New Phytologist

Research

Development and organization of the central vacuole of *Acetabularia acetabulum*

Duc A. Ngo, Phillip A. Garland and Dina F. Mandoli

Department of Biology & Center for Developmental Biology, Box 355325, University of Washington, Seattle, WA 98195-5325, USA

Author for correspondence:
Dina F. Mandoli
Tel: +1 206 543 8917
Fax: +1 206 685 1726
Email: mandoli@u.washington.edu

Received: *6 July 2004*
Accepted: *28 September 2004*

Summary

• Here we analyzed the shape of the central vacuole of *Acetabularia acetabulum* by visualizing its development during diplophase (from juvenility through reproduction) and haplophase (from meiosis through mating).

• Light microscopy and whole-organism applications of a pH-sensitive dye, neutral red, were used to visualize the anatomy of the central vacuole. We studied connectivity within the thallus by locally applying dye to morphologically distinct regions (rhizoid, stalk, apex, hairs) and observing dye movements.

• In vegetative thalli most of the rhizoid, stalk and young hairs stained with dye. In reproductive structures (caps, gametangia) dye also stained the majority of the interiors. When applied to small areas, dye moved at different rates through each region of the thallus (e.g. within the stalk). Dye moved from younger hairs, but not from older hairs, into the stalk. Errors in incorporation of central vacuole into gametangia occurred at $<10^{-5}$.

• These data indicate that the central vacuole of *A. acetabulum* is a ramified polar organelle with, potentially, a gel-like sap that actively remodels its morphology during development.

Key words: *Acetabularia acetabulum*, central vacuole, development, dye movement, morphogenesis, organelle.

New Phytologist (2005) **165**: 731–746

© *New Phytologist* (2005) **doi**: 10.1111/j.1469-8137.2004.01287.x

Introduction

The central vacuole is a multifunctional organelle essential for the viability of plant cells (Rojo *et al.*, 2001). Vacuoles of plant cells have numerous functions: storing proteins, amino acids and ions; maintaining cytoplasmic homeostasis; sequestering toxins, pigments and metabolites; providing an optimally acidic compartment for hydrolytic enzymes; and generating turgor for cell expansion (Marty, 1999). Occupying 90–95% of the volume of the cells of plants (Weibe, 1978), the central vacuole maximizes both the surface-to-volume ratio of the cytoplasm (Weibe, 1978) and the surface area of cytoplasm in contact with the environment. As part of the endomembrane system, vacuoles have important roles in many aspects of development and signal transduction (Surpin & Raikhel, 2004). Vacuole biogenesis, protein targeting and trafficking mechanisms (Di Sansebastiano *et al.*, 1998; Frigerio *et al.*, 2001; Gao *et al.*, 2004; Hicks *et al.*, 2004), as well as more classical studies of ion flux across the tonoplast, are important areas of study in multicellular plants. There is now broad recognition of the complexity of this endomembrane system and acceptance that there are at least two functionally distinct vacuolar compartments, protein storage and lytic vacuoles, that are, respectively, pH neutral or acidic (Paris *et al.*, 1996; Di Sansebastiano *et al.*, 1998, 2001) and that are marked by specific tonoplast intrinsic proteins (Paris *et al.*, 1996; Jauh *et al.*, 1999; Jiang *et al.*, 2000). The two or three subcellular routes (e.g. passage through Golgi or endoplasmic reticulum) by which these vacuoles are made, maintained and merged during the development of various tissues have begun to be elucidated (Matsuoka *et al.*, 1995; Jiang & Rogers, 1997; Jauh *et al.*, 1999; Jiang *et al.*, 2000, 2001; Di Sansebastiano *et al.*, 2001).

As part of our interest in mechanisms of regional morphogenesis (Dumais *et al.*, 2000; von Dassow *et al.*, 2000) and RNA localization in *Acetabularia acetabulum* (Serikawa *et al.*, 2000; Henry *et al.*, 2004), in this paper we explore suborganellar

New Phytologist

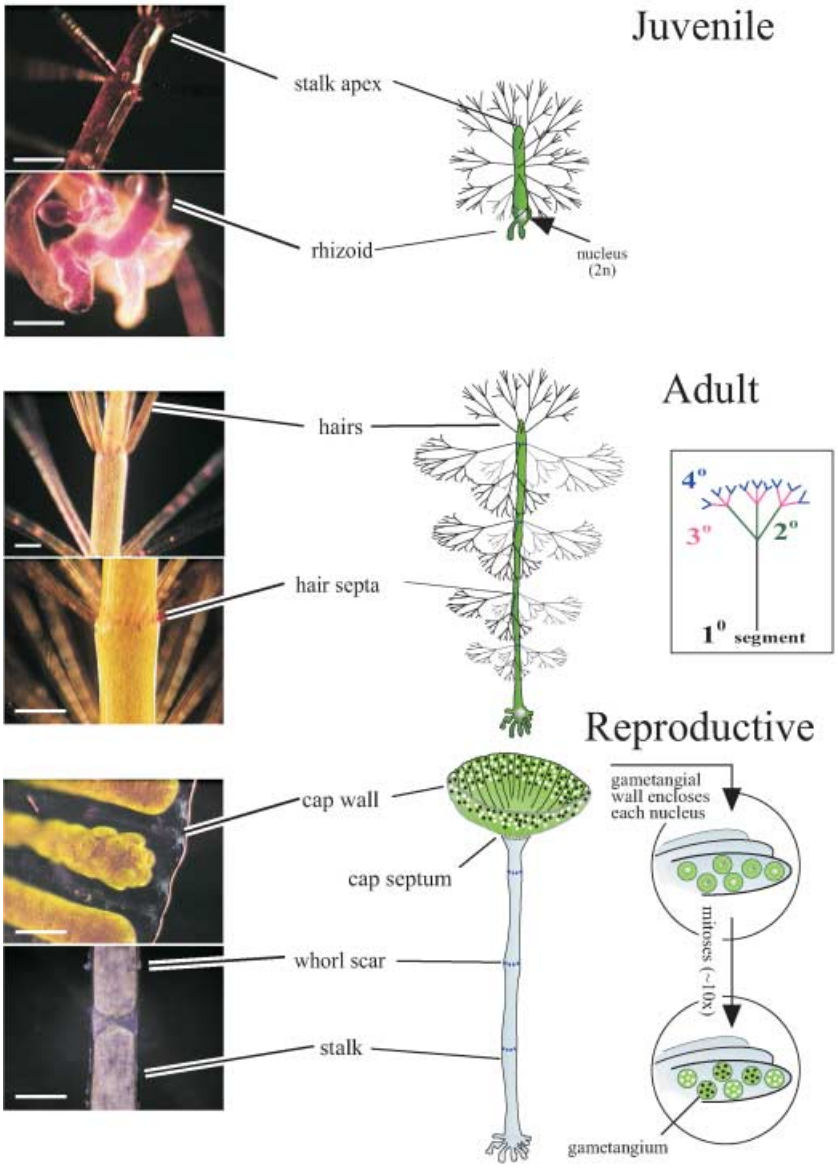


Fig. 1 Anatomy of *Acetabularia acetabulum* during development. The giant central vacuole was visualized with ≈0.1% (w/v) neutral red dye. The entire thallus of all individuals stained, except for the stalk of some very old reproductive algae that had completed gametangia formation but retained the central vacuole (lowest panel). Bars, 0.125 mm.

differentiation and morphogenesis of what we initially hypothesized might be a giant, central vacuole in an organism that builds and maintains a complex shape, despite being a giant unicell. Is this central vacuole regionally differentiated, fulfilling the myriad of functions and developmental behaviors of the central vacuoles in multicellular plants (Di Sansebastiano *et al.*, 2001; Flückiger *et al.*, 2003; Hicks *et al.*, 2004) – or is it a simple central vacuole that merely serves as a hydrostatic driver of growth? To answer these questions we needed to know if this really was a single morphologically complex central vacuole, and the degree to which it changes shape during development.

During development *A. acetabulum* (Fig. 1), a giant unicellular marine green alga, grows from 10 μm to 35 mm high (Nishimura & Mandoli, 1992b), a 3500-fold increase in length. Stalk elongation and hair initiation in the vegetative alga occur primarily at the stalk apex (von Dassow *et al.*, 2000). Both kinds of laterals, vegetative hairs and reproductive gametophores, are merely extensions of the stalk wall; in the adult phase cytoplasm streams from the stalk and into young hairs and back in healthy thalli, whereas older hairs become transparent and are eventually shed (Nishimura & Mandoli, 1992b), leaving a scar on the cell wall (Berger & Kaever, 1992). During reproduction a cap is generated by radial growth of specialized laterals, the gametophores, where they join the stalk (Serikawa & Mandoli, 1998). After meiosis, haploid nuclei undergo 10–11 rounds of mitosis and are transported to the cap (Nishimura & Mandoli, 1992b), probably on an actin cytoskeleton (Menzel, 1986). Once packaged into gametangia, these haploid nuclei undergo another 10–11 rounds of mitosis (Mandoli & Larsen, 1993). During formation

of gametangia the cytoplasm and nuclei are arranged by the cytoskeleton (Menzel, 1986, 1994; Menzel & Elsner-Menzel, 1990). Surprisingly, the basic anatomy and morphology of the central vacuole, the largest subcellular compartment, during growth and development have never been documented.

Prior studies of the *A. acetabulum* vacuole have focused on the electrophysiology of the tonoplast (Gradmann, 1975; Freudling & Gradmann, 1979; Mummert & Gradmann, 1991; Amtmann & Gradmann, 1994). The vacuole of *A. acetabulum* responds to wounds by retracting away from the wound site bidirectionally, then initiates healing via fusion of the vacuolar fragments across the wound site (Fester *et al.*, 1993). Hairs of *A. acetabulum* may increase the efficiency of both nutrient uptake and photosynthesis by virtue of their large surface area relative to the stalk (Gibor, 1973), but whether or not they are vacuolated is not known. To the best of our knowledge, there are no previously published data on the degree to which any portion of this giant unicell is vacuolated during its life cycle.

Our study is the first to examine the anatomy, development, and morphogenesis of the central vacuole of *A. acetabulum*. The central vacuole of *A. acetabulum* is easily accessible and manipulated, providing an excellent system in which to study vacuole morphology and development. In the long term, we are interested in the relationship between the central vacuole in the distinct functional regions of the unicell (e.g. stalk, hairs, cap and gametangia) and the anatomy and morphogenesis of the thallus as a whole during development.

To study the homogeneity and morphogenesis of the central vacuole, we measured the rates of dye movement through various regions of the thallus. Using light microscopy and a vacuole-specific vital dye, we determined the morphology of the central vacuole throughout the algal thallus during development, and observed morphological changes in the extent and shape of the central vacuole that occur in conjunction with hair shedding and packaging of nuclei and cytoplasm into the haplophase (gametangia).

Materials and Methods

Acetabularia culture

A genetically heterogeneous lineage of wild-type *Acetabularia acetabulum* (L.) Silva (Dasycladales), Aa0005 (a.k.a. Ladenburg 17), was used for most experiments. Additionally, individuals of two wild-type near-isogenic lines, QUχ6MSA4H23 and QUχ6MSI7X2, were also used to explore hair maturation and vacuolar volume, respectively (see Figs 3, 4 later). All phases of the life cycle were cultured according to Serikawa *et al.* (2000).

