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**Physiological and Bioinformatic Studies on Polarity Development
in *Ceratopteris richardii* Spores**

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**Physiological and Bioinformatic Studies on Polarity Development
in *Ceratopteris richardii* Spores**

by

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CHAPTER 1

INTRODUCTION

The establishment and orientation of cell polarity have significant developmental and physiological consequences in nearly all multicellular organisms. A cell's alignment to its surroundings, either to other cells in a tissue or to the environment, has obvious implications for its success. Consequently, the orientation of the polar axis is often responsive to positional or environmental signals. While a rudimentary framework of common mechanisms involved in establishing polarity has been identified, the molecular mechanisms connecting these external cues to axis alignment remain largely unknown.

In plants and algae, polarity establishment is integral to two types of growth and development: asymmetric cell division and polar growth. Polarization in single celled systems leading to an asymmetric cell division has been studied in the zygotes of *Arabidopsis thaliana* and two species of brown algae, *Fucus distichus* and *Pelvetia compressa*, as well as the haploid microspores of *Arabidopsis* and spores of the fern *Ceratopteris richardii* (reviewed by Scheres and Benfey, 1999; Cove, 2000; Souter and Lindsey, 2000). Polar growth, or tip growth, has been best characterized through investigations of pollen tube and root hair elongation in a variety of higher plant species (reviewed by Hepler et al., 2001).

Presumably, both asymmetric cell division and tip growth ultimately rely upon the polar or asymmetric distribution of proteins or other cellular components in order to establish the polar axis. A recent series of investigations into the localization of auxin influx and efflux transporters in *Arabidopsis* has provided clues regarding the cellular systems involved in establishing and maintaining polar protein localization in plants (reviewed by Friml and Palme, 2002).

Molecular genetic approaches, primarily in *Arabidopsis*, have begun to elucidate more detailed molecular mechanisms for polarity establishment and maintenance in the contexts of protein localization, asymmetric cell division, and polar growth. Despite there being a wealth of physiological information, a lack of molecular genetic tools has hampered detailed, molecular level investigations of polarity development in the single cell systems *Fucus*, *Pelvetia*, and *Ceratopteris*. However, because axis alignment in these systems is responsive to environmental signals like light and gravity, they offer a unique perspective on the perception of physical signals and the transduction of those signals into biological signals directing subsequent growth and development—all within a single cell.

MECHANISMS OF POLARITY ESTABLISHMENT AND MAINTENANCE

Across various external signals in a number of plant model systems, a common set of cellular processes or components has been identified to play a role in polarity establishment and maintenance. These include calcium ion channels, the actin cytoskeleton, and endocytic cycling (reviewed by Grebe et al., 2001).

Similar processes are also recognized to be important for polarization in other systems including yeast, nematode and fly (reviewed by Nelson, 2003; Roegiers and Jan, 2004).

The cytoskeleton, calcium, and secretion are involved in algal zygote polarization

In the zygotes of the furoid algae *Fucus* and *Pelvetia*, establishment of the rhizoid – thallus axis results in the initiation of polar growth at the presumptive rhizoid pole followed by an asymmetric cell division perpendicular to the initial growth axis. Although light is the primary signal guiding axis alignment in these systems, the position of sperm entry during fertilization provides the initial symmetry breaking cue for the zygote. A cortical filamentous actin (F-actin) patch marking the site of sperm entry is formed rapidly after fertilization, and without any subsequent orienting signal, this F-actin patch marks the future rhizoid pole (Hable and Kropf, 2000). Exposure to unilateral light, however, results in the reorientation of this default axis alignment, such that the rhizoid pole is localized to the shaded half of the zygote and the thallus is oriented toward the light vector. This realignment, or photopolarization, involves the disassembly of the original cortical F-actin patch and reassembly of a new actin patch at the new rhizoid pole (Alessa and Kropf, 1999).

F-actin may serve as scaffolding for the subsequent assembly of a polarity signaling complex of additional proteins that maintain and fix the orientation of

the growth axis. Fluorescently labeled dihydropyridine (DHP), which binds to L-type Ca^{2+} channels in mammalian systems, has been used to label putative calcium channels (DHP receptors) in *Fucus*. During photopolarization, labeled DHP receptors gradually localize to the actin patch at the rhizoid pole, and their localization coincides with an increase in intracellular Ca^{2+} concentration at the rhizoid pole. Because treatment with various actin filament destabilizers prevents localization of the receptors and Ca^{2+} gradient formation, these receptors may rely on the F-actin patch for their positioning (Shaw and Quatrano, 1996a; Pu et al., 2000).

Eventually, the zygotes are no longer responsive to changes in the orientation of the light vector, indicating that the orientation of the rhizoid – thallus axis becomes fixed during development. A number of treatments, including transient applications of actin filament destabilizers and brefeldin-A (BFA), an inhibitor of Golgi mediated secretion, extend the period of time that the growth axis is responsive to changes in the orientation of the light vector, indicating that these treatments prevent axis fixation (Shaw and Quatrano, 1996b; Love et al., 1997; Hable and Kropf, 1998). That BFA does not prevent localization of DHP receptors or F-actin suggests that Golgi mediated secretion is a key step in axis fixation and that actin polymerization mediates axis fixation upstream or independently of the secretion step.

Rop-GTPases mediate polar growth through regulation of the actin cytoskeleton

Small GTPases are involved in the development of polarity in a variety of systems (Pruyne and Bretscher, 2000; Wedlich-Soldner et al., 2003; Nelson, 2003; Etienne-Manneville and Hall, 2004). Although plants do not have any of the Rho GTPase subfamilies typically found in other eukaryotes, another Rho subfamily unique to plants, the Rop-GTPases, appears to play an important role in mediating cell expansion. Initially touted as a “master switch” of cell polarity (Fu and Yang, 2001), members of the Rop family localize to the tips of pollen tubes and root hairs where they regulate the formation of cortical F-actin (Fu et al., 2001; Molendijk et al., 2001; Jones et al., 2002). Rop proteins also localize to the sites of root hair initiation in a BFA-dependent but actin-independent manner prior to bulging of the root hair from the trichoblast (Molendijk et al., 2001). Disruption of Rop signaling in pollen tubes also disrupts the calcium influx at the pollen tube tip (Li et al., 1999), which is well known to be required for pollen tube growth (Pierson et al., 1994; Malho and Trewavas, 1996; Pierson et al., 1996).

As has been demonstrated in the fucoid algae (Shaw and Quatrano, 1996a; Pu et al., 2000), altered F-actin dynamics, due in this case to the manipulations of Rop signaling, could inhibit the tip localized calcium influx in pollen. Although this remains to be tested, actin depolymerization is known to inhibit pollen

germination and pollen tube growth (Gibbon et al., 1999). More recently, Rop-GTPases have been shown to also mediate initial cell expansion by regulating F-actin dynamics in a variety of additional cell types, including leaf epidermal cells (Fu and Yang, 2002). Consequently, it appears that in plants, as in most eukaryotes, small GTPases can regulate the actin cytoskeleton (reviewed by Hall, 1998; Mullins, 2000). However, precisely how cortical F-actin, downstream of Rop activity, induces cell expansion in plants, and the involvement of calcium in this process, is not clearly understood. Moreover, it should be clear that upstream regulators of Rop-GTPase activity and localization may be more determinative players in establishing cellular polarity.

Asymmetric distribution of auxin transporters requires cycling to the membrane

The polar localization of auxin transport proteins is essential for the normal transport of auxin from the shoot into the root. Additionally, in response to changes in shoot or root orientation with respect to light or gravity vectors, auxin is asymmetrically redistributed to modulate rates of cell elongation, thereby enabling the differential growth response to light and gravity signals (Muday, 2001).

Investigations into the localization mechanisms of the auxin transport regulators, the PIN proteins, have underscored the importance of actin dependent vesicle trafficking in maintaining the proper polar localization of these proteins. The auxin transport regulator PIN1, exhibits a striking basal localization in the

vascular cells of *Arabidopsis* that is important for maintaining basipetal auxin transport (Galweiler et al., 1998). Mutants defective in a membrane associated guanine-nucleotide exchange factor on ADP-ribosylation factor G protein (ARF GEF), GNOM, fail to properly localize PIN1, and show altered embryonic developmental patterns, including loss of the apical-basal axis. BFA, which is known to block auxin efflux, inhibits the GTP-GDP exchange activity of GNOM, and BFA treated lateral roots also fail to maintain a polar localization of PIN1 (Steinmann et al., 1999). ARFs are known to be involved in membrane trafficking from the Golgi, and it appears that GNOM regulates vesicle trafficking necessary for the polar localization of PIN1 (Steinmann et al., 1999; Geldner et al., 2002).

Further investigation revealed that PIN1 rapidly cycles between the plasma membrane and endosomal compartments in an actin-dependent manner (Geldner et al., 2001). Additionally, a number of polar auxin transport inhibitors were shown to broadly block membrane trafficking rather than having specific effects on auxin transporters, further highlighting the importance of efflux carrier cycling for polar auxin transport. Rapid cycling, endocytosis of plasma membrane proteins and their subsequent localized return to the membrane, counteracts the potential loss of localization due to protein lateral diffusion in the membrane. Endocytic recycling has been demonstrated to be a sufficient mechanism to maintain the polar localization of plasma membrane proteins in

yeast and is hypothesized to be particularly effective for proteins with transient or variable polarity (Valdez-Taubas and Pelham, 2003).

Because the direction of auxin transport must be responsive to gravity and light signals, proteins in the auxin transport machinery may be expected to be able to change their localization within the cell. PIN3, which is expressed in root columella cells and rapidly cycles between the plasma membrane and endosome, relocates laterally in response to gravistimulation, providing a possible mechanism for auxin redistribution (Friml et al., 2002). Similarly, PIN1 becomes delocalized from the basal plasma membrane in hypocotyl cells during the tropic bending towards blue light (Blakeslee et al., 2004). However, the mechanisms that allow and direct PIN3 and PIN1 redistribution are wholly unknown.

GRAVITY PERCEPTION AND SIGNAL TRANSDUCTION

Even given these common components of polarity development and maintenance, the specific mechanisms that connect them to gravity signal perception are not known at the molecular level. Two models, the starch – statolith and the gravitational pressure models, have been advanced to describe the mechanism of gravity perception (reviewed by Kiss, 2000).

In the starch – statolith model, the sedimentation of dense organelles, such as the starch filled plastids of the root columella cells, signals the direction of the vector of gravity. Conversely, in the gravitational pressure model, sedimentation of the entire protoplast within the extracellular matrix (ECM) generates opposing

tension and compression forces between the plasma membrane and ECM at the top and bottom of the cell, respectively. These forces are then transduced into a biological signal orienting the cell. The two theories are not necessarily mutually exclusive. Because of the constant evolutionary presence of gravity, redundant gravity sensing systems may exist. In plants that have evolved relatively recently, “primitive” gravity sensing mechanisms such as that described by the gravitational – pressure model, may coexist with or be refined by more recently evolved gravity sensing mechanisms involving statoliths (Barlow, 1995).

Starch-statolith hypothesis

The starch-statolith hypothesis has been most extensively studied through experiments with the amyloplast-containing columella cells of the root tip. Upon reorientation of the root tip, the amyloplasts sediment towards the new bottom of the columella cell, signaling the new orientation to the plant. The movement of these amyloplasts is central to the root gravitropic response. Starchless and starch-deficient mutants, as well as root tips with laser-ablated columella cells, show severely reduced yet residual gravity responses (Kiss et al., 1989, 1996; Blancaflor et al., 1998). Additionally, manipulations of the position of statoliths in other systems induce the expected gravitropic responses (Kuznetsov et al., 1999; Weise et al., 2000; Braun, 2002).

What remains almost entirely unclear, however, is how statolith movement is translated into the other known downstream gravity responses, like

relocalization of the auxin transporters. Yoder et al. (2001) proposed that amyloplast sedimentation in columella cells disrupts the actin cytoskeleton, and that the resulting altered tension distribution within the cytoskeletal network spatially propagates the gravity signal to mechanosensitive ion channels. However, the actin cytoskeleton has recently been shown to be unnecessary for the initial perception of gravity in *Arabidopsis* roots. Depolymerization of actin with Latrunculin B led to an enhanced gravitropic response, suggesting that actin may be an essential component for the downregulation of gravity signaling (Hou et al., 2004).

Gravitational pressure model

The gravitational pressure model has been explicitly tested in three varied systems (algal internodal cells, rice roots, and moss protonemata) with conflicting results. Because the buoyancy of the protoplast relies upon the difference in density between the protoplast and the surrounding medium, varying the density of the external medium can alter the buoyancy of the protoplast within the ECM. In both *Chara* internodal cells and rice roots, gravity responses are dampened or reversed by raising the density of the external medium (Staves et al., 1997a; Staves et al., 1997b). However, in similar experiments with the moss *Ceratodon purpureus*, gravitropic curvature of the moss continued even at external densities greater than the density of the protoplast (Schwuchow et al., 2002). In both rice and *Ceratodon*, amyloplasts continue to sediment within the gravity sensing

tissue, but the opposing outcomes make it difficult to reach a broad conclusion about the gravitational pressure model.

GRAVITY DIRECTED POLARITY DEVELOPMENT IN *CERATOPTERIS RICHARDII*

The spores of the semi-aquatic fern *Ceratopteris richardii* are a useful model system for studying gravity perception and response (Chatterjee and Roux, 2000). Spore germination is triggered by light, and subsequent polarity development is directed by the vector of gravity during a limited window between 3 to 12 hours after initiation of germination (Edwards and Roux, 1994). To date, there are four events that indicate polarity development in the spore: a polar calcium current, downward nuclear migration, polar asymmetric cell division, and downward rhizoid emergence.

During the first 24 hours after light exposure, a significant calcium efflux is found at the top of the spore, opposite the vector of gravity, and a 20-fold smaller influx is found at the bottom of the spore. This calcium current peaks very strongly between four and seven hours into the germination sequence, concurrent with the polarity fixation window. Moreover, rotating the spore 180° before polarity fixation, results in the reorientation of the calcium current to the new correct orientation within five to ten minutes (Chatterjee et al., 2000). Subsequent to polarity fixation, the nucleus of the spore migrates downward, and the cell divides asymmetrically. The upper cell develops into the prothallus, while the lower gives rise to the primary rhizoid. Finally, in greater than 90% of

the spores, the rhizoid emerges from the spore in the down direction around 72 hours after light induction of germination.

Asymmetric cell divisions are typically established by asymmetric positioning of the nucleus (Sheres and Benfey, 1999). In clinostat experiments with *Ceratopteris*, although the direction of rhizoid emergence is random, it is typically predicted by the direction of nuclear migration (Edwards and Roux, 1998a). Spores germinated in microgravity also show a random orientation of rhizoid emergence, indicating that although gravity directs spore orientation, it is not required for polarity development (Roux et al., 2003).

Several pieces of evidence suggest the calcium current is a critical step in polarity fixation in the *Ceratopteris* system. Calcium signaling is a well-known part of many stimulus-response pathways and has often been implicated in gravitropism (Sinclair and Trewavas, 1997). More convincingly, calcium influxes marking cell polarity and subsequent asymmetric cell division are also found in zygotes of *Fucus* and *Pelvetia* (Roberts and Brownlee, 1995; Shaw and Quatrano, 1996). In *Ceratopteris*, as mentioned above, the calcium current peaks during the period of axis alignment in the spore system. Additionally, treatment of the spores with a calcium channel blocker significantly diminishes both the calcium current and the ability of the spore to reorient correctly to gravity (Chatterjee et al., 2000). These observations indicate that intact calcium signaling and/or homeostasis is required for polarity development in *Ceratopteris*.

SUMMARY

Given the role for calcium signaling in many plant polarization processes including the *Ceratopteris* gravity response, this dissertation uses bioinformatic, molecular, and physiological techniques to further investigate the involvement of calcium in gravity directed polarity development in *Ceratopteris*.

Because the *Ceratopteris* system has been limited by a lack of molecular-genetic tools, a secondary goal for this dissertation was the development of a system allowing the manipulation of selected genes in a reverse genetic manner. To that end, a collection of *Ceratopteris* ESTs were analyzed to identify the genes expressed during polarity development, and three genes involved in calcium signaling were selected for testing of an RNA interference (RNAi) approach that had been previously been applied to germinating fern spores (Klink and Wolniak, 2000; Klink and Wolniak, 2001; Tsai and Wolniak, 2001).

