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Membrane fusion process and assembly of cell wall during cytokinesis in the brown alga, *Silvetia babingtonii* (Fucales, Phaeophyceae)¹

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Running title: Cytokinesis in fucoid zygotes

Abstract

During cytokinesis in brown algal cells, Golgi derived vesicles (GVs) and flat cisternae (FCs) are involved in building the new cell partition membrane. In this study, we followed the membrane fusion process in *Silvetia babingtonii* zygotes using electron microscopy together with rapid freezing and freeze substitution. After mitosis, many FCs were formed around endoplasmic reticulum clusters and these then spread toward the future cytokinetic plane. Actin depolymerisation using latrunculin B prevented the appearance of the FCs. Fusion of GV's to FCs resulted in structures that were thicker and more elongated (EFCs; expanded flat cisternae). Some complicated membranous structures (MN; membranous network) were formed by interconnection of EFCs and following the arrival of additional GV's. The MN grew into membranous sacs (MSs) as gaps between the MNs disappeared. The MSs were observed in patches along the cytokinetic plane. Neighboring MSs were united to form the new cell partition membrane. An immunocytochemical analysis indicated that fucoidan was synthesized in Golgi bodies and transported by vesicles to the future cytokinetic plane, where the vesicles fused with the FCs. Alginate was not detected until the MS phase. Incubation of sections with cellulase-gold showed that the cellulose content of the new cross wall was not comparable to that of the parent cell wall.

Key words

brown algae · cell wall · cytokinesis · flat cisternae · Golgi derived vesicles

Abbreviations

EFC	Expanded flat cisterna
ER	Endoplasmic reticulum
FC	Flat cisterna
GV	Golgi derived vesicle
LB	Latrunculin B
MN	Membranous network
MS	Membranous sac
MT	Microtubule

Introduction

For more than 100 years, zygotes of the furoid genera *Fucus* and *Silvetia* have been used to investigate cellular polarity and asymmetric cell division. Just after fertilization furoid zygotes are still spherical but they produce the rhizoid in response to environmental stimuli such as light, gravity and ion gradient (Kropf 1992; Belanger and Quatrano 2000a) resulting in the establishment of asymmetry. The first cell division always arise perpendicular to the growth axis. Cytokinesis occurs where microtubules (MTs) from each centrosome intermingle (Nagasato and Motomura 2002a; Bisgrove et al. 2003), and the centrosomes rotate so that cytokinesis occurs across the appropriate plane after mitosis. As a result of the asymmetrical division of the zygote, the thallus and rhizoid cells are formed. Each daughter cell has a determined developmental fate, later giving rise to the thallus or the holdfast of the sporophyte organism. However, there has been controversy regarding the mechanism of cytokinesis. The first report based on ultrastructural observations in *Fucus vesiculosus* Linnaeus indicated that cytokinesis was proceeded by furrowing of the plasma membrane (Brawley et al. 1977), as has been observed in many other brown algal cells (Markey and Wilce 1975; La Claire II 1981; Katsaros et al. 1983). However, more recently, it has been reported that the new cell partition membrane starts forming from within the cytoplasm during cytokinesis in furoid algae (Belanger and Quatrano 2000b; Bisgrove and Kropf 2004). Belanger and Quatrano (2000b) showed that the new cell partition membrane formed centrifugally using FM4-64(N-(3-triethylammoniumpropyl)-4-(6-(4-diethylamino)phenyl) hexatrienyl) pyridium dibromide), which stains endomembrane systems. Bisgrove and Kropf (2004) using electron microscopy reported that initiation of the new cell partition membrane occurred in small patches or islands. The islands then fused to each other to attain the parent plasma membrane.

The mechanism of cytokinesis is also unclear in other brown algal species, because conventional chemical fixation and preparation for electron microscopy were often inadequate for the preservation of membrane structure and penetration of the resin. The use of rapid freezing and freeze substitution for ultrastructural has provided new insights into the process of cytokinesis in brown algae (Nagasato and Motomura 2002b, 2009; Katsaros et al. 2009). This approach allowed the observation of flat cisternae (FCs) and revealed the involvement of these structures, together with Golgi derived vesicles (GVs), in the building of the new septum. In zygotes of the isogamous brown alga, *Scytosiphon lomentaria* (Lyngbye) Link, GVs and FCs accumulated around the future cytokinetic plane and formed some membranous sacs (MSs) (Nagasato and Motomura 2002b, 2009). The MSs fused to each other and completed the

partition membrane. Fibrous cell wall material was then deposited within the partition membrane. It has also become clear that cytokinesis in *Dictyota dichotoma* var. *linearis* and *Halopteris congesta* (Reinke) Sauvageau (Katsaros et al. 2009) proceed as in *Scytosiphon lomentaria* zygotes. Although furrowing of plasma membrane was observed in *Sphacelaria rigidula* Kützing, FCs and GVs were supplied to the plasma membrane. Therefore, the involvement of two membrane systems, FCs and GVs, in cytokinesis appears to be a common feature among brown algae.

To date, the individual roles of the two membrane resources in the formation of the new septum have not been clear. In a previous report, we suggested differences on transportation system, origin, components and composition between FCs and GVs in *Scytosiphon* zygotes following an examination of the effects of brefeldin A and latrunculin B (LB) on cytokinesis (Nagasato and Motomura 2009). Following addition of the membrane trafficking inhibitor, brefeldin A, FCs appeared at the future cytokinetic plane, and a new cell partition membrane was formed without a supply of GVs. After completion of cytokinesis, the partition membrane became thicker, and it was filled with amorphous material rather than with fibrous cell wall material. On the other hand, treatment with LB, which depolymerizes actin filaments, prevented the formation of FCs in *Scytosiphon* zygotes. Transportation of GVs to the plasma membrane was not affected but recruitment of GVs to the future cytokinetic plane was insufficient to complete the new cell partition membrane. From these results, we concluded that a supply of FCs is necessary for the completion of the new cell partition membrane and that GVs may play an important role in the deposition of cell wall material.

In land plants, cytokinesis involves the phragmoplast-cell plate system, which consists of MTs, actin filaments and GVs (Hepler 1982; Schopfer and Hepler 1991; Samuels et al. 1995). A drastic transformation of membrane configuration has been observed in these organisms during cytokinesis (Samuels et al. 1995; Otegui and Staehelin 2000a, 2000b). The initial event is the fusion of GVs, and formation of a tubulo-vesicular network (TVN). Then a tubular network (TN) appears and extension of tubule areas gives rise to a fenestrated sheet (FS). The most advanced region of the cell plate is coincident with the center of the cell, because cell plate formation occurs centrifugally. The edges of the expanding cell plate are associated with the phragmoplast MTs, and many GVs transported by phragmoplast MTs are observed (Otegui et al. 2001). Abnormality of Golgi bodies caused by brefeldin A, affects cytokinesis by suppressing the membranous source for cell plate formation (Rutten and Knuiiman 1993; Yasuhara et al. 1995; Yasuhara and Shibaoka 2000). Plant cell walls contain abundant cellulose microfibrils synthesized by cellulose synthases embedded in the plasma membrane (Saxena and Brown 2005) together with non cellulosic cell wall polysaccharides, synthesized in Golgi bodies (Moore and Staehelin

1988), and protein. Observations following labeling with cellobiohydrolase I conjugated colloidal gold showed that cellulose does not deposit on the cell plate until the late TN phase (Samuels et al. 1995). During cell plate formation, callose is the predominant polysaccharide, being detected from TVN to TN. With the maturation of the cell plate, the composition of cell wall switches from callose to cellulose. Also, xyloglucan synthesized in Golgi bodies is detected on the forming cell plate (Moore and Staehelin 1988).

