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Ca^{2+} and Calmodulin Dynamics during Photopolarization in *Fucus serratus* Zygotes[1]

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The role of Ca^{2+} in zygote polarization in fucoid algae (*Fucus*, *Ascophyllum*, and *Pelvetia* species) zygote polarization is controversial. Using a local source of *Fucus serratus*, we established that zygotes form a polar axis relative to unilateral light (photopolarization) between 8 and 14 h after fertilization (AF), and become committed to this polarity at approximately 15 to 18 h AF. We investigated the role of Ca^{2+}, calmodulin, and actin during photopolarization by simultaneously exposing *F. serratus* zygotes to polarizing light and various inhibitors. Neither removal of Ca^{2+} from the culture medium or high concentrations of EGTA and $LaCl_3$ had any effect on photopolarization. Bepridil, 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester, nifedipine, and verapamil, all of which block intracellular Ca^{2+} release, reduced photopolarization from 75 to 30%. The calmodulin antagonists *N*-(6-aminohexyl)-5-chloro-L-naphthalenesulfonamide and trifluoperazine inhibited photopolarization in all zygotes, whereas *N*-(6-aminohexyl)-L-naphthalenesulfonamide had no effect. Cytochalasin B, cytochalasin D, and latrunculin B, all of which inhibit actin polymerization, had no effect on photopolarization, but arrested polar axis fixation. The role of calmodulin during polarization was investigated further. Calmodulin mRNA from the closely related brown alga *Macrocystis pyrifera* was cloned and the protein was expressed in bacteria. Photopolarization was enhanced following microinjections of this recombinant calmodulin into developing zygotes. Confocal imaging of fluorescein isothiocyanate-labeled recombinant calmodulin in photopolarized zygotes showed a homogenous signal distribution at 13 h AF, which localized to the presumptive rhizoid site at 15 h AF.

Zygotes of the fucoid algae (*Ascophyllum*, *Fucus*, and *Pelvetia* species) have been widely used to investigate the processes involved in generating and maintaining plant cell polarity. In the early stages of development fucoid zygotes are apolar and possess no inherent cytoplasmic order (Jaffe, 1968). Over several hours, during a period termed "axis formation" or "axis induction," a potential polar axis is generated within the zygote. The incipient polarity may be oriented by a number of environmental stimuli, including the direction of incident light (Whitaker and Lowrance, 1936), the position of neighboring zygotes (Jaffe and Neuscheler, 1969), water currents (Bentrup and Jaffe, 1968), chemical or ionic gradients (Bentrup et al., 1966; Robinson and Jaffe, 1976; Gibbon and Kropf, 1993), and electrical fields (Lünd, 1923; Novák and Bentrup, 1973).

Zygotes undergo successive periods of sensitivity to different polarizing cues (Jaffe, 1968). During axis induction, the polarity is labile and can be re-oriented by the subsequent exposure to a stimulus in a different direction. The loss of polar axis lability commits the zygote to a polarity and defines a second developmental phase termed "axis fixation." At this stage polarity is rapidly stabilized by interactions between cell wall components, cortical vesicles, and microfilaments, and is no longer susceptible to re-orientation by environmental cues (Kropf et al., 1988, 1989; Quatrano et al., 1991).

At the phase called "rhizoid germination," the spherical symmetry of the zygote is broken by the emergence of a polar bulge, the rhizoid, from the surface of the cell. The direction of rhizoid germination defines the direction of the polar axis. Unequal cytokinesis follows, perpendicular to the direction of rhizoid germination, yielding an embryo consisting of two highly differentiated cells: a large, rounded thallus cell, which is the precursor of the frond, and a smaller, elongated rhizoid cell, which generates the stipe and holdfast. The progression through each stage of embryogenesis is species-specific and broadly synchronous within a population of zygotes (Kropf and Quatrano, 1987). Nevertheless, the boundaries between each stage overlap and are blurred by individual variability (Kropf, 1992).

Little is known about the intracellular events that occur during axis induction. Unlike the cytoplasmic rearrangements of axis fixation (Kropf et al., 1989), only an asymmetry in ion transport and a small, localized secretion of polysaccharide at the future rhizoid pole have been detected in zygotes at this stage (Jaffe, 1966; Schröter, 1978) The polarized ion flux is the first discernible asymmetric event, detectable as early as 30 min AF in *Pelvetia fastigiata*, in which axis induction lasts approximately 8 h (Nuccitelli,

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Abbreviations: AF, after fertilization; ASW, artificial sea water; DASW, Ca^{2+}-depleted ASW; FITC, fluorescein isothiocyanate; TFP, trifluoperazine; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester; W5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

1988). The current is dependent on the microfilament network, and ceases following inhibition of actin polymerization (Brawley and Robinson, 1985). Moreover, it follows the developmental pattern of the zygote, being at first erratic, often with several influx sites, but then later stabilizing in alignment with polarizing stimuli (Nuccitelli and Jaffe, 1974, 1975). Ca^{2+} is an important component of the ionic current, and it has been suggested that asymmetric Ca^{2+} circulation from the medium, through the zygote, results in a dynamic, transcytoplasmic Ca^{2+} gradient. This gradient effectively constitutes a polar axis and transduces environmental stimuli into localization of cytoplasmic components (Jaffe et al., 1974; Robinson and Jaffe, 1974).

This model remains controversial. The presence of a transcellular Ca^{2+} gradient is supported by experiments using $^{45}Ca^{2+}$ tracers (Robinson and Jaffe, 1973), by indirect measurements of Ca^{2+} influx during polarization using the vibrating probe (Nuccitelli and Jaffe, 1976), by extracellular application of Ca^{2+} and Ca^{2+} ionophore gradients that polarize zygotes (Robinson and Jaffe, 1976; Robinson and Cone, 1979), and by microinjection of the Ca^{2+} chelator BAPTA (1,2-bis[*o*-aminophenoxy]ethane-*N*;*N*;*N*';*N*'-tetraacetic acid) into zygotes in the early stages of polarization, which inhibits normal embryogenesis (Speksnijder et al., 1989).

However, certain data are contradictory. First, zygotes respond normally to polarizing stimuli in media containing 10 to 100 pM Ca^{2+}, 10^9 times less than the $[Ca^{2+}]$ of sea water, suggesting that Ca^{2+} circulation is not essential for polar axis formation (Hurst and Kropf, 1991), although these findings have themselves been questioned by recent results (Robinson, 1996a). Second, chlorotetracycline fluorescence imaging of membrane-bound Ca^{2+} displayed no asymmetric distribution during polar induction (Kropf and Quatrano, 1987). Third, attempts to directly visualize free Ca^{2+} in polarizing zygotes have had little success (Berger and Brownlee, 1993), even though a Ca^{2+} gradient has been readily imaged in growing rhizoids (Brownlee and Wood, 1986).

The controversy may have several explanations. The wild algae that are the source of zygotes used in these experiments are inherently variable. Precise timing of the developmental phases of zygotes from each population is therefore of crucial importance to analyze results accurately, but is often overlooked. Moreover, developmental timing varies between fucoid species, making results between them difficult to compare (Kropf and Quatrano, 1987). The lack of inhibition of polar axis formation by EGTA may result from cytoplasmic Ca^{2+} mobilization from intracellular stores. Although BAPTA microinjections were designed to address this possibility, the effect of the compound was not reversible. Consequently, it was impossible to correlate a Ca^{2+} requirement with a specific developmental stage. Direct Ca^{2+} imaging has been difficult due to the poor resolution of the zygotes, which contain innumerable cytoplasmic vesicles, and possibly to the sensitivity of the Ca^{2+}-indicator dyes currently available. Finally, the involvement of cytosolic Ca^{2+} receptors such as calmodulin has been overlooked during fucoid zygote polarization. This oversight is of critical importance, because active calmodulin will amplify the effects of a small cytosolic Ca^{2+} release, which may explain many of the ambiguities in Ca^{2+} requirements during this developmental stage.

MATERIALS AND METHODS

Zygote Culture

To minimize variation, *Fucus serratus* plants were collected from a single site covering 100 m of shoreline near Barnsness, Scotland. Plants were segregated according to their sex. Ripe receptacles were cut from the fronds, washed in distilled water, blotted dry, and stored in the dark at 4°C. This treatment resulted in abundant gamete secretion within 24 h. Following purification, gametes were united and incubated at 14°C under fluorescent white light. Fertilization was considered complete 20 min after gamete unification (Brawley et al., 1976) and defined as time zero for subsequent experiments. Zygotes were then sparsely sown onto glass coverslips to which they adhered firmly, and, unless stated otherwise, were incubated in ASW (450 mM NaCl, 30 mM $MgCl_2$, 16 mM $MgSO_4$, 10 mM KCl, 9 mM $CaCl_2$, and 2 mM $NaHCO_3$) at 14°C in the dark. Under these conditions zygotes developed synchronously.

Characterization of the Period when *F. serratus* Zygotes Are Polarized by Unilateral Light

Zygotes were cultured as described previously, and were illuminated with 325 μmol m^{-2} s^{-1} unilateral white light (15-W Litegard warm white bulbs, Osran, UK) for 1 h, from fertilization to 21 h AF. Following germination the direction of rhizoid growth, i.e. the direction of polar axis formation, was determined for all zygotes that were separated by more than four egg diameters from their neighbors (Whitaker, 1931). At least 200 zygotes per treatment were scored, and each experiment was replicated 5 times. For n zygotes, the degree of photopolarization P was determined by the cosine of the angle θ between the direction of rhizoid germination and that of the unilateral light pulse such that $P = [(\Sigma\ cos\theta)/n] \times 100$. Axis fixation was determined by exposing zygotes to unilateral light in one direction for 1 h, then in the opposite direction for 1 h. The degree of photopolarization was calculated as described above.

