

**UNRAVELING THE MOLECULAR
PHOTOBIOLOGY OF THE EMERGING MODEL FERN,
*CERATOPTERIS RICHARDII***

by

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**A dissertation submitted to the Graduate Faculty in Biology in partial
fulfillment of the requirements for the degree of Doctor of Philosophy,
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ABSTRACT**UNRAVELING THE MOLECULAR PHOTOBIOLOGY OF THE EMERGING
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Advisor: Professor Timothy Short

A plant's survival depends on its ability to detect and react to light-mediated changes in its environment. One type of photoreceptors that enables a plant to modify its development and structure in response to light is the red light and far-red light photoreceptors, the phytochromes. Although many functions of phytochrome in mosses and angiosperms have been molecularly characterized, the molecular biology of phytochromes in ferns and gymnosperms are obscured by their relatively long lifecycles and the lack of available tools to knockout or knockdown specific target genes.

Ceratopteris richardii is a recently evolved polypod that is emerging as a model fern to study the process of photomorphogenesis because of its rapid lifecycle and the ability to reduce the expression of targeted genes in dry spores by RNA interference (RNAi). Previous physiological studies of photomorphogenesis in *Ceratopteris richardii* have provided circumstantial evidence that phytochromes and cryptochromes (blue light photoreceptors) are involved in the regulation of spore germination and gametophyte development, although no photoreceptors have been molecularly identified. In this study, five phytochrome sequences have been isolated: *CrPHY1A*, *CrPHY1B* (partial cDNA), *CrPHY2*, *CrPHY4A* and *CrPHY4B*. *CrPHY1B* and *CrPHY4A* have resulted from recent duplications, possibly by retro-transposition and gene conversion or homologous

recombination. Phylogenetic analyses show that the *PHY1* and *PHY2* genes of ferns evolved very early in the history of vascular plant, and that their divergence preceded the separation of ferns from seed plants. During the evolution of the leptosporangiate ferns, a gene duplication occurred coinciding with angiosperm radiation (approximately 260 mya), producing the orthologues *PHY2* and *PHY4*. Consistent with the phylogenetic analyses, physiological studies demonstrate that the responses mediated by the phytochromes of ferns show limited similarities to those of the phyA and the phyB of angiosperms. The phytochrome multigene family of *Ceratopteris richardii* act synergistically (perhaps redundantly) and the absolute dosage of the Pfr form of phytochrome regulates germination, cell division, rhizoid formation and chlorophyll production. Gene duplication of phytochromes in ferns and positive selection may have allowed some group of ferns to attain their current diversity as a response to the diversification of flowering plants. Further physiological and molecular studies have also shown that specific cryptochromes (e.g. *CrCRY4*) act antagonistically to phytochromes to regulate germination and prothallar development, while others may act synergistically with phytochromes to allow for greater development in specific light conditions.

TABLE OF CONTENTS

List of Tables.....	vii
List of Illustrations, Charts, Diagrams.....	viii
CHAPTER 1.....	1
CHAPTER 2.....	43
CHAPTER 3.....	81
CHAPTER 4.....	109
Tables and Figures.....	140
Appendix.....	210
Bibliography.....	218

LIST OF TABLES

Table1. Comparisons of unique amino acid differences between PHY1 and PHY2 of <i>Adiantum capillus veneris</i> (Ac) and <i>Ceratopteris richardii</i> (Cr).....	164
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LIST OF ILLUSTRATIONS, CHARTS, DIAGRAMS

CHAPTER 2

Figure 1	Sequence comparison of the phytochrome multigene Family in <i>Ceratopteris richardii</i> (Cr) with <i>Arabidopsis thaliana</i> (At) PHYA, PHYB and <i>Avena sativa</i> (As) PHYA using CLUSTALW.....	140
Figure 2	Nucleotide sequence comparison of <i>PHY1</i> , <i>PHY2</i> and <i>PHY4</i> from <i>Adiantum capillus-veneris</i> (Ac) and <i>Ceratopteris richardii</i> (Cr) using CLUSTALW.....	144
Figure 3	Comparison of the hydropathy plots of <i>Ceratopteris richardii</i> (Cr) PHY1 and PHY2 with those of <i>Arabidopsis thaliana</i> (At) PHYA and PHYB.....	154
Figure 4	CLUSTALW nucleotide alignment of the 3' end of the mRNA of <i>CrPHY4</i> and the phytochrome genomic DNA sequences of <i>PHY1</i> (AB01651), <i>PHY2</i> (AB016231) and <i>PHY4</i> (AB003364) <i>Adiantum capillus-veneris</i>	155
Figure 5	Comparison of <i>CrPHY4A</i> and <i>CrPHY4B</i> using CLUSTALW.....	156
Figure 6	Comparison of a phototropin-related fragment isolated from <i>Ceratopteris richardii</i> (CrPHOT) with <i>Adiantum capillus veneris</i> (Ac) PHOT1 (BAA95669), <i>AcPHOT2</i> (BAD16729) and <i>AcPHY3</i> (BAA36192) using CLUSTALW.....	158
Figure 7	Southern hybridization analysis of <i>Ceratopteris</i> genomic DNA....	159
Figure 8	Phylogenetic analysis of phytochromes isolated from <i>Ceratopteris richardii</i> using the Neighbor Joining Method.....	160
Figure 9	Phylogenetic analysis of cryptogam phytochromes using the Neighbor Joining Method.....	161
Figure 10	CLUSTALW alignment showing conserved intron position of fragments of <i>PHY1</i> and <i>PHY2</i> from the genomic DNA (g) of <i>Adiantum pedatum</i> (Ap), <i>Adiantum tenerum</i> (At), <i>Ceratopteris richardii</i> (Cr), <i>Ceratopteris thalictroides</i> (Ct), <i>Pteris cretica</i> and <i>Pteris multifida</i> (Pm).....	162
Figure 11	Phylogenetic analysis of the C-terminal end of <i>PHY1</i> and <i>PHY2</i> from <i>Adiantum capillus veneris</i> (Ac), <i>Adiantum pedatum</i> (Ap), <i>Adiantum tenerum</i> (At), <i>Ceratopteris richardii</i> (Cr), <i>Ceratopteris thalictroides</i> (Ct), <i>Pteris cretica</i> (Pc) and <i>Pteris multifida</i> (Pm) using the Neighbor Joining Method.....	165
Figure 12	RT-PCR of RNA isolated from 7 day-old gametophytes grown in continuous white (W), red (R), far red (FR), blue (B) light or from gametophytes that were exposed to white light for 1 day then grown in the dark (D) for 6 days.....	166

CHAPTER 3

Figure 13	Semi-quantitative RT-PCR of <i>CrPHY1</i> , <i>CrPHY2</i> and <i>CrPHY4B</i> from spores that were dark imbibed in <i>C-fern</i> media for 1 or 3 days.....	167
Figure 14	Percent germination of spores after dark imbibed for 1 or 3 days.....	168
Figure 15	High temperature and high fluence red (R) light inhibit germination of spores in <i>Ceratopteris richardii</i>	169
Figure 16	Prothallus development in darkness (D) or under continuous far-red (FR), red (R), blue (B) or white (W) light.....	170
Figure 17	Mean cell size of prothalli grown under different light regimes.....	171
Figure 18	Number of rhizoids and the relative amount of chlorophyll accumulation in gametophytes grown in continuous far-red (FR), red (R), blue (B), or white (W), or in darkness (D).....	172
Figure 19	Development of gametophytes in white light (5 days) after 14 days of continuous exposure to R or FR.....	173
Figure 20	The length of the primary rhizoids in darkness (D) or under continuous far-red (FR), red (R), blue (B) or white (W) light.....	174
Figure 21	RNAi of <i>CrPHY</i> in spores.....	176
Figure 22	RNAi stimulates of the transcript accumulation of <i>CrPHY1</i> , <i>CrPHY2</i> and <i>CrPHY4B</i>	177
Figure 23	The effect of dsRNA on the germination of spores in W_c or B_c	178
Figure 24	Development of the prothalli from spores treated with dsRNA in continuous white light.....	180
Figure 25	Development of the prothalli from spores treated with dsRNA in continuous white or red light.....	181
Figure 26	Chlorophyll accumulation in W_c -grown RNAi-treated prothalli.....	183
Figure 27	Development of the prothalli from spores treated with dsRNA in continuous red light.....	184
Figure 28	Blue light exposure after germination stimulates cell division and development.....	186
Figure 29	Semi-quantitative RT-PCR of <i>CrCRY4</i> from 7-day-old gametophytes grown in continuous white (W), red (R), far-red (FR), or blue (B) light, or from gametophytes that were exposed to white light for 1 day then grown in the dark (D) for 6 days.....	187
Figure 30	RNAi of <i>CrCRY4</i> in spores.....	188
Figure 31	dsRNA for <i>CrCRY4</i> stimulates germination of spores in B_c	189
Figure 32	dsRNA for <i>CrCRY4</i> stimulates prothalli development and chlorophyll production in W_c	190
Figure 33	Semi-quantitative RT-PCR of <i>CrPHY1</i> , <i>CrPHY2</i> , <i>CrPHY4A</i> and <i>CrPHY4B</i> from wild type <i>RNW1</i> and <i>dkg1</i> spores that were dark imbibed in <i>C-fern</i> media 5 d.....	192

CHAPTER 4

Figure 34	The isolation of putative light signaling factors from the EST library of <i>Ceratopteris richardii</i>	193
Figure 35	The isolation of putative light signaling factors by PCR amplification.....	196
Figure 36	RT-PCR of RNA isolated from 7-day-old gametophytes grown in continuous white light.....	200
Figure 37	Putative physical interaction of <i>CrNDPK</i> with the C-termini of <i>CrPHY1</i> and <i>CrPHY2</i>	201
Figure 38	Putative physical interaction of <i>CrPP2A</i> with the C-termini of <i>CrPHY1</i> and <i>CrPHY2</i>	202
Figure 39	Verification of yeast two-hybrid interaction.....	203
Figure 40	Presumptive phylogenetic analyses of <i>CrPP2A</i> isolated from <i>Ceratopteris richardii</i> using the Neighbor Joining Method.....	204
Figure 41	Semi-quantitative RT-PCR of <i>CrNDPK</i> and <i>CrPP2A</i> from 7 day-old gametophytes grown in continuous white (W), red (R), far red (FR), blue (B) light or from gametophytes that were exposed to white light for 1 day then grown in the dark (D) for 6 days.....	205
Figure 42	Development of the prothalli from spores treated with dsRNA in continuous white.....	206
Figure 43	RNAi of <i>CrCOP1</i>	207
Figure 44	Development of the etiolated prothalli from spores treated with dsRNA.....	208
Figure 45	dsRNA for <i>CrCOP1</i> stimulates chlorophyll production in W_c	209

CHAPTER 1: INTRODUCTION

- A. The Role of Light in Plant Life**
- B. The Phototropins**
- C. The Cryptochromes**
- D. The Phytochromes**
- E. Types of Phytochrome**
- F. Types of Phytochrome Responses**
- G. General Structural and Functional Domains of Phytochromes**
- H. Prokaryotic Phytochrome and Phytochrome Related Proteins**
- I. Fine Mapping of the Functional Regions of Plant Phytochrome**
- J. Phytochrome Localization**
- K. Plant Phytochromes**
- L. The Emergence of *Ceratopteris richardii***
- M. Life Cycle of *Ceratopteris richardii*.**
- N. Some Strengths of *Ceratopteris* as a Model System**
- O. Taxonomy of *Ceratopteris***
- P. Photobiology of Ferns**
- Q. Conclusions**

A. The Role of Light in Plant Life

Throughout its life cycle, the sessile plant must alter its physiology, morphology and development to withstand the barrage of environmental insults and stresses placed upon it. One of the major environmental stimuli affecting a plant in virtually every respect is light. Besides its role as the primary source of energy for a plant during photosynthesis, light provides a plant with positional information (e.g., sensing neighboring plants) and controls the mechanisms that underlie photomorphogenesis (growth in response to light), phototropism, circadian rhythms, and flowering time (Neff et al., 2000). In seedling development, the effect of light is dramatically exemplified in the phenotypes associated with skotomorphogenesis (growth in complete darkness) and photomorphogenesis. Seedlings grown in the light have short hypocotyls, open and expanded cotyledons, and differentiated chloroplasts, and express light-inducible genes. In contrast, dark grown seedlings have long hypocotyls, closed cotyledons, etioplasts, and an apical hook (von Arnim and Deng, 1996).

