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Cytomorphogenesis in coenocytic green algae. VI. Dynamic changes in the actin cytoskeleton during wound-induced contraction in *Valonia utricularis*

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The coenocytic green alga *Valonia utricularis* was used to investigate the induction of protoplasmic contractions and concurrent actin cytoskeletal changes. Immunofluorescence microscopy revealed in intact cells reticulate action filaments (AFs) that are distributed throughout a protoplasm zone containing chloroplasts. Upon wounding cells, protoplasm rapidly retracted from the wound, around which fluffy AFs were left. Rapid retraction was followed by two kinds of slow contractions. Centripetal contraction made the wound gradually close, and AFs appeared circumferentially at the edge of the contracting protoplasm. The other contraction occurred perpendicularly to the wound in parietal protoplasm, where longitudinally oriented AFs developed. Both circumferentilly and longitudinally oriented AFs disappeared after the wound was healed. Reticulate AFs were continuously present during and after wound healing. Mycalolide B destroyed AFs and completely inhibited wound-induced contraction, whereas a microtubule-depolymerizing agent APM had no effect on contraction. These results suggest that AFs are involved in wound-induced contraction in *Valonia utricularis*. The pattern of wound-induced contraction and the development of actin cytoskeletons in *Valonia utricularis* were compared with those in other siphonous green algae.

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Introduction

Siphonous green algae are composed of just a few very large cells or of a single giant cell. Such algal cells have the ability to heal wounds against various physical damages (for reviews, see Menzel 1988; La Claire 1992). In the orders Caulerpales and Dasycladales, wounded cells quickly seal the wound with insoluble plugs, while the protoplasm retracts from the wound sites (Burr & West 1971). La Claire (1982) reported several different types of wound responses in the Siphonocladales. In most of these algae, no wound plug forms. For example, in *Ernodesmis* mechanical wounding induces contraction of the protoplasm that retract it from the wound site and close the wound.

Immunofluorescence studies have shown that wound-induced contractions in siphonous green algae involve the actin cytoskeleton. Menzel & Elsner-Menzel (1989) reported in the dasycladalean alga *Acetabularia* that the actin cytoskeleton orients longitudinally to the cell axis in intact cells, but it is quickly rearranged upon wounding by forming bundles that orient circumferentially around the contracting area. In the siphonocladalean alga *Ernodesmis* and *Boergesenia*, La Claire (1989) reported that only fine punctate labeling of actin occurs in intact cells, but in cells upon wounding actin-containing arrays rapidly appear. These arrays develop during wound-induced contraction and disappear after healing is

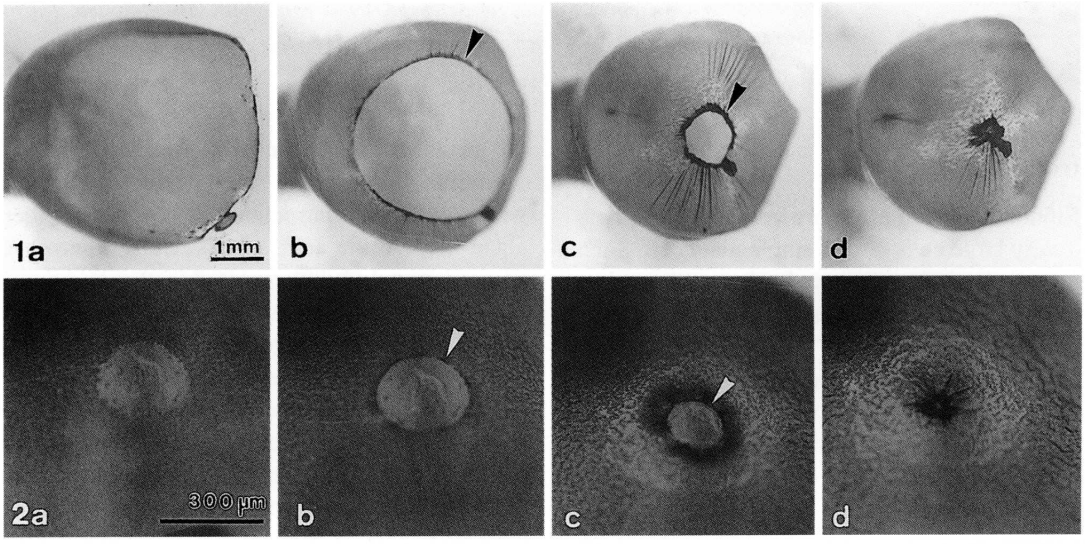
complete. La Claire (1991) reported that myosin also appears concomitantly with wound-induced contraction and becomes co-localized with the actin arrays, and suggested that an actin-myosin interaction may mediate the contraction.

