Phycologia (2005) Volume 44 (2), 156–162 Published 26 April 2005

Decalcification during epithallial cell turnover in *Jania adhaerens* (Corallinales, Rhodophyta)

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C.M. PUESCHEL, B.L. JUDSON AND S. WEGEBERG. 2005. Decalcification during epithallial cell turnover in *Jania adhaerens* (Corallinales, Rhodophyta). *Phycologia* 44: 156–162.

Epithallial cells of the coralline red algae are characterized by structural specializations that include deep invaginations of the distal cell surface, and by a unique developmental pattern that culminates in senescence, shedding and replacement of the cells. Combined scanning and transmission electron microscopic study of epithallial cell differentiation in the geniculate coralline alga *Jania adhaerens* suggests that some of the unusual features of epithallial cell structure may be related to the need for localized wall decalcification as part of epithallial cell replacement. Distal wall ingrowths begin to form on the cells whose cleavage eventually gives rise distally to new epithallial cells. After the distal wall ingrowths form, the overlying crosswall becomes rich in organic material. For this organic wall material to be deposited into the existing crosswall, the wall must first be decalcified; therefore, the presence of abundant organic material in the crosswall provides a marker of localized decalcification. The location and time of origin of distal wall ingrowths suggest a connection between the ingrowths and the coordinated processes of localized secretion of organic wall material and decalcification of the overlying cell wall in preparation for the movement of the young epithallial cell into a new location relative to the surrounding calcified matrix.

INTRODUCTION

The surfaces of most pseudoparenchymatous red algae are covered by apical cells that can divide to thicken and enlarge the thallus. By contrast, thalli of members of the Corallinales are covered by epithallial cells, which constitute one of the most distinctive cell types in the red algae. Epithallial cells develop a variety of unusual specializations before undergoing senescence and shedding in a programmatic fashion (Borowitzka & Vesk 1978; Pueschel *et al.* 1996; Wegeberg & Pueschel 2002). The origin of epithallial cells is also unlike that of apical cells; each epithallial cell is derived from a subtending initial cell on the same filament. Typically, the initial cells of separate filaments collectively form a laterally contiguous, one cell thick layer of initial cells in the outer cortex, and each initial cell cleaves cortex cells from its proximal face and epithallial cells from its distal face (Johansen 1981).

One of the most distinctive features of epithallial cells is the common occurrence of prominent invaginations of the distal cell surface (e.g. Borowitzka & Vesk 1978; Bressan *et al.* 1981). These invaginations are filled by wall material and hence are often referred to as distal wall ingrowths. The presence of ingrowths on the distal cell wall of senescent and dead epithallial cells (Pueschel & Miller 1996; Pueschel *et al.* 1996; Pueschel & Keats 1997) supports the interpretation of these distal wall features as persistent structures, rather than transient features associated with wall secretion (Giraud & Cabioch 1976; Cabioch & Giraud 1986).

The distinctive nature of epithallial cell walls has led to several proposals of possible functions of the ingrowths and these ideas are strongly connected to particular notions about the function of epithallial cells. Epithallial cells have been compared to transfer cells of higher plants (Borowitzka & Vesk 1978), whose highly infolded cell surfaces provide increased area for nutrient uptake and transfer (Pate & Gunning 1972; Gunning 1977). Borowitzka & Vesk (1978) proposed that convolutions enhance nutrient uptake by the distal walls of epithallial cells. Bressan *et al.* (1981) agreed that epithallial cells function as transfer cells and suggested that the cell invaginations were involved in calcium absorption from seawater. In addition to the physiological functions postulated for epithallial cells, ecological roles have been proposed, such as sloughing to remove surface fouling (e.g. Steneck 1982) and protection of initial cells from grazing (Steneck 1985, 1986). Some field observations and experimental evidence are consistent with antifouling activity (Masaki *et al.* 1984; Johnson & Mann 1986; Keats *et al.* 1997). Although these various ecological functions are consistent with epithallial cell behaviour, they offer no explanation of the distinctive morphological features of epithallial cells.

