Sperm entry induces polarity in fucoid zygotes

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SUMMARY

Fucoid zygotes establish a rhizoid-thallus growth axis in response to environmental signals; however, these extrinsic cues are not necessary for polarization, suggesting that zygotes may have inherent polarity. The hypothesis that sperm entry provides a default pathway for polarization of zygotes cultured in the absence of environmental signals was tested, and was supported by several lines of evidence. First, an F-actin patch, a cortical marker of the rhizoid pole, formed at the sperm entry site within minutes of fertilization. Second, the sperm entry site predicted the site of polar adhesive secretion (the first morphological manifestation of the rhizoid pole) and the position of rhizoid outgrowth. Third, when fertilization was restricted to one hemisphere of the egg, rhizoid outgrowth always occurred from that hemisphere. Fourth, delivery of sperm to one location within a population of eggs resulted in polarization of both adhesive secretion and rhizoid outgrowth toward the sperm source. Finally, induction of polyspermy using low sodium seawater increased the frequency of formation of two rhizoids. Sperm entry therefore provides an immediate default axis that can later be overridden by environmental cues.

Key words: Localized F-actin, Pelvetia compressa, Polar adhesive, Polarity establishment, Sperm entry, Zygotic polarity

INTRODUCTION

The mechanisms by which fertilization initiates development are best understood from studies in animals, where sperm entry rapidly induces a cascade of ionic and biochemical events that activate the egg, prevent fertilization by additional sperm, and in some cases provide cues for developmental patterning. As a fast block to polyspermy, the egg membrane of sea urchins and frogs transiently depolarizes immediately after sperm binding (Steinhardt et al., 1971; Jaffe, 1976; Cross and Elinson, 1980), temporarily preventing fusion of other sperm. In most animal eggs studied, sperm binding also induces a large, transient, increase in Ca²⁺ ion concentration within the egg, which begins at the site of fertilization and moves across the egg as a wave (Whitaker and Swann, 1993). Although the mechanism by which sperm induce Ca²⁺ waves is widely debated (Swann and Lai, 1997; Jones et al., 1998), it is clear that the wave induces cortical vesicle fusion with the plasma membrane resulting in the elevation of the fertilization envelope, a mechanical barrier to further sperm penetration (slow block to polyspermy).

In some animal eggs, sperm entry also provides developmental patterning cues. For example, in the tunicate, Styela partita, fertilization induces cytoplasmic rearrangements that are important for determining the positions of the future mesenchyme and muscles (Conklin, 1905). In Xenopus eggs, sperm entry induces a microtubule-dependent rotation of cortical cytoplasm relative to the inner cytoplasm, which initiates a cascade of events resulting in formation of the dorsoventral axis, with the ventral pole at the sperm entry site (Gerhart et al., 1989). In plants much less is known about fertilization and its role in egg activation and developmental patterning. In large part, this lack is due to the relative inaccessibility of eggs and developing embryos as they are buried deep within maternal (gametophytic and sporophytic) tissues of higher plants. Gametes and zygotes of the fucoid algae, in contrast, are well-suited for studies of early development.

Fertilization in the fucoid algae exhibits some similarities to that of animal systems. Fusion of the sperm with the wall-less egg (Jaffe, 1958) induces a rapid, sodium-dependent membrane depolarization which is a fast block to polyspermy (Brawley, 1987). The depolarization activates voltage-gated Ca²⁺ channels (Taylor and Brownlee, 1993) resulting in Ca²⁺ influx that leads to a further depolarization and a slight local elevation of Ca²⁺ beneath the plasma membrane (Roberts et al., 1994; Roberts and Brownlee, 1995). Unlike animal cells, no wave of elevated cytosolic Ca²⁺ has been detected in fucoid eggs. Elevated Ca²⁺ appears to induce exocytosis of cell wall materials within minutes of fertilization, and the nascent wall is thought to function as a slow block to polyspermy (Roberts et al., 1994).

The unfertilized egg is an apolar cell and patterning is
established following fertilization. In the unfertilized egg, organelles are distributed uniformly throughout the cytoplasm (Brawley et al., 1976), microtubules nucleate uniformly from the egg pronucleus and extend to the cortex (Swope and Kropf, 1993), and actin filaments are evenly dispersed throughout the cortex (Kropf et al., 1989). There is also no evidence of parthenogenetic development in fucoid eggs. Treatments that typically activate mammalian eggs, including calcium injection (Roberts et al., 1994), application of calcium ionophores (Brawley and Bell, 1987) or injection of inositol triphosphate (Roberts and Brownlee, 1995), do not induce fucoid development. Instead, fertilization appears to be required to initiate development.