Inhibition of Photopolarization

Inhibitors used were bepridil, cytochalasin-B, cytochalasin-D, EGTA, $LaCl_3$, latrunculin-B, nifedipine, TFP, TMB-8, verapamil, W5, and W7. All inhibitors were purchased from Sigma, except latrunculin-B, which was from Calbiochem. One-molar stock solutions in DMSO were diluted to working concentrations in DASW, in which $CaCl_2$ was replaced by Suc, and the solutions were cooled to 14°C. EGTA stock was prepared in distilled water. As a toxicity control, 1 mL of nifedipine stock was irradiated with 10 J of UV light.

ASW was replaced by inhibitor solutions at 9.5 h AF (Fig. 2A). Zygotes were exposed to unilateral white light for 3 h,

between 10 and 13 h AF. Inhibitor solutions were then discarded and zygotes were rinsed three times in ASW. Following incubation in the dark for a further 36 h, the degree of photopolarization was assessed. The maximum concentration of each inhibitor applied to zygotes was that beyond which over 10% of treated cells lysed.

The effect of cytochalasin-B, cytochalasin-D, and latrunculin-B on polarization was also tested in a second experiment: Zygotes were cultured in ASW and exposed to unilateral light from 10 to 13 h AF. ASW was then replaced by solutions of cytochalasin-B, and cultures were incubated in darkness until 21 h AF. The inhibitor was then substituted by ASW, and zygotes were exposed to a second unilateral light pulse in the opposite direction to the first for 3 h. Following rhizoid germination the degree of photopolarization relative to the direction of the first illumination was determined.

Expression, Purification, and Labeling of Recombinant Calmodulin from *Macrocystis pyrifera*

Using PCR, a *Nde*I restriction site was engineered into the cDNA encoding *M. pyrifera* calmodulin (Love et al., 1995) to coincide with the start codon. The PCR yielded an expected 920-bp fragment, which was cloned into pBluescript SK$^-$ at the *Sma*I site. The engineered calmodulin sequence was excised from this clone using *Nde*I and *Eco*RI, and ligated in frame with the T7 promoter in the expression vector pRSET A.

Recombinant calmodulin was expressed in *Escherichia coli* strain BL21-ΔDE3. Transformed bacteria were cultured on a shaker in 40 mL of Luria-Bertani broth (Sambrook et al., 1989) at 37°C until the A_{600} of the cell suspension reached between 0.8 and 0.9. Two replicate cultures were prepared. In one culture, recombinant protein expression was induced by adding isopropyl β-D-thiogalactoside to a final concentration of 4 mM, and incubating cultures for 1 h at 37°C; in the other culture, this compound was not added. Rifampicin was then added to both cultures at a final concentration of 200 mg mL^{-1} and both were incubated for 1 h at 37°C.

Cultures were chilled and bacteria were pelleted by centrifugation at 4000*g* for 20 min at 4°C. Cells were resuspended in 5 mL of ice-cold buffer L (200 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM β-mercaptoethanol), frozen in liquid N_2, thawed on ice, and sonicated in an ice-cold water bath for 30 s. The lysate was cleared by centrifugation and loaded onto a calmodulin-affinity chromatography column containing 3 mL of W7-agarose (Sigma) at 4°C. Unbound proteins were flushed from the column with buffer L in 0.5-mL fractions until the A_{280} was below 0.05. Calmodulin was eluted in 0.5-mL fractions with buffer E (200 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EGTA, and 1 mM β-mercaptoethanol). Fractions containing calmodulin were pooled and buffer E was replaced with a microinjection buffer containing the main ions present in the fucoid cytoplasm (320 mM KCl, 40 mM NaCl, 25 mM $MgCl_2$, and 4 mM $CaCl_2$; Allen et al., 1972) using a column with a 10-kD filter (Centricon, Amicon, Beverly, MA). Calmodulin was concentrated to 5 mg mL^{-1} in a 10-kD column (Amicon), aliquoted into 10-mL volumes, and stored at −80°C.

Recombinant calmodulin was labeled with FITC following the method of Rinderknecht (1962). Unconjugated FITC was removed from the solution by filtration through a Sephadex G25 column (Pharmacia). FITC-calmodulin was concentrated to 5 mg mL^{-1} in microinjection buffer using a 10-kD microfiltration column (Amicon). The activities of FITC-labeled and unlabeled calmodulin were determined by the calmodulin-dependent phosphodiesterase assay devised by Cheung (1971) and compared with that of spinach (*Spinacia oleracea*) calmodulin (Sigma).

Calmodulin Microinjection into Developing *F. serratus* Zygotes

F. serratus zygotes were allowed to settle onto coverslips that formed the base of a chamber filled with ASW, and were cultured at 14°C. Zygotes were divided into two groups: one was cultured in darkness, and the other was exposed to unilateral light from 10 to 13 h AF. Zygotes of both groups were microinjected with recombinant *M. pyrifera* calmodulin containing 1 mM calcium green (Molecular Probes, Eugene, OR) from 13 to 14 h AF. Calcium green was used as a tracer dye because its fluorescence is clearly visible when microinjected into the *F. serratus* cytoplasm, and the excitation and emission wavelengths of the dye are similar to that of FITC. Controls containing no calmodulin or those in which calmodulin was replaced by 5 mg mL^{-1} protease-free BSA were also performed.

From the amount of tracer dye injected with the calmodulin, we estimated that between 1 and 10% of the cytoplasmic volume was introduced into *F. serratus* zygotes. The average diameter of a fucoid egg is 60 nm, so a solution of 5 mg mL^{-1} calmodulin will increase the cell's calmodulin content by 3.2 to 32 pg (0.57–5.7 fmol), approximately 10 to 100 times the calmodulin content of plant cells (Marmé and Dieter, 1983). Following microinjection, zygotes were cultured for 36 to 48 h in darkness at 14°C, and the direction of rhizoid germination was scored. To avoid confusion between potential polarizing stimuli, the direction of impalement and of the polarizing light pulse were perpendicular.

Zygotes were resolved using an inverted microscope (Diphot, Nikon) with a ×10 (Plan 10/0.25 Dic, Nikon), ×20 (PlanApo 20/0.75, Nikon), or ×40 (40 Dic, Nikon) objective. Microinjection was performed at 15°C using a hydraulic pressure probe (Oparka et al., 1991). Injection electrodes were pulled from filamented borosilicate glass capillaries (Clark EMI, Reading, UK) using an electrode puller (model PB-7, Narishige, Tokyo). Electrodes were back-filled with 1 μL of injection solution, completely filled with degassed silicone oil, and inserted into a stainless steel injection holder (model H1–4A, Narishige). A three-axis joystick hydraulic micromanipulator with extra fourth-axis movement (Narishige) was used to impale zygotes with the injection electrode.

Zygotes were held in place with a micropipette made from flexible, fused-silica capillary tubing (TSP/025/150, Composite Metal Services, UK). Microinjection was monitored by calcium green or FITC fluorescence with a 510-nm

dichroic mirror (Nikon) coupled to a 520-nm long-pass filter (Nikon). The excitation wavelength was determined by a 490-nm interference filter (Ealing Electro-Optics, UK) in front of a super high-pressure mercury bulb.

Recombinant, FITC-labeled *M. pyrifera* calmodulin was microinjected into zygotes between 5 and 8 h AF. Control zygotes were microinjected with 20-kD FITC-dextran (Sigma). Zygotes were exposed to unilateral light from 10 to 13 h AF, and the distribution of the fluorescent signal was monitored at various times thereafter using a laser confocal imaging system (model MRC 600, Bio-Rad) powered by an argon laser. The excitation wavelength was obtained by a 488-nm excitation filter. A primary filter block containing a 510-nm long-pass dichroic reflector and a 515-nm long-pass emission filter was used to monitor FITC fluorescence. A secondary filter block containing a 565-nm long-pass dichroic mirror, a 600-nm long-pass barrier filter, and a 540-nm barrier filter was used to measure chloroplast autofluorescence. Images of both green and red fluorescence were accumulated simultaneously using two photomultiplier tubes, and were analyzed using computer software (Comos, Bio-Rad).

RESULTS

Timing of Polar Axis Formation and Fixation in *F. serratus* Zygotes

The light sensitivity of zygotes collected from the local, wild *F. serratus* population was investigated to determine the timing of the different phases of embryogenesis (Fig. 1). Zygotes were broadly synchronous, with 60 to 75% of them being most responsive to exposure to 1 h of unilateral light between 8 and 14 h AF. However, the population was