The ability to detect intensity, direction, spectral quality and periodicity of light, as well as interpret and transduce these light signals, requires the presence of multiple photoreceptors. These photoreceptors, extensively characterized in higher plants such as *Arabidopsis thaliana*, include the phytochromes (PHYA, B, C, D and E; (Sharrock and Quail, 1989), cryptochromes (CRY1 and CRY2; (Ahmad and Cashmore, 1993), phototropins (PHOT1 and PHOT2; (Short and Briggs, 1994; Huala et al., 1997; Imaizumi and Wada, 2001) and the molecularly uncharacterized UV-B receptors. Each group of photoreceptors is classified by the predominant wavelength of light absorbed. Phytochromes absorb light primarily in the 600nm-750 nm range (red and far red light,

respectively), with some absorption in the blue and long ultraviolet region (350nm-500nm), and the cryptochromes and the phototropins absorb light predominantly in the 350-500 nm region of the electromagnetic spectrum.

B. The Phototropins

To optimize photosynthesis, plants have evolved a variety of responses such as the photorelocation of chloroplast for optimum light capture, the differential stem growth towards a unidirectional light (phototropism), and the opening of the stomata for gas exchange (Chen et al., 2004). In *Arabidopsis thaliana*, these responses are mediated by blue light that is detected in part by two photoreceptors, PHOT1 and PHOT2. PHOT1 is credited for responses to low fluence rates of blue light and PHOT2 is responsible for reactions to higher fluence-rate blue light, such as photoprotective chloroplast migration. In high fluence rate blue light, there is also a redundancy of activity displayed by the both phototropins. In the fern *Adiantum capillus veneris*, phototropism and chloroplast photorelocation is mediated by red and blue light. In addition to having PHOT1 and PHOT2, which are similar to those of *Arabidopsis*, *Adiantum* also has an unconventional photoreceptor, PHY3. The *PHY3* gene encodes a chimeric protein with its amino-terminus resembling a phytochrome-like chromophore binding domain and a complete phototropin as its carboxy-terminus (Nozue et al., 1998). *PHY3* has been described in only ferns of the polypodiaceae, such as *Dryopteris-felix mas*, *Onoclea sensibilis*, *Pteridium aquilinum* and *Matteuccia struthiopteris*. Mutant analyses show that *PHY3* is responsible for red light-induced phototropism and chloroplast movement. Corollary with its role, *PHY3* could not be detected in primitive ferns that do not have red light

phototropism and chloroplast movement, such as *Osmunda japonica* and *Lygodium japonicum* (Kawai et al., 2003). Recently, it was demonstrated in the moss *Physcomitrella patens* that phototropins may be downstream components in the phytochrome signaling pathway, explaining why *Physcomitrella* displays red-light induced chloroplast movement but has no homologues of *PHY3* (Kasahara et al., 2004).

C. The Cryptochromes

Cryptochromes are UV-A/Blue light photoreceptors that have structural similarities to DNA photolyases and are found in all kingdoms of organisms. They do not, however, have typical photolyase DNA repair activity. In plants, the most significant functions of cryptochromes were determined from studies on *Arabidopsis thaliana* (*At*) CRY1 and CRY2. Together with phytochromes—either through direct or indirect interactions—*AtCRY1* and *AtCRY2* mediate photomorphogenic responses such as inhibition of hypocotyl/stem elongation, stimulation of leaf expansion, control of photoperiodic flowering, entrainment of the circadian clock, and regulation of gene expression (Cashmore et al., 1999). In addition to phototropism and chloroplast photorelocation, many other physiological responses of lower plants such as ferns are mediated by blue light, for example germination, gametophyte tip growth, apical swelling, and cell division. In *Adiantum capillus-veneris* (*Ac*), five cryptochromes have been identified (*AcCRY1-5*). Of the five cryptochromes, only two are nuclear proteins and as such, behaviorally similar to *Arabidopsis* CRY1 and CRY2. *AcCRY3* is imported to the nucleus in the dark or in red light but is mostly cytosolic in blue light, in contrast to *AcCRY4* that accumulates constitutively in the nucleus (Imaizumi et al., 2000). Either

one or both of these cryptochromes are believed to mediate the inhibition of spore germination exhibited by many ferns.

D. The Phytochromes

Phytochrome in the cell is a soluble protein that may exist as a homodimer or a heterodimer. Each subunit or monomer consists of a protein component (apoprotein) covalently bound to a light-absorbing chromophore molecule. The complex of the apoprotein and its chromophore forms the holoprotein. The chromophore of plants is an open chain linear tetrapyrrole (a bile pigment) known as phytochromobilin (Smith, 2000). It is synthesized in the plastids from 5-aminolevulinic acid via a branching pathway off chlorophyll anabolism (Rudiger, 1992). The synthesis of the chromophore requires the products of the nuclear-encoded chloroplast enzymes *heme oxygenase 1* and *photochromobilin synthase* (Fankhauser, 2001). Loss of function of either of these genes in *Arabidopsis thaliana* result in mutants (*hy1* and *hy2*, respectively) that grow tall in white (W), red (R), or far-red (FR) light and show other characteristics of multiple phytochrome apoprotein mutants (Koornneef et al., 1980). In higher plants, attachment of the chromophore to the apoprotein occurs via a thio-ether linkage at an invariant cysteine residue within the N-terminal half of the polypeptide. This attachment process results from the bilin lyase activity of the apoprotein; hence, it is an autocatalytic event and occurs spontaneously in the cytosol. In the mature protein, the chromophore of the holoprotein undergoes a cis-trans isomerization by rotation around the double bond between carbons 15 and 16 when a photon is absorbed. The holophytochrome is synthesized in the form that absorbs maximally in the red spectral region, **Pr**, and

remains in that form if kept in the dark. If the etiolated plant is illuminated by red light, most of the Pr is converted into the far-red light-absorbing form, **Pfr**. When the Pfr absorbs far-red light, the Pfr is converted back to the Pr form. Conversion of one form of the phytochrome to another requires conformational change in the chromophore as well as the apoprotein. It has been demonstrated that each form of phytochrome can bind different monoclonal antibodies and that Pr and Pfr forms differ in their susceptibilities to proteases and in their phosphorylation by exogenous protein kinases (Furuya and Song, 1994). Changes in the apoprotein are believed to occur in the N-terminal domain.

Spectral analysis of the two forms of phytochromes shows that the absorbance spectra of Pr and Pfr overlap significantly. The photoreversible nature of phytochrome establishes a state of dynamic equilibrium, with the ratio of the phytochrome forms (Pfr/Pr) dependent upon the relative amount of red and far-red light. Since Pfr also absorbs red photons and only a small amount of far-red light is absorbed by Pr, and both forms absorb blue photons, the photoequilibrium of Pfr/P_{total} is about 80% in red (660nm), 3% in far-red (730nm) and 40% in blue (450nm) light (Batschauer, 1999). Moreover, since red light converts the bulk of phytochrome from Pr to Pfr, and morphogenic responses have been demonstrated to correlate with theoretical Pfr levels or Pfr/P_{tot} ratios, Pfr is believed to be the active form of phytochrome in all but few responses. Studies of the development of null mutants that are unable to synthesize individual phytochromes have provided further evidence that Pfr is the physiologically active form of phytochrome. Although the Pr form is thought to be biologically inactive, there is some evidence that the cycled Pr* form (i.e. phytochrome that has been converted to Pfr and back to Pr) also has a functional role (Shinomura et al., 2000)) and this form

behaves differently from the naïve Pr (Shinomura et al., 2000; Schafer and Bowle, 2002). The magnitude of the phytochrome response may depend on the Pfr/Pr (Pr*) ratio or the Pfr/P_{tot} ratio. Changes in the R:FR ratio of ambient light lead to large changes in the ratio of Pfr/P_{tot}, which may serve in the natural environment to signal changes in shade or in density of competitors (Smith, 2000).

E. Types of Phytochrome

In *Arabidopsis thaliana*, the five holoproteins (phyA-phyE) have been grouped into two types of phytochromes based on their light stabilities. In etiolated tissue, phyA is 50-100 fold more abundant than any of the other phytochromes (Somers et al., 1991). The Pfr form of phyA (PfrA) holoprotein is unstable and undergoes rapid proteolytic destruction by the ubiquitin-mediated system; hence phyA has been termed light labile and is classified as a *Type I* phytochrome. In addition to the rapid degradation of the PfrA holoprotein, the transcription of the *PHYA* gene is rapidly down-regulated and the *PHYA* mRNA has a high turnover rate, so its cellular concentration dramatically decreases following the transition from darkness to a light environment (Casal et al., 2002). Thus, the level of a *Type I* phytochrome in a cell can be regulated by transcriptional inhibition, mRNA instability, and targeted degradation of the holoprotein. PHYB-E are the most abundant phytochromes in green plants (Quail et al., 1995) because they are light stable, and thus are classified as *Type II* phytochromes. *Type I* phytochromes have been shown to regulate early photomorphogenic responses associated with the development of young seedlings such as inhibition of hypocotyl elongation, expansion of cotyledons, and the block of chlorophyll accumulation. As the plant grows

and becomes exposed to more light, *Type II* phytochromes begin to regulate later responses such as red light-dependent hypocotyl growth inhibition, entrainment and resetting of the circadian clock, and induction of flowering. In both *Type I* and *II* classes, the subunits of each phytochrome can exist as homodimers; however, heterodimers are only observed between members of the *Type II* phytochromes (Sharrock and Clack, 2004).

F. Types of Phytochrome Responses

Phytochrome responses are dependent in part on the quantity of absorbed photons, and can be classified by the total fluence and the fluence rate of light required to induce them. Fluence is defined as the number of photons impinging on a unit surface area, typically measured as $\mu\text{moles of quanta per square meter}$ ($\mu\text{mol m}^{-2}$). Fluence rate is the moles of quanta per square meter per unit time, generally $\mu\text{mol m}^{-2} \text{ s}^{-1}$. Based on the amount of light required to elicit a response, phytochrome responses are divided into three major categories: very low fluence responses (VLFR), low fluence responses (LFR), and high irradiance responses (HIR).