When, following fertilization, polarity is first acquired is unclear. A few hours after fertilization fucoid zygotes send environmental cues and, in accordance with these cues, select a rhizoid-thallus axis, with growth occurring from the rhizoid pole (Kropf, 1992, 1997). However, the intertidal environment is unpredictable and appropriate cues are not always present during polarization. Indeed, these cues are not necessary for polarization (Jaffe, 1968). In the dark, zygotes will polarize and grow in random orientations. (These zygotes are not sensitive to gravity (Henderson et al., 1998); therefore, dark-grown zygotes develop essentially in the absence of environmental cues.) The mechanism of axis selection under these conditions is not known. One possibility is that the axis forms by stochastic localization of molecules involved in the polarization pathway; alternatively, the sperm entry site may specify a default axis during fertilization, as in some animal cells.

We have investigated the role of sperm entry in polarization of *F. compressa* zygotes. We show that an axis marked by F-actin is induced by the sperm at fertilization such that the rhizoid pole forms at the site of sperm entry. This axis can be overridden by environmental cues, such as light, and in nature this is probably most common. The sperm-induced axis provides the zygote with a default polarization pathway in the event that environmental signals are not present during its window of sensitivity, a successful strategy for living in an unpredictable environment.

**MATERIALS AND METHODS**

**Plant material and culture**

Sexually mature receptacles of the monoeocious species *Pelvetia compressa* (J. Agardh) de Toni, were collected near Pigeon Point Lighthouse (north of Santa Cruz, CA) or from Monterey, CA. Receptacles were shipped cold and stored in the dark at 4°C for up to 2 weeks. To induce the release of gametes, receptacles were potentiated by placing them in uniform light (100 μmol/m²·second) at 16°C in artificial seawater (ASW; 0.45 M NaCl, 10 mM KCl, 9 mM CaCl₂·2H₂O, 30 mM MgCl₂·6H₂O, 16 mM MgSO₄, and 40 μg/ml chloramphenicol, buffered to pH 8.2 with 10 mM Tris base). Elevated K⁺ in this medium depolarizes the egg membrane potential and thereby prevents fertilization (Brawley, 1991), and the absence of Mg²⁺ and Ca²⁺ retards release of eggs from oogonial sacs and sperm from antheridia (Wakana and Abe, 1992). Receptacles in fresh solution were placed in the dark for 15 to 30 minutes to release oogonia and antheridia. The solution was filtered 3 times through 90 μm mesh, to collect intact oogonia (eggs, sperm, and antheridia passed through this filter), and 5 times through 44 μm mesh, to collect oogonia and any released eggs (sperm and antheridia passed through). Eggs and oogonia were then rinsed twice in ASW and left in the dark for 1 to 5 hours, during which time eggs were released from the oogonia and the membrane potential repolarized. All steps were carried out at 16°C. To assess the yield of eggs versus zygotes, fertilization was assayed by the synthesis of the cell wall using calcofluor white staining (Fluorescent Brightener 28, Sigma, St. Louis, MO; 0.00035% in ASW, 5 minutes). Experiments were done only with populations containing greater than 90% eggs.

**Labeling of F-actin, sperm pronucleus and polar adhesive**

To label F-actin and the sperm pronucleus in the same cell, F-actin patches were labeled in zygotes 30 to 60 minutes after fertilization (AF) according to the ‘non-vital’ staining protocol of Alessa and Kropf (Alessa and Kropf, 1999), except that cells were permeabilized with 0.2 or 0.25 mg/ml saponin (Sigma) in ASW for 15 minutes and labeled with 12 μM rhodamine phalloidin (Molecular Probes, Eugene, OR) in 25% methanol, 75% ASW for 30 minutes. Sperm pronuclei were then labeled with 5 μg/ml Hoechst (stock was 1 mg/ml in DMSO; Calbiochem, San Diego, CA) in ASW for 15 minutes, and were rinsed 5 times with ASW. The angle defined by the sperm pronucleus, center of the zygote, and F-actin patch was measured using a protractor on a reticle in the eyepiece of a fluorescence microscope. An angle of 0° indicates strict colocalization and 180° indicates that sperm and F-actin were at opposite poles of the zygote. Similar angles were measured in subsequent experiments and are hereafter referred to as ‘polarization angles’ (defined as the angle formed by the sperm entry site, the center of the cell, and the rhizoid pole). Polarization angles were always measured to the nearest 5° and mean polarization angles with the standard error of the mean were calculated.