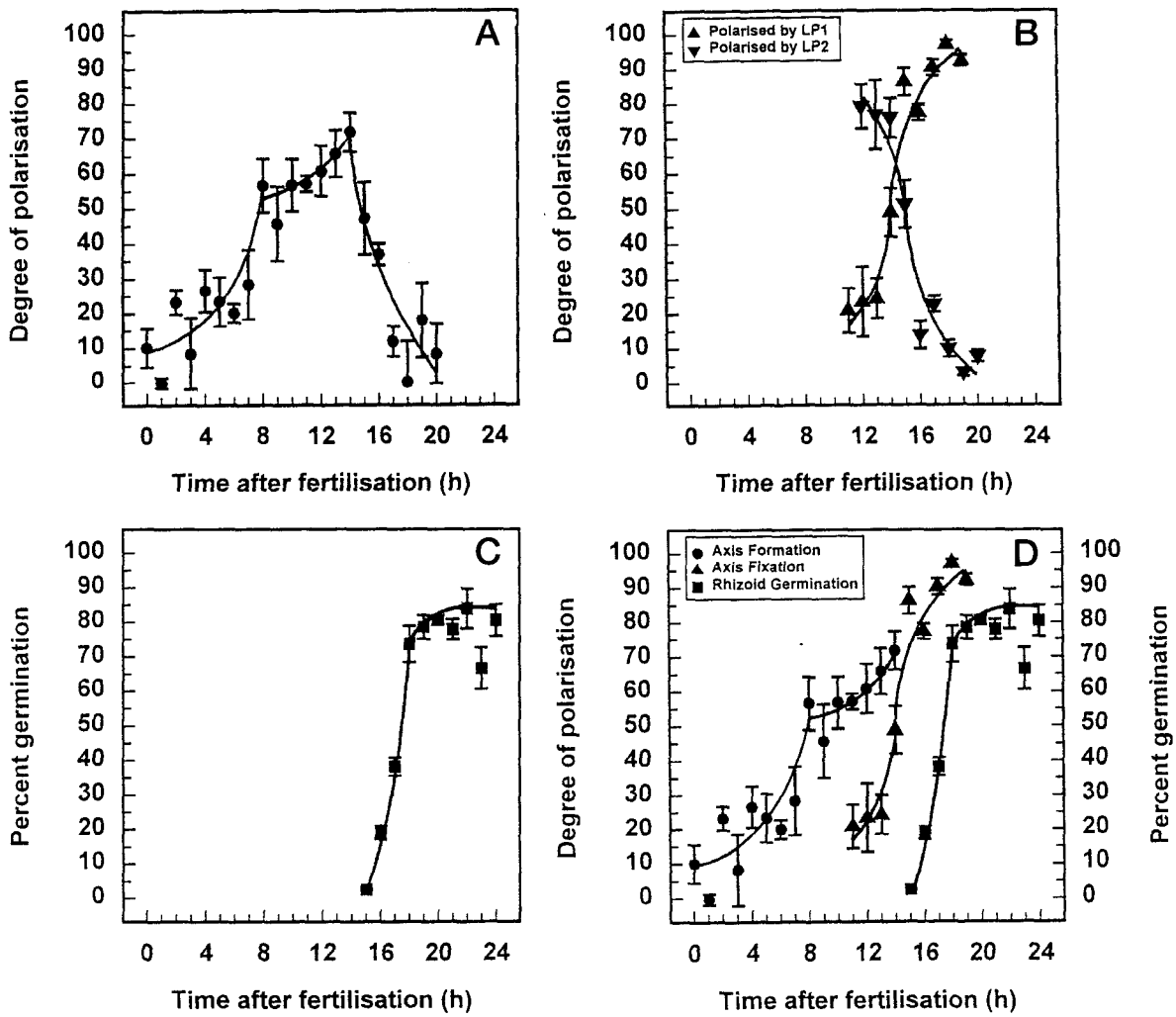

Figure 1. Time course of polar axis formation, fixation, and germination in synchronously developing *F. serratus* zygotes. A, Photopolarization of zygotes exposed to 1 h of unilateral light from fertilization (0 h) to 20 h AF. Each point represents the mean of five replicate experiments, for which at least 200 zygotes were scored. SE bars are shown. B, Photopolarization of zygotes exposed to two consecutive 1-h light pulses in opposite directions (LP1 and LP2). LP1 begins at 10 h AF. The axis is said to be fixed when it is no longer oriented by LP2. C, Germination of zygotes exposed to a 3-h white light pulse from 10 to 13 h AF. D, Superposition of the three phases of polar establishment: Axis formation, fixation, and germination.

clearly heterogeneous, with some zygotes responsive to light earlier than others, as illustrated by the gradual increase in sensitivity to polarizing light from 1 to 8 h AF.

The timing of axis fixation was also determined. Immediately after a polar axis was induced in one direction by exposure to 1 h of unilateral light (LP1), zygotes were administered a second 1-h-long pulse of unilateral light in the opposite direction (LP2). If the axis were labile, polar axis formation would be relative to LP2; if, however, the polar axis were fixed and could not be reoriented, polarity would be relative to LP1. Until 14 h AF, 70% of the zygotes were oriented by LP2, indicating that axis re-orientation was possible. At 15 to 16 h AF, following a rapid decline, less than 10% of zygotes were polarized relative to LP2 but were instead polarized by LP1. The commitment to polarize according to unilateral light therefore occurs from approximately 14 to 16 h AF, and coincides with the loss of sensitivity to light. All zygotes were fully committed to a polar axis from approximately 16 h AF. At 16 h AF a small proportion of zygotes exhibited polar bulges indicating the onset of rhizoid germination and the morphological expression of polarity. Almost all zygotes had germinated by 19 h AF.

Ca^{2+} Involvement during Polar Axis Formation in *F. serratus*

To address the controversy concerning the role of a Ca^{2+} gradient during polar axis formation in *F. serratus* zygotes, we devised a simple series of experiments in which Ca^{2+}-channel blockers and other inhibitors of intracellular Ca^{2+} release were transiently applied to developing zygotes from 10 to 13 h AF (Fig. 1A). Only zygotes that developed and produced rhizoids within the normal time frame of embryogenesis (and were therefore perturbed by the experimental procedure only during the targeted developmental phase) were scored.

Illumination for 3 h increased the degree of photopolarization from 60 to 70% obtained following only 1 h of illumination during the same period to 75 to 90%. No significant difference in the degree of photopolarization was observed between zygotes incubated in ASW (77.47 ± 3.17%), DASW (77.40 ± 6.21%), or in solutions of EGTA to concentrations of 10 mM (74.26 ± 3.70%) during the 3-h period (Fig. 2B). EGTA was applied to zygotes in DASW, reducing the concentration of free Ca^{2+} in the medium so low as to be effectively negligible, especially when considered relative to the 9 mM Ca^{2+} content of normal sea water.

$LaCl_3$, a well-characterized inhibitor of plasma membrane Ca^{2+} channels (Dos Remedios, 1981; Taylor and Brownlee, 1993), reduced the degree of photopolarization from 65% to just below 50% (Fig. 2B). However, concentrations in excess of 1 mM $LaCl_3$ resulted in zygotes becoming unstuck from the culture dishes, which made accurate scoring of polarization impossible and casts doubt on the specificity of La^{3+} at these high concentrations. Indeed, La^{3+} is usually active at micromolar concentrations, three orders of magnitude less than in the experiments described here (Lonergan, 1990; Natawa, 1992; Herrmann and Felle, 1995; Sinclair et al., 1996).

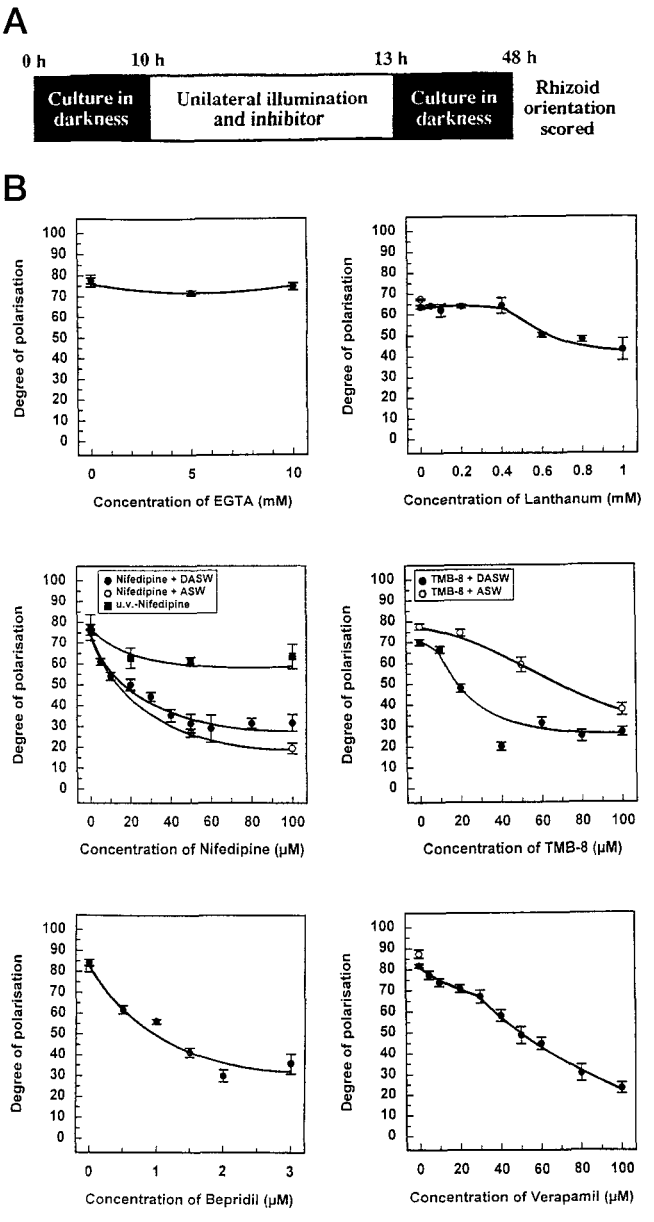

Figure 2. Effect of Ca^{2+} inhibitors on photopolarization of *F. serratus* zygotes. A, Diagram of the experimental protocol. Culture conditions and the method of calculating the degree of polarization are identical to Figure 1. Zygotes were simultaneously exposed to unilateral light and inhibitors from 10 to 13 h AF. B, Effect of transient exposure to the Ca^{2+} chelator EGTA, the plasma membrane Ca^{2+}-channel inhibitor La^{3+}, and the inhibitors of cytoplasmic Ca^{2+} mobilization nifedipine, TMB-8, bepridil, and verapamil on photopolarization. ●, Solutions in DASW; ○, solutions in ASW. Experimental conditions and the method of calculating the degree of polarization are described in Figure 1. Each point is the mean of five replicates. SE bars are shown.

Nifedipine, bepridil, and verapamil each inhibited photopolarization at a similar concentration range and in a dose-dependent manner (Fig. 2B). Although the specificity of these compounds has not been fully determined in plant cells, they are considered to be effective inhibitors of Ca^{2+} channels and therefore of cytosolic Ca^{2+} mobilization

(Malagodi and Chiou, 1974; Schramm et al., 1983; Lehtonen, 1984; Reiss and Herth, 1985). No single compound had a complete inhibitory effect, although the degree of photopolarization was reduced from 75 to 80% to under 30% in each case. Moreover, the effect of nifedipine was approximately the same when applied to zygotes in medium either containing 9 mM Ca^{2+} or depleted of Ca^{2+}, indicating a specific inhibition of plasma membrane Ca^{2+} channels by this compound.