Very low fluence responses (VLFRs) are induced with very little light, within the range of approximately 10^{-6} - $10^{-2} \mu\text{mol m}^{-2}$ of red light. Thus, only small amount of Pfr (<0.02%) is required to initiate these responses. Since FR conversion of Pfr to Pr is never complete (97%), and there is more of the Pfr (3%) than is needed for VLFR, FR cannot reverse VLFRs and can actually induce VLFRs in the absence of red light.

Low fluence responses (LFRs) typically have a threshold fluence of around $0.1 \mu\text{mol m}^{-2}$ and are saturated near $10^3 \mu\text{mol m}^{-2}$ of red light. A high Pfr/ P_{total} ratio (beyond

the critical threshold) is therefore required for a physiological response. Red light-initiated LFRs can be reversed by subsequent FR because the amount of Pfr is returned to a level below the threshold concentration required for response. VLFRs and LFRs both obey the law of reciprocity and are dependent on the total fluence. If the fluence rate is low (i.e. low light intensity), then the duration of exposure must be long; likewise, if the intensity of light is high, then the exposure can be brief.

High irradiance responses (HIRs) require prolonged or continuous exposure to light, and the degree of the response is dependent on the fluence rate. Since neither brief exposure to bright light nor long but transient exposure to dim light can induce responses, HIRs do not obey the law of reciprocity. Periodic pulses of light can mimic the continuous light responses, but the intervening dark periods must be short to elicit comparable responses (Hamazato et al., 1997). HIRs can be further subdivided into red (R) and far-red (FR) HIRs.

In *Arabidopsis*, mutational analyses have revealed that VLFR, LFR, R-HIR and FR-HIR are elicited by specific phytochromes, although redundancy of activity does exist among different phytochromes. VLFR are mediated by phyA, LFR and R-HIR primarily by phyB, and FR-HIR by the photocycled form of phyA* (Shinomura et al., 2000).

G. General Structural and Functional Domains of Phytochromes

The apoprotein sequence of plant phytochromes has been strongly conserved in the course of plant evolution and consists of approximately 1120 amino acids or 120 kDa. The first 65 kDa has been termed the N-terminal 'photosensory-input' domain. This domain has a greater than 50% sequence identity among all plant phytochromes. The

latter 55 kDa C-terminus has been termed the ‘regulatory output’ domain and is highly variable among phytochromes. These two domains are connected via a hinge segment that allows for the necessary proximal interactions between both termini.

The N-terminus of plant phytochromes can be further divided in four sub-domains; P1-P4 (Montgomery and Lagarias, 2002). The P1 domain is serine rich and is relatively short. It consists of the region referred to as the N-terminal extension region (NTE) that is poorly conserved among phytochromes (Fankhauser, 2001). The NTE region is believed to regulate the stability of the Pfr form. The P2 domain is related to the PAS domains that are found in a large family of transcriptional regulatory proteins including the *period clock* (PER) protein, the *aromatic hydrocarbon receptor nuclear translocator* (ARNT) and the *single minded* (SIM) protein of *Drosophila* (Aravind and Ponting, 1997). The P3 domain is the tetrapyrrole-binding GAF domain. This domain is present in vertebrate *cGMP-specific phosphodiesterases*, in cyanobacterial *adenylate cyclases* and in the *formate hydrogen lyase* transcriptional activator FhlA (Aravind and Ponting, 1997). This domain is also defined as a bilin lyase domain (BLD), the minimal GAF domain capable of autocatalytic assembly of the chromophore to the apoprotein (Wu and Lagarias, 2000). The BLD domain is approximately 200 amino acids (aa) long and lies within residues 200aa-400aa. Within the P3 domain (near residue 322 of oat phyA), there are five conserved amino acids that form the site for the chromophore attachment (Lamparter, 2004). The P4 domain is referred to as the PHY domain and is required for the stabilization of the Pfr chromophore (Wu and Lagarias, 2000).

The carboxyl terminus of plant phytochromes consists of a PAS-related domain (PRD) containing two PAS repeats (PAS₁ and PAS₂) and a histidine-kinase-related

domain (HKRD). The PAS domains are believed to function in protein-protein interactions and ligand binding (Park et al., 2000). Loss of function by missense mutations within the PRD suggested that intact PAS domains are critical for phytochrome signaling (Quail et al., 1995). Overlapping the PRD are two regulatory core sequences: the “Quail Box” and one of two dimerization motifs (D1), which specifically overlaps the PAS₁ domain. The histidine (His) kinase motifs are not functional in plants but display instead Ser/Thr protein kinase activity. Within the HKRD is a loosely defined second dimerization motif, D2 (Wang and Deng, 2002).

H. Prokaryotic Phytochrome and Phytochrome-Related Proteins

Proteins with a bilin lyase domain can be grouped into four families: eukaryotic phytochromes (*PHY*), cyanobacterial phytochrome 1 (*Cph1*), cyanobacterial phytochrome 2 (*Cph2*) and phytochrome –related (*Phr*) families (Montgomery and Lagarias, 2002). The *Cph1* family lacks the serine-rich P1 domain that is dispensable for bilin binding and photochromic activity. This family uses phycocyanobilin (PCB) as the chromophore instead of phytochromobilin (PΦB) found in eukaryotic phytochromes. Many members of the *Cph1* family lack the conserved cysteine residue in the P3 domain of eukaryotic phytochromes. The chromophore instead attaches to a conserved histidine residue that lies immediately next to the conserved cysteine of a eukaryotic phytochrome (Lamparter, 2004). The *Cph1* phytochromes have been demonstrated to be functional light-regulated histidine kinase (Yeh et al., 1997). As with classic bacterial two-component signaling systems, *Cph1* autophosphorylates on a histidine residue and then transfers this phosphate to an aspartate on a second, receiver protein. Unlike plant

phytochromes, Cph1 activities (autophosphorylation and phosphorylation of its substrates) are greater in the Pr form than the Pfr form (Hughes and Lamparter, 1999).

The cyanobacterial *Cph2* family lacks the P1 and P2 domains found in plant phytochromes. Members of this family have multiple GAF domains and contain the conserved cysteine present in the P3 domain. *In vivo*, this family binds PCB instead of PΦB and, characteristic of this chromophore, displays an approximately 20-nm blue shift in the absorption spectra of both Pr and Pfr. The C-terminus of the Cph2 family also has a histidine-kinase transmitter domain.

The *Phr* family members possess GAF domains closely related to plant phytochromes. Little is known about this family other than they may have photosensory roles in cyanobacteria. Interestingly, there is a proliferation of this particular family with the cyanobacterial genome.

Unlike the cyanobacterial phytochromes, the eukaryotic phytochromes, PHY, can autophosphorylate on serine residues *in vivo* in a light-dependent manner, and have been demonstrated to be serine/threonine kinases *in vitro*. The kinase domain has not been ascertained precisely, and there is conflicting evidence about whether the HKRD has functionally shifted to a ser/thr kinase, or whether the kinase domain resides elsewhere in the molecule.

I. Fine Mapping of the Functional Regions of Plant Phytochrome

Mutational and biochemical analyses have mapped many functional regions within the plant phytochromes as exemplified by oat, *Avena sativa* (*As*) PHYA and *Arabidopsis thaliana* (*At*) PHYB. The minimum region necessary in both phytochromes

for the autocatalytic chromophore attachment is between positions 115 and 450 (Boylan and Quail, 1991; Deforce et al., 1991; Cherry et al., 1993; Vierstra, 1994; Aravind and Ponting, 1997; Quail, 1997). For the maintenance of photoreversibility and absorption spectra in both Pr and Pfr form unaltered from those characteristics displayed by the native phytochrome, the complete NH₂-terminal structural domain (amino acids 1-673) is required. Deletion of segments within the first 115 positions (P1 and P2 sub-domains) or between positions 524-673 (P4 sub-domain) of the NH₂ terminus altered the stability and spectral integrity of the Pfr and Pr forms (Quail, 1997). Deletion of the first 69 residues of oat phyA produced blue shifted absorption maxima of the Pr and Pfr form and an increase in the rate of dark reversion of Pfr to the Pr form when expressed in transgenic plants. Deletions of the serine-rich region between amino acids 6-12 of oat phyA (Δ 6-12) resulted in a phytochrome that was hyperactive in VLFR components of hypocotyl growth and cotyledon growth when expressed in tobacco. When expressed in *Arabidopsis*, the same Δ 6-12 deletion mutant was as active as the full length PHYA in VLFR for hypocotyls growth and cotyledon growth as well as in inducing a VLF blocking of subsequent greening under white light (Casal et al., 2002), which is not found in wild type or full-length oat phyA-overexpressing *Arabidopsis* plants. Oat Δ 6-12 phyA also showed a dominant-negative suppression of FR-HIR when expressed in tobacco or *Arabidopsis*. The deletion of the serine rich region also affects nuclear accumulation when expressed in *Arabidopsis* (Casal et al., 2002).

During the photoconversion between the Pr and Pfr forms, region 37-136 (P1-P2) and region 684-731 (PRD) undergo conformational changes. For dimerization of PHYA, regions within PRD (652-741) and the HKRD (1116-1195) may be necessary.

Implicated in the degradation of Pfr form of PHYA (PfrA) is a motif close to the chromophore-binding domain of PHYA, the PEST sequence motif. The Proline (P), glutamate (E), serine (S) and threonine (T) sequence is hypothesized to be involved in photo-degradation of phyA. The presence of a PEST sequence is not found in PHYB-E and there is no experimental evidence to link the PEST sequence to PfrA degradation.

Domain swapping experiments have demonstrated that photosensory responses specific to PHYA and PHYB reside in the NH₂ terminal domains (Wagner et al., 1996). Until recently, the emerging view of a phytochrome is that it is a two-component regulatory system with a photosensory domain and a regulatory region. When light is absorbed, not only does the chromophore undergo a conformational change but the apoprotein also changes. Autophosphorylation events occur at various sites within the protein (e.g. Ser 7, Ser 17, Ser 598, and others). Phosphorylation may result in subtle conformation changes that mediate interactions between the N terminus and the C terminus, or between the C-terminus and other downstream interacting factors (e.g. PKS1, PIF3, NDPK2). Moreover, phosphorylation may unmask subcellular localization signals (i.e. nuclear localization signal), facilitate degradation of the protein, or alter the photoconversion efficiency between Pr and Pfr. This view has come under scrutiny since the demonstration that the C-terminus, specifically the histidine kinase- related domain (HKRD), is dispensable for biological activity in PHYB and is not required for signal transduction so long as there are a functional dimerization domains and a nuclear localization signal. The C-terminal domain of PHYB may be necessary only for attenuation of the activity of PHYB, rather than positive transduction of signals to other factors (Krall and Reed, 2000; Matsushita et al., 2003).

