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Gravity-directed calcium current in germinating spores of *Ceratopteris richardii*

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Abstract. Gravity directs the early polar development in single cells of *Ceratopteris richardii* Brogn. It acts over a limited period of time during which it irreversibly determines the axis of the spore cell's development. A self-referencing calcium selective electrode was utilized to record the net movement of calcium across the cell membrane at different positions around the periphery of the spore during the period in which gravity orients the polarity of the spore. A movement of calcium into the cell along the bottom and out of the cell along the top was detected. This movement was specific, polarized, and strongest in a direction that opposed the vector of gravity. Treatment with nifedipine, a calcium-channel blocker, diminished the calcium current and caused the cell to lose its responsiveness to the orienting influence of gravity. Results shown suggest that calcium plays a crucial role in the ability of a single cell to respond to gravity and in the subsequent establishment of its polarity.

Key words: Calcium – *Ceratopteris* (Ca, gravity) – Developmental polarity – Gravity – Ion current – Nifedipine

Introduction

The force of gravity can induce a variety of developmental events and growth responses in many different kinds of cells. The cellular mechanisms by which gravity acts, particularly how individual cells sense gravity and transduce this stimulus into an oriented response, remains a mystery. Although prior reports have elucidated the role of the intracellular signaling ion, calcium, in gravitropism (Roux 1995), its role in gravity perception and response in single cells remains to be determined.

Edwards and Roux (1994, 1998) recently reported on a unique gravity response seen in spores of *Ceratopteris richardii*, an aquatic fern that is useful as a model system for studying plant growth and development (Chasan 1992; Hickok et al. 1987). After the spores are induced to germinate by light, there is a limited period of time, usually 24 h, during which gravity can set their developmental polarity. The results of this directive action are that the cell nucleus migrates downward, the plane of the first cell division is positioned asymmetrically, and the two cell types produced grow in opposite directions parallel to the vector of gravity. Here we report first-time evidence that gravity can polarize the flow of calcium into and out of single spore cells of *Ceratopteris* early during the period when it is orienting the cell's axis of growth, and that this polarization is crucial in the early development of the gametophyte.

Materials and methods

Plant material and growth conditions. Spores of the fern, *Ceratopteris richardii* Brogn. were of an inbred diploid strain, Hn-n. Spores were surface-sterilized and then soaked in complete darkness for a period of 7 d to enhance synchronization of spore germination (Warne et al. 1986; Cooke et al. 1987).

Sowing of spores for window determination. Pre-sterilized and soaked spores were sown in a 0.5% MS (Murashige and Skoog 1962)/agarose solution onto microscope slides and then placed in a directional manner in several humid slide chambers. Each slide chamber was oriented on its side in a 29 °C lighted (35 μmol · $m^{-2} \cdot s^{-1}$) incubator. Each slide chamber was turned 180° at a designated time. Spores were viewed under a light microscope using a 10× objective for changes in development as well as to the final orientation (up or down) of the primary rhizoid, according to a method described in Edwards and Roux (1994).

Ion flux measurements during the period of polarity fixation. Ion-selective electrodes specific for Ca^{2+} and H^+ were utilized to record the net movement of these ions across the cell membrane at different points along the cell with respect to the vector of gravity. This was accomplished using the self-referencing microelectrode

