J Plant Growth Regul (2000) 19:284–289
DOI: 10.1007/s003440000032

J P G R
Journal of Plant Growth Regulation

Ceratopteris richardii: A Productive Model for Revealing Secrets of Signaling and Development

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Abstract

Ceratopteris richardii is an aquatic fern grown in tropical and subtropical regions of the world. It is proven to be a productive model system for studies in the genetics, biochemistry, and cell biology of basic biologic processes that occur in early gametophytic development. It provides several advantages to biologists, especially those interested in gravitational biology, polarity development, and in the genetics of sexual development. It is easy to culture, has a relatively short life cycle, and offers an array of attractive features that facilitate genetic studies. The germination and early development of large populations of genetically identical spores are easy to synchronize, and both the direction of polarity development and cell-level gravity responses can be measured and readily manipulated within the first 24 h of spore development. Although there is no reliable transformation system available yet in *Ceratopteris,* recent studies suggest that the technique of RNA interference can be used to block translation of specific genes in a related fern, *Marsilea,* and current studies will soon reveal the applicability of this approach, as well as of other transformation approaches, in *Ceratopteris.* A recently completed expressed sequence tag (EST) sequencing project makes available the partial sequence of more than 2000 cDNAs, representing a significant percentage of the genes being expressed during the first 24 h of spore germination, when many developmentally interesting processes are occurring. A microarray of these ESTs is being constructed, so especially for those scientists interested in basic cellular phenomena that occur early in spore germination, the availability of the ESTs and of the microarray will make *Ceratopteris* an even more attractive model system.

Key words: Cell polarity; Expressed sequence tag; Fern gametophytes; Gravity responses

General Introduction

Ceratopteris richardii is an aquatic fern grown in tropical and subtropical regions of the world (Lloyd 1974), particularly in aquatic and grassy areas such as ponds, lagoons, marshes, rivers, ditches, and rice paddies (Tryon and Tryon 1982). It has been identified in Africa, Southeastern Asia, Japan, Australia, Fiji, and the Hawaiian Islands. Its highest concentration is in the Americas, where it is found mostly in the southern United States between Texas and Florida, in Mexico, Central America, and Argentina. It was once cultivated as a crop plant in the Philippines (Hickok and others 1995), but never became economically important. *Ceratopteris* is classified in the Class Polypodiatae (Figure 1) and placed either in its own familiy, Parkeriaceae, or included with members of the Pteridaceae (Cronquist and others 1966; Tryon and Tryon 1982). It is composed of four

Online publication 9 March 2001
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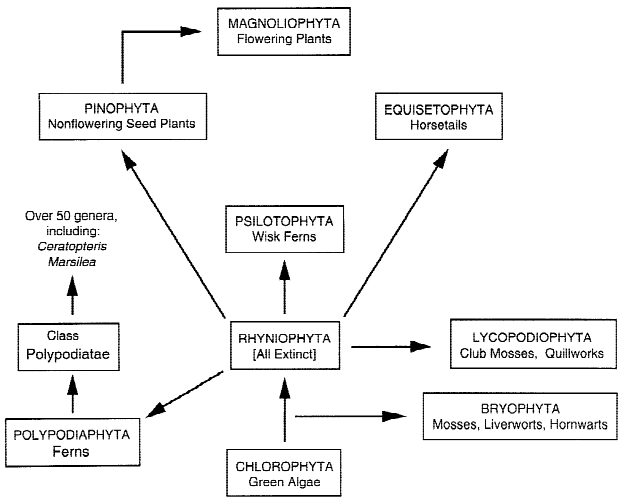

Figure 1. A proposed phylogeny of plants, adapted from the publications of the late Arthur Cronquist, showing the position of ferns and two genera of ferns featured in this special feature, *Ceratopteris* and *Marsilea.*

species, *cornuta, pteridoides, richardii,* and *thalictroides,* which differ primarily in terms of their geographic location and number of spores per sporangia. Unlike most classical ferns, which are perennial with a long-lived woody rhizome, *Ceratopteris* grows as an annual with a short upright rhizome. It has slender but strong dimorphic leaves and divergent branches. Young simple or trilobed fronds are sterile but become fertile and dissected as development progresses. Common to household aquariums, it is known as water sprite, and plastic representations of it are often sold as aquarium decorations.