J. Phytochrome Localization

The apoproteins of phytochromes are synthesized within the cytosol and assemble autocatalytically with photochromobilin pigment produced in plastids. In their newly synthesized Pr form, the phytochrome holoproteins are predominantly localized outside the nucleus. In *Arabidopsis*, light triggers the translocation of the photo-converted Pfr form into the nucleus and induces the formation of nuclear speckles. The nuclear localization signal (NLS) is believed to be in the C-terminus and its accessibility may be mediated by the photo-state of the phytochrome. However, translocation of PHYC-E is not strictly light dependent, suggesting that the Pfr form is necessary but not sufficient for some phytochromes (PHYA and PHYB). PHYC-E are detected in the cytosol and the nucleus in etiolated seedlings. However, the formation of nuclear speckles (functional significance is presently controversial) by PHYC-E is light dependent (Wang and Deng, 2002).

Although never demonstrated conclusively, it is hypothesized that the phytochromes of cryptogams are localized continuously in the cytoplasm and that nuclear localization is an adaptive facet of seeded plant phytochromes. Cryptogam phytochromes in the Pr form are believed to be bound to the plasma membrane, and the conformational change to Pfr may lead to association or direct interaction of phytochrome with the cytoskeleton. This interaction, in turn, can affect chloroplast positioning (if red light mediated) or changes in growth direction. If the Pr or Pfr forms of cryptogam phytochromes are localized only to the cytosol, then they must interact with transcription factors that are initially present there or they must activate second messengers and/or signaling intermediates that allow indirect transduction of the light signal to the nucleus.

Productive interactions would lead to nuclear localization of these transcription factors, which can then induce light-responsive genes mediated by phytochrome activation. In *Arabidopsis*, phytochromes can interact with transcription factors in the cytoplasm (e.g. NDPK2) and in the nucleus (e.g. PIF3). Sub-cellular localization, sequestration within areas of cytosol or nucleus, and migration kinetics affected by different fluences of light are several means plants have evolved to regulate phytochrome activity. It is not known whether these features have evolved gradually or are integral to the structure of eukaryotic phytochromes. Studies of phytochromes localization in lower plants may provide insights into these adaptations.

K. Plant Phytochromes

Moss Phytochromes

In the moss *Ceratodon purpureus*, three full-length phytochromes have been reported: *CpPHY1*-*CpPHY3* or CP1-CP3. *CpPHY2* and *CpPHY3* are conventional phytochromes, each with a HKRD at its C-terminus. *CpPHY1*, however, is a unique phytochrome with a serine/threonine/tyrosine kinase at its C-terminus that has little homology to the kinase domain of PHY3 in the fern *Adiantum capillus-veneris*. The C-terminus of *CpPHY1* has the most sequence similarity to a tyrosine kinase from *Dictyostelium discoideum* (Thummler et al., 1992). *In vitro* phosphorylation experiments have demonstrated that *CpPHY1* has a red-light-dependent kinase activity (Algarra et al., 1993). *CpPHY1* and *CpPHY2* mRNA appear to be constitutively transcribed in a light independent manner, though *CpPHY2* messages accumulate to a higher level than those of *CpPHY1* (Pasentsis et al., 1998). At the protein level, *CpPHY2* (and possibly

CpPHY3) is the predominant phytochrome in *Ceratodon*, consistent with the observed mRNA levels. Similar to the phytochrome of the green algae *Mesotaenium caldariorum*, *CpPHY2* accumulation is influenced by photosynthesis. The light-dependent depletion of dark-accumulated phytochrome could be inhibited by the photosynthesis inhibitor DCMU (Morand et al., 1993). The amount of *CpPHY2* is believed thus to be regulated at the post-transcriptional level. Little is known about the individual molecular role of *CpPHY1*-*CpPHY3* in *Ceratodon* growth and development.

In another moss, *Physcomitrella patens*, four canonical phytochromes have been identified (PP1-4). The absence of a *CpPHY1*-like homologue in *Physcomitrella* suggests that *CpPHY1* has recently diverged from a relatively recent duplication and gene rearrangement of *CpPHY2*-like gene. Analysis of mRNA shows that the transcripts of *PP1* and *PP3* are lower in light than in darkness, and the transcripts of *PP2* and *PP4* are higher in light than in darkness (Mittmann et al., 2004). Knockouts of *PP1*-*PP4* by homologous recombination showed a specific defect in red light-mediated phototropism, polarotropism and chloroplast photorelocation. These phytochromes have been demonstrated to act upstream to modulate the phototropin-signaling pathway in *Physcomitrella*.

Phylogenetic analysis shows that the phytochromes of moss form a distinct slowly evolving clade from those of vascular plants, including ferns and seed-bearing plants. In mosses, it can be surmised that there are two basic phytochrome groups, represented by *PP1* (*CpPHY1*/*CpPHY2*) and *PP2* (*CpPHY3*). *PP3* and *PP4* may have resulted from recent duplications (after separation from *Ceratodon*) of *PP1* and *PP2*, respectively. Although there is a redundancy of function, each phytochrome has evolved distinct

functions that could only be discerned by exposure to different fluences of light. PP4 has the most pronounced role in cytoplasmic perception/response in *Physcomitrella* (Mittmann et al., 2004).

Fern Phytochromes

In all ferns where it has been tested, phytochromes are represented by a multigene family. In an absolute sense, phytochrome sequences have been reported from the following ferns: *Adiantum capillus-veneris*, *Anemia phyllitidis*, *Angiopteris evecta*, *Anthoceros punctatus*, *Azolla filiculoides*, *Blechnum spicant*, *Botrychium lunaria*, *Dicksonia Antarctica*, *Dryopteris-felix mas*, *Equisetum arvense*, *Huperzia selago*, *Marattia laevis*, *Marsilea quadrifolia*, *Ophioglossum vulgatum*, *Osmunda regalis*, *Pilularia globulifera*, *Platynerium angolense*, *Polypodium vulgare*, *Psilotum nudum*, *Salvinia natans*, *Selaginella martensii*, and *Vittaria lineate* (ncbi.nlm.nih.gov). From among these ferns, full-length phytochrome sequences have only been reported in *Adiantum*, *Marrattia*, *Marsilea*, and *Selaginella*, and only from *Adiantum* have full-length phytochrome paralogues been reported. In *Anemia phyllitidis*, four phytochrome gene fragments (*AphPHY1*- *AphPHY4*) have been reported, though not all have been made publicly available. In *Adiantum capillus-veneris* (*Ac*), three complete phytochromes (*AcPHY1*- *AcPHY3*) have been identified from genomic DNA or cDNAs (Okamoto *et al*, 1993; Nozue, Fukuda, Kanegae and Wada, unpublished results). Although screening of a genomic DNA library in *Adiantum* has resulted in the identification of an *AcPHY4*, only a partial sequence (exon1) of this phytochrome is available. Moreover, a Ty3/gypsy type retrotransposon (ARET-1) has been found within

a 10 kb proposed intron downstream of the *AcPHY4* phytochrome exon1-homologous sequence (Nozue K, 1997), suggesting that this phytochrome may be nonfunctional.

Structurally *AcPHY1* and *AcPHY2* have all the canonical features of a typical plant phytochrome. *AcPHY3* is an unconventional phytochrome in that it contains a phytochrome chromophore-binding domain at its amino terminus and a full-length phototropin at its carboxyl end (Kawai et al., 2003). *AcPHY3* has been demonstrated through mutational analysis and complementation to be involved in red light chloroplast relocation and red light mediated phototropism (Kawai et al., 2003)—functions that are controlled exclusively by blue light through phototropins in higher plants—and has been isolated only in a subset of the order Polypodiales, to which *Adiantum* and *Ceratopteris* belong. From the available sequences, it is generally believed that most ferns have a phytochrome gene family of three or four genes (Wada M, 1997).

In *Adiantum*, *AcPHY1* message was first reported by Okamoto *et al* (1983) and was isolated only from dark-grown gametophytes and sporophytes. In the liverwort *Marchantia paleacea* var. *diptera*, *MpdPHY1* transcript accumulated in the dark and is suppressed in the light. Red light irradiation resulted in the degradation of the *MpdPHY1* transcript and far-red light suppressed this degradation (Suzuki et al., 2001).

Adiantum PHY2 cDNA is similar in sequence to previously cloned cryptogam phytochromes and less similar to those from seed plants (Nozue K, 1998). RT-PCR analysis showed that the *AcPHY2* gene is expressed in all developmental stages examined (imbibed spore, protonema, dark- or light-grown young leaf), suggesting that *AcPHY2* might function constitutively (Nozue K, 1998). In *Anemia*, it was reported that *PHY1* and *PHY4* mRNA accumulated during spore imbibition, and that those transcript levels

decreased when spores were transferred to continuous red light. On the other hand, *PHY3* mRNA could not be detected in spores during the 48 h of darkness, but increased when spores were transferred to red light. The interpretation of these data is that *PHY1* and *PHY4* are responsible for inducing germination, and *PHY3* for protonemal development (Maucher, Schraudolf and Scheuerlein, unpublished result). Since the sequences of these four fragments of *Anemia* were never provided, it is not possible to determine how similar these gene sequences are to those of *AcPHY1*-*AcPHY4*, or to determine if there is any correlation between the expression patterns of each *Anemia* gene and its closest *Adiantum* homologue.

At the protein level, crude phytochrome extracts have been prepared from the ferns *Adiantum*, *Lygodium*, and *Anemia*. Spectrophotometric quantification revealed that phytochrome levels increased 4 to 5-fold in the dark for several days, then decreased when the sample was exposed to red or white light (Wada M, 1997). With the exception of *Adiantum PHY3*, little is known about the function of *PHY1* and *PHY2* as no mutant deficient in either gene has been isolated or characterized. Moreover, there have never been any correlative studies explaining the evolutionary relationship between *Adiantum PHY1* and *PHY2* with phytochromes of other ferns and groups of plants. Preliminary analysis by Kolukisaoglu *et al* (1994) using partial phytochrome sequences from various species, however, shows that the phytochromes of *Selaginella* and *Equisetum* are more closely related to those of moss than to those of present-day ferns, and phytochrome sequence from *Psilotum* is quite distinct, suggesting that *Psilotum* may not be a near relative of the ancestors of all vascular plants as had been supposed. The phytochrome sequences from *Psilotum*, *Adiantum* and other ferns form a monophyletic cluster,

separated from the basal group of moss and the rapidly evolving group of seed-bearing plants. It has been suggested that the phytochrome sequences of ferns have more similarity to those of higher plant *PHYB* than to *PHYA* (Okamoto et al., 1993; Thümmeler et al., 1990; Kolukisaoglu et al., 1993), though no significant data comparing full-length sequences has been produced to support this claim.