Being encased in mineralized cell walls provides an obstacle to cell growth and to movement of young epithallial cells towards the thallus surface during replacement of senescent epithallial cells. Localized decalcification is necessary to accommodate epithallial cell dynamics, and we found that the sites of decalcification in the geniculate alga *Jania adhaerens* J.V. Lamouroux are made visible by the secretion of organic wall material to fill the decalcified wall space. Our study identifies the developmental origin of ingrowths and shows their spatial and temporal association with inferred decalcification of the overlying crosswalls.

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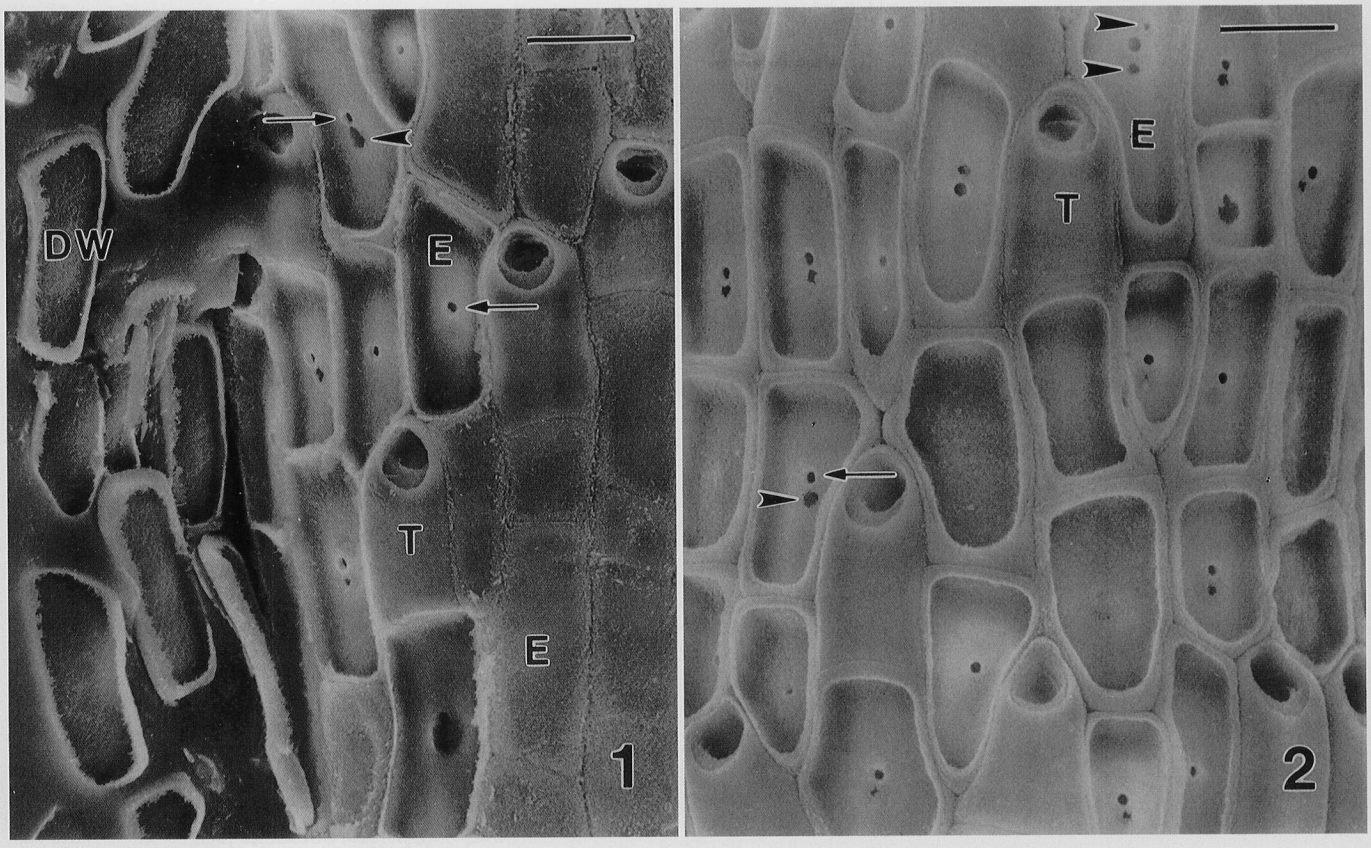


Figs 1, 2. Apical segment of *Jania adhaerens* treated with bleach to remove the organic material; SEM. The calcified distal wall of each trichocyte complex (T) remains intact. Loss of the distal calcified wall of the primary epithallial cells (E) reveals concavities, which result from calcification of the lateral and proximal cell walls. Pit plug apertures (arrows) within the concavities are smooth-rimmed and uniform in diameter, but more variable supernumerary perforations (arrowheads) are also often present. The pit plug aperture is usually to the apical side of the supernumerary perforation, but some perforations merge with the pit plug apertures. The apex of the thallus is towards the top of each figure. Scale bars = 10 μm.