Strengths of the *Ceratopteris* System

Ease of Culture: Relatively Short Life Cycle

C. richardii is relatively easy to culture. Tens of thousands of spores or gametophytes can be cultured on a 14-cm Petri dish, thus making large mutant screens possible. Spores remain viable for years and are amenable to long periods of storage. Spores are produced through meiosis on sporangia of fertile fronds, yielding approximately 16 spores/sporangia in *richardii.* Mature sporophytes produce approximately 1×10^6 spores from 1 plant in approximately 1 month. Large populations of spores can be generated from genetically identical sporophytes under controlled growth conditions.

Because of the small size of the adult sporophyte (typically less than 2 feet tall, spread over a diameter of less than 1 foot), a large number of plants can be propagated in a small growth chamber or greenhouse in standard pots under humid conditions. *Ceratopteris* sporophytes have an outstanding capacity for vegetative propagation. Buds found on senescing fronds can easily be grown into plants. A procedure has been developed for isolating protoplasts from the green aerial tissue of the gametophyte (prothallial cells). These cells can be cultured and are capable of regeneration (Edwards and Roux 1998a), following a developmental pattern similar to that of spores.

Many of the advantages of using *Ceratopteris* come from the features of its life cycle. The life cycle is relatively short, encompassing the gametophytic and the sporophytic stages in typically 120 days. *Ceratopteris* has a developmentally simple haploid gametophytic stage and a more complex vascular diploid sporophytic stage. The two stages exist independently of one another. Thus, either stage of the cycle can be studied without artificial manipulations. The development of the gametophytic stage progresses rapidly after the germination process is initiated on exposure to red light and water. Germination is first observed after 3 days with the emergence of the primary rhizoid. By 6 to 8 days a fully mature gametophyte is developed containing a prothallus with rhizoids, vegetative cells, and mature sexual organs. At this point, sexual differentiation occurs under the control of the mating pheromone antheridiogen. In the absence of antheridiogen, hermaphroditic gametophytes that have both the male and female sexual organs (antheridia and archegonia) develop rapidly and possess a meristem. They secrete antheridiogen to the slower developing spores, which will become ameristematic males. A sporophyte is formed when an egg is fertilized by swimming sperm that were released by antheridia. These sporophytes take about 3 months to mature and give rise to millions of spores.

Advantages for Genetic Studies

As reviewed by Chasan (1992), Hickok and colleagues (1995), and Banks (1999), the unique features of the life cycle of *Ceratopteris* offer numerous benefits to the geneticist. Haploid gametophytes can be selfed, thus sporophytes that are homozygous can be produced in just one generation. These sporophytes will give rise to genetically identical haploid spores. Crosses can be made easily to produce heterozygotes (Hickok 1985). Spores are subject to x-ray and chemical mutagenesis. Dominant and recessive mutations both give phenotypes in gametophytes, because the gametophytes are haploid. A variety of selection agents can be used, including growth regulators, toxins, herbicides, and extreme conditions such as temperature and oxygen stress.

Another aid in genetic analysis of *Ceratopteris* is dioecy—the separation of male and female individuals. This feature has been used to advance an understanding of the mechanisms underlying sex determination. Because the gametophyte can be controlled hormonally to develop as a male or as a hermaphrodite, it is readily amenable to experimental analysis of the genes that control maleness and femaleness (Banks 1994). Mutants have been identified in the areas of sex determination, photomorphogenesis, and environmental stresses (Banks 1999).

The preceding touches only briefly on the many advantages of *Ceratopteris* for genetic analyses. These themes are developed much more at length in an excellent review by Banks (1999) and in a series of papers published in the October 1995 issue of the *International Journal of Cytology* (Vol. 156, issue 3). The reader is referred to these recent reviews for more details on this topic.

Advantages for Studies of Biochemical Aspects of Development

Often, a limiting feature for biochemical studies on an organism is the quantity of tissue that is readily available. Certainly, for the sporophyte of *Ceratopteris* this is not a problem. One 5.5 sq ft shelf in a growth chamber can hold 24 plants, each of which can yield 40 g fresh weight of tissue. Hundreds of additional plants can be propagated vegetatively (see earlier) and grown into mature plants from cuttings in 3 months or less.