Gymnosperm Phytochromes

In gymnosperm, there are at least two types of phytochromes, *PHYO/N* (*PHYA*-like) and *PHYP* (*PHYB*-like), which represent the descendants of two of the most ancient phytochrome lineages of seeded plants (Schmidt and Schneider-Poetsch, 2002). Since *PHYO* and *PHYN* of present gymnosperms resulted from a gene duplication event, there is some controversy as to which phytochrome arose first, hence the progenitor of these two phytochromes is referred to as *PHYO/N*. Depending on the group of gymnosperms, different numbers of phytochrome gene family members have been found. In *Gnetatae*, there are generally only two gene types (*PHYO/N* and *PHYP*). In *Ginkgo* and *Cycadatae*, there are three gene types (*PHYO*, *PHYP* and *PHYN*). In conifers, there are four gene types (*PHYO*, *PHYP*, *PHYN₁* and *PHYN₂* resulting from a second duplication event). Their long life, large seeds and the fact that the de-etiolation process is uncoupled from light signals have stymied characterization of molecular role of individual phytochrome family members in gymnosperms. Many gymnosperms (except the *Ginkgos*) are constitutive photomorphogenic in the dark (Bogorad, 1950; Mukai et al., 1992). In *Pinus sylvestris*, it was demonstrated that only R or W could reduce hypocotyl length but not FRc, suggesting that the *PHYA* homologue (*PHYN* or *O*) does not have all the properties of the angiosperm *PHYA*. In *Pinus elliotii*, low levels of FRc causes slight height

increases over D controls (Burgin et al., 1999). There is, however, a limited de-etiolation response in FR-HIR-grown seedlings such as greater cotyledon and leaf development than those observed in dark grown seedlings. Quantitative RT-PCR of *Picea abies* *PHYO* and *PHYN* show that the steady-state transcript levels of these *PHYA* homologues slightly decrease following light irradiation (Clapham et al., 1999), further supporting the functional divergence of angiosperm *PHYA* from the *PHYO/PHYN* of gymnosperms.

Unlike other gymnosperm, *Ginkgo biloba* can etiolate in a manner similar to angiosperms when grown in D. At low fluences, neither R nor B yielded reduced stem length. A fluence threshold determines the magnitude of the stem growth response to irradiation. High fluence rate W_c causes a greater suppression of stem elongation than does low fluence rate W_c (promotes stem elongation) but a lesser increase in total chlorophyll accumulation. High fluence rate continuous monochromatic light did not reduce stem elongation, but simultaneous high fluence rate irradiation with R_c and B_c was as effective as W_c in mediating the response. High fluence rate B_c yielded the greatest increase in chlorophyll accumulation in the bases or lower stems of the seedlings than any other monochromatic light irradiation treatment or W_c . Unlike the response in angiosperms, if *Ginkgo* seedlings are grown in FR_c for 30 days, these seedlings are still able to accumulate chlorophyll when subsequently exposed to W_c . The amount of chlorophyll accumulated, however, is only ~30-40% that of seedlings where a 30 day dark pretreatment was substituted for the FR_c pretreatment prior to the W_c exposure. If seedlings are pretreated with 20 days or less of FR_c , these seedling show more than a two fold increase in chlorophyll than the D controls. Like the conifers, *Ginkgo* also has limited ability to de-etiolate in FR-HIR, exhibiting greater cotyledon and leaf

development than do dark-grown seedlings. These results have led to the conclusion that *Ginkgo biloba* has both *Type I* and *Type II* phytochromes, and speculation that its PHYA homologue has some of the functions of the angiosperm PHYA (Christensen et al., 2002). Molecular evidence for such claims has not been provided. The lack of FR-HIR de-etiolation responses in many gymnosperms also suggests that FR-HIRs of angiosperm and other seeded plants may have evolved independent functions.

Angiosperm Phytochromes

All angiosperms characterized to date contain a small gene family of phytochromes. At least two of the phytochrome lineages (*PHYA* and *PHYB*) appear to have been established before the divergence of angiosperms from gymnosperms. In angiosperms, the earliest gene duplication gave rise to a lineage comprising of *PHYA* and *PHYC* (Mathews and Sharrock, 1997). Following separation of monocots from eudicots, a second gene duplication gave rise to the lineage comprising *PHYB* and *PHYE*. In some eudicots, sporadic losses of *PHYE* have occurred (Mathews and Sharrock, 1997). *PHYD* is a recent duplication of *PHYB* (80% amino acid identity to *PHYB*) and has been described only in *Arabidopsis* and its relatives. In some eudicots such as *Solanum*, *PHYC* has duplicated to produce the orthologue *PHYF*. It can therefore be surmised that the phytochromes of angiosperms are of a monophyletic group that fall into four subfamilies (A, B/D, C/F, and E) in eudicots and three subfamilies in monocots (A, B, and C). PHYA is a light labile Type I phytochrome that is most abundant in etiolated seedling. In VLFR mode, PHYA promotes germination of seeds. Through the FR-HIR, PHYA suppresses hypocotyl growth, promotes cotyledon unfolding, inhibits chlorophyll synthesis, and

promotes anthocyanin accumulation. When *Arabidopsis* seedlings are grown under high fluence rate FR_c for 4 days, they fail to green upon subsequent transfer to W_c because of the PHYA-mediated block of chlorophyll biosynthesis. Besides *Ginkgo biloba*, no other groups of plants outside of angiosperm group have shown a FR block on chlorophyll. PHYB, PHYC and PHYL are Type II phytochromes that are more or less unregulated by light at the mRNA or protein levels. Through the LFR and R-HIR, PHYB has been shown to be primarily responsible for hypocotyl growth inhibition, chloroplast maturation, regulation of flowering time, shade avoidance, stem and leaf expansion, and circadian rhythmicity. Little is known about role of other phytochromes. PHYC has a primary role in leaf expansion and probably in daylight perception in short-day plants (Halliday et al., 1997; Qin et al., 1997). PHYC has also supplemental role with PHYB in regulating de-etiolation in red light. PHYL acts together with PHYB in regulating many aspects of shade avoidance (Aukerman et al., 1997) in red light, and can substitute partially for *phyB* loss of function mutations. In the *Wassilewskija* (Ws) ecotype of *Arabidopsis thaliana*, most of the *PHYL* gene has been lost through a chromosomal deletion, however its functional maintenance in other ecotypes suggests that PHYL is important. Under FR, PHYE performs supplemental roles to PHYA in inducing germination, whereas under R it overlaps some roles of PHYB in mediating the shade avoidance responses.

The evolution of angiosperms has been closely linked with the evolution of their phytochromes. Angiosperm diversity far exceeds the collective diversity of all other plants. Comparisons of angiosperm phytochromes with those of other groups of plants have shown that the phytochrome gene family in flowering plants has increased in size

and complexity (Kolukisaoglu et al., 1995), and the degree of these changes differs significantly among the families of angiosperms. Regardless, it is believed that the evolution of *PHYA* and *PHYC* (which occurred early in the angiosperm lineage) has conferred a competitive advantage on plants growing in dense shade (Yanovsky et al., 1995). In recent evolutionary history, *PHYA* has been selectively maintained while *PHYC* has been evolving under less stringent selective constraints, as supported by evidence that the nucleotide substitution rate of *PHYA* differs significantly from that of *PHYC* (Mathews and Sharrock, 1997). Comparison of phytochromes from eudicots and monocots has shown that *PHYC* has evolved faster than *PHYA*, which in turn has evolved faster than *PHYB*. In grasses, however, the photosensory domain of *PHYA* has evolved more rapidly than that of *PHYC*, indicating that an episode of positive selection has occurred on *PHYA* (Mathews et al., 2003). Diversification of *PHYA* and *PHYC* may involve both the relaxation of selective constraints and positive selection (Mathews et al., 2003). With the exceptions of grasses (specialized monocots), the C-terminal domain of angiosperm phytochromes is evolving at least twice as fast as the N-terminal domain, which is under more strict evolutionary constraints. The C-terminal half of PHYs has thus been associated with the functional divergence of phytochromes.

The rise of angiosperms to dominate the terrestrial landscape has led to the decline of many non-seed-bearing vascular plants. Ferns were believed to be an evolutionary “cul-de-sac,” until Schneider *et al* (2004) provided evidence that ferns today have attained their current diversity as a response to the diversification of flowering plants. Radiation of the angiosperms created new and complex ecospace where only opportunistic species could thrive. To grow under the low-light canopy conditions

present in angiosperm forests, it was proposed that the evolution of ferns would correlate or coincide with the evolution of their photoreceptors such as phytochromes. *PHY3* was cited as one example evolved by *polypods* ferns to cope with the low-light canopy conditions. However, not all polypods show red-light mediated phototropism and chloroplast relocation of light-grown and dark-adapted gametophytes, including *Pteris* and *Ceratopteris*; thus it seems that other changes in photoreceptors would also be involved. It has never been determined whether the typical phytochromes of the recently diverged polypods show the signs of rapid molecular evolution that has been seen with *PHYA* and *PHYC*.

L. The Emergence of *Ceratopteris richardii*

Although the functions of phytochrome in mosses (e.g. *Physcomitrella patens* and *Ceratodon purpureus*) and angiosperms (e.g. *Arabidopsis thaliana*) have been molecularly characterized, the molecular biology of phytochromes in ferns and gymnosperms are obscured by their relatively long lifecycles and the lack of available tools to knockout or knockdown specific target genes.

Ceratopteris richardii is a recently evolved polypod that is emerging as a model fern for studying a variety of biological phenomena in plants, such as photomorphogenesis, spore germination, development, differentiation, and hormone and pheromone responses. The advantage of using this organism stems from the unique properties of its development and life cycle. *Ceratopteris* can exist independently as either a haploid (gametophyte) or a diploid (sporophyte) autotrophic generation. This biphasic life cycle allows for observations and experimental manipulations at the cellular or whole organism level without artificial manipulation of the life cycle (Hickok et al.,

1995). Within 120 days, *Ceratopteris* can develop from a spore to a mature gametophyte to a young sporophyte that produces new spores. Since ferns represent the branching point of the vascular plant evolutionary tree, *Ceratopteris* combines features of both higher and lower plants systems. In contrast to other models, it provides a link between the more “primitive” and “advanced” plants.

Recently, it has been demonstrated that incubation of sterilized dry spores with sequence-specific double stranded RNA (dsRNA) can reduce the expression of targeted genes in *Ceratopteris richardii* (Stout et al., 2003). The availability of an expressed sequence tag (EST) library, the ease of generating and delivering dsRNA, and the convenience of this RNA interference (RNAi) approach (Stout et al., 2003) has greatly increase the utility and value of *Ceratopteris* to continue studying the process of photomorphogenesis in this fern. If homologues of *Adiantum PHY1-PHY4* were present in *Ceratopteris*, it would be possible to characterize for the first time the role of individual phytochromes in regulating such processes as spore germination, rhizoid development, and the growth and development of fern gametophytes. Moreover, it would be also be possible to determine if there is any evidence of adaptive evolution displayed by the phytochrome multi-gene family of ferns.

