Coordination of cellular events that precede reproductive onset in

*Acetabularia acetabulum*: evidence for a ‘loop’ in development

Linda L. Runft† and Dina F. Mandoli*

Department of Botany 355325, University of Washington, Seattle, WA 98195-5325, USA

*Author for correspondence
†Present address: The University of Connecticut Health Center, Department of Physiology, Farmington, CT, USA

SUMMARY

Amputated apices from vegetative wildtype cells of the uninucleate green alga *Acetabularia acetabulum* can differentiate a reproductive structure or ‘cap’ in the absence of the nucleus (Hämmerling, J. (1932) *Biologisches Zentralblatt* 52, 42-61). To define the limits of the ability of wildtype cells to control reproductive differentiation, we determined when during development apices from wildtype cells first acquired the ability to make a cap in the absence of the nucleus and, conversely, when cells with a nucleus lost the ability to recover from the loss of their apices. To see when the apex acquired the ability to make a cap without the nucleus, we removed apices from cells varying either the developmental age of the cells or the cellular volume left with the apex. Cells must have attained the adult phase of development before the enucleate apex could survive amputation and make a cap. Apices removed from cells early in adult growth required more cell volume to make a cap without the nucleus than did apices removed from cells late in adult growth. To define the limits of the cell to recapitulate development when reproduction falters, we analyzed development in cells whose caps either had been amputated or had spontaneously aborted. After loss of the first cap, cells repeated part of vegetative growth and then made a second cap. The ability to make a second cap after amputation of the first one was lost 15-20 days after cap initiation. Our data suggest that internal cues, cell age and size, are used to regulate reproductive onset in *Acetabularia acetabulum* and add to our understanding of how reproduction is coordinated in this giant cell.

Key words: *Acetabularia acetabulum*, competence, differentiation, reproduction, gametangium

INTRODUCTION

Coordination of cellular events at reproductive onset is important for the unicellular alga, *Acetabularia acetabulum*: since virtually all of the contents of the cell are donated to its progeny, if the parent cell fails to coordinate nuclear and cytoplasmic events it will die without producing offspring. Thus, reproductive onset in *A. acetabulum* may be more tightly regulated than in organisms whose commitment to reproduction does not entail such drastic consequences for the species.

Accordingly, the cell has evolved strategies both to prevent premature entry into reproduction and to reiterate reproductive development. For example, it survives winters by retracting the cytoplasm into the rhizoid (Berger and Kaever, 1992; Dao, 1962), it can stall development between the phases in vegetative growth (Mandoli, unpublished data), and the cell can execute a ‘developmental loop’ by aborting reproduction (*A. crenulata*: Puiseux-Dao, 1963; *A. mediterranea*: Declève et al., 1972) if environmental conditions are not optimal or the original apex is lost. The strategies used by the cell to ensure reproductive success are not well understood.

This organism is attractive for developmental studies for many reasons. First, in contrast to multicellular organisms, reproductive onset in *A. acetabulum* is regulated only by interactions within the cell and with its environs, not by signals in or from multicellular tissues or organs. The cellular dialog between the nucleus, which is located in the basal rhizoid of the cell, and the cell apex, which is the site of differentiation, occurs over a long distance (3-4 cm). Second, the age of a cell can be assessed because the stalk is decorated with rings of hairs, or ‘whorls’, that leave scars whose spacing is characteristic of the phase of development (Nishimura et al., 1992b). Third, it’s giant size and ability to heal wounds (Fester et al., 1993; Menzel, 1980) facilitates manipulations such as cell grafting (Berger et al., 1987; Menzel, 1994) and allows the developmental potential of pieces of the cell to be analyzed. Finally, the cell can be stably transformed (Neuhaus et al., 1984), expresses and correctly targets proteins encoded by heterologous or foreign DNA readily (Neuhaus et al., 1984), and is amenable to biochemical (Berger et al., 1987) and genetic manipulations (Mandoli and Larsen, 1993). Comprehensive knowledge of development in wildtype cells is important to make full use of these features.