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technique (Kuhtreiber and Jaffe 1990; Smith et al. 1994). Spores were placed in the medium and the relative flux was measured. The bottom of the spore was measured using a plastic holding pipette to suspend the spore in place. For calcium measurements the concentration of calcium was lowered to between 20 and 50 μM in order to enhance the capacity of the system to measure the calcium flux (Smith et al. 1994). Ion-selective electrodes were constructed from silanized glass micropipettes with a tip diameter of between 1 and 3 μm using a specific ionophore cocktail (Fluka) according to Smith and Shipley (1990). For calcium measurements the electrodes were backfilled with 100 mM $CaCl_2$ and for proton measurements the electrodes were backfilled with 100 mM KCl. These electrodes were operated with an Ag/AgCl reference electrode which completed the circuit in solution via a 3 M KCl/5% agar bridge. The electrodes were calibrated in three different concentrations of the ion of interest. The electrode rig was constructed around an Axiovert 100 (Ziess) inverted microscope fitted with a stage plate upon which the head-stage and translational motion control system were mounted. The latter consisted of Newport 310 series translation stages arranged in an orthogonal array and driven by size 23 stepper motors. This arrangement provided nano-to-millimeter resolution of movement of the head-stage in either an oscillating or static mode. Control over movement was achieved by computer control using IonView software developed at the BioCurrents Research Center (MBL, Woods Hole, Mass., USA). The entire assembly was mounted on an anti-vibration table and housed within a Faraday cage equipped with a temperature control system. Data were collected by using IonView software. Data were collected at a rate of 1000 data points per second and signal averaged into 10 values consisting of 166 data points each. Average flux was calculated from the electrode output values using the Fick equation (Porterfield et al. 1998).

Reversal of orientation. Sterilized spores were passed through a 1″ wire mesh that had pores of approximately 150 μm. Those spores that were suspended in the mesh were then transferred to Ca^{2+}-free medium and ion flux measurements were then taken at the top of the spore as described above. The same probe was then moved away from the spore, the mesh was rotated 180°, and the measurement was retaken.

Inhibitor studies. For growth polarity measurements, spores that had been sterilized and presoaked in water for 7 d in darkness were exposed to room light to initiate germination, then sown in a solution of 0.5% agarose ±100 μM nifedipine onto microscope slides as described above. Spores were observed for position of rhizoid emergence according to a method described by Edwards and Roux (1994). For ion current measurements, spores that had been sterilized and presoaked in water in darkness were irradiated and transferred to medium ±100 μM nifedipine. Those spores that had been soaked for 9.5 h in 100 μM nifedipine were rinsed in calcium-free medium several times before being measured. The relative calcium efflux of both nifedipine-treated and untreated spores was measured at 10.5 ± 0.7 h after the spores were first exposed to light.

Results and discussion

Polarized calcium movement coincides with the period of polarity fixation by gravity. We utilized a self-referencing calcium-selective electrode to record the net movement of calcium across the cell membrane at the top, side, and bottom of the spore after germination was initiated. Figure 1A shows the period of polarity fixation by gravity as well as the approximate sequence of events in the early polar development of spores of *Ceratopteris*. Figure 1B shows, during the corresponding period, the movement of calcium at the top, side, and bottom of the spore. A strong efflux of calcium was seen at the top of the spore that increased sharply at approximately 6 h after germination was initiated. The movement of calcium was seen only in the first 23 h after germination was initiated, after which it declined at all three points to low steady-state levels. There was also a calcium efflux from the sides of the spore, but it was 20-fold smaller than the top efflux. An influx of calcium was seen at the