With the advent of improved techniques and hardware capabilities in gas chromatography-mass spectrometry, microsequencing, and nucleotide analyses, it has become feasible to biochemically analyze even the microscopic spores and gametophytes. This point can be illustrated with two brief examples from our laboratory. The first is that it was possible to construct a cDNA library from 1 mg total RNA isolated from 10 g of germinating spores that had developed for 24 h after irradiation. The yield of mRNA was 3 µg and the library had 3.8×10^7 colony-forming units with an average insert size of 1.5 kb. The company contracted to do the library (Life Technologies, Rockville, MD, USA) could have constructed the same library from one-tenth the starting amount of RNA. This library will be an invaluable resource for studying the biochemistry of mRNA expression, processing, and turnover in *Ceratopteris.* A second example is that we were able to purify from 60 g of 4-day-old gametophytes (grown from 40 g of dry spores) 8 µg of the calcium-binding protein annexin. This yield, in turn, was sufficient to

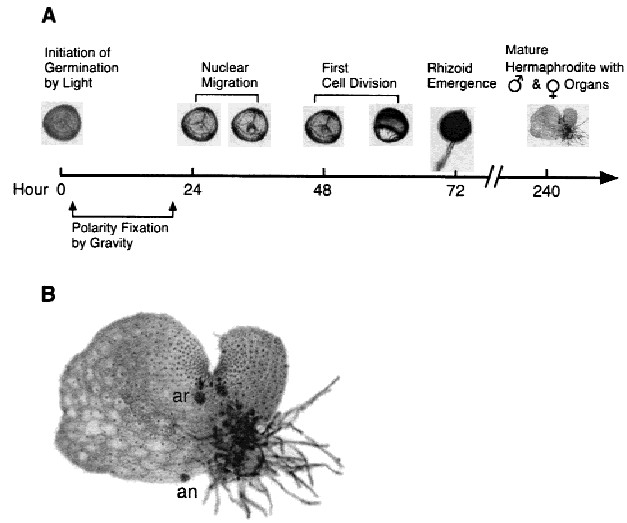

Figure 2. **(A)** Timeline of gametophyte development of *Ceratopteris richardii,* highlighting the stages of early polar development and of gamete development that have been the foci of studies among the laboratories currently using *Ceratopteris* as a model system for investigation. **(B)** Gametophyte at 8-d old stage showing the position of antheridia (an) and archegonia (ar).

generate peptides that could be microsequenced, yielding more than six different peptide sequences (H. Ren and S. Roux, unpublished). Similar results could have been obtained with half the amount of starting material.

A special advantage of using germinating spores for studying biochemical aspects of development is the ease of synchronizing their growth and development. Spores can be soaked in darkness for weeks and remain dormant until they are activated by light by means of the photoreceptor, phytochrome. When dark-soaked spores are irradiated with red or white light, more than 98% of them enter into a developmental pathway leading to nuclear migration, polarity development, the first cell division and differentiation of rhizoid and prothallus, all of which happens sequentially during the following 72 h (Figure 2). Very large populations of cells can be induced to progress through this pathway with a high level of synchrony, and this synchrony of development can be further improved by lengthening by up to a week the time the spores are soaked in darkness before being irradiated (E. Edwards, A. Chatterjee and S. Roux, unpublished). Chatterjee (1999) took advantage of this feature to document genes that were differentially expressed during the period of polarity fixation, which in *Ceratopteris* is directed by gravity and happens in the first 24 h after the spores are light activated (Edwards and Roux 1994).

For plant scientists studying biochemical aspects

of polarity development, gravity responses, phytochrome signaling, or cell division, being able to study these phenomena in a genetically and developmentally homogeneous, synchronously growing population of cells before their first mitosis offers significant advantages. Signal/noise ratios for any measured biochemical parameter will surely be much lower in these cells than in any of the multicellular plant systems often used for these studies.

Advantages for Cell Biological Studies

Many of the rewards of using *Ceratopteris* for cell biologic studies come from the attributes of the spore. *Ceratopteris* possesses a large spore, approximately 70–150 µm in diameter, which is the largest recorded among homosporous ferns. The spore is a single cell, thus many components of signal transduction pathways can be studied without the complexities of multicellularity. For example, Edwards and Roux (1994, 1998b) determined the influence of gravity and light on the direction of development both in single-celled spores and in protoplasts derived from prothallial cells of *Ceratopteris*. They found that the polarity of nuclear migration and subsequent cell division and rhizoid formation were directed more strongly by gravity than by light in germinating spores. More recently, Chatterjee and Roux used videomicroscopy on a NASA space shuttle flight to show that nuclear migration, polarity development, cell division, and rhizoid development all occurred normally in microgravity, although the polarity of this development was random in space in contrast to the gravity-directed "downward" polarity of these events on earth (Unpublished). Certainly the large size of the *Ceratopteris* spore made it a more attractive subject for the videomicroscopy analyses on the shuttle.