The percentage of zygotes for which both F-actin and the sperm pronucleus were labeled was low, approximately 1% of the population. The low level of double labeling had at least three causes. First, the dense pigmentation of the egg cytoplasm made the sperm pronucleus difficult to visualize once it had migrated greater than 5 μm inward. Second, the F-actin patch labeled in only approximately 5% of the population, as previously reported (Alessa and Kropf, 1999), third, only one hemisphere of the zygote was visible through the microscope; it was not possible to observe fertilization occurring on the hemisphere opposite the objective lens.

To compare the fertilization site to the position of polar adhesive, zygotes were treated with oryzalin, a microtubule inhibitor, at 30 minutes AF. Oryzalin treatment halted pronuclear migration but had no effect on the subsequent occurrence or timing of polarization of the zygotes (Hable and Kropf, 1998), allowing the sperm pronucleus to be used as a marker of the sperm entry site. Zygotes were then cultured in the dark for 5 to 6 hours, after which time adhesive was labeled. Polar adhesive was outlined with YG fluorescent microspheres (Polysciences, Warrington, PA) as described by Hable and Kropf (1998), and the sperm pronucleus was labeled with Hoechst as described above. To reduce the likelihood that zygotes were polarized by neighboring zygotes (group effect; Rosenvinge, 1888), only zygotes that were separated two cell diameters (approximately 200 μm) or greater away from any neighbor were assayed. To compare the sperm entry site to the position of rhizoid outgrowth, oryzalin-treated
zygotes were cultured in the dark for 24 hours before the sperm pronucleus was labeled.

**Polyspermy**

Polyspermy was induced in ASW in which Na⁺ had been reduced to 0 or 2.5 mM and substituted with N-methyl-glucamine (Sigma; Brawley, 1987). Receptacles that had been potentiated in ASW were rinsed 5 times in reduced-Na⁺ ASW and placed in the dark for 30 minutes. At 1 hour AF, zygotes were rinsed 5 times in normal ASW, plated sparsely, and cultured in the dark. To determine the number of sperm pronuclei per cell in polyspermy experiments, cells were fixed and stained with Mithramycin A (Fluka, Ronkonkoma, NY) according to the method of Francois-Yves Bouget (personal communication). Cells were fixed in 0.1 M citric acid, 0.1% Triton X-100 (fixation buffer) overnight and cleared with methanol for a minimum of 24 hours. Prior to staining, cells were rehydrated with 1 volume fixation buffer, 4 volumes 0.4 M NaH₂PO₄ for 30 minutes, incubated in 1 M LiCl for 20 minutes and rinsed with dH₂O. Cells were then stained with 0.2 mg/ml Evans Blue for 5 minutes, rinsed with dH₂O, and stained with 0.1 mg/ml Mithramycin A in 50 mM MgCl₂ for a minimum of 1 hour. Stained cells were rinsed and mounted in 50 mM LiCl for 20 minutes and rinsed with dH₂O. Cells were then stained and with Hoechst staining were observed with an ultraviolet filter set (Chroma Technology). Slides were taken with a Contax 167MT and Hoechst staining were observed with an epifluorescence microscope (Carl Zeiss Co., Oberkochen, FRG) equipped with epifluorescence. Rhodamine phalloidin and Mithramycin A staining were visualized with Texas Red/Rhodamine and fluorescein filter sets (Chroma Technology, Brattleboro, VT), respectively. Fluorescent microspheres were visualized with 0.1 mg/ml Mithramycin A in 50 mM MgCl₂ for a minimum of 2 days before counting the number of rhizoids present. Low Na⁺ inhibits rhizoid outgrowth (unpublished observation), so as a control for the effective removal of Na⁺, a subset of zygotes was left in low-Na⁺ ASW. In all experiments, none of the zygotes cultured continuously in 0 or 2.5 mM Na⁺ ASW grew rhizoids.