M. Life Cycle of *Ceratopteris richardii*.

The life cycle of *Ceratopteris richardii* (C-Fern) is characterized by the plant's two independent autotrophic phases: the gametophytic and the sporophytic stages. The haploid gametophyte (N=39 chromosomes) is structurally simple and develops mitotically from the single-celled spore after germination. Upon exposure to W (or R) and water, germination occurs within 3-4 days with the emergence of the primary rhizoid

(a single, elongated, non-photosynthetic cell used for anchorage and nutrient absorption). Within 6-8 days of germination, a gametophyte develops, possessing a two-dimensional sheet of vegetative cells making up the prothallus and rhizoids. At maturity, sex organs including antheridia and, in hermaphrodites archegonia are fully developed. Sexual differentiation of the gametophyte is regulated by the mating pheromone antheridiogen, with hermaphroditic gametophytes (containing both male and female sexual organs) developing under low antheridiogen concentrations. As the meristematic hermaphrodites develop, they secrete antheridiogen to cause the subsequently germinating spores to become ameristematic males. Within a few days of the fertilization of the egg in the hermaphrodite archegonium by a motile sperm of the antheridium, the zygote develops into a sporophyte. Through the process of apogamy, a haploid sporophyte can also develop. The mature sporophyte can then produce spores on fertile fronds (more than 10^6 spores per plant is common) to complete the sexual cycle. The sporophytes commonly reproduce asexually through production of plantlets directly on the sporophyte frond margins, and the sporophytes can also be induced to produce diploid gametophytes directly via apospory (Banks, 1999), which involves the generation of gametophytes from diploid sporophyte leaves without meiotic cell division. Therefore, *Ceratopteris* allows for a great deal of genetic and developmental plasticity as an experimental system.

N. Some Strengths of *Ceratopteris* as a Model System.

Since the spores produced are genetically identical and are generated in large quantities, rapid isolation of mutants is possible. Mutations generated by X-ray irradiation or ethylmethanesulfonate (EMS) treatment can be identified in gametophytes

within 2 weeks. Because the spore contains only one haploid cell, a mutation would be propagated to all cells of the haploid gametophyte and can be identified without regard to the relative dominance of a mutant allele. Thousands of spores can be plated at high density on the appropriate medium in a Petri-dish, allowing for easy screening for mutants. Gametophytes can then be self-fertilized to generate, within one generation, homozygous sporophytes. Because the archegonia mature slightly ahead of the corresponding hermaphrodite antheridia, crosses can also be made to produce heterozygote sporophytes (Hickok et al., 1995). Spores are produced by the sporophytes within 3 months, at which time mutant gametophytes can be retested (Chasan, 1992).

O. Taxonomy

Placement of *Ceratopteris* into definite taxa is a bone of contention among phylogenists. *Ceratopteris* may be classified in the Class Filicopsida (Banks, 1999) or the Class Polypodiatae (Chatterjee and Roux, 2000). Using the latter classification scheme, *Ceratopteris* may then be placed either in its own family, Parkeriaceae, or included with members of Pteridaceae. Within the genus *Ceratopteris* are four species: *C. cornuta*, *C. pteridoides*, *C. richardii*, and *C. thalictroides*. Species differ in their geographical distributions, number of spores per sporangium, and chromosome number (e.g., diploid versus tetraploid), but fertile hybrids have been described among different species in this family.

P. Photobiology of Ferns

Spore Germination

In most of the ferns where it has been studied, including *Lygodium japonicum*, *Pteris vittata*, *Anemia phyllitidis*, *Adiantum capillus-veneris*, and *Ceratopteris richardii*, spore germination is induced by a brief irradiation with R light and this promotive effect can be inhibited by subsequent FR light treatment. This low fluence photoreversible effect is diagnostic for the role of phytochromes in mediating spore germination as has been demonstrated in seed-bearing plants. In *Lygodium*, it has been demonstrated spectrophotometrically that the amount of phytochrome in the spore increases during imbibition, and such an increase correlates with the spore's sensitivity to R (Kendrick and Kronenberg, 1994). This increased responsiveness to R was also characterized in *Ceratopteris*, which required at least 7-10 days of dark incubation after imbibition to achieve maximum sensitivity. This lag was also attributed to the spectrophotometrically determined increase of phytochrome content (Cooke, 1993). Moreover, spores of *Ceratopteris* with 10 days D pretreatment displayed fluence-dependent sensitivity towards FR (Cooke et al., 1987). Cooke *et al* dismissed this far-red response to the age of that particular population of spores. Recently it was demonstrated that R-HIR resulted in a delay of germination (Mohamed, unpublished studies). This delayed germination was not reported by others and contradicts data that showed an increase in germination with an increased fluence of R in *Ceratopteris*. In other ferns however such as the filicales *Rumora adiantumtformis*, and *Dicksonia sellowiana*, it was demonstrated that R-HIR could partially inhibit spore germination. This inhibition became more pronounced if the temperature was also increased to 30°C or greater (Blum, 2002). Temperature- and

R-HIR-dependent inhibition was also observed in the spores of *Onoclea sensibilis*, *Dryopteris paleacea* and *Dryopteris felix-mas*. Raghavan (1989) proposed that the high temperature uncouples the phytochrome Pfr-dependent signal from the processes leading to germination. Although the elevated temperature inhibits germination if Pfr is continuously present, the inhibition is neither total nor permanent (Haupt, 1990). It is believed that this inhibition is adaptive, ensuring that gametophyte growth and reproduction occur only in the appropriate environmental conditions where abundant water supply, low irradiance, and mild temperature are available (Blum and Randi, 2002). Therefore, the R-HIR inhibition of germination that Mohamed observed in *Ceratopteris* may have resulted from the combined effects of light and high growth temperatures as has been observed in other species.

Cooke and colleagues (date) also showed the effects of various combinations of R and B light on the germination responses. B alone (without any R pre-treatment) does not induce germination and inhibits R-induced germination ($10^3 \mu\text{mol m}^{-2}$ R followed by $10^4 \mu\text{mol m}^{-2}$ B) to less than 10% that of the R-only control. If spores are exposed to B first then to R, the resulting germination is 25% that of the R control. Escape from the inhibitory effects of B begins if a dark period of approximately 1.7 h is inserted between the inductive R pulse and the inhibitory B. Complete escape occurs if the intervening D period exceeds 24 h. The inhibitory and antagonistic effects observed from the B treatment suggest that cryptochromes may be also involved in the photomorphogenic signaling pathways, as has been observed in other ferns and plants. It has been asserted that CRY3 or CRY4 (or both) are responsible for this inhibition because of their

translocation to the nucleus, although phytochromes are known to act in the cytosol as well.

A mutant of *Ceratopteris richardii*, designated *dark germinator 1* (*dkg1*) was identified from a population of EMS-treated spores for its ability to germinate in complete darkness (Cooke et al., 1995). Moreover, germination of the *dkg1* mutant is inhibited by white light, specifically wavelengths between 550-640 nm that normally induce germination in flowering plants. Genetic crosses revealed that the *dkg1* mutation segregates as a single nuclear recessive gene. Further analysis of the *dkg1* mutant suggests that the DKG1 protein may couple the phytochrome signaling process to the germination process. The DKG1 protein blocks the germination process unless the appropriate phytochrome photostationary states (P_{fr}/P_{total}) is achieved. It has been demonstrated in many ferns that Ca^{2+} , gibberellins (GA), or antheridiogens (structurally similar to GA) may be downstream signaling factors of the photosensory networks that can induce spore germination in the dark. It may be also that DKG1 is pleiotropic in how it affects these signaling pathways.

Protonema and Prothallus Development

In some fern species such as *Adiantum* and *Pteris*, a distinct two-dimensional filamentous stage called the protonema exists between spore germination and development of the prothallus. Although the structure and the development of protonemata are quite variable between species, both of these features are strongly affected by environmental light conditions.

In *Adiantum*, R stimulates the first cell division through phytochromes, which can be blocked by brief irradiation with B, presumably perceived through cryptochromes. Following the initial cytokinesis, however, B promotes subsequent cell division and development of the protonema to a greater extent than R (Furuya et al., 1997). B can induce apical swelling of the protonema and can re-arrange the cortical cytoskeleton of the protonemal apex, thereby affecting subsequent patterning and cell division.

In ferns without a visible protonema stage (e.g. *Ceratopteris* and *Lygodium*), the effect of continuous R, FR or B light on cell division after germination is largely unknown. The germinating spores of ferns develop directly into the two dimensional prothalli under R or W. In the etiolated gametophytes of *Ceratopteris*, light exposure can alter the rate and pattern of cell division, cell elongation, and cell differentiation. Brief exposure of the etiolated gametophyte to B inhibits cell elongation of the subapical elongation zone. R or FR has no effect on elongation (Murata et al., 1997). Exposure of the dark grown gametophyte to R and B light can alter the meristem morphology, increasing cell division and differentiation, resulting in a broadened notch meristem and the transition from filamentous to prothallial growth.

In an unpublished study, Mohamed reported that R_c severely reduces the size of the gametophytes of *Ceratopteris* whereas the cell number is indistinguishable from that of D-grown gametophytes. However, it is important to note that these gametophytes were grown in same conditions where a R-HIR inhibition of germination was observed, and therefore may have been affected by elevated temperatures. FR_c -grown gametophytes developed elongated prothalli similar to those grown in darkness, however they were larger and broader than those grown in the dark. Moreover, FR_c -grown

gametophytes had more cell files constituting the subapical region than were observed in dark grown gametophytes. Gametophytes grown under FR_c had at least twice the number of cells per gametophyte as R_c - or D-grown gametophytes. If gametophytes were grown in B_c or R_c+FR_c , the prothalli had more cells than FR_c , R_c , or dark grown gametophytes but fewer than those grown under W_c . Only the B_c or W_c treatment produced gametophytes with the typical heart-shaped prothalli. Those grown under R_c+FR_c were irregularly shaped. Mohamed concluded that R_c (high Pfr) inhibits cell elongation but not cell expansion and cell division in gametophytes. FR_c promotes elongation of subapical cells to produce the strap-shaped prothalli and encourages two-dimensional cell expansion in apical cells, results previously seen when pulse of blue and red light are applied to dark-grown prothalli. Under R_c+FR_c , cell elongation is intermediate between that of plants grown in R_c or FR_c , and cell expansion increased more in R_c+FR_c than in FR_c . B_c inhibits cell elongation but induces more cell division than R_c+FR_c . Cryptochromes and phytochromes are believed to act synergistically to inhibit unidirectional cell elongation, and to promote more cell expansion and cell division.

In etiolated *Arabidopsis* seedling, R_c , FR_c or B_c can reduce the elongation of hypocotyls and induce cell division in the cotyledons and in the shoot apex (von Arnim, 1996). Phytochromes and cryptochromes have been demonstrated to act collectively to inhibit cell elongation and to induce cell division (Lin et al., 1995) at the molecular level. Although the roles of phytochromes and cryptochromes have never been molecularly characterized in ferns, it has been deduced through physiological studies that phytochrome and cryptochromes are involved in regulating in cell division and cell elongation during early development of *Ceratopteris*. In the *dkg1* mutant of

Ceratopteris, it was found that cell division and cell expansion could occur independent of light stimulation.

The DKG1 protein may be involved in several-phytochrome-mediated responses (Kamachi et al., 2004). Recently, it has been demonstrated that cytokinins can induce photomorphogenic development in dark-grown gametophytes of *Ceratopteris*, a response seen in seed-bearing plants (Spiro et al., 2004). Cytokinins can induce a subset of responses mediated by B and R, such as formation of rhizoid initials and the development of the notch meristem. These reports suggest that photosensory activation of phytochromes and cryptochromes of skotomorphogenic gametophytes may induce cytokinin-signaling pathways that, in turn, produce the altered morphologies observed in the dark-light transition. Cytokinins, however, cannot induce germination, sexual maturation of the hermaphrodites, rhizoid elongation, or increased chlorophyll synthesis; processes that are light-regulated.