The cell wall of brown algae consists of cellulose, acidic polysaccharides (alginate and sulfated fucans), phenolic compounds and a small amount of protein (Quatrano and Stevens 1976; Kloareg and Quatrano 1988; Schoenwaelder and Clayton 1998a, 1998b, 1999). The alginates, which are a major component of cell wall in brown algae, are linear heteropolysaccharides composed of α -1,4 linked L-guluronic acid and β -1,4 D-mannuronic acid (Haug et al. 1966). Algal sulfated fucans are mainly composed of sulfated α -L-fucose and small proportions of other sugars such as mannose and galactose (Kloareg et al. 1986). Phenolic compounds are enclosed in vesicles known as physodes (Regan 1976), and the size of a physode varies from 0.1-10 μm in diameter (Schoenwaelder and Clayton 1998a, 1998b). It has been suggested that physodes participate in cytokinesis and cell wall assembly in brown algae (Schoenwaelder and Clayton 1998a). However, there is no information as to how other cell wall components are deposited at the new septum during cytokinesis of brown algae.

In furoid algae, cytokinesis has been investigated only by conventional chemical fixation (Brawley et al. 1977; Bisgrove and Kropf 2004). In this study, we reexamined cytokinesis in a furoid alga, *Silvetia babingtonii* zygotes using rapid freezing and freeze substitution. This method allowed the membrane fusion process and the transient membrane conformations within the new cell partition membrane to be observed. Moreover, deposition of cell wall material such as cellulose, alginate and fucoidan during the construction of the new septum was examined using an immunocytochemical approach. This study is the first to use immunoelectron microscopy to identify the site of synthesis and transportation of fucoidans. Rapid freezing and freeze substitution can be used to immunolocalize both alginate and fucoidan, since these polysaccharides are easily extracted by the procedures of conventional chemical fixation.

Materials and methods

Materials

Receptacles of mature thalli of *Silvetia babingtonii* (J. Agardh) E. Serrão, T. O. Cho, S. M. Boo and Brawley, which is a monoecious species, were collected from September to November 2006 - 2009, at Charatsunai, Muroran, Hokkaido (42° 19' N, 140° 59' E). To induce the release of eggs and sperm, thalli were placed in the light (30-40 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) at 18°C overnight. Before release of gametes, thalli were transferred to 4°C in a dark room and left for 2 h. Chilled PES medium (Tatewaki 1966) was poured into dishes containing the thalli to induce release of gametes and fertilization. Zygotes on gel support films (ATTO Co. Tokyo, Japan) were washed with distilled water and then attached to Petri dishes by adhesive tape where they were cultured in PES medium containing 40 $\mu\text{g mL}^{-1}$ chloramphenicol at 18°C, continuous light.

Treatment with latrunculin B

Latrunculin B (10 μM ; Sigma Inc, St. Louis, Mo, USA), which disrupts actin filaments, was added to zygotes from 14 h to 24 h after fertilization. Latrunculin B was stored in ethanol at 50 mM, and further diluted in PES medium before use.

Sample preparation for electron microscopy

Zygotes on the gel support films were rapidly frozen by transferring the films into liquid propane previously cooled to -180°C by liquid nitrogen. They were then immediately transferred into liquid nitrogen. From the liquid nitrogen storage, the samples were transferred into cooled acetone (-85°C) containing 2% osmium tetroxide, and stored at -85°C for about 2 days. After that, samples were kept at -20°C for 2 h and 4°C for 2 h. 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's epoxy resin on dishes of aluminum foil. 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 using a diamond knife on a 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 JEM-1011 electron microscope (JEOL, Tokyo, Japan). Sets of serial sections representing almost complete zygotes were observed.

Immunolabeling of alginate and fucoidan

Localization of alginate and fucoidan was examined using rabbit polyclonal antibody against alginate (Chi et al. 1999) and mouse monoclonal antibody against fucoidan (Mizuno et al. 2009). Sections picked up on formvar coated nickel grids were floated on a 10 μ L drop of 5% H_2O_2 diluted with distilled water for 5 min. After washing with distilled water, grids were transferred to blocking solution (2.5% skimmed milk, 5% normal goat serum and 0.05% NaN_3 in PBS; 137 mM NaCl, 2.7 mM KCl, 4.9 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4). This procedure was applied for 1 h at room temperature. Afterwards, they were incubated with anti-alginate antibody diluted 1:500 in PBS or anti-fucoidan antibody diluted 1:10 in PBS for 2 h at room temperature. They were washed with PBS, and incubated for 60 min at room temperature with a goat anti-rabbit IgG conjugated with 15 nm colloidal gold particles (BBinternational, Cardiff, UK) or a goat anti-mouse IgG+IgM conjugated with 10 nm and 15 nm colloidal gold particles (BBinternational, Cardiff, UK). After washing with distilled water, the grids were stained with uranyl acetate. To confirm specific binding of colloidal gold particles, each antibody was preincubated with 1mg/mL alginate (sodium salt) extracted from *Macrocystis pyrifera* (L.) C. Agardh (Sigma Chemical Co., St Louis, MO, USA) or fucoidan extracted from *Laminaria japonica* Areschoug instead of the normal primary antibody.

Preparation of cellulase-gold probe

Cellulase extracted from *Trichoderma reesei* (Sigma Inc, St. Louis, Mo, USA) was conjugated to colloidal gold as follows (Bonfante-Fasalo et al. 1990; Samuels et al. 1995). After the pH was adjusted to 4.7 using HCl, 5 μ L of 2 mg mL^{-1} cellulase was added to 500 μ L of 10 nm colloidal gold (BBinternational, Cardiff, UK), and the sample was incubated in an ice bath for 5 min with stirring. Then 25 μ L of 1% PEG was added for stability. The conjugation of cellulase-gold was centrifuged at 15,100g (Kokusan RM-190, Tokyo, Japan) for 60 min. The clear supernatant was discarded and the red mobile pellet resuspended in 250 μ L of 0.05 M citrate buffer, pH 4.5, with 0.02% PEG and recentrifuged. The red mobile pellet was recovered and resuspended in 250 μ L of 0.05M citrate buffer, pH 4.9. After edging by H_2O_2 and blocking, the sections were floated on a drop of citrate buffer, pH 4.5, for 10 min. The cellulase-gold conjugate was then added to sections for 20 min at 37°C. The sections were washed with distilled water, and stained with uranyl acetate.

Results

In *Silvetia babingtonii*, the first mitosis was observed in 14 hour-old zygotes and cytokinesis was complete within 6 h after mitosis under 18°C and continuous light conditions. The cytokinetic plane was orientated at right angle to the long axis of the germinated cell. The second mitosis and cytokinesis events occurred from 22 – 24 h after fertilization, in both the thallus and rhizoid cells. We observed both the first and second cytokinesis.