TMB-8, an inhibitor of intracellular Ca^{2+} release (Lonergan, 1990), inhibited photopolarization from 70% to approximately 30% at a concentration of 40 to 50 μM. Unlike nifedipine, the effect of TMB-8 was attenuated when applied to zygotes in ASW, suggesting that extracellular Ca^{2+} influx may compensate for the inhibition of intracellular Ca^{2+} release.

The Role of Actin during Polarization

It has been reported that actin polymerization is essential to the transcytoplasmic current observed during polar axis formation (Brawley and Robinson, 1985) and that cytochalasins prevent photopolarization (Kropf, 1992). However, cytochalasin-B, cytochalasin-D, and latrunculin-B, all of which are inhibitors of actin polymerization, had no effect on polar axis formation in *F. serratus* zygotes when applied from 10 to 13 h AF. We tested the effects of each inhibitor on axis fixation, which requires actin polymerization (Quatrano, 1973; Kropf et al., 1989). Zygotes were unilaterally illuminated from 10 to 13 h AF and then exposed to the inhibitors from 13 to 21 h AF. A second unilateral illumination in the opposite direction to the first was administered from 21 to 24 h AF, when, under normal conditions, rhizoid germination has occurred and polarity is no longer labile. Zygotes treated in this way predominantly polarized relative to the second light pulse (Fig. 3). Actin polymerization is therefore essential to polar axis fixation but is not required for polar axis formation.

Calmodulin Involvement in Polar Axis Formation

Many of the contradictory results concerning the Ca^{2+} requirements during axis formation might be explained by the involvement of calmodulin, which specifies an increased level of sensitivity to Ca^{2+}-dependent processes (Cox, 1986). The calmodulin antagonists W7 and TFP (Hidaka et al., 1981; Poovaiah and Reddy, 1987; Cook et al., 1994) inhibited photopolarization in a dose-dependent manner (Fig. 4) and yielded 0% photopolarization at 50 to 80 μM for W7 and at 3 μM for TFP, exceeding the effect observed with the Ca^{2+} inhibitors. W5, an inactive analog of W7, had no effect on photopolarization when applied at equal concentrations to W7, indicating that the observed effect is not due to simple toxicity. The concentrations at which the inhibitors were applied are comparable to similar studies on other plant systems (Sinclair et al., 1996) and indicate an important role for calmodulin during polar axis formation.

Cloning, Expression, and Purification of *M. pyrifera* Calmodulin for Microinjection

Little is known about calmodulin in the Fucales. A putative calmodulin has been identified in *P. fastigiata* (Brawley and Roberts, 1989), but has not been characterized further. Moreover, the only algal calmodulin sequence available in the databases, that of *Chlamydomonas reinhardtii* (Chlorophyceae), was sufficiently different from that of higher plant calmodulins to make us doubt whether the experimental use of either *C. reinhardtii* or higher plant calmodulins in *F. serratus* would be justified.

Initial attempts to isolate *F. serratus* calmodulin were unsatisfactory because of variable yields and uncertain quality of the protein. Molecular cloning of the calmodulin gene from *F. serratus* was hampered by the copious amounts of acidic polysaccharides contained in the algae. Instead, we isolated, sequenced, and expressed in bacteria

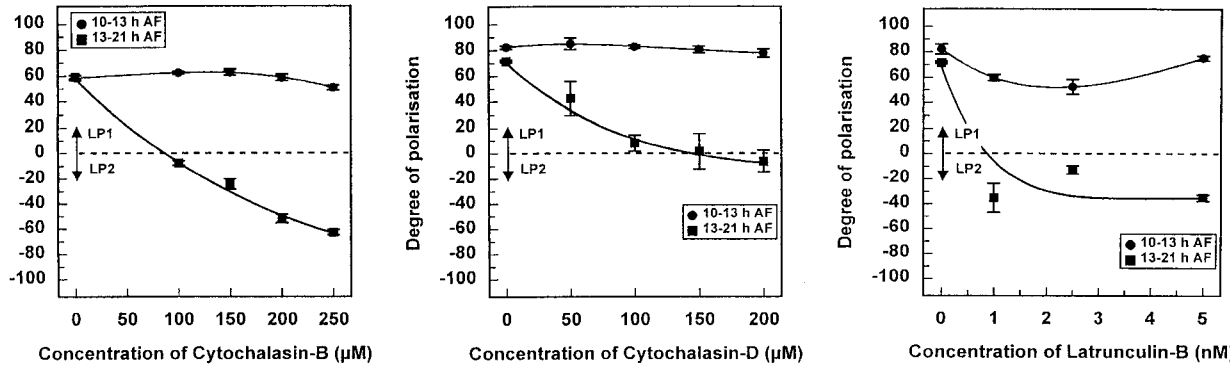

Figure 3. Effect of the inhibitors of actin polymerization cytochalasin-B, cytochalasin-D, and latrunculin-B on photopolarization. Each graph shows the data from two complementary experiments: First, zygotes were simultaneously exposed to the inhibitors and unilateral light from 10 to13 h AF (●). Second, zygotes were exposed to unilateral light from 10 to 13 h AF (LP1), in ASW. The medium was then replaced with inhibitor solutions, and zygotes were incubated in darkness from 13 to 21 h AF. Zygotes were rinsed in ASW and administered a 3-h pulse of unilateral light (LP2) in the opposite direction to the first (■). For both experiments the degree of polarization was calculated relative to the direction of LP1 and as described in Figure 1. A positive value indicates that zygotes polarized relative to LP1; a negative value indicates that zygotes polarized relative to LP2.

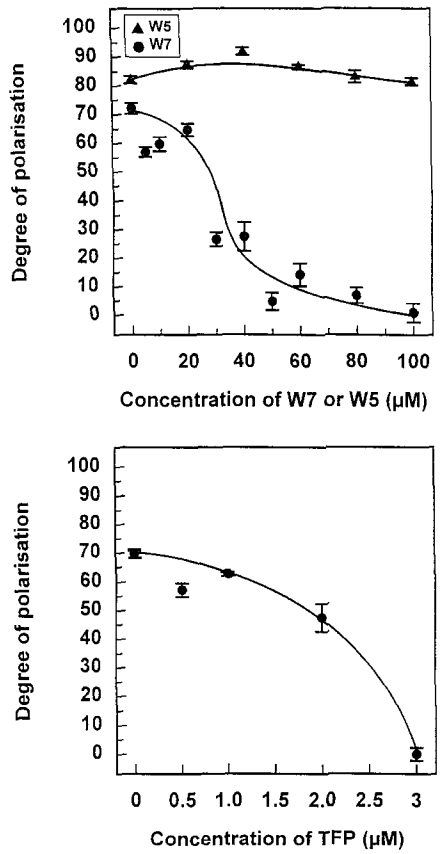

Figure 4. Effect of calmodulin inhibitors on photopolarization of *F. serratus* zygotes. Effect of transient exposure to the calmodulin antagonists W5, W7, and TFP on photopolarization of *F. serratus* zygotes. Experimental conditions and the method of calculating the degree of polarization are described in Figure 1. Inhibitors were dissolved in DASW. Each point is the mean of five replicates. SE bars are shown.

the calmodulin cDNA from the closely related brown alga *M. pyrifera* (Love et al., 1995).

The expressed product of the *M. pyrifera* calmodulin cDNA was identified as a functional calmodulin in three different ways. First, the protein was purified by W7 affinity chromatography (Fig. 5A). Second, mobility on SDS-PAGE of the purified protein compares to that of commercially produced *S. oleracea* calmodulin (Fig. 5B). Moreover, it migrates with an apparent molecular mass of 15 kD in the presence of Ca^{2+}, but displays an electrophoretic band shift to 18 kD when Ca^{2+} is chelated by EGTA, a characteristic of active calmodulin (Watterson et al., 1980). Third, the protein activated calmodulin-dependent phosphodiesterase in a manner similar to that of *S. oleracea* calmodulin (Fig. 5C). Labeling the recombinant *M. pyrifera* calmodulin with FITC did not affect its behavior on SDS-PAGE (Fig. 5B), and minimally reduced its capacity for activating phosphodiesterase compared with the unlabeled protein (Fig. 5C). Spectrophotometric analysis of FITC-calmodulin showed a 1:1 molar ratio between FITC and protein. A higher FITC:calmodulin ratio resulted in a loss of phosphodiesterase activity.

Increased Cytoplasmic Calmodulin Enhances Photopolarization

Calmodulin may be asymmetrically distributed during polar axis formation. In an attempt to swamp the putative gradient, and thereby disrupt polar axis formation, we microinjected recombinant *M. pyrifera* calmodulin into developing *F. serratus* zygotes. Zygotes cultured in darkness prior to microinjection with solutions of calcium green, calcium green and BSA, or calcium green and calmodulin tended to germinate opposite the site of impalement (Fig. 6). A similar effect was observed when zygotes were impaled only. Zygotes that had been exposed to unilateral light before microinjection with calcium green or calcium green and BSA were also inclined to germinate opposite the site of impalement. However, zygotes that had received a unilateral illumination prior to microinjection with calcium green and calmodulin skewed the position of rhizoid germination toward alignment with the direction of illumination (Fig. 6). Raising the cytoplasmic calmodulin concentration therefore had no effect dark-grown zygotes, but reinforced the light-induced polar axis of zygotes exposed to unilateral light.

Imaging Calmodulin Distribution in Photopolarized *F. serratus* Zygotes

The distribution of FITC-labeled *M. pyrifera* calmodulin in the cytoplasm of polarizing *F. serratus* zygotes was visualized in vivo following exposure to unilateral light from 10 to 13 h AF. The quantity of FITC-calmodulin microinjected was of critical importance for imaging: too much and zygotes failed to develop and lysed after approximately 24 h; too little and the FITC fluorescence was undetectable above background.