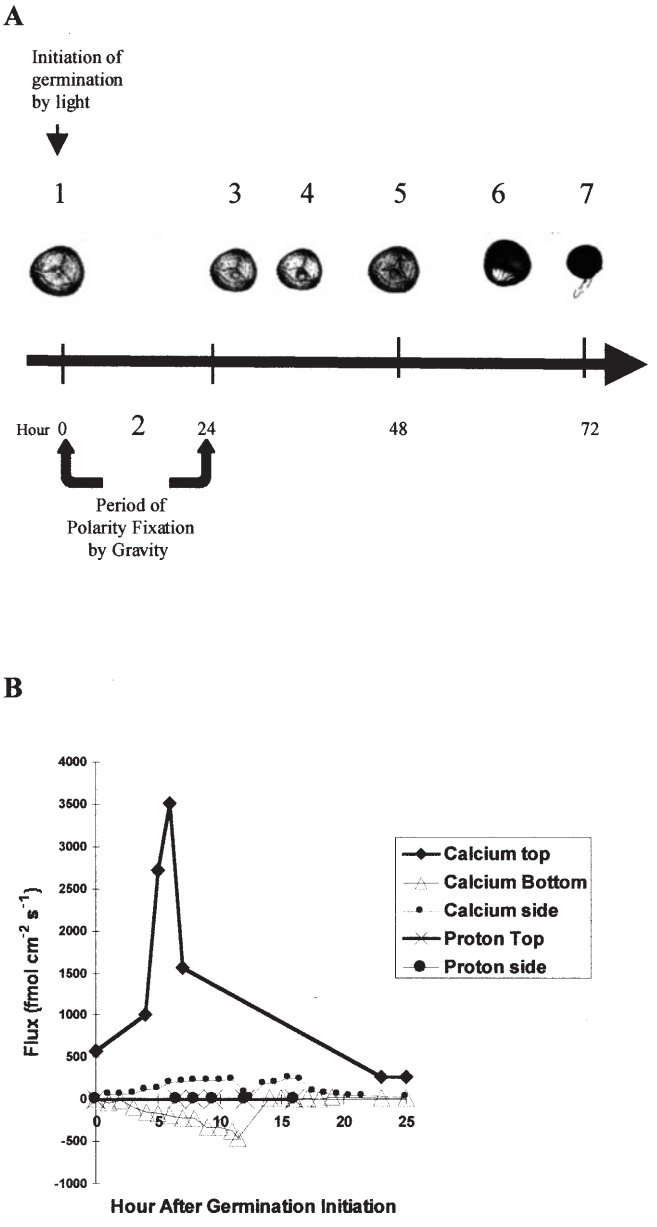

Fig. 1. A Sequence of key events in early polar development during the germination of *Ceratopteris* spores. Images shown are light micrographs at 10× magnification. Horizontal axis indicates hour after initiation of germination by light. *1*. Initiation of spore germination by light. Proximal face of single-celled spore is shown, and the nucleus is centered behind the trilete marking. *2*. Period of polarity fixation and emergence from dormancy. *3*, *4*. Nuclear migration from center to bottom of spore. *5*. Spore coat cracking. *6*. Asymmetric cell division. *7*. Rhizoid emergence downward. **B** Relative flux of calcium and proton currents in single-celled spore of *Ceratopteris* after its germination is initiated by light. Inset legend entries denote the position of the probe on the spore relative to gravity

bottom of the spore, peaking 6 h after the peak at the top. Thus the period of maximal polarization of the calcium current coincided with the period during which the developmental polarity of the spores was fixed by gravity.

This experiment was replicated for 10 different spores, with essentially similar results each time. Although there was spore-to-spore variation in the overall magnitude of the flux, the ratio of the top efflux to the side efflux was approximately the same in every spore.

Polarized movement of calcium is not paralleled by a proton current. To test the specificity of this response for calcium, we utilized a probe specific for H^+ and examined the relative flux during the same period of time. Figure 1B shows the relative flux of protons at the top and side of the spore as compared with calcium 1 h after germination was initiated. It demonstrates that there was no significant flux of H^+ as compared to the magnitude of the calcium current at this time, the relative flux for calcium being at least 10-fold greater than that for protons (Fig. 1B). The relative flux of protons at the top was similar to that seen at the side and thus these two lines overlap in the figure. The variation in the magnitude of the efflux from one figure to the next represents spore-to-spore variation. Although there was some variation in the overall magnitude, the ratio of the top efflux to the side efflux was approximately the same in every spore.

Direction of the calcium current reverses when the orientation of the cell is reversed. To verify that this polarized movement of calcium was indeed attributable to gravity and not due to the intrinsic polarity of the cell, spores were sown in a wire mesh to keep them suspended in a fixed orientation (Fig. 2). Measurements taken at the top of the spore approximately 1 h after the initiation of germination again revealed a large efflux of calcium. The wire mesh was then turned 180°, and within the 5–10 min that it took to establish a new stable recording, a new calcium polarity was established, showing the same high level of efflux at the newly positioned top of the cell (Fig. 2). Thus the polarized movement of calcium is directed by the vector of gravity.