Flat cisternae are closely associated with the endoplasmic reticulum

After mitosis, endoplasmic reticulum (ER) membranes were gathered between the two daughter nuclei, and were surrounded by Golgi bodies (Fig. 1a). At the mid-zone between the daughter nuclei, some FCs could be found parallel to the ER cluster (Fig. 1a, b). As cytokinesis approached, FCs formed a line along the future cytokinetic plane together with GVs (Fig. 1c). FCs were frequently aligned along the periphery of ER (Fig. 1a, b, d, e), and remained close to ER after fixation of the cytokinetic plane (Fig. 1d, e). The size of FCs was about 650 nm in diameter and 20 nm in height on average. On the other hand, the diameter of the GVs was about 60 nm. Tubule-like structures, which were located at the sides of FCs, were also observed (Fig. 1d, e).

Fusion of flat cisternae and Golgi-derived vesicles is the beginning of partition membrane formation

At the future cytokinetic plane, connections were found between GVs and FCs (Fig. 2a-c). Supply of GVs to FCs transformed FCs into expanded FCs (EFCs; Fig. 2d, e). Moreover, fusion of EFCs and addition of GVs resulted in the development of a membranous network (MN; Fig. 2d, e). Further development of the MN gave rise to membranous sacs (MS), which were lined up along the cytokinetic plane (Fig. 2e, f). Clathrin-coated pits and vesicles, which possess electron dense fuzzy covering, were frequently observed during the late MS phase, suggesting that excess membrane material may be recycled (Fig. 2g, h). Each MS became united with the one next to it by expansion and smoothing (Fig. 2i). After completion of a continuous new cell partition membrane, deposition of fibrous cell wall material within it was observed (Fig. 2j), and the formation of amorphous strands. FCs were positioned just beneath the cell partition membrane after completion of cytokinesis, similar to those found under the parent plasma membrane.

Latrunculin B prevents organization of flat cisternae

In order to elucidate the details of the cytokinetic mechanism, we examined the effect of LB, which depolymerizes actin filaments. Zygotes were incubated with 10 μM LB from 14 to 24 h after fertilization. Under these conditions, the rhizoid was slightly elongated, and mitosis was completed. The nucleus on the thallus side was abnormally positioned within the cell cortex (Fig. 3a). Time-lapse photographic observations indicated that nucleus on the thallus side constantly moved either to the cortex or to the middle area of the cell (date not shown), whereas the nucleus in the rhizoid side was static (Fig. 3b). Well-developed ER clusters existed between the two daughter nuclei as in normal zygotes (Figs. 1a, 3c). However, there was not any sign of initiation of the new cell partition membrane. In particular, the organization of FCs around ER clusters was strictly inhibited (Fig. 3c). MTs formed bundles (Fig. 3d) and gathering of numerous electron dense vesicles was observed at the periphery of centrioles on both nuclei (Fig. 3e). The electron dense vesicles were 0.05 – 0.15 μm in diameter.

Fucoidan transported by Golgi-derived vesicles is localized in both parental and septum cell walls

The localization of fucoidan within the parent and the septum cell wall was confirmed using an anti-fucoidan antibody (Fig. 4a). Colloidal gold never bound to sections incubated with the antibody when it had been preincubated with 1mg mL⁻¹ fucoidan (Fig. 4b). Within the cells, colloidal gold (indicating localization of fucoidan) was found on the *trans*-face of the Golgi body, the trans Golgi network (TGN) and GVs (Fig. 4c, d). This result indicates that fucoidan is synthesized within Golgi bodies and transported by GVs. Also, colloidal gold was detected in vesicular bodies containing granular material near Golgi bodies (Fig. 4c). Experiments using primary antibody preincubated with fucoidan clearly showed that this binding was specific (Fig. 4e). Alginate was detected at the parental and the septum cell walls (Fig. 4f), and in osmiophilic vesicles (Fig. 4g). Binding of colloidal gold to vacuoles was not abolished by preincubating the primary antibody with alginate (data not shown).

Modifications of cell wall composition within the cell partition membrane

Changes in cell wall composition during the formation of the new cell partition membrane were examined. Figure 5a shows that the edges of FCs contained fucoidan, caused by attachment of GVs containing fucoidan. On the other hand, there was no alginate within the FCs (Fig. 5b). Fucoidan was continuously detected on all transitional membrane configurations. At the MS phase, fucoidan

was found in both the lumen and shafts (Fig. 5c). It was not until the MS phase that alginate deposition was detected (Fig. 5d). After completion of the new septum, the frequency of colloidal gold indicating fucoidan and alginate increased (Fig. 5e, f).

Cellulase-colloidal gold didn't bind to the MS and the new septum (Fig. 5g, h), although the parental cell wall had a lot of gold particles (Fig. 5i).

Fucoidan is a predominant polysaccharide in the new septum

The distribution of fucoidan and alginate within the parental cell wall and within the new septum was examined by measuring the label density of colloidal gold (Table 1). Particular attention was given to the junction of the new septum and the parental cell wall. Colloidal gold within 0.25 μm square of each location was counted for three spots and the average was calculated. This calculation showed that the fucoidan content in the new cross wall was not significant different from that of the parental cell wall. On the other hand, the alginate content of the new cross wall was lower than that of the parental cell wall.

Discussion

In this study, we clarified the transitional membrane configuration and the process of cell wall assembly during cytokinesis in *Silvetia* zygotes. Figure 6 shows a model of how the new septum develops in brown algae, based on our electron microscope observations. Firstly, the fusion of GVs to FCs occurs and FCs assume a more expanded shape (EFCs; expanded flat cisternae; Fig. 6a). At this time, some GVs transfer fucoidan into FCs. Secondly, fusion of EFCs and further supply of GVs produce complicated membranous structures (MN; membranous network; Fig. 6b). Thirdly, the MN develops into a broad membranous sac (MS) as gaps disappear (Fig. 6c). Alginate starts to be laid down, and clathrin coated pits are frequently detected on the MS surface. MSs unite to form the new cell partition membrane (Fig. 6d). Deposition of cellulose is not detected until this stage. Bisgrove and Kropf (2004) reported that MSs were formed in patches or islands. In this study, it was confirmed that the formation of the new septum involved a membrane fusion process that occurred in some places along the cytokinetic plane. Observations obtained using rapid freezing and freeze substitution showed details of the transitional membrane configuration during cytokinesis in fucoid zygotes.

Does the endoplasmic reticulum contribute to the cell partition membrane formation?

Previous studies have indicated that the maturation of the partition membrane during cytokinesis in *Silvetia* zygotes is similar to equivalent processes in Tobacco BY-2 and root cells (Samuels et al. 1995). However, there were differences in the membrane resource that produced the septum. In land plants, cell plate formation proceeded mainly by fusion of GVs, resulting in loading of the cell wall materials (Samuels et al. 1995), although a recent study showed recruitment of vesicles from endocytosis into the cell plate (Dhonukshe et al. 2006). Also, involvement of ER in the formation of the cell plate has been described. Hepler (1982) reported the distribution of the ER during formation of the cell plate and plasmodesmata in lettuce root cells, fixed by conventional chemical methods and selectively stained by osmium tetroxide-potassium ferricyanide (OsFeCN). He described a close association of a tubular ER network with the nascent cell plate, surrounding the latter structure. Recent electron tomographic analysis of cell plate formation in meristematic cells of *Arabidopsis* prepared by high-pressure freezing showed that few ER membranes associated with the cell plate during the initial stages of the cell plate formation, but that this increased towards cell plate completion (Seguí-Simarro et al. 2004). However, there is no direct evidence that ER contributes to the membranous structures involved in cell plate formation. In land plants, three possible functions have been suggested for the ER in cell plate formation (Hepler and Newcomb 1967; Hepler 1982);

