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Immunogold-labeling analysis of alginate distributions in the cell walls of chromophyte algae

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SUMMARY

The immunogold electron microscopy technique was employed to detect the presence of alginates in the cell walls of selected chromophyte species. Anti-alginate antiserum labeled the cell walls of *Sphacelaria* and *Scytosiphon* (Phaeophyceae), *Tribonema*, *Vaucheria*, *Botrydium, Botrydiopsis* (Xanthophyceae) and an 'undescribed filamentous species' (*incertae cedis*), but it did not label those of *Giraudyopsis*, *Phaeosaccion* (Chrysomeridales), *Antithamnion* (Rhodophyceae) and *Bryopsis* (Ulvophyceae). This is the first report of the occurrence of alginates in the chromophyte outside Phaeophyceae. The absence of alginates in Chrysomeridales, which has an unclear phylogenetic position, implies a rather distant phylogenetic relationship of the order Chrysomeridales from Phaeophyceae/Xanthophyceae.

Key words: alginate, cell wall, chromophyte, immunogold electron microscopy, Phaeophyceae, Xanthophyceae.

INTRODUCTION

The large majority of benthic photosynthetic organisms, especially multicellular algae and land plants, have cell walls composed of various polysaccharides. Cell walls furnish strength and give shape to the cells, as well as functioning as protective barriers against the attacks of viruses, bacteria and protozoa. They are essential for constructing multicellular upright thalli to compete with other organisms for light to support photosynthesis. Therefore, multicellular algal groups such as Rhodophyceae, Phaeophyceae and most of the Chlorophyta have well developed cell walls. However, these eukaryotic photosynthetic organisms are believed to have originated independently by independent endosymbiotic events between unicellular heterotrophic protozoa and photosynthetic prokaryotes (primary endosymbiosis), with further endosymbiosis between other protozoa and eukaryotic photosynthetic organisms (secondary endosymbiosis) (Kowallik 1993). The development of cell walls is considered to have occurred after the establishment of each algal lineage, hence, cell walls are indubitably polyphyletic.

Chromophyta constitute a monophyletic algal lineage evolved by the secondary endosymbiosis between a heterokontic protozoan (stramenopile) and a chlorophyll *c*-containing eukaryotic alga (Kowallik 1993; Andersen *et al*. 1998; Bailey *et al*. 1998). They include Chrysophyceae, Eustigmatophyceae, Raphidophyceae, Pelagophyceae, Phaeophyceae, Phaeothamniophyceae, Synurophyceae and Xanthophyceae. Among them, Chrysophyceae, Phaeophyceae, Phaeothamniophyceae and Xanthophyceae include multicellular forms, and have cell walls. Cell walls of Phaeophyceae contain alginates and cellulose as major components. Alginates are linear heteropolysaccharides composed of α-1,4 linked L-guluronic acid and β-1,4 D-mannuronic acid residues in varying proportions, and they are widely distributed among brown algae (Painter 1983). Brown algae have alginates in the form of the mixed calcium-sodium-magnesium salt of alginic acid in their cell walls and intercellular matrix, which may provide structural reinforcement and ion-exchange properties (Frei and Preston 1962). The presence of alginates in Phaeophyceae has been believed to be unique in the chromophytes, and has been used as one of the distinctive characters to separate Phaeophyceae from related groups such as Chrysophyceae and Xanthophyceae, together with some other morphological features (e.g. the presence of plasmodesmata connecting adjacent cells [Clayton 1989]).

Unlike most other phylogenetic lineages containing multicellular benthic thalli (e.g. Ulvophyceae, Rhodophyceae), no unicellular forms have been found in the Phaeophyceae. Therefore, the origin of the Phaeophyceae and its phylogenetic relationship with other classes have been controversial. Based on fine-structural

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features, especially those of flagellar root systems, Chrysomeridales including *Giraudyopsis stellifera* Dangeard were suggested to be a direct ancestor of the Phaeophyceae (O'Kelly and Floyd 1985; O'Kelly 1989). However, the absence of alginates in *Phaeosaccion collinsii* Farlow (Craigie *et al*. 1971) does not support this notion, and furthermore, recent molecular studies have indicated a closer relationship to the Phaeophyceae and Xanthophyceae (Ariztia *et al*. 1991; Andersen *et al*. 1993, 1998).

