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Rhizoid formation in *Valonia* (Siphonocladales, Chlorophyceae)

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Rhizoids were artificially induced by the contact or approach of substrata toward *Valonia macrophysa*, *V. fastigiata* and *V. aegagropila* cell surfaces. A single, spherical cell produced rhizoids locally at the portion that contacted with a glass coverslip, a sheet of cellophane or surface of another *Valonia* cell. Local induction of rhizoid formation did not always require direct contact with substrata: occurring when two living cells were placed apart but closer than 0.5 mm. Induction of rhizoid formation required continuous contactless exposure to a substratum for at least 48 h. In these cases, amorphous materials were secreted to external surfaces and accumulated in the space between two adjacent cells, but when washed out, the number of rhizoids induced decreased remarkably. The amorphous materials were stained with periodic-acid Schiff's and Alcian Blue and conjugated fluorescein isothiocyanate–labeled lectins that recognize β-D-glucose, α-D-mannose, β-D-galactose, N-acetyl-D-galactosamine and N-acetyl-glucosamine residues. Further analysis using thin-layer chromatography confirmed the presence of galactose, N-galactosamine and N-glucosamine in the amorphous materials; in addition, a high-R_f monosaccharide was also detected. When rhizoid formation was induced, a local aggregation of protoplasm began concomitant with cortical microtubules changing in arrangement from parallel to radial and the contraction of actin filaments. This was followed by disassembly of perinuclear and cortical microtubules in the protoplasmic aggregation. The protoplasmic aggregation was then split from the cell by a septum to become a small lenticular cell, which eventually elongated toward a substratum, differentiating into a rhizoid.

Key Words: Actin, FITC-labeled lectin, Microtubule, Polysaccharide, Rhizoid induction, *Valonia*

INTRODUCTION

Cell differentiation is a vital step for the formation of specialized parts of a multicellular organism. It starts with the recognition of a signal from inside or outside the cell followed by transduction of such signal to the area of interest wherein a coordinated action of genes facilitates reorganization of cytoskeletons and organelles toward creation of an entirely different cell (Kost *et al.* 2002). Stimuli include hormones and external physical and chemical signals (Dornelas 2003; Ramirez-Parra *et al.* 2005). Understanding cell differentiation provides important information regarding multicellularity, adaptation and diversity. In order to do this, basic morphological development in relatively simple organisms such as algae are used as models; one fine example is rhizoid formation.

Algal rhizoids, structures that serve as anchorage of cells or thalli to substrata, have been shown to be influenced by various factors such as gravity in *Chara* (Braun *et al.* 1999; Ackers *et al.* 2000; Kuznetsov & Hasenstein 2001), substratum in *Fucus* (Hardy & Moss 1979), wounding in *Caulerpa prolifera* (Friedlander *et al.* 2006) and light in *Boergesenia* (Ishizawa & Wada 1979). It may contribute to promoting our understanding of plant cell morphogenesis and differentiation to identify regulating mechanisms in rhizoid formation shared by most of these species, but little is known about how cells perceive the above environmental factors.

Members of the genus *Valonia* are marine coenocytic green algae, consisting of large multinucleate cells. Primary cells produce lateral cells through lenticular cell formation. Several lateral branch cells can arise from a single mother cell and in turn become the origin of younger cells. During germination, a primary holdfast serves as the initial attachment but is later followed by numerous rhizoids mostly confined around the basal area of cells (Chihara 1959; Bold & Wynne 1985). Tenacular cells, connective structures resembling rhizoids, form sporadically in clusters along lateral surfaces of adjacent vesicles (Fritsch 1935; Kanda 1940), and such features are present in all *Valonia* species according to Olsen & West (1988). These morphological characteristics make *Valonia* an ideal specimen in investigating the properties of rhizoids as they relate to those of tenacular cells. In addition, the large size and the conspicuous formation of lenticular cells offer ease of observation and manipulation during experiments.

In this article we report in three species of *Valonia* the ability to produce rhizoids on any region of the cell surface and factors inducing rhizoid formation with distinct substrata. In addition, using indirect immunofluorescence and fluorescent lectin microscopy coupled with thin-layer chromatography (TLC), we describe changes in the localization of microtubules, actin filaments and sugars involved in rhizoid formation.

MATERIAL AND METHODS

Algal materials

Specimens used in this study were *Valonia macrophysa* Kützing, *V. fastigiata* Harvey *ex* J. Agardh and *V.*

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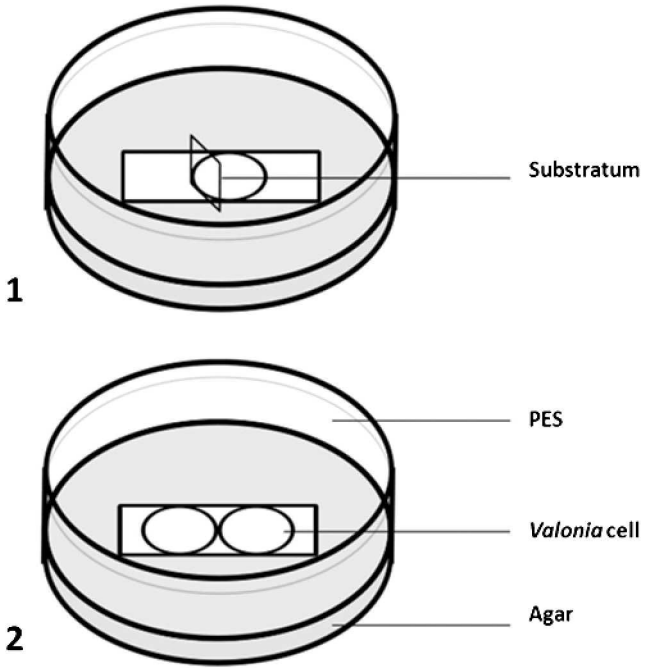
aegagropila C. Agardh. Thalli of *V. macrophysa* were obtained from the southern coast of Yokonami Peninsula, Susaki, Kochi, Japan, on 24 May 2009, while those of *V. fastigiata* were collected from waters off Otsuki, Kochi, Japan, on 8 June 2009. Thallus of *V. aegagropila* (strain no. 7) was collected from the coast of Sta. Ana, Cagayan, Philippines, on 21 February 2006. Zoospores or parthenogametes released from thalli of these three species were isolated according to methods described by Kawai *et al.* (2005) and cultured in Petri dishes containing *c.* 150 ml of quarter-strength PES medium (Provasoli 1968) at 22°C in long-day conditions (14:10 light:dark) under fluorescent lamps (40–50 μmol photons m^{-2} s^{-1}). Each of the germlings developed into a single spherical cell 3–4 mm in diameter within 3 wk and used for experiments on rhizoid induction. When a vegetative cell cultured for a month was cut and squeezed to release its protoplasm in culture medium, the protoplasm became a protoplast and subsequently regenerated into a new small cell (cf. Enomoto & Hirose 1972). Such regenerated cells 0.3–0.4 mm in diameter were also used for the other experiments.