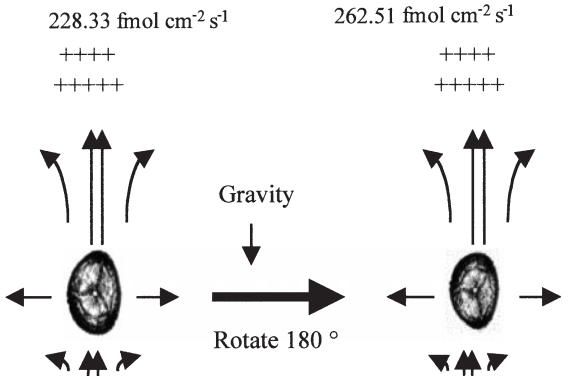

Fig. 2. Schematic diagram of gravity-induced polarity of calcium current. *Ceratopteris* spores were sown in a wire mesh and flux measurements were taken at the top of the spore approximately 1 h after germination was initiated. The mesh was flipped 180° and flux was re-measured. *Arrows* indicate direction and relative magnitude of current. Relative flux is indicated above the respective arrows

Treatment with calcium-channel blocker suppresses the gravity-directed calcium current. Figure 3 shows the relative flux of untreated spores versus nifedipine-treated spores at the top and side of the spore. The concentration of nifedipine used was the same as that used by Staves et al. (1992) to inhibit cytoplasmic streaming responses to gravity in single cells of the unicellular alga *Chara*. The relative efflux of calcium from the top of untreated spores (160 ± 22 fmol $\cdot$ cm^{-2} $\cdot$ sec^{-1}) was 40× greater than from the top of nifedipine-treated spores (4.5 ± 5.6 fmol $\cdot$ cm^{-2} $\cdot$ sec^{-1}), and the efflux was 15× greater from the sides of untreated (33 ± 4.9 fmol $\cdot$ cm^{-2} $\cdot$ sec^{-1}) versus treated spores (2.5 ± 3.5 fmol $\cdot$ cm^{-2} $\cdot$ sec^{-1}). Addition of nifedipine almost completely suppressed the calcium efflux.

Treatment with calcium-channel blocker disrupts gravity-directed developmental polarity. The effect of nifedipine on the ability of gravity to direct the developmental polarity of the spores was measured by recording the direction of the emergence and growth of the treated cells' primary rhizoids (Table 1). The criteria for evaluating the oriented growth response of rhizoids are described in Edwards and Roux (1994). Of the nifedipine-treated spores, only 42% showed gravity-oriented polarity downward versus 92% of the untreated spores.

Fig. 3. Relative flux of calcium after treatment with nifedipine. Flux was measured at the top and side of *Ceratopteris* spores treated with medium alone or medium + nifedipine at approximately 10.5 h after germination was initiated. Data shown represent the mean ± SD for $n = 7$ measurements

Table 1. Nifedipine blocks the ability of gravity to orient the direction of rhizoid growth and inhibits rhizoid formation in germinating *Ceratopteris* spores

Sample[a]	% of primary rhizoids growing		% of germinated spores with no rhizoid
	Upward	Downward	
Untreated	8	92	0
+ Nifedipine[b]	25	42	33

[a] $n = 50$ for treated and untreated samples, respectively
[b] [nifedipine] = 100 μM

Of the nifedipine treated spores, 25% did not orient with respect to the vector of gravity and grew upward versus 8% of the untreated spores. It is noteworthy that 33% of the spores treated with nifedipine actually showed no rhizoid at all, and of these, 13% had a prothallus which grew downward, a phenotype not seen in untreated spores (data not shown). Statistical analyses of the data in Table 1 were done by a χ^2 test of independence utilizing a contingency table, and they support the conclusions that polarized development differs depending on the type of treatment (nifedipine vs. no treatment; $P < 0.001$), and that the percentage of rhizoids growing downward in treated cells is not significantly different from random ($P < 0.05$). The nifedipine experiments were repeated and yielded results that were statistically evaluated as not significantly different from the first set of results.