Fig. 1. Distal wall (DW) of primary epithallial cells is calcified but adhesion to the specimen-mounting material mechanically removed this portion, showing concavities.

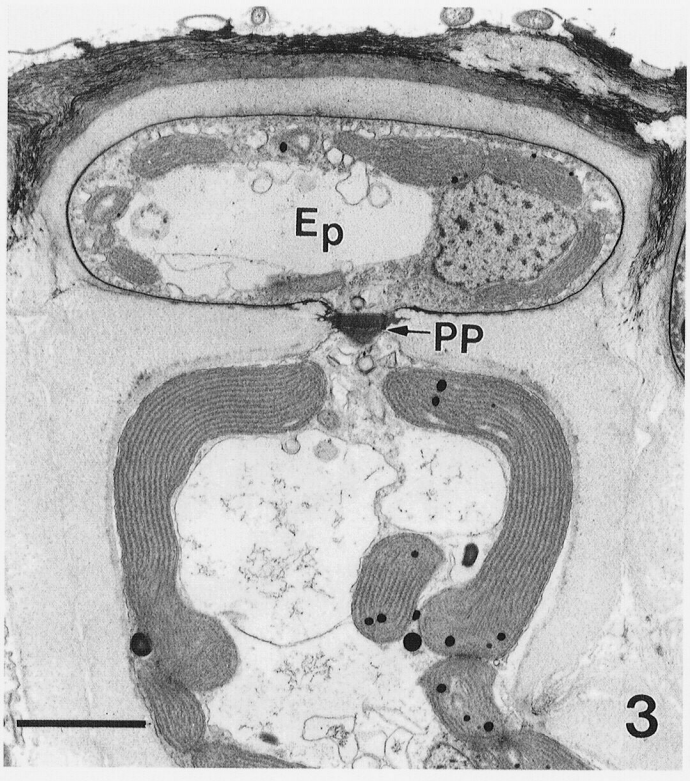
Fig. 2. Primary epithallial cells have lost their calcified distal wall, revealing perforations in most proximal walls. The pit plug aperture in some concavities is obscured or sealed. Some cells have multiple supernumerary perforations.