Rhizoid induction

To study rhizoid induction, six-welled sterile plastic plates (Cell Culture Plate no. 3506; Corning Inc., Corning, NY, USA) were used. Each well (35 mm in diameter) was filled with 3 ml 1% agar solution left to solidify in room temperature. A horizontal 3–4 × 5–8-mm slot was carved in the gel using a scalpel. The well was then filled with 10 ml PES. Using forceps, a cell approximately 3–4 mm in diameter was transferred from stock cultures to the well and settled on this agar slot without touching the bottom of the well (Fig. 1). Substrata used for testing effects on rhizoid differentiation were glass coverslips, sheets of cellophane (dialysis cellulose membrane; Sanko Co. Ltd, Tokyo, Japan), other living cells or pieces of agar gel. Glass and cellophane are two hydrophobic materials, glass being a commonly used substratum as culture vessels. On the other hand, agar gel was chosen to determine the effect of a hydrophilic substratum. Use of a living cell simulates the conditions for the formation of tenaculae and should provide interesting information regarding the induction of both rhizoids and tenaculae. The substratum was set to directly contact with the surface of a cell placed in a slot as shown in Fig. 1. In the case where another living cell was used as a substratum, six different distances between two cells (0, 0.3, 0.5, 0.7, 0.9 and 1.2 mm) were adjusted using an optical micrometer (Fig. 2). These plastic plates were placed under the same culture conditions as those described above and checked to see if rhizoids were produced between a cell and a substratum every 2 d for 12 d using an inverted (CKX41; Olympus Optical Co., Ltd, Tokyo, Japan) or a dissecting (SZX7; Olympus Optical Co.) microscope equipped with a digital camera for documentation. Experiments were also carried out to determine the duration of the presence of a substratum required for the establishment of rhizoid induction. Two cells were arranged at a distance of 0.3 mm in an agar slot and incubated in a well of the plastic plate under the culture conditions. One of the two cells was transferred from the original position into a vacant agar slot



Figs 1, 2. Illustration of incubation chambers for rhizoid induction.
Fig. 1. A slot was carved on an agar gel in a plastic plate filled with PES medium. A cell was inserted in the slot to anchor and placed in contact with a distinct type of substratum.
Fig. 2. A cell was put next to another cell side by side at defined distances.

12, 24, 36, 48, 60 or 72 h after the start of experiments. These cells were continuously cultured until 12 d after the start of experiments. Exposed surfaces, the areas facing each other cell surface, were observed under the inverted microscope for induction of rhizoids. Means were determined after dividing the number of rhizoids between each pair of cells by two since each cell may be capable of producing rhizoids on its own. An attempt to diffuse possible materials localized in the space between two adjacent cells was made. A pair of cells was placed 0.3 mm apart so that they might be allowed to produce rhizoids. The surfaces of these cells were rinsed once per day with culture medium by generating a water stream through a Pasteur pipette at the narrow space between two cells. The number of rhizoids in 20 cells was counted 12 d after the beginning of experiments.

Staining of cell surfaces

Amorphous materials on cell surfaces were stained using acid fuchsin (AF), periodic acid-Schiff's (PAS) reaction, Alcian Blue (AB) and Sudan Black (SB) according to McCully *et al.* (1980). Young cells *c.* 300 μm in diameter that regenerated from protoplasts were fixed with 2.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 10 min and stained with these dyes.

Application of fluorescein isothiocyanate–labeled lectins

The fluorescein isothiocyanate (FITC)–labeled lectins (Vector Laboratories, Inc., Burlingame, CA, USA) were mixed with PES medium to a final concentration of

5 μg ml^{-1} to make lectin solutions. Twenty cells that regenerated from protoplasts were transferred into the depression of a depression-glass slide containing 1.5 ml lectin solution and incubated for 3 d at room temperature. After incubation, cells were rinsed with PES three times, mounted on glass slides with the medium, sealed with silicone grease (G-30M; Shin-Etsu Chemical Co. Ltd, Tokyo, Japan) and then observed with an Olympus BX-51 epifluorescence microscope (Olympus Optical Co.). The following lectins were tested: *Pisum sativum* agglutinin (PSA), jacalin (jac), *Ulex europaeus* agglutinin I (UEA I), *Sophora japonica* agglutinin (SJA) and *Griffonia (Bandeiraea) simplicifolia* II (GSII). Controls were set by adding a 0.1 M competing sugar to each lectin solution: glucose (Glc) and mannose (Man) for PSA, galactose (Gal) for jac, fucose (Fuc) for UEA I, N-acetyl galactosamine (GalNAc) for SJA and N-acetyl glucosamine (GlcNAc) for GS II. To visualize amorphous materials localized in the space between two adjacent cells, cells that were placed next to each other were cultured under normal culture conditions for 3 d. Effects of sugars and lectins on the induction of rhizoids were examined by culturing cells with these compounds. Two cells placed 0.3 mm apart were incubated with PES containing 10 mM GalNAc, 10 mM GlcNAc, 100 mM GlcNAc, 10 mM Gal or 20 μg ml^{-1} GS II, PSA, jac, SJA and UEAI (not conjugated with FITC) under normal culture conditions for 12 d, and the number of rhizoids formed was counted in 10 cells per treatment.

TLC

Sample preparation and TLC were performed according to Fry (1988) with some modifications. Amorphous materials were collected from approximately 100 cells that were recently transferred (3 d) to a clean 50-ml plastic centrifuge tube where cells were in close contact with each other to simulate the conditions for the induction of rhizoids. Cell surfaces were washed by filling and emptying the tube containing the cells three times with sterile distilled water. The surfaces of individual cells were then lightly rubbed against a 10 × 30-mm strip of sterile filter paper using a pair of forceps. The piece of filter paper was left to dry, cut into shorter strips and placed inside 1.5-ml centrifuge tubes that were filled with distilled water to wash any trace of the culture medium. Tubes were finally filled with 300 μl distilled water and then placed in a warm bath (60–70°C) for 30 min. The contents of the tube were filtered by centrifugation in a column (Nihon Millipore, Tokyo, Japan). The eluent was then dried by vacuum with centrifugation. The resulting gel-like pellet was suspended in 425 μl distilled water and 75 μl pure trifluoroacetic acid in a tube with a tightly fitting cap lined with Teflon pads. Samples were placed in an oven at 120°C for 1 h. After cooling, samples were centrifuged and then dried. Samples were dissolved in water-saturated butanol for TLC. Cellulose powder TLC plates on glass (Merck, Darmstadt, Germany) were used for separation of the amorphous material sugar residues. The solvent used was butanol:acetic acid:water (3:1:1). Staining of monosaccharides was done using aniline hydrogen-phthalate (stock: 1.6 g phthalic acid in 49 ml acetone, 49 ml diethyl ether and 2 ml water; added aniline to 0.5% [v/v] before use). For staining of amino sugars, 0.1% ninhydrine in acetone was used. Plates were dipped quickly in the staining solutions, dried for 3–5 min and then incubated in an oven at 105°C for 5 min.

