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Limited period of graviresponsiveness in germinating spores of *Ceratopteris richardii*

Erin S. Edwards[1], **Stanley J. Roux**[2*]

[1] Department of Botany, The University of Texas at Austin, Austin, TX 78713, USA
[2] Department of Plant Physiology, Wageningen Agricultural University, 6703 BD Wageningen, The Netherlands

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Abstract. Rhizoids of the fern *Ceratopteris richardii* Brogn. usually emerge 40 h after germination is initiated by light, and more than 90% of them emerge growing in a downward direction. However, when the spores are germinated on a clinostat, the emerging rhizoids show no preferential orientation. This indicates that under normal $1 \cdot g$ conditions the initial growth direction of rhizoids can be oriented by gravity. If the orientation of the spores is changed 3 h or less after the start of germination, the growth direction of most emerging rhizoids becomes downward relative to the new orientation. However, if the orientation of the spores is changed by 180° 8 h or more after germination is initiated by light, most rhizoids emerge growing upward; i.e., the same direction as if there had been no orientation change. Emerged rhizoids also do not change their direction of growth if their orientation is changed. These results indicate that the growth direction of emerging rhizoids is set by gravity prior to actual emergence, and that the time of full orientation responsiveness is limited to a period ranging from the initiation of germination to about 3–4 h after the start of germination. There is a gravity-oriented nuclear movement beginning at about 13 h after germination, and this movement appears to predict the initial growth direction of rhizoids.

Key words: Cell polarity – *Ceratopteris* – Fern gametophyte – Gravity – Nuclear migration – Rhizoid

Ceratopteris richardii Brogn. is a homosporous fern that is endemic to pantropical regions. Both the gametophyte and sporophyte can grow on land or submerged in water. Hickok et al. (1987) have described the distinct advantages of using this fern as a model for studying growth and development in plants, and more recently Chasan (1992) has echoed this theme. When germination is induced in *C. richardii*, the nucleus migrates in the spore to allow for an asymmetric division. This division produces two cells, one giving rise to the primary rhizoid, and the other giving rise to the protonema. So, this simple cellular division not only produces two cells, but it also creates two different cell types, or differentiation of cells. The movement of the nucleus and subsequent asymmetric division are evidence of spore polarity, and the factors controlling this polarity are not fully understood. Here we report that in *C. richardii*, the direction of spore nucleus migration and of rhizoid growth when it emerges from the spore is controlled in large part by gravity.

Spores and spore preparation. Spores of the fern *Ceratopteris richardii* Brogn., a gift from Leslie Hickok at the University of Tennessee, are of an inbred diploid strain designated Hn-n. Spores were surface-sterilized by a method adapted from Warne et al. (1986). After the spores were soaked 3 min in 0.875% sodium hypochlorite [1 part bleach (5.25% sodium hypochlorite) to 5 parts distilled water], they were rinsed three times in sterile deionized water. To enhance synchronization of spore germination, spores were then soaked in complete darkness for 6 d. Germination requires light-activated phytochrome (Cooke et al. 1987).

Culture conditions for fixed-orientation, clinostat, and orientation-change experiments. Most of the water used for soaking was removed from the spore suspension and the spores were resuspended in warm media solidified with 0.5% agarose to a concentration of 25–30 spores per drop of a Pasteur pipet. The media contained 4.4% Murashige and Skoog Basal Salt Mixture, 0.3 mM $MgSO_4$, 2.2 mM KH_2PO_4, 0.1 mM $FeSO_4.7H_2O$, and 0.1 mM Na_2EDTA adjusted to pH 6.5. The suspended spores were sown onto the surface of sterile microscope slides.

For fixed-orientation and orientation-change experiments, slides were placed vertically in clear plastic staining jars that had been sterilized by rinsing them in 50% bleach solution containing one drop per 100 ml Tween-20 (BioRad, Hercules, Cal., USA) then rinsing them three times with sterile deionized water. To decrease evaporation and still allow for air circulation, 2 ml sterile distilled water covered the bottom of each jar, half of the jar lid was sealed with Parafilm, and the jar was loosely wrapped in a clear plastic bag. Hours of germination were measured from the first light exposure of spores. Sowing was complete in about 25 min and jars were placed in an incubator at 30–31° C with continuous white light illumination. Because the germinating spores were in clear chambers, they received some illumination from all directions. The source of illumination was located at the side of the chamber, and so the direction of illumination was perpendicular to the direction of gravity at all times, whether the spores remained in one orientation or were rotated by 180°.

For clinostat experiments, the slides were taped to the inside of sterile Petri dishes (100 mm diameter, 15 mm deep) on top of a wet

* *Permanent address:* Department of Botany, The University of Texas at Austin, Austin, TX 78713, USA

Correspondence to: Stanley J. Roux; FAX: 1(512)471-3878; E-mail: sjrx@hpcf.cc.utexas.edu

Kimwipe (Kimberley-Clark; Roswell, Ga., USA), lids were partially sealed with Parafilm and the petri dishes were wrapped loosely in clear plastic bags. The clinostat rotated at 2 rpm, stationary controls were placed vertically, and the spores were incubated at 30–31° C.