Nawata et al. (1993) reported two distinct wound responses in the single species *Ventricaria ventricosa*: a centripetal aggregation of protoplasm around the wound resulting in healing; a conversion of protoplasm into numerous small protoplasts. Which occurs depends on the size of the wound in this case. In the present study, we used *Valonia utricularis* that is a coenocytic green algal species belonging to the Siphonocladales. We examined how wound-induced contraction occurs when the cells are cut or when they are punctured by a fine needle. We demonstrated immunofluorescence of actin in intact, contracting and healed cells and found three distinct actin cytoskeletal systems present in this species.

Materials and Methods

Valonia utricularis (strain LB 2357) was obtained from UTEX, the algal culture collection at the University of Texas at Austin. The Thalli were cultured in nutrient-enriched sea water medium (PES medium) (Starr and Zeikus 1993), under 22℃ and a 14:10 h light : dark cycle. Cool white fluorescent lamps, with an intensity of ca. 1 W/m^2, were used. A cell was wounded either with a microdissecting scissor by cutting it into two pieces or with a fine needle tip by perforating it. The wounded cell was allowed to contract in the medium.

Chemical fixation, removal and adhesion of the protoplasm to coverslips, immunolabeling and microscopy procedures were essentially the same as those previously described for tubulin localization (Okuda et al. 1997). Double staining for actin and tubulin was employed in the present study (see also Okuda et al. 2000). A 50 μL primary antibody mixture containing rabbit anti-actin antibodies (A2066, Sigma Chemical Co., St. Louis, Mo., USA, diluted 1 : 50) and rat anti-tubulin antibodies (YL1/2, Sera Labs, Crawley Down, England, diluted 1 : 50) was applied to fixed specimens on coverslips. The secondary antibodies used for these primary antibodies were FITC-conjugated goat anti-rabbit IgG (F0382, Sigma Chemical Co., diluted 1 : 50) and TRITC-conjugated goat anti-rat IgG (55763, Cappel Products, USA, diluted 1 : 50). Fluores-



Figs. 1 & 2. Time courses of wound healing in *Valonia* cells. 1, one of two pieces into which a cell was cut one (a), 15 (b), 20 (c) and 50 min (d) after wounding. Arrowheads showing a dark green belt. 2, A cell wounded by perforation. one (a), 5 (b), 10 (c), 30 min (d) after wounding. Arrowheads showing a dark green ring.

cence from FITC and TRITC was observed with an epifluorescence microscope (BX50-FLA, Olympus, Tokyo, Japan). The filter set for FITC consists of a 460-490-nm band pass excitation filter and a 515-550-nm barrier filter, and that for TRITC is a 520-550-nm exciter with a 590/35-nm barrier filter.

Mycalolide B (ML-B) is a toxin to irreversibly destroy the organization of actin filaments in plants (Simmen et al. 1995). A 10 mM stock solution of ML-B (Wako Pure Chemical Industries, Ltd. Osaka, Japan) was prepared in dimethyl sulfoxide. The stock solution was diluted with the culture medium to give a final ML-B concentration of 50 μM. Cells were pre-incubated in 10 mL test solution (or in control solutions containing medium and 0.5% dimethyl sulfoxide) for 30 min prior to wounded by a fine needle. Wounded cells were allowed to contract for 30 min and then fixed for immunofluorescence microscopy. Similar experiments were carried out with amiprophos methyl (APM) that depolymerizes microtubules. The stock solution was prepared as described by Okuda et al. (1993). Cells were pre-incubated in culture medium containing 10 μM APM for 72 h to achieve maximal depolymerization of the microtubules prior to wounding.