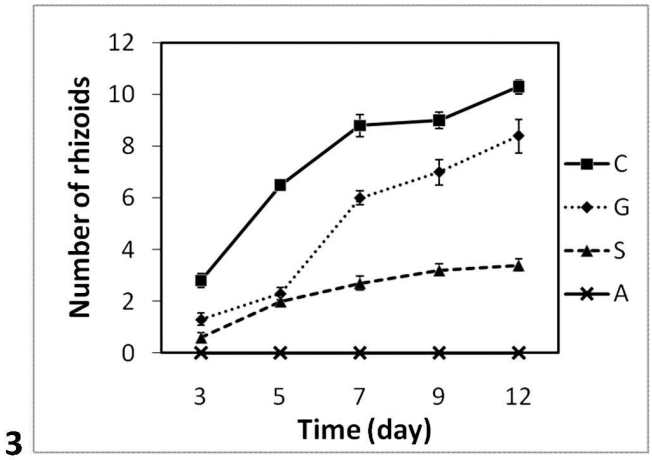
Staining of microtubules and nuclei

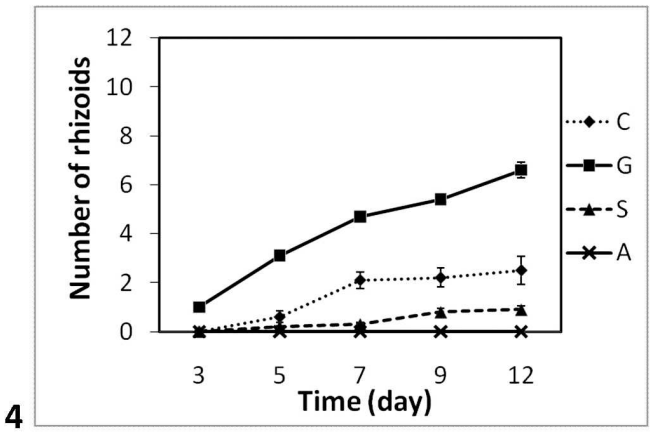
Indirect immunofluorescence microscopy for microtubules was essentially the same as that described by Okuda *et al.* (2000). Briefly, a cell was fixed in a buffer (MTSB: 587 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 5 mM ethylene glycol-bis-[β-aminoethyl ether]- N,N,N′,N′-tetraacetic acid and 50 mM piperazine-N,N′-bis-[2-ethanosulfonic acid], pH 7.0) containing 4% paraformaldehyde and 1% glutaraldehyde. A part of protoplasm of the fixed cell was mounted on a poly-L-lysine–coated coverslip, incubated with a primary antibody (a rat monoclonal anti-β-tubulin antibody, YL1/2; Sera Labs, Crawley Down, UK) followed by treatment with a secondary antibody (a goat polyclonal anti-rat IgG antibody conjugated with FITC, F6258; Sigma Chemical, St. Louis, MO, USA) to detect microtubules. For actin, a primary antibody (rabbit anti-actin antibody, A2066; Sigma) and a secondary antibody (goat anti-rabbit IgG antibody conjugated with FITC, F0382; Sigma) were used. Samples were mounted on a glass slide with VECTASHIELD Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI; at a concentration of 1.5 μg ml^{-1}; Vector Laboratories). Fluorescence was observed using the epifluorescence microscope.

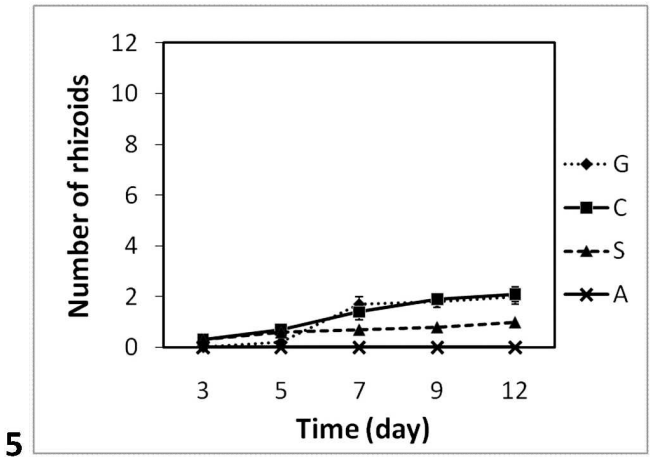
RESULTS

Rhizoid formation induced by the presence of a substratum

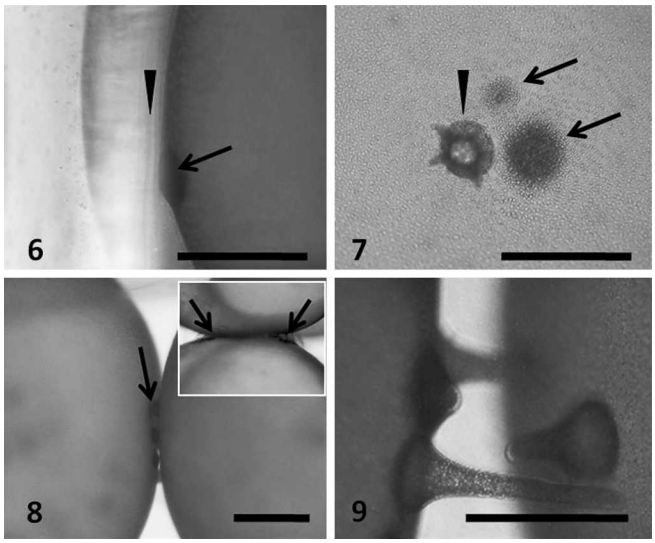
Cells were settled to be in contact with distinct substrata and cultured for 12 d to examine if rhizoid formation can be induced on adjacent cell surfaces. Rhizoids began to form 3 d after the beginning of experiments when glass coverslips, sheets of cellophane and other living cells were used as substrata for *V. macrophysa* (Fig. 3), *V. fastigiata* (Fig. 4) and *V. aegagropila* (Fig. 5). *Valonia macrophysa* produced the highest number of rhizoids among three species during culture of 12 d, responding strongest against the substrata that had an effect to induce rhizoids. No induction of rhizoids occurred in cells in contact with pieces of agar and in control (without any substrata). This was the reason why we used agar gel to anchor cells for the experiments. An initial rhizoid started to protrude from the cell surface at a contact point with the surface of the substratum (Fig. 6). After the initial rhizoid formed, other rhizoids appeared around the base of the initial rhizoid (Fig. 7). Therefore, the number of rhizoids increased with time as the cell was cultured. In cases where two living cells were placed in direct contact (Fig. 8), both cells produced rhizoids toward the opposite cells. However, mechanical stimulus from full contact did not induce rhizoid formation (Fig. 8, inset). Instead, rhizoids formed around but not on







Figs 3–5. Effect of distinct types of substratum on rhizoid formation.
Fig. 3. The number of rhizoids induced by four different types of substratum, glass coverslip (G), cellophane (C), another cell surface (S) and agar gel (A) recorded during incubation of *V. macrophysa* for 12 d. Data: means ± SE (n = 30).
Fig. 4. The number of rhizoids induced by four different types of substratum, glass coverslip (G), cellophane (C), another cell surface (S) and agar gel (A) recorded during incubation of *V. fastigiata.* Data: means ± SE (n = 30).
Fig. 5. The number of rhizoids induced by four different types of substratum, glass coverslip (G), cellophane (C), another cell surface (S) and agar gel (A) recorded during incubation of *V. aegagropila.* Data: means ± SE (n = 30).