A fluorescent signal was detectable in 14 of the 18 zygotes that were successfully microinjected. At 13 h AF the signal was distributed homogeneously throughout the cytoplasm in 11 zygotes (Fig. 7). In the remaining three, fluorescence was concentrated at the site of microinjection, but these cells lysed at a later stage. At 15 h AF, FITC-calmodulin fluorescence was localized in the cortical cytoplasmic region of the shaded hemisphere, to the site of presumptive rhizoid germination, in five zygotes. The image presented in Figure 7 is typical of the localization of the fluorescent signal that occurred in these zygotes at 15 h AF. In two zygotes the fluorescence was cortical but not localized, and was homogeneously distributed throughout the cytoplasm in the other four zygotes. At 20 h AF, FITC-calmodulin fluorescence was observed in the germinating rhizoids but the spatial resolution was insufficient to distinguish a calmodulin gradient.

Zygotes were microinjected with 20-kD FITC-dextran to determine whether localization of fluorescence was due to hydrolysis of the label from the support molecule and subsequent localization of the label alone. Seven zygotes were injected with FITC-dextran, and a random distribution of fluorescence was observed in all zygotes at 13 and 15 h AF. (Fig. 8).

DISCUSSION

The Origin and Role of Ca^{2+} in Polar Axis Formation

We investigated the role and origin of Ca^{2+} during polar axis formation in *F. serratus* zygotes by simultaneously exposing zygotes to unilateral light and various inhibitors during axis formation. The requirement for extracellular Ca^{2+} was examined by chelating free Ca^{2+} in the medium with EGTA, by directly blocking Ca^{2+} import into zygotes with various chemically unrelated channel blockers, and by destabilizing the actin cytoskeleton to inhibit the transcellular Ca^{2+} current. The role of intracellular Ca^{2+} release was investigated with TMB-8.

Removal of free Ca^{2+} from the culture medium and the addition of high concentrations of EGTA had no significant effect on photopolarization. This confirms previous data (Hurst and Kropf, 1991), but is different from a recent study on the photopolarization of *P. fastigiata* zygotes (Robinson, 1996a). Using a protocol similar to ours, that study simultaneously exposed zygotes to 4 mM EGTA in Ca^{2+}-depleted ASW and to unilateral light and found that photopolarization was inhibited; in our study 10 mM EGTA had no effect on polar axis formation. However, in the previous study (Robinson, 1996) the developmental progression of *P. fastigiata* zygotes was not clearly defined: Gametes were released over a 30-min period and, because *P. fastigiata* is monoecious, fertilization may have occurred during that time. Synchronicity between zygotes was therefore reduced by design and required adjustment of the data relative to controls. Furthermore, axis fixation in *P. fastigiata* occurs at approximately 8 h AF (Kropf and Quatrano, 1987). Zygotes were exposed to unilateral light and inhibitors between 6 and 7 h AF until 7.5 to 8.5 h AF in the first experiment, or until 8.5 to 9.5 h AF in the second experiment. Consequently, axis formation and axis fixation overlapped, resulting in considerable confusion as to whether inhibition indeed targeted the developmental phase under investigation. The protocol we have used targets polar axis formation more precisely.

It remains possible that the presence of EGTA in the medium might not completely inhibit Ca^{2+} flux into zygotes. Indeed, plant cells possess a rich and poorly understood reserve of extracellular Ca^{2+} bound to the cell wall, and several hours of exposure to EGTA is required to remove this Ca^{2+} pool (Saunders and Hepler, 1983). Such a procedure was not applicable to the experiments described here, nor was it used in the experiments of Hurst and Kropf (1991). Instead, this eventuality was investigated by direct inhibition of plasma membrane Ca^{2+} channels. The effects of the various Ca^{2+}-channel inhibitors used in this study

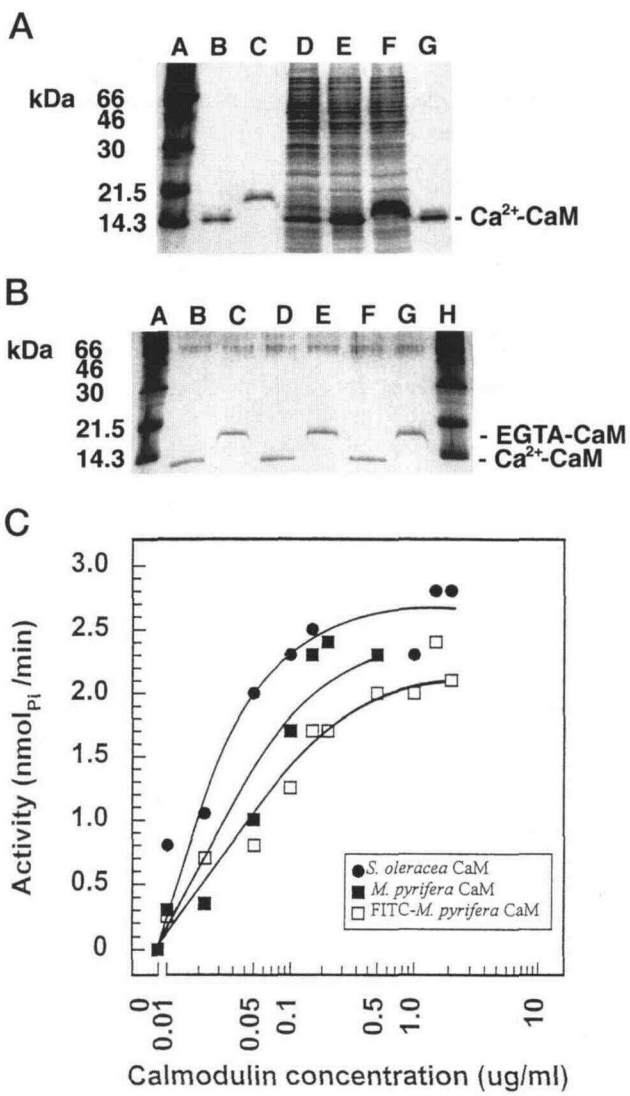

Figure 5. Production and purification of recombinant *M. pyrifera* calmodulin. A, SDS-PAGE of recombinant calmodulin expression in bacteria. The calmodulin cDNA from the brown alga *M. pyrifera* was cloned and expressed in *E. coli* strain BL21-ΔDE3. Recombinant protein expression was induced by isopropyl-β-thiogalactopyranoside (IPTG). Calmodulin (CaM) was purified from bacterial lysates by W7-affinity chromatography. Lane A, 1 μg of rainbow markers (Amersham); lane B, 2 μg of *S. oleracea* calmodulin in 10 mM $CaCl_2$ (Sigma); lane C, 2 μg of *S. oleracea* calmodulin in 10 mM EGTA (Sigma); lane D, 1 μL of bacterial lysate from an uninduced culture; lane E, 1 μL of bacterial lysate from a culture in which recombinant protein expression was induced by 4 mM IPTG prior to purification by affinity chromatography; lane F, 1 μL of bacterial lysate from a culture in which recombinant protein expression was induced by 4 mM IPTG in 10 mM EGTA; lane G, 5 μg of recombinant *M. pyrifera* calmodulin in microinjection buffer. Note the characteristic band shift between the calmodulin in the presence (lanes B and E) and absence (lanes C and F) of Ca^{2+}. B, SDS-PAGE of purified recombinant *M. pyrifera* calmodulin showing the characteristic band shift in the presence and absence of Ca^{2+}. Lane A, 1 μg of rainbow markers (Amersham); lane B, 1 μg *S. oleracea* calmodulin in 10 mM $CaCl_2$ (Sigma); lane C, 1 μg of *S. oleracea* calmodulin in 10 mM EGTA (Sigma); lane D, 1 μg of *M. pyrifera* calmodulin in 10 mM $CaCl_2$; lane E, 1 μg of *M. pyrifera* calmodulin in 10 mM EGTA; lane F, 1 μg of FITC-labeled *M. pyrifera* calmodulin in 10 mM $CaCl_2$; lane G, 1 μg of FITC-labeled *M. pyrifera* calmodulin in 10 mM EGTA; lane H, 1 μg of rainbow markers (Amersham). C, Phosphodiesterase activity of *S. oleracea* calmodulin, recombinant *M. pyrifera* calmodulin, and FITC-labeled *M. pyrifera* calmodulin. Activity is expressed in nanomoles of Pi produced minute per microgram of calmodulin.

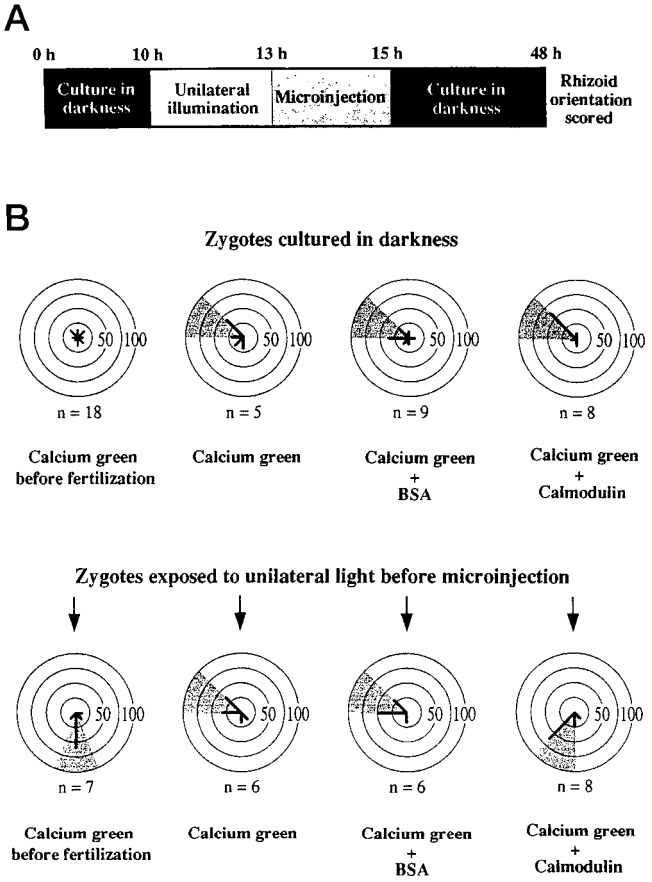

Figure 6. Polarization of *F. serratus* zygotes following microinjection from 13 to 15 h AF. A, Diagram of the experimental protocol. B, Direction of polarization of zygotes microinjected with calcium green, calcium green and BSA, or calcium green and recombinant *M. pyrifera* calmodulin. Treatments and numbers of zygotes are indicated for each diagram. Bars represent the percentage of rhizoids germinated in a particular direction on a concentric scale. Shaded areas highlight the direction of rhizoid germination of the majority of zygotes. The direction of unilateral illumination (10–13 h AF) is indicated. The site of microinjection is at three o'clock on the images.

varied according to the site of action and, although the specificity of the inhibitors has not been fully determined in plant cells, their effects are useful pointers to possible events involved in photopolarization.

