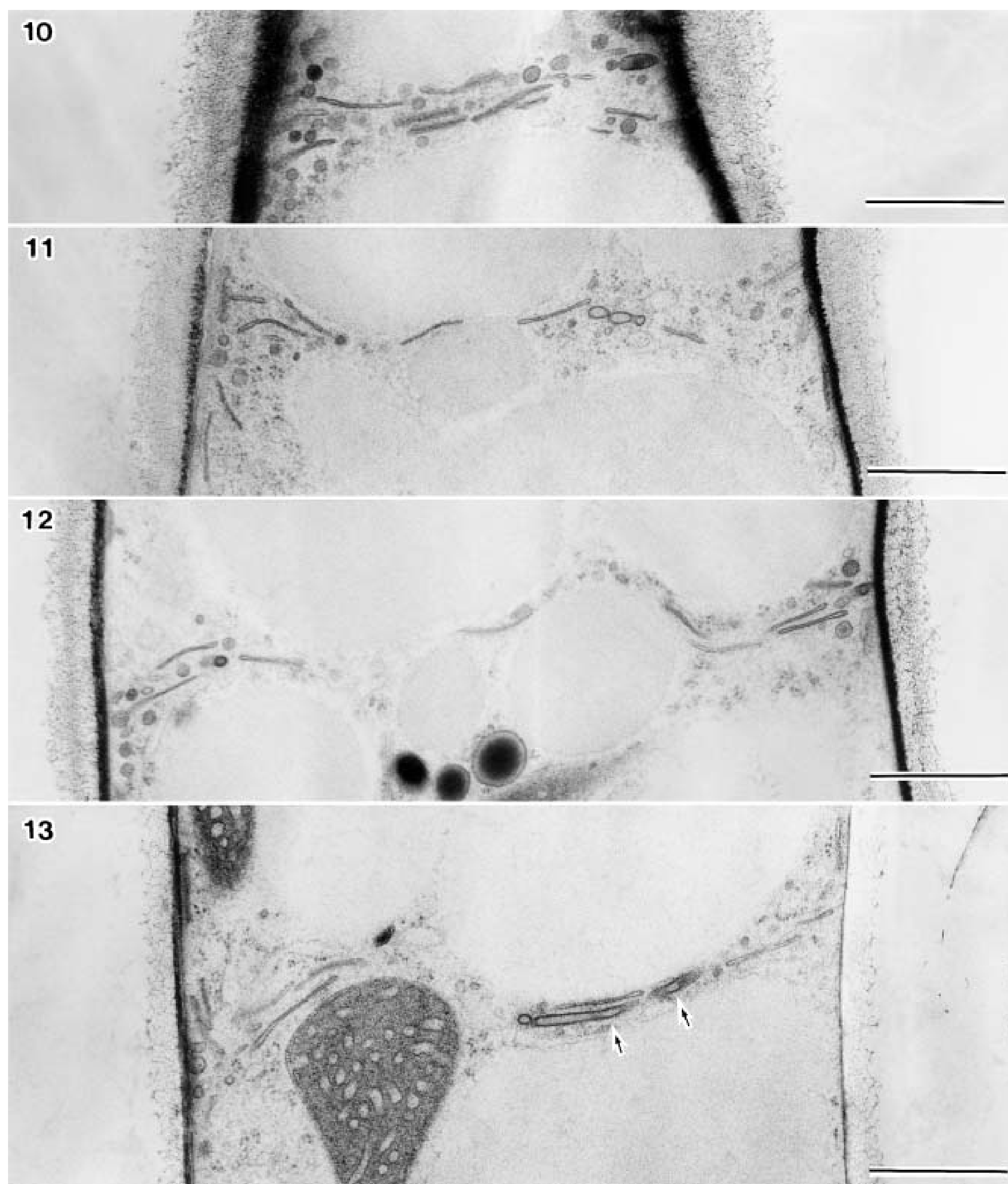
Fig. 7. A complex of centrioles and Golgi body (*G*) after mitosis. Golgi body becomes activated and produces numerous vesicles. Some vesicles seem to be attached on MTs strands (arrowheads). Bar: 1 µm

Fig. 8. MTs (arrowheads) toward the mid-plane of cell from around centriole (arrow). A pyrenoid (*P*) is located near the centriole. Note migration of Golgi-derived vesicles along with MTs. Bar: 1 µm