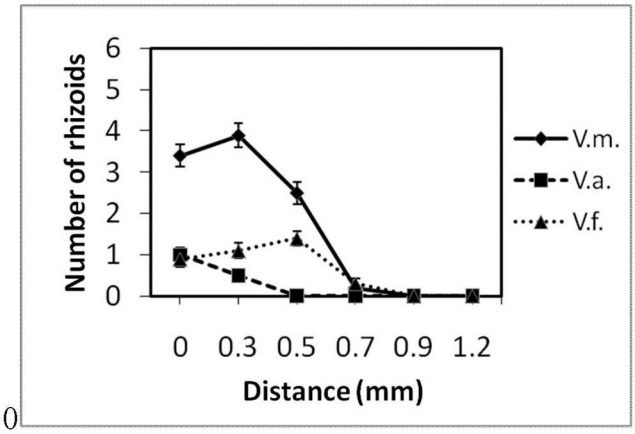


Figs 6–9. Rhizoid formation in *V. macrophysa.*
Fig. 6. Side view of a small lens-shaped cell (arrow) that protruded toward a coverslip (arrowhead). Scale bar = 100 μm.
Fig. 7. Tip of a rhizoid resembling a hapteron (arrowhead) attached to the surface of a coverslip when viewed from the back side of the coverslip. New rhizoids (arrows) initiating near the developed rhizoid. Scale bar = 100 μm.
Fig. 8. Rhizoids (arrow) formed on the surface where two cells touched each other. When cells were pressed together (inset), rhizoids formed around the tightly contacting region (arrows). Scale bar = 100 μm.
Fig. 9. Rhizoids produced between two adjacent cells. Scale bar = 100 μm.

the tightly pressed areas. In addition, cells near each other also produced rhizoids from either opposite directions (Fig. 9).

As described above, rhizoids were also induced on the cell surfaces that a substratum, such as a coverslip, had not directly touched. In fact, induction of rhizoid did not always require a direct contact between a cell and a substratum. When a cell was placed in close proximity to another cell as a substratum, both cells produced rhizoids on their surfaces in the narrow space between them (Fig. 9). Rhizoids were induced to form when two cells were left a space between them shorter than 0.3 mm in *V. aegagropila* or 0.7 mm in *V. macrophysa* and *V. fastigiata* (Fig. 10). When two cells were placed 0.9 and 1.2 mm apart, they never produced any rhizoid.

Rhizoid cells were divided from the mother cells and began to elongate about 3 d after the mother cells were in contact with or in proximity to effective substrata (Fig. 9). The length of time required for the establishment of induction of rhizoids by continuous exposure to substrata was examined. Two cells were placed 0.3 mm apart to induce rhizoids, and then one of the cells was removed to leave the other cell on its own position at a definite time. Cells left alone 12, 24 and 36 h after the beginning of experiments did not produce any rhizoid (Fig. 11). Continuous exposure to an opposite cell for more than 48 h in *V. macrophysa* and *V. fastigiata* or for more than 60 h in *V. aegagropila* was required for the induction of rhizoids. The number of induced rhizoids increased with exposure time.



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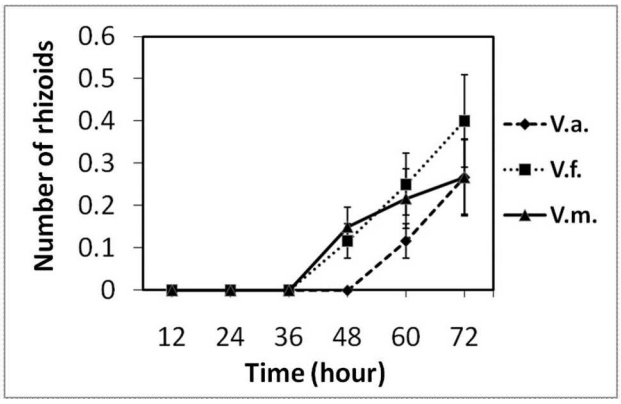

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Figs 10, 11. Effects of distance between cells and length of time of exposure on the induction of rhizoid formation.

Fig. 10. Distances between two adjacent cells required for the induction of rhizoid formation. The number of rhizoids formed was counted 12 d after two cells were placed at the distance of 0, 0.3, 0.5, 0.7, 0.9 or 1.2 mm in *V. macrophysa* (V.m.), *V. fastigiata* (V.f.) and *V. aegagropila* (V.a.). Data: means ± SE (n = 30).

Fig. 11. Hours of exposure to substrata required for establishing the induction of rhizoid formation. Two cells were placed side by side at the distance of 0.3 mm to induce rhizoid formation and separated from each other 12, 24, 36, 48, 60 or 72 h after the beginning of each experiment. The number of rhizoids formed was counted 12 d after the beginning of the experiments in *V. macrophysa* (V.m.), *V. fastigiata* (V.f.) and *V. aegagropila* (V.a.). Data: means ± SE (n = 30).

Characterization of materials on cell surfaces

It was found that rinsing the cell surfaces had an effect on rhizoid formation (Fig. 12). About 24 h after two cells were placed in close proximity to induce rhizoids, hyaline, amorphous materials accumulated in spaces between the cells in *V. macrophysa* (Fig. 13). The amorphous materials adhered on outer surfaces of cell walls. Unless cells were exposed to any substrata, no such local accumulations of amorphous materials were observed on cell wall surfaces. To prevent amorphous materials from accumulating in the narrow space between two adjacent cells, cell surfaces were rinsed with culture medium by generating water flow using a Pasteur pipette. This helped visible amorphous materials disappear (Fig. 14). The induction of rhizoids was affected by rinsing out amorphous materials. The number of induced rhizoids decreased when cells were rinsed as