Reproductive onset is marked by an explicit shape change at the cell apex, initiation of a cup-shaped structure or ‘cap’, and is regulated by both internal and external cues. The internal cues are the age and size of the cell (Nishimura et al., 1992b), the presence of a putative population of cap-specific mRNAs...
concentrated at the cell apex (Hämmerling, 1963a), and the absence of a putative, cytosolic inhibitor of the expression of these mRNAs (Bannwarth et al., 1991; Beth, 1953; Li-Weber et al., 1985; Shoeman et al., 1983; Zetsche, 1966). The external cues include the nutrient environment in which the cell grew (Hunt et al., 1995) and probably blue light (Clauss, 1970). Cells reproduce only once all the internal and the external cues are met.

Vegetative development of *Acetabularia acutabulum*, which must be completed prior to reproductive onset, has morphologically distinct juvenile and adult phases (see Materials and Methods) which are temporally sequential and spatially stacked (Nishimura et al., 1992b). In Fig. 1, the vegetative phases are delineated on a mature or ‘late adult’ cell which is ready to make a cap. After the cap initiates, it expands laterally (Fig. 1). During cap expansion the diploid nucleus probably undergoes meiosis (Green, 1973; Koop, 1975) followed by 9-10 rounds of mitosis (Nishimura et al., 1992a). The resulting haploid ‘secondary’ nuclei are transported up the stalk along with most of the parent cell contents into the cap. After nuclear transport, the junction or ‘septum’ between the cell stalk and the cap closes (Fig. 1, far right, center cap). The stalk is now optically clear yet retains its shape. Gametangial cell walls enclose each haploid nucleus with some parental cell contents (Fig. 1, far right, bottom cap). Gametangial formation is fairly independent in the individual partitions or gametophores of the cap (Berger et al., 1992; Shihiira-Ishikawa, 1989). Temporal coordination between and interdependence of these developmental events in wildtype cells is not known.

Our goal was to analyze the age and size requirements for cap initiation and to begin to assess the roles played by the nucleus and apex in regulating reproductive onset in wildtype cells. Our data define the spatial and temporal limits to reproductive development both in intact cells and in cells regenerating from cap amputation. The ‘developmental loop’ described by these data will be a useful experimental tool in analyzing how reproductive onset is regulated in *Acetabularia*.

**MATERIALS AND METHODS**

**Cell strain and culture**

*Acetabularia acutabulum* (L.) Silva (Chlorophyta), strain Ao0005 (Ladenburg #17), was used in all experiments. This laboratory strain has been axenically propagated for 3 generations using all the gametangia from >200 individuals per generation to prevent loss of alleles. Gametangia-bearing caps were decontaminated (Hunt et al., 1992) and then stored at 10°C in the dark until use. Following mating (Mandoli et al., 1993), the axenic zygote stock was stored in the dark at 10°C. Zygotcs were grown at 1 cell/3 ml until they made the first whorl (Zeller et al., 1993). Then, each cell was grown to reproductive maturity in a polystyrene Petri dish with 20 ml of seawater at 21±1°C under cool-white fluorescent lights (40-70 μmol photons/m²/second) on a 14:10 hour photoperiod. One version of a novel artificial seawater (Hunt et al., 1995) which is based chemically on a derivation of Müller’s medium (Müller, 1962) as modified by Schweiger et al. (1977) was used here except in some of the experiments involving amputation (Figs 5 and 6) in which a slightly different, earlier version of the recipe was used.

**Cell developmental age**

The phase of vegetative development was assessed for all cells (Nishimura et al., 1992b). ‘Juveniles’ were threadlike, 0.1-1.0 cm tall, and had 1-5 whors consisting of clear, ephemeral hairs that branched 0-3 times each. ‘Early adults’ had thin stalks, were 1-3 cm tall and had more than 6 green whors comprised of hairs that branched three times each. ‘Late adults’ had thick robust stalks and persistent green whors composed of hairs that branched four times each. The ‘reproductive phase of development’ includes cap initiation and expansion, gametogenesis, and mating. Cells whose caps had reached their maximum diameters and contained gametangia were termed ‘mature’ cells or caps.

**Measurements of structures, amputation of cells, and data analysis**

Cap diameters were measured on a dissecting microscope fitted with an ocular micrometer. An isolation-fixation solution containing 4′-6-diamidino-2-phenylindole (DAPI) was used to stain nuclei (De et al., 1990). Plastic and glassware were sterilized with ethylene oxide (Zeller et al., 1993). All chemicals were purchased from Sigma (St. Louis, MO, USA).