La^{3+}, a potent competitive inhibitor of Ca^{2+} uptake (Dos Remedios, 1981; Tlalka and Gabrys, 1993) that blocks Ca^{2+} import in *F. serratus* zygotes (Brownlee and Wood, 1986; Taylor and Brownlee, 1993), caused a small reduction in the degree of photopolarization within the zygote population. Moreover, La^{3+} concentrations exceeded the usual inhibitory range for plant cells even though the inhibitor was applied in Ca^{2+}-depleted ASW. Thus, it appears that Ca^{2+} influx from the medium is not a fundamental requirement of polar axis formation. It does, however, remain possible that sufficient Ca^{2+} flux into zygotes occurred to polarize zygotes even in the presence of La^{3+}, implying that a small Ca^{2+} asymmetry, rather than a transcytoplasmic gradient, may be sufficient for polarization. As previously noted, this last possibility may also explain the lack of effect by EGTA.

Ca^{2+} channels were directly inhibited during photopolarization with bepridil, nifedipine, and verapamil (Lee and Tsien, 1983; Schramm et al., 1983; Lehtonen, 1984; Reiss and Herth, 1985; Lonergan, 1990). Each compound reduced the degree of photopolarization by approximately 40%. Although the specificity of these inhibitors may be questioned, they are considered to be efficient Ca^{2+}-channel inhibitors and are chemically unrelated. Moreover, they have similar effects on *F. serratus* zygotes, especially at lower concentrations, which may be argued as arising from the specific effect of each compound on Ca^{2+}-channel transport. Nifedipine inhibited photopolarization by approximately 40% whether applied to zygotes in ASW or in DASW, indicating a direct and specific effect on plasma membrane-bound Ca^{2+} channels (Saunders and Hepler, 1983).

UV-irradiated nifedipine inhibited photopolarization in at most 10% of the zygote population, showing that compound toxicity alone had little involvement in the inhibitory effect. It may be significant that each Ca^{2+}-channel inhibitor failed to inhibit photopolarization in approximately 30% of the zygote population, especially at concentrations that might be considered specific for Ca^{2+} channels. This might be due to several types of Ca^{2+} channels being involved in Ca^{2+} import and/or to Ca^{2+} being mobilized from intracellular stores during photopolarization. A requirement for intracellular Ca^{2+} release during polar axis formation was shown by the application of TMB-8 (Saunders and Hepler, 1983; Lonergan, 1990), which inhibited photopolarization in approximately 70% of the zygote population. The effect was reduced in the presence of extracellular Ca^{2+}, indicating that Ca^{2+} influx from the extracellular medium may compensate for the inhibition of intracellular Ca^{2+} release. It therefore appears that a small cytosolic Ca^{2+} mobilization is important during photopolarization.

It has been shown that the microfilament inhibitor cytochalasin-B disrupts the transcytoplasmic current in *Fucus vesiculosus*, *Fucus disticaus*, and *P. fastigiata* zygotes (Brawley and Robinson, 1985). In this study, cytochalasin-B, cytochalasin-D, and latrunculin-B had no effect on axis formation in *F. serratus* zygotes, although each compound inhibited axis fixation, which is known to require actin polymerization (Quatrano, 1973; Kropf et al., 1989). F-actin polymerization, and consequently the transcellular Ca^{2+} current, are therefore not essential components of axis formation. These observations are contrary to the current model of polarization, which states that asymmetric Ca^{2+} influx and export at opposite hemispheres of fucoid zygotes generates a transcytoplasmic Ca^{2+} gradient that polarizes the cell. The role of Ca^{2+} during polar axis formation must therefore be re-assessed and a different model proposed.

Photopolarization results from unilateral illumination and might be achieved by a localized and self-sustaining asymmetry in cytoplasmic free Ca^{2+}, rather than a transcellular Ca^{2+} gradient. Light is asymmetrically focused in fucoid zygotes so that the maximum difference between

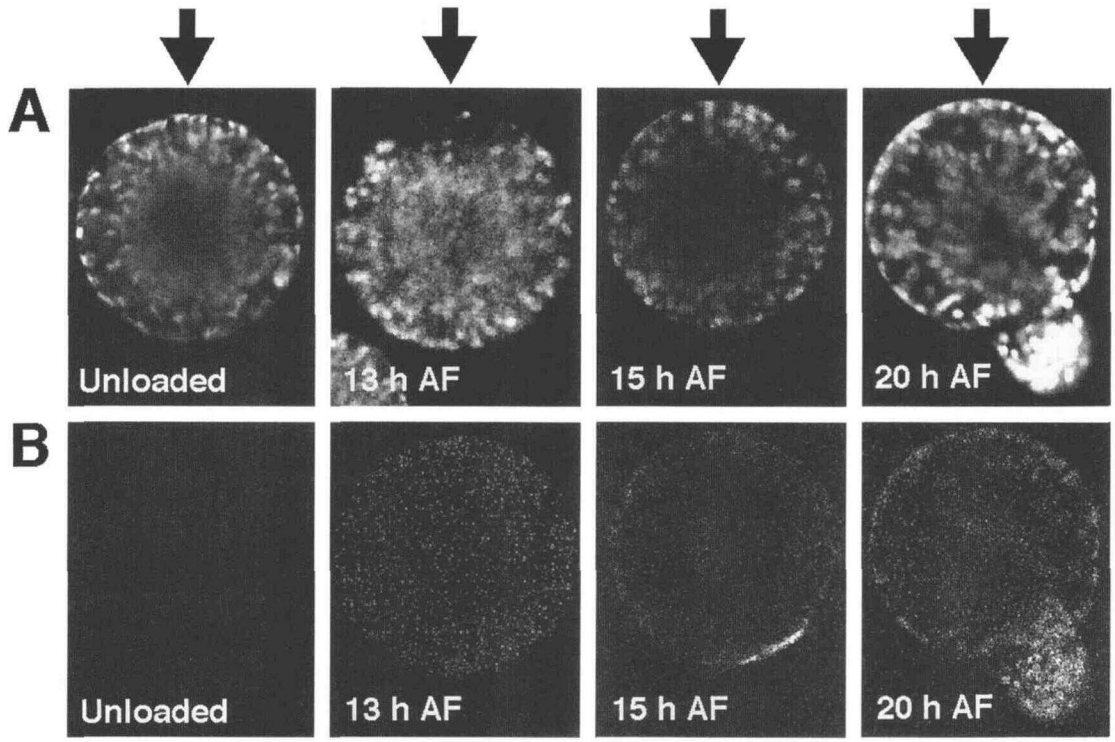

Figure 7. Fluorescence confocal imaging of recombinant *M. pyrifera* FITC-calmodulin in polarizing *F. serratus* zygotes. Zygotes were microinjected with FITC-calmodulin between 5 and 8 h AF and exposed to unilateral light from 10 to 13 h AF (indicated by the arrows). The site of microinjection is at three o'clock on the images. A, Autofluorescence of the chloroplasts; B, fluorescence of FITC-calmodulin. Uninjected zygotes show no FITC signal (images marked "unloaded"). Injected zygotes were imaged at 13, 15, and 20 h AF, during axis formation, fixation, and germination, respectively. At 13 h AF, the distribution of fluorescence is typically uniform. At 15 h AF, an intense signal is visible as a white crescent at the site of future rhizoid germination. At 20 h AF, a tip-to-base gradient of fluorescence is present in the growing rhizoid. Population numbers are given in the text.

light and dark (the maximum stimulus) occurs, paradoxically, in the cortical cytoplasm of the shaded hemisphere (Jaffe, 1968; Berger and Brownlee, 1994). This view has recently been challenged (Robinson, 1996b), but the data describe light absorption through the zygote and do not address the position of absorption within the cell. From the inhibitor data presented here, we can speculate that unilateral illumination causes a localized Ca^{2+} mobilization from both extracellular and intracellular sources in the cortical cytoplasm of the presumptive rhizoid. It has recently been shown that a cytoplasmic dihydropyridine receptor, which may correspond to a Ca^{2+} channel, is localized to the putative rhizoid pole at axis fixation and coincides with the establishment of a detectable cytoplasmic Ca^{2+} gradient (Shaw and Quatrano, 1996a). Distribution of the receptor is uniform during axis formation, and, like the data presented in this paper, suggests that a transcytoplasmic Ca^{2+} gradient is not involved during polar axis formation.

Our data also indicate that the transcytoplasmic current is not required for polarization. Instead, it is possible that rather than initiating polarity, the Ca^{2+} current is involved in the maintenance and reinforcement of a potential polar axis. For instance, zygotes and embryos of *Bithinia tentaculata* generate a polar, transcytoplasmic Ca^{2+} current, the intensity and direction of which has been shown to reinforce the polar organization of the developing organism (Zivkóvic and Dohmen, 1991). Such a process might occur in fucoid zygotes, in which the timing of the transcytoplasmic current and of polarized secretion coincide with photopolar axis formation (Nuccitelli and Jaffe, 1975; Schröter, 1978). Like the current, polar secretion appears to be essential only at axis fixation (Shaw and Quatrano, 1996b), even though it is observed during axis formation and might reflect the gradual nature of polarization.