firstly, the ER may trap the vesicles for cell plate formation in certain positions and promote their fusion. Secondly, the ER network might regulate the ionic conditions, especially the concentration of Ca^{2+} , allowing vesicle fusion to occur. Thirdly, plasmodesmata appear to be derived from ER around cell plate. In brown algae, both FCs and GVs are necessary for completion of cytokinesis (Nagasato and Motomura 2002b, 2009; Katsaros et al. 2009). FCs are unique structures found at the cytokinetic plane just beneath the plasma membrane in brown algae. In the green alga, *Micrasterias denticulata*, flat vesicles (similar structures to flat cisternae) were observed, originating from Golgi cisternae. These flat vesicles are involved in synthesis and orientation of secondary wall microfibrils (Kiermayer 1981). At present, it remains obscure whether the function of FCs in brown algae is similar or different to that of flat vesicles in *Micrasterias*. The fact that FCs occur in the vicinity of ER clusters lying between the two daughter nuclei, and ER membranes appear juxtaposed to FC along the cytokinetic plane, may suggest an ER origin of FCs. However, more work is necessary to clarify the origin and characterization of FCs in order to understand cytokinesis in brown algae.

Actin is contributes in transportation of cell wall material and organization of flat cisternae

In brown algae, the role of actin in cytokinesis has been examined in pharmacological experiments using actin depolymerizing and stabilizing reagents, such as LB, cytochalasin B or D and jasplakinokide (Allen and Kropf 1992; Bisgrove and Kropf 1998; Karyophyllis et al. 2000; Hable et al. 2003). These studies have shown that actin plays a critical role in the initiation of cytokinesis. However, it is unclear what happens in the cells treated with actin inhibitors at the ultrastructural level. Our previous study (Nagasato and Motomura 2009) and this study revealed that LB inhibited the organization of FCs and preventing the progression of cytokinesis. Moreover, an accumulation of numerous electron dense vesicles in the perinuclear region was commonly observed in *Scytosiphon* and *Silvetia* zygotes (Nagasato and Motomura 2009). There are two candidates identities for the electron dense vesicles, i.e. they are either osmiophilic vesicles with a positive reaction against the anti-alginate antibody or physodes. In other furoid algae, a remarkable alignment of phenolic vesicles (physodes) was reported at the beginning of cytokinesis (Phillips et al. 1994; Schoenwaelder and Clayton 1998a; Schoenwaelder and Wiencke 2000). Since physodes remained in the perinuclear region following LB treatment, it was suggested that actin filaments are involved in the movement of physodes to the appropriate location (Schoenwaelder and Clayton 1998b). Moreover, it was suggested that MTs are also involved in the transportation of physodes (Schoenwaelder and Clayton 1998b). In the present study the appearance of physodes after freeze fixation was not similar to that in

chemically fixed material. The electron dense vesicles, which were observed in *Scytosiphon* and *Silvetia* zygotes treated with LB, may correspond to the physodes found following chemical fixation. In *Silvetia* zygotes, MTs became bundles and never intermingled in the middle region of the cell. Aggregation of electron dense vesicles in the perinuclear region was promoted by an abnormal arrangement of MTs, which was indirectly affected by depolymerization of actin filaments.

The appearance of an actin plate at the future cytokinetic plane is a distinctive feature in brown algae (Brawley and Robinson 1985; Bouget et al. 1996; Karyophyllis et al. 2000; Bisgrove et al. 2003; Hable et al. 2003; Bisgrove and Kropf 2004; Nagasato and Motomura 2009). In *Silvetia* and furoid zygotes, the actin plate expanded centrifugally, and this expansion correlates with the outgrowth of the new cell partition membrane (Belanger and Quatrano 2000; Bisgrove and Kropf 2004). LB affected the formation of flat cisternae, which is the initial stage of membrane fusion process during cytokinesis (this study; Nagasato and Motomura 2009). However, the actin plate may also be involved in the later stages of cytokinesis such as arrangement of FCs around the cytokinetic plane, fusion of FCs and GVs and recycling of membranous material from the nascent new cell partition membrane, a process similar to the endocytotic pathway in yeast and mammalian cells (Engqvist-Goldstein and Drubin 2003). Further investigation is required to elucidate the function of actin filaments in membrane configuration and membrane trafficking in brown algae.

Sites of unstable membrane configuration are rich in fucoidan

Localization of polysaccharides within the cell wall of furoid zygotes, revealed that the first detectable cell wall component after fertilization is alginate, and that the proportion of cellulose in the cell wall becomes equal to that of alginate within 1 h after fertilization (Quatrano and Crayton 1973; Novotny and Forman 1974; Quatrano and Stevens 1976; Callow et al. 1978; Brawley and Quatrano 1979). The fucoidan contents increase from initiation of rhizoid formation and the rhizoid cell has fucoidan-rich cell wall (Novotny and Forman 1974; Quatrano and Stevens 1976; Callow et al. 1978). Localization of fucoidan has been examined by analysis using toluidine blue O staining or autoradiographic techniques, which indicated that sulfated polysaccharides may be synthesized in the Golgi bodies and transported to the plasma membrane via vesicles (Evans et al. 1973; Callow et al. 1978; Brawley and Quatrano 1979). In this study, fucoidan was detected in the *trans*-face Golgi body, the trans Golgi network (TGN) and in GVs by immunoelectron microscopy using an anti-fucoidan antibody. On the other hand, alginate was not detected in GVs and Golgi bodies. Instead, osmiophilic vesicles were found to react positively with the anti-alginate antibody. In the case of cross wall formation,

secretion of fucoidan into the new cytokinetic plane occurred rapidly compared with the timing of alginate deposition. As a result, the content of fucoidan in the cross wall and in the parental cell wall were almost equal by cytokinesis (Table. 1). Manipulation of MASSUE/AtGSL8, which encodes a putative callose synthase, indicated that timely deposition of callose is essential for cell plate formation in *Arabidopsis* (Thiele et al. 2009). Callose is thought to protect the fragile nascent cell plate structure. From the above it could be suggested that fucoidan in cytokinetic cells of brown algae may play a role similar to that of callose in flowering plant cells in supporting the unstable membrane configuration at the nascent cell plate.

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Legends of figures

Fig. 1 Arrangement of ER and FCs around the future cytokinetic plane during the second division of zygotes.

These images were examined in the rhizoid cells (a-c) and the thallus cell (d, e) of 2-celled zygotes.

- a) ER lies between the two daughter nuclei. Golgi bodies surround ER cluster. FCs are developing around it. The upper nucleus is found in other sections.
- b) Enlargement of FCs in A in another section. FCs are in a layer.
- c) FCs move towards the future cytokinetic plane and form a line along together with GVs (arrows).
- d, e) Consecutive serial sections. Note electron transparent structures are ER (arrowheads), and FCs (arrows) arranged close to ER. Tubule-like structures (TB) side with FCs.

Ch: chloroplast, ER: endoplasmic reticulum, FC: flat cisterna, G: Golgi body, N: nucleus. Bar 1 μm (a), 200 nm (b), 2 μm (c) or 500 nm (d, e).