We also employed physiological approaches to complement the expected development of a molecular-genetic manipulation technique. We used a self-referencing ion-selective microelectrode to further characterize the mechanism that generates the basal-to-apical polar calcium current during polarity fixation in *Ceratopteris*. Specifically, we attempted to answer the question of how the calcium current reorients by minimizing the timeframe in which we were able to measure the calcium current after reorientation of the spore, reasoning that if the current were able to reorient faster than some threshold, we could eliminate

cellular mechanisms which would require a longer timeframe than that threshold. These studies have added to our resources for *Ceratopteris* research and have advanced our understanding of a single celled system in which the alignment of its polar axis is governed by a pervasive external signal, gravity.

CHAPTER 2

RAPID REORIENTATION OF A GRAVITY DIRECTED CALCIUM CURRENT

INTRODUCTION

Previous reports from this lab have described a polar bottom-to-top calcium current present during approximately the first 20 h after initiation of spore germination by light (Chatterjee et al., 2000). Several lines of evidence suggest that the calcium current is important to polarity development in *Ceratopteris*. The current is the first detectable response in *Ceratopteris* of gravity perception and response and is able to reorient in response to changes in the orientation of the spore relative to the vector of gravity. The current magnitude, particularly the efflux from the top of the spore, peaks strongly between 5 and 10 h after induction of germination, concurrent with the period of axis fixation, when spores lose their responsiveness to changes in their orientation relative to the vector of gravity (Edwards and Roux, 1994). Finally, ablation of the current, with a calcium channel blocker, alters polarity development in the spore and reduces the ability of the spores to orient correctly.

Given the typically low cytoplasmic concentration of calcium in plant cells relative to their extracellular environment, we expect that the reported influx at the spore bottom and the efflux at the spore top, could be mediated by calcium channels and pumps respectively. A significant question raised by this earlier

work is, what is the mechanism for reorientation of the calcium current? We propose two alternative hypotheses, a localized activation model and a component redistribution model for reorientation of the calcium current. In the localized activation model, the current components are uniformly distributed in or near the plasma membrane and are locally activated at the spore top and bottom in response to the gravity signal. The alternative hypothesis is that the components of the current are localized to the spore top and bottom and must be moved through the membrane from one pole to the other in response to spore reorientation. We expect that these two hypotheses may be distinguished by the time it would take either one to occur, with the localized activation model working, potentially, near instantaneously, and the component redistribution model requiring some extended period of time to transport the current mediators from one pole of the spore to the other.

Previous observations of calcium current reversal, measuring the magnitude and direction of the current before and after rotation, had a time resolution of 5 to 10 minutes after spore rotation. The goal of the present work was to measure the calcium current as quickly as possible after rotation, in an effort to eliminate one of the potential models for calcium current reversal.

As in the previous studies, a self-referencing ion-selective electrode system was used to make calcium flux measurements at various positions around the spore (Kuhntreiber and Jaffe, 1990; Smith et al., 1999). This approach utilizes

a single calcium-selective microelectrode oscillated relatively slowly (0.2 – 0.3 Hz) between two points. The calcium ion concentration is measured at each extreme, permitting detection of a concentration gradient between the two points, and consequently, calculation of the ion flux along that vector.

Because a single microelectrode is used to make both measurements, electrode drift is common to both measurements, enabling better sensitivity than measurement with two independent electrodes (Kuhntreiber and Jaffe, 1990; Smith et al., 1999). However, this self-referencing approach sacrifices time resolution for measurement of ion currents, because of the period of measurement oscillation. Consequently, an additional goal of this report was to characterize the variability observed in the *Ceratopteris* system, in an effort to better define the system requirements for the development of new calcium ion sensor technology.

MATERIALS AND METHODS

Spore culture

Ceratopteris richardii spores were surface sterilized by washing in 20% bleach for 90 s followed by 3 washes with sterile water. The sterilized spores were resuspended at a rate of 10 mg ml⁻¹ in half-strength MS medium modified to contain 25 µM CaCl₂. The spores were dark imbibed for 4 to 8 days at 29° C before initiation of germination with white light.

Self-referencing ion selective electrode system

Calcium flux measurements were made with a self referencing ion-selective electrode system. Patch-clamp style microelectrodes with tips approximately 5 microns in diameter were pulled with a micropipette puller (Sutter P-80/PC; Sutter Instrument Co., Novato, CA) and silanized. The electrodes were backfilled with 100 mM CaCl_2 and then front filled with calcium selective liquid ion exchanger (Sigma; St. Louis, MO) to a column height of 30 to 40 microns. Electrodes were seated in a silver-silver chloride wire junction half cell. Reference electrodes were made with saturated 3 M KCl in 3% agar. Before use for flux measurements, electrodes were calibrated in standard solutions of CaCl_2 and used if the resulting Nerstian slope was within 10% of theoretical. The system assembly was mounted on a vibration isolation table and surrounded by a Faraday cage. Experiments were performed at both the University of Missouri at Rolla and the University of Texas at Austin on equivalent systems.

Calcium flux measurements

Flux measurements were made in a modified half-strength MS medium containing 25 μM CaCl_2 . Spores were held in place with a glass holder pipette and were typically used within the first 12 to 15 h after light initiation of germination. The spores were rotated 180° within about 3 s by motorized rotation of the holder pipette, and in any given rotation experiment, calcium flux was measured at two positions around the spore as well as background measurements away from the spore.

For the measurements, electrodes were oscillated at 0.2 Hz. Reported averages at each position (top, side, bottom) were typically made over 10 or more oscillations. Background voltage differences were subtracted from the position measurements to correct for system offsets.

Calculations

The voltage measurements at each sampling point were used to calculate the corresponding $[Ca^{2+}]$ at that position with the following equation:

$$[Ca^{2+}] = 10^{S(V-b)}$$

where, S is the inverse of the Nernst slope of the electrode, V is the voltage measurement, and b is the Nernst intercept.

The differences in $[Ca^{2+}]$ at each position were then used to calculate the flux values with the following equation:

$$J = -D (C_{av}^{10S\Delta V} - C_{av}) \Delta r^{-1}$$

where, J is flux ($\text{mol cm}^{-2} \text{ s}^{-1}$), D is the diffusion coefficient for calcium ($0.79 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$), C_{av} is the average of the $[Ca^{2+}]$ at both measurement positions, S is the inverse of the Nernst slope, ΔV is the voltage difference between the two measurement positions, and Δr is the distance between the two positions.

We follow the convention of reporting positive values for ion efflux from the sample and negative values for ion influx regardless of the actual direction of the vector along the axis.

RESULTS

Polar calcium current reorients in less than 44 s

Over a series of separate 29 rotation experiments, calcium fluxes were measured at two of three positions around the spore before and after rotation. Examples of flux recordings from several representative individual rotation experiments are shown in Figs. 2.1 - 2.4. Because the overall magnitude of the current varied between spores, composite data from all of the experiments are presented relative to the flux measured from the top prior to rotation. Data from one rotation experiment was discarded because a relatively small pre-rotation efflux from the top substantially inflated the other relative measurements. Prior to rotation, calcium effluxes at the sides and bottom were 3 and 5 fold smaller respectively, than the efflux measured from the top (Fig. 2.5). No significant differences were seen within a measurement position before and after rotation (Fig. 2.5). In all but one of the 28 presented experiments, the post-rotation current measured at the spore top was greater than the current measured from the side or bottom, either before or after rotation. These data confirm the presence of a gravity directed polar calcium current in *Ceratopteris* spores with a relatively large efflux found at the spore top.

The time lag between rotation of the spore and current measurement was typically on the order of 100 s (Figs. 2.1 – 2.4), however in 3 experiments we were able to begin recording within 50 s of rotation, with a minimum resumption of recording under 44 s (Table 2.1). In each of these experiments, the top current

had reoriented to at least 69% pre-rotation values and was substantially (4 to 10 fold) greater than the efflux measured from the side.

DISCUSSION

The motivation for these experiments was to minimize the time required to measure the calcium current after reorientation of the spore from the 5 to 10 minutes previously reported. The minimization of this timeframe has theoretical implications for the potential mechanism of current reorientation for which we propose two general models: localized activation or component redistribution.

Reorientation time favors localized activation model

In the localized activation model, we propose that the components mediating the calcium current are uniformly distributed in or near the spore plasma membrane and are locally activated to generate the polar calcium current. The alternative hypothesis demands that the current components have a localized distribution which is moved through the membrane or cell upon reorientation.

Ceratopteris spores are typically 100 to 150 μm in diameter, and consequently, have a circumference of around 314 μm or more. In order to travel in the membrane, from one pole to the opposite, the current components would have to move at a velocity of approximately $3.5 \mu\text{m s}^{-1}$ to reorient the calcium current in less than 44 s.

Protein mobility in cell membranes is usually characterized through lateral diffusion rates, which typically range from approximately $1.2 \mu\text{m}^2 \text{s}^{-1}$ to $0.1 \mu\text{m}^2$

s^{-1} (Kenworthy et al., 2004). However reorientation of the current components would require directed motion, not simple diffusion. Directed motion of membrane proteins in advancing lamellipodia has been measured at velocities of approximately $1 \mu\text{m s}^{-1}$ (Kucik et al., 1989). More recently, directed motion of actin patches in yeast has been reported to range from 0.28 to $0.49 \mu\text{m s}^{-1}$ (Waddle et al., 1996; Pelham and Chang, 2001; Carlsson et al., 2002). These numbers suggest it would be unusual for a protein to move at the velocity required to reorient the calcium current within the 44 s timeframe reported here.

The component redistribution model places these velocity requirements for directed motion not just on a single protein type, but potentially on proteins regulating both the calcium influx and efflux activities. Furthermore, we would expect that entire groups of proteins from each pole would have to be moved coordinately at this velocity. These additional stipulations make it more unlikely that the component redistribution model explains the mechanism of current reorientation. Consequently, the results from this experiment better support the localized activation model for calcium current reorientation.

One of the possible outcomes of the localized activation model is that activation of the calcium current components may be very closely associated with the gravity perception mechanism in *Ceratopteris*. Indeed, the participation of mechanosensitive ion channels directly in gravity perception has been invoked by proponents of both the starch-statolith hypothesis and the gravitational-pressure

hypothesis (Yoder et al., 2001; Staves et al., 1997a). Alternatively, the channel and pump components could be delivered to the membrane in a localized fashion utilizing cycling mechanisms similar to those reported for polar auxin transport regulators (Geldner et al., 2001).

Calcium has long been implicated in gravity signal transduction in plants, but the involvement in the early signal transduction events has only recently been demonstrated in plant systems, including the previous *Ceratopteris* studies. Experiments with the luminescent calcium indicator aequorin indicate a near instantaneous increase in cytoplasmic $[Ca^{2+}]$ of whole seedlings in response to gravistimulation (Plieth and Trewavas, 2002). Notably the gravity response kinetics in these experiments were different than those induced by mechanical stimulation. These results are consistent with the findings of Plieth and Trewavas (2002), who found that gravistimulation of *Arabidopsis* seedlings by reorienting them induces cytosolic calcium transients. They contradict earlier findings of no changes in cytoplasmic $[Ca^{2+}]$ in response to gravistimulation (Legue et al., 1997), however the technique used in this earlier study may have lacked the sensitivity or resolution to detect the subtle changes reported above.

Does calcium current establish an internal calcium gradient?

We speculate that the physiological consequence of the calcium current is to alter the internal distribution of calcium within the spore. Greater calcium efflux from the spore top relative to other regions of the cell would be expected to

generate relatively reduced cytoplasmic $[Ca^{2+}]$ in the apical region of the spore and relatively increased cytoplasmic $[Ca^{2+}]$ in the basal region of the spore. Regions of increased $[Ca^{2+}]$ mark sites of tip growth in *Fucus* zygotes and plant pollen and root hair systems (Shaw and Quatrano, 1996a; Pu et al., 2000; Hepler et al., 2001). In *Ceratopteris*, this internal calcium gradient may be involved in orienting subsequent polarity development events, including nuclear migration. Because the direction of nuclear migration predicts the rhizoid pole, nuclear migration represents the first committed step of polarity development in *Ceratopteris*, and as such, mechanisms directing nuclear migration should be central to gravity signal perception and transduction in *Ceratopteris*.

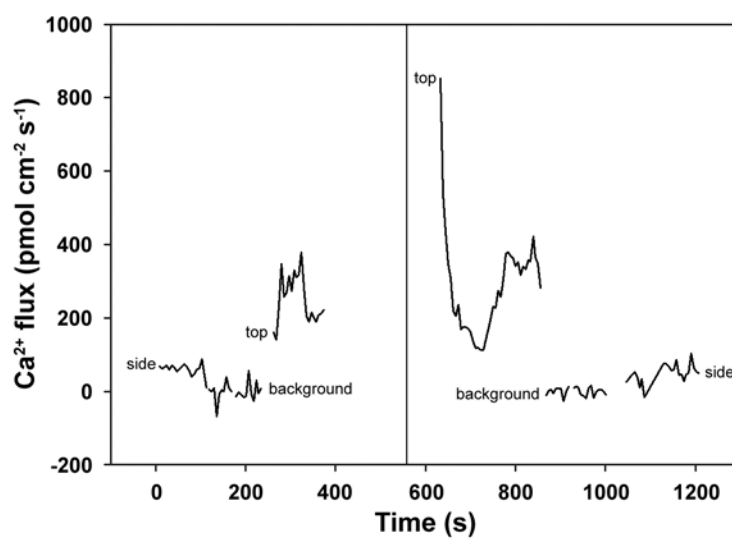


Fig. 2.1 Representative recording of calcium flux before and after spore rotation. Position of the measurements is indicated in the figure. Vertical line indicates the time the spore was rotated.

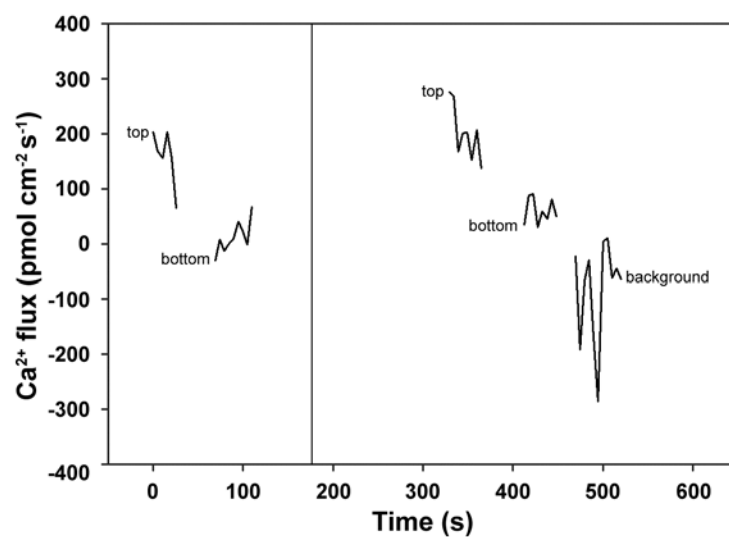


Fig. 2.2 Representative recording of calcium flux before and after spore rotation. Position of the measurements is indicated in the figure. Vertical line indicates the time the spore was rotated.

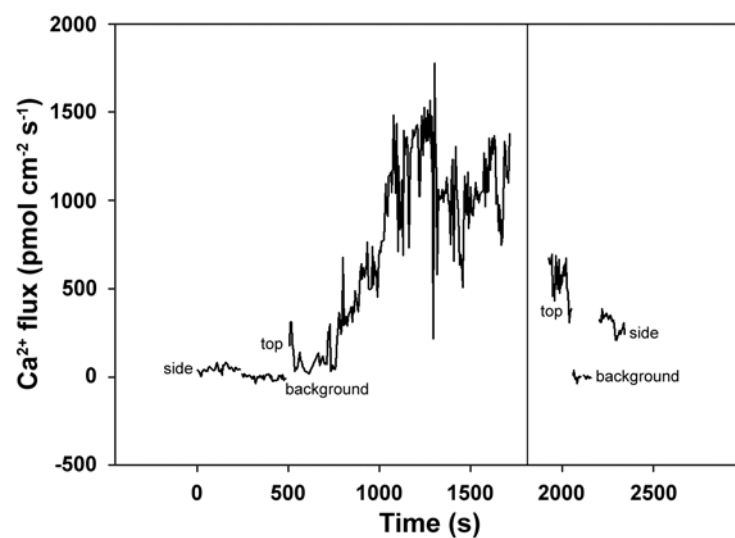


Fig. 2.3 Representative recording of calcium flux before and after spore rotation. Position of the measurements is indicated in the figure. Vertical line indicates the time the spore was rotated.