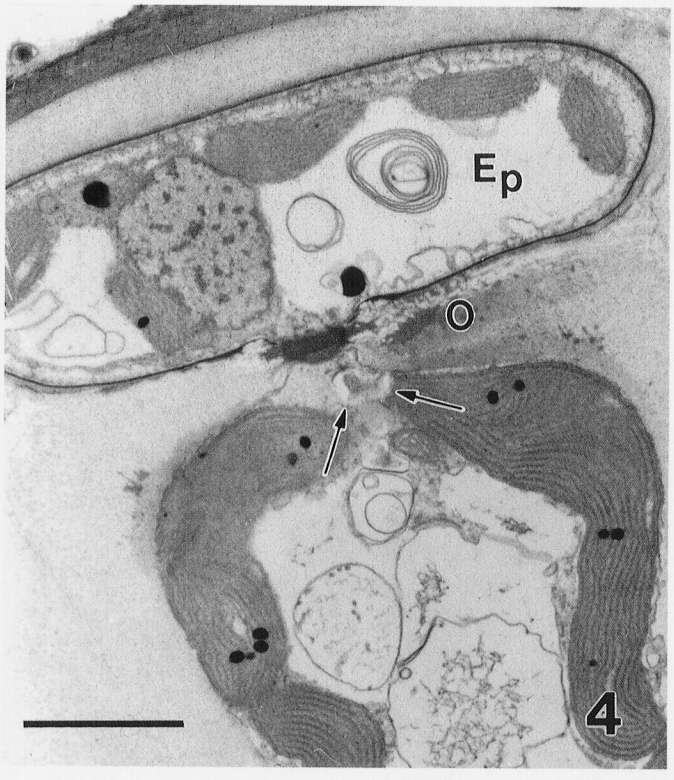
MATERIAL AND METHODS

Detailed collection and specimen preparation information for *J. adhaerens* is given in Judson & Pueschel (2002). Briefly, specimens were collected near St Ann's Bay, Jamaica and Key West, Florida, USA. For scanning electron microscopy (SEM) of the calcified skeleton, specimens fixed in 10% formalin in seawater were treated in 5% sodium hypochlorite (bleach) for 1–3 days in a 60°C oven to remove the organic material. The disarticulated segments were washed in water, rinsed in methanol, air dried, and placed on SEM stubs covered with adhesive. The specimens were coated with gold in a sputter coater (Denton Vacuum, Cherry Hill, NJ, USA) and then viewed on an Autoscan SEM (ETEC, Hayward, CA, USA) at 20 kV. Specimens for transmission electron microscopy (TEM) were fixed in 5% glutaraldehyde, 0.1 M Na cacodylate buffer (pH 7.0), and 0.2 M sucrose. The material was rinsed in buffer and decalcified in numerous changes of 5% ethylene diamine tetraacetate. After buffer rinses, the specimens were fixed in aqueous 1% osmium tetroxide, then rinsed in buffer and distilled water. Samples were dehydrated in acetone, then infiltrated and embedded in EMbed-812 resin (Electron Microscopy Sciences, Fort Washington, PA, USA). Ultrathin sections were stained in 2% uranyl acetate followed by Reynolds' lead citrate (Reynolds 1963) and examined on a Hitachi 7000 TEM (Hitachi, Tokyo, Japan).

RESULTS

Scanning electron microscopy and TEM images of *J. adhaerens* cell walls were complementary. Scanning electron microscopy images of bleach-treated specimens had organic material removed, exposing the mineral skeleton (Figs 1, 2). By contrast, the calcium carbonate, which typically occupies most of the volume of cell walls, is removed by decalcification during specimen preparation for TEM. The remaining organic component of the wall appeared as diffuse electron-dense fibrils with little electron density (Figs 3–7). However, walls that were not heavily calcified in the living thallus typically were densely fibrillar and more electron-dense after staining (Figs 4–7). The change in appearance of an existing cell wall, from having low electron density and sparse microfibrils to being rich in microfibrils, was reasoned to be the result of





Figs 3, 4. Transverse sections of *Jania adhaerens* showing primary epithallial cells (E_p) and their subtending cells; TEM. The wall immediately surrounding the epithallial cells is electron-transparent because the mineral has been removed and little stained organic material is present in this space. The absence of density differences between distal and proximal portions of the wall of the primary epithallial cells suggests that the entire wall had been calcified. Scale bars = 2 μm.

Fig. 3. Epithallial cell and its subtending cell share a pit plug (PP) and are separated by a uniformly electron-transparent crosswall.

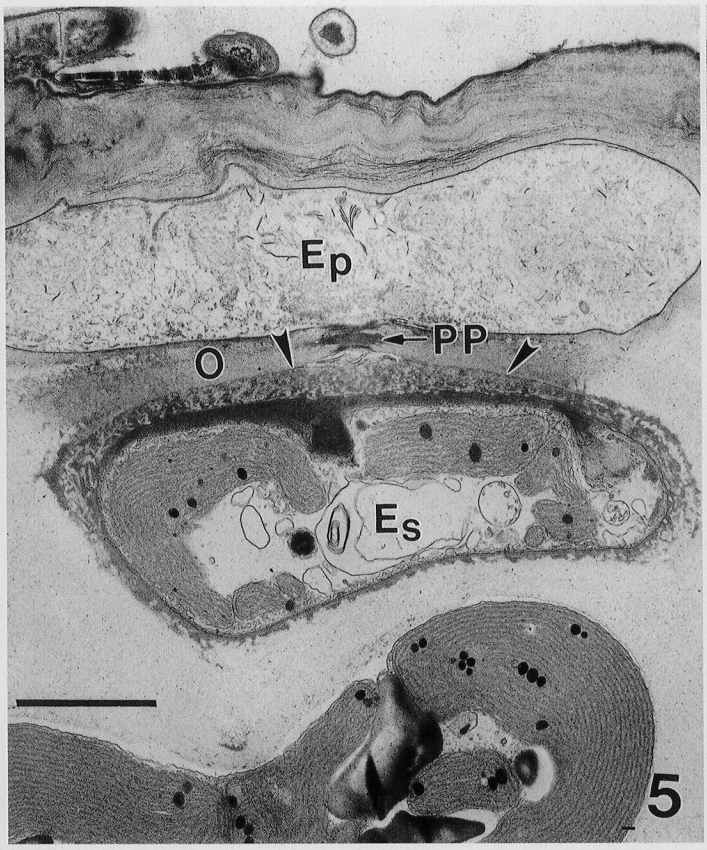
Fig. 4. The subepithallial cell has deep invaginations (arrows) on its distal surface, near the pit plug. Near the invagination, the crosswall between the epithallial cell and its subtending cell is electron-dense due to the presence of densely stained organic wall constituents (O).