Results

A *Valonia* cell was wounded by cutting it into two pieces. In each of these pieces, wound-induced contraction began at the cut edge of the protoplasm (Fig. 1a-d). Rapid contraction occurred within a few seconds (Fig.1a), and then simultaneous, symmetrical retraction of the cut edge gradually led to the formation of a dark green belt by accumulation of chloroplasts (Fig. 1b). The belt then detached from the cell wall and contracted centripetally (Fig. 1b), while parietal protoplasm contracted longitudinally from the wound site. When the diameter of the contracting belt decreased, protoplasm adjacent to the belt was pulled off the cell wall and stretched toward the belt (Fig. 1c). It took about 50 min under a room temperature condition before a continuous layer of protoplasm closed the opening of the wound (Fig. 1d). In a cell that was wounded by inserting a needle tip, contraction occurred in a relatively small area (Fig. 2a-d). Soon after perforation, protoplasm retracted radially from the hole and a round clear zone appeared (Fig. 2a). Chloroplasts aggregated and encircled the clear zone, forming a dark green ring (Fig. 2b). The ring contracted centripetally (Fig. 2c) until the wound was closed (Fig. 2d). A whole protoplasm layer of the

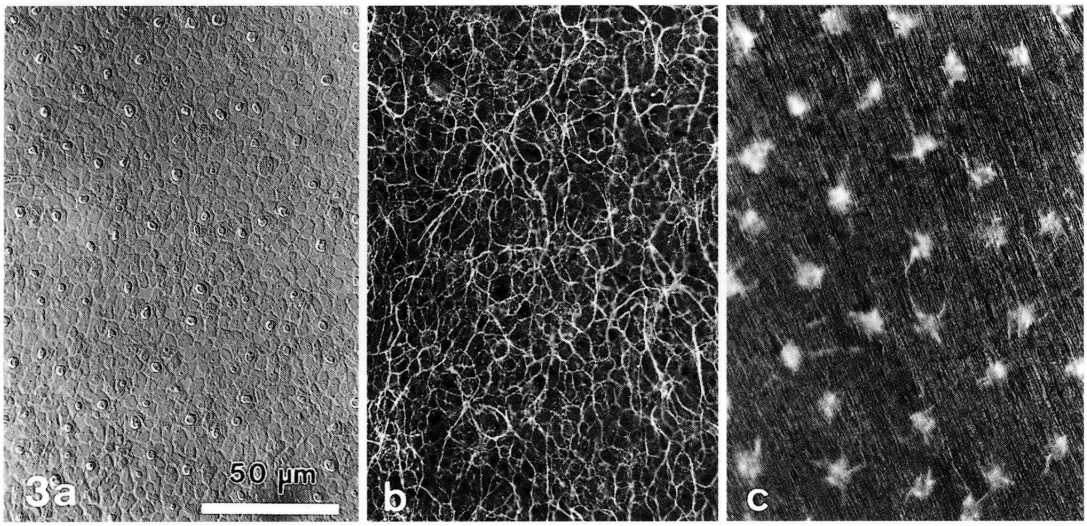
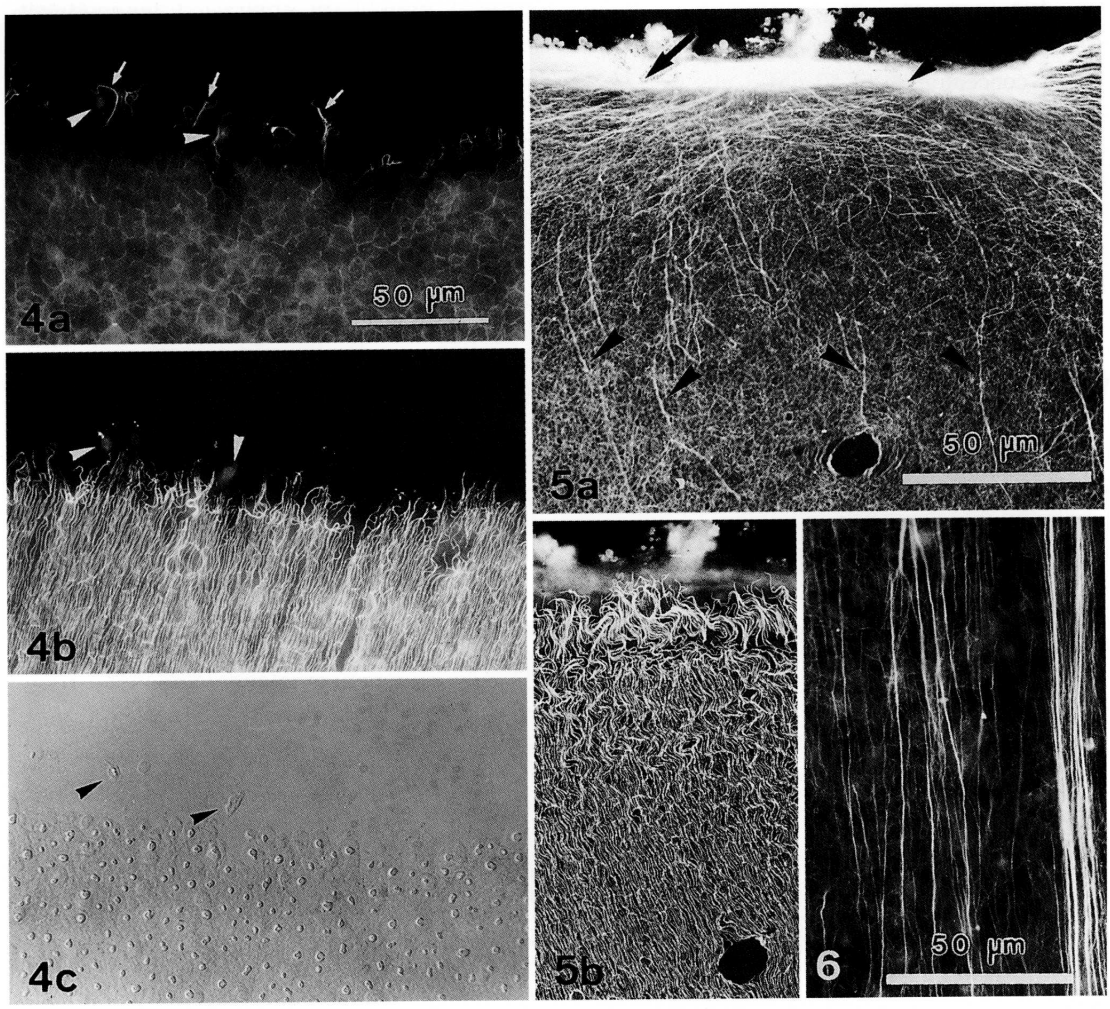


Fig. 3. Images of differential interference contrast (a) and double-label immunofluorescence of actin (b) and tubulin (c) in the same area of an intact *Valonia* cell.