**Fluorescence microscopy**

Zygotes were observed on a Zeiss Axiostar microscope (Carl Zeiss Co., Oberkochen, FRG) equipped with epifluorescence. Slides were taken with a Contax 167MT camera (Kyocera Corp., Glendale, CA) on Kodak Ektachrome film, ASA 400 and were scanned with a DuoScan AGFA scanner.

**Spatial control of fertilization**

To control the position of fertilization, a single egg (approximately 100 μm in diameter) was loaded into a glass micropipet (14478-048, Van Waters and Rogers, Seattle, WA) that had been pulled and fire-polished to a tip diameter of 15 to 75 μm. Eggs in ASW were loaded either by drawing them into the micropipet through the tip, or by loading them through the back of the pipet. During pipet loading, flexible, wall-less eggs were easily distinguished from rigid, walled zygotes. Because the loaded pipet was tapered, when it was positioned vertically in a beaker of ASW, the egg settled and became loosely wedged in place, effectively partitioning the pipet into two chambers, one above and one below the egg (see Fig. 5). Sperm were added either to the ASW in the top of the pipet, or to the ASW in the beaker. Zygotes were allowed to develop within each pipet in the dark for 24 to 36 hours and were then scored for the direction of rhizoid outgrowth. Since any gap between the egg and the pipet wall would allow sperm to swim by and fertilize either side, rhizoid outgrowth was assayed only in cases where the egg had been wedged prior to the addition of sperm.

To control the position of fertilization in a population of zygotes, eggs were sparsely plated on a coverslip and sperm were locally delivered. A glass micropipet fire-polished to a tip diameter of approximately 15 to 30 μm was front-filled with ASW and back-filled with sperm. Sperm were then delivered to one point on the edge of the coverslip containing eggs by allowing sperm to swim out of the micropipet. Zygotes were allowed to develop in the dark for 5 to 6 hours, at which time adhesive was labeled. The polarization angle was measured, as was the distance between the zygote and the sperm source (see Fig. 6A). To correlate the positions of sperm delivery and rhizoid outgrowth, zygotes were allowed to develop in the dark for 24 hours, at which time the polarization angle and distance between the sperm source and rhizoid outgrowth were measured.

**RESULTS**

**Sperm entry induces formation of a rhizoid pole**

The sperm entry site predicts the position of polar adhesive secretion in dark-grown zygotes.

Sperm entry induces formation of a rhizoid pole. The sperm entry site predicts the position of polar adhesive secretion in dark-grown zygotes. Thirty-minute-old zygotes were treated with the microtubule depolymerizing agent, oryzalin, to prevent pronuclear migration, and the position of the sperm pronucleus was used as a marker of the sperm entry site. Oryzalin treatment had no effect on the subsequent occurrence or timing of polarization of the zygotes, including deposition of adhesive (Hable and Kropf, 1998). The spatial relationship between the cortically localized sperm pronucleus and polar adhesive was examined after 5 to 6 hours of development in the dark.

Sperm adhesive was secreted very near the position of sperm entry (Figs 1, 2A). The mean polarization angle was 34±4° and was significantly different from the mean (90°) of a population in which sperm pronuclei and adhesive were randomly positioned (P<0.0001, Student’s t-test). The spatial relationship between the cortically localized sperm pronucleus and polar adhesive could be overridden by application of subsequent environmental cues, such as unidirectional light. When the polarization angle was measured in oryzalin-treated zygotes grown in unidirectional light from 1 to 6 hours AF (Fig. 2B),
the angles were uniformly distributed from 0 to 180°, with a mean of 89±5°, which was not significantly different from 90° (P=0.83, Student’s t-test). Instead, for 100% of the 118 zygotes examined, adhesive was secreted on the shaded hemisphere, in accordance with the light vector. Thus, colocalization of polar adhesive and the sperm pronucleus was dependent on development occurring in the absence of environmental cues.

The sperm entry site predicts the rhizoid outgrowth position in dark-grown zygotes

The role of sperm entry in polarity establishment was further tested by examining the spatial relationship between the sperm pronucleus and rhizoid outgrowth in oryzalin-treated, dark-grown zygotes. In many instances, the rhizoid was found to colocalize with the sperm pronucleus (Fig. 3). Quantification of these data showed a less precise correlation than observed for polar adhesive (Fig. 4A); however, the mean polarization angle (θ) was 69±6°, and was significantly different from 90° (P=0.0003, Student’s t-test).