Experimental manipulations are depicted by cartoons on the relevant figures. Before amputation, a ‘pressure-wound’ was made at the prospective cut site with a dental tool (small excavator spoon, CG-AMERICAN Model #UW A 46, GC International Corp., Scottsdale, AZ, USA) so as to minimize loss of cell contents. Pressure wounds bisected the cell contents, caused them to retract away from the wound site, and created a region devoid of both cytoplasm and vacuole (Fester et al., 1993). The cell wall was cut with a scalpel (type 21, Bard Parker, Division of Becton, Dickinson, and Company Ruther-
ford, NJ) perpendicular to the long axis of the stalk 5-7 seconds after pressure-wounding.

StatView® 4.0 was used to analyze the data (Abacus Concepts, Inc.). Standard errors of the mean are given when possible.

RESULTS

Ability of the apex to differentiate: dependence on the nucleus

Although it was shown over 60 years ago that vegetative *A. acetabulum* apices can differentiate a cap without a nucleus (Hämmerling, 1932, 1934, 1963b), the developmental age when the apex first becomes independent of the nucleus has never been discerned. Cells used for amputation experiments by previous researchers (Beth, 1953; Hämmerling, 1932, 1934) were probably late adults (see Materials and Methods).

To see whether the ability of the apex to initiate a cap depended on the age of the cell when the rhizoid was removed, whole cells were bisected at different times after formation of the first whorl leaving all of the stalk and about 90% of the cell contents with the apex. Fig. 2 shows that apices whose rhizoids had been amputated ≤ 20 days after the intact cells had made the first whorl of hairs had <50% probability of surviving the procedure whereas apices amputated ≥20 days after making the first whorl had an 80-100% chance of surviving. The youngest apices that could make a cap without the nucleus were amputated from cells ≥35 days after formation of the first whorl (Fig. 2, arrow), which corresponds to the early adult portion of vegetative growth. In contrast to apices from juvenile cells, apices from adult cells can make caps without the nucleus as shown in Fig. 3. When about 90% of the cell

Fig. 2. Ability of the apex to survive without the rhizoid depends on the age of the cell. The ages of the populations (e.g. those at 25 and 20 days) at the time of amputation are approximate as the different experimental populations were not developmentally synchronous. Cells whose tips were amputated at 0 to 25 days after time zero were juveniles, at 25 to 45 days were early adults, and at 45 to 60 days were late adults (see definitions in Materials and Methods). Apices amputated from cells at 35 days after formation of the first whorl were the youngest to subsequently initiate caps (vertical arrow). Each point represents from 6 to 20 cells with a mean of 15.6±1.6 cells. Intact cells made caps starting 45 days and enucleate apices 55.8±0.8 days after formation of the first whorl. Cartoon to the right of the question mark indicates the terminal morphology of apices that survive amputation but fail to make a cap.

Fig. 3. Apices that made a cap versus the fraction of the cell left with the apex. The percentages of cap initiation in 4 populations of intact, wild-type cells are indicated by the symbols on the right. These control populations were grown under identical conditions and were pressure-wounded but not amputated either during the day (open circles) or night (solid circles). Cells amputated during the day are indicated by open symbols connected with a line. Cells amputated during the night are indicated by solid symbols connected with a line. The mean number of cells represented by data from several experiments was 14.9±0.1 except for the control cell populations (n = 60 cells total).

Fig. 4. Comparison of the diameters reached by aborted and mature caps. Only those cells that eventually bore gametangia were used in two wild-type, laboratory strains (*Aa* 0005: 85 out of 98 cells, *Aa* 0006: 68 out of 121 cells). The average cap diameter at abortion was 58.5±4.1% for *Aa* 0005 or 50.0±3.3% for *Aa* 0006 of the mature, gametangia-bearing cap. The average diameter of the mature gametangia-bearing cap was 5.7±0.2 mm for *Aa* 0005 or 4.7±0.2 mm for *Aa* 0006. Between successive caps, cells made an average of 4.0±0.4 whorls for *Aa* 0005 or 3.1±0.4 whorls for *Aa* 0006. The inset shows the same data analyzed in a ‘box plot’ format. The five horizontal lines in each box plot represent 10, 25, 50, 75, and 90% of the population. The symbols at the top and bottom of each box plot show ‘outliers’ or individual cells which are the 10% at the population extremes.
was left with the apex, apices from late adults were 3 times more likely to initiate a cap than apices from early adults (Fig. 3). These results suggest that for an apex to initiate a cap in the absence of the nucleus, the cell from which it was taken must have reached the early adult phase of development (Figs 2 and 3).