The large size of the spore also makes it readily subject to electrophysiologic studies. Chatterjee and others (2000) used a self-referencing calcium-selective electrode to record the net movement of calcium across the cell membrane at different positions around the periphery of the spore during the period in which the cell is sensing gravity. They found that germinating spores had a calcium current that predominately moved in at the cell bottom and out from the top. This movement was specific, polarized, and strongest in a direction that opposed the vector of gravity. The magnitude of this current was greatest in the middle of the period during which gravity fixed the polarity of the cell and fell to near baseline levels at the end of this period (Chatterjee and others 2000). Rotating the spore 180° rapidly reversed the direction of the current. Treatment with nifedipine, a calcium channel blocker, diminished the calcium current and caused the cell to lose its responsiveness to the orienting influence of gravity. These results strongly indicate that calcium plays a crucial role in the ability of a single cell to respond to gravity and in the subsequent establishment of its polarity, and they illustrate how readily amenable this model cell system is to electrophysiologic studies. Certainly, for studying the cellular basis of gravity effects, the rapidly reversible calcium current would have to rank as one of the earliest cell-level responses to changed orientation in plants.

Cell biology studies are certainly aided by cytologic staining procedures and by metabolic inhibitors that selectively interfere with certain metabolic pathways or signal transduction chains. Until recently, those using *Ceratopteris* spores as a single-cell model system were hampered by the relative impermeability of the coat of germinating spores both to applied reagents and to glass needles standardly used for microinjection experiments. Then it was observed that the coat of dry spores remains very permeable to reagents during most of the first hour of imbibition (A. Chatterjee, M. Salmi, and S. Roux, unpublished). We now know that dry spores readily take up such large reagents as actinomycin D, cycloheximide, and rhodamine phalloidin. Chatterjee (1999) took advantage of this feature to stain the actin cytoskeleton of *Ceratopteris* spores and follow its dynamic changes during the period preceding the spores' first cell division. Figure 3 illustrates not only the efficacy of rhodamine phalloidin staining of actin bundles but also the strong autofluorescence of the spore coat, a distinct disadvantage that is only partially overcome by confocal imaging. Salmi and Roux are now effectively using actinomycin-D and cycloheximide-treated spores to study the role of new mRNA and protein synthesis in gravity-directed polarity development in germinating spores. Klink and Wolniak (2000) recently described how dry spores of the fern *Marsilea* efficiently take up both antisense constructs and RNA interference constructs and how this feature can be used to achieve gene-specific blocks to translation. In the next section we will discuss current efforts to test whether dry spores of *Ceratopteris* can also take up polynucleotides and how this could help solve a key weakness of the *Ceratopteris* system.

Current Weaknesses of the System (. . . but stay tuned.)

As the reader will readily appreciate, breakthroughs in technology are a frequent occurrence, and road-

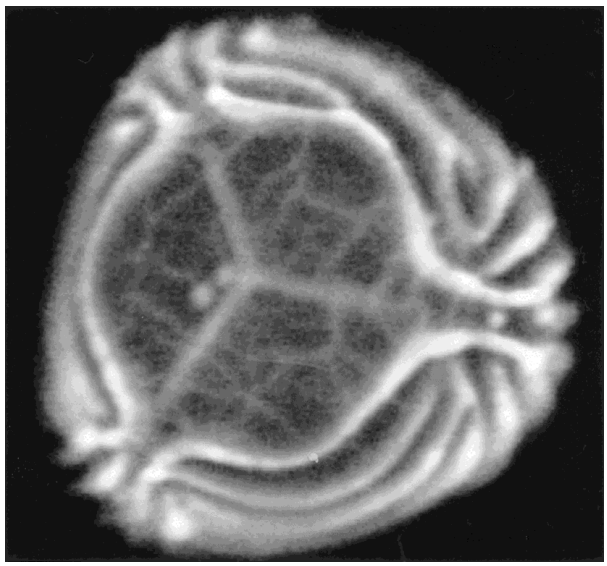

Figure 3. Confocal image of a *Ceratopteris* spore stained with rhodamine phalloidin 10 h after activation of germination by light. The depth of field of this image is about 10 µm, and the network of thin actin bundles can be visualized in the center of the image just below the brightly autofluorescent spore coat.

blocks that seem insurmountable today may be of no consequence tomorrow. In this context, we review here two current limitations of the *Ceratopteris* system, knowing full well that within a year of the publication of this review one or more of these limitations may have been overcome. Nonetheless, at present, these limitations reduce the attractiveness of *Ceratopteris* as a model system and overcoming them will surely encourage more plant scientists to use it to solve basic problems in genetics, cell biology, and development.