In oryzalin-treated, unidirectional light-grown zygotes, 99% of rhizoids (97 of 98) grew preferentially on the shaded hemisphere, independent of the position of sperm entry (Fig. 4B). The mean polarization angle was 95±7°, which was not significantly different from 90° (P=0.47, Student’s t-test). Thus, this population showed no preference for polar growth near the site of fertilization.

Local sperm delivery orients the polar growth axis in dark-grown zygotes

We next determined whether experimentally manipulating the position of sperm entry orients the growth axis in dark-grown zygotes. To control the site of sperm entry, a single egg in ASW was loosely wedged in a glass micropipet, and the micropipet was positioned vertically in a beaker of ASW (Fig. 5). The egg effectively partitioned the pipet into two chambers, one above and one below the egg. Sperm were added either to the top of the pipet, or to the beaker, so fertilization was restricted to one hemisphere of the egg. After 24 to 36 hours of development in the dark, zygotes were scored for the direction of rhizoid outgrowth relative to an arbitrary, downward vector (germination angle; Fig. 5). Many zygotes did not grow a rhizoid (ungerminated zygotes); some zygotes had lysed, and others had not been fertilized, judging by their lack of adhesion to the inside of the pipet (Table 1). Those that did grow a rhizoid (germinated zygotes), however, always grew toward the source of applied sperm (12/12; P=0.00024, one-tailed binomial test).

As a second approach to limit the site of sperm entry, sperm were delivered to one point on the edge of the coverslip containing sparsely plated eggs (Fig. 6A). Local sperm delivery was accomplished by allowing them to swim out of a glass micropipet. After 5 to 6 hours in the dark, two parameters were scored for each zygote (Fig. 6A): (1) the polarization angle (the angle between the positions of the sperm source and polar adhesive), and 2) the distance between the zygote and the sperm source. Alternatively, after 24 hours, the same parameters were scored, except in relation to rhizoid outgrowth. The percent polarization toward the sperm source was calculated from the angle data (see Fig. 6 legend) such that 0% reflects random polarization and 100% reflects polarization of all zygotes toward the sperm source. The Student’s t-test was used to calculate all P values for angle measurements.
For zygotes that lay within 8 mm of the sperm source, the population was 71% polarized toward that sperm source with respect to adhesive secretion (Fig. 6B) and was 36% polarized toward the sperm source with respect to polar growth (Fig. 6C). The mean polarization angle between the sperm source and polar adhesive was $67^{\circ} - 10^{\circ}$ and was significantly different from the 90° mean of a randomly polarized population ($P = 0.03$). For the polarization angle between the sperm source and rhizoid outgrowth, the mean was $69^{\circ} - 9^{\circ}$ and was also significantly different from 90° ($P = 0.028$).

Fucoid zygotes are highly sensitive to gradients of ions, temperature and other factors. As a control for the specificity of sperm as the polarizing cue, ASW from which sperm had been filtered was locally delivered to a coverslip containing young zygotes. The population of zygotes within 8 mm of the source was $-12$% polarized in the secretion of adhesive (Fig. 6B). The mean polarization angle between the sperm source and polar adhesive was $67^{\circ} - 10^{\circ}$ and was significantly different from the 90° mean of a randomly polarized population ($P = 0.03$). For the polarization angle between the sperm source and rhizoid outgrowth, the mean was $69^{\circ} - 9^{\circ}$ and was also significantly different from 90° ($P = 0.028$).

A prediction of this experiment is that eggs lying closer to the sperm entry site are more likely to be fertilized on the side toward the sperm source; with more distant eggs, random swimming of sperm is more likely to result in fertilization on either hemisphere. When zygotes were greater than 8 mm from the sperm source, the population polarized essentially randomly; 7% polarized with respect to adhesive with a mean polarization angle of $100^{\circ} - 15^{\circ}$, and $-7$% polarized with respect to rhizoid outgrowth, with a mean of $95^{\circ} - 15^{\circ}$. Neither of these means were significantly different from 90° ($P = 0.65$ and 0.52, respectively). Similarly, the population of zygotes greater than 8 mm from the source in the control polarized randomly.