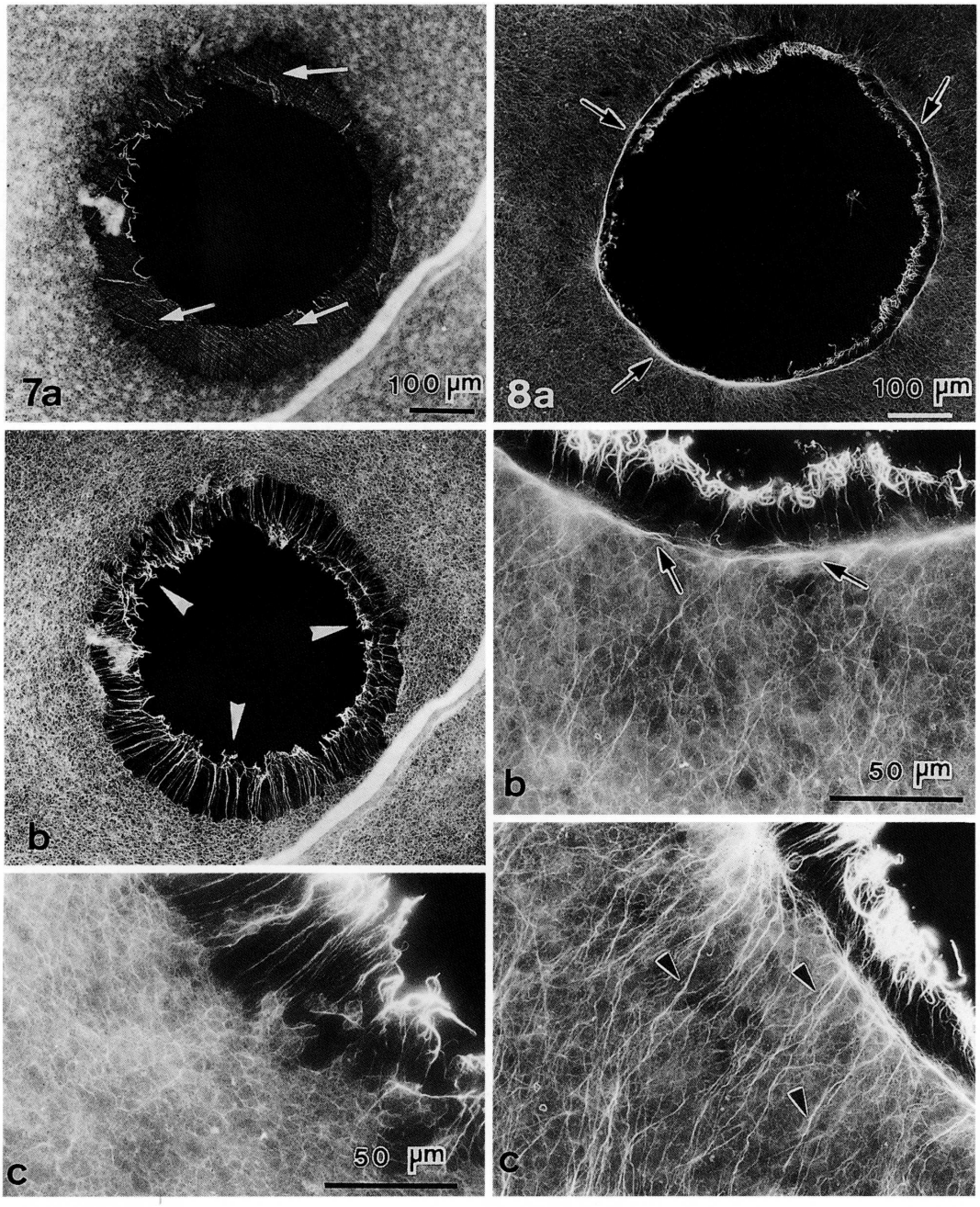
wounded cell detached slightly from the cell wall in places and appeared to corrugate, probably due to the loss of turgor. In the above two cases, no wound plug was formed.

In intact cells, chloroplasts were distributed evenly under the plasma membrane and formed a layer (Fig. 3a). Nuclei were distributed in the zone of protoplasm below the zone containing the chloroplast layer. Immunofluorescence of actin exhibited reticulate and convoluted patterns in the zone containing the chloroplast layer (Fig. 3b). Actin filaments (AFs) were contact with the chloroplasts, ramified extensively and thus formed a three-dimensional network. Two distinct arrays of microtubules (MTs) were present (Fig. 3c), parallel cortical MTs just on the inner side of the plasma membrane and perinuclear MTs surrounding the nucleus.