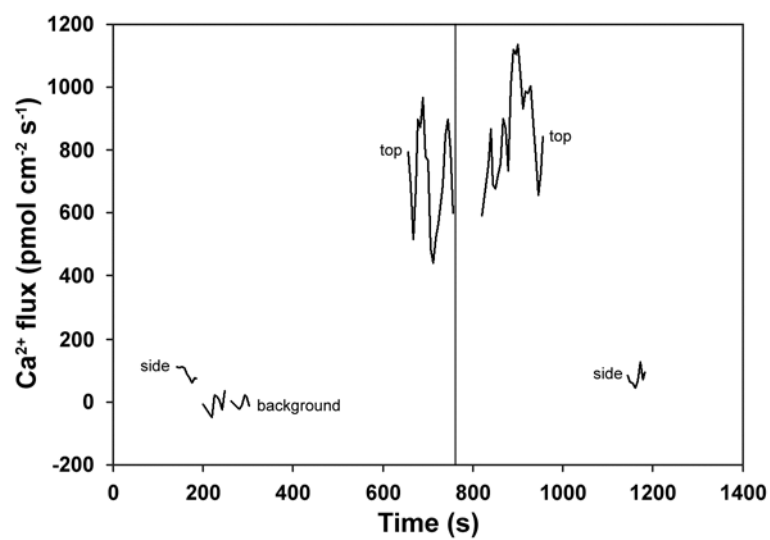


Fig. 2.4 Representative recording of calcium flux before and after spore rotation. Position of the measurements is indicated in the figure. Vertical line indicates the time the spore was rotated.

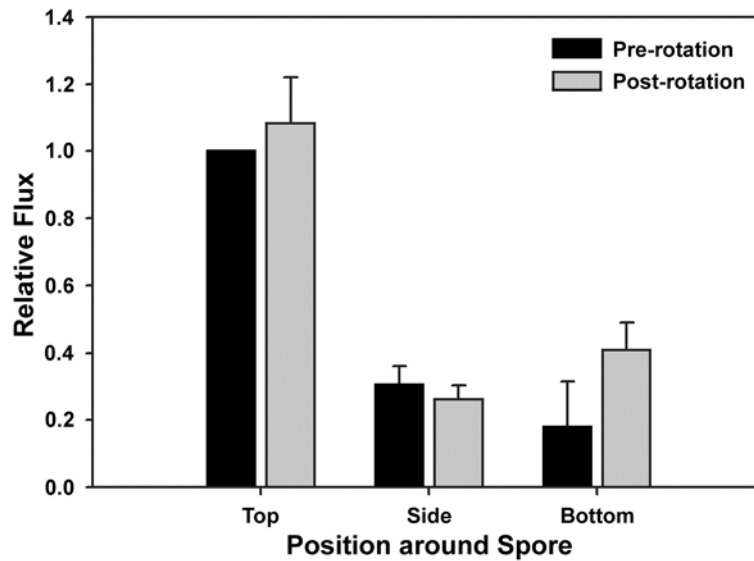


Fig. 2.5 Relative Ca^{2+} flux before and after spore rotation. For each rotation recorded, measurements taken at each position before and after rotation were averaged and then normalized to the Ca^{2+} flux recorded at the top of the spore prior to rotation. The top efflux was significantly greater than the measured efflux from the side or bottom (Student's *t*-Test, $P < 0.05$). There were no significant differences within a position before or after rotation (Paired *t*-Test, $P > 0.05$). For top measurements, $n = 28$; for side, $n \geq 16$; for bottom, $n = 3$. Error bars represent standard error.

Table 2.1 Rapid reorientation of calcium current. The time elapsed between spore rotation and collection of the first subsequent data point is indicated. Average of measurements collected before and after rotation at the top and side are presented.

ΔT (s)	Flux ($\text{pmol cm}^{-2} \text{s}^{-1}$)			
	Top		Side	
	Pre-	Post-	Pre-	Post-
49.8	699.91	487.99	29.02	94.45
43.8	704.59	864.46	94.45	80.17
46.5	2532.41	3035.37	47.36	46.37

CHAPTER 3

BIOINFORMATIC ANALYSIS OF AN EXPRESSED SEQUENCE TAG LIBRARY FROM GERMINATING SPORES OF *CERATOPTERIS RICHARDII*.

INTRODUCTION

Partial sequencing of cDNA clones as expressed sequence tags (ESTs) is an alternative to more extensive genome sequencing efforts. While the genomes of two model systems, *Arabidopsis* and rice, have been completely sequenced, large scale EST sequencing projects, (>40,000 ESTs) have been described in a number of plant systems including corn, tomato, moss, soybean, and clover (Fernandes et al., 2002; Van der Hoeven et al., 2002; Rensing et al., 2002; Shoemaker et al., 2002; Sawbridge et al., 2003).

Because ESTs are sequenced from cDNAs, EST sequencing has the advantage of rapidly and directly identifying expressed genes, any of which may later be completely characterized. Additionally, when cDNA clones are randomly selected for sequencing, the abundance of ESTs for the same gene is related to the expression level of that gene in the cDNA library. This relationship enables expression analysis of genes within a sample or comparisons between libraries constructed from biologically distinct samples using only the EST abundance data generated from sequencing and analysis (Fernandes et al., 2002). Finally, cDNA

sequencing is necessary for other expression analysis techniques, including cDNA microarrays, which rely upon known sequence information.

We used this approach to identify genes expressed during spore germination in the fern *Ceratopteris richardii*. Although the developing gametophytes of *Ceratopteris* have been a productive model system for studies of gravity and light regulated development as well as the development of sex differentiation (Banks, 1997; Chatterjee and Roux, 2000), a lack of molecular sequence information has limited investigations in this system. Because of the demonstrated importance of calcium signaling in *Ceratopteris* polarity development, our efforts focused on identifying and cloning genes related to calcium signaling and homeostasis. Analysis of the library has yielded insights into the complexity of this single-celled system and provided targets for the study of gravity directed polarity development.

MATERIALS AND METHODS

cDNA library construction and EST sequencing

RNA isolated from spores 20 h after light initiation of germination (~24 h before the first cell division) was used for a commercially prepared cDNA library (Life Technologies, Rockville, MD). Randomly chosen clones were sequenced at the Purdue Agricultural Genomics Facility, Purdue University (West Lafayette, IN), and 5085 of the resulting single pass sequences were used for further analysis. To date, 3587 of these expressed sequence tags (ESTs) have been submitted to

dbEST (GenBank accession numbers BE640669-BE643506 and BQ086920-BQ087668).

EST assembly

Prior to assembly, a contaminating linker sequence present in a large percentage of the ESTs was removed. Additionally, the ESTs were filtered for short entries or low complexity sequences using SeqClean (<http://www.tigr.org/tdb/tgi/software>). The resulting sequences were assembled with the TGIR Gene Indices clustering tools (TGICL; Pertea et al., 2003). Briefly, TGICL clusters all of the ESTs using minimum overlap length (40 bp) and percentage identity (95%) criteria. The initial clusters are then sent to an assembly program (CAP3; Huang and Madan, 1999) which attempts to create one or more contigs from each cluster.

The resulting sequences in the dataset are termed tentative unique genes (TUGs) and consist of two types of sequences: contigs and singletons. Contigs are two or more ESTs which are presumed to represent the same transcript, and singletons are ESTs without significant similarity to any other ESTs. Consequently, the set of TUGs, composed of the sets of contigs and singletons, represents the unique genes found in the EST collection.

TUG identification and functional analysis

The identities of the TUGS were determined using BLASTX (Aultschul et al., 1997) against the *Arabidopsis* proteome (ATH1_pep_cm_20040228;

<http://www.arabidopsis.org>). Functional and localization categories of the *Ceratopteris* TUGS were assigned using the TAIR Gene Ontology terms associated with the locus of the best *Arabidopsis* BLAST match (<http://www.arabidopsis.org>).

***Arabidopsis* tissue specific genes**

EST collections from *Arabidopsis* seed, leaf, root, and shoot cDNA libraries were downloaded from The Institute for Genomic Research (TIGR; www.tigr.org). The ESTs were pooled together and analyzed as above for production of clusters and singletons. The resulting TUGs were identified by BLAST analysis against the *Arabidopsis* transcriptome (ATH1_cDNA_cm_20040228; <http://www.arabidopsis.org>). Only TUGs with transcript matches longer than 100 bp and greater than 97% identity were retained. A set of *Arabidopsis* genes expressed in pollen was also obtained from Honys and Twell (2003).

Percent coverage calculation

An estimate of the total number of unique genes expressed in *Ceratopteris* spores was made using a nonparametric estimator typically used for the estimation of population size or species richness in ecological studies (Burnham et al., 1979; Brose et al., 2003). This calculation makes use of the number of random samples in which each species appears. In our studies we defined a sample as a single 384 well sequencing plate. For each TUG, the number of

samples it occurred in was tabulated from the number of plates containing its constituent ESTs. Given an expected percent coverage of approximately 25-30%, the fourth-order jackknife estimator was used (Brose et al., 2003), and the final percent coverage of expressed genes was determined by dividing the number of TUGs by the estimated number of unique genes.

Cloning of calcium related signaling genes

For *CrCaMI* and *CrProI*, cDNA clones with BLAST matches to calmodulin and profilin were selected from the EST library and completely sequenced. For *CrCPK1*, complete sequencing of the corresponding cDNA clone yielded only a partial 3' sequence. A modified chromosome walking approach was used to determine the 5' end. Briefly, *Ceratopteris* genomic DNA was isolated and separately digested with four restriction enzymes (Kpn I, Not I, Sac I, Xho I; New England BioLabs, Beverly, MA) in parallel to create four separate libraries of *Ceratopteris* genomic restriction fragments. These fragments were then ligated to plasmid digested with the same restriction enzyme (pCR2.1 TOPO; Invitrogen, Carlsbad, CA). PCR with combinations of gene-specific and plasmid-specific primers was used to amplify fragments putatively 5' of the genomic position of the known *CrCPK1* cDNA. These fragments were authenticated by nested PCR and sequenced, yielding the upstream genomic sequence of *CrCPK1*. Primers, varying in distance from the 3' known *CrCPK1* sequence, were then

used to amplify the full-length expressed *CrCPK1* gene from a *Ceratopteris* spore cDNA library.

The open reading frames for all three genes were determined from predicted translations of the full length cDNAs (transeq, EMBOSS package; <http://www.uk.embnet.org/Software/EMBOSS/>). The resulting sequences from these analyses were deposited in GenBank.

CrCaM1 and CrPro1 protein expression and purification

The coding regions for *CrCaM1* and *CrPro1* were each cloned into the pETBlue-1 vector and expressed as native proteins according to the manufacturer's directions (Novagen, Madison, WI). CrCaM1 was purified using phenyl-Sepharose affinity chromatography (Gopalakrishna and Anderson, 1982) and CrPro1 was purified using a poly-L-proline column as described previously (Gibbon et al., 1997).

Protein Isolation and Immunoblotting

Crude extracts were obtained by grinding 4 mg of spores in 50 μ l of homogenization buffer (50 mM Tris acetate, pH 7.9; 100 mM potassium acetate; 1 mM EDTA; 1 mM DDT; 20% (v/v) glycerol). In some experiments, 8 mg of spores were ground in 100 μ L of homogenization buffer. Cellular debris was removed by centrifugation ($2K \times G$, 5 min, 4° C) and the resulting supernatant was removed to a clean microcentrifuge tube and either further processed to enrich for CaM or used directly for gel electrophoresis for profilin immunoblots.

To enrich for CaM, an equal volume of 100% ethanol was added and allowed to incubate for 15 minutes on ice. The samples were then centrifuged ($15K \times G$, 15 minutes, $4^{\circ} C$), the supernatant was removed to a clean microcentrifuge tube, and 2.5 volumes of 100% ethanol were added and allowed to incubate on ice for 15 minutes. The samples were then centrifuged ($15K \times G$, 15 minutes, $4^{\circ} C$), the supernatant was discarded, and the resulting pellet was allowed to air dry briefly and then resuspended in 15 μ l of homogenization buffer.

The proteins were separated by 10-20% gradient PAGE and transferred to nitrocellulose (Protran, 0.2 μ pore size; Schleicher and Schuell BioScience, Keene, NH). After transfer, the membranes were briefly rinsed in phosphate buffered saline (PBS) then fixed in 0.20% glutaraldehyde in PBS for 1 h on a rocker. Membranes were then blocked for 1 h in 0.10% Tween-20, 1% milk, in PBS. Primary antibody was diluted 1:1000 in 0.10% Tween-20 in PBS and incubated overnight at $4^{\circ} C$ on a rocker. For CrCaM1, commercial mouse monoclonal anti-Calmodulin (Zymed Laboratories Inc., South San Francisco, CA) was used as the primary antibody. For CrPro1, rabbit polyclonal sera raised against two maize profilins, anti-ZmPro3 or anti-ZmPro5 were used (Karakesisoglou et al., 1996). The membranes were then extensively washed in PBS and the secondary antibody (ImmunoPure Goat Anti-Mouse IgG or ImmunoPure Goat Anti-Rabbit IgG; Pierce Biotechnology, Rockford, IL), diluted 1:20,000 in 0.10% Tween-20 in PBS, was added and rocked at room temperature for 1 h. After they were extensively

washed with PBS, membranes were used for ECL (SuperSignal West Pico Chemiluminescent Substrate; Pierce Biotechnology) according to manufacturer directions.

RNA Isolation and reverse-transcriptase (RT-PCR)

Spores were ground in 25 μ l RNA grinding buffer (1 M Tris, pH 7.3; 5 mM EDTA; 1% (w/v) SDS; 1 mM DTT) and 25 μ l phenol:chloroform:IAA (25:24:1, pH 5.2) in microcentrifuge tubes with a disposable plastic pestle and handheld motor. After this grinding step, an additional 25 μ l each of grinding buffer and phenol:chloroform were added, the tubes were vortexed and centrifuged (15K \times G; 20 minutes, 4° C). The resulting aqueous phase was removed and extracted with an equal volume of chloroform:isoamyl alcohol (24:1). After centrifugation (15K \times G; 10 minutes, 4° C), the aqueous phase was retained, and RNA was precipitated overnight at -20° C with 1/10 volume 3 M sodium acetate and 2.5 volumes 100% ethanol using 5 μ g of glycogen as a coprecipitant. After centrifugation (15K \times G, 20 minutes, 4° C), the resulting RNA pellet was washed with 500 μ l 70% ethanol (v/v), resuspended in 9 μ l DEPC treated water and 1 μ L RNase-free DNase (1 U μ l⁻¹; Epicentre Technologies, Madison, WI), and incubated at 42° C for 15 minutes. The DNase was inactivated by incubation at 75° C for 10 minutes.

Reverse transcription was carried out using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. After first strand cDNA synthesis, 1/20 volumes were used as template for PCR (Taq MasterMix; Qiagen, Valencia, CA) according to manufacturers instructions.

RESULTS

Identification of unique *Ceratopteris* genes

Manual inspection of the EST collection revealed the presence of a linker (5'-CCACGCGTCCGC-3') used during construction of the cDNA library in most of the EST sequences. After removal of the linker and additional quality filtering, 5059 ESTs with an average read length of 785 nucleotides were selected for additional analysis.

Clustering and assembly of the ESTs yielded 3930 TUGs composed of 513 contigs and 3417 singletons (Table 3.1). Contigs are consensus sequences generated from two or more ESTs that are determined to represent the same expressed gene, while singletons are ESTs with no strict homology to other ESTs in the collection. Contig assembly increased the average length of the resulting consensus sequences over 30% from the average length of the individual ESTs (Table 3.1).