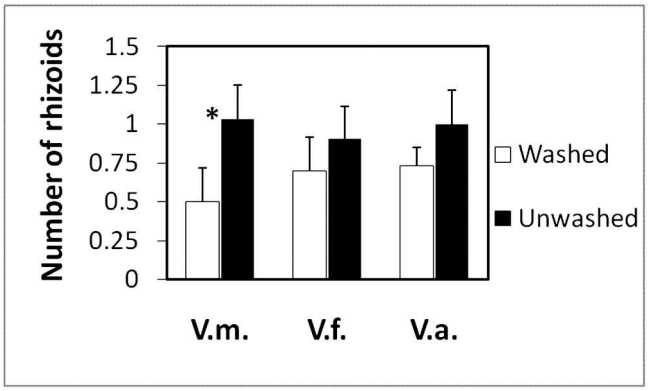
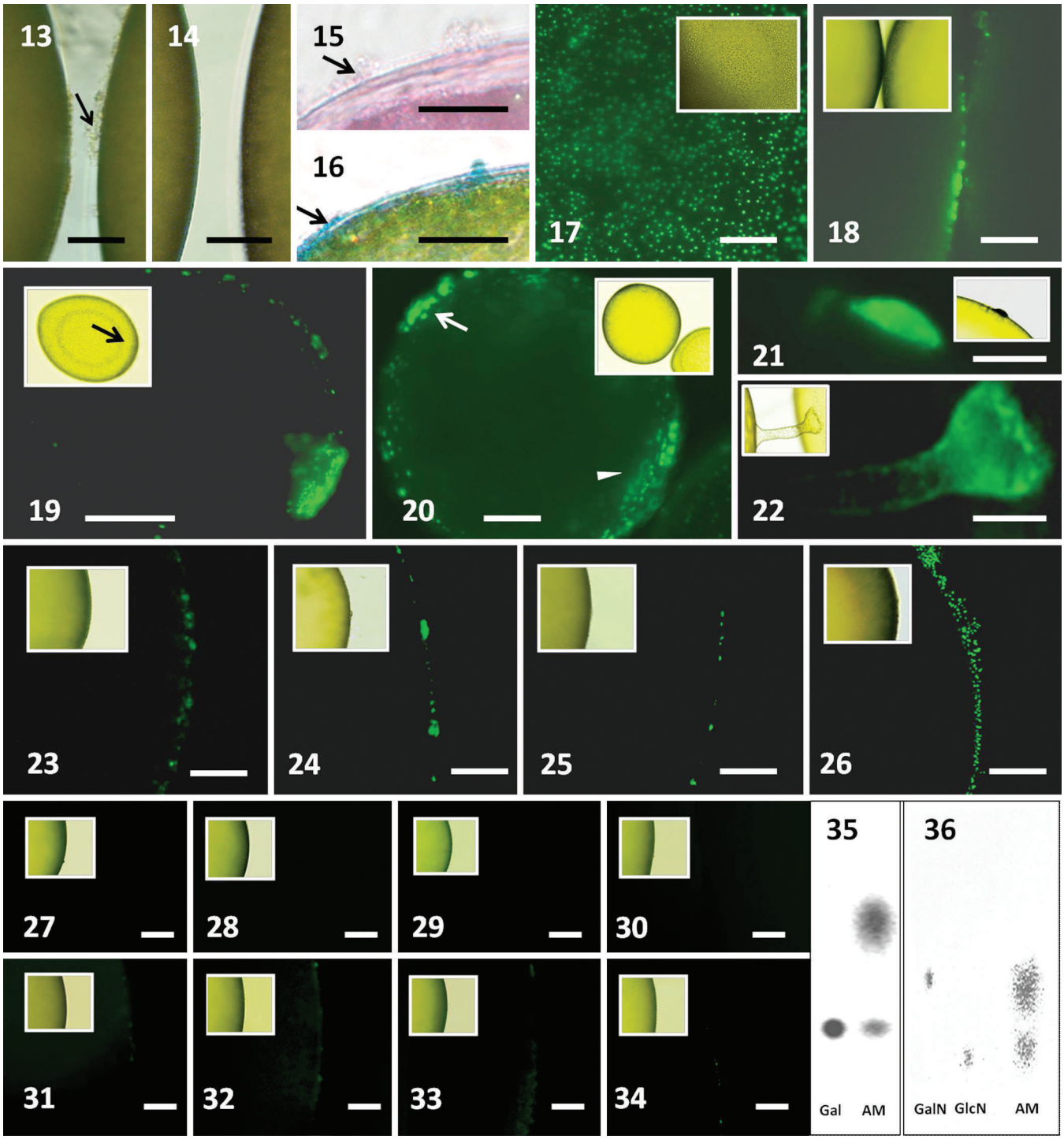


Fig. 12. Effect of washing cell surface areas between two adjacent cells with culture medium on the induction of rhizoid formation. The number of rhizoids formed on washed and unwashed cells was counted 12 d after the beginning of experiments in *V. macrophysa* (V.m.), *V. fastigiata* (V.f.) and *V. aegagropila* (V.a.). The bars represent mean values with standard errors (n = 60). * = significant difference between washed and unwashed means, $P < 0.05$, Student's t test.

compared with controls but kept more than half as much as in controls. There was a statistically significant difference in the number of rhizoids between rinsed cells and controls in *V. macrophysa*. This signifies that amorphous materials accumulating between two adjacent cells are involved in the induction of rhizoids. Although rinsing may cause mechanical stress that may inhibit rhizoid formation, other options of removing the amorphous materials, such as brushing off or applying enzymes, were deemed more intrusive. Thus, washing was done with minimal stress and disturbance to the cell. Prior tests wherein cells were applied a stream of culture medium without removing the amorphous materials showed no inhibitory effect on rhizoid formation.

Amorphous materials deposited on cell wall surfaces were characterized by histochemical staining. Positive staining did not occur with AF and SB, suggesting the absence of proteins and lipids in amorphous materials. However, amorphous materials were stained pink and blue with a PAS reaction and AB, respectively (Figs 15, 16). These results indicated that amorphous materials might contain polysaccharides. FITC-labeled lectins were then applied to investigate the composition of the polysaccharides. Young cells regenerated from protoplasts were used for experiments using lectins because they were regarded to have new cell walls without any visible exterior accumulations or debris observed under a light field-microscope. Solitary cells were tested first. Four lectins, including PSA, jac, SJA and GS II, except UEA I, which is specific for L-fucose, were bound to the cell surfaces (Table 1; see also below). Fluorescence of the FITC labeled–lectins was distributed over areas of outermost cell walls and not localized at definite sites, that is, FITC-GSII (Fig. 17). This indicated that sugar moieties combining with their complementary lectins were already present on cell surfaces even in cells without any substrata. In addition, it was verified that amorphous materials accumulating between two cells, which were placed in close vicinity to each other for 3 d, were also stained with FITC-GSII (Fig. 18). In this case, strong fluorescence was detected



Figs 13–36. Accumulation, staining and analysis of extracellular amorphous materials in *V. macrophysa*.
Figs 13, 14. Accumulation and rinsing of amorphous materials.
Fig. 13. Amorphous materials (arrow) accumulated in a narrow space between two cells. Scale bar = 100 μm.
Fig. 14. Amorphous materials disappeared after rinsing cell surfaces with culture medium. Scale bar = 100 μm.
Figs 15, 16. Cytochemical staining of cell surfaces.
Fig. 15. Amorphous materials (arrow) on cell surface treated with periodic acid Schiff's reaction. Scale bar = 50 μm.
Fig. 16. Amorphous materials (arrow) on cell surface treated with Alcian Blue. Scale bar: 50 μm.
Figs 17–22. Localized buildup of amorphous materials stained with FITC-GSII during rhizoid formation.
Fig. 17. A surface view showing fluorescent residues over a cell wall. Inset: light photomicrograph of the same area. Scale bar = 10 μm.
Fig. 18. Lateral view of labeling on the cell surface where another cell was placed in close vicinity for 3 d. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Fig. 19. Labeling (arrow) on the basal area of a young cell. Inset: light photomicrograph of the same area. Scale bar = 100 μm.

Table 1. FITC-labeled lectin staining of amorphous materials on cell surfaces.[1]

Lectin	Specificity	Result
Pisum sativum agglutinin (PSA)	β-D-glucose, α-D-mannose	+
Jacalin (jac)	β-D-galactose	+
Ulex europaeus agglutinin I (UEA I)	L-fucose	−
Sophora japonica agglutinin (SJA)	N-acetyl-D-galactosamine	+
Griffonia (Bandeiraea) simplicifolia II (GSII)	N-acetyl-glucosamine	+

[1] FITC = flourescein isothiocyanate.

on the cell surfaces where two cells were made to face each other.