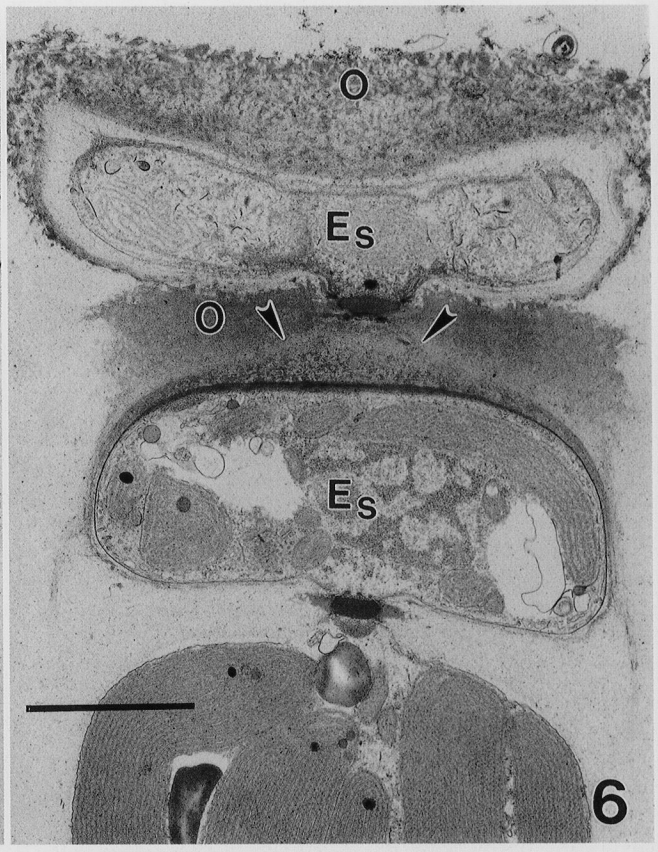
localized decalcification and secretion of new organic wall components. To place the formation of distal wall ingrowths and the decalcification–secretion episodes into proper developmental sequence, the course of epithallial cell turnover is described in brief.

The epithallial cells that formed immediately behind the apex of the axis were primary epithallial cells, in contrast to secondary epithallial cells, which were produced after sloughing of the primary epithallial cells. The distal cell walls of primary epithallial cells survived bleach treatment (Fig. 1), indicating that they were calcified while the alga was living. Figure 1 shows removal of the distal portion of some primary epithallial cell walls by mechanical manipulation, but farther from the apex, this outer wall was lost without additional disruption (Fig. 2). In the absence of the distal epithallial cell walls, air-dried or bleach-treated thalli appeared to be covered by concavities (Fig. 2). The appearance of concavities results from the persistent calcification of lateral and proximal walls of the epithallial cells. Only near the branch apices, where the calcified distal wall of the primary epithallial cells were still present, was the thallus surface relatively smooth (Fig. 1).