Thirty seconds after cells were cut, the time when the protoplasm rapidly retracted from the



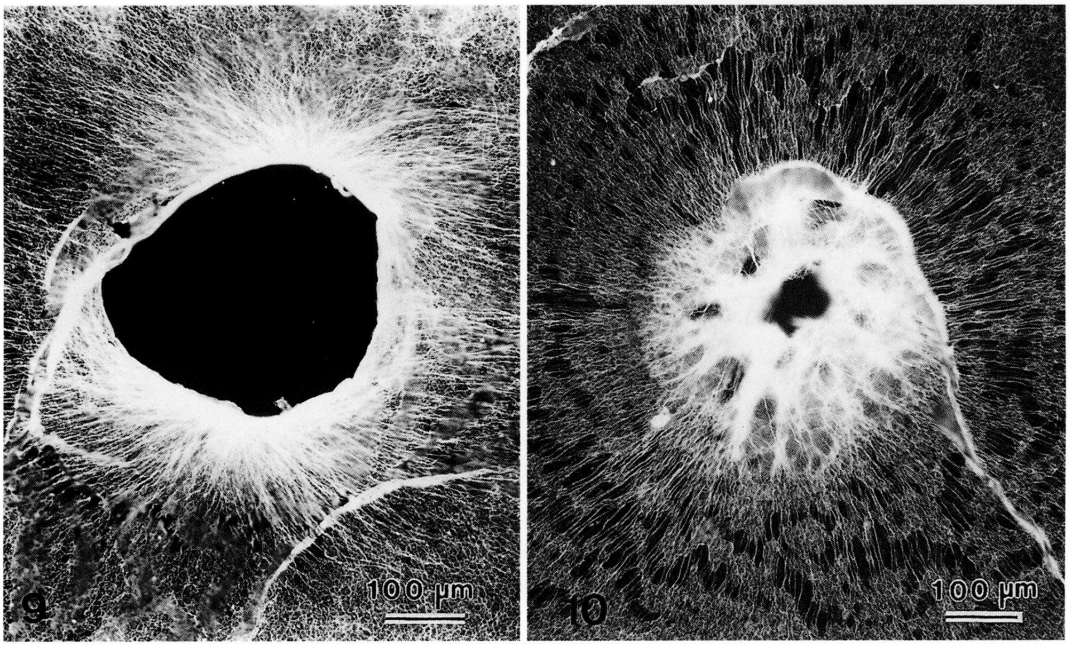
Figs. 4-6. Immunofluorescence images of AFs and MTs in *Valonia* cells wounded by cutting. 4, Images of double-label immunofluorescence of actin (a) and tubulin (b) and differential interference contrast (c) in the same area of a wounded cell 30 sec after wounding. Isolated chloroplasts (arrowheads) associated with fluffy AFs (arrows). 5, Double-label immunofluorescence of actin (a) and tubulin (b) in a wounded cell 10 min after wounding. Circumferentially oriented AFs (arrows) along the edge of contracting protoplasm and longitudinally oriented AFs (arrowheads). 6, Longitudinal bundles of AFs in parietal protoplasm of a wounded cell 20 min after wounding.



Figs. 7 & 8. Immunofluorescence images of AFs and MTs in *Valonia* cells wounded by perforation. 7, Double-label immnunofluorescence of tubulin (a) and actin (b) in a wounded cell 30 sec after wounding. Cortical MTs (arrows), and fluffy AFs (arrowheads) connecting with reticulate AFs (c). 8a–c, Actin immunofluorescence in a wounded cell 10 min after wounding. AFs orienting circumferentially around the wound (arrows) and AFs orienting perpendicularly to the wound (arrowheads). b & c in the same magnification.