Fig. 2 Formation of the new cell partition membrane during the second division of zygotes. These images were examined in cytokinetic plane of the thallus (a-c) and the rhizoid cells (d, i), and the mature septum (j) of 2-celled zygotes.

- a-c) Consecutive serial sections. GVs are attached to FC (arrows).
- d) EFCs are formed by fusion of FCs and GVs. The EFCs intertwine each other, becoming a MN.
- e) More advanced stages of MS development from MN.
- f) MSs appear in patches at the future cytokinetic plane.
- g, h) Serial sections of the same cell. Clathrin coated- pits (arrows) are frequently observed on MS.
- i) Expanding MS has irregular surface.
- j) Mature septum of the first division. After completion of new cell partition membrane, fibrous cell wall material deposit within it. FCs exist at the beneath of the new cross wall. Amorphous strands (arrows) are found within it.

EFC: expanded flat cisterna, FC: flat cisterna, MN: membranous network, MS: membranous sac. Bar 200 nm (a-e, g-j) or 1 μm (f).

Fig. 3 Effects of latrunculin B on cytokinesis.

- a, b) Daughter nuclei after the first mitosis in thallus (a) and rhizoid (b) sides. Note that thallus nucleus is located at the cell cortex.
- c) ER cluster is recognized between the two daughter nuclei. Organization of FCs is not observed. The formation of new cell partition membrane never initiates.
- d) MTs become bundles and wavy (arrows).
- e) Numerous electron dense vesicles gather around the periphery of centriole.

C: centriole, ER: endoplasmic reticulum, N: nucleus. Bar 10 μm (a-c) or 1 μm (d, e).

Fig. 4 Immunogold localization of fucoidan and alginate within two celled zygotes.

- a) Localization of fucoidan within parent and the first septum cell wall.
- b) Section incubated with preincubated antibody with fucoidan. Colloidal gold never binds with the cell wall.
- c) Localization of fucoidan to *trans*-face Golgi body, trans Golgi network (TGN) and a vesicular body contained granular material (VB).
- d) Transportation of fucoidan to the rhizoid site by GVs.
- e) Section incubated with preincubated antibody with fucoidan. Colloidal gold never binds to Golgi bodies.
- f) Localization of alginate within the parent and the first septum cell wall.
- g) Localization of alginate in osmiophilic vesicles.

G: Golgi body, M: mitochondrion, Os: osmiophilic vesicle, and V: vacuole. Bar 500 nm (a, b, e-g) or 200 nm (c, d).

Fig. 5 Transformation of the cell wall composition during the formation of new cell partition membrane during the second division of zygotes.

- a) These images were examined in cytokinetic plane of the thallus (a-d, g) and the first septums (e, f, h, i) of 2-celled zygotes. Fusion of GVs containing fucoidan with edges of FCs (arrows).
- b) No detection of alginate within FC.
- c) Localization of fucoidan in the lumen and shafts of MS.
- d) Localization of alginate on the lumen and shafts of MS.

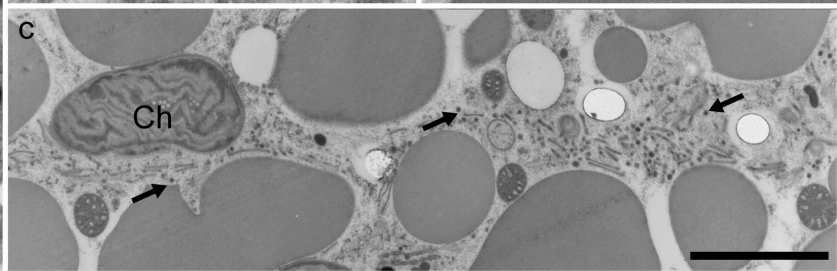
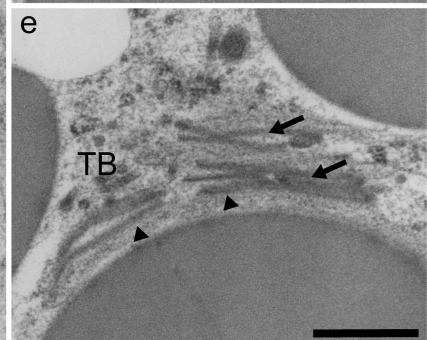
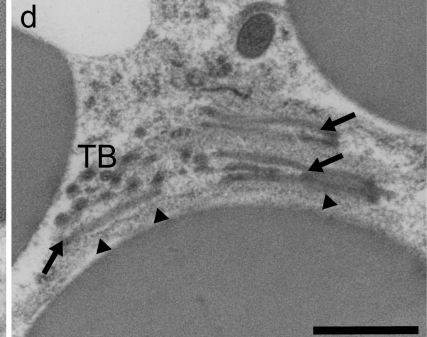
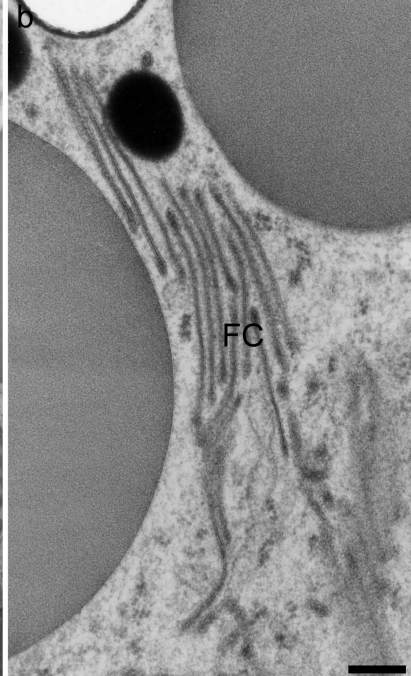
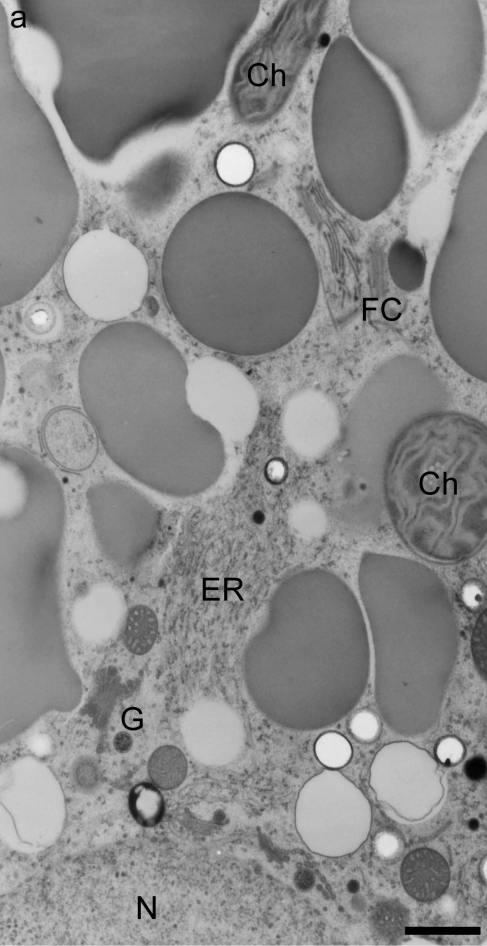
- e) Increase of colloidal gold labeled with anti-fucoidan on the complete septum.
- f) Increase of colloidal gold labeled with anti-alginate on the complete septum.
- g) No detection of cellulase-colloidal gold within MN and MS.
- h) No detection of cellulase-colloidal gold within the complete septum.
- i) Localization of cellulase-colloidal gold within the mother cell wall.