Culture conditions for prolonged time-lapse observations. Lab-Tek Chamber Slide Culture Chambers (Nunc Inc., Naperville, Ill., USA) were filled almost completely with media solidified with 0.8% agarose. Just before the agarose solidified completely, spores suspended in sterile distilled water were sown on the surface and the water removed so that the spores could adhere to the agarose. To eliminate the problem of water condensation on the top coverslip which would prevent viewing, as much sterile distilled water as possible was then added and a coverslip sealed to the chamber with Parafilm. When the chamber was vertical, the water filled half the remaining space in the chamber. The spores viewed were therefore submerged. A vertical microscope was converted to a horizontal microscope and the video camera was focused on submerged spores. Images collected through the video camera were digitized and stored by a software program (RasterOps MediaGrabber; RasterOps Corp., Santa Clara, Cal., USA) every 30 min. Temperature could not be closely controlled, but room temperature averaged approx. 22° C.

Criteria for evaluating the oriented growth response of rhizoids. About 20 h before rhizoid emergence, the spore cracks open at the laesura, a trilete marking on the proximal face of the spore. The rhizoid emerges through the split laesura. To evaluate the direction or orientation of rhizoid growth when it emerged, the following criteria were used. Rhizoids emerging below the mid-line of the spore and not growing up past the mid-line of the spore were designated as growing down in response to gravity. Rhizoids which emerged above the mid-line and did not grow down below the mid-line and rhizoids which emerged on level with the mid-line and grew straight out to the side were designated non-gravity responsive. The rhizoids emerging from spores which had their laesura on the side or top would have to grow for a short time before it could be determined whether they would bend "downward" after their emergence (see Fig. 1), and so these rhizoids were counted only after they had grown more than approx. 20 μm.

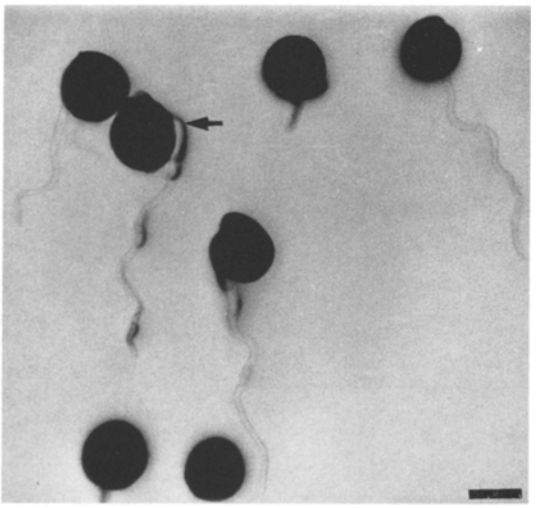

Fig. 1. Germinating spores of the fern *Ceratopteris richardii* showing primary rhizoids growing down with respect to gravity. *Arrow* indicates a spore which has its laesura positioned at the top of the spore, and shows the emerging rhizoid curving downward. ×68; bar = 100 μm

Table 1. Direction of growth of *C. richardii* rhizoids when spores are germinated on a clinostat. An average of 150 spores was assayed for each data set

	Clinostat.		Control	
	% direction 1	% direction 2	% down	% up
Experiment 1	52	48	88	12
Experiment 2	58	42	99	1
	55	45	96	4
Mean ± SD	55 ± 2.0	45 ± 2.0	94 ± 4.3	6 ± 4.3

Direction of rhizoid growth when spores are germinated with fixed orientation. Using the criteria for orientation determination described in *Materials and methods*, over 90% of the rhizoids grew in the direction of down in response to gravity (Fig. 1). Because the illumination source during germination and early growth was perpendicular to the direction of gravity, light could not have influenced the rhizoids to grow preferentially up or down. Whether the 10% or so of spores that did not clearly show a gravity response are truly agravitropic cannot be ascertained by the data collected. Gametophytes from these spores have been selfed and the spores from resulting sporophytes will have to be tested to determine if the apparently agravitropic phenotype is heritable.

Clinostat germination. To help determine if the oriented growth observed was the result of a response to gravity, spores were germinated on a clinostat rotating continually at 2 rpm so that the direction of gravity was continually changing. For clinostat germination, opposite directions, 1 and 2, were arbitrarily assigned for counting purposes (since there is no up or down on a clinostat) and the same parameters, previously described, were used to determine the direction of growth. Since the measurement categories were opposite directions, rather than graviresponsive and non-graviresponsive, rhizoids emerging on line with the mid-point and growing straight to the side could not be classified in either category and so were not counted. Rhizoids of spores germinated on a clinostat grew about 55% in one direction and about 45% in the other direction, as opposed to 94% down and 6% up for their stationary controls (Table 1). This distribution near 50:50 shows an approximately random orientation for spores germinated on the clinostat, while spores germinated under stationary conditions show oriented growth.