Photography

Digital images, taken through a dissecting microscope, were captured directly either into a computer or onto a memory card of a Nikon Coolpix 990 digital camera. Images were manipulated in Adobe PHOTOSHOP 4.0 and made into figures in Macromedia FREEHAND 9.0. No modifications were made that altered the data in any image. Individual algae were photographed either before or after staining.

Because it was more accurate, we measured the lower angle described by the hairs and the stalk (between hairs and stalk below each whorl when the apex was pointing up) from photographs using the public domain NIH IMAGE computer program (developed at the US National Institutes of Health and available at http://rsb.info.nih.gov/nih-image) on a Macintosh computer, then calculated the complementary (upper) angle.

Measurements and calculation of vacuolar volume and surface area

Measurements of cell dimensions (and distance of dye movement) were determined using an ocular micrometer on a Nikon dissecting microscope that had been calibrated with a laser-etched stage micrometer.

Vacuolar volume and surface area of vegetative individuals were calculated using the following formulae:

$$\text{Volume of vacuole} = [\pi(\text{segment radius} - \text{thickness of cell wall \& cytoplasm})^2 \times (\text{segment length})] \times \text{number of segments per whorl}$$

$$\text{Surface area of vacuole} = [2\pi(\text{segment radius} - \text{thickness of cell wall \& cytoplasm}) \times (\text{segment length})] \times \text{number of segments per whorl}$$

The cytoplasm in the stalk, which was about as thick as the cell wall (10 μm) (Schweiger *et al.*, 1974, von Dassow *et al.*, 2000), was taken into account in our volume calculations. All segments of each hair were treated as perfect cylinders; although hair tips and stalk apices are shaped as inverted cones, we assumed that those regions were quite small relative to the segments to which they belonged.

We approximated gametophores in reproductive individuals as cones joined at their sides to form a cap, and calculated the volume and surface area of the cap as follows:

$$\text{Volume of cap} = \text{number of rays} \times [(\pi/3) \times (\text{radius of ray})^2] \times \text{length of ray}$$

$$\text{Surface area of cap} = \text{number of rays} \times [\pi(\text{radius of ray})] \times [(\text{radius of ray})^2 + (\text{radius of cap})^2]^{1/2}$$

Dye preparation and staining techniques

Saturated solutions of neutral red were made in artificial seawater with a pH of 7.8 (Hunt & Mandoli, 1996). Undissolved neutral red was removed from solution by centrifugation. The supernatant, ≈0.1% (w/v) solution, was used in all staining experiments. We estimated the concentration of this solution

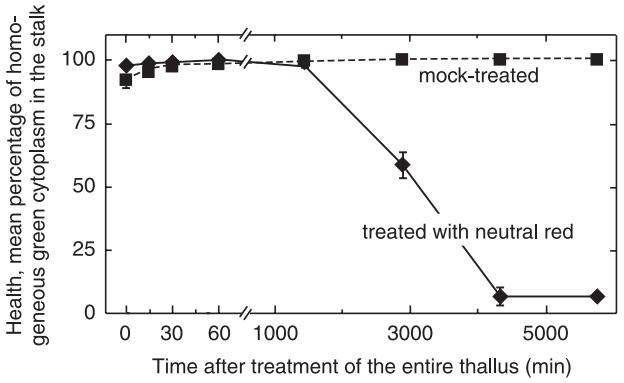


Fig. 2 Effects of 0.1% neutral red on cytoplasmic appearance after brief staining of the entire thallus of *Acetabularia acetabulum*. Ten individuals were used per experiment and the experiment was repeated three times: each datum is the mean of response of 30 individuals. Each individual was observed at each time point from 0 to 5760 min after staining.

by making a dilution series of completely solubilized neutral red and comparing the color by eye with the supernatant from the saturated solution. The value we obtained in this way, ≈0.1%, is 10-fold less than in fresh water (Budavari, 1996).

Two staining techniques were used: one to stain whole organisms, the other to stain small regions of individuals. In whole-organism staining, individuals were immersed in 1 ml saturated neutral red solution for periods ranging from 2 min for a juvenile up to 1 h for a lightly calcified reproductive alga. Stained individuals were rinsed in fresh artificial seawater (5–7 ml per individual) for 5–15 s, then placed in 20 ml fresh artificial seawater for 1 h (Fig. 1) or for 4 d (Fig. 2) before observation or measurement.

To stain specific anatomical regions of individuals locally, a small, clear piece of tygon tubing was used to restrict dye to the desired region. Tygon tubing (5 mm diameter) was cut into a small hollow cylinder 3–4 mm tall. Before use these cylinders were sterilized in ethanol (95%) for 15 min and then air-dried. A glass syringe (1 mm tip diameter) was used to apply vacuum grease to one cut surface of the cylindrical rings of tubing. The greased end of a ring was then placed over the region of the alga to be stained, thus securing the rings to the Petri dish and retaining dye inside the ring. Specific anatomical regions were stained for 10–15 min, then individuals were transferred to fresh artificial seawater for 18–24 h (see Figs 7, 8) or a more specific time interval (Fig. 7) before observation.

Measuring the rate of dye movement

Regions of apex, hairs, stalk and rhizoid were locally stained using rings of tubing and vacuum grease as described above. In the stalk, we defined the distance the dye moved (see Fig. 5) in one direction as one-half the total distance moved, since dye diffused both up and down the stalk simultaneously. At the rhizoid, hair tips and apex, the dye could move in only one direction, so the distance the dye moved was simply the distance the dye had traveled from the point of application.

Hair maturation

Groups of 10 late adults were transferred from culture boxes (Sigma Catalog No. C8062) into round 20 × 60 mm Petri dishes containing 5–7 ml artificial seawater. One whorl of hairs per individual, *c.* 20% of the thallus (Figs 3, 4), was selected for staining with neutral red. Two to five hairs of the same whorl,

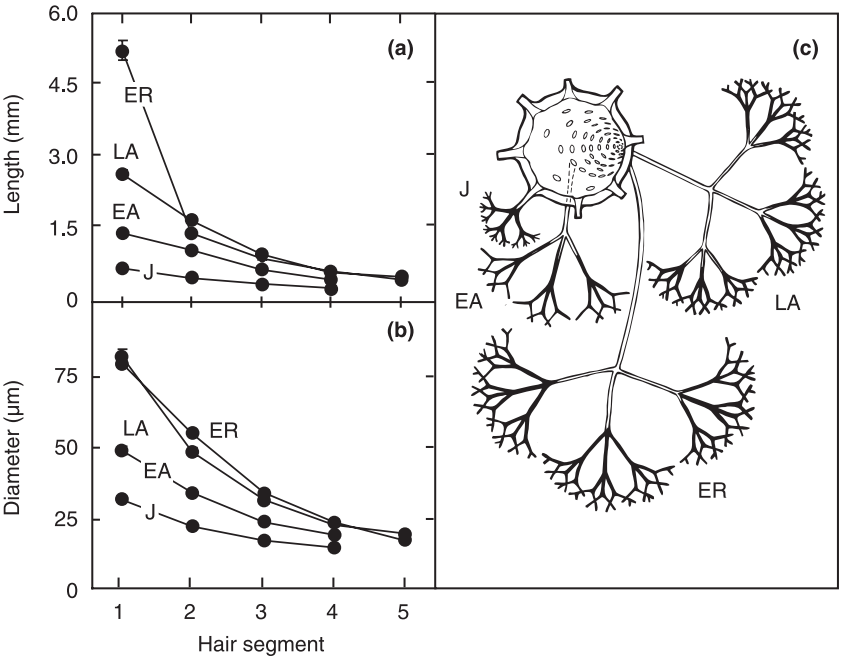


Fig. 3 Hair length and width during development was measured on unstained individuals of *Acetabularia acetabulum* (no dye was used for these measurements). Averages ± SE of hair length and width were obtained from measurements of two hairs per whorl per individual and 10 individuals per developmental phase (total 40 individuals). Average numbers of intact whorls per alga were 3.3, 4.6, 4.7 and 1 for juvenile (J), early adult (EA), late adult (LA) and early reproductive (ER), respectively. Diagrams are drawn to scale with respect to average hair length.

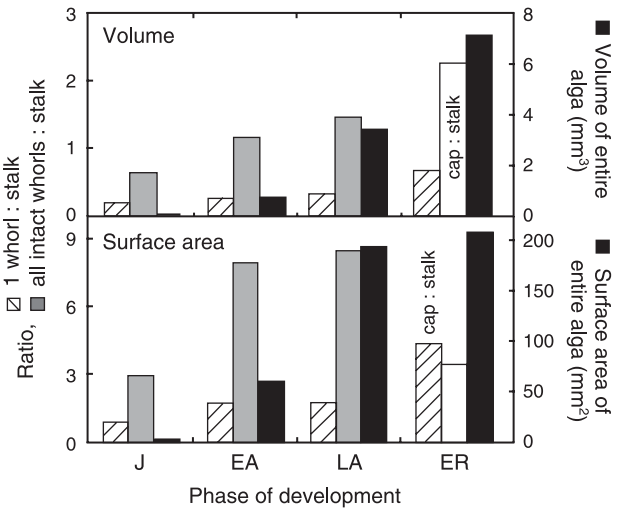


Fig. 4 Calculated volumes and surface areas during development of *Acetabularia acetabulum* (see Materials and Methods). J, juvenile; EA, early adult; LA, late adult; ER, early reproductive. Total volume and surface area include both stalk and all intact whorls (solid black bars).

from secondary segments to the very tip of the hairs (Fig. 1), were isolated with rings of tubing and vacuum grease. Saturated neutral red (10 μl) was pipetted into the sealed ring of tubing. After 5–10 min the dye was removed and the whole individual was transferred into 5–7 ml fresh artificial seawater. To allow dye movement through the thallus, these stained and washed late adults were left for 18–24 h in fresh artificial seawater before observation and measurement.

Results

Why neutral red was chosen as a dye

We were surprised to discover that the central vacuole of *A. acetabulum* is highly resistant to gross perturbations, including penetration by microinjection (J. Graff, N. Fiana and D.F.M., unpublished results). Although we could readily inject and follow the movement of beads or dyes in the cytoplasm with video microscopy, any and all attempts to penetrate the central vacuole were frustrated by passage of the needle through, or quasi-active movement of, the vacuole away from the needle (not shown). A second kind of experiment also indicated that the central vacuole is very resilient: we could remove all the cytoplasm of the stalk from around the central vacuole with a needle which, when the cytoplasm was almost completely removed, caused the central vacuole to collapse in volume suddenly. We then injected the cytoplasm back and watched the central vacuole instantaneously resume its form, size and shape. The sudden changes in vacuolar volume we recorded are reminiscent of phase transitions of a gel (Chin *et al.*, 2004). Remarkably, *A. acetabulum* grew and developed normally after this treatment. Thus in *A. acetabulum* dyes that require microinjection into the central vacuole cannot yet be used to study vacuole dynamics unless some means can be found to make this organelle more penetrable.