Fig. 9. Periphery of the future division plane. Note that Golgi-derived globular vesicles and tubular cisternae are gathering there along the inside of the plasma membrane. Rough ER (arrowheads) is observed. Bar: 1 µm

branes and organelles. The plasma membrane closely adhered to the cell wall and contents in various vesicles were well preserved. Phenolic substances in vacuoles (physode granules) appear electron dense after chemical fixation (McCully 1968, Pellegrini 1980, Ragan 1981, Clayton and Beakes 1983), but these were not detected after freeze substitution methods (Schoenwaelder and Clayton 2000). Electron-dense phenolic material was observed in vacuoles of chemically fixed Scytosiphon cells (Clayton and Beakes 1983, Nagasato et al. 2000) but was lost during freeze substitution with acetone.

Many ultrastructural studies have described mitosis and cytokinesis in brown algae by conventional chemical fixation (Rawlence 1973; Markey and Wilce 1975; Brawley et al. 1977; La Claire 1982; Katsaros et al. 1983; Katsaros and Galatis 1988, 1992; Motomura and Sakai 1985; Motomura 1994; Nagasato et al. 2000). Our observations on mitosis by freeze substitution are quite similar to those in previous reports. As in other brown-algal species, a distinctive kinetochore structure could not be observed in zygotes of *S. lomentaria*, although attachment of spindle MTs from the centrosome to the chromosomes was confirmed from observations of serial sections. Fine fibrous kinetochore-like structures have been distinguished only in apical cells of *Sphacelaria tribuloides* (Katsaros et al. 1983) and zygotes of *Fucus distichus* (Motomura 1994).



Figs. 10–13. Aggregation of Golgi-derived globular and tubular cisternae at the future division plane. Figures 10–12 are nonconsecutive serial sections. Bars: 0.5 µm

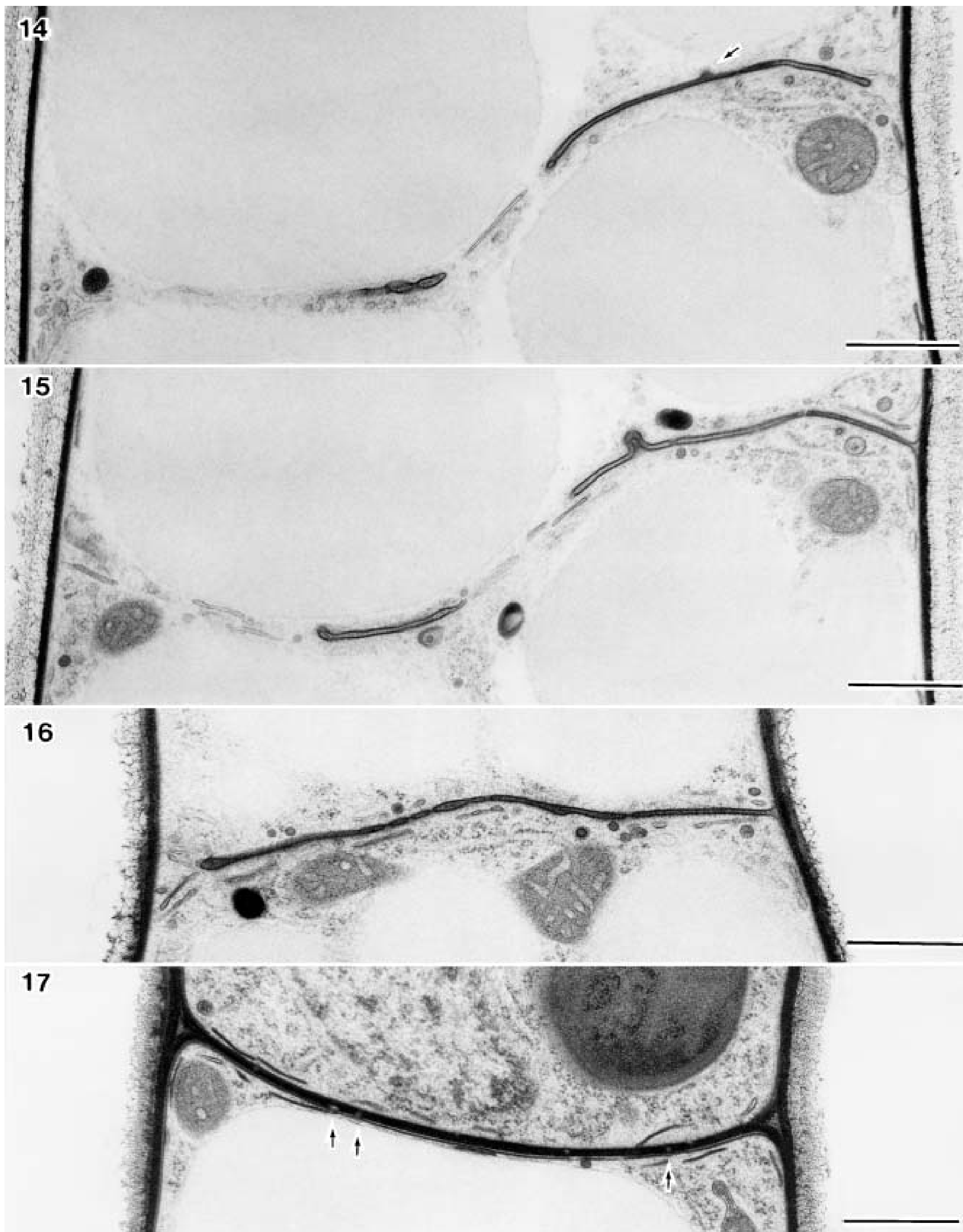
Fig. 10. Periphery of the future division plane. Many globular and tubular cisternae form a horizontal line at the future division plane