Chemical analysis of alginates requires a relatively large quantity of material, and the localization in the cell cannot be investigated by such methods. However, the immuno-labeling method using gold probes conjugated with anti-alginate antisera is highly sensitive in detecting the presence of alginates in cell walls, and can provide information about their localization within cell walls. Therefore, this study aimed to examine the occurrence of alginates in multicellular Chromophyta and also to provide information about the localization of alginates in cell walls using the immunogold labeling method.

MATERIALS AND METHODS

Unialgal cultures of 11 species were used in the present study (Table 1). *Sphacelaria rigidura*, *Scytosiphon lomentaria*, *Phaeosaccion collinsii*, *Giraudyopsis stellifera* and the 'undescribed filamentous species' were cultured in PESI medium (Tatewaki 1966), and those of *Antithamnion nipponicum* and *Bryopsis plumosa* were cultured in PES medium (Provasoli 1966). The freshwater algae *Vaucheria geminata*, *Tribonema* sp., *Botrydium stoloniferum* and *Botrydiopsis arhiza* were cultured in 3N+ medium (Starr and Zeikus 1993). These cultures were maintained under illumination of approximately 1 W m^{-2} provided by cool-white fluorescent lamps with a 14:10 h light:dark photoperiod at 18°C, except for *Phaeosaccion collinsii* which was kept at 5°C (Kawai 1989). The 'undescribed filamentous species' is an undescribed taxon of marine multicellular filamentous alga, which we are preparing to publish as a new species. The cells have lobed chloroplasts containing chlorophyll *a* and *c*, but they do not have H-shaped cell wall components characteristic of some members of multicellular xanthophytes, nor plasmodesmata characteristic of the phaeophytes. The species could be assigned to Chrysophyceae or the recently described Phaeothamniophyceae (Andersen *et al*. 1998; Bailey *et al*. 1998).

Fixation procedures in the electron microscopy for *Sph. rigidula*, *Scy. lomentaria*, *G. stellifera*, *P. collinsii* and the 'undescribed filamentous species' were essentially the same as those as described by Tamura *et al*. (1996). The thalli were fixed with 3% glutaraldehyde in 0.1 mol L^{-1} sodium cacodylate buffer (pH 7.2) containing 2% NaCl, 0.1% $CaCl_2$ and 0.5% caffeine anhydride at 4°C for 2 h. The fixed thalli were rinsed with the buffer and postfixed in buffer containing 2% osmium tetroxide, 2% NaCl and 0.1% $CaCl_2$ at 4°C for 2 h. The thalli of *B. plumosa* and *A. nipponicum* were also fixed as mentioned above, but the fixatives contained no $CaCl_2$ or caffeine anhydride. For the freshwater algae, the thalli were fixed with 3% glutaraldehyde in 0.05 mol L^{-1} sodium cacodylate buffer containing 0.1% $CaCl_2$ and postfixed in the buffer containing 1.5% osmium tetroxide. Each of these postfixed specimens was dehydrated with ethanol and embedded in LR white. Thin sections were made on a Reichert Om U2 ultramicrotome (Reichert, Vienna, Austria) and mounted on Formvar-coated grids.

For immunogold electron microscopy, labeling was performed in approximately 100 µL droplets on Parafilm (American National Can Co., Greenwich, CT, USA) in a moist chamber at room temperature. Grids

Table 1. List of algal species examined

Taxa	Origin of cultures
Phaeophyceae (Chromophyta)	
Scytosiphon lomentaria (Lyngbye) Link	H. Kawai culture, Oshoro, Hokkaido, Japan
Sphacelaria rigidula Kützing	T. Motomura culture, Muroan, Hokkaido, Japan
Xanthophyceae (Chromophyta)	
Botrydiopsis arhiza Borzi	UWCC FW 131 (= UTEX 87)
Botrydium stoloniferum Mitra	UWCC FW 132 (= UTEX 156)
Tribonema sp.	UWCC FW 135
Vaucheria geminata (Vauch.) de Candolle	UTEX LB1035
Chrysophyceae (Chromophyta)	
Giraudyopsis stellifera Dangeard	H. Kawai culture
Phaeosaccion collinsii Farlow	H. Kawai culture, Isoya, Hokkaido, Japan
Incertae cedis undescribed filamentous species	E. Henry culture, Naples, Italy
Rhodophyceae (Rhodophyta)	
Antithamnion nipponicum Yamada et Inagaki	M. Kamiya culture, Shimokita, Aomori, Japan
Ulvophyceae (Chlorophyta)	
Bryopsis plumosa (Hudson) C. Agardh	K. Okuda culture, Muroran, Hokkaido, Japan