Effects of orientation change at different time periods. Spores were soaked for 6 d in the dark to enhance the synchronization of their germination. After this dark incubation they were repositioned randomly when they were sown in the light. Apparently the initial direction of rhizoid growth is not set during this dark preincubation period. If it had been set, then the rhizoids growing out from the spores sown in the light would have emerged growing in random directions, since the spores were repositioned during sowing. However, over 90% of the rhizoids emerged growing downward. Because this downward orientation is fixed only after the spores are sown in the light, the same light signal that initiates germination must also induce a condition that allows the initial direction of rhizoid growth to be fixed by gravity.

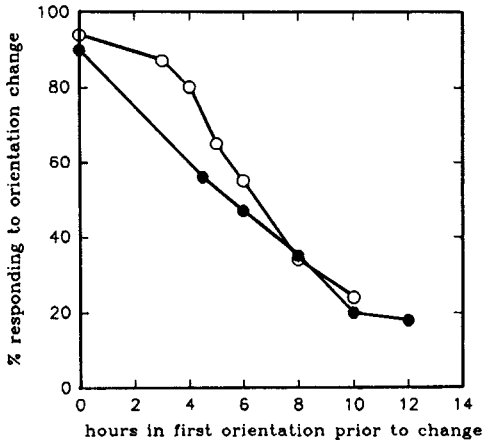

Fig. 2. Two experiments showing a decreasing response of *C. richardii* rhizoids to orientation change with increasing time after induction of spore germination by light. Time = 0 represents spores whose orientation has not been changed. An average of 150 spores was assayed for each datum point given

The direction of rhizoid growth seems to be determined irreversibly several hours before spore cracking and rhizoid emergence. When the orientation of germinating spores was changed 180° at different time periods, not all of the spores responded to the orientation change. For example, in one set of data from an experiment in which the orientation was changed 6 h after the first light exposure (Fig. 2), only 55% of the spores responded, and rhizoids grew in the direction of the second, or new, down. That is 39% less than grew down when their orientation was not changed, i.e. 39% had already set their direction of down before their orientation was changed. The percentage of spores which responded to that change decreased with increasing time after light initiated the germination process; i.e., with increasing time an increasing number of spores have set their gravity direction and have ceased responding to gravity. Determination of down begins to occur well before spore cracking, which does not begin to occur until around 20–25 h at this temperature, and rhizoid emergence, around 40 h. It is also substantially long before nuclear movement, which begins to occur around 13–18 h. After about 12 h, no reorientation of rhizoids toward a new direction of down occurs whether the orientation is changed before or after rhizoid emergence.

Nuclear migration. The nuclear migration seen in germinating *C. richardii* spores indicates that gravity has had an effect on spore polarity. The nucleus is visible through the transparent spore coat as a clear area in the intact spore. The direction which the rhizoid will grow when it emerges is predicted by a prior movement of the nucleus. Before germination induction and for about a dozen or so hours after induction, the nucleus is centered behind the laesura. After the direction of rhizoid emergence has been set, the nucleus migrates from its central location to the bottom perimeter of the laesura. This movement of the nucleus is the first visible response to gravity that we have been able to detect in unstained spores using the light microscope. Higher-resolution studies may reveal precedent orientation changes, for the displacement movement of the nucleus cannot be the initiator of the gravity response, which is set prior to the movement.

Nuclear migration determines the position of the first asymmetric division, which determines the differentiation of the primary rhizoid and protonema. To the extent that the polarity of this division is determined by the gravity-directed migration of the nucleus, gravity is regulating developmental potential as well as oriented growth in germinating spores of *C. richardii.*

In summary, the results described above indicate that the initial direction of growth of the rhizoid is set by gravity prior to actual emergence, and that the time of full orientation responsiveness to 1 *g* of a population of spores is limited to a period of only about the first 5 h after the start of germination. To our knowledge, this is the only report of a gravity effect being observed in fern spores. The only other report of a gravity response in a fern gametophyte generation is that of Bloom and Nichols (1972), who showed that gravity affected the direction of growth of rhizoids emerging from the multicellular megagametophyte of *Marsilea.* The oriented growth response in germinating *C. richardii* spores recommend this system as one with outstanding potential for revealing fundamental aspects of gravity-initiated signal transduction. In these spores the entire signalling pathway from stimulus perception to the final response of fixing the direction of rhizoid emergence occurs during a specified period of time after germination has been initiated by activated phytochrome. Once this period is over, spore graviresponsiveness is apparently lost. Even the emerged rhizoid is not gravitropic. By contrasting the cytological, biochemical and genetic events that occur in spores just before and just after their period of graviresponsiveness, one should be able to identify key events in the stimulus-response pathway. Such results would create a valuable new information base for understanding gravity responses in single cells, and for formulating new testable hypotheses for explaining how oriented growth responses occur in more complicated systems.

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