Fig. 11. Inner region of Fig. 10

Fig. 12. Section cut at about the middle of the zygote. Vesicles are not conspicuous yet at the center of the future division plane compared to both edges

Fig. 13. Initiation of cell partition. Vesicles and cisternae fused and formed flat membranous sacs (arrows). Furrowing of plasma membrane cannot be observed

This study shows some new findings in cytokinesis of brown algae. Previous ultrastructural observations in brown algae have shown that cytokinesis starts by a furrowing of the plasma membrane centripetally and proceeds by addition of vesicles to the edge of the furrowing (Markey and Wilce 1975, Brawley et al. 1977, La Claire 1981, Katsaros et al. 1983). Only Rawlence (1973) has reported that cytokinesis was accomplished



Figs. 14–17. Outgrowing of the flat membranous sacs and completion of cytokinesis. Bars: 0.5 μm

Fig. 14. The flat membranous sacs appear in several places very close to each other. Globular vesicles are added to the sacs (arrow)

Fig. 15. Inner part region of Fig. 14. A part of the flat membranous sac reaches the plasma membrane

Fig. 16. New cell partition is near completion

Fig. 17. After completion of cell partition, cell wall material is deposited between the plasma membranes. There are translucent parts in some places (arrows), but plasmodesmata cannot be observed at this stage

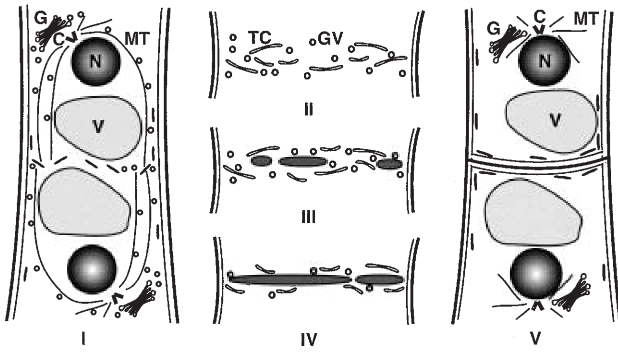


Fig. 18. Diagrammatic summary of the cytokinesis of *S. lomentaria* zygotes. MTs from the centrosome elongate toward the future division plane and Golgi-derived vesicles migrate along MTs after completion of mitosis (I). Tubular cisternae and Golgi-derived vesicles gather at the cell division plane (II), where they fuse (III). As a result of vesicle fusion, flat membranous sacs are formed. The flat sacs grow by addition of vesicles (IV). The flat sacs fuse and reach the plasma membrane. After that, cell wall material is deposited (V). Tubular cisternae continue to exist after completion of cytokinesis. *C* Centriole, *G* Golgi body, *GV* Golgi-derived vesicle, *TC* tubular cisterna, *N* nucleus, *MT* microtubule, *V* vacuole

by centrifugal outgrowth of membranous structures by fusion of vesicles. In Scytosiphon zygotes, it becomes clear that two kinds of vesicles, i.e., Golgi-derived globular vesicles and tubular cisternae, are involved in cytokinesis, since their membranes were well preserved by freeze substitution. TEM studies using conventional chemical fixation have been unable to show the presence of these vesicles. In Scytosiphon zygotes, cytokinesis was initiated by the aggregation of the vesicles at the mid-plane between daughter nuclei. Next, fusion of the vesicles formed thin membranous sacs which grew and reached the plasma membrane. Finally, cytokinesis was completed with deposition of cell wall material. Cleavage furrowing of plasma membrane was never observed in *S. lomentaria* zygotes.