In bleach-treated apical segments viewed by SEM, a pore was visible in the proximal wall of the epithallial concavities (Figs 1, 2). The pit plug shared by the primary epithallial cell and its subtending cell formerly occupied this aperture. In some concavities, a second hole was present near the pit plug aperture, usually to its basipetal side (Figs 1, 2). In contrast to the smooth lip and uniform diameter of pit plug apertures, these supernumerary perforations were irregular in size and shape. Some cells had two supernumerary perforations (Fig. 2), whereas others had a single hole that was the product of the supernumerary perforation merging with or encompassing the pit plug aperture (Figs 1, 2). The supernumerary perforations result from the thinning and decalcification of the wall originally laid down between the primary epithallial cell and its subtending cell. Open pit plug apertures were not apparent in some concavities (Fig. 2), perhaps because the organic material was not fully removed by bleach, or because the aperture was occluded from below. When viewed obliquely, proximal walls without easily visible apertures had slightly convex contours in the area near the pit plug aperture, and this bulge in the wall may have been the result of a new epithallial cell pressing from below.

Transmission electron microscopy images of primary epithallial cells showed walls of uniformly low electron density surrounding the cells (Fig. 3). Presumably, this wall layer, like walls of similar density surrounding subtending cells, was calcified in the living thallus. A layer of distinctive fibrillar material covered the entire primary thallus surface (Figs 3, 4); therefore, the integrity of this continuous layer was an indication that no epithallial cell turnover had occurred. By contrast, in areas in which epithallial cells had been replaced (Figs 6, 7), the original surface layer was lost and the wall material distal to the secondary epithallial cells was dense and different





Figs 5–6. Transverse sections showing epithallial cell turnover in *Jania adhaerens*; TEM. Secondary epithallial cells (E_s) have been cleaved from the cells subtending the dead epithallial cells. The pit plug (PP) between the old and new epithallial cells has been sealed proximally by dense organic material deposited by the young epithallial cell (arrowheads); the formerly calcified crosswall between the two epithallial cells is now rich in organic material (O). Scale bars = 2 μm.

Fig. 5. The thallus surface is still intact, indicating that the dead cell being replaced was a primary epithallial cell (E_p).

Fig. 6. The dead cell has richly organic wall material on the distal surface, showing that it was a secondary epithallial cell. In turn, it is being replaced by another secondarily formed epithallial cell.

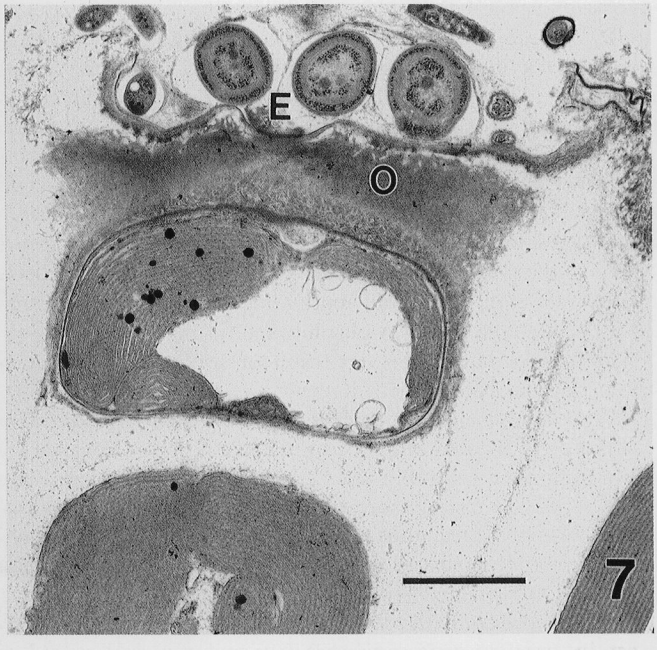


Fig. 7. A young epithallial cell is capped on its distal surface by thick layers of densely stained organic wall material (O) and by the proximal wall and bacteria-filled remnants of an older epithallial cell (E).

in texture from that of primary epithallial cells (Figs 3, 4). The lateral walls of secondary epithallial cells retained their electron transparency (Fig. 7), indicating that they remained calcified, which is also consistent with the existence of concavities seen in SEM (Figs 1, 2).

Unlike the typical corallinalean anatomy, a cell identifiable as an initial cell may not be present in *J. adhaerens* (Pueschel *et al.* 2002). Instead, the epithallial cell shares a pit plug with a medullary cell and, owing to cell fusions, several epithallial cells may share the same subtending cell. Replacement of an epithallial cell requires a highly asymmetrical division of the large subtending cell, which cleaves off a new epithallial cell bearing the pit connection to the old epithallial cell. The lobe from which a new epithallial cell is cut may begin to form distal wall ingrowths even before the new cell is cleaved off (Fig. 4).