Estimating the number of genes expressed in germinating *Ceratopteris* spores

In order to determine the EST library's percent coverage of the actual population of genes expressed during spore germination, we estimated the number

of unique genes present in the population by applying an approach used to estimate species richness in ecological studies (Burnham and Overton, 1979; Brose et al., 2003). We estimate 14,275 unique genes to be expressed 20 h after light initiation of spore germination, suggesting that the current 3930 TUGs represent approximately 27% of those sequences.

In addition to estimating the EST library's percent coverage, determining the expected rate of discovery of new genes is important for evaluating the benefit of continued sequencing efforts. Examination of the accumulation of observed TUGs in each of the samples (384 well plate units) indicated approximately 64% of the ESTs from the last plate sequenced represented new TUGs (Fig. 3.1). This demonstrates that the sampling is far from complete, in agreement with the percent coverage estimate above.

Functional and compartmental categorization of the *Ceratopteris* TUGs

The TUGs were identified by BLAST analysis against the *Arabidopsis* proteome, yielding 2710 TUGs with significant homology (E-value $\leq 1.0 \times 10^{-10}$) to *Arabidopsis* proteins. Using the Gene Ontology terms of the matching *Arabidopsis* loci (Berardini et al., 2004), functional and localization classifications were made for each of the *Ceratopteris* TUGs, and only genes with an assignable function or compartmentalization are presented. In order to determine what may represent typical functional and compartmental distributions,

as well as provide a basis for comparison, loci expressed in *Arabidopsis* seed, pollen, and leaf tissue were similarly analyzed.

The functional patterns seen in *Ceratopteris* spores were generally similar to those found in the *Arabidopsis* tissues sampled (Fig. 3.2). In each of the sets of loci, the functional classifications of metabolism and protein metabolism were the most abundant, accounting for more than 45% of the genes with assignable functions (Fig. 3.2). The compartmental distribution of genes was more variable between the *Ceratopteris* EST collection and the *Arabidopsis* tissues (Fig 3.3). The largest difference was seen in the number of genes with their localization classified as “Other Membranes,” where *Ceratopteris* spores had a proportion 5 to 10% smaller than those typically seen in *Arabidopsis*. The “Other Membranes” compartment is defined as membrane proteins, excluding those that localize to the plasma membrane. The only other substantial difference between the *Ceratopteris* spores and the three *Arabidopsis* tissues was seen for genes associated with the ribosome, which occurred 1.5 to 4-fold more frequently in the spores than in the *Arabidopsis* tissues (Fig. 3.3).

Abundant ESTs

More than 95% of the *Ceratopteris* TUGs are represented by three or fewer spore ESTs (Fig. 3.4). Given the 3930 TUGs found in the library of 5059 ESTs, a naïve prediction would be that the average gene would be represented by approximately 1.3 ESTs. However, 15 contigs were assembled from at least 9

ESTs each (Table 3.2). Over 3.5% of the ESTs in the library belong to one of these 15 TUGs, which represent under 0.4% of the total number of TUGs (Fig. 3.4). The presence of multiple entries for the same *Arabidopsis* protein elongation factor 1- α (EF1- α) most likely indicates the simultaneous expression of several closely related family members in *Ceratopteris* spores. Attempted alignments of the various EF1- α contigs indicate that the sequences clearly do not belong to the same transcript even though their predicted translations encode highly similar amino acid sequences (data not shown).

Comparison of genes expressed in *Ceratopteris* spores and *Arabidopsis* pollen and seeds

Ceratopteris spores share biological and physiological characteristics with *Arabidopsis* pollen and seeds. We therefore examined sets of genes expressed in those tissues in an effort to identify shared genes which may be involved in those similarities. In order to limit our comparison to genes that show relatively specific patterns of expression rather than genes that are broadly expressed, we first screened each of these gene sets with a set of vegetatively expressed genes derived from analysis of over 32,000 ESTs from *Arabidopsis* roots, shoots, and leaves. Between 50 to 60% of the genes included in the seed, spore, or pollen sets were also expressed in vegetative tissues (Fig. 3.5). The genes exhibiting tissue specific expression were then compared to determine which genes were shared amongst seeds, spores, and pollen. Nearly 9% of the genes expressed in seeds or

pollen are also expressed in *Ceratopteris* spores (Fig. 3.5) and eight genes are expressed in all three tissues (Table 3.3).

Calcium signaling genes expressed during germination

Because of the importance of calcium signaling to normal polarity development in *Ceratopteris*, we selected TUGs corresponding to three calcium signaling genes, calmodulin (CaM), calmodulin domain protein kinase (CDPK), and profilin, to fully sequence and characterize (Table 3.4). Complete sequences for these genes were determined and deposited in GenBank as follows: CaM, *CrCaM1* (AF510075); CDPK, *CrCPK1* (AY138479); and profilin, *CrPro1* (AY102169).

CrCam1 is encoded by an 801 base pair (bp) cDNA, containing a 53 bp 5' untranslated region (UTR) and a 300 bp 3' UTR. The open reading frame translates into a 149 amino acid long protein with over 90% identity to other higher plant CaMs. Heterologous expression of the coding region followed by phenyl-sepharose affinity column purification produced a 15 kD protein recognized by monoclonal antibodies raised to bovine brain calmodulin (Fig. 3.6). *CrCPK1* is a 2368 bp transcript with a 224 bp 5' UTR and a 575 bp 3'UTR. *CrCPK1* encodes a 522 amino acid protein with approximately 60% identity to other plant CDPKs. CrPro1 is 133 amino acids long and is greater than 55% identical to other plant profilins. Heterologously expressed CrPro1, purified by poly-L-proline affinity chromatography, produced a single band of approximately

14 kD that is recognized by antibodies raised against two maize profilins, anti-ZmPRO3 (Fig. 3.7) and anti-ZmPRO5 (data not shown).

The expression of pattern of these genes during spore germination was also evaluated. All three genes are detectable by RT-PCR through the first 96 h of germination, indicating their expression at the mRNA level over this time period. We further examined the expression pattern of *CrCaM1* and *CrPro1* at the protein level. Both proteins are present in desiccated spores, but their relative abundance increases during germination (Fig. 3.6, Fig. 3.7). These experiments also permitted an evaluation of the overall pattern of protein expression through examination of the PAGE separated protein extracts (Fig. 3.6, Fig. 3.7). In dry spores, there appear to be five major bands (approximately 50, 30, 27, 20, and 15 kD), but the abundance of these bands decreases during germination. At 96 h after light initiation of germination, the distribution of protein abundance appears to be more uniform.

DISCUSSION

Current library represents only a portion expressed genes

We report the identification of nearly 4000 unique genes from analysis of over 5000 ESTs collected from germinating spores of the fern *Ceratopteris richardii*. A number of our results indicate that sampling of the EST population is far from complete, supporting our estimate of the EST library's coverage as 27% percent of the actual genes expressed at this stage of development. The

accumulation of identified TUGs is largely linear over the first 14 sequencing plates and doesn't indicate any saturation (Fig. 3.1). Also, over the last four samples, the discovery rate of TUGs remained relatively constant, around 64%, suggesting that continued sequencing would continue to reveal a substantial number of new genes and that the discovery rate may continue to decline relatively slowly.

Additionally, of the TUGs identified, nearly 87% of them are represented as singletons. In plant EST libraries with much greater sampling drawn from multiple cDNA libraries, singletons typically represent approximately 50% of the identified TUGs. Sampling of over 73,000 ESTs from maize yielded 22,532 TUGs, 51.4% of which occurred as singletons (Fernandes et al., 2002). Very similar results have been reported in soybean and tomato where, with over 100,000 ESTs sequenced each, 50.6% and 51.5% of the identified TUGs are singletons respectively (Shoemaker et al., 2002; Van de Hoeven et al., 2002). These comparisons suggest that as the depth of EST sampling increases, the proportion of TUGs found as singletons decreases.

Alternatively, the large percentage of singletons may simply represent the expression of a greater proportion of low abundance transcripts than are typically expressed in other species. This hypothesis is only modestly supported by examination of individual cDNA libraries included in the above large scale analyses. In several soybean libraries with approximately equal sampling as the

Ceratopteris library, the representation of singletons was around 5% less than the 87% reported here (Shoemaker et al., 2002). However, additional analysis is needed to draw a stronger conclusion.

Comparison of gene expression in spores, seeds, and pollen

While the number of ESTs analyzed in this project is similar in scale with other libraries of specific tissues and developmental stages (Fernandes et al., 2002; Sawbridge et al., 2003; Shoemaker et al., 2003), this library is unique, because its biological source is a single cell and represents the plant gametophytic generation. The first cell division in *Ceratopteris* typically takes place 48 h after light initiation of germination, approximately 24 h after the gravity directed downward migration of the spore nucleus. Because the cDNA used for EST sequencing was collected 20 h after initiation of germination, we expect this collection of ESTs to represent *Ceratopteris* gene expression at a single-celled stage of development.

Family members of several of the genes highly expressed in *Ceratopteris* spores are among those most highly and broadly expressed in *Arabidopsis*, including heat shock protein 70, S-adenosyl-L-homocysteine hydrolase, EF1- α , and a variety of ribosomal proteins (<http://mpss.udel.edu/at/>). However, two of the most abundant ESTs in the *Ceratopteris* library show restricted and differing patterns of expression in *Arabidopsis*.

The Late Embryogenesis Abundant (LEA) proteins are expressed during seed desiccation and also in response to abscisic acid, osmotic or water stress (Delseny et al., 1994; Skriver and Mundy, 1990) and are thought to protect the tissue from desiccation stress (Bray, 1993). In contrast, Protodermal Factor 1 (PDF1) is specifically expressed in the L1 layer of the shoot apical meristem and the protoderm of organ primordia (Abe et al., 1999). Although not much is known about PDF1's cellular function, its expression in *Ceratopteris* spores may be an indicator of the developmental pattern of the germinating spores and the subsequent planar, two dimensional growth of the protonema. Despite the differing expression pattern of these two genes in *Arabidopsis*, their expression is coincident in the single celled *Ceratopteris* spores.

Spores at this stage of development are transitioning from a dormant, desiccated state to a metabolically active one, analogous to the process of seed germination in angiosperms. This similarity may be more than superficial as both processes appear to involve similar and relatively specifically expressed proteins including desiccation and dormancy related proteins, as well as several aspartic or cysteine proteases (Table 3.3). In developing seeds, storage proteins are processed into mature subunits by aspartic and cysteine proteases upon their transport to specialized protein storage vacuoles (PSV), and proteases are further involved during seed germination to initiate and complete metabolism of the storage proteins (Muntz, 1996; Gruis et al., 2002; Gruis et al., 2004). Because

transport to the PSV is critical, the shared expression of a seed specific vacuolar processing enzyme may also be an indicator that a similar protein storage strategy operates in both seeds and spores. It has previously been hypothesized that seed plants could have co-opted genes previously used for spore dormancy to develop post-embryonic seed dormancy (Banks, 1999). The common, tissue specific expression of genes in both *Ceratopteris* spores and *Arabidopsis* seeds supports this hypothesis.

Biologically, fern spores are part of the haploid gametophytic generation and are equivalent to angiosperm microspores and megaspores, which divide to produce mature pollen grains and embryo sacs, respectively. Using a microarray approach, approximately 1000 pollen expressed genes have been identified in *Arabidopsis*, leading to an estimate of the total number of pollen expressed genes to be over 3500 (Honys and Twell, 2003), and a similar number has also been predicted for *Arabidopsis* pollen using a Serial Analysis of Gene Expression approach (Lee and Lee, 2003). Estimates from our EST analysis place this number at over 14,000 in germinating *Ceratopteris* spores. The substantial difference in estimated gene diversity between these two biologically equivalent samples may reflect the relative complexity of the independent gametophytes in homosporous ferns as compared to the reduced gametophytes of angiosperms. Alternatively, it is possible that a greater number of genes may be expressed throughout the *Ceratopteris* life cycle. Completion of a *Ceratopteris* bacterial

artificial chromosome library resulted in the estimate that the *Ceratopteris* genome is approximately 100 times larger than the *Arabidopsis* genome, making this alternative likely to be at least partially responsible (Green Plant BAC Library Project; http://www.genome.arizona.edu/BAC_special_projects/).

Cloning of calcium signaling genes demonstrates utility of *Ceratopteris* EST library

Despite the physiologically and biologically significant relationships between *Ceratopteris* spores and *Arabidopsis* seeds and pollen, our focus on the system has been as a model of gravity directed polarity development. Because previous studies have demonstrated a necessary role of calcium signaling during this process in *Ceratopteris* (Chatterjee et al., 2000), we used the library to identify and clone several calcium signaling related genes expressed during spore polarity development: a CaM, *CrCaM1*; a CDPK, *CrCPK1*; and a profilin, *CrPro1*.

Changes in the cytoplasmic calcium concentration are involved in the transduction of many plant environmental responses. CaM is a well described calcium binding protein which transduces those signals by binding to downstream target proteins in a calcium-dependent manner (reviewed by Zielinski, 1998). Similarly, CDPKs are involved in the transduction of calcium transients to downstream proteins through calcium-dependent phosphorylation of those target proteins. The CDPKs represent a large multigene family in plants (>30 members

in *Arabidopsis*) and they appear to be involved in a diverse set of environmental responses and developmental programs (reviewed by Harmon et al., 2000; Cheng et al., 2002). Profilins are calcium-dependent monomeric actin-binding proteins that may be involved in regulating actin polymerization (reviewed by McCurdy et al., 2001). Because actin dynamics appear to be involved in polarity development across a variety of models, profilin represents a possible mechanism by which calcium, through regulation of profilin, could play a role in polarity development.

As predicted by their inclusion in the EST library, each of these genes is indeed expressed during spore germination at the mRNA level. For *CrCaM1* and *CrPro1*, we were also able to examine their expression at the protein level, and both proteins show increasing levels from desiccated spores through germination. This may reflect, in part, a shift from the presence of a few relatively abundant proteins, to a more uniform pattern of protein abundance (Fig. 3.6, Fig. 3.7).

Conclusion

The relatively direct identification and cloning of three calcium signaling genes validates the development of the *Ceratopteris* EST library as an approach to identify expressed genes. Given the large predicted size of the *Ceratopteris* genome, as well as the relatively shallow sampling evidenced in the current EST dataset, EST sequencing will likely continue to be the most efficient approach to produce additional genetic sequence information in this model system. The

identification of additional genes also permits targeted, reverse genetics approaches to investigate the function of an increasing number known genes.

ACKNOWLEDGEMENTS

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Table 3.1 Analysis of an EST library from germinating spores of the fern *Ceratopteris richardii*.

	No. of Sequences	Average Length
EST library	5059	785
TUGs	3930	825
Contigs	513	1091
Singletons	3417	785

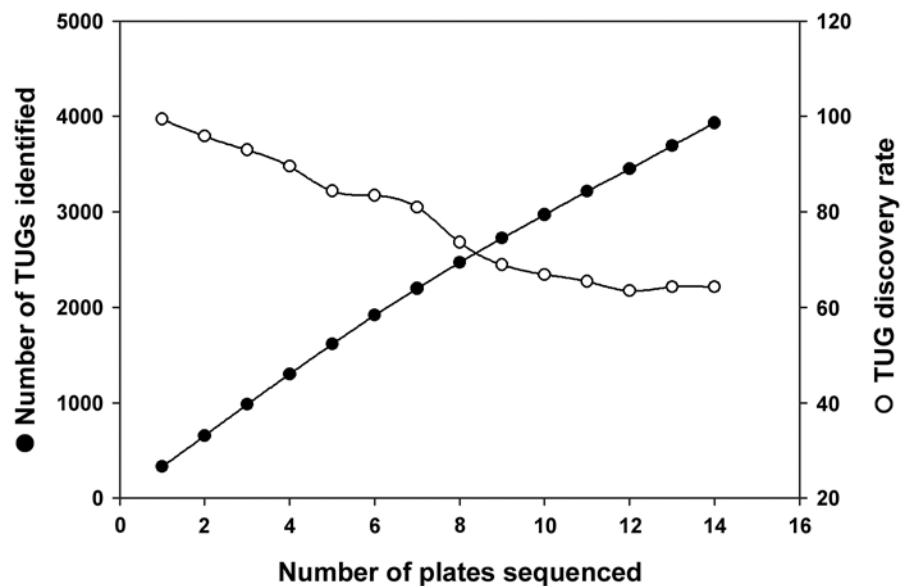


Fig. 3.1 Accumulation of new TUGs during EST sequencing. (●) The cumulative number of unique genes identified from each 384-well sequencing plate. (○) The discovery rate, the percentage of ESTs that represent new unique genes, is shown for each plate.