Table 1. Position of rhizoid outgrowth with respect to position of sperm delivery

<table>
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<tr>
<th>Position of sperm delivery</th>
<th>Ungerminated zygotes</th>
<th>Germinated zygotes</th>
<th>Germination angle</th>
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<tr>
<td>Bottom (0°)</td>
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<td>Top (180°)</td>
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Fig. 4. Preferential outgrowth of the rhizoid near the sperm entry site in dark-grown (A), but not in unidirectional light-grown (B), zygotes. The polarization angle ($\Theta$) was $<90^{\circ}$ in two-thirds of dark-grown zygotes ($n = 108$, for 4 replicates) and was random in light-grown zygotes ($n = 98$, for 2 replicates).

Fig. 5. Schematic illustration of strategy for localizing sperm delivery. See text for details. The germination angle was measured relative to a downward vector; 0° refers to growth straight down and 180° refers to growth straight up.
Alessa and Kropf (1999) demonstrated that in zygotes polarized by unidirectional light or neighbors (group effect), F-actin is an early marker of the rhizoid pole. They speculated that F-actin may be a universal marker of the rhizoid pole, regardless of polarizing cue. If so, F-actin should also localize to the sperm entry site. To test this prediction, the sperm pronucleus and F-actin were labeled in recently fertilized zygotes. F-actin is uniformly distributed in the cortex of eggs (Kropf et al., 1989), but this cytoskeletal symmetry is broken at fertilization. As early as 30 minutes AF (the earliest time that could be tested), F-actin had localized around the sperm pronucleus (Fig. 7). F-actin was generally organized as a small patch, but often a small region devoid of F-actin was observed in the center of the patch with the sperm pronucleus occupying that region. The spatial relationship between the F-actin patch and sperm pronucleus was investigated in greater detail by measuring the polarization angle (θ) (Fig. 8). In 90% of the double-labeled zygotes, an angle of 0° was measured, indicating that the F-actin patch and sperm pronucleus were tightly colocalized. The mean polarization angle was 7°–4° (significantly less than 90°, P<0.0001, Student’s t-test).

Polyspermy induces double rhizoids

In rare instances, zygotes with two F-actin patches that colocalized with two sperm pronuclei were observed (Fig. 9). The low frequency of polyspermy in the natural population is due to a fast electrical block to polyspermy that is Na+ dependent (Brawley, 1987, 1991). When Na+ in ASW is omitted or greatly decreased, the frequency of polyspermy increases (Brawley, 1987). We used low Na+ ASW to induce polyspermy and tested whether polyspermic zygotes, when cultured in the dark, produced multiple rhizoids. Low Na+ ASW induced fertilization by more than one sperm in...
approximately 40% of zygotes (Fig. 10) and the number of sperm pronuclei per zygote ranged from 2 to 6. In contrast, only 10% of controls were polyspermic.

Low Na⁺ ASW also increased the frequency of double rhizoids, but zygotes with 3 or more rhizoids were never observed. The control level of double rhizoids was extremely small, approximately 0.4%, and treatment with low Na⁺ ASW significantly increased this value to 2.5% for 2.5 mM added Na⁺, and 4% for 0 mM added Na⁺ ($P<0.001$ for both, Chi Square test; Fig. 10). The concurrent increase in polyspermy and double rhizoids suggests that multiple fertilization sites occasionally result in two rhizoid growth sites. That not every sperm entry site becomes a rhizoid pole may reflect a limitation in some component necessary for marking the axis, or may indicate that multiple axes somehow are resolved into one or two axes. At present, how some sperm entry sites prevail over others is unclear.

**DISCUSSION**

**Sperm entry orients the growth axis**

One of the most important events in development is the acquisition of asymmetry. Fucoid zygotes become exquisitely sensitive to a number of external cues several hours after fertilization and use this information to orient their growth axes. However, as environmental cues are quite variable, there are circumstances in nature in which a zygote may never receive a constant signal during the period of polarity establishment. In the absence of these cues, zygotes will establish and express polarity with approximately the same timing as those polarized by light (Kropf, 1992).

At least two possibilities account for this default pathway. Polarization could arise stochastically, through random localization of molecules in the polarization pathway; alternatively, sperm entry could provide positional information that orients the axis. Support for the sperm-induced default pathway comes from experiments in another brown alga, *Cystosira barbata*, which indicate that the sperm entry point defines the rhizoid pole in the absence of environmental cues (Knapp, 1931). Whether this finding is relevant to fucoid algae has remained unclear for over sixty five years. *P. compressa* is not a close relative of *C. barbata*; *P. compressa* is in the family Fucaceae, whereas *C. barbata* is in the Cystoseiraceae family. Moreover, in *C. barbata* a local change in plasma membrane morphology was observed at the sperm entry site, allowing it to be used as a natural marker of this site (Knapp, 1931). No such morphological change at the plasma membrane occurs in fucoid algae, and no visible markers of the sperm entry site are known, indicating that the physiology of fertilization may be different in *C. barbata* and fucoid algae.