Since a glass coverslip served as an effective substratum to induce rhizoids, other glassware, such as Petri dishes, functioned for rhizoid induction as well. During stationary culture of protoplasts in glass Petri dishes, most cells regenerated from the protoplasts formed primary rhizoids directly toward the bottom of Petri dishes. The primary rhizoids had their tips adhered to the surfaces of glass with a hapteron, providing the initial anchorage of the cells. The surface region of a cell where primary rhizoids are to form is referred to as the basal area, while the directly opposite surface region is called the apical area in the present study. When some young regenerated cells without any rhizoids and grown in stationary culture conditions for about 7 d were treated with a culture medium containing 5 μg ml^{-1} FITC-GSII for 1 h, intense fluorescence was detected at the basal area (Fig. 19, arrow), beneath which chloroplasts gathered. In the other young regenerated cells, the apical area of the cell was placed facing another cell as a substratum, incubated for more than 2 d after stationary culture and then stained with FITC-GSII. Fluorescent materials were detected both on the apical and on the basal regions of the regenerated cell (Fig. 20). Materials stained with FITC-GSII were also localized on the surface of an early rhizoid cell (Fig. 21) and on the tip/hapteron surfaces of an elongated rhizoid, which adhered to the surface of a substratum (Fig. 22). These results suggested that materials bound by lectins such as GSII accumulate at the site where a rhizoid is to form and continue to be localized at the tip of a developing rhizoid. FITC-GSII was used as a representative to show staining in various stages of rhizoid formation since it showed the most intense signal among the other FITC-lectins when applied on cell surfaces of mother cells (Figs 23–26). Photomicrographs of negative controls for each FITC-lectin test showed no detectable staining (Figs 27–34).

The composition of the amorphous materials was analyzed through TLC. Amorphous materials were transferred onto filter papers by lightly rubbing off cell surfaces on the filter paper and then extracting the materials from the filter paper by incubating in warm water. After separation on a cellulose powder TLC plate and staining with aniline hydrogen-phthalate, two spots were visualized

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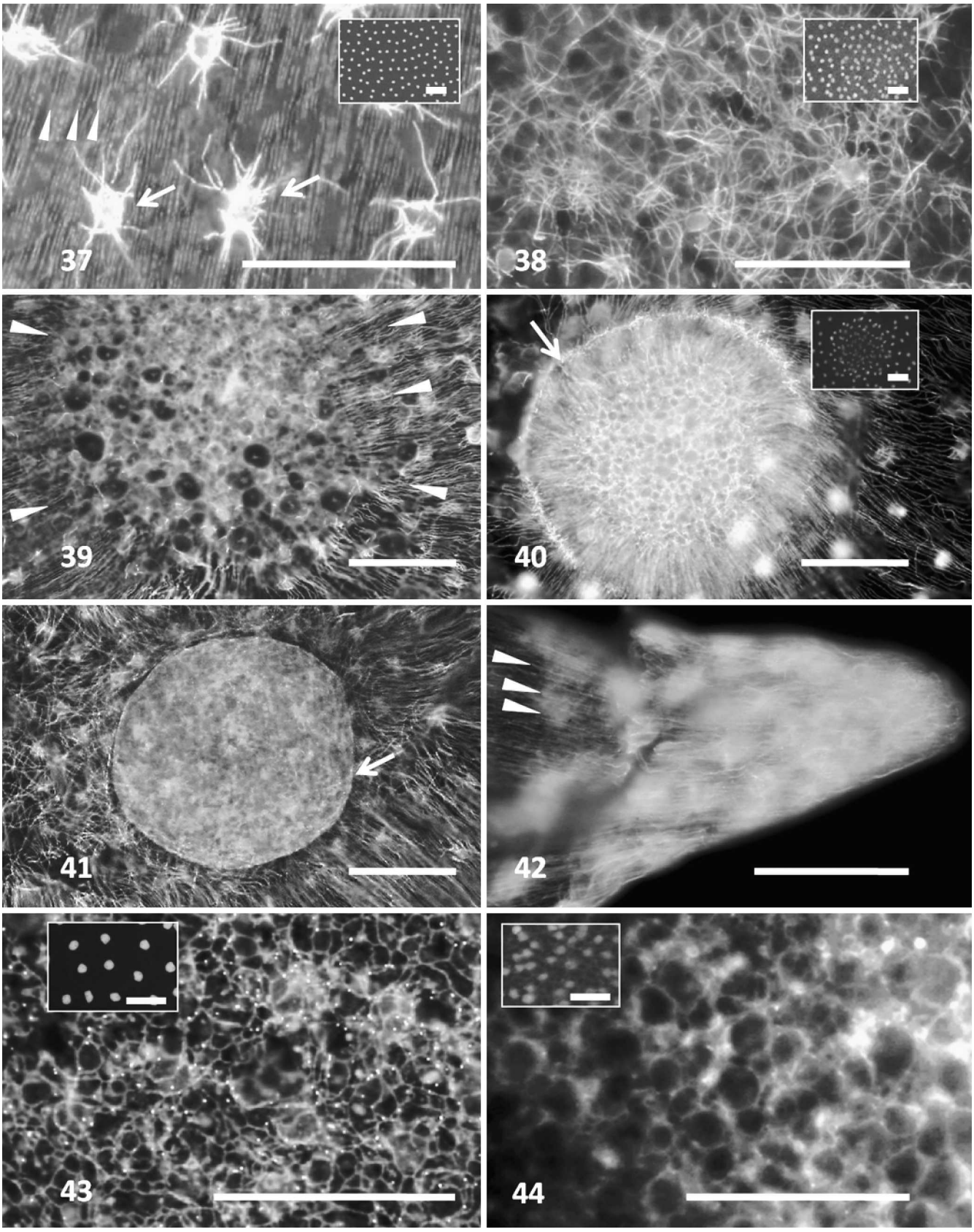
Fig. 20. A young cell showing fluorescence both on the basal area (arrow) and on the apical area (arrowhead) where another cell was placed in close vicinity for 3 d. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Fig. 21. A newly formed rhizoid cell (a lenticular cell) covered with materials labeled with FITC-GSII. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Fig. 22. A rhizoid reaching the surface of another cell with only the tip stained. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Figs 23–34. Cell surfaces stained with FITC-conjugated lectins and corresponding controls.
Figs 23–26. *V. macrophysa* samples treated with FITC-conjugated lectins.
Fig. 23. Cell surface stained with FITC-jac. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Fig. 24. Cell surface stained with FITC-PSA. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Fig. 25. Cell surface stained with FITC-SJA. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Fig. 26. Cell surface stained with FITC-GSII. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Figs 27–30. *V. macrophysa* samples incubated in the medium with a monosaccharide and stained with a specific FITC-lectin.
Fig. 27. Photomicrograph of a cell surface treated with 0.1 M galactose and FITC-jac. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Fig. 28. Photomicrograph of a cell surface treated with 0.1 M mannose and FITC-PSA. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Fig. 29. Photomicrograph of a cell surface treated with 0.1 M N-acetyl-galactosamine and FITC-SJA. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Fig. 30. Photomicrograph of a cell surface treated with N-acetyl-glucosamine and FITC-GSII. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Figs 31–34. *V. macrophysa* samples rinsed with the culture medium before staining with FITC-lectins.
Fig. 31. Photomicrograph of a cell surface rinsed with the culture medium and stained with FITC-jac. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Fig. 32. Photomicrograph of a cell surface rinsed with the culture medium and stained with FITC-PSA. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Fig. 33. Photomicrograph of a cell surface rinsed with the culture medium and stained with FITC-SJA. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Fig. 34. Photomicrograph of a cell surface rinsed with the culture medium and stained with FITC-GSII. Inset: light photomicrograph of the same area. Scale bar = 100 μm.
Figs 35, 36. Thin-layer chromatograms of amorphous materials from cell surfaces.
Fig. 35. Monosaccharides from the amorphous material (AM) were run alongside galactose (Gal) on a cellulose powder TLC plate using butanol:acetic acid:water (3:1:1) as solvent and then stained with aniline hydrogen-phthalate.
Fig. 36. Amino-sugars from the amorphous material (AM) were run alongside N-acetyl-D-galactosamine and N-acetyl-glucosamine on a cellulose powder TLC plate using butanol:acetic acid:water (3:1:1) as solvent and then stained with ninhydrine.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(Fig. 35). One spot ran the same distance with the galactose control. The other spot had a high R_f (retention value) of around 0.7–0.8. Ninhydrine staining revealed two spots that travelled distances matching those of either N-acetyl-galactosamine or N-acetyl-glucosamine (Fig. 36).