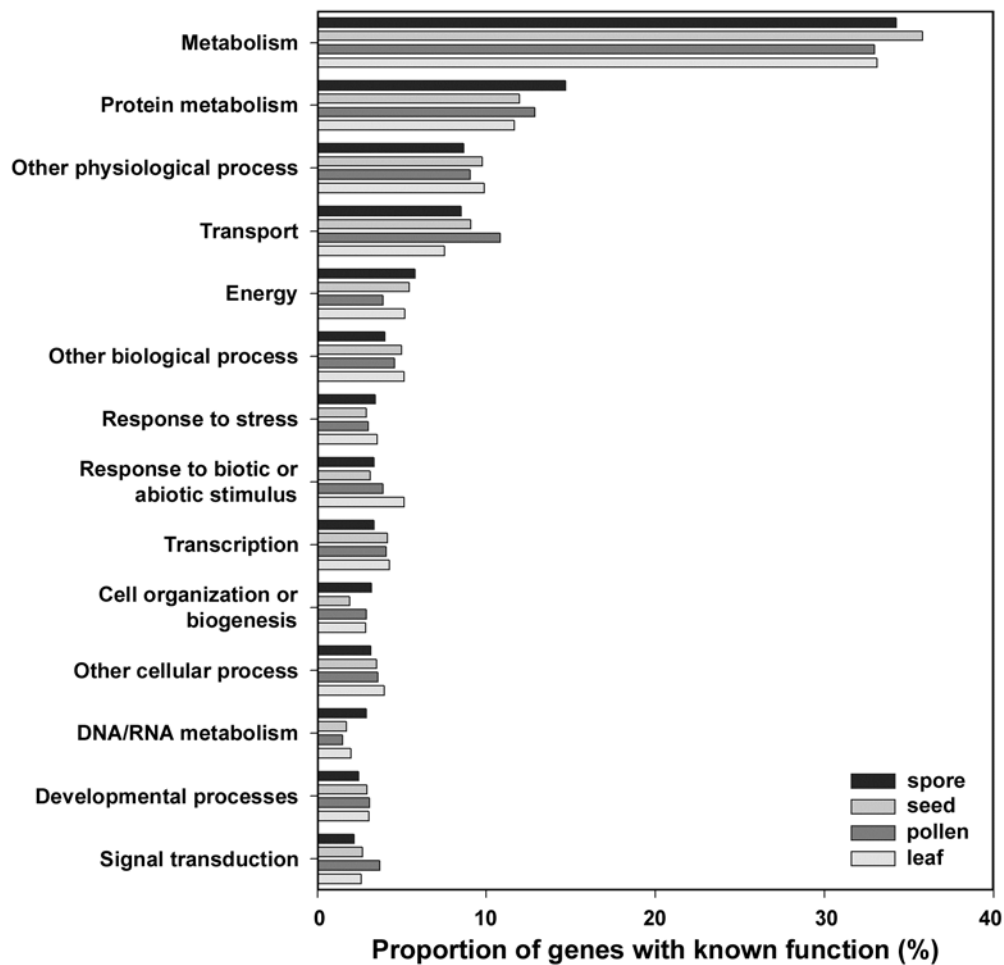


Fig. 3.2 Functional classification of genes expressed in *Ceratopteris* spores. *Ceratopteris* TUGs were annotated by BLAST comparison with the *Arabidopsis* proteome and the functional classification of each TUG was done according to the TAIR Gene Ontology database of the resulting best BLAST match. The functional classification distribution of genes expressed in *Arabidopsis* seed, pollen, and leaf tissue are also indicated.

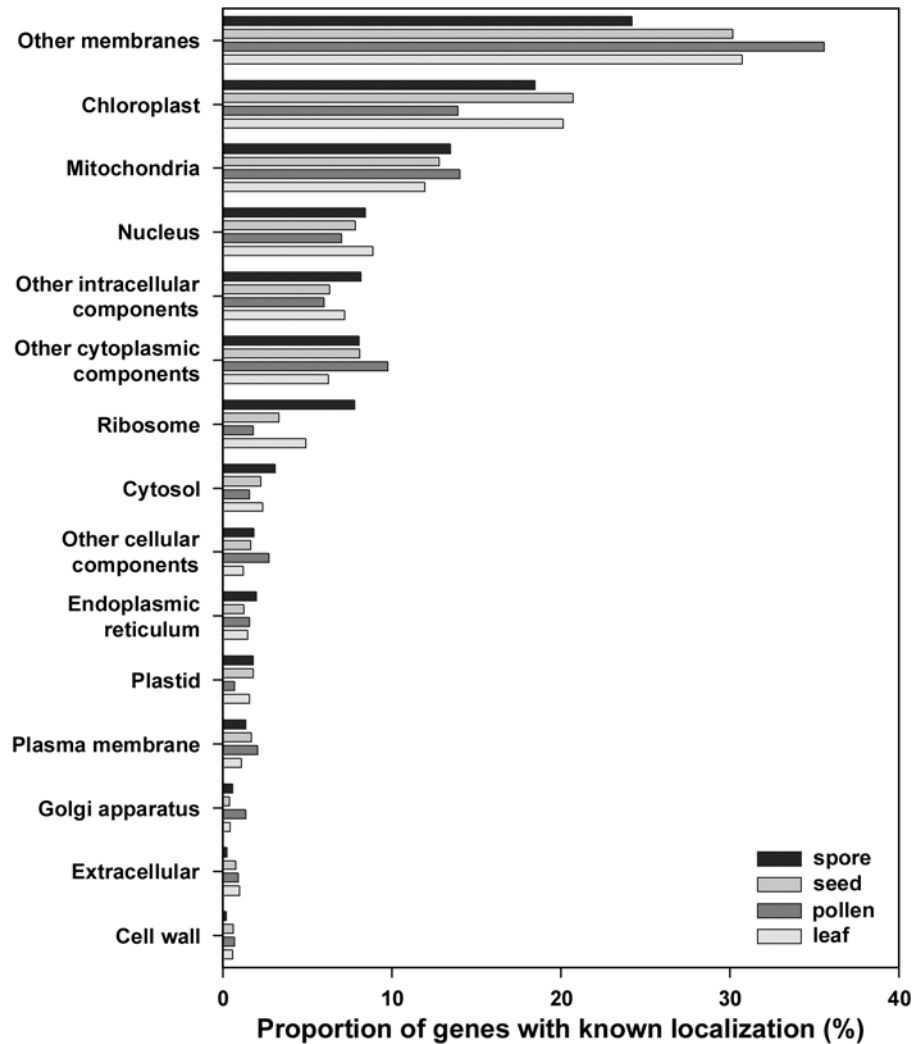


Fig. 3.3 Localization of genes expressed in *Ceratopteris* spores. *Ceratopteris* TUGs were annotated by BLAST comparison with the *Arabidopsis* proteome and the compartmental classification of each TUG was done according to the TAIR Gene Ontology database of the resulting best BLAST match. The compartmental distribution of genes expressed in *Arabidopsis* seed, pollen, and leaf tissue are also indicated.

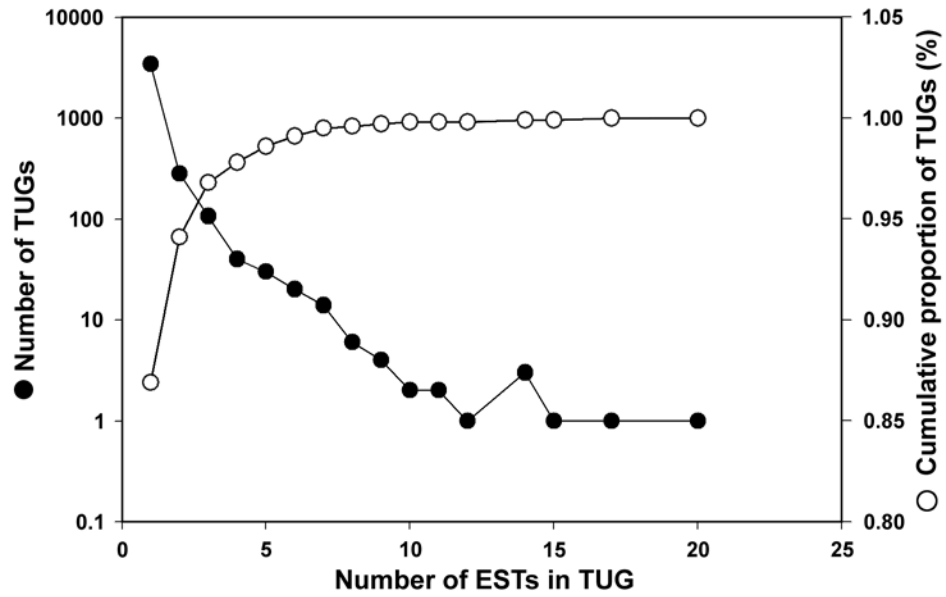


Fig. 3.4 Graph of the number of ESTs included in the *Ceratopteris* TUGs. (●) The number of ESTs in each TUGs was tabulated and the frequency of the resulting size classes is shown. (○) The cumulate number of the various sized TUGs as a proportion of the number of TUGs.

Table 3.2 Highly expressed genes identified in *Ceratopteris* spores. TUGs containing 9 or more ESTs are listed.

Unigene ID	Description	No. of included ESTs
CriU1	elongation factor 1- α	20
CriU3	protodermal factor 1 (PDF1)	17
CriU5	peroxidase	15
CriU7	S-adenosyl-L-homocysteine hydrolase	14
CriU4	polyubiquitin (UBQ10)	14
CriU6	elongation factor 1- α	14
CriU8	polyubiquitin (UBQ11)	12
CriU9	cysteine proteinase	11
CriU2	elongation factor 1-a	11
CriU12	late embryogenesis abundant protein	10
CriU10	catalase 2	10
CriU13	fructose-bisphosphate aldolase	9
CriU14	heat shock protein 70	9
CriU11	n.s. ¹	9
CriU15	40S ribosomal protein S26 (RPS26B)	9

¹ No significant BLAST match at the $E \leq 1 \times 10^{-10}$ level.

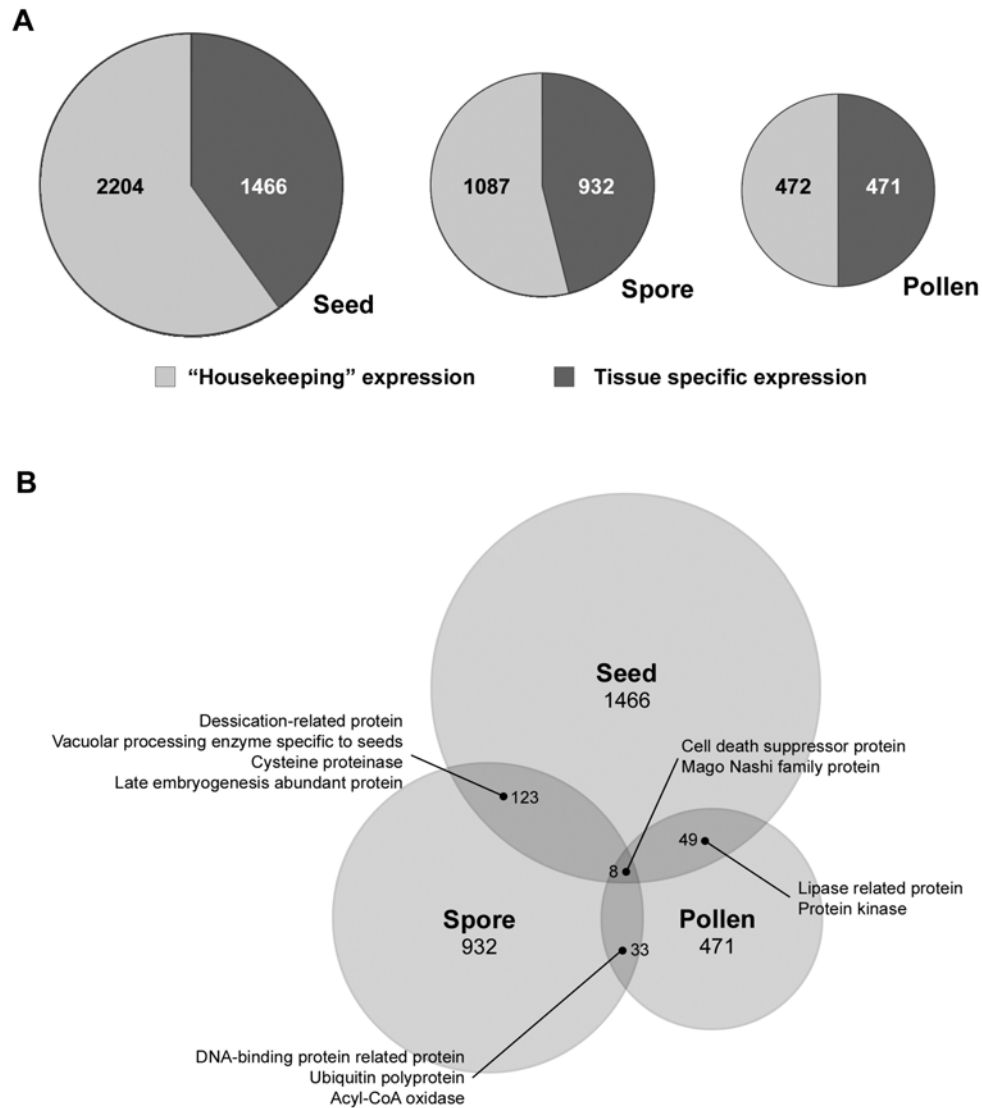


Fig. 3.5 Comparison of the *Ceratopteris* spore TUGs to genes expressed in *Arabidopsis* seeds and pollen. (A) Identification of tissue specific gene expression in *Ceratopteris* spores and *Arabidopsis* seeds and pollen. The proportion of genes present in the seed, spore, and pollen sets that were also expressed in vegetative tissue are indicated in light gray. The remaining tissue specific genes, indicated in dark gray, are used for the expression comparisons. Numbers indicate the number of genes included in each proportion. (B) Overlapping expression of genes expressed in seeds, spores, and pollen. Numbers in overlapping regions indicate number of genes shared between the respective sets. Several relatively abundant genes present in each overlap are also indicated.

Table 3.3 Genes expressed in *Ceratopteris* spores and *Arabidopsis* pollen and seeds. Genes were selected on the basis of abundance in the respective tissues.

AGI Number	Description	No. of ESTs (Seed/Spore) ¹
<i>Expressed in seed, spore, and pollen</i>		
AT1G02140	Mago nashi family protein	2/2
AT1G54290	Eukaryotic translation initiation factor SUI1	1/1
AT2G02050	NADH-ubiquinone oxidoreductase B18 subunit	1/1
AT2G17370	HMG-CoA reductase 2 (HMGR2)	3/1
AT2G25110	MIR domain-containing protein	2/1
AT2G32670	Synaptobrevin family protein	1/1
AT4G25650	Similar to cell death suppressor protein	4/2
AT5G56290	Peroxisomal targeting signal type 1 receptor	2/1
<i>Expressed in seed and spore</i>		
AT3G62730	Dessication related protein	56/1
AT1G62290	Aspartyl protease family protein	33/2
AT1G54870	Short-chain dehydrogenase/reductase (SDR) family protein, C-terminal similar to dormancy related protein	13/4
AT1G62710	Vacuolar processing enzyme specific to seeds	9/2
AT3G54940	Cysteine proteinase	5/7
AT5G50260	Cysteine proteinase	4/11
AT3G15670	Late embryogenesis abundant protein	2/10
AT5G03860	Malate synthase, strong similarity to glyoxysomal malate synthase	1/8
<i>Expressed in spore and pollen</i>		
AT2G21870	Expressed protein	-/4
AT2G32910	Expressed protein	-/4
AT5G20620	Ubiquitin polyprotein	-/4
AT1G04290	Thioesterase family protein	-/3
AT1G13950	Eukaryotic translation initiation factor 5A-1	-/3
AT1G23750	DNA-binding protein-related protein	-/3
AT3G51840	Short-chain acyl-CoA oxidase	-/2
AT1G51260	Acyl-CoA:1-acylglycerol-3-phosphate acyltransferase	-/1

¹Only abundance of seed and spore ESTs are shown because the set of pollen expressed genes was obtained from microarray data (Honys and Twell, 2003).