wound site, short, fluffy AFs were observed along the cut edge (Fig. 4a). Some isolated chloroplasts were associated with the AFs (Fig. 4a, c). The AFs extended from parts of a reticulate actin network distributed in the retracting protoplasm. They disappeared about 20 min after cells were cut. In this early contracting stage, arrangement of cortical MTs was perturbed only at the cut edge (Fig. 4b). When the contracting belt formed 3 min after cells were cut (cf. Fig. 1b), AFs began to appear circumferentially along the belt. They developed into bundles remarkably at the rim of the belt as centripetal contraction proceeded (Fig. 5a). At the same time, another type of AF arrays also appeared to orient longitudinally to the cut end. The AFs occurred in parietal protoplasm first and then extended to the vicinity of the contracting belt (Fig. 5a). Longitudinally oriented AFs increased in density and developed into thicker bundles during longitudinal contraction (Fig. 6). Longitudinal AFs were superimposed over the reticulate AFs and situated in almost the same level as cortical MTs lay. Cortical MTs were highly convoluted in the contracting protoplasm (Fig. 5b). Longitudinal and circumferential AFs disappeared about 50 min and 4 h after wound closure, respectively. A reticulate actin network was present continuously during and after contraction.

Fluffy AFs and two types of AF arrays distinct in distribution appeared also in cells wounded by perforation. Immediately upon perforation, the round clear zone is formed by radial contraction as mentioned above (cf. Fig. 2a). At that time, most of protoplasmic contents retracted from a hole of the wound (Fig. 7a). However, the plasma membrane was actually left in the peripheral area of the hole, since parallel cortical MTs were present in the area (Fig. 7a). This indicates that the plasma membrane is not directly involved in rapid contraction occurring in this initial stage. Fluffy AFs radially oriented in the peripheral area (Fig. 7b) and extended from reticulate AFs in rapidly retracting protoplasm (Fig. 7c). Fluffy AFs were still present 10 min after perforation, although their tip portions became short and convoluted (Fig. 8a–c). Circumferentially oriented AFs appeared along the rim of centripetally contracting protoplasm (Fig. 8b). AFs that oriented perpendicularly to



Figs. 9 & 10. Actin immunofluorescence in *Valonia* cells wounded by perforation. Wounded cells 20 min (9) and 40 min (10) after wounding.

the rim also appeared (Fig. 8c). As the diameter of the hole decreased by centripetal contraction, these AFs increased in density and developed into bundles (Fig. 9). When the wound almost closed, circumferential AFs almost disappeared, but AFs orienting radially from the site of wound closure remained for several hours (Fig. 10). A reticulate actin network in the chloroplast zone was persistently present during and after contraction.

No wound-induced contraction occurred in cells pre-incubated with ML-B. In these cells, MTs were observed normally, but AFs were destroyed completely (not shown). APM had no effect on contraction. In cells pre-incubated with APM, MTs were depolymerized completely, but development of AFs during wound-induced contraction was identical to controls (not shown).

Discussion

Wound response in *Valonia utricularis* essentially corresponds to that in *Ernodesmis verticillata* and *Struvea* spp. described by La Claire (1982). The wound-induced contraction in *Valonia utricularis* involves at least three different motions of protoplasm: rapid retraction from the wound that stops within several seconds; slow, centripetal contraction that closes the opening of the wound; slow longitudinal contraction that occurs perpendicularly to the wound. The third motion does not remarkably occur in the cells wounded by puncturing. In wounded cells of *Valonia*, the protoplasm never divides into small protoplasts, unlike in *Boergesenia* (Enomoto & Hirose 1972) and in *Ventricaria ventricosa* (Nawata et al. 1993). Apart from centripetal and longitudinal contractions in *Ernodesmis* described by La Claire (1982), the present study showed the occurrence of rapid contraction immediately after wounding in *Valonia*. Centripetal and longitudinal contractile motions have reported also in members of the Caulerpales and Dasycladales such as *Bryopsis* (Burr & West 1971), *Caulerpa* (Dreher et al. 1978) and *Acetabularia* (Menzel & Elsner-Menzel 1989). In these cases, wound-induced contraction takes only a few seconds or less than three minutes (Menzel 1988). However, in the siphonocladalean algae including *Valonia utricularis* (the present study) and *Ernodesmis* (La Claire 1982), contraction proceeds much slowly and it takes about an hour to close the wound.