Introduction of varying concentrations of sugars and lectins to the culture medium showed no specific effect on the induction of rhizoid formation (data not shown). When GS II, PSA, jac, SJA and UEAI were supplemented to the culture medium at 20 μg ml^{-1}, rhizoids were inhibited. However, lower concentrations of all lectins had no inhibitory effect on rhizoid formation.

Evaluation of protoplasmic organization during induction and initial formation of a rhizoid

A *Valonia* cell grown in stock cultures generally had a thin layer of protoplasm between a cell wall and a central vacuole where chloroplasts and nuclei were evenly distributed. Cortical microtubules (CMTs) were arranged parallel to each other beneath the plasma membrane; whereas, perinuclear microtubules (PMTs) surrounded each of interphase nuclei (Fig. 37). About 24 h after a cell was exposed by a substratum to induce rhizoid formation, changes in the local distribution of protoplasmic structures began to occur. The incipient sign of rhizoid formation was the occurrence of a small assembly of chloroplasts beneath the cell surface site where the cell faced a substratum (Fig. 7). In the protoplasmic area where chloroplasts aggregated, nuclei also gathered, and the parallel arrangement of CMTs was disoriented (Fig. 38).

Protoplasmic assembly still continued 36–48 h after a cell was placed next to a substratum, and this assembly attained a diameter approximately 60 μm. CMTs became fragmentary and random within a disk-shaped area where chloroplasts and nuclei aggregated but became arranged radially from the periphery of the disk-shaped area (Fig. 39). Aggregated nuclei had no PMTs. A part of cell wall just above the disk-shaped area started to slightly protrude, concomitant with the dense aggregation of brushlike short microtubules on the outer boundary of the disk-shaped area (Fig. 40).

Septum formation occurred 60 h after a cell was exposed to a substratum, so that a small lenticular cell was divided from the mother cell (Fig. 41). The lenticular cell packed chloroplasts and nuclei that had accumulated before septum formation and had no CMTs. CMTs distributed in mother cells seemed to terminate at the edge of a septum. Subsequently, a cone-shaped rhizoid began to elongate from the apical part of the lenticular cell. Longitudinal CMTs were observed in elongating rhizoids (Fig. 42).

An intricate network of actin filaments was observed to span the protoplasm in control cells (Fig. 43). These were evenly distributed throughout the protoplasm with the circular dark gaps indicating the space occupied by the organelles. Actin filaments were thin, fine and continuous, interconnecting with those around immediate areas. After 24 h of induction of rhizoid formation, the exposed area showed actin filaments as short, thick, contracted and compacted filaments (Fig. 44) concomitant with the accumulation of chloroplasts and nuclei. Because of the density of the stained actin filaments, bright signal obscured the detail of the actin network structure at the center of the aggregating protoplasm. This bright staining increased in intensity as the protoplasm continued to accumulate, with the compacted organelles occupying several layers on top of each other. As such, the short contracted actin filaments were not clearly visualized in the succeeding stages.

DISCUSSION

The present study revealed that *Valonia* cells were induced to produce rhizoids when made to face an effective substratum. Effective substrata included cellophane, glass and a living cell; whereas, agar did not work for such as an inducer. Whereas agar is highly hydrophilic, the surfaces of cellophane, glass and a living cell may have hydrophobic properties.

←

Figs 37–44. Changes in microtubule and actin arrangement during rhizoid formation.

Figs 37–42. Microtubule rearrangement during rhizoid formation.

Fig. 37. Parallel orientation of cortical microtubules (arrowheads) and perinuclear microtubules (arrows) before the start of rhizoid formation. Scale bar = 20 μm. Inset: nuclei evenly distributed over the cytoplasm. Scale bar = 20 μm.

Fig. 38. Random arrangement of cortical microtubules in area where nuclei began to aggregate (inset) about 24 h after the cell was exposed to a coverslip. Scale bars = 20 μm.

Fig. 39. Radial distribution of cortical microtubules (arrowheads) around a disklike protoplasmic assembly 36 h after the cell was exposed to a coverslip. Scale bar = 20 μm.

Fig. 40. A disklike protoplasmic assembly where nuclei gathered remarkably (inset) was bounded by dense, brushlike short microtubules (arrow) 48 h after the cell was exposed to a coverslip. Scale bars = 20 μm.

Fig. 41. A new rhizoid cell (a lenticular cell) divided by a septum from the mother cell where cortical microtubules terminated at the edge of the rhizoid cell (arrow) about 60 h after exposure to a coverslip. Scale bar = 20 μm.

Fig. 42. Cortical microtubules (arrowheads) oriented longitudinally to an elongating cone-shaped rhizoid about 5 d after the rhizoid was produced. Scale bars = 20 μm.

Figs 43, 44. Actin network before and during initiation of rhizoid formation.

Fig. 43. A complex network of actin filaments in the protoplasm before the induction of rhizoid formation (control). Scale bar = 20 μm. Inset: even distribution of nuclei in the protoplasm. Scale bar = 20 μm.

Fig. 44. Compact arrangement of a network composed of contracted actin filaments concomitant with the dense aggregation of protoplasmic mass in the exposed area after 24 hours of induction of rhizoid formation. Scale bar = 20 μm. Inset: aggregation of nuclei. Scale bar = 20 μm.

Even without the cell surface directly contacting with the substratum, rhizoids were still induced to form toward the hydrophobic substratum surface. At a distance, a cell cannot directly recognize the substratum surface; however, because of the accumulation of amorphous materials between the cell surface and the substratum, the gap can be bridged and provide a continuous mechanical stimulus to the cell. Since the amorphous materials have been shown to be comprised of monosaccharides, the type of substratum appeared to affect the amorphous materials' solubility and, consequently, their local accumulation. A hydrophilic substratum such as agar could cause dissolution of the amorphous materials upon contact, leaving little chance for the materials to accumulate between the two surfaces. On the other hand, hydrophobic materials such as glass and cellophane can allow deposition and accumulation of polysaccharides, without immediate dissolution, on substratum surfaces. This buildup and binding of amorphous materials can help the cell recognize the presence of an appropriate substratum without direct contact for the induction of rhizoid formation. As these amorphous materials are continuously produced on all surfaces of the cell, it is highly likely that without their local accumulation arising from continuous exposure to a hydrophobic substratum, no induction of rhizoid formation can be expected. On the other hand, an isolated cell wall or its extract may not work for inducing a rhizoid except if these are placed specifically in between the cell surface and a hydrophobic substratum for an extended period of time in such a way that any insoluble material placed in the same manner may serve a similar purpose of inducing rhizoid formation.