Each epithallial cell that had normal, healthy-looking organelles was joined to its subtending cell by a pit plug that was in direct contact with the cytoplasm of both cells (Fig. 3). At a stage when the epithallial cell still appeared vital and its pit plug was in contact with cytoplasm, an invagination developed on the distal surface of the subepithallial cell, next to the pit plug (Fig. 4). This invagination, which might have been initiated by secretory vesicles, was the first sign of the

development of a distal wall ingrowth. In connection with the formation of such invaginations, the crosswall near the ingrowths became richer in densely staining organic material (Fig. 4). The great enrichment of organic material within the crosswall was an indication of previous or simultaneous removal of inorganic material from the same wall space. Although the epithallial cell at this stage appears to have been fully functional, with numerous intact chloroplasts, the decalcifying crosswall suggests that a replacement epithallial cell would soon be formed, at which time the distal wall ingrowths would become features of the newly formed epithallial cell.

By the time that a new epithallial cell was cleaved from the medullary cells, the crosswalls between the two generations of epithallial cells were richly organic (Figs 5, 6). The pit plugs that once joined the young secondary epithallial cells and the older cells that they would replace were sealed by the deposition of a thick layer of material on the proximal end of the pit plugs (Figs 5, 6). As in other corallines (Pueschel *et al.* 1996), angular and needle-shaped electron-transparent outlines were sometime apparent within the dense organic matrix of this wall material, indicating the presence of unconsolidated carbonate crystals (Fig. 5). Although the layer of material beneath the pit plug was only weakly mineralized, it may have been calcified enough to explain why SEM images failed to show an obvious, unobstructed pit plug aperture in the proximal wall of some epithallial cells that had been treated in bleach (Fig. 2).

Distal wall ingrowths in secondary epithallial cells were usually present beneath the broad cap of material that proximally sealed the pit plug shared with the dead or dying distal cell (Fig. 5). Not every plane of section through a cell would be expected to encounter an ingrowth, and they are more commonly seen in longitudinal than in transverse views, so absence of ingrowths cannot be inferred on the basis of individual sections (Fig. 6). Eventually, the dead epithallial cells were lost, and only a layer of wall material from the proximal face of the old epithallial cells persisted (Fig. 7).

DISCUSSION

Pervasive wall calcification in corallinalean algae creates developmental challenges not shared by uncalcified or weakly calcified red algae. A unique component of calcification in corallines is that, for most of the thallus, meristematic activity is subapical, rather than apical on filaments forming the cortex. For a newly formed epithallial cell to move to the thallus surface, the mineral skeleton between the old and new epithallial cells first must be removed. The principal goal of this study was to demonstrate that this process can be detected by the appearance of densely microfibrillar wall material in what had been a fully mineralized crosswall. Wegeberg & Pueschel (2002) briefly noted such demineralization of crosswalls in *Lithothamnion glaciale* Kjellman.

The present study also demonstrates that primary and secondary epithallial cells differ in wall calcification. Judson & Pueschel (2002) distinguished between primary and secondary epithallial cells of *J. adhaerens,* but this distinction has not been made in other ultrastructural studies of epithallial cells (e.g. Giraud & Cabioch 1976; Borowitzka & Vesk 1978; Cabioch & Giraud 1986; Pueschel & Miller 1996; Pueschel *et al.* 1996). Future studies should address whether this distinction is important.

The specializations of corallinalean epithallial cells are unique within the red algae and therefore probably have a unique function. As noted by Borowitzka & Vesk (1978), the distal wall ingrowths of epithallial cells bear some structural resemblance to transfer cells of land plants (Pate & Gunning 1972) and some green algae (Graham & Wilcox 1983). Transfer cells are implicated in nutrient uptake or transfer (Pate & Gunning 1972; Gunning 1977). However, the specimens of *J. adhaerens* examined in this study had abundant hair cells, which could be over 150 μm long (Judson & Pueschel 2002). The projecting morphology and large surface area of hair cells in many red algae provide access to substantial volumes of moving water, and very probably are involved in nutrient uptake (e.g. Oates & Cole 1994). Infolding of the distal epithallial cell membrane provides large surface area, but the volume of water within the folds from which nutrients may be extracted is miniscule and replenishment of nutrients absorbed from this volume would be exclusively by diffusion. The formation of distal wall ingrowths by the initial cells and epithallial cells occurs while these cells are still distant from the thallus surface (Wegeberg & Pueschel 2002; this study), so they are not yet in a location for enhanced nutrient uptake. Furthermore, if the function of the invaginations is nutrient uptake, the restriction of these structures to only the distal face of the epithallial cell would seem to limit their uptake capacity, especially in species with multicellular epithallial filaments (Pueschel & Miller 1996; Pueschel *et al.* 1996; Pueschel & Keats 1997).