Instead of a model in which polarity arises from a movement of Ca^{2+} through the zygote, we conceive of polarization as a consequence of a localized Ca^{2+} flux into the cytoplasm in response to unilateral light. The cytoplasmic Ca^{2+} asymmetry need not be large to produce a substantial physiological effect (Robinson and Jaffe, 1974, 1976), and would consequently be invisible with current Ca^{2+}-imaging technology. The perturbation may then be amplified by several processes: activation of plasma membrane Ca^{2+} channels, intracellular Ca^{2+}-induced Ca^{2+} release, Ca^{2+}-dependent protein kinase activation, or activation of Ca^{2+} receptors. To test this idea we investigated the role of calmodulin, the main receptor for intracellular Ca^{2+} in plant cells, during polarization.

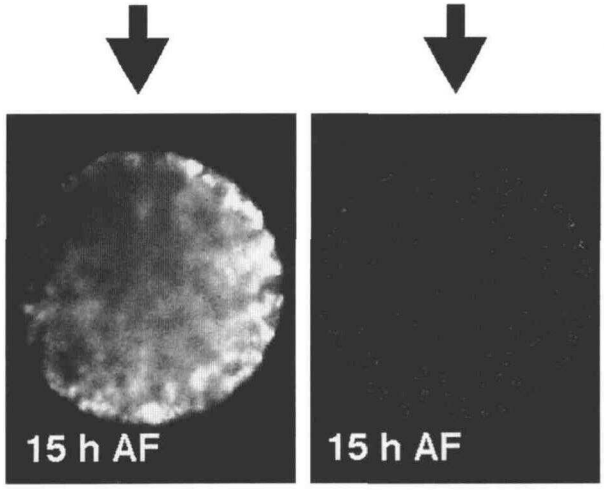

Figure 8. Fluorescence confocal imaging of 20-kD FITC-dextran at 15 h AF. Zygotes were microinjected with 20-kD FITC-dextran between 5 to 8 h AF, and exposed to unilateral light from 10 to 13 h AF (indicated by the arrows). The site of microinjection was at three o'clock. Fluorescence is uniformly distributed in the cytoplasm, and no localization of fluorescence, as shown in Figure 7, was ever observed. Population numbers are given in the text.

Calmodulin Involvement in Polar Axis Formation

The inhibition of photopolarization by calmodulin antagonists and the effects of artificially increasing cytoplasmic calmodulin concentration in developing *F. serratus* zygotes present a compelling case for calmodulin involvement during polar axis formation. Exposure to the calmodulin antagonists W7 and TFP (Poovaiah and Reddy, 1987; Hidaka et al., 1981; Hidaka and Ishikawa, 1992) during polar axis formation inhibited the alignment of the polar axis relative to unilateral light in all zygotes. W5, used as a control for W7, had no effect. Although it has been reported that W7 has no effect on photopolarization of *P. fastigiata* zygotes (Robinson, 1996a), in that study zygotes were exposed to only 4 μM W7. In our experiments 10 μM W7 had no effect on polar axis formation, either; it was maximally effective from 60 to 80 μM, thus explaining the discrepancy.

The role of calmodulin in polarization was investigated further by microinjecting recombinant *M. pyrifera* calmodulin into developing *F. serratus* zygotes. These species are closely related, and given the high degree of homology between the calmodulin sequence of *M. pyrifera* and higher plants, it is reasonable to assume few differences between the calmodulins of both algae. Zygotes cultured in darkness prior to microinjection always polarized relative to the site of impalement, whether microinjected with recombinant algal calmodulin, or with control solutions of calcium green or BSA. The manner in which impalement orients polarity is unknown, but may be due to cell damage at the site of wounding causing an incapacity for growth in that region. This effect has not previously been reported, and may be due to the late stage at which zygotes were injected. Alternatively, in this particular series of experiments impalement was the last asymmetric event perceived by the zygotes. It is therefore possible that zygotes responded, as they would normally, to the last polarizing stimulus (Jaffe, 1968). Zygotes exposed to unilateral light prior to microinjection polarized relative to the position of impalement when microinjected with control solutions, but in an intermediate position between the direction of impalement and that of unilateral light following calmodulin microinjections. The photopolar axis was therefore enhanced by an increase in cytoplasmic calmodulin concentration.

Two possible explanations might account for this result. First, during photopolarization calmodulin becomes asymmetrically localized. Microinjected calmodulin may then be recruited by the photopolar axis and asymmetrically distributed in the zygote, thereby intensifying photopolarization. The second possibility proposes no localization of calmodulin, but depends on the interplay between Ca^{2+}, calmodulin concentration, and the target enzymes of calmodulin, first reported by Cox (1986), who found that increasing the amount of available calmodulin enhances the activity of calmodulin-activated enzymes, and therefore the sensitivity to Ca^{2+}-related processes. It is possible that increasing the calmodulin concentration throughout the cytoplasm in photopolarized zygotes would further sensitize zygotes to a small Ca^{2+} asymmetry, and yield polar axes even more firmly biased toward the direction of illumination. Real-time visualization of FITC-labeled calmodulin in polarizing zygotes was used to decide between these two possible solutions.

At 13 h AF, FITC-calmodulin was evenly distributed throughout the cytoplasm (Fig. 8); therefore, calmodulin is not polarized during axis formation and the second of the two options described above is preferred: Cytoplasmic asymmetry generated by a localized activation of calmodulin and calmodulin-dependent enzymes following cytoplasmic Ca^{2+} release is the possible scenario for the polar axis formation suggested by our results.

The Role of Actin during Polar Axis Formation

Although it has been shown that actin is fundamental to polarity in fucoid zygotes (Quatrano, 1973; Kropf et al., 1989), the role of the microfilament cytoskeleton during the early stages of polar axis formation is unclear. Exposure to cytochalasin-B disrupts polarization (Quatrano, 1973) and the transcytoplasmic Ca^{2+} current that circulates through developing fucoid zygotes (Brawley and Robinson, 1985). This, as previously explained, led to the suggestion that F-actin may be involved in initiating the Ca^{2+} current, and therefore the polar axis (Kropf, 1992). However, the data presented in this paper show that photopolarization proceeds normally in the presence of cytochalasin-B, cytochalasin-D, and latrunculin-B, but that axis fixation is inhibited by these compounds. Actin polymerization is therefore not required for polar axis formation but is essential to polar axis fixation.

Moreover, at axis fixation accumulations of cortical F-actin have been observed in the presumptive rhizoid pole of *Fucus distichus* zygotes using rhodamine-phalloidin staining (Kropf et al., 1989). During axis formation, however, F-actin staining was symmetrically distributed throughout the cortex. F-actin might therefore be localized

to the presumptive rhizoid at axis fixation in response to the intracellular asymmetries generated during axis formation, but is not directly involved in producing these asymmetries at the earlier stage of embryogenesis. The polar axis is later stabilized during axis fixation through interactions between F-actin, the plasma membrane, secretory pathways, and the cell wall (Kropf et al., 1988; Quatrano et al., 1991; Shaw and Quatrano, 1996b).

Calmodulin Dynamics during Axis Fixation

At 15 h AF during axis fixation, a cortical localization of FITC-calmodulin was observed at the presumptive rhizoid pole of approximately one-half of the zygotes monitored. Similar calmodulin localizations have been described in other budding and tip-growing cells, such as at the bud site of *Saccharomyces cerevisiae* (Brockerhoff and Davis, 1992; Sun et al., 1992) or to the expanding region of *Micrasterias* lobes (Kiermayer, 1981). By complementing the *S. cerevisiae* calmodulin-deficient mutant CMD-1 with various altered calmodulin genes, Ohya and Botstein (1994) demonstrated many facets of calmodulin function during budding and cytokinesis. Four complementation groups were analyzed, showing defects in calmodulin localization to the bud site, actin organization, nuclear division, and bud germination. Most importantly, the accumulation of calmodulin at the bud site was essential for the subsequent assembly of F-actin and intermediate filaments in that region (Ohya and Botstein, 1994).

As previously noted, F-actin accumulates at the rhizoid pole of fucoid zygotes during axis fixation (Quatrano and Kropf, 1989). Moreover, homologs to yeast proteins have been cloned from *F. distichus*, although their function in vivo is unknown (Goodner and Quatrano, 1993). It is possible that the calmodulin accumulation observed during axis fixation in *F. serratus* has a similar function, and guides the asymmetric distribution of the cytoskeleton at axis fixation. The manner in which calmodulin is translocated and the proteins with which it interacts are still a mystery in *Fucus* as well as in yeast. However, *Fucus* zygotes constitute an excellent system with which to investigate in plants the functional role of calmodulin-binding and bud site-associated proteins that are more easily identified in simpler genetic systems.

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LITERATURE CITED

Allen RD, Jacobsen L, Joaquin J, Jaffe LF (1972) Ionic concentrations in developing *Pelvetia* eggs. Dev Biol **27:** 538–545

Bentrup F, Sandan T, Jaffe L (1966) Induction of polarity in *Fucus* eggs by potassium ion gradients. Protoplasma **64:** 254–266

Bentrup FW, Jaffe LF (1968) Analyzing the 'group effect': rheotropic responses of developing *Fucus* eggs. Protoplasma **65:** 25–35

Berger F, Brownlee C (1993) Ratio confocal imaging of free cytoplasmic calcium gradients in polarizing and polarized *Fucus* zygotes. Zygote **1:** 9–15

Berger F, Brownlee C (1994) Photopolarization of the *Fucus* sp. zygote by blue light involves a plasma membrane redox chain. Plant Physiol **105:** 519–527

Brawley SH, Roberts DM (1989) Calmodulin binding proteins are developmentally regulated in gametes and embryos of fucoid algae. Dev Biol **131:** 313–320

Brawley SH, Robinson KR (1985) Cytochalasin treatment disrupts the endogenous currents associated with cell polarization in fucoid zygotes: studies of the role of F-actin in embryogenesis. J Cell Biol **100:** 1173–1184

Brawley SH, Wetherbee R, Quatrano RS (1976) Fine structural studies of the gametes and embryo of *Fucus vesiculosus* L. (Phaeophyta). I. Fertilization and pronuclear fusion. J Cell Sci **20:** 233–254