Support for the alternative stochastic possibility was surmised from treatment with polarized light. Plane polarized light induces two rhizoids in accordance with the electrical vector (Jaffe, 1958), indicating that there is not a preformed sperm-induced axis that is rotated in response to environmental cues. The induction of two rhizoids suggests that a developmental axis is established de novo in response to external cues. This observation does not, however, obviate a
role for sperm entry in polarity establishment; rather, it addresses the mechanism by which a zygote establishes a new axis in response to environmental cues.

We have investigated the role of sperm entry in polarity establishment and conclude that the sperm entry site defines the rhizoid pole in \textit{P. compressa} zygotes. Several lines of evidence support this conclusion. Sperm entry induces a local accumulation of F-actin, a cortical marker of the rhizoid pole, at the fertilization site no more than 30 minutes AF (as early as can be sampled) (Figs 7, 8). Polar adhesive, an early morphological marker of the rhizoid pole, is secreted preferentially near the site of sperm entry in dark-grown zygotes (Figs 2A, 6B). This colocalization is disrupted in the presence of unidirectional light; zygotes secrete adhesive in accordance with the light vector, rather than the fertilization site, confirming that the sperm-induced axis can be overridden by environmental cues (Fig. 2B).

Four lines of evidence show that the sperm-induced default pathway also orients rhizoid outgrowth itself. First, in experiments where the position of fertilization is restricted to one hemisphere of the egg, polar growth always occurs on that hemisphere (Table 1). Second, when sperm are applied from a single point to a population of eggs, the rhizoid outgrowth is usually on the egg hemisphere closest to the sperm source (Fig. 6B). Third, in experiments where the fertilization site is not limited, rhizoid outgrowth occurs preferentially near the site where the sperm entered (Fig. 4A). Fourth, when polyspermy is induced in low Na\(^+\) ASW, the frequency of double rhizoids also increases (Fig. 10).

Taken together, these data support a model whereby sperm entry establishes a labile, default growth axis at fertilization which is overridden by light, and presumably other external cues. The formation of an axis at fertilization ensures that polarity establishment occurs regardless of whether environmental signals are present. Such a strategy would be advantageous in the unpredictable intertidal environment, and may be common in brown algae, most of which are marine.

As development proceeds, the colocalization of the sperm entry site and the rhizoid pole becomes more variable (compare Figs 8, 2A, 4A). For most zygotes, F-actin is localized precisely at the sperm entry point shortly after fertilization. Four to five hours later localization of adhesive is not as precise, and by the time of rhizoid outgrowth (12 hours AF) the population shows only a general two-fold preference for initiating rhizoid growth near the site of fertilization. There are at least two possibilities, not mutually exclusive, that may explain this phenomenon.

First, the use of a microtubule inhibitor to block pronuclear migration may have caused the sperm pronucleus to drift slowly away from the sperm entry site. Microtubules are required to anchor the egg pronucleus in the center of the cell, and in the absence of microtubules the pronucleus is easily displaced from the cell center (Swope and Kropf, 1993). Similarly, in the absence of microtubules, the sperm pronucleus may not only fail to migrate, but also may drift within the cytoplasm over time. As a consequence, more drifting would occur as development progresses and the measured colocalizations would get weaker with time.

A second possibility is that despite our efforts, other environmental cues were present during these experiments, and these cues overrode the sperm-induced axis with time. When sperm application was spatially limited to a single point within a population of eggs, adhesive secretion was better polarized toward the sperm source than was rhizoid outgrowth (Fig. 6B). The sperm pronucleus was not used to mark the sperm entry site in these experiments, and some factor other than drifting must be operating. With time, some zygotes may have sensed and polarized according to weak vectors, such as neighbors. Indeed, when single cells were placed in a micropipet, having no neighbors, rhizoid outgrowth always occurred on the same hemisphere to which sperm were applied (Table 1).