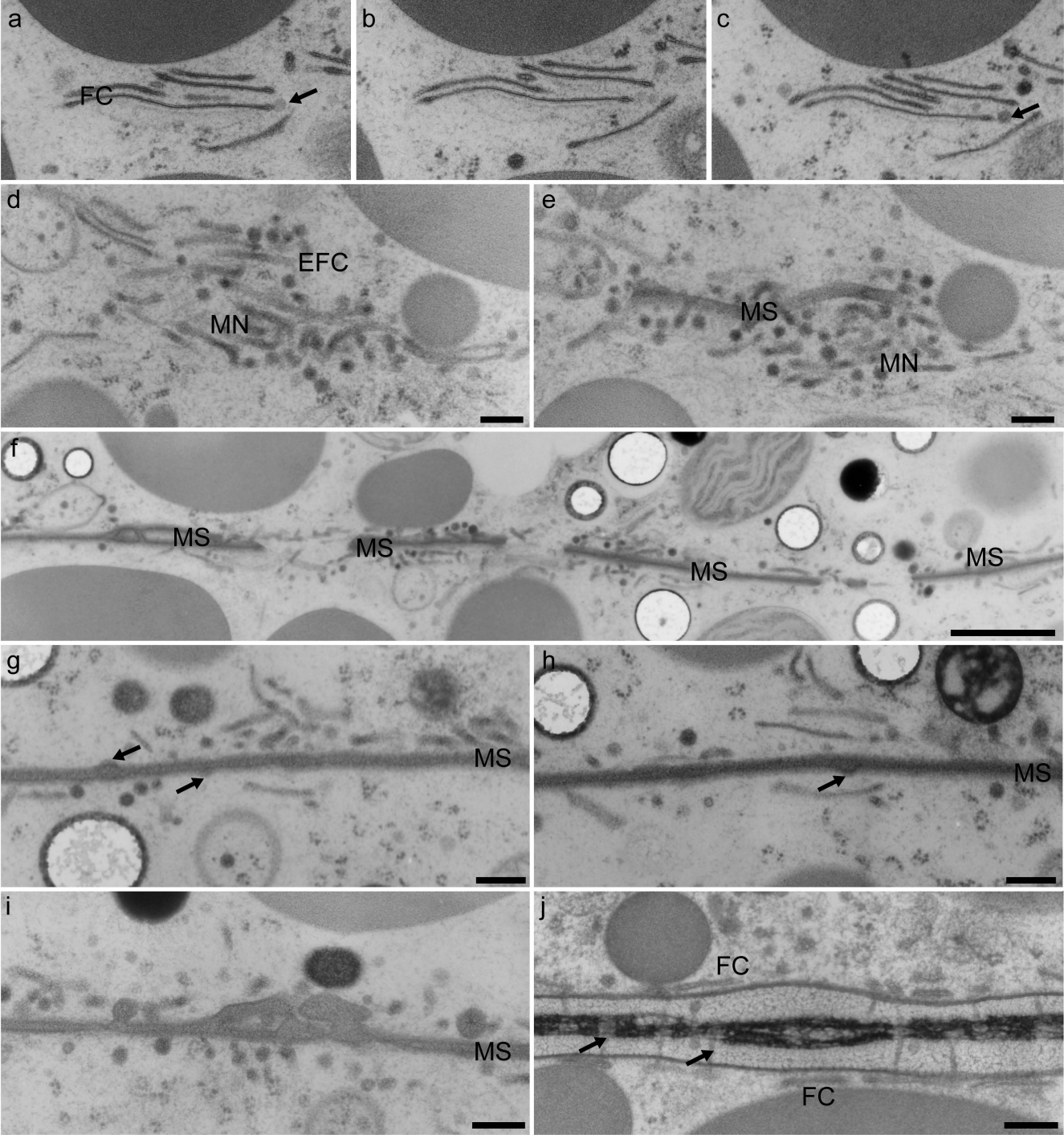
FC : flat cisterna, MN : membranous network, MS : membranous sac. Bar 200 nm (a-d) or 500 nm (e-i).

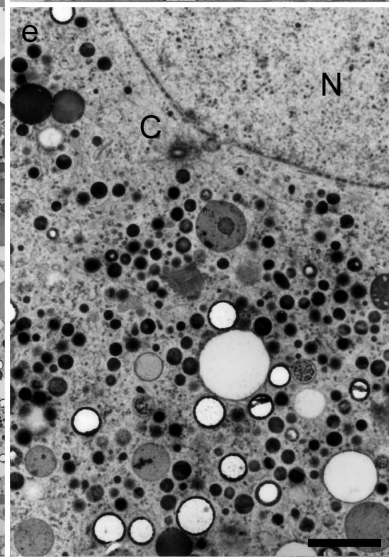
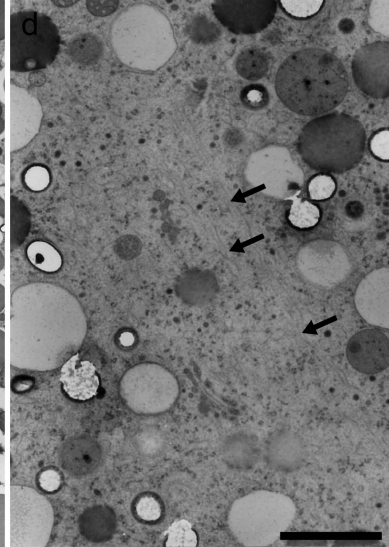
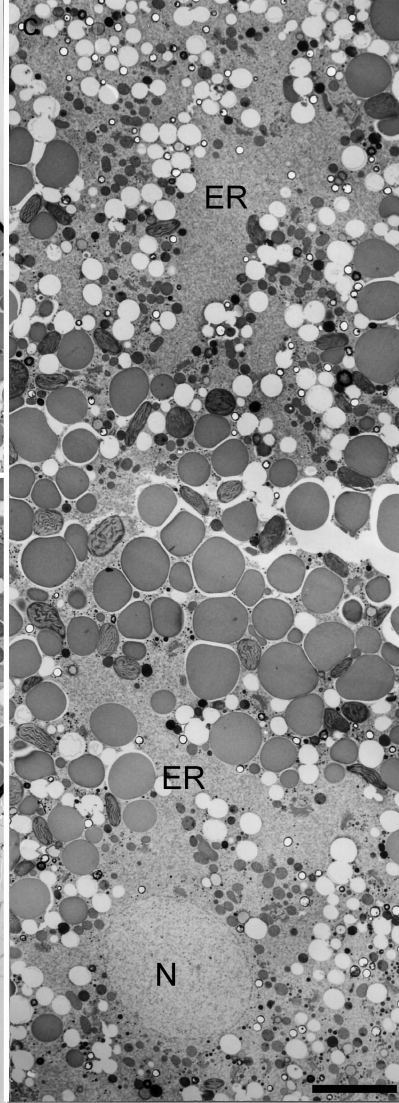
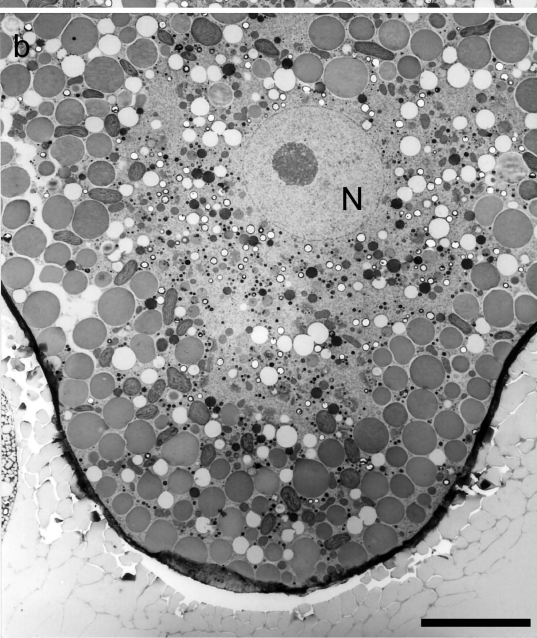
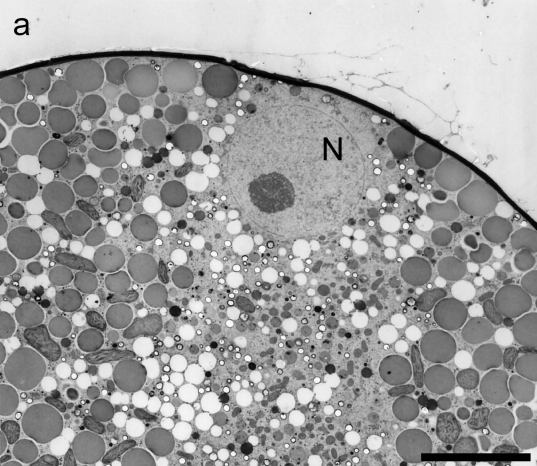
Fig. 6 Schematic diagram of transitional membrane configuration during cytokinesis in brown algae.