To establish how cell volume affected the ability of an enucleate apex taken from adult cells to initiate a cap, early and late adult cells were amputated at varying points along the stalk (Fig. 3). Apices varied in length by increments of 1/8 of a cell. The rhizoid comprised the lower or distal 1/8 of the total cell length. Since cell height is genetically determined (Nishimura et al., 1992b; Mandoli and Hunt, unpublished data), relative rather than absolute length was used. Two thirds of the intact controls (40 out of 60 cells) initiated caps (Fig. 3). The apex itself (upper quarter of the cell) was rarely capable of making a cap without the nucleus. Clearly, early adult apices required more cell volume than late adult apices to make a cap without the nucleus.

To see how cap initiation depended on the number of chloroplasts in the apex, cell populations were amputated both during the day, when the chloroplasts stream continuously in the stalk, and during the night, when most of the chloroplasts stop streaming and ‘rest’ in the rhizoid (Schweiger et al., 1964; Wollum, 1991). This circadian rhythm in chloroplast movement results in a gross asymmetry of organelles so that amputation of cells at night yields apices with fewer chloroplasts and rhizoids which are highly chloroplast enriched. Conversely, amputation of cells during the day yields cell pieces with fairly equal chloroplast densities. Apices amputated from early adult cells during the day needed more cell body to make a cap than those amputated at night (Fig. 3, top) whereas apices amputated from late adult cells needed less than half of the cell body to initiate a cap regardless of time of day at which amputation occurred (Fig. 3, bottom). Hence, the ability of an enucleate apex to make a cap was not strictly dependent on the number of chloroplasts in the apex (Fig. 3).

The ability to make more than 1 cap: a ‘loop’ in reproduction

Cells can repeat the transition from vegetative to reproductive development: a cell will cease expansion of a cap, produce more stalk, and then initiate a new cap as diagrammed in Fig. 4. The cell contents are withdrawn from the ‘aborted’ cap back into the stalk so that the aborted cap eventually appears white. This ‘loop’ in development is repeated until the youngest (i.e., uppermost) cap formed at the cell apex completes gametogenesis. A cell that has aborted resembles a stack of 2 or more umbrellas which are fused top to bottom (Fig. 4, top right). Abortion occurs both in the ocean and in the laboratory.

Cells with aborted caps were isolated from wildtype laboratory strains in which they had spontaneously appeared. Cap abortion occurred in about 5% of a population (6 out of 130 cells). In two cell lines, only 56.2 to 86.7% of the cells that had aborted a cap made gametangia (see Fig. 4 legend). Cells that failed to make gametangia died and were excluded from this analysis. The diameters of aborted caps were measured and then normalized to the diameter reached by the mature gametangia-bearing cap eventually made by the same cell in order to nullify genetic variation in cap diameter (Koop, 1977).

Note that we could not tell how much the aborted cap expanded after growth of the stalk recommenced because we can’t predict when and in which cells cap abortion will occur. The diameters of the aborted caps varied: the mean diameter of the aborted caps was about 50% of the mature, gametangia-bearing caps with 3-4 whorls made between successive caps (details in Fig. 4 legend). Although cap diameter at abortion varied widely, all cells of a strain made the same number of whorls before reproduction was attempted again.

To estimate the time of abortion, the final diameter of the aborted caps (Fig. 4) was correlated with the cap diameter in cells that developed normally. For this estimate to be valid, cap expansion must be linear and similar regardless of whether a cell is making a first or second cap. First, as Fig. 5 illustrates, caps expanded at a linear rate until they reach their final mature diameter about 25 days after initiation. Second, the rate of cap expansion is similar for intact cells and the second cap of whose first cap had been removed (Fig. 5: intact, 3.6%; amputated, 3.9% of the final cap diameter per day). Note that although 13.3% (10 out of 75) of the mature gametangia-bearing caps were smaller in diameter than the immature caps that had been aborted by the same cell (Fig. 4), this does not affect the calculation of the rate of cap expansion since the data were normalized. These data predict that cap abortion occurred about 15 days after a cap initiated: at 3.6% expansion of the final cap diameter per day, caps reach 55.4% of the mature diameter 15.4 days post cap initiation (Fig. 4, inset box plots).