As an alternative to microinjection, we considered dyes that are taken up readily from the external environment. Some dyes did not label the central vacuole at all. For example, LysoSensor Green labeled only a dynamic, mobile population of small, highly acidic vesicles in the cytoplasm (not shown), and so was inappropriate for this study. Most candidate dyes are fluorescent and require a confocal or epifluorescent microscope for imaging. The giant size of the thallus rendered most of these dyes inappropriate for visualizing the shape of the entire central vacuole: we needed to image on a large scale before moving to the finer one. A final major flaw of fluorescent dyes is their narrow effective pH range (Molecular Probes, Inc., Eugene, OR, USA).

We chose neutral red, an inexpensive, pH-sensitive dye, to visualize gross vacuolar morphology during development and also as a crude assay of acidity. Neutral red, N^8,N^8,3 trimethyl-2,8-phenazine diamine monohydrochloride, is yellow at pH ≥ 7–8 and dark red at pH ≤ 6.8 (Budavari, 1996). Neutral red partitions rapidly into acidic compartments rendering the anatomy and morphology of native or regenerated lytic vacuoles visible with light microscopy (Strugger, 1935; Hörtensteiner *et al.*, 1992; Ehara, 1996; Di Sansebastiano *et al.*, 2001). Our measurements of crude extracts from regions of the thallus suggest that most regions of this central vacuole are acidic, except for the rhizoid region that is basic (A. Altares and D.F.M., unpublished results; Fig. 1), thus indicating that neutral red would enable us to visualize the vacuole in the entire thallus. Neutral red alters aspects of cell physiology including K^+ efflux and current-voltage changes (Ullrich *et al.*, 1990). Neutral red will also bind to DNA in a temperature-dependent fashion (Wang *et al.*, 2003). Despite these shortcomings, we reasoned that, as long as we carefully documented its effects, neutral red would be the fastest and least expensive way to map the gross anatomy of the central vacuole in this giant unicell.

Morphology of the central vacuole

Whole organism staining of vegetative and reproductive individuals clearly indicated that the vacuole ramified into all laterals: from the stalk into each hair in the whorls of vegetative individuals, and into each gametophore in the cap of reproductive ones. Hairs, stalks and apices of juveniles took up dye after ≤2 min of staining (Fig. 1) and stained dark red. In the rhizoid of juveniles, the dye was yellow or pinkish and discontinuous. Red dye was evident in the stalk, hairs, apex and rhizoid of adults (Fig. 1). In reproductive thalli immediately after nuclear transport, the central vacuole remained intact within the stalk (Fig. 1), but as the organism aged the vacuole in the stalk became increasing fragmented, particularly near the rhizoid. Neutral red also stained all gametophores of the cap (Fig. 1).

These data suggest that the central vacuole in *A. acetabulum* is a giant, ramified organelle that extends throughout vegetative and reproductive thalli.

To convince ourselves that neutral red could be used in this system as a vital dye to visualize the extent of the central vacuole as described above, we stained the entire thallus of populations of whole organisms for varying amounts of time, washed them extensively in fresh seawater, then assessed the 'health' of each individual 24 h later (Fig. 2). 'Healthy' thalli had cytoplasm that was homogeneous green in color, whereas unhealthy cytoplasm was mottled white and green, and appeared clumped. Health was scored both as a percentage of the length of the stalk that was a homogeneous green (Fig. 2) and also as the percentage of the population that recovered fully from exposure to dye, that is, grew and developed rather than died (not shown). After incubation of whole thalli in the dye, nearly 100% of the cytoplasm was homogeneous, values comparable to or better than those of controls mock-treated but not exposed to neutral red (Fig. 2). Only after 24 ± 1 h exposures to dye did the percentage of homogeneous cytoplasm decline. Once an individual had >90% clumped cytoplasm (<10% homogeneous green cytoplasm in the stalk), it died. For subsequent analyses of anatomy and morphology of the central vacuole of this species, the maximum exposure of whole thalli (arrow, Fig. 2) was to 0.1% neutral red for 10–15 min (Fig. 1, left panels; portions of Figs 10–12 as noted in legends). Note that the majority of the subsequent experiments used only brief exposures to dye applied to a small region of the thallus (Figs 5–8) or no dye at all (Figs 3, 4, 9). Finally, we never grew or developed alga in the presence of neutral red: we used neutral red solely as a terminal assay to visualize development that had already occurred in the absence of dye.

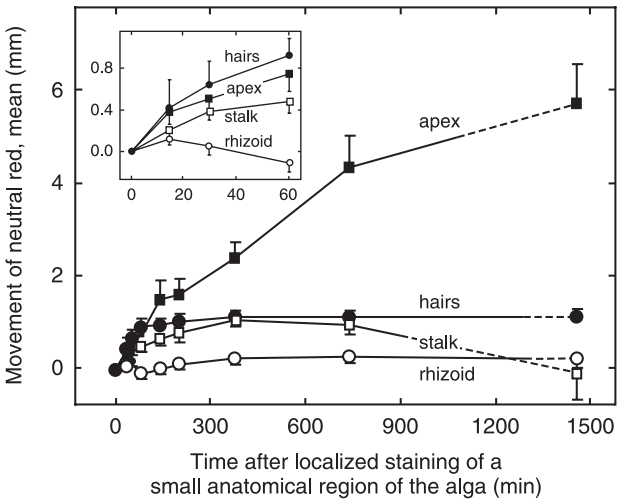


Fig. 5 Rates of dye movement within regions of the vacuolar sap of *Acetabularia acetabulum*. Hairs were not usually longer than 1 cm so the dye did not move after it reached the end of the hair (≈60 min; see inset). Inset shows times <1 h. Solid lines indicate times for which neutral red caused no visible changes in the cytoplasm; dashed lines indicate times for which neutral red caused harm to homogeneity of the cytoplasm (Fig. 6).

Volume and surface area of stalk and laterals in unstained organisms

Using our length and width measurements of unstained algal stalks and whorls (Fig. 3), we calculated the volume and surface area of the stalk and whorls at different developmental ages (see Materials and Methods for formulae). We defined an 'intact' whorl of hairs as one that retained ≥90% of all its hair segments. Both the mean total volume and surface area increased throughout development (Fig. 4, black solid bars).

The ratio of the volume of a single whorl of hairs to the volume of the stalk remained constant during vegetative development, increasing only during reproduction (Fig. 4, hatched bars). The volume of one whorl of hairs was as little as one-fifth the volume of the stalk in juvenile, and one-fourth, one-third and two-thirds the volume of the stalk in early adult, late adult and reproductive, respectively. Similarly, the surface area of a single whorl of hairs was equal to that of the stalk in juveniles, was twofold greater during adult development (early adult and late adult), and nearly fivefold greater than that of the stalk during reproduction (Fig. 4, hatched bars).

Similar calculations for all intact whorls (those not being shed) revealed the pattern of the organism as a whole more clearly. The ratio of the stalk to all intact whorls (those not being shed) increased during the vegetative phase (Fig. 4, grey bars). Note that there are no calculations for this relationship for reproductive individuals (there are no grey bars for the reproductive phase; Fig. 4). During reproduction, all whorls of hairs below the cap are shed. Only a single tall and elaborate whorl of hairs emanating from the superior corona of the cap was retained during reproduction (Fig. 4, hatched bar for early reproductive).

The reproductive laterals or gametophores of the cap have more than twice the volume of the stalk and about four times the surface area of the stalk (Fig. 4, white bars).

Differentiation within the central vacuole

To assess how distinct the central vacuole was in various anatomical regions of the thallus in vegetative adults, we applied dye locally to each portion of the anatomy (see Materials and Methods) and measured rates of movement of the dye within the thallus. Rates of dye movement depended on the anatomical region. During the first 60 min after exposure to neutral red, the rate of dye movement was greater in the hairs > apex > mid-stalk, traveling nearly twice as fast in the hairs than in the stalk (Fig. 5, inset). In the rhizoid, the rate of dye movement was nearly negligible (Fig. 5 and inset). One hour after the initial application of dye, the rate of dye movement was negligible in all regions except the apex (Fig. 5). In contrast, dye applied at the apex maintained its rate of movement,

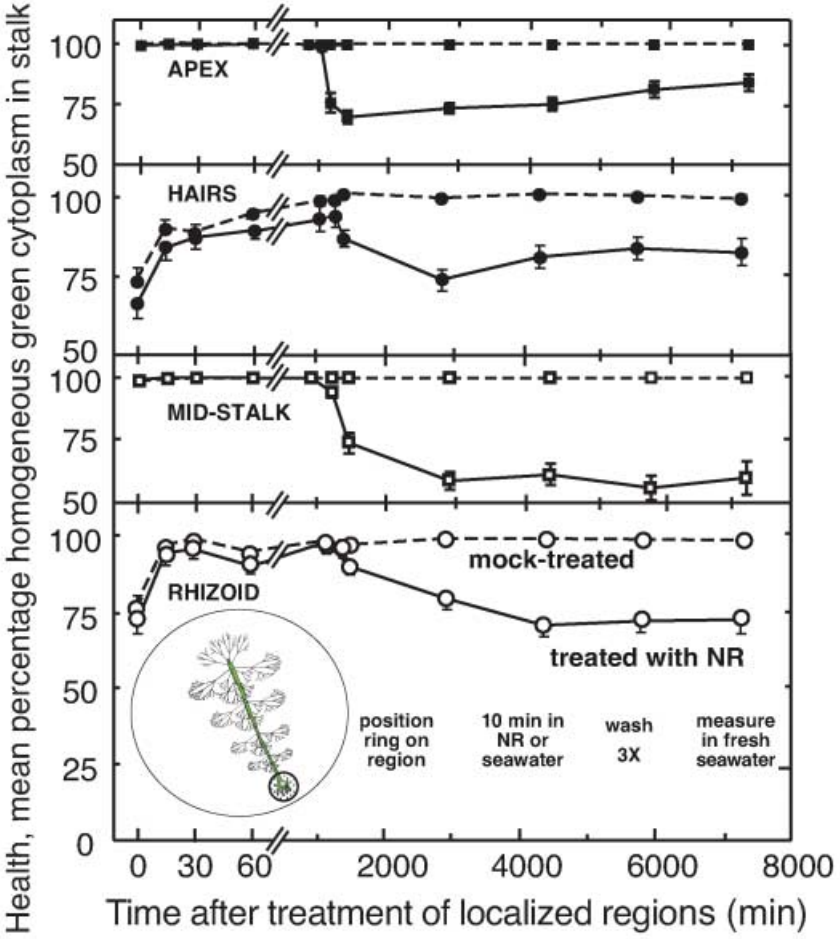


Fig. 6 Appearance of the cytoplasm of *Acetabularia acetabulum* after localized regions were stained with neutral red or artificial seawater. Anatomical regions were exposed either to 0.1% neutral red (solid line) or artificial seawater (dashed line) inside a small ring (left inset) for 10 min, followed by three washes and placement of the treated individual in fresh artificial seawater (right inset). Health of the organisms was determined by measuring the percentage of the length of the thallus that did not become clumped or heterogeneous in color because of a treatment.

traveling a total of 4 mm in 11 h, about one-sixth of the length of the stalk. Because hairs are not usually longer than 1 cm, the dye either entered the stalk or did not move after it reached the end of the hair (≈60 min). Note that as dye moved farther from its point of application, the concentration of the dye declined and this decreased the certainty of accurate measurement after the longest time intervals.

Monitoring diffusion of dye through water- or agar-filled capillary tubes, we could mimic the range of rates of dye movement (not shown). However, serious attempts at *in vitro* modeling must wait until we know the composition and biophysical nature of the vacuolar sap in this species.