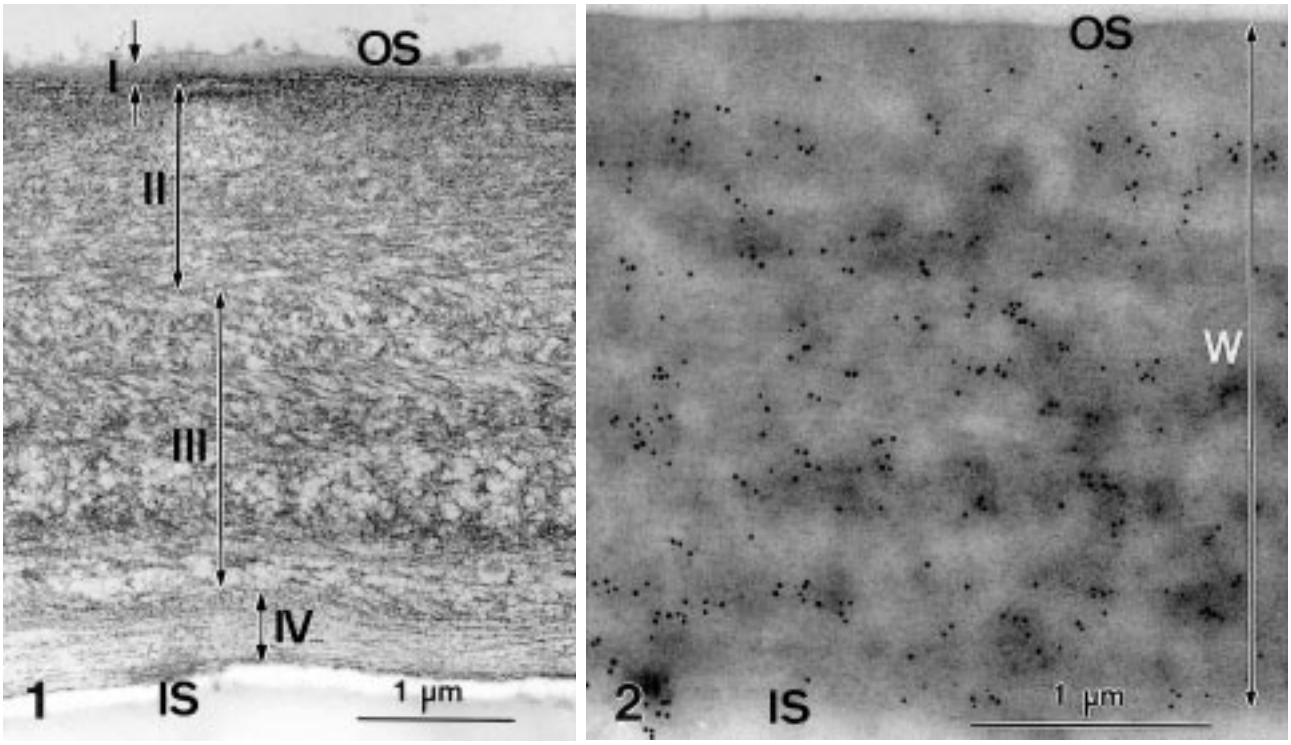
were floated, section-side down, on the following solutions: (i) a block solution of 2% (w/v) gelatin in phosphate-buffered saline (GPBS) for 10 min; (ii) an antialginate serum diluted 1/500 in GPBS for 2 h; (iii) GPBS, three times for 2 min each; (iv) a goat antirabbit IgG conjugated with 10 nm gold particles (Sigma BioSciences, St Louis, MO, USA) and diluted 1/10 in 1% bovine serum albumin in PBS (BPBS) for 1 h; (v) BPBS three times for 2 min each. Controls were incubated with pre-immune rabbit serum instead of the antialginate serum. After the incubation, the grids were washed with distilled water. Some sections were stained with 1% uranyl acetate and Reynold's lead citrate. Unstained and stained sections were observed with a JEOL JEM1010T electron microscope (JOEL Co. Ltd, Tokyo, Japan).