Fig. 5. Rate of cap expansion for amputated and intact cells. The linear regression for days 0-25 was y = 9.3x+4.0x (R=0.98) for the amputated cells (mean cells per datum for days 0-25=11.2±1.2, mean cells per datum point for days 25-45=22±1.2) and was y=11.9+3.6x (R=0.99) for the intact cells (mean cells per datum point = 28.8±1.3). The average diameter of the mature gametangia-bearing cap was 5.6±0.1 mm for intact cells and 6.02±0.2 mm for the second cap of amputated cells. Data for the amputated population at days 45 and 50 are missing since the cells had formed gametangia and so could not make a second cap. Other developmental events which occur during this time frame are shown in Fig. 10.

Triggering the loop in reproduction with cap amputation

To determine the limits of cells to reiterate reproductive onset, apices of reproductive cells were amputated until all cells in
Coordinating reproduction in *Acetabularia*

The population stopped initiating caps. Apices were amputated on the day of cap initiation and were discarded. All cells initiated a second and then a third cap following amputation of the first and second caps respectively (Fig. 6). The probability that a cell would initiate 4 or more caps was inversely related to the number of apices that had been previously amputated. On average, cells made 9.0±1.0 caps after successive apex amputations before losing the ability to reiterate reproductive onset. One cell initiated 23 caps in succession, failing to make a new cap only after the 23rd apex amputation. Fig. 7 shows that new caps were initiated an average of 11 days after amputation of the previous cap. Although the intervals needed to reinitiate a cap following amputation of caps 3 through 23 ranged from 7 to 17 days there was no pattern in the time required: one cell might take 17 days to make the 10th cap but only 7 days for the 11th suggesting that the amount of time required to initiate a new cap was not a function of the amputation history of the cell but of that particular amputation.

These data show that *A. acetabulum* has a remarkable capacity to recover from wounds and can repeat reproductive onset if the apex is lost.

We wondered whether the cells were returning to the same earlier point in development regardless of how the cap was lost. That is, was the loop in development the same in aborted and amputated cells? We measured both the time from loss of the cap to initiation of a new one and the cell structure of the stalk made during this developmental loop. A quantitative temporal comparison of the abortion and amputation data (Figs 4 vs. 6) was not possible since we could not induce cap abortion but only see that a cap had aborted retrospectively. Data in Fig. 8 show that cells whose apices were removed on the day of cap initiation made a new cap 5 days post amputation with 1 whorl between the amputation site and the new cap. Cells whose apices were amputated 5-20 days post cap initiation took longer to make a new cap and made more whorls than those amputated on the day of cap initiation (Fig. 8). From 0-20 days these increases are nearly linear. When apices were removed >20 days post cap initiation, most of the cells could not make a new cap but the few that could did so 8-11 days after amputation. The time required for healing the wounds was not assessed. These data suggest that a cell can repeat reproductive development at almost any time during cap expansion if the cell returns to a point in vegetative growth at least 5 days prior to cap initiation and suggests that the amount of vegetative growth after cap amputation depends strongly on the amount of cell contents that were removed.

**Point of no return: loss of ability to repeat reproductive onset**

If there existed a point when a cell had to finish reproduction with the last cap made and could no longer repeat reproductive onset, then cells whose apices were amputated after this would not be able to form a new cap. As shown in Fig. 9, cell populations were amputated at several times post cap initiation to see if such a point exists. All cells that were amputated ≤15
and 50% of the cells completed septum closure at 32 days post cap initiation. Septum closure first occurred about 24 days after cap initiation (Fig. 1). Septum closure marks the end of nuclear transport (Fig. 1). Gametangia formation about 55 days post cap initiation (Fig. 10). Cells completed cap expansion, septum closure and gametangial formation about 1 week apart (Figs 5, 10).

**DISCUSSION**

Our data suggest that for *A. acetabulum* to reproduce it must be old and/or big enough and establishes the limits of the cell’s ability to reproduce when the reproductive apex aborts or is lost. Based primarily on our data, Fig. 11 summarizes the relative timing of the developmental events or interactions that surround reproductive onset in *A. acetabulum*. This is the first time that events preceding and during reproductive development have been quantified with the amputations feasible in *A. acetabulum*.