To assess the health of thalli exposed briefly and locally to neutral red (see Materials and Methods), we exposed one of four regions to 0.1% neutral red inside a small ring of tygon tubing for 10 min, removed the dye, washed the exposed region three times with fresh seawater, removed the ring which had held the dye, then monitored the health of treated and mock-treated individuals for the next several days (Fig. 6). Again, we measured the percentage of the length of the thallus that became heterogeneous in color in response to this treatment as an assessment of the health of the algae, and compared these data with those for thalli mock-treated for each region. The stalk region reacted most quickly to local, brief exposure to dye, beginning to clump its cytoplasm 900 min (15 h) after dye removal (Fig. 6). Apices, hairs and rhizoids reacted more slowly, initiating cytoplasmic clumping only 1080, 1320 and 1320 min (18 or 22 h), respectively, after dye removal (Fig. 6). The response reached a maximum for apices 1440 min later, and for mid-stalks and hairs or rhizoids 2880 or 4260 min (48 or 71 h) later. Rhizoids were the least sensitive region, with rhizoids < apices = hairs = mid-stalk. Apices exposed briefly to dye were able to recover half their cytoplasmic homogeneity over a period of 5 d (from 70 to 84%; Fig. 6). Based on these data, all individuals subjected to localized dye applications (Figs 5, 7) either were measured before clumping of the cytoplasm, or we clearly indicated which measurements were taken after initiation of the clumping reaction (dashed lines, Fig. 5).

Hair development and vacuolar partitioning

We hypothesized that, if separation of the central vacuole between hairs and stalk occurred as a function of the age of the hairs, then when dye was applied to the tips of hairs, we would either see dye move into the stalk (connected) or retention of dye at the hair septum (disconnected) depending on the age

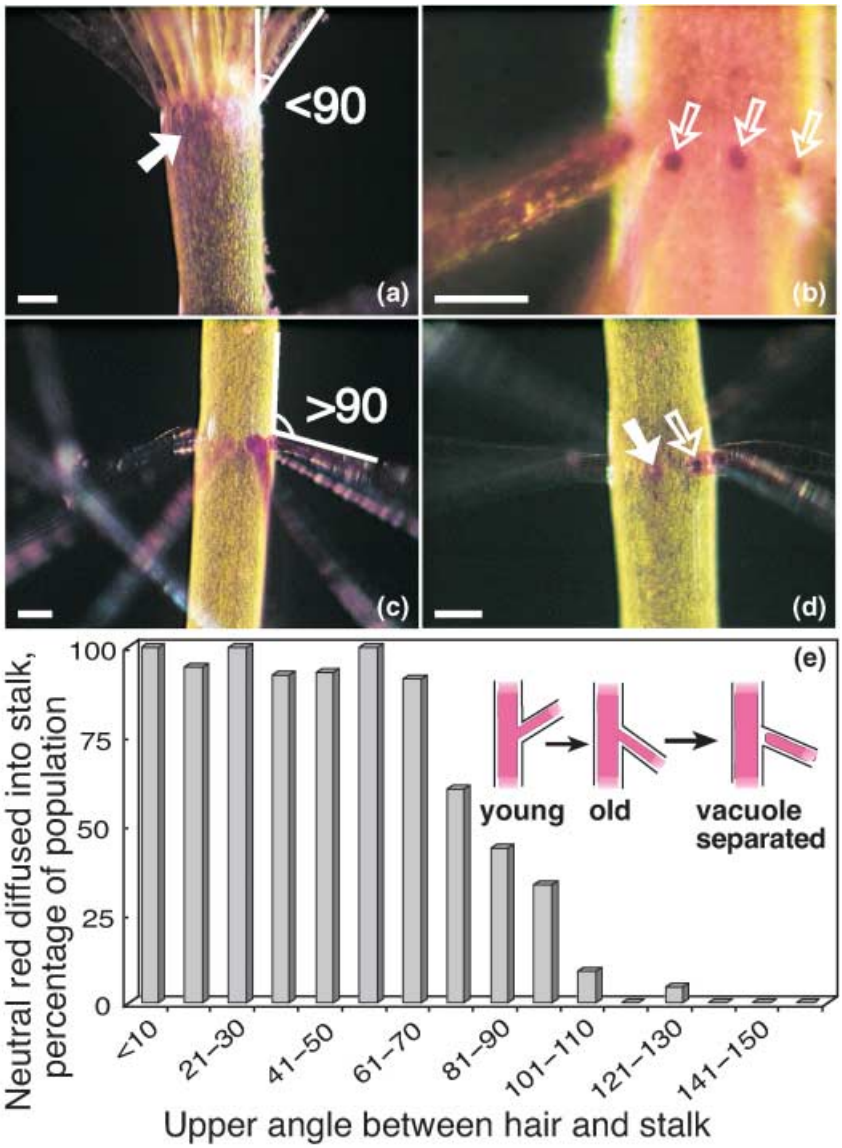


Fig. 7 Vacuolar connectivity during development of *Acetabularia acetabulum*. Hairs of late adults were locally stained between 2° segments and the tip of the hair. Dye applied to whole organism (b). Bars 0.125 mm in all panels. (a) 1 d after staining, neutral red in young hair (<90°) had moved into the stalk. (b) Open arrows mark open hair septa. (c) Older hairs in middle of stalk (>90°) retained all dye 24 h after staining. Note the sharp color junction between stalk and hair. (d) Two septa of the same whorl where dark sphere (open arrow) was seen in one septum, but not another (closed arrow, hair points out of the page). (e) In populations, the percentage of individuals in which dye moved into the stalk from locally stained hairs decreased when hairs drooped >90°. Independent assays of dye movement used 190 late adults with just one whorl of hairs dyed per unicell.

of the whorls. Regions of a particular whorl of hairs, between secondary hair segments (see definitions, Fig. 1) and the tips of that hair, were isolated with rings of tubing and stained by adding neutral red inside the ring. Neutral red could diffuse up to 1 mm in 1 h (Fig. 5, inset) and hairs are typically less than 1 cm long. Therefore leaving stained individuals overnight before making observations allowed adequate time for the dye to move along the length of a hair and into the stalk if the path between the hair and stalk was open to the dye.

When the upper angle of the hair with respect to the stalk (see Materials and Methods) was ≤90°, dye was observed in the stalk 1 d after hairs were stained (Fig. 7a). In these hairs we observed a red, translucent, circular area at each hair septum, suggesting that this septum remained open to the stalk and was where the central vacuole in the stalk ramified into the hair (Fig. 7b, open arrows).

By contrast, when the angle of the hair with respect to the stalk was ≥90°, dye was seen only in the hairs and we observed a sharp color junction at the hair septa (Fig. 7c). The lack of translucent circular area at the septum was associated with a closed septum (Fig. 7d, closed arrow). Note that another hair of the same whorl contained a red circular area at its septum (Fig. 7d, open arrow), indicating that the septa of individual hairs in a whorl can close asynchronously.

Populations of individuals were assayed to determine if the angle between the stalk and the whorl correlated with hair septa open to dye. Most (91.4–100%) individuals assayed with angles ≤70° had dye in their stalk 1 d after staining (Fig. 7e). The percentage of the population in which dye was observed in the stalk 1 d after staining decreased as hairs exceeded 71°. Except for one population (121–130°, Fig. 7e), no individuals in populations with angles ≥111° had dye in the stalk 1 d after staining.

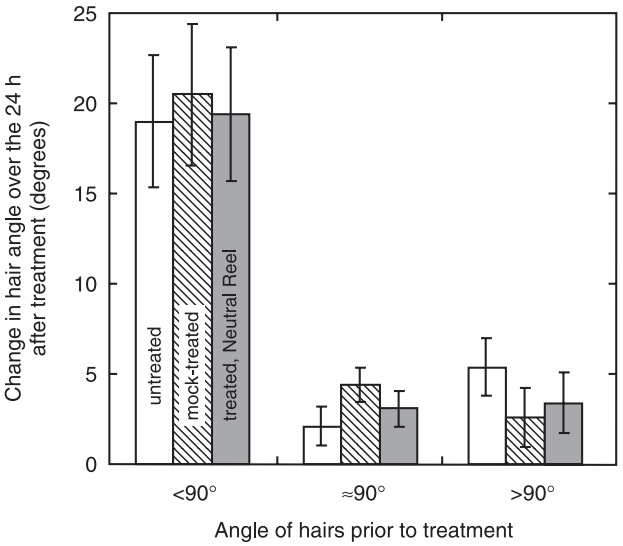


Fig. 8 Change in angle of *Acetabularia acetabulum* hairs with respect to the stalk over 24 h following a brief, localized exposure to dye or mock treatment. A single whorl of hairs from each late adult was treated per individual. The tip down to the 2° segments of a hair (Fig. 1) was trapped underneath a ring of tygon tubing (see Materials and Methods). This ring was either filled with artificial seawater (mocked-treated individuals, patterned bars) or 0.1% neutral red in seawater (gray bars) for 10 min. To assess the effect of the mechanical treatment alone, another cohort of individuals was placed in artificial seawater, but their hairs were not trapped by tygon rings (open bars). For each bar of <90° and ≈90°, n = 26–27. For populations >90°, n = 16–19. Values of t-test comparing neutral red-treated and mock-treated populations for each angle cohort were <90°, t = 0.47, df = 26, $P > 0.50$; ≈90°, t = 0.97, df = 26, $P > 0.15$; >90°, t = 0.33, df = 18, $P > 0.5$.

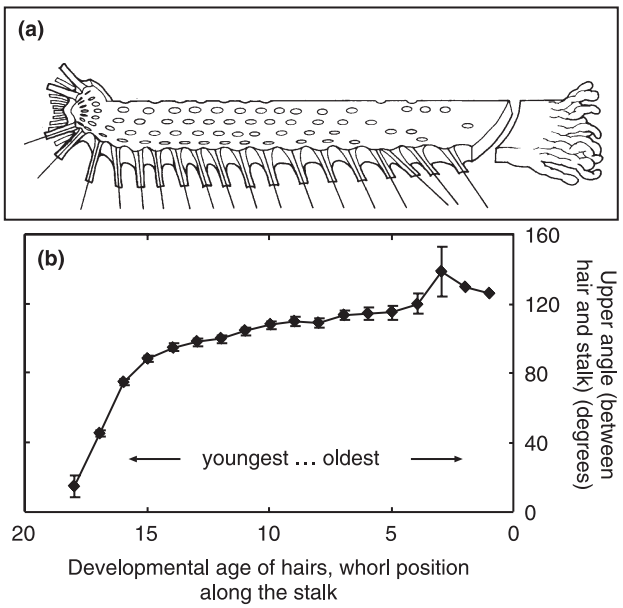


Fig. 9 (a) Relationships between angles of hairs to whorl positions along the stalk of *Acetabularia acetabulum*. Drawings of angles for all whorls of hairs reflect the angles measured. (b) Hairs in a whorl drooped as a function of developmental age. Angles of two hairs per whorl of 80 late adults were measured (n = 1471). Youngest hairs (position 17) were at the apex and oldest were near the rhizoid (position 1). Each value consists of a number of data points (11–158) for whorl positions 4–17. Values of whorl positions 1 and 2 have no standard errors because they are the mean of two individuals. Whorl position 3 is the mean of six hairs. Equations from linear regression for the data points of young whorls (positions 15–18) and old whorls (positions 1–14) are $y = -25.1x + 469.4$ ($R^2 = 0.97$) and $y = -2.78x + 133.6$ ($R^2 = 0.87$), respectively.