Close gaps between neighboring cells are common especially among some coenocytic algae that develop into reticulate thalli. Small tenacular cells form on neighbouring large cells and link them tightly. Since rhizoid induction was effective up to a distance of 0.7 mm between *Valonia* cells, there is a possibility that rhizoids and tenaculae might share common induction mechanisms. Several tenacular cell types are observed to occur in cladophoralean and siphonocladalean algae, such as *Microdictyon*, *Dictyospheria*, *Boodlea*, *Apjohnia* and *Valonia*, (Leliaert *et al.* 2007). In *V. fastigiata* tenacular cells, which Agardh referred to as 'fibulae' (Murray & Boodle 1888), are numerous, highly conspicuous and are produced between lateral cells. According to these reports, tenaculae are for attachment of branches to each other to provide close packing and reinforcement. On the other hand, Ikegaya *et al*. (2008) reported that absence of contact stimulation failed to promote rhizoids in *Spirogyra*; however, distance of the cell from the substratum probably exceeded the range needed for rhizoid induction.

Various subtle changes transpire during prerhizoid formation, such as tip growth in *Spirogyra* (Yoshida *et al.* 2003) and the development of actin patches in *Pelvetia compressa* zygotes (Alessa & Kropf 1999). However, the reversibility of such initial changes is unclear. It took 36–48 h of continuous contactless exposure for *Valonia* rhizoids to form, coinciding with the occurrence of radial arrangement of CMTs and dense aggregation of chloroplasts and nuclei. Removal of substratum before this time span and consequent failure to produce rhizoids indicates that accumulation of chloroplasts and nuclei is reversible provided that CMT rearrangement has not started.

CMTs were noted to function in maintaining cellular polarity in the tip growing coenocytic green alga *Chamaedoris* (Okuda *et al.* 1997a). However, in *Valonia*, CMTs seem to play distinct roles in both lateral cell and rhizoid differentiation. CMTs changed in arrangement from parallel to meridional (radial) before protoplasmic aggregation became remarkable during rhizoid formation in the present study. The fact that protoplasm aggregates within the area where meridional CMTs were arranged was also reported during the formation of lateral branch cells in *V. utricularis* (Okuda *et al.* 1997b). These suggest that the organization of meridional CMTs may define the area where protoplasmic movement occurs. In species of *Valonia*, lateral branch cells and rhizoids develop from large and small lenticular cells, respectively (Bold and Wynne 1985). Protoplasm of a lenticular cell is divided from that of the mother cell by a septum (Okuda *et al.* 1997b). However, lateral cells were never induced by placing substrata, unlike in the case of rhizoids in the present study.

Actin filaments appeared to facilitate the aggregation of the chloroplasts and nuclei during rhizoid initiation wherein thin actin filaments contracted and formed short bundles as the protoplasm around the rhizoid formation area became dense and compacted. Although microtubules are also known to function in protoplasmic streaming (Sato *et al.* 2001), numerous reports illustrate the central role of actin in organelle movement (Kachar & Reese 1988; Menzel & Elsner-Menzel 1989; Takagi 2003; Shimmen 2006). In *Spirogyra*, a meshlike actin structure has been suggested to be crucial in rhizoid elongation (Yoshida & Shimmen 2009). In characean algae, the actin cytoskeleton has been shown to be responsible for controlling cell shape and growth direction of protonemata and rhizoids (Braun & Wasteneys 1998).

While these protoplasmic changes occur, the surfaces of cells also undergo transformation through secretion of amorphous materials containing sugars that constitute a large proportion of mucilage as reported previously in various algal species. Extracellular sulfated and carboxylated polysaccharides have been detected in *Laurencia arbuscula* (Bouzon & Ouriques 2007) and 31 other species of freshwater red algae (Sheath & Cole 1990). According to Forbes & Hallam (1979), a sulfated polysaccharide serves as an adhesive and is constantly released during rhizoid formation in *Hormosira banksii*. Polysaccharides with vicinal hydroxyl groups were also reported in previous studies on red algae (Bhatia & Vijayaraghavan 1995; Bouzon & Ouriques 2007). At least four sugar residues were detected by lectin-FITC, but only three were confirmed by TLC. Glucose or mannose were not detected by TLC but may have been present only in trace amounts that were faintly stained by PSA-FITC. Presence of glucose, mannose, galactose, N-acetyl-galactosamine and N-acetyl-glucosamine has been previously described in the above studies on red algae mucilage, although the detection of an additional monosaccharide with a high R_f in the present study is not entirely unexpected, as other high-R_f mono-

saccharides, such as ribose or rhamnose, have been detected in the mucilage of another green alga, *Chlorella sorokiniana* (Watanabe *et al.* 2006). Nevertheless, these sugars appeared to comprise the bulk of the amorphous materials that accumulated between the surfaces of the cell and the substratum. These accumulated sugars could have provided a continuous stimulus for cell surfaces even without directly contacting with substrata during induction of rhizoid formation.

When each of the detected sugars was applied to the culture medium for cells placed next to each other, no inductive or inhibitory effect on rhizoid formation was observed. This could be attributed to the fact that these sugars were dissolved and did not form a localized accumulation of materials between the adjacent cell surfaces that could have served as a continuous mechanical stimulus for the induction of rhizoid formation. A high concentration of unspecific lectins inhibited rhizoid formation, possibly by interfering with the deposition and accumulation of the polysaccharides on cell surfaces, while very low concentrations showed no specific inductive effect on rhizoid formation. In *Micrasterias*, production of sugars transported by vesicles from Golgi bodies to the primary cell wall was traced using lectins (Brosch-Salomon *et al.* 1998). Another report illustrated that two pathways from the dictyosome are involved: (1) through the cell wall pore apparatus via mucilage vesicle (MV) and (2) directly through the cell wall via MV and dark vesicle (Oertel *et al.* 2004). Algal mucilage is essential not only in the attachment of rhizoids to substratum (Braten 1975; Fletcher & Callow 1992; Vreeland *et al.* 1993; Bouzon *et al.* 2006) but also in numerous other vegetative and reproductive functions (Boney 1981). Moreover, it appears that mucilage can be directed anywhere toward a nearby substratum. Some of these sugars were retained on the surface of lenticular cells before and after hapteron formation in the present study.

In summary, a cell's proximity to a substratum results in the local accumulation of amorphous materials followed by the determination of the position of rhizoid induction. The determined spot undergoes local aggregation of protoplasm concomitant with CMT rearrangement and actin filament contraction followed by the production of more amorphous materials and, eventually, formation of a complete rhizoid. This sequence of processes provides a comprehensive perspective on *Valonia* rhizoid formation that can be useful in understanding algal cell differentiation and morphogenesis.

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