Ion currents also influence polarity in Fucales. Other plants in which polarized development in single cells has been well characterized are the brown algae *Fucus* and *Pelvetia*, members of the order Fucales. In these organisms, fertilization initiates polarization process, and the sperm entry point defines the developmental axis of the embryo. The first division of the zygote, similar to the first division in *Ceratopteris* spores, is asymmetric, with the smaller cell giving rise to the rhizoid and the larger cell giving rise to the thallus (Kropf 1997). For this polarized development, ion currents appear to have an important role. Several hours after fertilization, gradients of H^+ and Ca^{2+} are detected at the presumptive rhizoid pole. Unlike the case in *Ceratopteris*, protons appear to play as important a role as calcium in polarity development, with both currents believed to help stabilize the pole where the rhizoid will be formed.

Summary and conclusion. The results reported here demonstrate that during the first day after *Ceratopteris* spores are induced to germinate, the movement of calcium out of the cells is polarized and is strongest in a direction that opposes the vector of gravity. The magnitude of the calcium current is highest during the period of polarity fixation by gravity, and the direction of the current is dictated by gravity, for it reverses rapidly when the cells are turned upside down. The current is not indicative of a generic ion movement for there is no H^+ current of a comparable magnitude during the same period of time. These results are consistent with the conclusion that while gravity is fixing the polarity of the cells, it is also activating calcium pumps along the top and sides of the cell and inducing calcium channels to open along the bottom, resulting in a calcium current that moves primarily from the bottom to the top of the cell. Because the efflux from the top and sides is much greater than the influx from the bottom, some release of internal calcium stores probably participates in sustaining the current.

In the presence of the calcium-channel blocker, nifedipine, the calcium current at the top and sides of the spores is significantly diminished. This suggests that the calcium transport disrupted by nifedipine is needed to sustain the current. Because this polarized current develops so rapidly after reorientation, it is probably one of the earliest cell-level responses induced by gravity and plays a key role in guiding subsequent polar events. Because nifedipine disrupts both the polarizing influence of gravity and normal rhizoid development, it is likely that calcium channels participate in the regulation of these two events. To our knowledge, this is the first evidence that calcium currents help regulate the response to gravity in single cells. Further studies are needed to define more specifically the role of calcium in the perception/response pathway leading to gravity-directed development in *Ceratopteris* spores.

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References

Chasan R (1992) *Ceratopteris*: A model plant for the 90's. Plant Cell 2: 113–115

Cooke T, Racusen R, Hickok L, Warne T (1987) The photocontrol of spore germination in the photocontrol of spore germination in the fern *Ceratopteris richardii*. Plant Cell Physiol 28: 753–759

Edwards ES, Roux SJ (1994) Limited period of graviresponsiveness in germinating spores of *Ceratopteris richardii*. Planta 195: 150–152

Edwards ES, Roux SJ (1998) Influence of gravity and light on the developmental polarity of *Ceratopteris richardii* fern spores. Planta 205: 553–560

Hickok L, Warne T, Slocum M (1987) *Ceratopteris richardii*: applications for experimental plant biology. Am J Bot 74: 1304–1316

Kropf DL (1997) Induction of polarity in fucoid zygotes. Plant Cell 9: 1011–1020

Kuhtreiber WM, Jaffe LF (1990) Detection of extracellular calcium gradients with a calcium-specific vibrating electrode. J Cell Biol 110: 1565–1573

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473–497

Porterfield DM, Trimarchi J, Keefe DL, Smith PJS (1998) Metabolism and calcium homeostasis during development of the mouse embryo to the blastocyst stage in M2 culture medium. Biol Bull 195: 208–209

Roux SJ (1995) Assessing potential targets of calcium action in light-modulated gravitropism. ASGSB Bull 9: 83–92

Smith PJS, Sanger R, Jaffe L (1994) The vibrating Ca^{++} electrode: a new technique for detecting plasma membrane regions of Ca^{++} influx and efflux. Methods Cell Biol 40: 115–134

Smith PJ, Shipley A (1990) Regional variation in the current flow across an insect blood-brain barrier. J Exp Biol 154: 371–382

Staves MP, Wayne R, Leopold AC (1992) Hydrostatic pressure mimics gravitational pressure in characean cells. Protoplasma 168: 141–152

Warne T, Walker G, Hickok L (1986) A novel method for surface sterilizing and sowing fern spores. Am Fern J 76: 187–188