To determine if neutral red had altered the angle of the hairs during these experiments, we exposed just the tips of hairs at various angles to dye, washed them, removed the ring, then remeasured the angles of the same hairs 24 ± 1 h later. Exposure to dye did not significantly alter the angle of the hairs with respect to the stalk (Fig. 8) over the duration of these measurements. Over the subsequent 24 h, when exposed to dye, hairs at <90° changed in angle by ≈20°, but hairs ≤90° changed only by ≤5° (Fig. 8), suggesting that the changes in angle were a function of developmental age rather than a result of exposure to a localized pulse of dye.

To assess connectivity of the vacuolar extensions in relation to hair development, we measured the angle of hairs of each position along the thallus by measuring the mean angles of hairs at each whorl along the stalk (Fig. 9). The position of a whorl was plotted against hair age. As the alga elongates, a whorl of hairs initiates at the apex: older whorls are basipetal to younger whorls on the stalk. The oldest whorl of hairs on a stalk is in position no. 1 (Fig. 9) (Nishimura & Mandoli, 1992b). Although all hairs in a whorl are initiated simultaneously, they mature somewhat asynchronously. To quantify the relative age of the whorl of hairs, we plotted each angle in a whorl against the position of that whorl along the stalk. Although the number of measurable whorls of hairs per individual varied because hairs are shed from the stalk as an individual ages (Gibor, 1973), on average late adults made 16–19 whorls of hairs (Nishimura & Mandoli, 1992b). Hairs initiating at the apex sometimes had negative angles because hairs sometimes grew towards each other from opposite sides of the stalk. To reduce error, we measured the angle of two hairs of each whorl (one on opposite side of the stalk). All values (n = 1471) are averages of the angles at each whorl position along the stalk. As hairs aged, the angle between hair and stalk increased. Mean angles of the youngest four whorls of hairs changed from 14 to 88° most rapidly. Hairs older than the four youngest changed from 94 to 126° very slowly (Fig. 9). These data are consistent with our measurements of the change over 24 h in the angle of whorls of hairs: young hairs at <90° changed angle fourfold faster than older hairs at ≈90° or >90° (Fig. 8).

In reproductive individuals, cytoplasmic streaming in the interwhorls and between each hair was uniform (Koop & Kiermayer, 1980; Menzel, 1994). In contrast, cytoplasmic streaming was absent above and below the youngest two whorls of hairs (not shown). The strands of cytoplasm that carry the chloroplasts above and below these hair septa were relatively devoid of chloroplasts, and the hairs themselves were

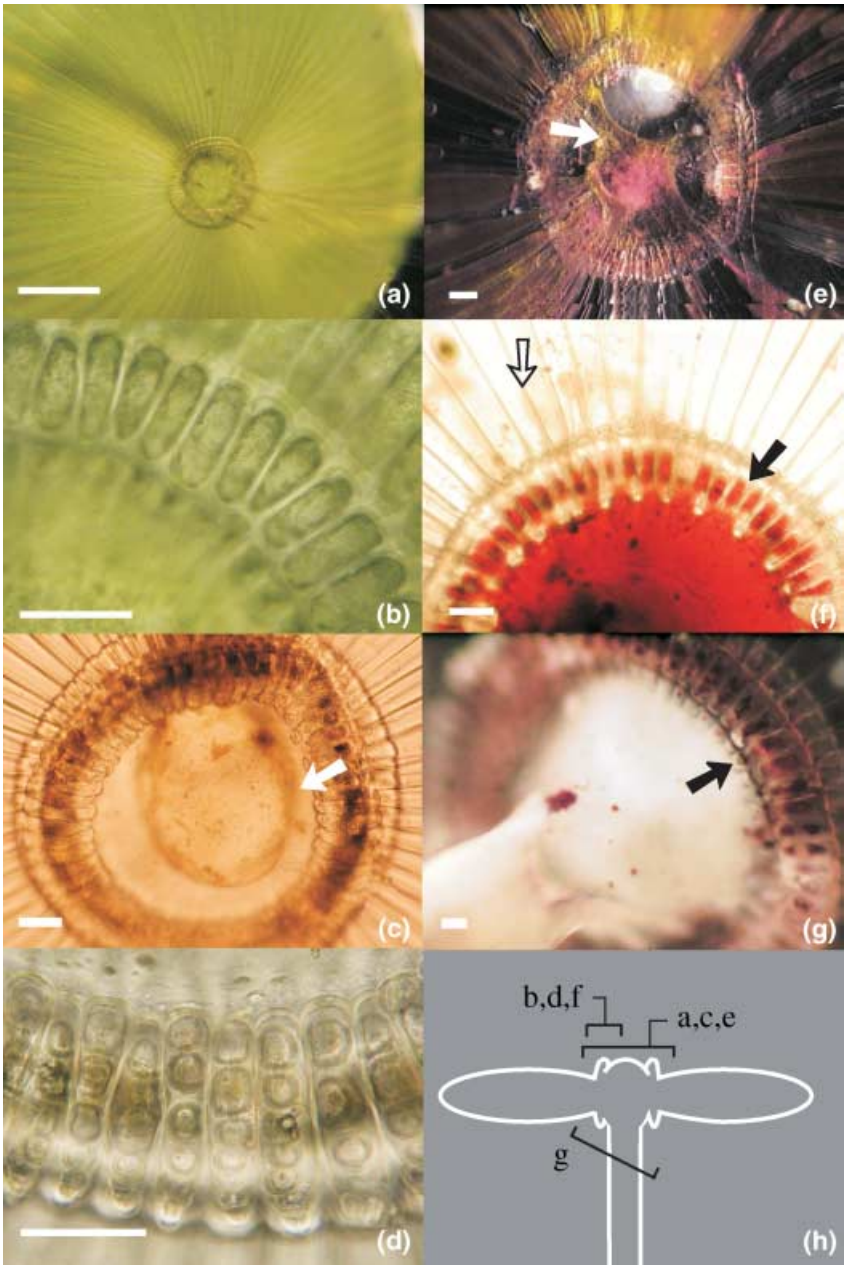


Fig. 10 Vacuolar reorganization at the cap septum during reproduction of *Acetabularia acetabulum*. Bars, 0.1 mm except where indicated. (a–d) Unstained caps; (e–g) individuals whose thalli were completely immersed in 1 ml 0.1% neutral red then washed for 10–15 s in 40 ml fresh seawater, and photographed. (a) Septum and superior corona of cap before nuclear transport. (b) Inferior corona of cap before transport of haploid nuclei and cytoplasm. (c) Unstained septum and superior corona after gametangia have formed. Vacuole in septum region retracts radially (arrows) away from stalk wall. (d) Superior corona of cap after gametangial formation. The circular structures visible on the corona are features of the cell wall that mark where hairs emerge from the cap. (e) Cytoplasm and vacuole from gametophores partitioning off from cytoplasm and vacuole in the septum (arrows) just before gametangial initiation. (f) Septum and superior corona after gametangial formation. In most lobes of the superior corona, the septum was still ramified into the superior corona (closed arrow) and the vacuole retracted away from the septum, rendering the gametophores free of dye (open arrow). (g) Septum and inferior corona following gametangial formation. Most lobes have retained vacuoles (arrow). (h) Diagram showing the anatomical positions of the images in a–g.

filled with chloroplasts. However, in older whorls of hairs and also across whorl scars, cytoplasmic streaming was uniform in the stalk and the hairs themselves had few or no chloroplasts inside them. These observations suggest that the cytoskeleton was remodeled in the stalk near hair septa during normal development of hairs.

Reorganization of the central vacuole during reproduction

Morphogenesis of the vacuole during reproduction has consequences for the pathway haploid nuclei take as they migrate from the rhizoid into the cap. As a first step in elucidating this pathway, we visualized the fate of the vacuole during reproductive morphogenesis using neutral red. Gametangia initiate just after nuclear transport. Cap septa and the superior coronae of caps contained both cytoplasm (Fig. 10a,b) and vacuole (Fig. 10c) before gametangia formed. However, in preparation for gametangial initiation the vacuole retracted toward the center of the cap septum, sealing off the stalk from the cap (Fig. 10e,f, arrows). This indicates that the cytoplasm and vacuole underwent physical partitioning at the cap septum when nuclear transport was complete. After gametangial formation, dark red dye was visible in each lobe of the superior (Fig. 10f, closed arrow) and inferior corona (Fig. 10g), but no dye was left in the gametophores (Fig. 10f, open arrow). The vacuole

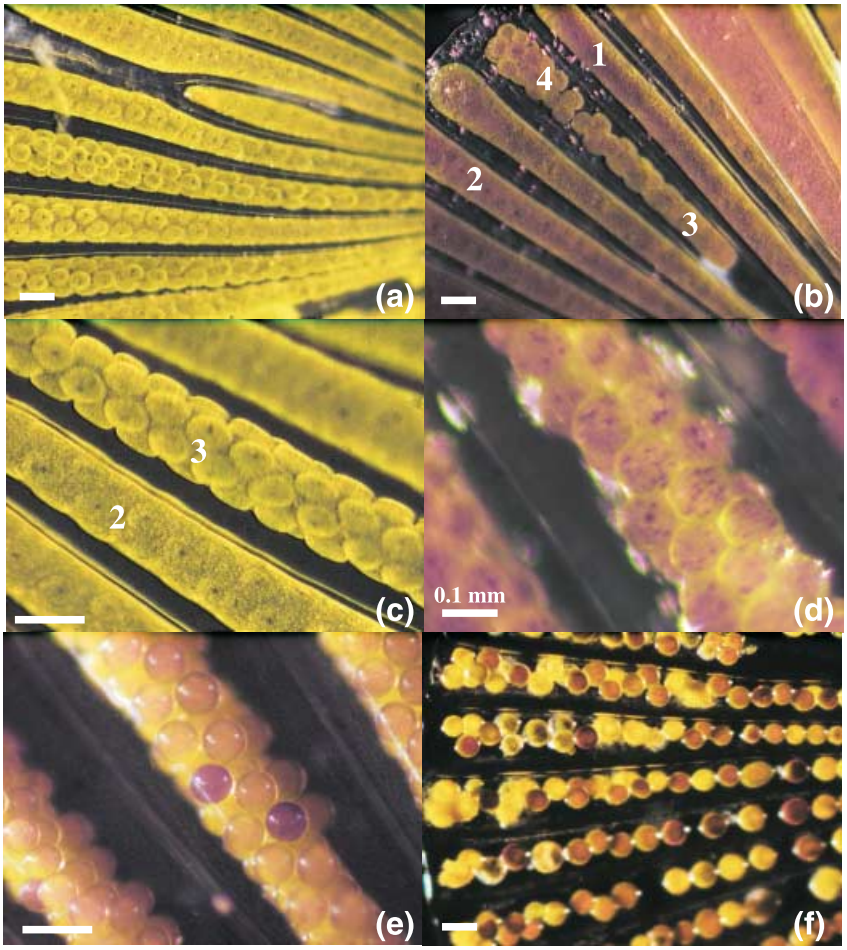


Fig. 11 Behavior of the vacuole during reproduction of *Acetabularia acetabulum*. For all panels, after caps had reached the desired developmental age, neutral red was used to visualize the vacuole in caps and the gametangia they bore (no dye was present during development in either unstained controls or stained caps). To illustrate the color difference ± dye, both unstained (a,c) and stained (b,d,e,f) anatomies are imaged. Bars, 0.25 mm except where indicated. (a,c) Unstained control caps during formation of gametangia. (b,c) In wild-type strains gametangia normally formed at different times in the gametophores of a single cap. Numbers in these panels indicate the order of gametogenesis: 1, least developed; 4, most developed gametophore. (d) Small vesicles also stain with neutral red. (e,f) Gametangia differed in color after staining.

extended into each lobe of the superior corona. Apparently, like the central vacuole in the stalk (Fig. 1), the vacuole in the corona is left behind in the thallus of the diploid parent after gametangial formation.