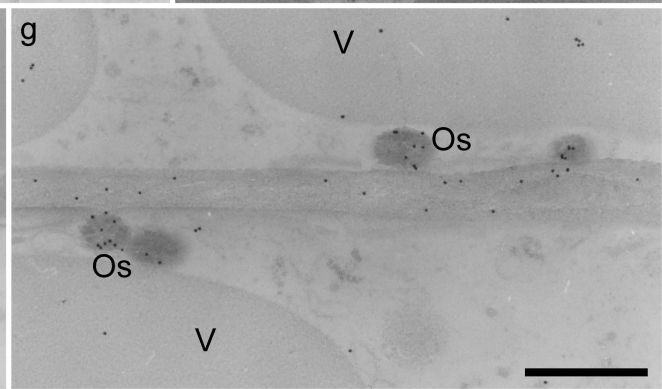
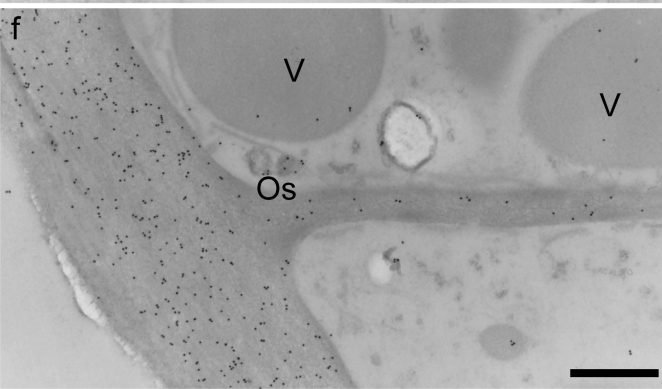
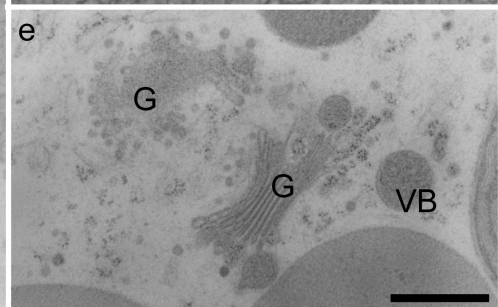
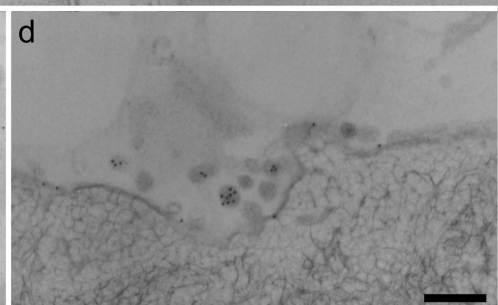
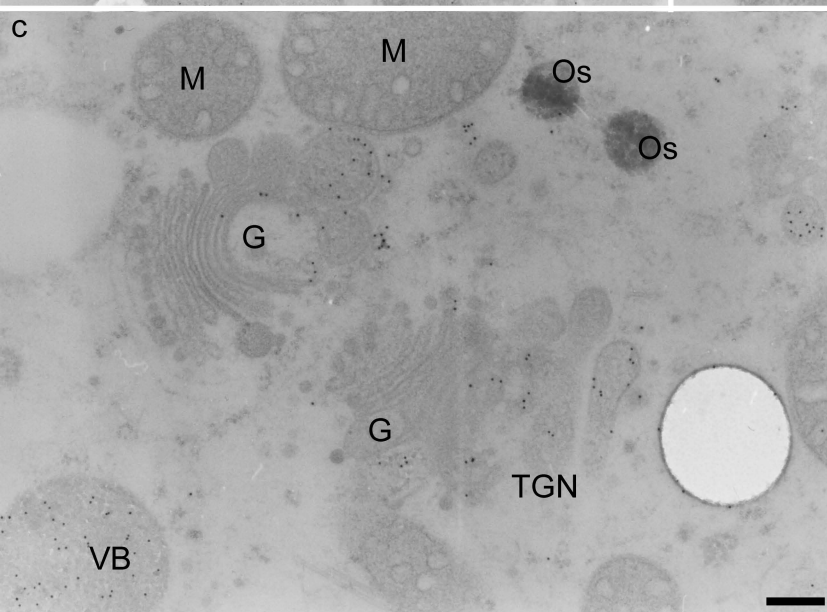
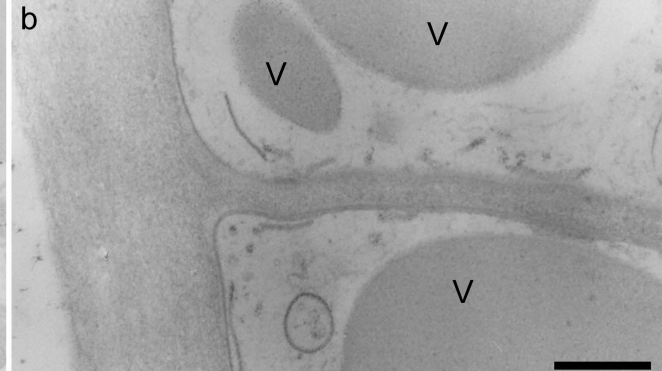
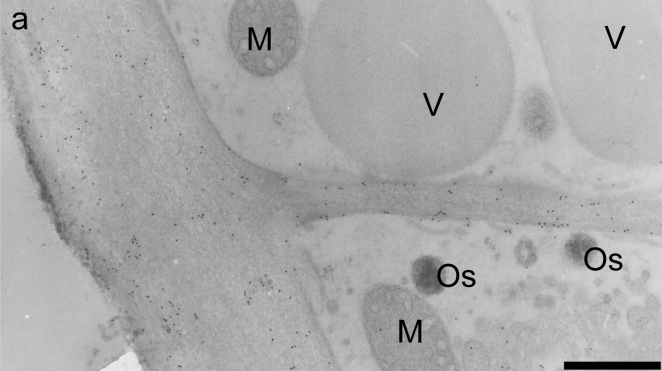
- a) Fusion of GVs to FCs transforms FCs into EFCs. GVs put fucoidan into FCs. Dots show accumulation of fucoidan.
- b) Fusion of EFCs and supply of GVs produces MN.
- c) MN grows into MS with disappearance of gaps in the MN. MSs appear in patches. Clathrin coated pits (CP) are detected on the MS. Alginate indicated by open squares start to be accumulated.
- d) MSs become a continuous new cell partition membrane. Crystalline cell wall material deposits within it.