Rabbit polyclonal antibodies against alginates (43085 and 43086) were prepared according to the methods described by Vreeland (1970) at SAWADY Technology Co. Ltd (Tokyo, Japan). Alginic acid (sodium salt) extracted from *Macrocystis pyrifera* (L.) C. Agardh (Sigma Chemical Co., St Louis, MO, USA) was used as an antigen. The antialginate antibody titer was determined with an enzyme linked immunosorbent assay (ELISA) with antigen on the solid phase (1 µg per well). The pre-immune serum was tested at the same time as the production bleed to assess the effectiveness of the purification. Titer values were expressed as the reciprocal of the serum dilution that results in an OD492 of 0.2 (detection with goat-antirabbit IgG HRP conjugate and peroxidase dye). Titers in pre-immune serum, antibody 43085 and 43086 were less than 50, 235400 and 3500, respectively. In the present study, antibody 43085 was used for immunogold electron microscopy because of its stronger activity towards the antigen.

RESULTS

Cross sections of cells in each algal thallus were applied for immunogold labeling. Some sections were stained to observe fine structures of cell walls with or without previous immunogold labeling. On all control sections, no labeling was found when using the pre-immune serum instead of the antialginate antibody (not shown).

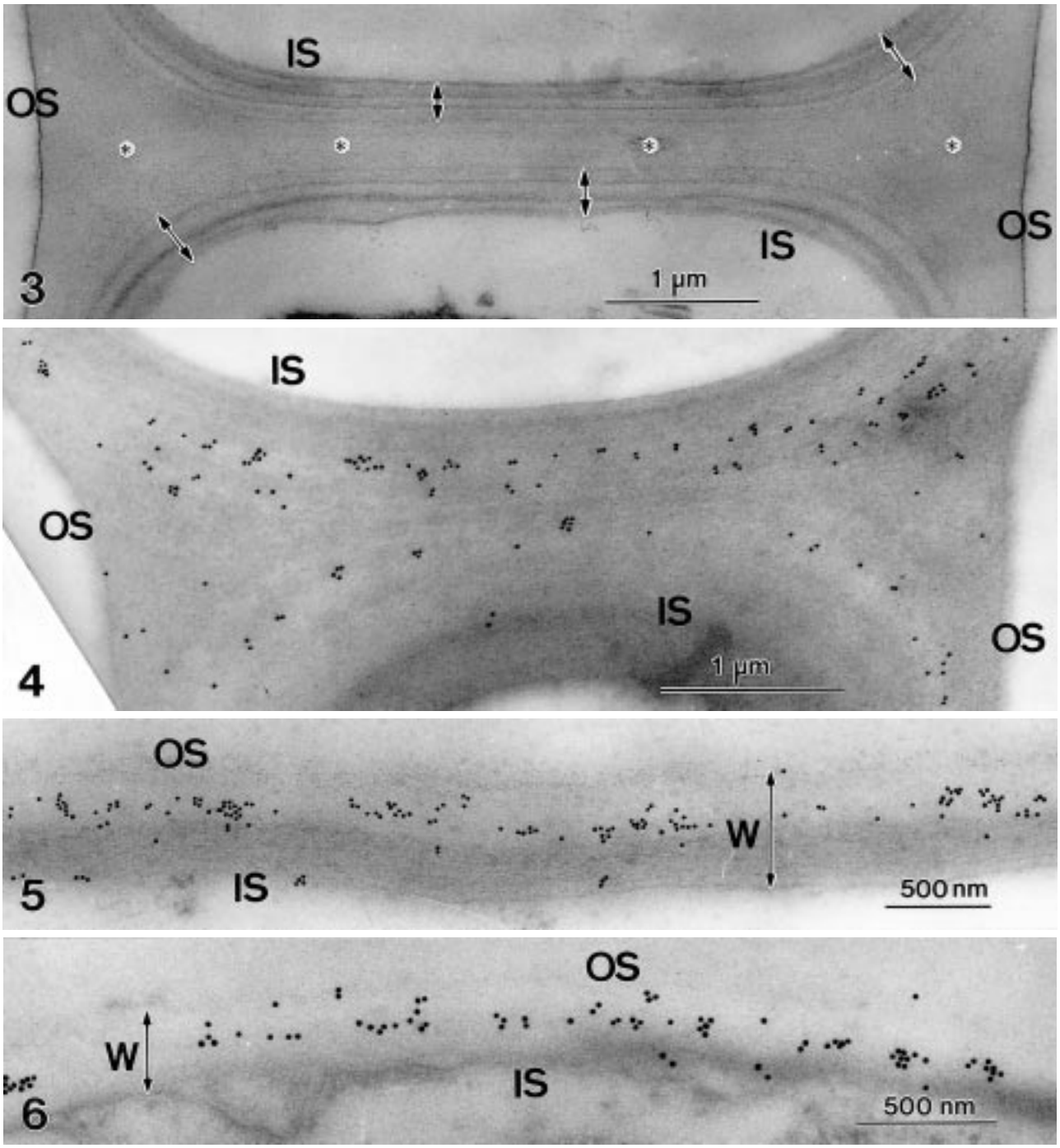
In the Phaeophyceae, the cell wall of *Sphacelaria rigidura* was composed of an outermost thin, non-fibrous layer and three other distinct layers when observed in stained sections (Fig. 1). Fibrillar structures were observed in the third layer from the outside of the wall. By immunogold microscopy, gold particles were observed to be distributed all over the cell wall of *Sphacelaria*, although the labeling was scanty near the outermost layer (Fig. 2). *Scytosiphon lomentaria* also showed even distribution of gold particles on the cross wall (data not