After nuclear transport, gametangia form within each gametophore, packaging nuclei, cytoplasm, and vacuole in the gametophores inside gametangia (Fig. 11). Gametangial initiation was far more synchronous within a gametophore (Fig. 11a) than between gametophores (Fig. 11a–c). Also, initiation of gametangia within a gametophore was more synchronous than their subsequent development (compare Fig. 11a,c to Fig. 11b, 3, 4).

Staining revealed small, dark red areas in nascent gametangia (Fig. 11d), suggesting the presence of small vacuoles in addition to the large central vacuole in each gametangium. When stained with neutral red, mature gametangia within a gametophore varied in color from purple to red to yellow (Fig. 11e,f), suggesting that the central vacuole in these gametangia differed in pH.

After the completion of gametangial development, staining with neutral red also revealed abnormalities of vacuole packaging into gametangia. Note again that dye was not present during development, but was used only as a terminal assay. One dark red vacuole was observed in a gametangium that lacked cytoplasm (Fig. 12a,b, single asterisk). Other gametangia contained cytoplasm but no trace of neutral red (Fig. 12a,b, double asterisk) and were proximal to membrane-bound but wall-less, darkly stained vacuoles (Fig. 12a,b, #).

To quantify the frequency of abnormal vacuolar phenotypes, we stained 50 caps that bore gametangia and counted all errors in vacuole packaging. These errors could not have been induced by the dye since neutral red was applied only after gametangia had fully developed. There was an average of 60 ± 8 gametophores per cap and 90 ± 39 gametangia per gametophore, giving a total of 2.70×10^5 gametangia in our 50 caps. These values are comparable to those found previously (Nishimura & Mandoli, 1992a). We observed a total of 19 errors in all gametophores (rays) for wall-less (unpackaged) cytoplasm (Fig. 12c, ##; Table 1) and 19 occurrences of wall-less vacuole (Fig. 12a, #; Table 1). Overall, 4 and 16% of all caps had the former and latter phenotypes, respectively (Table 1). Although early in our study of the *A. acetabulum* central vacuole we found one cap containing gametangia that lacked a vacuole (did not obtain a portion of the central vacuole from its diploid parent), as none of the gametangia later counted lacked a vacuole the frequency of vacuole-excluded gametangia was $<10^{-5}$ (Table 1).

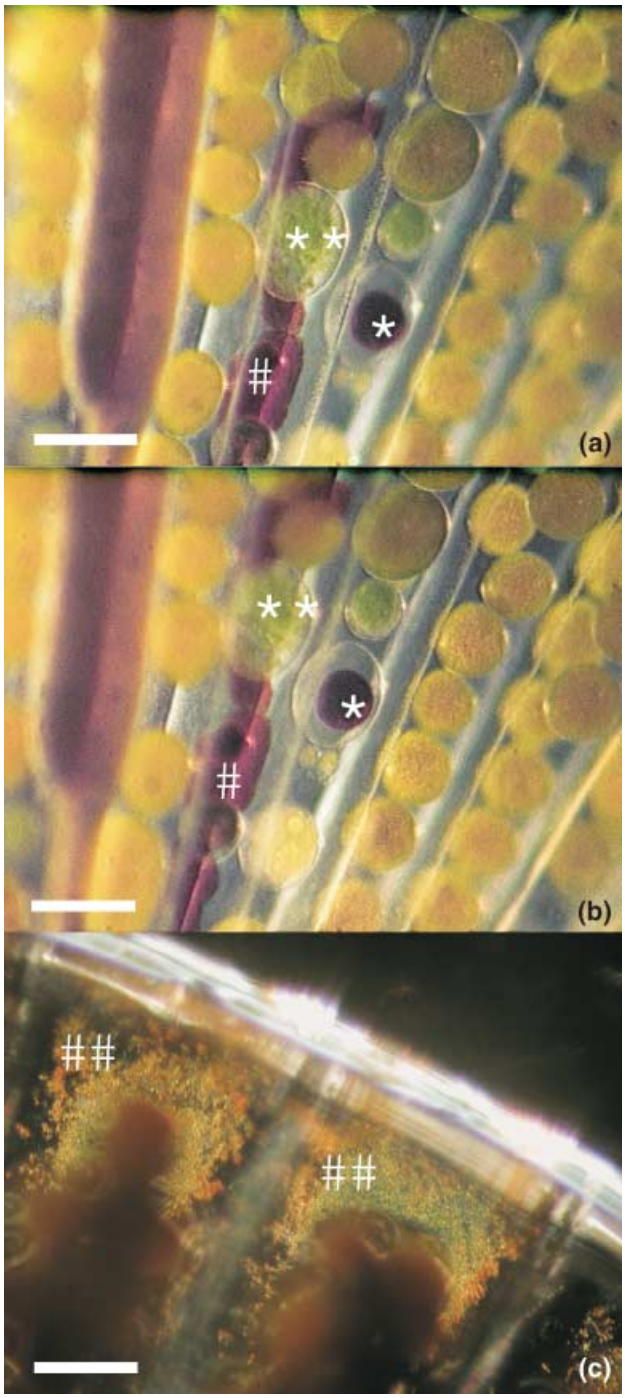


Fig. 12 Packaging errors during reproduction of *Acetabularia acetabulum*. As in Fig. 10, neutral red was applied only after gametangia bearing caps were fully developed: dye was used as a terminal assay to visualize the vacuole(s) in gametangia. (a,b) Same cap but at different planes of focus. #, Portions of vacuole remained in a gametophore outside the gametangia; *, gametangium containing a vacuole but no cytoplasm; **, gametangium containing cytoplasm but no vacuole. (c) Wall-less cytoplasm remained unpackaged after gametangial formation (##).

Errors counted as vacuole exclusion in fully developed gametangia that were subsequently dyed were qualitatively defined to have a heterogeneous and fragmented appearance of cytoplasm inside the gametangia (Fig. 12a, **) and may appear proximal to an area of wall-less, unpackaged vacuole.

Discussion

Our experiments are the first to describe the anatomy, development and morphogenesis of the central vacuole of *A. acetabulum*. Using the highly controlled conditions defined here (Figs 2, 6, 8), neutral red was useful for visualizing the extent and shape of this acidic central vacuole (Figs 1, 9–12). In addition, brief, localized exposures visualized dye movement within the thallus (Figs 5, 7).

We found that *A. acetabulum* has a dynamic, polar central vacuole that ramifies throughout the thallus (Fig. 1). This central vacuole increases dramatically in surface area and volume (Figs 3, 4) and is actively remodeled during development (Figs 7, 10). The location, development and morphology of the central vacuole have implications for routes of migration of the haploid nuclei. Finally, the central vacuole is actively packaged into the haplophase (Table 1; Figs 11, 12).

Like the vacuolar remodeling we observed here (Figs 7, 10), ramification or 'invagination' of the central vacuole into new extensions of cells also occurs in growing pollen and in root hairs of *Arabidopsis* (Flückiger *et al.*, 2003; Hicks *et al.*, 2004). If *A. acetabulum* also contains smaller, pH-neutral, 'peripheral' vacuoles, as do vascular plants (Di Sansebastiano *et al.*, 1998, 2001), remains to be explored.

Acetabularia has a dynamic, apparently polar central vacuole

How are we to interpret the differences in rates of movement of neutral red through the thallus (Fig. 5)? The vacuole is clearly a major site of dye accumulation after localized regions of the thallus were briefly exposed to neutral red (Fig. 7), and it is tempting to conclude that there is a direct relationship between dye movement through the thallus and dye accumulation in the vacuole – that the dye moves directly and predominantly through the vacuolar sap. However, in interpreting what causes the rate of dye movement through the thallus to vary, we need to consider the routes of dye travel within all portions of the cell. Note, in contrast, that interpretation of connectivity between the hairs and the stalk (Fig. 7) does not depend on the route of dye movement *per se*.

To reach the vacuolar sap from the external environs, a molecule must pass through the cell wall, the cytoplasm and at least two membranes: plasma membrane and tonoplast. In a multicellular organism this pathway becomes far more complex. Before we can visually detect neutral red in any cell space, it must accumulate until it reaches a concentration and hue we can detect. Although in cross-section we could

Table 1 Packaging errors within gametangia of *Acetabularia acetabulum*

Component	Symbol in image	Number of errors in: Gametangia	Gametophores	Gametophores with errors	Caps with errors
Wall-less cytoplasm	##	–	19	19	2
Wall-less vacuole	#	–	19	14	4
Cytoplasm excluded	*	3	–	3	2
Vacuole excluded	**	0	–	–	–

Total 50 caps.

not see yellow dye in the pH-neutral cytoplasm, we have only this visual assay. Variables that will affect how subcellular compartments/regions within the thallus compete for dye include pH; relative speeds of adjacent compartments (cytoplasm vs central vacuole); position with respect to the point of application; binding constants for the dye; polymer topology, etc. An active mechanism, such as vesicle trafficking, that buds off and re-fuses vesicles in another site might also move neutral red within the thallus.

Considering one of many alternate routes for dye movement more concretely is illustrative. Cytoplasmic streaming through all regions of the stalk was 1–2 μm s^{-1} (Koop & Kiermayer, 1980; Koop, 1981), four- to eightfold faster than the rate of neutral red movement in the fastest regions of the thallus (Fig. 5). Direct observations of the stalk under a microscope clearly revealed dye only in the central vacuole (not shown). In addition, the chemistry of neutral red means that it strongly partitions into the acidic compartments, not into neutral ones such as the cytoplasm. Taken together, these data suggest that if the dye is traveling in the cytoplasm, it does so at low concentration, with rapid passage or exchange into other compartments.

If the major route of dye movement through the thallus is the vacuole itself, one intriguing interpretation of the distinct rates of dye movement in different regions of this unicell (Fig. 5) is that the vacuolar sap has a viscous, gel-like composition that varies throughout the thallus. There are many precedents for the existence of biological gels, particularly in animal systems (Klymkowsky, 2001; Pollack, 2001). The discovery that varying pH and ion concentrations alter the flow rate in the xylem of vascular plants suggests that plant cells contain hydrogels that undergo phase transitions (Zwieniecki *et al.*, 2001). Algal vesicles also contain biological gels important in many processes (Chin *et al.*, 2004).