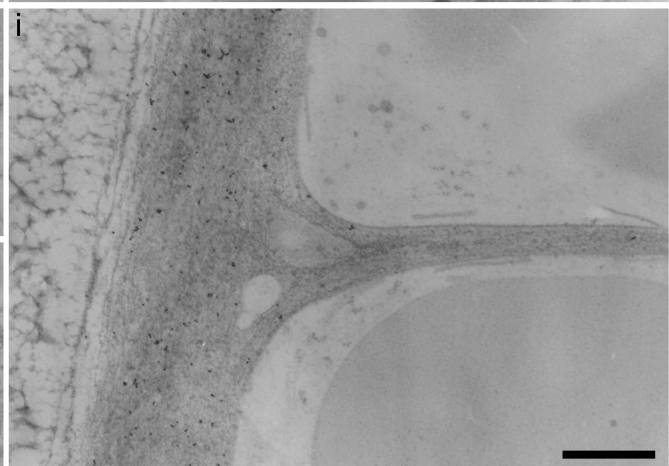
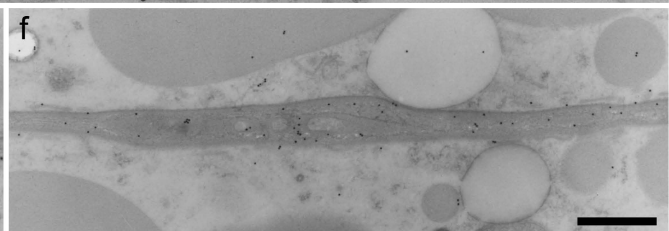
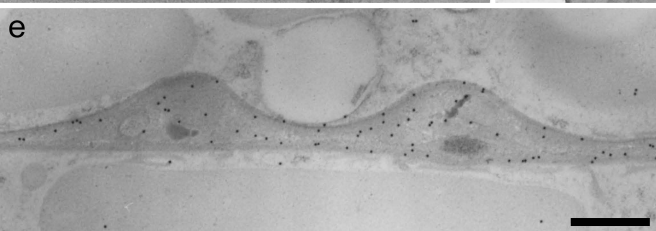
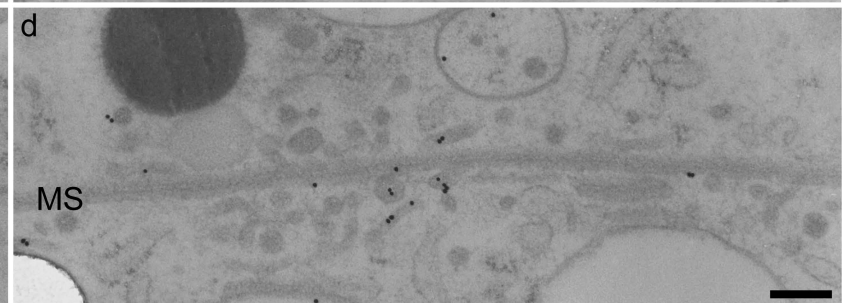
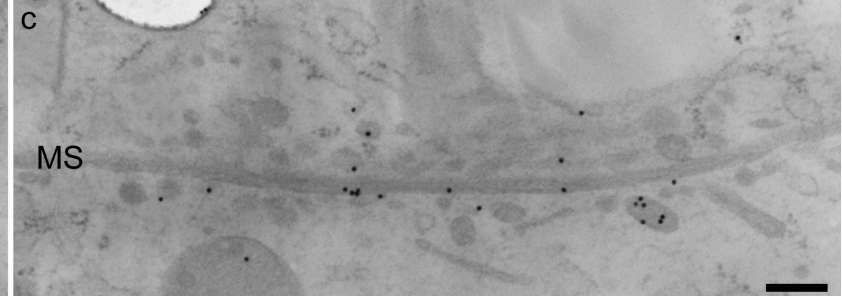
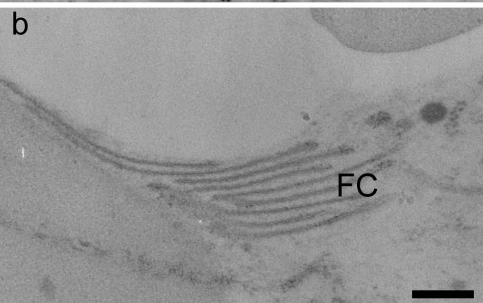
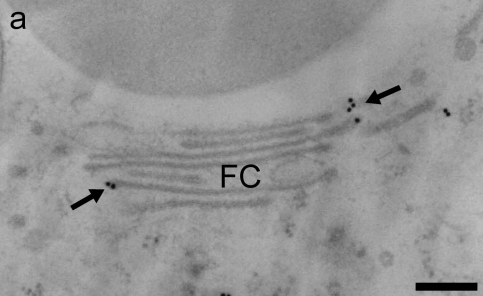
EFC: expanded flat cisterna, FC: flat cisterna, GV: Golgi derived vesicle, MN: membranous network, MS: membranous sac.











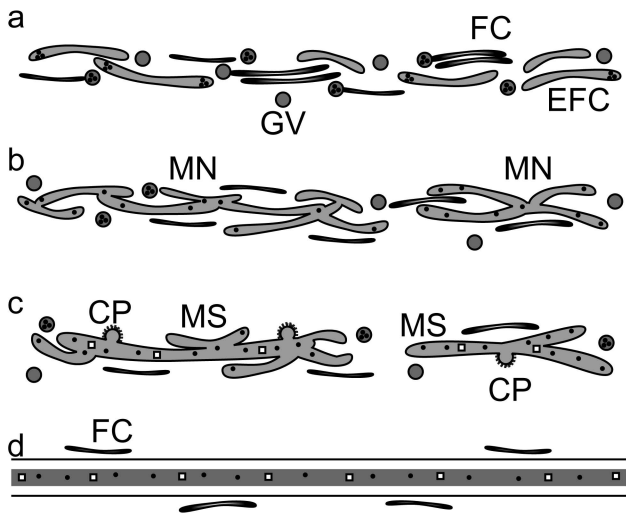


Table 1. Distributions of fucoidan and alginate within the mother cell wall and new septum.

Label density (Gold particles / 0.25 μm^2)		
Location within CW	fucoidan	alginic acid
New septum	26 \pm 3	12.7 \pm 4
Mother CW	21 \pm 8.3	39.3 \pm 5.7

Value show average and standard deviations.