Brockerhoff SE, Davis TN (1992) Calmodulin concentrates at regions of cell growth in *Saccharomyces cerevisiae*. J Cell Biol **118:** 619–629

Brownlee C, Wood JW (1986) A gradient of cytoplasmic free calcium in growing rhizoid cells of *Fucus serratus*. Nature **320:** 624–626

Cook WJ, Walter LJ, Walter MR (1994) Drug binding by calmodulin: crystal structure of a calmodulin-trifluoperazine complex. Biochemistry **33:** 15259–15265

Cox JA (1986) Calcium-calmodulin interaction and cellular function. J Cardiovasc Pharmacol (Suppl.) **8:** S48–S51

Cheung WY (1971) Cyclic 3′5′-nucleotide phosphodiesterase: evidence for and properties of a protein activator. J Biol Chem **246:** 2859–2869

Dos Remedios CG (1981) Lanthanide ion probes of calcium binding sites on cellular membranes. Cell Calcium **2:** 29–51

Gibbon BC, Kropf DL (1993) Intracellular pH and its regulation in *Pelvetia* zygotes. Dev Biol **156:** 259–268

Goodner B, Quatrano RS (1993) Conservation of proteins involved in polar axis formation—*Fucus* meets Yeast. J Cell Biochem **17B:** 39

Herrmann A, Felle HH (1995) Tip growth in the root hair cells of *Sinapsis alba* L.: significance of internal and external Ca^{2+} and pH. New Phytol **129:** 523–533

Hidaka H, Ishikawa T (1992) Molecular pharmacology of calmodulin pathways in cell functions. Cell Calcium **13:** 465–472

Hidaka H, Sasaki Y, Tanaka T, Endo T, Ohno S, Fujii Y, Nagata T (1981) *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation. Proc Natl Acad Sci USA **78:** 4354–4357

Hurst SR, Kropf DL (1991) Ionic requirements for the establishment of an embryonic axis in *Pelvetia* zygotes. Planta **185:** 27–33

Jaffe LF (1966) Electrical currents through the developing *Fucus* egg. Proc Natl Acad Sci USA **56:** 1102–1109

Jaffe LF (1968) Localization in the developing *Fucus* egg and the general role of localizing currents. Adv Morphog **7:** 295–328

Jaffe LF, Neuscheler W (1969) On the mutual polarization of nearby pairs of Fucaceous eggs. Dev Biol **19:** 549–565

Jaffe LF, Robinson KR, Nuccitelli R (1974) Local cation entry and self electrophoresis as an intracellular localization mechanism. Ann NY Acad Sci **238:** 372–389

Kiermayer O (1981) Cytoplasmic basis of morphogenesis in *Micrasterias*. Cell Biol Mono **8:** 147–189

Kropf DL (1992) Establishment and expression of cellular polarity in fucoid zygotes. Microbiol Rev **56:** 316–339

Kropf DL, Berge SK, Quatrano RS (1989) Actin localization during *Fucus* embryogenesis. Plant Cell **1:** 191–200

Kropf DL, Kloareg B, Quatrano RS (1988) Cell wall is required for fixation of the embryonic axis in *Fucus* zygotes. Science **239:** 187–189

Kropf DL, Quatrano RS (1987) Localization of membrane associated calcium during development of fucoid algae using chlorotetracycline. Planta **171:** 158–170

Lee KS, Tsien RW (1983) Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. Nature **302:** 790–794

Lehtonen J (1984) The significance of Ca^{2+} in the morphogenesis of *Micrasterias* studied with EGTA, verapamil, $LaCl_3$ and calcium ionophore A23187. Plant Sci Lett **33:** 53–60

Lonergan TA (1990) Steps linking the photosynthetic light reactions to the biological clock require calcium. Plant Physiol **93:** 110–115

Love J, Oliver IR, Trewavas AJ (1995) The nucleotide sequence of *Macrocystis pyrifera*: calmodulin cDNA (GenBank X85019) (PGR 95–029). Plant Physiol **108:** 1748

Lünd EJ (1923) Electrical control of organic polarity in the eggs of *Fucus*. Bot Gaz **76:** 288–331

Malagodi MH, Chiou CY (1974) Pharmacological evaluation of a new Ca^{2+} antagonist, *H*-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8): studies in smooth muscle. Eur J Pharmacol **27:** 25–33

Marmé D, Dieter P (1983) Role of Ca^{2+} and calmodulin in plants. *In* W-Y Cheung, ed, Calcium and Cell Function, Vol 4. Academic Press, New York, pp 264–311

Natawa T (1992) A major role for transcellular Ca^{2+} ion currents in cell elongation in the unicellular green alga *Closterium*. Plant Cell Physiol **33:** 881–888

Novák B, Bentrup FW (1973) Orientation of *Fucus* egg polarity by electric AC and DC fields. Biophisik **9:** 253–260

Nuccitelli R (1988) Ion currents in morphogenesis. Experientia **44:** 657–666

Nuccitelli R, Jaffe LF (1974) Spontaneous current pulses through developing *Fucus* eggs. Proc Natl Acad Sci USA **71:** 4855–4859

Nuccitelli R, Jaffe LF (1975) The pulse current pattern generated by developing fucoid eggs. J Cell Biol **64:** 636–643

Nuccitelli R, Jaffe LF (1976) The ionic components of the current pulses generated by developing *Fucus* eggs. Dev Biol **49:** 518–531

Ohya Y, Botstein D (1994) Diverse essential functions revealed by complementing yeast calmodulin mutants. Science **263:** 963–966

Oparka KJ, Murphy R, Derrick PM, Prior DAM, Smith JAC (1991) Modification of the pressure-probe technique permits controlled intracellular microinjection of fluorescent probes. J Cell Sci **98:** 539–544

Poovaiah BW, Reddy ASN (1987) Calcium messenger systems in plants. Crit Rev Plant Sci **6:** 47–103

Quatrano RS (1973) Separation of processes associated with differentiation of two celled *Fucus* embryos. Dev Biol **30:** 209–213

Quatrano RS, Brian L, Aldrige J, Schultz T (1991) Polar axis fixation in *Fucus* zygotes: components of the cytoskeleton and extracellular matrix. Development (Suppl.) **1:** 11–16

Quatrano RS, Kropf DL (1989) Polarization in *Fucus* (Phaeophyceae) zygotes: investigations of the role of calcium, microfilaments and cell wall. *In* AW Coleman, LJ Goff, JR Stein Taylor, eds, Algae as Experimental Systems. Alan R. Liss, New York, pp 111–119

Reiss H-D, Herth W (1985) Nifedipine-sensitive calcium channels are involved in polar growth of lily pollen tubes. J Cell Sci **76:** 247–254

Rinderknecht P (1962) Ultra-rapid labeling of proteins. Nature **193:** 167–168

Robinson KR (1996a) Calcium and the photopolarization of *Pelvetia* zygotes. Planta **198:** 378–384

Robinson KR (1996b) Fucoid zygotes germinate from their darkest regions, not their brightest ones. Plant Physiol **112:** 1401

Robinson KR, Cone R (1979) Polarization of fucoid eggs by a calcium ionophore gradient. Science **207:** 77–78

Robinson KR, Jaffe LF (1973) Ion movements in a developing fucoid egg. Dev Biol **35:** 349–361

Robinson KR, Jaffe LF (1974) Polarizing fucoid eggs drive a calcium current through themselves. Science **187:** 70–72

Robinson KR, Jaffe LF (1976) Calcium gradients ands cell polarity. J Cell Biol **70:** 30(a) 110

Sambrook J, Frisch EF, Maniatis T (1989) *In* N Ford, C Nolan, M Ferguson, eds, Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Saunders MJ, Hepler PK (1983) Calcium antagonists and calmodulin inhibitors block cytokinin-induced bud formation in *Funaria*. Dev Biol **99:** 41–49

Schramm M, Thomas G, Towart R, Franckowiak G (1983) Novel dihidropyridines with positive inotropic action through activation of Ca^{2+} channels. Nature **303:** 535–537

Schröter K (1978) Asymmetrical jelly secretion of zygotes of *Pelvetia* and *Fucus*: an early polarization event. Planta **140:** 69–73

Shaw SL, Quatrano RS (1996a) Polar localization of a dihydropyridine receptor on living *Fucus* zygotes. J Cell Sci **109:** 335–342

Shaw SL, Quatrano RS (1996b) The role of targeted secretion in the establishment of cell polarity and the orientation of the division plane in *Fucus* zygotes. Development **122:** 2623–2630

Sinclair W, Oliver I, Maher P, Trewavas A (1996)The role of calmodulin in the gravitropic response of the *Arabidopsis thaliana agr-3* mutant. Planta **199:** 343–351

Speksnijder JE, Miller A, Weisenseel MH, Chen TH, Jaffe LF (1989) Calcium buffer injections block fucoid egg development by facilitating calcium diffusion. Proc Natl Acad Sci USA **86:** 6607–6611

Sun G-H, Ohya Y, Anraku Y (1992) Yeast calmodulin localizes to sites of cell growth. Protoplasma **64:** 110–113

Taylor AR, Brownlee C (1993) Calcium and potassium currents in the *Fucus* egg. Planta **189:** 109–119

Tlalka M, Gabrys H (1993) Influence of calcium on blue light-induced chloroplast movement in *Lemna triscula* L. Planta **189:** 491–498

Watterson DM, Iversom DB, van Eldik LJ (1980) Spinach calmodulin: isolation, characterization and comparison with vertebrate calmodulins. Biochemistry **19:** 5762–5768

Whitaker DM (1931) Some observations on the eggs of *Fucus* and upon their mutual influence in the determination of the developmental axis. Biol Bull **61:** 294–308

Whitaker DM, Lowrance EW (1936) On the period of susceptibility in the egg of *Fucus furcatus* when polarity is induced by brief exposure to directed white light. J Cell Comp Physiol **7:** 417–424

Zivkóvic D, Dohmen R (1991) Changes in transcellular currents associated with cytokinesis and polar lobe formation in embryos of *Bithinia tentaculata* (Mollusca). Development **112:** 451–459