To interpret the meaning of the variance in rates of dye movement through the thallus (Fig. 5), we need to use other methods complementary to the cell biology here. Use of fluorescent, cell-permeant dyes such as carboxyfluorescein derivatives will not resolve this issue because their pathways into the vacuole are just as complex as that of neutral red. At present we cannot microinject anything into the central vacuole, so membrane-impermeant dyes are not options. Classical biophysical analysis of regions of the vacuolar sap, lipophilic dyes and *in vivo* monitoring of cell lines transformed with green fluorescent protein (GFP)-fusion proteins will be important in understanding the biology of this gradient in dye mobility, regardless of the compartment in which the dye is moving.

Central vacuole increases dramatically in surface area and volume during development

Gibor (1974) proposed that maintenance of the chloroplast-containing younger vegetative whorls (Fig. 9) augmented photosynthesis and nutrient uptake. He found a ≈1 : 1 relationship between the surface area of a single whorl and the entire stalk. Our data show that the average surface area of a single whorl of hairs relative to the stalk increases during development from ≈1 : 1 in the juvenile phase to almost 5 : 1 in reproductive individuals. Gibor (1973) obtained a ratio of 0.80 : 1 for a single 'representative' individual of unknown age. The greater surface area of the whorls of hairs we observed probably results from our improvements in cell culture (Zeller & Mandoli, 1993; Hunt & Mandoli, 1996; Cooper & Mandoli, 1999) that foster more extensive lateral growth.

Our data suggest that an important function of development is to increase the surface area and volume of the thallus. The surface area and volume of all intact laterals relative to the algal stalk increase throughout development (Figs 3, 4). The surface area of all intact laterals relative to the stalk increases by a factor of ≈2.5, and the volume of these laterals compared with the stalk doubles between juvenile and early adult phase. This trend toward increased surface area and volume is even more evident in reproduction: the combined surface area of all laterals is nearly ninefold greater, and the combined volume of all laterals is nearly threefold greater than the volume of the stalk (Fig. 4, hatched and white bars). Clearly, the central vacuole plays an essential role in increasing both cellular volume and surface area during development.

The central vacuole is actively remodeled during development

Our data suggest that the *A. acetabulum* central vacuole is actively remodeled during hair development (Fig. 7). Vacuole movements

and changes in vacuole morphology occur in stomatal cells of *Allium* (Palevitz *et al.*, 1981). 'Mobile invaginations' of the vacuole undergo dynamic changes during growth of pollen tubes or root hairs (Flückiger *et al.*, 2003; Hicks *et al.*, 2004). Although it is clear that the cell wall is essential for separation of older hairs from the stalk (Puiseaux-Dao, 1962), it is not known what role the central vacuole plays in partitioning-off vegetative hairs. Immunohistochemistry and phalloidin labeling suggest close association of the cytoskeleton and the tonoplast in *Arabidopsis* pollen tubes (Derksen *et al.*, 2002), but the coordination, if any, of the cytoskeleton, vacuole and cell wall during hair development (from initiation to shedding) in *A. acetabulum* requires further study.

Cap morphology has implications for routes of nuclear migration

Our observation that the vacuole completely filled the inferior corona (Fig. 10f,g) presents us with a puzzle in understanding the transport route of thousands of haploid nuclei into the gametophores of the cap. If the vacuole remains ramified into the inferior corona during transport, haploid nuclei would have two possible routes for moving into the gametophore: a long route following the edge of the inferior corona; or a direct route through the vacuole, either along a string of cytoplasm protected from the acidic vacuolar sap or through the vacuole sap itself. As in other plants, strands of cytoplasm pass through the central vacuole of *A. acetabulum* (in the coronae, Menzel, 1981). Alternatively the vacuole in the inferior corona could partition off from the rest of the vacuole (by rearranging the cytoskeleton?), shortening the distance nuclei have to travel to enter the gametophores. A plug is always formed at the entrance to each gametophore after nuclei have been transported into it, and another plug sometimes forms in the coronal pore that separates the stalk from the coronae (Menzel, 1981). Callose plugs partition off the growing vacuole in pollen tubes (Laitiainen *et al.*, 2002). Further research using other methods is needed to reveal the positioning of the cytoskeleton and its interactions with the vacuole in the coronae and cap septa of this alga during nuclear migration.

The central vacuole is actively packaged into the haplophase

Here we documented the morphology of the giant central vacuole before and after gametangial formation, but the fate and function of the central vacuole, once incorporated into the gametangia, is unknown. In the green alga *Bryopsis plumosa*, the tonoplast in the gametangia disintegrates before the forced discharge of gametes, and the gametes then occupy the space the vacuole had previously filled (Mine *et al.*, 1996).

It is not known if, or how, each *A. acetabulum* gametangium divides its central vacuole among its gametes. The origin, function and development of the brightly stained, small red bodies within each gametangium (Fig. 11d) require clarification. Whether they are at all similar to 'dispersed vacuoles' found in hydrated pollen (Kuang & Musgrave, 1996), and have a particular function during reproduction, is not yet clear.

Vacuole packaging errors (Table 1) could result from genetic and environmental factors. The cause and fate of such rare phenotypes, for example, do they make and mature gametes, remains to be determined. We did not find gametangia lacking both cytoplasm and vacuoles (empty gametangial walls). Assuming that the exclusion of either component is independent of the other, such a phenotype would occur at a low enough frequency that it would have an extremely low probability of being present at our sample size. Alternatively, errors leading to this phenotype could be unable to initiate or complete the formation of gametangia.

Mistakes in packaging the vacuole and cytoplasm into gametangia could reflect errors in interactions between the cytoskeleton and the vacuole, although other mechanisms for changes in vacuole shape are also possible (Campbell & Garber, 1980; Fleurat-Lessard *et al.*, 1997). Actin filaments and microtubules have essential roles in the formation of gametangia (Menzel, 1986; Menzel & Elsner-Menzel, 1990). It is plausible that cytoplasm is associated with each haploid nucleus during nuclear transport, and that gametangial formation does not occur until the associated cytoplasm is properly arranged in the cap (Mandoli, 1996). However, nothing is known about the partitioning of the parent cytoplasm during reproduction in *Acetabularia* (Mandoli, 1996). Microtubules linking nuclei and chloroplasts have been observed in interphase cells in the green alga *Valonia ventricosa* (Shihira-Ishikawa, 1987). Gametangia are capable of completing morphogenesis correctly even when cytoplasm or vacuole is excluded, suggesting that packaging errors could derive from incorrect structure or mislocalization of molecules that mediate the association of the cytoskeleton with the organelles. One possible regulator of the cytoskeleton, a Rop GTPase, has been localized to the tonoplast of *Arabidopsis thaliana* (Lin *et al.*, 2001), but it is unknown if there are homologues in *A. acetabulum*.

In summary, our data indicate that the central vacuole of *A. acetabulum* is a dynamic organelle with a complex morphology and possibly a heterogeneous composition that undergoes morphogenesis of its vegetative and reproductive whorls during development. Regional applications of lipophilic dyes in conjunction with confocal microscopy will enable *in vivo* real-time studies of the endomembrane system in regions of particular interest during development of this complex unicell. Transformation with GFP constructs targeted to each of the two major types of vacuole (Ehara, 1996; Di Sansebastiano *et al.*, 1998, 2001; Neuhaus, 2000; Frigerio *et al.*, 2001; Moseyko & Feldman, 2001; Darnowski & Vodkin, 2002; Gao *et al.*, 2004) will begin to test the evolutionary conservation of these systems in a unicell phylogenetically distant from multicellular vascular land plants. Finally, classical biophysical analyses of the regional properties of this giant

central vacuole will tell us if the vacuolar sap is regionally differentiated and is a sol or a gel.

Acknowledgements

This work was supported in part by Friday Harbor Laboratory (FHL) Undergraduate Research Apprenticeships to D.A.N. and P.A.G. and in part by NSF MCB 0317151 to D.F.M. We thank Patrick Luethe for data in Fig. 5 that he gathered as an FHL Undergraduate Research Apprentice in the *Acetabularia* class in 2000. We thank Charles Woods for anatomically correct scale drawings in Figs 3 and 9.

References

Amtmann A, Gradmann D. 1994. Na^+ transport in *Acetabularia* bypasses conductance of the plasmalemma. *Journal of Membrane Biology* **139**: 117–125.

Berger S, Kaever MJ. 1992. *Dasycladales: An Illustrated Monograph of a Fascinating Algal Order*. London: Elsevier.

Budavari S. 1996. Neutral Red. In: O'Neil MJ, Smith A, Heckelman PE, Kinneary JF, eds. *Merck Index*. White House Station, NJ, USA: Merck Research Laboratories, 1741.

Campbell NA, Garber RC. 1980. Vacuolar reorganization in the motor cells of *Albizzia* during leaf movement. *Planta* **148**: 251–255.

Chin W-C, Orellana MV, Quesada I, Verdugo P. 2004. Secretion in unicellular marine phytoplankton: demonstration of regulated exocytosis in *Phaeocystis globosa*. *Plant and Cell Physiology* **45**: 535–542.

Cooper J, Mandoli DF. 1999. Physiological factors that aid differentiation of zygotes and early juveniles of *Acetabularia acetabulum* (Chlorophyta). *Journal of Phycology* **35**: 143–151.

Darnowski DW, Vodkin L. 2002. A soybean lectin–GFP fusion labels the vacuoles in developing *Arabidopsis thaliana* embryos. *Plant Cell Reports* **20**: 1033–1038.

von Dassow M, Odell G, Mandoli DF. 2000. Relationships between growth, morphology and wall stress in the stalk of *Acetabularia acetabulum*. *Planta* **213**: 659–666.

Derksen J, Knuiman B, Hoedemaekers K, Guyon A, Bonhomme S, Pierson ES. 2002. Growth and cellular organization of *Arabidopsis* pollen tubes *in vitro*. *Sexual Plant Reproduction* **15**: 133–139.

Di Sansebastiano G-P, Paris N, Marc-Martin S, Neuhaus J-M. 1998. Specific accumulation of GFP in a non-acidic vacuolar compartment via a C-terminal propeptide-mediated sorting pathway. *Plant Journal* **14**: 449–457.

Di Sansebastiano G-P, Paris N, Marc-Martin S, Neuhaus J-M. 2001. Regeneration of a lytic central vacuole and of neutral peripheral vacuoles can be visualized by green fluorescent proteins targeted to either type of vacuoles. *Plant Physiology* **126**: 78–86.

Dumais J, Serikawa KA, Mandoli DF. 2000. *Acetabularia*: a unicellular model for understanding subcellular localization and morphogenesis during development. *Journal of Plant Growth Regulation* **19**: 253–264.

Ehara M. 1996. Uptake of Neutral Red by the vacuoles of a green alga, *Micrasterias pinnatifida*. *Plant and Cell Physiology* **37**: 734–741.

Fester R, Hopkins C, Mandoli DF. 1993. Wounds, incurred during routine cell culture, prolong the life cycle of *Acetabularia acetabulum* and require K^+ to heal. *Protoplasma* **177**: 123–131.

Fleurat-Lessard P, Frangne N, Maeshima M, Ratajczak R, Bonnemain Martinoia E. 1997. Increased expression of vacuolar aquaporin and H^+-ATPase related to motor cell function in *Mimosa pudica* L. *Plant Physiology* **114**: 827–834.