Belanger and Quatrano (2000) reported that in zygotes of *Fucus distichus*, membrane recycling was examined with the vital stain FM4-64 (N-(3-triethylammoniumpropyl)-4-(6-(4-diethylamino)phenyl)hexatrienyl)pyridinium dibromide) taken into the cytoplasm by endocytosis and which stains membrane-bound organelles linked to the secretory pathway. Structures stained by FM4-64 initially appeared at the periphery of the nucleus during interphase and between the daughter nuclei after mitosis; they expanded centrifugally as cytokinesis progressed and eventually contacted the cell wall. Moreover, brefeldin A, which blocks Golgi-mediated secretion, inhibits cytokinesis (Shaw and Quatrano 1996, Belanger and Quatrano 2000). Similarly, Schoenwaelder and Clayton (1998, 2000) reported that physode granules were produced in ER and Golgi bodies in the perinuclear region, transferred to the future cytokinetic plane, and were involved in the cell plate formation in Australian fucoid algae, *Acrocarpia paniculata*, *Hormosira banksii*, and *Phyllospora comosa*. These observations fit in with our ultrastructural results. We found that Golgi-derived vesicles actively participate in cytokinesis. MTs from the centrosomes near the nuclei may function in transportation of Golgi vesicles to the future division plane.

In land plants, vesicle fusion during cell plate formation has become clear by high-pressure freeze substitution (Samuels et al. 1995, Verma and Gu 1996), starting with "the dumbbell-shaped vesicle–tubule–vesicle configuration" of membranes. This configuration appears to arise from the fusion of Golgi-derived globular vesicles with a tube extending from another Golgi vesicle. Phragmoplastin, a GTP-ase, appears to work on the modification of this vesicle configuration (Staehelin and Hepler 1996, Gu and Verma 1997). These Golgi vesicles form a membrane network. Eventually, the pores of the membrane network close and the mature cell plate is complete. Vesicle fusion similar to that in land plants occurred during cytokinesis in brown algae. In brown algae, two types of vesicles, the Golgi-derived globular vesicles and tubular cisternae, coexist at first. The origin of tubular cisternae remained unclear. However, they did not appear to be transformed from Golgi-derived vesicles accumulating at the cell division plane. In zygotes of *S. lomentaria*, tubular cisternae lie beneath the plasma membrane throughout zygote development, not just during cytokinesis. We believe the tubular cisternae are somehow related to the growth of the cell surface, and not only involved in cytokinesis.

Only Brawley et al. (1977) reported on the basis of ultrastructure studies the possible existence of MFs near the partition membrane in cytokinesis of *Fucus vesiculosus* zygotes. In our study, we could not detect MF arrays in cytokinesis of Scytosiphon zygotes even after using freeze substitution technique. This might be a result of Scytosiphon zygotes (ca. 5 μm diameter) being considerably smaller than Fucus zygotes (ca. 60 μm diameter) and the arrays of MFs might not be well developed during cytokinesis. Previous studies have confirmed that MFs participate in cytokinesis because cytochalasin B and D inhibit cytokinesis of *Fucus vesiculosus*, *Pelvetia fastigiata*, and *Sphacelaria*

rigidula (Brawley and Robinson 1985, Kropf et al. 1990, Karyophyllis et al. 2000). In Sphacelaria cells, MFs form a conspicuous noncontractile disklike structure at the future division plane. Therefore, this MF structure in brown-algal cytokinesis is quite different from the actin contractile ring of animal cells. We now think that overlapping of MTs from both centrosomes after telophase determines the position of MFs which assemble into the actin disk there (Katsaros and Galatis 1992, Karyophyllis et al. 2000). Afterwards, MTs from the centrosomes transport Golgi-derived vesicles to the division plane. MFs may also be involved in the disposition of the tubular cisternae in the division plane. Future ultrastructural observations on MF arrays will be necessary to fully understand cytokinesis of brown algae.

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