Rhizoid Production and Elongation

Unlike the fern *Onoclea*, in which rhizoid development is regulated by the internal delivery of photosynthetic products from green cells, growth and development of the rhizoids of *Ceratopteris* are believed to be regulated by light through photomorphogenic photoreceptors. R has been shown to induce cells at the margins of the elongation zone of etiolated gametophytes to divide asymmetrically, resulting in cells that develop into secondary rhizoids (Murata and Sugai, 2000). The effect of R can be cancelled by subsequent FR, indicating that phytochromes regulate rhizoid initiation and that the effect can be classified as a LFR. Although a R pulse induces the rhizoid initials, elongation of the rhizoid requires continuous irradiation of W, B, or R indicative of a

high fluence response. This differential suggests that secondary rhizoid formation and rhizoid elongation involve separate signal transduction pathways. Murata and Sugai hypothesized that a multi-gene family of phytochromes may exist in *Ceratopteris*, which may mediate the multiple photosensitivities of these phytochrome-mediated LFR and HIR. It was demonstrated by Mohamed that the number of rhizoids produced could be correlated directly with the number of cells per gametophyte, which was dependent on the light treatment. A R_c -grown gametophyte has the lowest cell number and also the fewest rhizoids, in contrast with a W_c -grown gametophyte which has the greatest cell number and the highest number of rhizoids. The elongation of the primary rhizoid was greatly inhibited by B_c and, to a lesser extent, by W_c or R_c , suggesting that cryptochromes have a greater role in inhibiting rhizoid elongation. Moreover, since B_c supplied after germination stimulates cell division and increases the number of rhizoids, the claim by Murata and Sugai that rhizoid elongation is regulated independently of rhizoid production is further supported. Mohamed showed that primary rhizoid elongation is closely associated with cell elongation, which is greatly inhibited by B and may require the collective activity of cryptochromes and phytochromes.

Chlorophyll Accumulation in Gametophytes

Unlike most flowering plants, gymnosperms and ferns are able to produce chlorophyll in the dark. Chlorophyll synthesis in angiosperms is mediated by the enzyme protochlorophyllide oxidoreductase (POR) whose activity is induced by R. Gymnosperm and lower plants contain both the light-dependent POR pathway and a light-independent pathway (Schoefs, 1999), the latter explaining why these plants can green in the dark.

Molecularly and physiologically, little is known about the light-regulated chlorophyll biosynthesis pathway in these plants. In the fern *Adiantum*, one of the genes in the chlorophyll biosynthetic pathway, chlorophyll a/b-binding (CAB) protein, has been shown to be transcriptionally regulated by light. R increases the level of mRNA, which can be retarded by B (Christensen and Silverthorne, 2001). Mohamed reported that R_c-grown gametophytes had less chlorophyll accumulation than those grown in the dark or under other light conditions for 14 days. FR_c-grown gametophytes had a marginal increase in chlorophyll content over R_c-grown gametophytes, and the combined effect of R_c+FR_c yielded pigment levels near those observed in W_c-grown tissues. The greatest increase in chlorophyll accumulation resulted from B_c. It should be noted that in all light conditions except for B, there is a direct relationship between cell number and chlorophyll accumulation, suggesting that the level of chlorophyll is only indirectly mediated by light.

Several hypotheses have been proposed to explained chlorophyll production in *Ceratopteris*. These hypotheses required at least one phytochrome and one cryptochrome affecting both the cell division and chlorophyll synthesis pathways. A high level of P_{fr} was hypothesized to suppress chlorophyll accumulation as well as to inhibit cell division. Lower levels of P_{fr} (produced by FR_c or R_c+FR_c) and the activities of cryptochromes promoted chlorophyll accumulation and cell division. Alternatively, it was suggested that the rapid P_r↔P_{fr} cycling of phytochromes that occurs in R_c+FR_c induces greater activation of the pathway than the slower cycling rates expected under monochromatic R_c or FR_c.

Antheridia and Archegonia Development

In the ferns where it has been examined, it has been demonstrated that antheridium development is mediated by plant pheromones called antheridiogens, or by environmental factors such as light. In *Polypodium aquilinum*, phytochromes have been implicated in the development of antheridia, as R can inhibit initiation of the antheridia and this inhibition is reversed by subsequent irradiation by FR (Schraudolf, 1967).

Antheridia are formed only under darkness or FR_c irradiation. In *Pteris vittata*, males are produced more frequently when germinating spores are grown in the darkness. If dark-grown *P. vittata* gametophytes are treated with a pulse of R or B, there is a reduction in the number of antheridia, whereas a FR pulse increases the number of antheridia. FR-grown gametophytes, however, have fewer antheridia than will dark-grown prothalli.

Thus, it seems that the P_{fr} form inhibits antheridia formation in *Pteris*.

In *Ceratopteris*, it has been demonstrated that antheridium development is induced by the antheridiogens (Banks, 1994). Although maleness has not been shown to be light-regulated, R can induce archegonia formation in dark-grown *Ceratopteris* prothalli (Kamachi et al., 2004). The effect of R can be cancelled by subsequent irradiation by FR, suggesting the role of phytochrome in a classical LFR. Neither a FR pulse nor a B pulse could induce archegonia in the dark-grown prothalli. Similar results were also obtained by Mohamed (unpublished), who demonstrated that R_c stimulated archegonial development. Moreover, co-illumination with R_c and FR_c yielded many more archegonia than either R_c or FR_c alone. W_c produced hermaphrodites with the highest numbers of archegonia, and archegonia on W_c-grown gametophytes matured much earlier than those on gametophytes from all other light treatments. It has been

hypothesized that archegonial development may result synchronously with the differentiated lateral meristem, which is induced by R (Kamachi et al., 2004).

Sporophyte Development

Light regulation of sporophyte development in ferns is poorly characterized. Mohamed has demonstrated that supplementary FR slows archegonia and sporophyte development (unpublished results). Only gametophytes grown in W_c and no other light treatment were able to produce mature archegonia and well-developed sporophytes within the time period studied. W_c supplemented with FR_c delayed—but did not prevent—sporophyte development. Therefore, the supplementary FR was suggested to maintain a greater fraction of the pool of a hypothetical light-labile phytochrome in its P_r form, stabilizing the level of inhibitory P_{fr} over a longer period and leading to delayed sporophyte development.

End of Day Far-Red Light (EOD-FR) Response

When angiosperms growing under diurnal day-night cycles are exposed to a pulse of FR shortly after the end of the light period, they elongate faster than those without the EOD-FR. This response can be reverse by subsequent treatment with R, demonstrating that the EOD-FR response is a LFR. EOD-FR responses have been shown to be mediated by a stable and long-lived form of P_{fr} form of PHYB (Smith and Whitelam, 1990). In ferns, there is no report of EOR-FR response, possibly because none can be detected. In her studies, Mohamed found no EOD-FR effect on germination, growth rates, archegonia

development, timing and extent of sporophyte initiation, chlorophyll content and the number and extent of fertile and infertile frond growth.

Conclusion

Although the photobiology of ferns is well characterized at the physiological level, little is known at the molecular level. The varied phenotypes displayed by *Ceratopteris* in LFR, FR-HIR and R-HIR modes suggest that it has multiple phytochromes, consistent with the multiple phytochromes found in most other plants. Finding these phytochromes would allow for characterization at many levels (i.e. phylogenetically, molecularly and biochemically), and would further unravel the webbed tapestry of phytochrome signaling in plants.

OUTLINE OF RESEARCH

To date, no phytochrome or related genes in the photomorphogenesis signaling pathways have been molecularly identified nor characterized in *Ceratopteris richardii*.

This research attempts to answer the following questions:

CHAPTER 2: THE PHYLOGENY OF PHYTOCHROMES OF FERNS

1. Does *Ceratopteris* possess a small multi-gene family of phytochromes, as is found in other plants?
2. How many phytochrome loci are found in the genome of *Ceratopteris richardii*?
3. What are the structural similarities and differences between the phytochromes of ferns and those of other groups of plants?
4. What is the evolutionary relationship between the phytochromes of ferns and those of other groups of plants?
5. Is there any evidence of adaptive evolution at the molecular level displayed by the phytochromes of ferns?
6. Are the putative phytochrome genes transcriptionally expressed in *Ceratopteris*, and if so, is the expression of these genes affected by light?

CHAPTER 3: THE ROLE OF PHYTOCHROMES IN *CERATOPTERIS*

7. How does the photobiology of *Ceratopteris* relate to the previous phylogenetic analyses of its phytochromes?

8. Are there functional similarities between phytochromes of *Ceratopteris* and phyA and phyB of angiosperms? How do the gametophytes develop with respect to R-HIR or FR-HIR, responses mediated by phyA and phyB?
9. Does the transcriptional level of specific phytochromes change during morphogenic events such as germination and prothallar development, and can these changes help explain the observed physiological phenomena?
10. What aspect of photomorphogenesis does each phytochrome regulate in *Ceratopteris*?
11. What are the roles of individual phytochromes in mediating such processes as germination, rhizoid development, cell division, and chlorophyll production?
12. Are phytochromes sufficient for regulating such processes as germination and gametophyte development in *Ceratopteris*?

CHAPTER 4: OTHER LIGHT SIGNALING FACTORS IN *CERATOPTERIS*

13. How evolutionarily conserved are the light signaling pathways in plants?
14. What light signaling factors other than phytochrome can be identified from *Ceratopteris*?
15. What factors interact *in vivo* with the phytochromes of ferns? Are there novel factors found in ferns but not in other groups of plants?
16. How do the isolated putative light-signaling factors affect germination and gametophyte development in *Ceratopteris*?