Flückiger R, De Caroli M, Dalessandro G, Di Neuhaus J-M, Di Sansebastiano G-P. 2003. Vacuolar system distribution in *Arabidopsis* tissues, visualized using GFP fusion proteins. *Journal of Experimental Botany* **54**: 1577–1584.

Freudling C, Gradmann D. 1979. Cable properties and compartmentation in *Acetabularia*. *Biochimica et Biophysica Acta* **552**: 358–365.

Frigerio L, Foresti O, Felipe DH, Neuhaus J-M, Vitale A. 2001. The c-terminal tetrapeptide of phaseolin is sufficient to target green fluorescent protein to the vacuole. *Journal of Plant Physiology* **158**: 499–503.

Gao D, Knight MR, Trewavas AJ, Sattelmacher B, Plieth C. 2004. Self-reporting *Arabidopsis* expressing pH and $[Ca^{2+}]$ indicators unveil ion dynamics in the cytoplasm and in the apoplast under abiotic stress. *Plant Physiology* **134**: 898–908.

Gibor A. 1973. Observations on the sterile whorls of *Acetabularia*. *Protoplasma* **78**: 195–202.

Gibor A. 1974. Cell development in *Acetabularia*. *Portugaliae Acta Biologica, Serie A* **14**: 295–302.

Gradmann D. 1975. Analog circuit of the *Acetabularia* membrane. *Journal of Membrane Biology* **25**: 183–208.

Henry IM, Wilkinson MD, Hernandez JM, Schwarz-Sommer Z, Grotewold E, Mandoli DF. 2004. Comparison of ESTs from juvenile and adult phases of the giant unicellular green alga *Acetabularia acetabulum*. *BMC Plant Biology* **4**: 3.

Hicks GR, Rojo E, Hong S, Carter DG, Raikhel NV. 2004. Germinating pollen has tubular vacuoles, displays highly dynamic vacuole biogenesis, and requires *VACUOLESS1* for proper function. *Plant Physiology* **134**: 1227–1239.

Hörtensteiner S, Martinoia E, Amrhein N. 1992. Reappearance of hydrolytic activities and tonoplast proteins in the regenerated vacuole of evacuolated protoplasts. *Planta* **187**: 113–121.

Hunt BE, Mandoli DF. 1996. A new, artificial seawater that facilitates growth of large numbers of cells of *Acetabularia acetabulum* (Chlorophyta) and reduces the labor inherent in cell culture. *Journal of Phycology* **32**: 483–495.

Jauh G-Y, Phillips TE, Rogers JC. 1999. Tonoplast intrinsic protein isoforms as markers for vacuolar functions. *Plant Cell* **11**: 1867–1882.

Jiang L, Rogers JC. 1997. Integral membrane protein sorting to vacuoles in plant cells: evidence for two pathways. *Journal of Cell Biology* **1443**: 1183–1199.

Jiang L, Phillips TE, Rogers SW, Rogers JC. 2000. Biogenesis of the protein storage vacuole crystalloid. *Journal of Cell Biology* **150**: 755–769.

Jiang L, Phillips TE, Hamm CA, Drozdowicz YM, Rea PA, Maeshima M, Rogers SW, Rogers JC. 2001. The protein storage vacuole: a unique compound organelle. *Journal of Cell Biology* **155**: 991–1002.

Klymkowsky MW. 2001. Selling cellular gels. *Nature Cell Biology* **3**: E213.

Koop HU. 1981. Protoplasmic streaming in *Acetabularia*. *Protoplasma* **109**: 143–157.

Koop HU, Kiermayer O. 1980. Protoplasmic streaming in the giant unicellular green alga *Acetabularia mediterranea* I. Formation of intracellular transport systems in the course of cell differentiation. *Protoplasma* **102**: 147–166.

Kuang A, Musgrave ME. 1996. Dynamics of vegetative cytoplasm during generative cell formation and pollen maturation in *Arabidopsis thaliana*. *Protoplasma* **194**: 81–90.

Laitiainen E, Nieminen KM, Vihinen H, Raudaskoski M. 2002. Movement of generative cell and vegetative cell nucleus in tobacco pollen tubes is dependent on microtubule cytoskeleton but independent of the synthesis of callose plugs. *Sexual Plant Reproduction* **15**: 195–204.

Lin Y, Seals DF, Randall SK, Yang Z. 2001. Dynamic localization of Rop GTPases to the tonoplast during vacuole development. *Plant Physiology* **125**: 241–251.

Mandoli DF. 1996. Establishing and maintaining the body plan of *Acetabularia acetabulum* in the absence of cellularization. *Seminars in Cell and Developmental Biology* **7**: 891–901.

Mandoli DF, Larsen T. 1993. Improved mating efficiency in *Acetabularia acetabulum*: recovery of 48–100% of the expected zygotes. *Protoplasma* **176**: 53–63.

Marty F. 1999. Plant vacuoles. *Plant Cell* **11**: 587–599.

Matsuoka K, Bassham DC, Raikhel NV, Nakamura K. 1995. Different sensitivity to wortmannin of two vacuolar sorting signals indicates the presence of distinct sorting machineries in tobacco cells. *Journal of Cell Biology* **130**: 1307–1318.

Menzel D. 1981. Development and fine structure of plugs in the cap rays of *Acetabularia acetabulum* (*mediterranea*) (L.) Silva (Dasycladales). *Phycology* **20**: 56–64.

Menzel D. 1986. Visualization of cytoskeletal changes through the life cycle in *Acetabularia. Protoplasma* **134**: 30–42.

Menzel D. 1994. Cell differentiation and the cytoskeleton in *Acetabularia*. *New Phytology* **128**: 369–393.

Menzel D, Elsner-Menzel C. 1990. The microtubule cytoskeleton in developing cysts of the green alga *Acetabularia*: involvement in cell wall differentiation. *Protoplasma* **157**: 52–63.

Mine I, Okuda K, Tatewaki M. 1996. Gamete discharge by *Bryopsis plumosa* (Codiales, Chlorophyta) induced by blue and UV-A light. *Phycology Research* **44**: 185–191.

Moseyko N, Feldman LJ. 2001. Expression of pH-sensitive green fluorescent protein in *Arabidopsis thaliana. Plant, Cell & Environment* **24**: 557–563.

Mummert H, Gradmann D. 1991. Ion fluxes in *Acetabularia*–vesicular shuttle. *Journal of Membrane Biology* **124**: 255–263.

Neuhaus JM. 2000. GFP as a marker for vacuoles in plants. In: Robinson DG, Rogers JC, eds. *Vacuolar Compartments.* Sheffield, UK: Sheffield Academic Press, 254–269.

Nishimura NJ, Mandoli DF. 1992a. Population analysis of reproductive cell structures of *Acetabularia acetabulum. Phycologia* **31**: 351–358.

Nishimura NJ, Mandoli DF. 1992b. Vegetative growth of *Acetabularia acetabulum* (Chlorophyta): structural evidence for juvenile and adult phases in development. *Journal of Phycology* **28**: 669–677.

Palevitz BA, O'Kane RE, Raikhel NV. 1981. The vacuole system in stomatal cells of *Allium.* Vacuole movements and changes in morphology in differentiating cells as revealed by epifluorescence, video and electron microscopy. *Protoplasma* **109**: 23–55.

Paris N, Stanley CM, Jones RL, Rogers JC. 1996. Plant cells contain two functionally distinct vacuolar compartments. *Cell* **85**: 5563–5572.

Pollack, GH. 2001. *Cells, Gels and the Engines of Life.* Seattle, WA, USA: Ebner and Sons.

Puiseaux-Dao S. 1962. Recherchés biologiques et physiologiques sur quelques Dasycladacées, en particulier, le *Bataphora oerstedii* J. Ag. et l'*Acetabularia mediterranea* (Lamouroux). *Revue Générale de Botanique* **69**: 409–519.

Rojo E, Gillmor CS, Kovaleva V, Somerville CR, Raikhel NV. 2001. *VACUOLELESS1* is an essential gene required for vacuole formation and morphogenesis. *Developmental Cell* **1**: 303–310.

Schweiger HG, Berger S, Bonotto S, Focken H. 1974. *Acetabularia and other Dascladaceae: Bibliography of the Reprint Collection of the Max-Planck-Institut für Zellbiologie.* Wilhelmshaven, Germany: Max-Planck-Institut für Zellbiologie, 1–81.

Serikawa KA, Mandoli DF. 1998. An analysis of morphogenesis of the reproductive whorl of *Acetabularia acetabulum. Planta* **207**: 96–104.

Serikawa KA, Porterfield DM, Smith PJS, Mandoli DF. 2000. Calcification and measurements of net proton and oxygen flux reveal subcellular domains in *Acetabularia acetabulum. Planta* **211**: 474–483.

Shihira-Ishikawa I. 1987. Cytoskeleton in cell morphogenesis of the coencytic green alga *Valonia ventricosa* I. Two microtubule systems and their roles in positioning of chloroplasts and nuclei. *Japanese Journal of Phycology* **35**: 251–258.

Strugger S. 1935. Beiträge zur Gewebephysiologie der Wurzel. Zur Analyse und Methodik der Vitafärbung pflanzlicher Zellen mit Neutralrot. *Protoplasma* **24**: 108–127.

Surpin M, Raikhel NV. 2004. Traffic jams affect plant development and signal transduction. *Nature Reviews: Molecular Cell Biology* **5**: 100–109.

Ullrich CI, Köhler K, Baier M, Förester B, Hartung W. 1990. Neutral red as a redox dye induces K^+ efflux and current-voltage changes in *Eremosphaera, Lemna, and* guard cells. *Botanica Acta* **103**: 214–221.

Wang Z, Zhang Z, Liu D, Dong S. 2003. A temperature-dependent interaction of Neutral Red with calf thymus DNA. *Spectrochimica Acta Part A* **39**: 949–956.

Weibe HH. 1978. The significance of plant vacuoles. *Bioscience* **28**: 327–331.

Zeller A, Mandoli DF. 1993. Growth of *Acetabularia acetabulum* on solid substrata at specific cell densities. *Phycologia* **32**: 136–142.

Zwieniecki MA, Melcher PJ, Holbrook NM. 2001. Hydrogel control of xylem hydraulic resistance in plants. *Science* **291**: 1059–1062.



About *New Phytologist*

- *New Phytologist* is owned by a non-profit-making **charitable trust** dedicated to the promotion of plant science, facilitating projects from symposia to open access for our Tansley reviews. Complete information is available at **www.newphytologist.org**.
- Regular papers, Letters, Research reviews, Rapid reports and Methods papers are encouraged. We are committed to rapid processing, from online submission through to publication 'as-ready' via *OnlineEarly* – the 2003 average submission to decision time was just 35 days. Online-only colour is **free**, and essential print colour costs will be met if necessary. We also provide 25 offprints as well as a PDF for each article.
- For online summaries and ToC alerts, go to the website and click on 'Journal online'. You can take out a **personal subscription** to the journal for a fraction of the institutional price. Rates start at £109 in Europe/$202 in the USA & Canada for the online edition (click on 'Subscribe' at the website).
- If you have any questions, do get in touch with Central Office (**newphytol@lancaster.ac.uk**; tel +44 1524 592918) or, for a local contact in North America, the USA Office (**newphytol@ornl.gov**; tel 865 576 5261).