Figs 1,2. Cross-section of the cell wall of *Sphacelaria rigidula* (Phaeophyceae). 1. Cell wall stained with uranyl acetate and lead citrate, consisting of four layers (I, II, III and IV). 2. Cell wall with immunogold labeling (no uranyl acetate/lead citrate staining). W, cell wall; OS and IS, outer and inner surfaces of the wall, respectively.

shown). Thus, in these species of Phaeophyceae the cell walls were labeled with antialginate antiserum, indicating the presence of alginate epitopes.

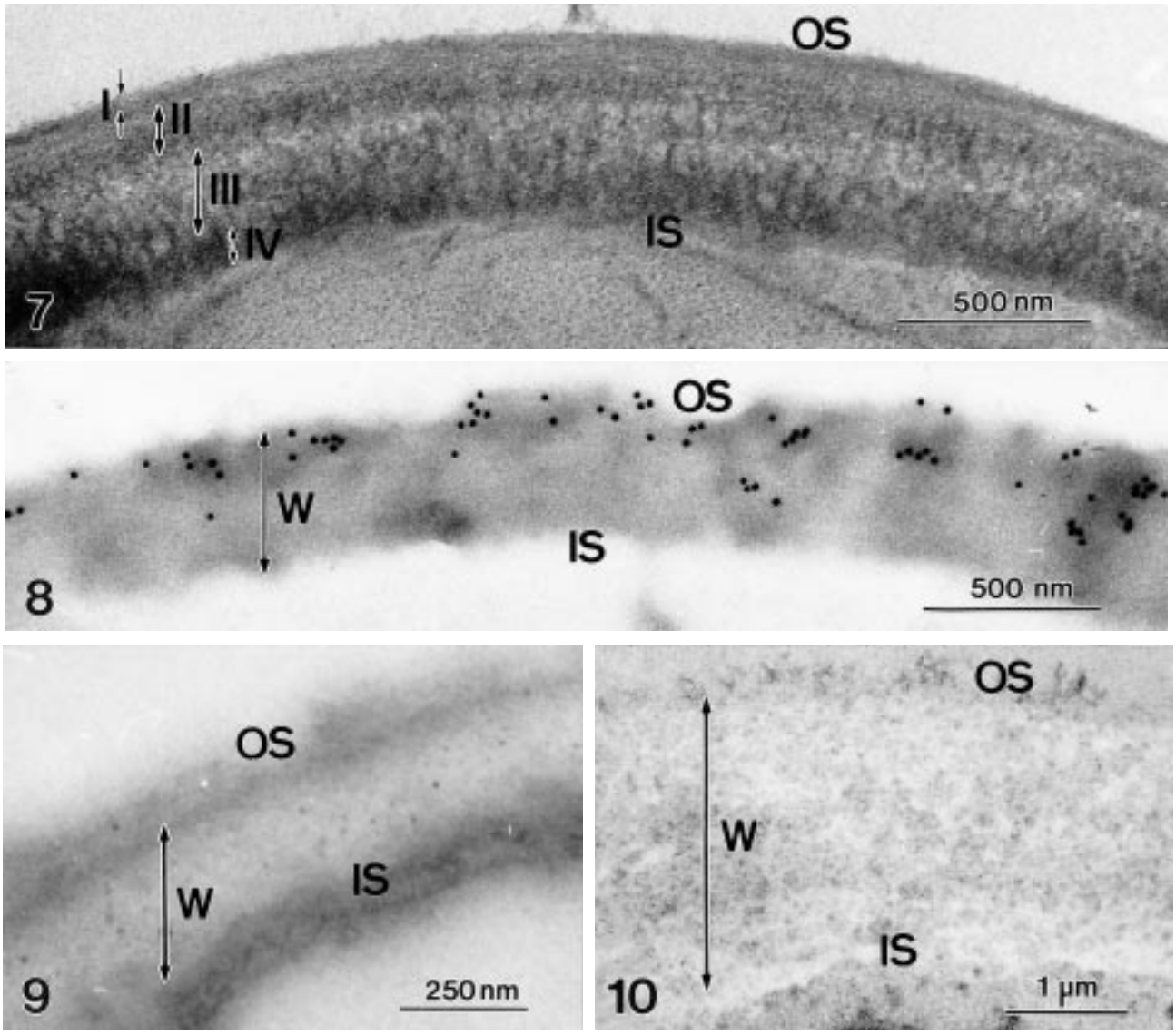
In *Tribonema* sp., a member of the Xanthophyceae, adjacent cells were separated by H-shaped walls (Fig. 3). The H-shaped wall was composed of non-layered homogeneous portion and layered portions. The former included the middle part of the transverse septum and the outer part of the lateral wall of the adjacent cells, while the latter was deposited on the inner surface of these cells. Gold particles tended to be distributed in the regions between the non-layered and layered portions of the H-shaped walls (Fig. 4). Localized distribution of gold particles was also observed in the cell walls of *Vaucheria geminata*, which consisted of two layers, an outer and a more electron-dense inner