No Reliable Transformation System Yet

Antisense transformation and T-DNA knockout analyses have dramatically increased our knowledge of gene function in *Arabidopsis,* maize, and many other plants. As of this writing, successful stable transformation in *Ceratopteris* has not been achieved. Certainly the focus of the relatively few laboratories using *Ceratopteris* as a model system has thus far been developmental, cytologic, and genetic studies rather than transformation studies. Thus, the lack of a reliable transformation system may be related as much to a lack of an intense, concerted effort, as to any intrinsic technical problem with transforming this fern. Indeed, some attempts have been made to transform the fern, but because these attempts have not resulted in dramatic successes as yet, their documentation is anecdotal rather than in the published literature. Here we will recount the unpublished efforts of our laboratory, which likely resemble those of others in the field.

Chatterjee has tested the particle bombardment method in spores and gametophytes, using two constructs, PBI221 (cauliflower mosaic virus promoter) and pACT-D1 (actin promoter), both linked to a GUS reporter gene. Both constructs gave significant transient expression staining in both tissues. The actin promoter was stronger, giving a higher level of GUS staining. Follow-up verification experiments with constructs carrying fern genes have not yet been completed, but the initial reporter gene findings seem promising, at least for achieving transient expression. Although stable transformation is certainly preferred, transient transformation can be useful for assaying the role of genes in functions that occur during a defined short period of time.

As mentioned earlier Klink and Wolniak (1999, 2000) have reported success in using double-stranded RNA to direct gene-specific posttranscriptional silencing in the water fern *Marsilea vestita.* This method of directly inhibiting gene function at the RNA level without knockout genetics is known as RNA interference (RNAi), and it has been used successfully in many organisms, including higher plants, mammals, zebra fish, fruit flies, nematodes, planaria, and trypanosomes (Fire 1999). Klink and Wolniak (2000) found that single and double-strand pieces of RNA up to 450 nt long were quickly taken up into the cytoplasm of *Marsilea* gametophytes at the time of spore imbibition and that RNAi effects were gene-specific and concentration-dependent. Specifically, they found that translation of the protein centrin, which is important for basal body synthesis, could be blocked by the presence of double-stranded RNA encoding a part of centrin.

At the time of this writing, our laboratory is testing whether dry *Ceratopteris* spores are as efficient as dry *Marsilea* spores in taking up double-stranded RNA. We are encouraged to carry out these tests by our results showing that *Ceratopteris* spores readily take up rhodamine phalloidin and other large molecules. Surely, if RNAi can be made to work in *Ceratopteris* as it does in *Marsilea,* the disadvantage of not having a transformation system in *Ceratopteris* will become of little consequence, and *Ceratopteris* will become an even more attractive model system for cellular, biochemical, and genetic studies.

Little Sequence Information

The completion of the *Arabidopsis* genome sequencing project this year will surely make this humble

weed an even more attractive model system for plant scientists. In contrast to this most valuable asset in *Arabidopsis,* there is very little sequence information on *Ceratopteris* genes. Several cDNA libraries have been constructed (for example, Münster and others, 1997; J. Banks, unpublished; S. Roux, unpublished), and recently a small EST project was completed at Purdue University (J. Banks and S. Roux, unpublished), providing about 3000 partial cDNA sequences from a library based on RNA isolated from germinating *Ceratopteris* spores 24 h after they were induced to germinate by light. Over 2,100 different genes were represented on the list of ESTs. A selection of some of the genes on this list that are considered important for signal transduction would include calmodulin, annexin, CDPK, CKII, 14-3-3, and G-proteins, to name just a few. All the partial cDNAs sequenced represent genes that were being expressed at a time when the polarity of the cell has been fixed by gravity (Edwards and Roux 1994), and all of the earliest transcription responses induced by phytochrome would have been activated (Cooke and others 1995). A microarray of these ESTs is currently being constructed at the University of Texas. The point to be made here is that, although the absolute number of gene sequences on this list of ESTs is low, the ones that are there would represent a significant percentage of the genes involved in polarity fixation and graviresponsiveness, as well as a large number of phytochrome-regulated genes. Thus, for those scientists interested in these phenomena, the availability of the cDNA clones and sequences and of the microarray will make *Ceratopteris* an even more attractive model system.

ACKNOWLEDGMENTS

We thank Homer Luna for expert technical assistance in growing *Ceratopteris* sporophytes, Hong Ren for providing valuable information on protein analyses, and Stephen Stout for helping to assemble Figure 1. Much of the authors' work discussed in this review was made possible by research grants to SJR from NASA (NAG2-1347 and NAG10-0202). AC was supported by a NASA training grant (NGT5-50099) and by a David J. Bruton Scholarship.

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