**F-actin localization may be early in a common pathway for axis establishment**

Alessa and Kropf showed that F-actin marks the rhizoid pole of photopolarized zygotes as early as 4 hours AF. Younger zygotes were not investigated because they are not sensitive to directional light cues until approximately 2 hours AF and take an additional hour to photopolarize (Hable and Kropf, 1998). Here we show that an F-actin patch is present as early as 30 minutes following fertilization, and that it marks the rhizoid pole of the sperm-induced axis.

Although sperm entry by its nature breaks the symmetry of the egg cytoplasm, the spatial cue provided by the sperm that causes F-actin assembly is unknown. Although Ca\(^{2+}\) entry at the sperm entry site could initiate local F-actin assembly, a local Ca\(^{2+}\) rise has not been detected and there is no evidence for a Ca\(^{2+}\) wave initiating at the sperm entry site (Roberts et al., 1994) and Ca\(^{2+}\) is not a likely marker of this axis. Moreover, no stable ion currents are present at the time of fertilization, so fluxes through the egg are not likely involved (Nuccitelli, 1978). Instead, proteins involved in regulating F-actin arrays, such as rho GTPases (Drubin and Nelson, 1996), or actin binding proteins, are candidates to be part of the spatial cue that localizes F-actin. During sperm entry, proteins like these may be introduced to the egg cortex from the sperm cytosol (Whitaker and Swann, 1993) where they recruit F-actin to the sperm entry site.

Fucoid algae are sensitive to several different signals for polarity establishment; but it is not clear where (and if) these signaling pathways converge in a common pathway of polarization (see Kropf, 1999). We have shown that at least two localizations, F-actin and adhesive secretion, previously known to be in the light-induced pathway (Alessa and Kropf, 1999; Hable and Kropf, 1998), are also part of the sperm-induced pathway. This finding suggests that these two pathways converge. The polarization of other molecules known to be part of the light-induced pathway (e.g. stable ion fluxes (Brawley and Robinson, 1985), Ca\(^{2+}\) gradients (Pu and Robinson, 1998), dihydropyridine receptors (Shaw and Quatrano, 1996)) have not yet been examined in response to the sperm-induced pathway.

F-actin localization appears to be an early step in the common pathway, and may provide a target site for subsequent localization of other molecules and processes. In support of this postulate, F-actin is localized rapidly in response to a polarizing cue (sperm or light); in fact, no other molecule or process is known to localize more quickly. Furthermore, in the light-induced pathway, inhibition of F-actin function through the use of pharmacological agents (cytochalasin D, latrunculin B) blocks localization of molecules and processes known to be
involved in polarity establishment in every case tested, including stable ion fluxes (Brawley and Robinson, 1985), adhesive secretion (Hable and Kropf, 1998) and dihydropyridine receptors (Shaw and Quatrano, 1996), suggesting that their localization is downstream of F-actin localization.

Temporal relationships

The zygote is sensitive to polarizing cues for the first 10 to 12 hours AF, and F-actin localizes immediately (within one hour) following polarization. Therefore, F-actin localization can occur at varied times in young zygotes. In contrast, other developmental processes, such as polar adhesive secretion and polar growth, appear to be regulated by a developmental clock. For example, localization of adhesive begins approximately 4 to 5 hours AF and rhizoid outgrowth begins 10 to 12 hours AF, regardless of the timing and nature of the polarizing signal. These observations indicate that the formation of the F-actin patch does not initiate polar adhesive secretion or rhizoid outgrowth; instead, developmentally regulated factors independent of F-actin localization must control the timing of these processes.

The timing between F-actin localization and these clock-regulated events can vary widely. For example, adhesive secretion can occur as early as 1 to 2 hours after F-actin localization in the case of a photopolarized zygote, or as long as 4 to 5 hours after F-actin localization for a zygote bearing a sperm-induced axis. This apparent pause after sperm-induced F-actin localization may function to allow zygotes time to monitor their local environment before continuing the polarization pathway.

Based on the data presented here and in other work, we propose the following scenario for polarity establishment. At fertilization, the sperm polarizes the egg cell, rapidly inducing the F-actin patch is disassembled and rapidly reassembled at fertilization, the sperm polarizes the egg cell, rapidly inducing F-actin localization for a zygote bearing a sperm-induced axis. This apparent pause after sperm-induced F-actin localization must control the timing of these processes.

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