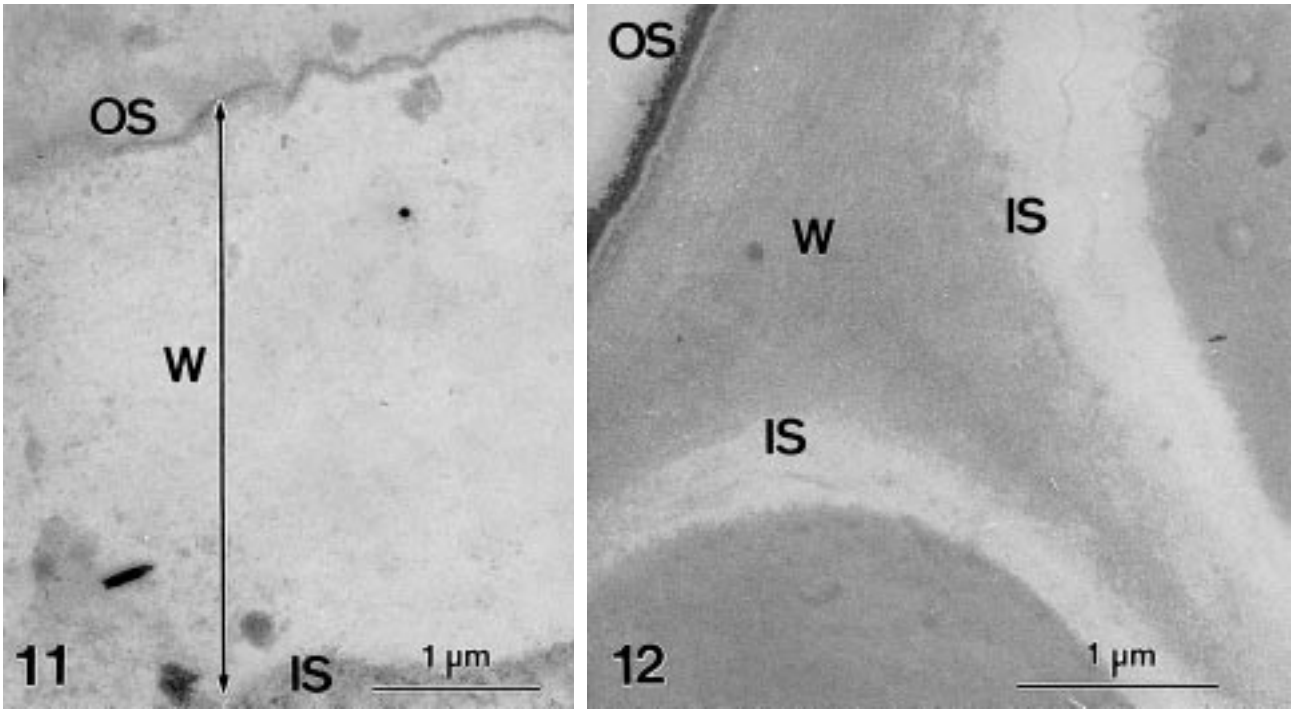
Figs 3–6. Cross section of cell wall in Xanthophyceae. 3. *Tribonema* sp. H-shaped cell wall stained with uranyl acetate and lead citrate. Asterisks indicate non-layered homogeneous portion of the wall; arrows indicate layered portions of the wall. 4–6. Immunogold labeling in the cell wall of *Tribonema* sp.: (4) *Vaucheria geminata*; (5) *Botrydiopsis arhiza*; and (6) without uranyl acetate/lead citrate staining. W, cell wall; OS and IS, outer and inner surfaces of the wall, respectively.

one (Fig. 5). The outer layer of the cell walls was labeled more densely. The cell walls of *Botrydiopsis arhiza* (Fig. 6) and *Botrydium stoloniferum* (not shown) were evenly labeled with anti-alginate antiserum.

In the 'undescribed filamentous species' the cell walls seemed to be composed of four distinct layers (Fig. 7). Below a thin outermost layer, three fibrous layers were present. The second and fourth from the outside of the walls were electron-dense layers, between which the third, relatively electron-transparent layer was sandwiched. Labeling appeared at the outer parts of the cell walls, probably including the outermost and the second layers (Fig. 8). In *Giraudyopsis steriferra* (Fig. 9) and *Phaeosaccion collinsii* (Fig. 10), granular or amorphous materials were homogeneously distributed in their cell walls, which were not labeled with anti-alginate antiserum. No labeling was observed in the cell walls of *Bryopsis plumosa* (Fig. 11) and *Antithamnion nipponicum* (Fig. 12). Table 2 summarizes the presence and absence of alginates in the species examined in the present study by means of immunolabeling.



Figs 7–10. Cross section of cell wall. 7. 'Undescribed filamentous species' cell wall stained with uranyl acetate and lead citrate. The cell wall consists of four layers: outermost (I); second (II); third (III); fourth (IV). 8. Immunogold labeling in the 'undescribed filamentous species' cell wall. 9, 10. Cell wall of *Giraudyopsis stellifer* (9) and *Phaeosaccion collinsii* (10), which were not labeled with gold particles. Sections were stained with uranyl acetate/lead citrate after immunogold labeling. W, cell wall; OS and IS, outer and inner surfaces of the wall, respectively.



Figs 11,12. Cross section of cell wall. No labeling was observed in *Bryopsis* (11) and *Antithamnion* (12). Sections were stained after immunogold labeling. W, cell wall; OS and IS, outer and inner surfaces of the wall, respectively.