The invaginations of the epithallial cells also bear some resemblance to charasomes, plasmalemmal elaborations present in some charophyte algae (Franceschi & Lucas 1980). The structural complexity of charasomes (Franceschi & Lucas 1980) is greater than that of distal wall ingrowths of epithallial cells, but the possible association of charasomes with adenosine triphosphatase (ATPase) activity (Franceschi & Lucas 1982; Price & Whitecross 1983), proton effluxes and calcification of *Chara* Linnaeus (McConnaughey 1991) provides an intriguing comparison. The precise role of the charasomes is still unresolved (Lucas *et al.* 1989), because even in the absence of charasomes, charophytes exhibit localization of ion transmembrane transport systems in such a way that bands of acidity and alkalinity form at the cell surface (Price & Whitecross 1983). Nevertheless, the charasome may provide a useful comparison for epithallial wall ingrowths, and a connection between distal wall ingrowths and ion fluxes, particularly protons, should be considered.

We demonstrated that wall decalcification is associated with epithallial cell replacement and we suggest that this process involves protons emerging from the distal face of the initial or young epithallial cell, thereby dissolving the carbonate in the overlying crosswall. The appearance of distal wall ingrowths at the time of demineralization is consistent with this suggestion. Presumably, the increased surface area of the newly formed epithallial cell (or its parent cell) provides greater proton pumping activity on the distal face of the cell, so that the overlying crosswall can be locally decalcified. After the efflux of protons on the distal cell face removes overlaying mineralization, enlargement of the initial cell moves the new epithallial cell (or distal portion of the initial cell) past where

previously there was a layer of calcification. These arguments are based on correlation and the ingrowths could also be associated with secretion of organic wall components, but highly localized decalcification of existing walls would appear to be an essential part of the process of epithallial cell turnover.

We do not consider the presence of distal wall ingrowths to be a prerequisite for decalcification, but the plasmalemmal invaginations may enhance rates of decalcification by providing increased surface area or sites in which particular membrane proteins are enriched. Mori *et al.* (1996) demonstrated that Ca^{2+}-dependent ATPase activity was associated with isolated plasmalemmal fragments of the geniculate coralline alga, *Serraticardia maxima* (Yendo) P.C. Silva, and they suggested that this enzyme could generate an H^+ efflux. The methods used in their study did not allow discrimination of cell types. Understanding of proton pumping and calcification at a level of resolution that distinguishes cell types would be a large step forward in understanding how these algae generate and cope with pervasive calcification. It might also give insight into the specializations of epithallial cells and the mechanisms involved in epithallial cell turnover. The decalcifying function of the distal wall invagination may occur early in the cell's life, when it is beneath a layer of calcification; by the time it becomes superficial, it would have already completed this function. It is noteworthy that these same structures and mechanisms could also function in bicarbonate utilization associated with trans-calcification (McConnaughey 1997). The distal wall ingrowths form a partially enclosed volume in which acidification by proton pumping could convert bicarbonate to carbon dioxide. A single physiological mechanism could underlie seemingly disparate morphogenetic and nutrient acquisition functions.

In summary, the time of origin of the localized epithallial cell wall ingrowths, the occurrence of ingrowths on only the distal cell face, and the concurrent or subsequent appearance of richly organic material in a wall that had been occupied by mineral are all consistent with the possibility that distal wall ingrowths have a role in decalcifying the overlying cell wall in corallinalean red algae.

ACKNOWLEDGEMENTS

The authors wish to thank Henry Eichelberger for his technical assistance. By her support and encouragement, Dr Kathleen Cole has greatly aided the first author throughout his career.

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Received 20 March 2004; accepted 10 October 2004
Communicating editor: R. Sheath (Guest Associate Editor)