Table 3.4 Characteristics of three calcium signaling and homeostasis genes in *Ceratopteris richardii*. The percentage of identical or similar bases with each protein's best BLAST match is indicated.

Gene	cDNA length (nt)	Protein length (aa)	Identity/Similarity (%)
<i>CrCaM1</i>	801	149	91/98
<i>CrCPK1</i>	2368	522	64/78
<i>CrPro1</i>	814	133	60/76

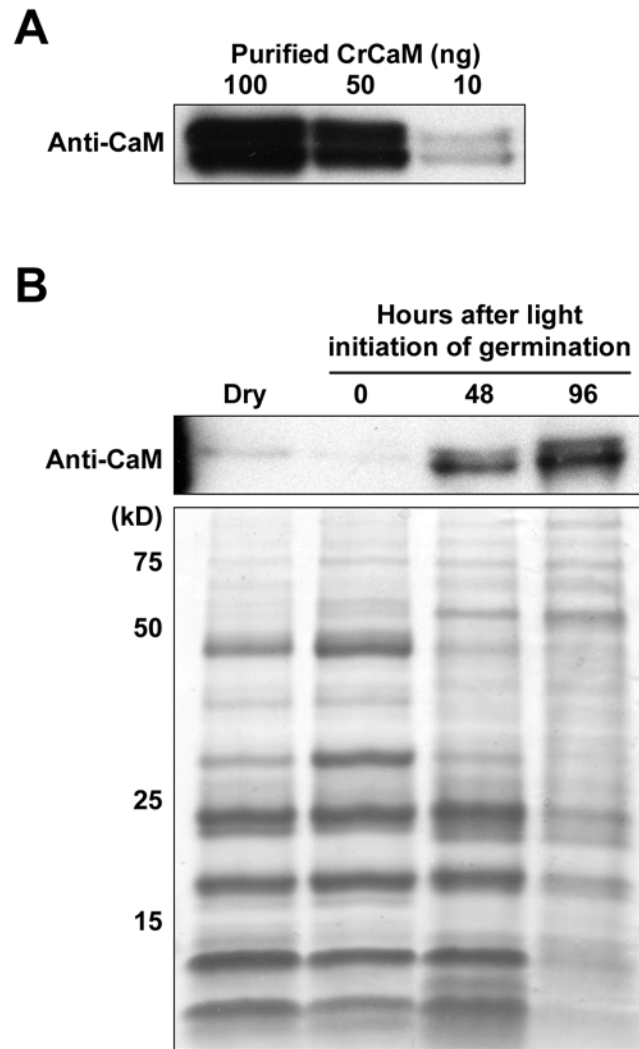


Fig. 3.6 CrCaM1 protein expression during spore germination. (A) Purified CrCaM1 is recognized by antibodies to bovine brain CaM. (B) CaM-enriched protein fractions collected from *Ceratopteris* spores at various times during germination. Equal loading shown by Coomassie staining gel after transfer.

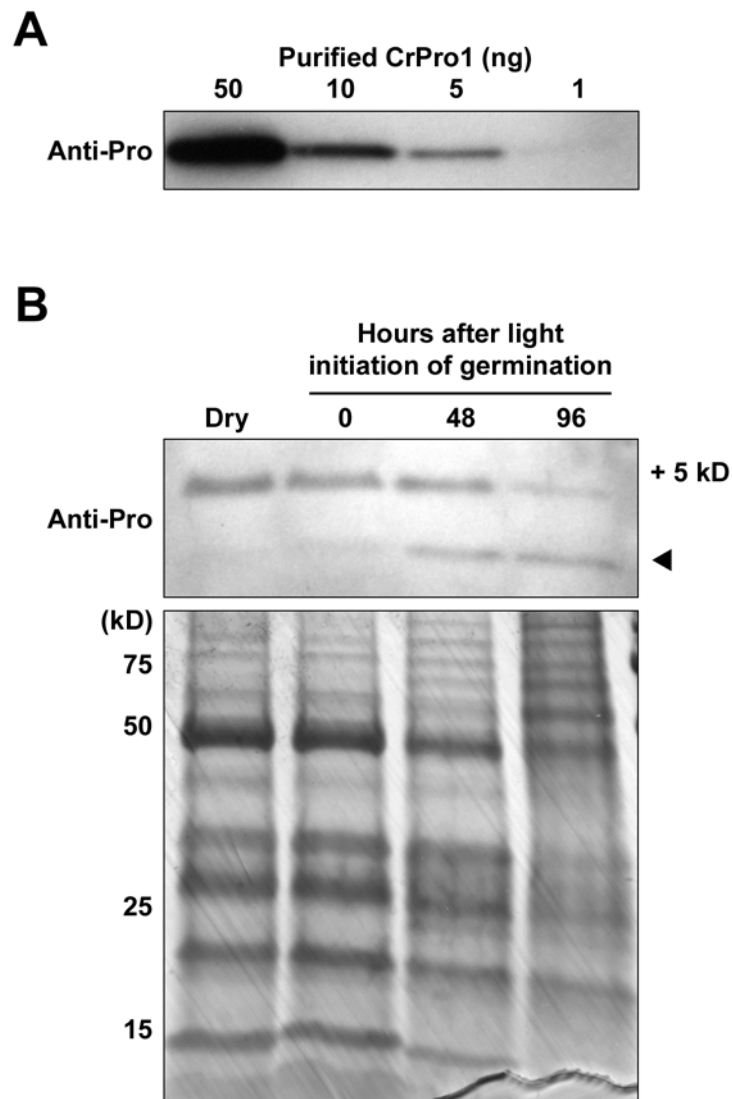


Fig. 3.7 CrPro1 protein expression during spore germination. (A) Purified CrPro1 is recognized by antibodies to *Zea mays* profilins. (B) Soluble protein extracts from *Ceratopteris* spores. The arrow indicates the expected position of the profilin protein determined from the molecular weight of the purified protein. The higher molecular weight band is approximately 5 kD larger in size than the expected size of CrPro1.

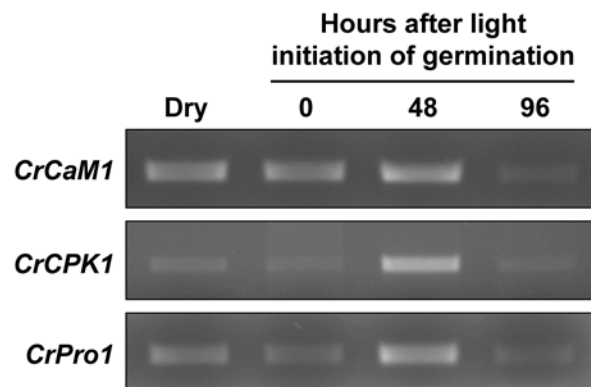


Fig. 3.8 Expression of calcium signaling genes during spore germination.
Gene expression at the transcript level as assayed by RT-PCR.

CHAPTER 4

TESTS OF AN RNAi SYSTEM IN *CERATOPTERIS RICHARDII*.

INTRODUCTION

RNA silencing or RNA interference (RNAi) has emerged as a convenient approach to knock-down or suppress the expression of genes in many eukaryotic systems. Conserved in eukaryotes, RNAi is initiated by the introduction of double stranded RNA (dsRNA) into the cell and results in the sequence-specific degradation of near identical endogenous RNA. The initially introduced dsRNA is processed into 21-25 bp length fragments, termed short interfering RNAs (siRNA), by Dicer, an RNase III-like enzyme. These siRNAs are subsequently loaded into an RNA-induced silencing complex (RISC) which mediates the degradation of matching cellular RNAs (reviewed by Hannon, 2002).

RNA silencing appears to be part of an ancient system employed in eukaryotic organisms for regulation of genes involved in development and defense against viruses or transposons. In the former case, genes encoding stem-loop RNAs, are transcribed and processed by Dicer to produce microRNAs (miRNAs) which regulate the expression of genes containing complimentary sequences either through translational suppression or message degradation (reviewed by Bartel and Bartel, 2003). In the latter case, viruses replicating through dsRNA intermediates may trigger the RNAi response, leading to the

targeting of viral gene products (Waterhouse et al., 2001). Additionally, endogenous RNA-dependent RNA polymerases (RdRP) may synthesize dsRNA from aberrant RNA transcripts produced by transposons or highly expressed transgenes, triggering the RNAi response and silencing of transcript (Vaistij et al., 2002).

RNAi has been used successfully as an experimental tool to knock down gene expression in many eukaryotic systems including *C. elegans*, *Drosophila*, and *Arabidopsis*. RNA silencing has also been used in another fern system, *Marsilea vestita*, to study the role of the cytoskeleton in microspore spermiogenesis (Klink and Wolniak, 2000; Klink and Wolniak, 2001; Tsai and Wolniak, 2001; Klink and Wolniak, 2003; Tsai et al., 2004). In the *Marsilea* system, dsRNA is simply added directly to the spore germination medium at the time of imbibition, and decreases in the abundance of targeted messages can be seen within 30 minutes of imbibition with dsRNA containing media (Tsai and Wolniak, 2001).

With the development of EST library described in Chapter 3, a reverse genetics approach to knock out or suppress the expression of known, sequenced target genes became feasible. Because of the relative ease of synthesizing dsRNA constructs and introducing the dsRNA into the spore, RNAi seemed attractive as a potentially rapid and efficient approach to suppress the expression of genes potentially involved in gravity perception and response in *Ceratopteris*.

MATERIALS AND METHODS

dsRNA Synthesis and RNAi Treatments

DNA templates for the transcription reactions were made by PCR using forward and reverse primers that included a 5' T7 RNA polymerase promoter sequence (5'-TAATACGACTCACTATAGGGAGACCAC-3'). The resulting DNA fragments included about 200 base pairs from the deduced open reading frame of the target gene, and were used for *in vitro* transcription reactions (Ampliscribe T7, Epicentre Technologies, Madison WI) in which both the sense and antisense strands were transcribed simultaneously and self annealed during the reaction incubation (Kennerdell and Carthew, 1998).

To introduce these dsRNAs into the *Ceratopteris* spore, 4 mg of dry, unsynchronized spores were surface sterilized spores and resuspended in 250 μ L liquid spore germination media (25 mM MES, pH 6.0; half-strength MS) containing the dsRNA (Klink and Wolniak, 2001). The spores were immediately placed in light to initiate the germination process, and unless otherwise indicated, allowed to develop for 24 h before RNA isolation for gene expression analysis.

Gene expression analysis

Total RNA was isolated from spores and used for RT-PCR as described in Chapter 3. The primers used for PCR are indicated in Table 4.1. Protein isolation and immunoblotting are also as described in Chapter 3.

Radiolabeled dsRNA experiments

Radiolabeled dsRNA for *CrPro1* was transcribed using *in vitro* transcription reactions as described above with the addition of α ^{32}P -UTP during transcription (PerkinElmer; Wellesley, MA). The resulting purified dsRNA was labeled to a specific activity of $0.117 \mu\text{Ci}\mu\text{g}^{-1}$. Prior to RNA isolation, spores were either extensively washed or used directly for RNA isolation as described above. Washing consisted of three rinses each of high (0.4 M NaCl) followed by low (0.004 M NaCl) salt solutions. After RNA isolation, activity was measured by liquid scintillation counting of duplicate aliquots of the resuspended RNA, and the mass of radiolabeled dsRNA present was calculated by comparison with a standard curve of the original purified dsRNA.

RESULTS

Sequence specific and concentration dependent suppression of gene expression assessed by RT-PCR

To test the whether or not inclusion of dsRNA in the germination medium could suppress gene expression, we synthesized dsRNA constructs for three calcium signaling genes previously cloned from *Ceratopteris* and assessed gene expression levels of spores treated with these dsRNAs by RT-PCR. Treatments with constructs of dsRNA derived from *CrCaM1*, *CrCPK1*, and *CrPro1*, all at a concentration of 0.1 mg mL^{-1} , specifically reduced amplification of the corresponding gene without altering the expression pattern of any of the other genes assayed (Fig. 4.1).

We next examined the concentration of dsRNA required to knock down the expression levels of the respective genes. While concentrations of dsRNA down to 0.01 mg mL^{-1} were effective in reducing amplification of the targeted gene, the most consistent suppression was seen at 10-fold higher concentrations of 0.1 mg mL^{-1} (Fig. 4.2).

Suppression can persist through polarity development in the constant presence of dsRNA

Because the major events in polarity development occur within the first 96 h of gametophyte development, the duration of the apparent suppression effect was examined. Spores continuously treated with dsRNA for CaM exhibit suppression through at least 120 h after light initiation of germination (Fig. 4.3).

In some systems, the RNAi demonstrates an ability to amplify or spread systemically from a localized introduction of dsRNA (Fire et al., 1998; Palauqui et al., 1997). We conducted pulse-chase type experiments, varying the dsRNA imbibition times and chasing by transfer to fresh dsRNA-free media for 24 h to determine whether or not the RNAi response persists in *Ceratopteris* spores in the absence of exogenous dsRNA. For incubations in dsRNA up to 36 h, transfer to dsRNA-free media relieves the reduced amplification of the targeted gene (Fig. 4.4).

Marsilea spores are permeable to large molecules for the first 3 h after imbibition, and dsRNA is apparently able to enter the spore during that time

period (Tsai and Wolniak, 2001). We tested to determine if *Ceratopteris* spores were similarly permeable to dsRNA for only a limited period of time by imbibing the spores for varying periods of time before addition of the dsRNA. Hydration of the spores for up to 36 h seemed to have no affect of the ability of exogenous dsRNA to prevent gene expression as assayed by RT-PCR (Fig. 4.4). Combined, these results suggest that *Ceratopteris* spores need a continual presence of dsRNA to suppress gene expression, and that regardless of the hydration status of the spore, the dsRNA is able to suppress gene expression.

Apparent suppression of gene expression does not result in a phenotype or observable reduction of protein levels

Despite the apparent complete suppression of steady state mRNA levels for the three calcium signaling and homeostasis genes described above, these treatments did not produce an observable, gene specific phenotype, even when a variety of imbibition strategies were tried (data not shown). Consequently, we examined expression of *CrCaM1* at the protein level, to determine if, despite apparent suppression of mRNA levels, the protein levels were in dsRNA treated spores were unaffected. Because dry spores already contain CaM protein (Fig. 4.5), we imbibed the spores in dsRNA containing media in the dark for up to 8 d in an effort to deplete the stored protein supply prior to initiation of germination. These treatments did not reduce protein expression levels in the spores (Fig. 4.5).

Success of RT-PCR depends upon location of PCR primers relative to dsRNA target

In contrast to the results presented above, other members of our lab observed a failure of exogenous dsRNA to suppress gene expression as assessed by RT-PCR. In the above experiments, the primer pairs used during RT-PCR spanned the dsRNA target region of the mRNA. However, the primer pairs used in these other experiments amplified a region entirely 5' of the dsRNA target, leading to the hypothesis that the position of the primers, relative to the dsRNA target region could affect the success of RT-PCR.

We tested this hypothesis by designing new primers for the 5' region of the *CrCaMI* transcript and comparing their performance with the primer we had been using above. While primers spanning the region of the mRNA targeted by the dsRNA failed to amplify *CrCaMI*, the primers 5' of this region were able to amplify *CrCaMI* from the same cDNA pool (Fig. 4.6A). As this was the method used to assess gene expression in the previous experiments, the former result would have been interpreted as a suppression of gene expression, while the later would have been interpreted as positive gene expression.

We further tested this hypothesis by varying the position of the dsRNA target between the 3' end of the open reading frame (ORF) and the 3' untranslated region (UTR). Regardless of the position of the dsRNA target, when primers spanning that region were used, they failed to amplify a product during PCR,

while primers outside of that region were successful using the same cDNA pool (Fig 4.6B).