**Regulation of cap initiation: putative mRNAs and a putative inhibitor**

The ability of the apex to make a cap without the nucleus has been attributed to the presence of a putative population of stable mRNAs (Berger et al., 1987; Beth, 1953; Hämmerling, 1963a). This ability is largely confined to the apex since isolated midsections of the cell rarely initiate caps (Hämmerling, 1932, 1934). Furthermore, Beth (1953) reasoned that differentiation of the apex was inhibited by the nucleus until the cell was ready to reproduce since the enucleate apex differentiated sooner than it would have in the presence of the nucleus. This hypothesis has been supported by comparison of the kinetics of cap initiation and the activity of developmentally regulated enzymes between intact and enucleate *Acetabularia* cells (Bannwarth et al., 1977, 1982; de Groot and Schweiger, 1983, 1985). The existence of the putative mRNAs and the role and identity of the putative cytosolic inhibitor of cap initiation...
Coordinating reproduction in Acetabularia

Repetition of reproductive development: abortion versus amputation

To make a new cap after abortion a cell repeats part of vegetative growth (Figs 4-6) that has been previously defined as late adult on a morphological basis (Nishimura et al., 1992b). The portion of vegetative growth which is repeated is invariant within a given strain. Three possible causes for cap abortion are: unfavorable environmental conditions (Fester et al., 1993), an error in coordination between the nucleus and apex, or the presence of a lethal mutation. Short days may induce cap abortion (Puiseux-Dao, 1963). Abortion in cells that failed to bear gametangia (see Fig. 4, legend) suggests the presence of lethal mutations in some cells. Acetabularia cells that have aborted superficially resemble mutants in yeast (cde4 and 34: Fong et al., 1986; Goebl et al., 1988), Aspergillus nidulans (brlAb: Miller, 1993) and possibly Drosophila (polycomb: Lonie et al., 1994): in each case reproductive structures are reiterated several times, stacked one above another. Whether spontaneous abortion in A. acetabulum also has a genetic basis is not known.

Although apex loss via abortion and amputation both trigger initiation of a new cap, the developmental pattern that ensues is not the same. During abortion the pattern of development does not vary, there is no wound to heal, and all the cytoplasm is recovered from the old cap. In contrast, when a cap is physically removed, cells make an increasing number of whorls as the interval between cap initiation and cap amputation increased (Fig. 8) and later amputations remove an increasing proportion of the cell body as more of the parental cell contents are moved into the expanding cap. (Note that the apparently faster cap initiation and fewer whorls made in this experiment (Fig. 8) should not be overinterpreted because the population was small by this time.) In sum, although cap abortion or amputation both trigger cells to repeat reproductive onset, it is not clear how these phenomena are related.

Point of no return

Cells cannot enter the loop in reproductive development after the cap reaches a certain age, when it must finish reproduction or die (Fig. 9). We avoid the term ‘commitment’ because our assay is a loss of function, inability to make a new cap post amputation, rather than a gain of function or commitment to a defined event such as the beginning of cell division, called START (Hartwell et al., 1989). Chromosome behavior visualized with DAPI staining in A. calyculus suggests that premeiotic chromosome condensation started 3 days after cap initiation and finished in the cell population when the cap was about three quarters of its final diameter (Shihiira-Ishikawa, 1984) but these events have not been documented well in A. acetabulum. The relationship between meiosis and the point of no return is not obvious.

These data contribute to our understanding of which cellular activities characterize the different developmental ages of Acetabularia acetabulum and describe the limits of the ability of the organism to control reproductive onset. How the apex and the nucleus of this organism work together to coordinate progress through the phases in vegetative development and to correct errors in the onset of reproduction will be intriguing puzzles to unravel.

The authors contributed equally to this work. This research was supported by a Hughes Undergraduate Research Fellowship (L. L. R.), in part by a Project R/B-8 of the Washington Sea Grant Program with funding from Grant no. NA89AA-D-SG022 from the National Oceanic and Atmospheric Administration, U.S. Department of Commerce (D. F. M.), and in part by the National Science Founda-
REFERENCES


(Accepted 21 December 1995)