Table 2. Immunogold labeling of alginates in the cell walls of the species examined

Algal species	Immunogold labeling
Scytosiphon lomentaria	+
Sphacelaria rigidula	+
Botrydiopsis arhiza	+
Botrydium stoloniferum	+
Tribonema sp.	+
Vaucheria geminata	+
Giraudyopsis stellifera	–
Phaeosaccion collinsii	–
Undescribed filamentous species	+
Antithamnion nipponicum	–
Bryopsis plumosa	–

+, labeled; –, unlabeled.

DISCUSSION

The cell walls of two brown algae *Sphacelaria* and *Scytosiphon* were clearly labeled with gold particles, indicating the presence of alginates. In addition to these brown algae, cell walls of all xanthophycean species examined (*Tribonema*, *Vaucheria*, *Botrydium* and *Botrydiopsis* and an undescribed multicellular species, perhaps chrysophyte or phaeothamniophyte) were labeled with gold particles. This is the first report of the occurrence of alginates in the chromophytes outside the Phaeophyceae. In contrast, no labeling appeared in the cell walls of the multicellular chrysophytes *Giraudyopsis* and *Phaeosaccion*, as well as the species of the rhodophytes and ulvophytes.

The existence of alginates in brown algal cell walls has been confirmed by differential, histochemical staining and indicated to be localized in particular layers of the cell walls (McCully 1966; Evans and Holligan 1972; Burns *et al*. 1982). Alginic acid is composed of three different oligosaccharides, mannuronic acid (M) blocks, guluronic acid (G) blocks, and blocks with alternating mannuronic and guluronic (MG) units (Haug *et al*. 1974). Proportions of the block components in alginates vary with species of brown algae (Haug *et al*. 1974; Craigie *et al*. 1984). They also differ within a species depending on the cell types and age of the tissue. The anti-alginate antibody used in the present study is polyclonal, not monoclonal, therefore it does not recognize a distinct epitope of alginate molecules. In order to study the distinctive distribution of carbohydrate epitopes in algal cell walls, monoclonal antibodies have been effectively used (Vreeland *et al*. 1984). Furthermore, it should be noted that the intensity of immunogold labeling using polyvalent antisera to alginates is not correlated to the amount of alginate present in a particular region of the algal cell walls, as described by Vreeland (1970, 1972).

Recent molecular studies (e.g. 18s rDNA sequences) have shown that the members of Sarcinochrysidales formerly assigned to Chrysophyta are rather distant from other members of the order and placed in the new class Pelagophyceae (Andersen *et al*. 1993, 1998). Andersen *et al*. (1998) showed that Phaeophyceae,

Xanthophyceae and *Phaeothamnion confervicola*, formerly classified in the Chrysophyceae (Preisig 1995), form a clade, but Chrysophyceae is included in an independent clade together with Synurophyceae, Eustigmatophyceae, Raphidophyceae. They further suggested treating Phaeothamnion and some other taxa formerly placed in Xanthophyceae and Chrysophyceae as an independent class Phaeothamniophyceae (Andersen *et al*. 1998; Bailey *et al*. 1998). The present results, that both phaeophycean and xanthophycean species have alginates in their cell walls, support the notion that they are phylogenetically close. This means that the capacity for alginate production had been acquired before divergence of the clades Phaeophyceae and Xanthophyceae. The absence of alginates in Giraudyopsis and Phaeosaccion fits with the relatively distant phylogenetic relationship with Phaeophyceae/Phaeothamniophyceae/Xanthophyceae deduced from molecular data (Saunders *et al*. 1997; Andersen *et al*. 1998).

Apart from chromophyte algae, alginates are also produced by prokaryotes such as *Pseudomonas aeruginosa* (Linker and Jones 1966) and *Azotobacter vinelandii* (Gorin and Spencer 1966), secreted as capsular material. In addition, Okazaki *et al*. (1982) reported that alginates occur in corallinacean red algae and may be involved in the uptake of calcium ions from sea water to serve as nucleation sites for calcite crystals. The phylogenetic implications of the occurrence of alginates in these groups, and occurrence only in Xanthophyceae/Phaeophyceae among Chromophyta are unclear.

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