Because the reverse transcription reactions utilized oligo-d(T) primers, cDNAs were synthesized from the 3' end of their mRNA templates towards the 5' end. Any degradation of the message in the 3' region of the transcript targeted by the dsRNA, even as part of a partial RNAi like response, would prevent synthesis of cDNA beyond that point. Therefore, the success of primer pairs at amplifying regions 5' of the dsRNA target sequence indicates that the mRNA templates for cDNA synthesis were present and intact.

Given the presence of complete cDNAs for the genes being assayed, these results focused our attention on the PCR step. The ineffectiveness of primers spanning the dsRNA target led to the hypothesis that exogenous dsRNA, leftover from the dsRNA treatment of the spores, inhibited the RT-PCR based analysis of gene expression specifically during the PCR step.

Exogenous dsRNA blocks RT-PCR

In order to test the hypothesis that exogenous dsRNA inhibited RT-PCR assessment of gene expression, we first tested how much dsRNA was typically leftover from RNA isolation. Using radiolabeled dsRNA and following the procedures used for the previous experiments, approximately 0.6 µg of the exogenously applied dsRNA was passed through during the RNA isolation, while virtually none remained if the spores were extensively washed prior to the RNA

isolation (Fig. 4.7B). Gel electrophoresis of the isolated RNA indicated that at 24 h, the input dsRNA was largely intact in the unwashed spores and confirmed the presence of virtually no dsRNA in the washed spores (Fig. 4.7A).

We next tested the effect of removal of the exogenous dsRNA, through washing the spores prior to RNA isolation, on PCR amplification of targets from dsRNA treated spores. Washing the spores prior to RNA isolation relieved the inhibition of PCR for primers spanning the dsRNA targeted region in dsRNA treated spores (Fig. 4.8). We also performed the converse experiment of adding dsRNA to untreated spores immediately prior to RNA isolation. The addition of 0.5 μ g dsRNA at this step resulted in inhibition of PCR similar to that seen in the above experiments (Fig. 4.8). These results indicate that carryover of exogenous dsRNA alone is capable of inhibiting RT-PCR amplification of homologous cDNA, generating the apparent of suppression of gene expression described above.

DISCUSSION

The data presented above lead to two conclusions: 1) the direct addition of dsRNA to the spore germination medium, in our hands, is not an effective approach in *Ceratopteris* spores for induction of RNA silencing and 2) dsRNA, homologous to target mRNA, interferes with RT-PCR based evaluation of gene expression.

In contrast to the relatively easy and direct induction of RNA silencing in *Marsilea*, the simple addition of dsRNA to the spore germination medium upon imbibition was ineffective with *Ceratopteris* spores. These results cannot be explained by the absence of the RNA silencing phenomenon in *Ceratopteris*. RNA silencing has recently been demonstrated in developing *Ceratopteris* gametophytes using biolistic delivery of an inverted repeat expressing construct (Rutherford et al., 2004). Bombardment of 6 d old gametophytes with constructs targeting genes involved in chlorophyll biosynthesis produced a colorless phenotype that spread throughout the gametophyte prothallus 7 days later in a high percentage of bombarded gametophytes.

Additionally, evolutionary studies into the involvement of miRNA regulation of plant development genes have indicated that this system of gene regulation also operates in *Ceratopteris*. A class-III homeodomain-leucine zipper transcript containing a region unusually well conserved among all land plant lineages has been identified in *Ceratopteris*. This region has been identified as a miRNA binding site and post-transcriptional cleavage within this region has been confirmed in *Ceratopteris* (Floyd and Bowman, 2004).

These reports indicate that both siRNA and miRNA mediated post-transcriptional gene silencing pathways are operational in *Ceratopteris*. However, the failure to induce silencing could be spore specific, rather than a general characteristic of the organism.

Given that direct addition of dsRNA to the spore germination medium has proven to be very efficient for induction of RNA silencing in *Marsilea* spores, it is unclear why this approach would not also work in *Ceratopteris*. However, there are substantial differences between *Marsilea* and *Ceratopteris* development that may be related to their different responses to treatment with dsRNA. *Marsilea* is a heterosporous fern, so both microspores (sperm producing spores) and megaspores (egg producing spores) are produced by the sporophytic generation, whereas in *Ceratopteris*, a homosporous fern, all spores are at least initially capable of production of both egg and sperm. Furthermore, *Marsilea* is endosporous, meaning that gametophyte development takes place almost entirely within the original spore cell wall, while *Ceratopteris* gametophytes are free-living and exosporic (Klink and Wolniak, 2000).

Gametophyte development also occurs much more rapidly in *Marsilea* microspores, undergoing 9 mitotic divisions within the first 5.5 h of germination (Klink and Wolniak, 2000). In contrast, the first cell division in *Ceratopteris* does not take place until 3 d after initiation of germination. The substantial difference in cellular activity during hydration and initiation of germination between *Marsilea* and *Ceratopteris* may result in differing abilities of the spores to import and/or process exogenous dsRNA.

Distinguishing between an inability to import exogenous dsRNA and an inability to process it may be difficult in *Ceratopteris*. From the radiolabelled

dsRNA experiments described here, although the amount of dsRNA retained in washed spores is not significantly different than zero, the mean (1.39 ng) still corresponds to the retention of hundreds of thousands of dsRNA molecules per spore. We expect only some proportion of these dsRNAs to genuinely be inside the spore, and additional experimental efforts might more solidly specify that proportion. However, given that only a few dsRNAs per cell can initiate RNAi in *C. elegans* (Fire and et al., 1998), it may be difficult to experimentally reduce the estimated number of intracellular dsRNAs per spore to a level below which RNAi should theoretically not occur, thereby defining the problem as one of dsRNA loading into the spore. Consequently, the best indicator that sufficient dsRNA import occurred might just simply be initiation of RNA silencing itself. However, without the certainty that RNA silencing is possible at this specific stage of development, the argument becomes circular.

Because the determinative events of gravity-directed polarity occur within the first 24 h of initiation of germination, the biolistic approach described above would not be a suitable gene silencing approach for our interest in *Ceratopteris*. Protoplasts generated from prothallial cells of mature gametophytes recapitulate the spore polarity development process, raising the possibility of generating “knockout” protoplasts from silencing induced gametophytes (Edwards and Roux, 1998b). However, these protoplasts are less responsive to gravity as an axis orienting signal.

The second novel finding from these studies was that the carryover of dsRNA through RNA isolation leads to the sequence specific suppression of RT-PCR amplification of expressed genes. These results imply that during PCR, the RNA strands anneal to their complimentary target cDNAs, preventing the polymerase from completing synthesis of a new daughter strand. dsRNA, with its ability to base pair with both strands of the DNA target, may be particularly effective at inhibiting amplification, because it would also be able to bind any second strand cDNA that was successfully synthesized.

Unintended heteroduplex formation can occur in competitive RT-PCR assays, in which coamplification of a known amount of competitor RNA is used as a reference to estimate the amount of target transcript present. Because the competitor and target sequences are typically identical, with the exception of a deletion within the competitor, heteroduplex formation between the competitor and target is often a problem in these assays, interfering with the accuracy and sensitivity of transcript quantification (Henley et al., 1996). In the situation presented here, however, the “competitor” dsRNA is not reverse transcribed and is not amplified during the PCR, but simply blocks amplification of the complete target transcript. The ability of dsRNA to prevent PCR in a sequence specific manner may have limited practical applications. For transcripts with splicing or allelic variants, dsRNA could enable specific amplification of one variant by preventing amplification of the second.

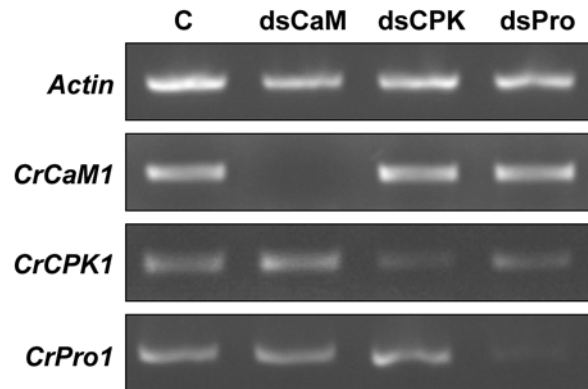


Fig. 4.1 Sequence specific suppression of gene expression in *Ceratopteris richardii* by treatment with dsRNA. Spores were treated with either 0.1 mg mL⁻¹ of various dsRNA constructs or were untreated, C for 24 h. The constructs targeted the gene for which expression was assessed.

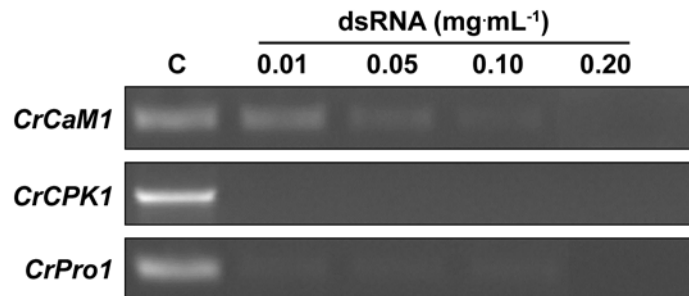


Fig. 4.2 Suppression of gene expression is concentration dependent. Spores with varying concentrations of the indicated constructs were treated for 24 h and gene expression was assayed with RT-PCR.

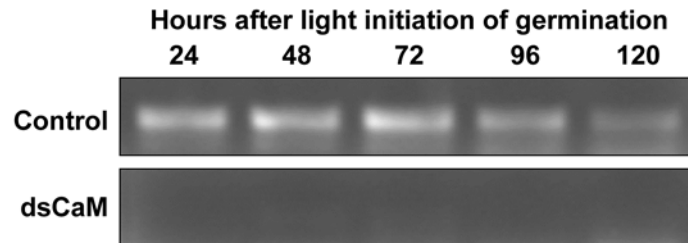


Fig. 4.3 Suppression of gene expression can persist through key polarity development timepoints. Spores were treated with continuously dsCaM or left untreated for varying times and gene expression was evaluated with RT-PCR.

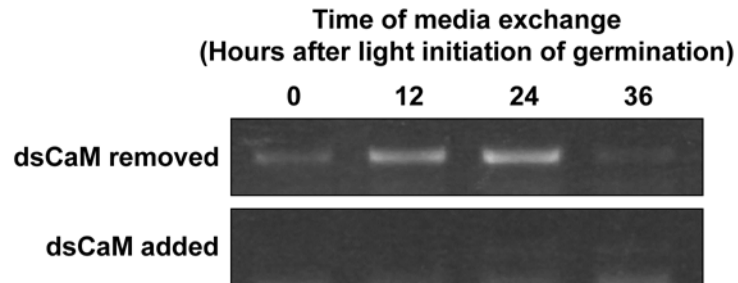


Fig. 4.4 Suppression requires constant presence of dsRNA. Spores were imbibed in media containing dsCaM, and at the indicated times the germination medium was removed and replaced with fresh media not containing dsRNA, or dsCaM was added to dsRNA-free media at the indicated times. Gene expression was assayed by RT-PCR 24 h after the media exchange.

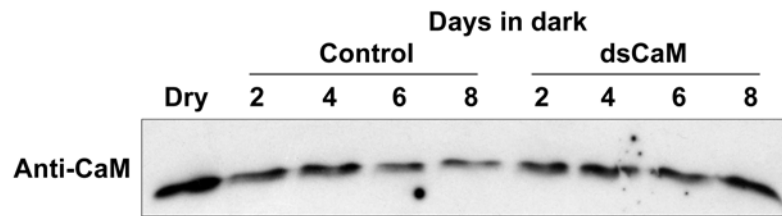


Fig. 4.5 Protein levels in response to dsRNA treatment. Spores were treated with $0.1 \mu\text{g } \mu\text{L}^{-1}$ dsCaM continuously or were left untreated in the dark for up to 8 days.

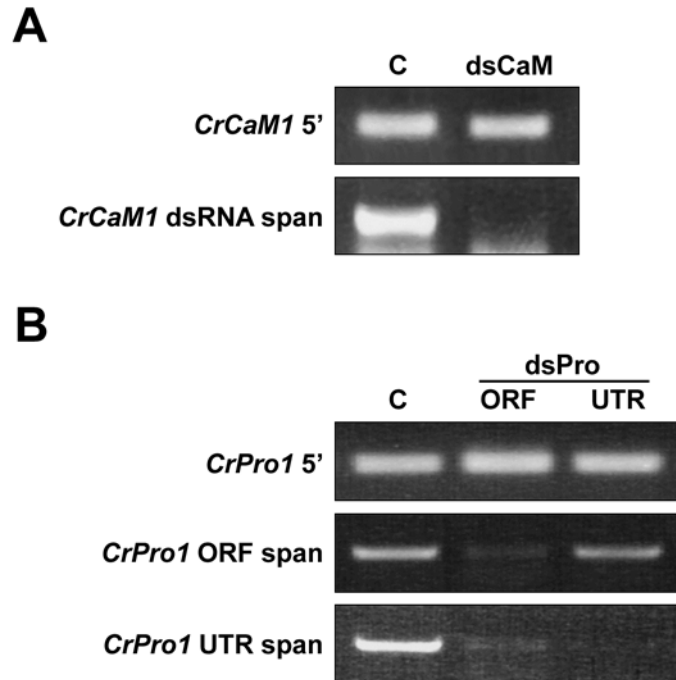


Fig. 4.6 Primers spanning the dsRNA fail to amplify the target cDNA. (A) Primers pairs for *CrCaM1* either 5' or spanning the dsRNA target region of the transcript were used to measure gene expression in response to treatment with dsCaM. (B) dsRNAs targeting two different regions of the *CrPro1* transcript were similarly tested with primer pairs overlapping neither, one or both dsRNAs.

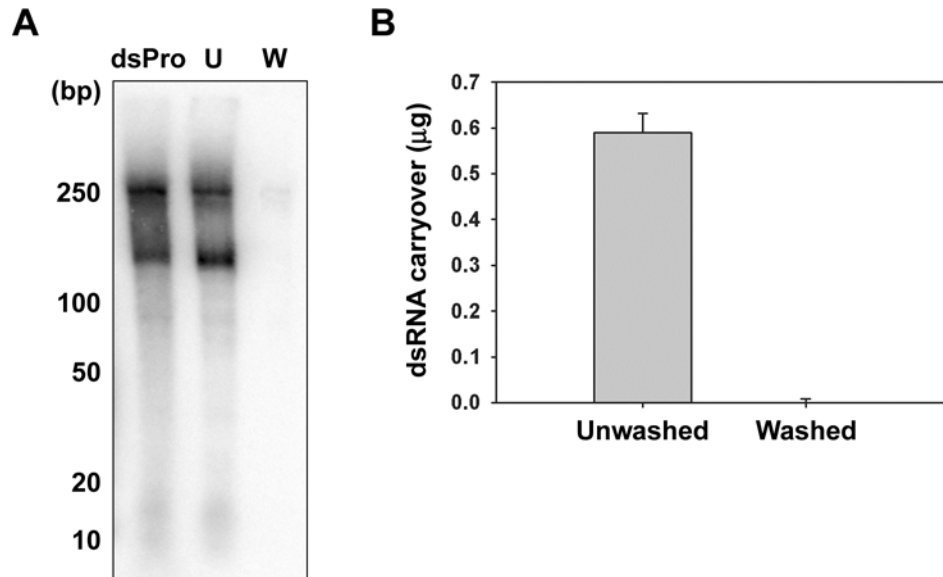


Fig. 4.7 Exogenous radiolabeled dsRNA experiments. (A) Gel electrophoresis of the radiolabeled dsRNA that was carried over after RNA isolation in (U) unwashed and (W) washed spores compared to (dsPro) 0.5 μg of dsRNA for *CrPro1* before imbibition. (B) Liquid scintillation counter quantification of carried over dsRNA in unwashed and washed spores.