It is evident from the absence of any effect of APM, an MT-depolymerizing agent, that MTs are not required for wound-induced contraction in *Valonia utricularis*. This corresponds to the results reported in *Ernodesmis* (La Claire 1987) and *Acetabularia* (Menzel & Elsner-Menzel 1989). Cytochalasin D that is known to cause depolymerization of AFs inhibits wound-induced contraction in *Acetabularia* (Menzel & Elsner-Menzel 1989), but it has no effect on contraction in *Ernodesmis* (La Claire 1989). Cytochalasin D modifies the organization of AFs and may not depolymerize AFs in some siphonocladalean algae (unpublished data). In the present study, we showed that ML-B destroys AFs and eventually inhibits wound-induced contraction in *Valonia utricularis*. This indicates that AFs are essential for contraction in this species.

It has been reported that conspicuous AFs appear during wound-induced contraction in the siphonocladalean algae *Ernodesmis* and *Boergesenia* (La Claire 1989) and in the dasycladalean alga *Acetabularia* (Menzel & Elsner-Menzel 1989). In *Acetabularia* restructuring of preexisting AFs is followed by the formation of AF bundles around the wound, whereas in *Ernodesmis* and *Boergesenia* AFs are not present till the cells are wounded. In the present study, we showed that in *Valonia* a reticulate actin cytoskeleton is distributed in the chloroplast zone in the intact cells. Immediately after the cells are wounded, fluffy AFs occur at the peripheries of rapid retracting protoplasm. Parts of reticulate AFs might be extended by the initial, rapid retraction and left as fluffy AFs on the plasma membrane. The present study showed circumferentially oriented AFs occurring in the Siphonocladales first. Circumferential AFs in *Valonia* appear in the edge of centripetally contracting protoplasm. This is also in the case of *Acetabularia* (Menzel & Elsner-Menzel 1989). It is unclear in *Valonia* whether circumferential AFs are converted from preexisting reticulate AFs. However, longitudinal AFs in *Valonia* that orient perpendicularly to the wound seem to develop independently on reticulate AFs, since

longitudinal AFs are superimposed over reticulate AFs and develop on the plasma membrane. In *Valonia* and *Ernodesmis* (La Claire 1989), longitudinal contraction occurs in an integral part of the cells after the cells are cut. Longitudinal AFs are distributed widely in the longitudinally contracting protoplasm in these species. However, in *Acetabularia* no such longitudinal AFs are induced by wounding, although in the intact cells other longitudinal AFs are involved in organelle movement (Menzel & Elsner-Menzel 1989). Wound response in *Acetabularia* seems to involve only centripetally contraction and to be restricted to the wound site where circumferential AFs are organized.

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佐藤　健・櫻井納美・奥田一雄：多核緑藻の細胞形態形成．VI．バロニアにおいて傷害によって誘導される原形質収縮の間におこるアクチン細胞骨格の動的な変化

細胞の切断，または細胞に小孔を開けることによって原形質の収縮が誘導された．原形質の収縮は3つの過程があった．傷害直後で原形質が傷害部から急速に退行し，それに続いて，傷害部の孔を閉じる求心的な収縮と傷害部から遠ざかる縦方向の収縮が徐々に起こった．しかし，小孔が開けられた細胞の原形質は，ほとんど縦方向に収縮しなかった．抗アクチン抗体を用いてアクチンフィラメント（AFs）を間接蛍光抗体法によって観察した．無傷および傷害を受けた細胞では，葉緑体を含む原形質の層に網目状に分布するAFsが常に存在した．傷害直後の傷害部の周縁部には短いAFsが残った．傷害後2種類のAFsが出現した．1つは求心的に収縮する原形質の周辺を取り巻き，もう1つは傷害部に対して縦に配向した．これら分布と配向が異なる2種類のAFsは，傷害部が閉塞した後に消失した．ミカライドBはAFsを破壊

して原形質の収縮を阻害したが，微小管破壊剤アミプロフォスメチルは原形質の収縮に対して影響を及ぼさなかった．以上の結果は，傷害によって誘導される原形質の収縮にAFsが関与することを示唆する．