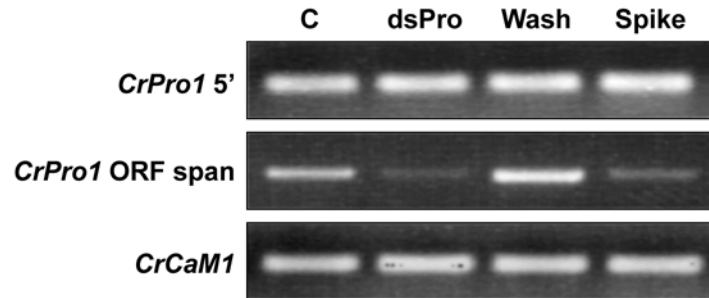


Fig. 4.8 Exogenous dsRNA inhibits RT-PCR amplification of expressed genes. RT-PCR analysis of gene expression for *CrPro1* using primers that either spanned the region targeted by the dsRNA (*CrPro1* ORF span) or were 5' of the targeted region (*CrPro1* 5'). Spores were either untreated (C), or treated with dsRNA targeting *CrPro1* (dsPRO) and washed prior to RNA isolation (Wash). dsRNA was also added to untreated spores immediately prior to RNA isolation (Spike).

Table 4.1 Primers used for RT-PCR experiments. Primer sequences are specified in the 5' to 3' orientation for both the forward and reverse primers

Amplicon	Forward	Reverse
CrCam1	ATGGCTGAGCAACTCACCCT	TCACCTTGAAAGCATCATCCTCA
CrCPK1	AATACATTTGGACGTGAAGAG	GAAACAGCCACGAAAAGCTGCT
CrPro1	ATGTCTTGGAATGGGTATGTTGA	GTACCAAAATCAAGAGCCGTTC
Actin	TGCATTGGACTATGAACAGGA	GTATGACGAGTCAGGGCCAT
CrCaM1 5'	ATGGCTGAGCAACTCACCCTG	ACTCAGCCTCGGTTGGATTCTG
CrPro1 5'	TGTCTTGGAATGCGTATGTTG	TAAGCCCACATAGCCTGGTCGT

CHAPTER 5

CONCLUSION

At the initiation of these studies, the primary limitation of the *Ceratopteris* system as a model for gravity perception and response was a lack of the molecular—genetic toolkit available to researchers in other systems. Consequently, we focused on enabling and developing those approaches in an effort to extend our understanding of the events of spore polarity development and germination.

The analysis of over 5000 ESTs provides sequence information for nearly 4000 genes expressed during germination of *Ceratopteris* spores. Prior to the release of this information, only a handful of genes had been described in *Ceratopteris* through a variety of individual approaches. The availability of this gene set enables a number of experimental approaches that were previously unavailable to researchers in the *Ceratopteris* system.

We utilized the library in two different approaches to learn about the development of germinating spores. Examining the library itself, we found that a surprisingly large number of genes may be expressed, especially considering that the ESTs represent genes expressed in a single cell approximately 24 h before division of that cell. Comparing the genes expressed in *Ceratopteris* spores to genes expressed in a physiologically related tissue, seeds, revealed conservation

of a set of seed storage, desiccation and dormancy related proteins between the two tissues. This is especially significant considering that fern spores represent the plant gametophytic generation, while seeds are part of the sporophytic generation in higher plants. The common set of dormancy related proteins suggests that the evolutionary switch from gametophytic dormancy in ferns to post-embryonic sporophytic dormancy in seed plants is accomplished by altering the timing of a discrete, conserved genetic program mediating dormancy.

The availability of gene sequence information enables targeted, reverse genetic approaches to investigate the function specific genes. Given the already described role of calcium signaling in *Ceratopteris* polarity development, we selected three calcium signaling genes for further characterization. The relatively direct, rapid cloning of these genes and their expression through spore germination validates the utility of the library as a rich source of expressed genes.

We next tried to develop an approach to modulate the expression of these genes to test their involvement in gravity-directed polarity development in *Ceratopteris*. Because of the success of RNA silencing as an approach to study the cytoskeleton in germination *Marsilea* spores, we attempted to develop a similar system of RNA silencing in *Ceratopteris* spores. The potential induction of RNA silencing in *Ceratopteris* spores through the simple addition of dsRNA into the germination medium, combined with the relative ease of *in vitro* synthesis

of dsRNA for any gene present in the EST library, made this an attractive, and potentially powerful approach for knock-out type studies.

RNA silencing, initiated using a biolistic approach, is now known to be effective in mature *Ceratopteris* gametophytes (Rutherford et al., 2004). We do not know why the direct introduction of dsRNA approach used here was not successful at a slightly earlier time point in development. Given the apparent ubiquity of RNA silencing in eukaryotes and its effectiveness in mature gametophytes, it would be surprising if germinating spores were unable to carry out RNA silencing. However, a failure to successfully introduce dsRNA into the spore may be the limiting factor in the extension of RNAi, as it is induced in *Marsilea*, to *Ceratopteris*.

Our experiments with dsRNA also led to the unexpected finding that exogenous dsRNA inhibits RT-PCR in a sequence-specific fashion. This result may have practical applications for distinguishing or preventing amplification of one of two similar transcripts. Many additional experiments need to be done to better understand specifically how dsRNA blocks RT-PCR amplification of transcripts.

Finally, we applied an electrophysiological approach to further characterize the polar calcium current. By reducing the timeframe in which measurements were made after reorientation of the spore, our results suggest that the components of the calcium current are distributed uniformly in or near the

plasma membrane and are locally activated in response to the gravity signal. This insight raises the possibility that the polar calcium current may be closely related to the gravity perception mechanism of *Ceratopteris* spores.

APPENDIX

MEASUREMENT OF ATP IN ARABIDOPSIS WOUND SITES

INTRODUCTION

Given the well established role of extracellular ATP and ADP (xATP and xADP) as signaling molecules in animal systems (reviewed by Ralevic and Burnstock, 1998), the possibility of extracellular nucleotide signaling in plants has recently been addressed in several studies (Lew and Dearnaley, 2000; Demidchik et al., 2003; Jeter et al., 2004). Data from these studies consistently indicate that sub-millimolar concentrations of xATP and xADP may indeed function as signaling molecules in plants.

The application of xATP or xADP has been shown to induce membrane depolarization in *Arabidopsis* root hairs (Lew and Dearnaley, 2000). In these studies ATP and ADP were effective at much different concentration ranges, approximately 250 μ M to 1.0 mM for ATP and 20 μ M to 100 μ M for ADP. Using the cytoplasmic Ca^{2+} reporter aequorin, Demidchik et al. (2003), found that application of xATP resulted in up to 70-fold increases in the cytoplasmic $[\text{Ca}^{2+}]$ in *Arabidopsis* roots. ATP concentrations as low as 300 nM caused measurable Ca^{2+} transients, and the response saturated at about 300 μ M. Similar results have been reported for whole seedlings with a range of responsiveness between 50 μ M and 800 μ M (Jeter et al., 2004). In addition to membrane depolarization and

induction of Ca^{2+} signaling, infiltration of sub-micromolar xATP into leaves can also induce the accumulation of superoxide, which, like Ca^{2+} , is involved in a number of physiological responses (Song and Roux, unpublished data). Additionally, both the Ca^{2+} and superoxide responses can be blocked by the application of animal purinoceptor inhibitors, raising the possibility of receptor mediated signaling for nucleotides in plants (Demidchik et al., 2003; Jeter et al., 2004; Song and Roux, unpublished data).

Application of xATP also results in downstream changes in gene expression. A number of genes involved in plant responses to wounding, touch or osmotic stress are upregulated in response to xATP treatment, including several MAP kinase family members and genes involved in ethylene and jasmonic acid biosynthesis (Jeter et al., 2004; Song and Roux, unpublished data). These observations, along with xATP induction of Ca^{2+} and superoxide signaling, invite the hypothesis that xATP signaling participates in the early steps of a variety of stress responses.

Any xATP signaling hypothesis, however, requires both the presence of ATP in the extracellular matrix (ECM) and a change in its concentration in response to a stimulus. Although the presence of this valuable “energy currency” outside of the cell is counterintuitive, relative increases in extracellular [ATP] have been documented in response to mechanical stimulation, osmotic stress, and wounding (Jeter et al., 2004; Song and Roux, unpublished data). However, the

absolute extracellular [ATP] in these studies is unknown, making it difficult to assess the physiological relevance of the sub-millimolar ATP treatments used to elicit the responses discussed above.

In an effort to complement the already documented relative changes in xATP, we carried out measurements of the absolute concentration of xATP present at *Arabidopsis* wound sites. These measurements suggest that significant quantities of ATP are present in the ECM after wounding and provide support for the hypothesis that xATP can act as a signaling molecule.

MATERIALS AND METHODS

Rosette leaves of mature *Arabidopsis* plants were detached, placed on a microscope slide, and wounded with a micropipette. Wounds were typically 3-4 mm long at the edge of the leaf and cut completely through the leaf.

Fluid from the wound site was collected with a micropipette positioned with a manual micromanipulator. The volume of fluid collected was calculated from the height of column of fluid in the micropipette and the measured dimensions of the tip of the micropipette. Fluid volumes typically ranged between 0.1 and 7.0 nl. Immediately after collection, the tip of the micropipette was snapped off in a 1.5 ml microcentrifuge tube and plunged in liquid N₂. Typically less than 3 minutes elapsed between wounding and freezing of the collected sample. A new wound site was created for each fluid collection, although the same leaf was used for more than one wound. Two to four

collections were pooled together in the same microcentrifuge tube and stored at -80° C for [ATP] determination.

The concentration of ATP present in each of the samples was determined using a bioluminescent detection reagent (ENLITEN rLuciferase/Luciferin; Promega, Madison WI). Frozen pooled collections were resuspended in 10 µl of sterile buffer (10 mM HEPES, pH 7.7), vortexed, spun down quickly, and transferred to a 12 x 50 mm test tube for measurement. 50 µl of rLuciferase/Luciferin Reagent was added to the resuspended sample and luminescence was measured in a luminometer (Turner Designs 20/20; Turner BioSystems, Inc., Sunnyvale CA) using a 2 s delay and a 10 s integration. The amount of ATP present in the sample was calculated from the measured relative light units (RLU) using a standard curve spanning the RLU range obtained from the samples, and the concentration of each sample was calculated by dividing the amount of ATP present by the volume of fluid collected.

The accuracy of this approach was validated by sampling from source pipettes containing known ATP standards. The methods for collection and concentration determination were the same as described above for the wound samples, and the calculated concentrations were compared against the known concentrations of the sample. The regression equation obtained from this comparison was used to correct the calculated concentrations obtained for the wound site samples.

RESULTS

The ATP concentrations of pooled nl volume samples of wound sap collected with a micropipette were measured using a bioluminescent detection reagent. To ensure the validity of the approach, samples of known ATP standards were collected and measured in the same manner used for the actual wound site samples and the calculated concentrations were compared against the known concentrations of the standard solutions (Fig. 1). The sampling and measuring procedure used here was linear over a range from 100 nM to 10 mM ATP and closely matched the actual values of the ATP standards over that range (Fig. 1). Fluid samples collected from *Arabidopsis* rosette leaf wound sites, measured with the same approach, had a mean ATP concentration of 15.86 μ M (Table 1).

DISCUSSION

Wounding is the most intuitively obvious source for ATP release into the ECM. Cytoplasmic ATP concentrations, across a variety of species, tissues and methods of analysis, have been reported to range from 50 μ M to 2.55 mM, with typical values of approximately 1 mM (Hampp et al., 1982; Blatt, 1987; Heinke et al., 1991; Farre et al., 2001; Borisjuk et al., 2003; Gajewski et al., 2003). Millimolar concentrations of ATP have also been reported in phloem sap of *Ricinus* seedlings (Geigenberger et al., 1993).

During wounding, at the moment of cell or phloem rupture, the surrounding tissue would be exposed to concentrations of ATP in the millimolar

range. The low micromolar concentrations reported here were determined from samples collected between 1 to 3 minutes after wounding. The substantial difference between our measurements and the millimolar levels of ATP in the cytoplasm and phloem is most likely due to the combined effects of nucleotide degradation (operating on the timescale of minutes) and dilution with the vacuolar compartment.

Additionally, we do not know the baseline or resting extracellular ATP concentration prior to wounding, making it difficult to gauge the absolute change that our measurements represent. However, because concentrations of ATP as low as 0.5 μM induce the accumulation of superoxide, we would expect the resting concentration in the ECM to be below this threshold (Song and Roux, unpublished data). If this were true, our measurements would represent at least a 30-fold change in extracellular [ATP] as a result of wounding.

The resting concentration of ATP could also be estimated with the present technique through repeated sampling of the wound site at defined intervals until a minimum, steady state level of ATP is reached. In addition, repeated sampling would provide data on the kinetics of ATP loss in the ECM, allowing an estimate of the maximal extracellular [ATP] experienced immediately after wounding. Nonetheless, our measurements of micromolar amounts of ATP in the extracellular fluid after wounding suggest that levels of ATP, within the range

that induces calcium and superoxide responses, persist for some period of time after wounding.

Aside from wounding, there are additional, less obvious, sources of ATP in the extracellular matrix. Plants may encounter xATP in the soil, where measurements of the [ATP] in a soil water fraction were approximately 40 nM (Thomas et al., 1999). The plant itself may also release ATP into the extracellular matrix. Plasma membrane proteins from the ABC transporter family can transport ATP into the extracellular matrix, and overexpression of an ABC transporter family member in *Arabidopsis* resulted in increased levels of ATP available on the surface of leaves compared to wild type plants (Thomas et al., 2000). Secretion of vesicles containing ATP has been shown to be a source of xATP in animal systems (Dubyak and El-Moatassim, 1993; Gordon, 1986), and this process could operate in plants as well. These mechanisms introduce the possibility of xATP acting as a signal of cellular activity which could be regulated in localized or spatial manner.

The technique used for the measurements reported here is obviously limited to the study of changes in [ATP] in response to wounding. Given the potential involvement of xATP signaling in other types of stress response, different techniques for ATP measurement need to be developed. Development of a non-invasive technique to measure the amount of ATP present in the ECM *in*

vivo would be a beneficial tool for investigating the dynamics of xATP signaling in a variety of physiological scenarios.

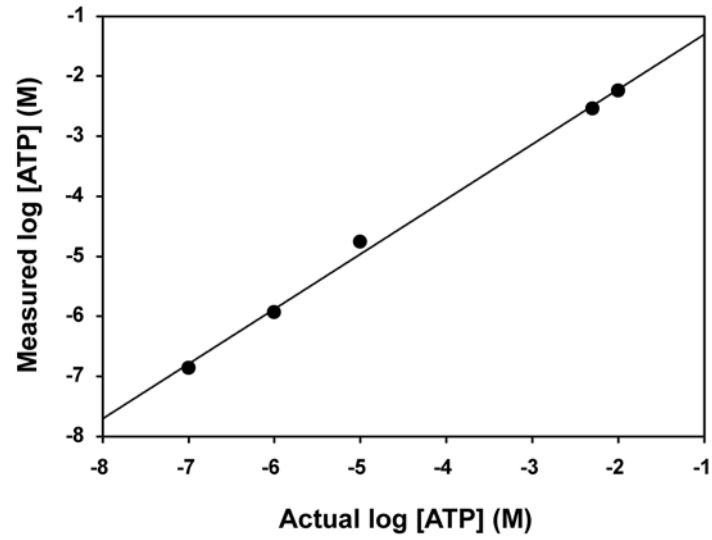


Fig 1. Validation of ATP measurement technique. The average of the measured concentration for each of the ATP standard solutions are plotted against the known, actual concentration ($1 \leq n \leq 3$). The regression equation of the measured versus actual concentrations ($[\text{ATP}]_{\text{measured}} = (0.9150 \times [\text{ATP}]_{\text{actual}}) - 0.3875$; $r^2 = 0.997$; $p < 0.0001$) was used to correct the samples from *Arabidopsis* wound sites.

Table I. Measurement of extracellular [ATP] at *Arabidopsis* wound sites. The [ATP] was determined for pooled nL samples of fluid collected from *Arabidopsis* leaf wound sites 1 to 3 minutes after wounding. For each of the samples, the calculated log molar concentration was corrected with the regression equation determined in Fig. 1 and converted to μM units for presentation.

Sample	[ATP] (μM)
1	4.61
2	5.27
3	0.98
4	45.86
5	25.31
6	23.13
7	5.88
Mean	15.86
Standard Deviation	16.34

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