CHAPTER 2: THE PHYLOGENY OF THE PHYTOCHROMES OF FERNS

A. ABSTRACT

B. INTRODUCTION

C. MATERIALS AND METHODS

D. RESULTS

- I. *Ceratopteris richardii* Has Homologues of *PHY1* and *PHY2*
- II. *Ceratopteris richardii* Has Two *PHY4* Loci
- III. Conserved Serines Among *CrPHYs*
- IV. PEST Sequences in *CrPHYs*
- V. A *PHOT2-Like* Protein, But Not a *PHY3*, Was Found in *Ceratopteris*
- VI. *Ceratopteris richardii* Contains a Small Gene Family of Phytochromes
- VII. *PHY1* and *PHY2* Evolved Before the Divergence of Seed-Bearing Plants From Ferns.
- VIII. *PHY3* Shares Ancestry With *PHYs* of Seed plants
- IX. *PHY2* and *PHY4* Resulted From a Gene Duplication That Occurred During the Rapid Angiosperm Radiation
- X. *PHY1* is Diverging More Rapidly Than *PHY2* or *PHY4*
- XI. Gene Duplication by Retro-Transposition in Ferns
- XII. Decrease of Transcript Level of *CrPHYs* in Rc

E. DISCUSSION

- I. Phytochrome is Represented by a Multi-Gene Family in Ferns

- II. Phytochrome Duplication and Species Divergence**
 - III. PHY1 is Evolving Faster in Ferns.**
 - IV. CrPHY4A and CrPHY4B**
 - V. Expression Pattern of the Phytochrome Gene Family in *Ceratopteris richardii***
 - VI. Conserved Serine Residues and PEST Motif**
- F. CONCLUSION**

A. ABSTRACT

Ceratopteris richardii is an annual homosporous fern that has a diploid sporophyte and a relatively short life cycle. Although its photobiology has been extensively characterized, no photoreceptors have been isolated. In this study, five phytochrome sequences have been identified: *CrPHY1A*, *CrPHY1B* (partial fragment), *CrPHY2*, *CrPHY4A* and *CrPHY4B*. *CrPHY1B* has recently duplicated by retro-transposition but has retained one of three introns located in the C-terminus. At least within the partial sequence obtained for *CrPHY1B*, the coding sequence is identical to that of *CrPHY1A*. *CrPHY4A* is also the result of a recent duplication of *CrPHY4B*, but it has lost the PAS₂ domain and HKRD. Both *CrPHY4A* and *CrPHY4B* are missing the P1 and P2 domains of the N-terminus and, other than the C-terminal truncation in *CrPHY4A*, are also identical in their coding sequences. RT-PCR demonstrates that *CrPHY1*, *CrPHY2*, *CrPHY4A* and *CrPHY4B* are transcribed and that red light lowers transcript accumulation of all four genes. Phylogenetic analyses show that ancestral *PHY1* and *PHY2* appeared very early in the history of vascular plants. Close homologues of *PHY1* and *PHY2* can be found in lycopods and in pteridophytes but not in bryophytes. Moreover, the evolution of *PHY1* and *PHY2* preceded the separation of ferns from seed-bearing plants. During the evolution of the leptosporangiate ferns, a gene duplication occurred coinciding with angiosperm radiation (approximately 260 mya), producing the orthologues *PHY2* and *PHY4*. In ferns, *PHY1* has accumulated more unique amino acid differences since the divergence of ferns from bryophytes and is evolving at a faster rate than *PHY2* or *PHY4*. Although believed to play an integral role in the evolution of some fern groups, a homologue of *PHY3* could not be detected by degenerate PCR. However,

a fragment corresponding to a *PHOT* was identified, consistent with physiological results that showed blue light-mediated but no signs of red light-mediated phototropism or chloroplast photorelocation in *Ceratopteris richardii*. Phylogenetic analyses of *Adiantum* *PHY3* also showed that the N-terminus of *PHY3* shares a common progenitor with the phytochromes of seed-bearing plants, representing the first direct linkage between the phytochromes of vascular cryptogams and seed plants. The N-terminus of *PHY3* is also evolving at a rate comparable to those of higher plant phytochromes and may have become lost in some species of ferns, including *Ceratopteris* and *Pteris*. In addition to the chimeric *PHY3*, the evolution of ferns can be associated with an increased number and divergence of its photoreceptors. Five cryptochromes have been identified in the *Adiantum capillus-veneris* and now five phytochromes have been isolated in *Ceratopteris richardii*.

B. INTRODUCTION

A plant's survival depends on its ability to react to light-mediated changes in its environment. The ability to detect intensity, direction, spectral quality and periodicity of light as well as interpret and transduce these light signals requires the presence of multiple photoreceptors. In seed plants and cryptogams, these photoreceptors include the phytochromes, cryptochromes, and phototropins (Smith, 2000). Of these photoreceptors, the best characterized are the phytochromes which are dimeric photoreversible chromoproteins that can absorb red light (Pr) and far red light (Pfr). In a model fern, *Adiantum capillus veneris* (Ac), which has been most characterized molecularly, phytochromes are encoded by a small gene family consisting of at least three members, designated *AcPHY1-3* (Okamoto, 1993; Nozue et al., 1998). *AcPHY1* and *AcPHY2* both contain chromophore-binding domains, PAS repeat domains (PRD) and histidine kinase related domains (HKRD) as seen in higher plants. *AcPHY3* is an unconventional phytochrome in that it contains a phytochrome chromophore binding domain at its amino terminus and a full-length phototropin at its carboxyl end (Kawai et al., 2003). Although screening of a genomic DNA library in *Adiantum* has resulted in the identification of an *AcPHY4*, only partial sequence (exon1) of this phytochrome is available. Moreover, a Ty3/gypsy type retrotransposon (ARET-1) has been found within a 10 kb proposed intron downstream of the *AcPHY4* phytochrome exon1-homologous sequence (Nozue K, 1997), suggesting that this phytochrome may be nonfunctional. With the exception of *PHY3*, little is known about the function of molecular biology of phytochromes in ferns

because of their relatively long lifecycles and the lack of available tools to knockout or knockdown specific target genes.

Ceratopteris richardii is a homosporous fern that is emerging as a model organism to study the process of photomorphogenesis at a cellular level without the experimental difficulties associated with complex multicellular organs (Cooke et al, 1995). Although no photoreceptors have been isolated, extensive studies on the photobiology of *Ceratopteris* have shown that red light at low fluence, stimulates germination of spores and a subsequent exposure to far-red light has reverse the effect, implicating the activity of phytochromes. Physiological characterization of wild type spores and the mutant *dark germinating1 (dkg1)* that does not require light for germination, has also implicated phytochrome to be involved in the development of apical and sub-apical zone of etiolated strap-shaped gametophytes, and the differentiation of rhizoid from the asymmetrical cell division in the sub-elongation zone (Marata and Sugai, 2000; Kamachi et al, 2003). Recently, it has been demonstrated that incubation of sterilized dry spores with sequence-specific double stranded RNA (dsRNA) can reduce the expression of targeted genes in *Ceratopteris richardii* (Stout et al., 2003). The availability of an expressed sequence tag (EST) library, the ease of generating and delivering dsRNA, and the convenience of this RNA interference (RNAi) approach (Stout et al., 2003) has greatly increase the utility and value of *Ceratopteris* to continue studying the process of photomorphogenesis in this fern. If homologues of *Adiantum PHY1-PHY4* were present in *Ceratopteris*, it would be possible to characterize for the first time the role of individual phytochromes in regulating such processes as spore germination, rhizoid development, and the growth and development of fern

gametophytes. Moreover, it would be also be possible to determine if there is any evidence of adaptive evolution displayed by the phytochrome multi-gene family of ferns and further elucidate the relationship between the phytochrome of ferns and other groups of plants. This study describes the isolation of phytochrome multigene family in *Ceratopteris richardii* and provides the initial characterization of these phytochromes at a structural and phylogenic level.

C. MATERIALS AND METHODS:

Isolation of Genomic DNA:

Genomic DNA was isolated from young sporophyte leaves using the cetyltrimethylammonium bromide method (CTAB) method. Five grams of frozen leaves were ground to a fine power in liquid nitrogen. The powder was transferred to a 50 ml centrifuge tube containing 25ml of CTAB buffer (140 mM sorbitol, 220 mM Tris, 22 mM EDTA, 800 mM NaCl, 1% Sarkosyl and 0.8% CTAB, pH 8.0) and incubated at 65°C for 20 min with occasional vigorous shaking. After incubating the lysate with 10ml of chloroform for 20 min at room temperature with gentle shaking, the aqueous and hydrophobic phases were separated by centrifugation at 1500xg for 5 min and the aqueous phase was transferred to another tube. DNA was precipitated by adding 17 ml isopropanol, incubated for 10 min on ice, and then centrifuged at 1500xg for 5 min. Pellet was dissolved in 4ml 1XTE (10mM Tris-Cl, pH 7.4; 1mM EDTA, pH 8.0), and 4ml of a 4M LiOAc was added. The suspension was placed on ice for 20 min then centrifuged at 1500xg for 10 min to remove residual RNA and polysaccharides. The supernatant was added to a fresh tube containing 16 ml ethanol, placed on ice for 20 min, then centrifuged at 1500xg to precipitate DNA. The pellet was dissolved in 0.9ml of TE, transferred to a 2ml microcentrifuge tube with 0.3 M NaOAc and the DNA was further extracted with successive phenol, phenol/chloroform, and then chloroform separations, retaining the aqueous phase in each. DNA was precipitated by adding 2 volumes of 100% ethanol to the aqueous phase, incubating on ice for 5 min then centrifuging for 5 min to collect the precipitate. The purified DNA pellet was dissolved in 0.1X TE.

Restriction Digestion and Southern Hybridization:

Ten micrograms of genomic DNA was digested in each of a series of restriction endonucleases *BamHI*, *BglI*, *EcoRI*, *HindIII*, and *PstI* for 20h at 37°C. Complete digestion required approximately 50 units of each enzyme in the buffer supplied by the manufacturer (New England Biolabs, Beverly, MA). Digested DNA was separated on a 1.0% agarose gel in TBE buffer (89 mM Tris base, 89mM boric acid, 2 mM EDTA) at 60V for 4 hrs. The agarose gel was blotted onto Hybond-XL nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ) with a VacuGene XL Vacuum Blotting System (Amersham Pharmacia Biotech) using 20X SSC (3M NaCl, 300mM Sodium Citrate, pH 7.0) as transfer buffer.

Membranes were probed with a 2488 bp ^{32}P labeled fragment of the *CrPHY2* (1×10^5 cpm/ml) possessing the highly conserved chromophore-binding domain. Probes were radiolabeled by PCR (1XPCR Buffer (Invitrogen), 1.5 mM MgCl_2 , 0.2mM dNTP mix (dATP, dTTP, dGTP), 0.002mM dCTP, $5\mu\text{Ci } \alpha^{32}\text{P dCTP}$, 1.0U *Taq Pol* (Invitrogen), using 0.2 μM primers (PHY2-184S: TAA GTT GCA CGC AGT TTT CG and PHY2-2672AS: ACC ATG GCG ATC AAA GAA AC) and 100ng of cDNA template of the *CrPHY2* gene sub-cloned into pGEM T-Easy (Promega, Madison, WI). The template was then amplified for 35 cycles at a denaturing temperature of 94°C for 30 s, an annealing temperature of 55°C for 30 s, an extension temperature of 72°C for 3 min. Unincorporated nucleotides were removed using the miniQuick Spin Column (Roche, Summerville, NJ). The DNA blot was incubated in *PerfectHyb Plus* hybridization buffer (Sigma, St Louis, MO) with 0.1mg/ml of single-stranded DNA probe (which was prepared by boiling for 10 min then placed immediately on ice for 5 min) for 12 h at

60°C. The blot was washed three times with 2xSSC, 0.1 %SDS for 20 min at 65°C, then a final wash containing 0.5XSSC and 0.1%SDS for 20 min at 65°C.

Light Sources:

General W light was emitted from cool white fluorescent bulbs at a fluence rate of $100\mu\text{mol m}^{-2} \text{ s}^{-1}$. FR_c light was supplied at a fluence rate of $4\mu\text{mol m}^{-2} \text{ s}^{-1}$ by FR LED light sources (Quantum Devices Inc., Barneveld, WI) with λ_{max} at 735 nm and filtered through far-red plastic resin (FRF700, Westlake plastics, Lenni mills, PA) to eliminate minor red-light emissions from the LED source. R_c was obtained from 667-nm Q-beam LED sources (Quantum Devices Inc., Barneveld, WI) at a fluence rate of $100\mu\text{mol m}^{-2} \text{ s}^{-1}$ and B_c was supplied from 464 nm Q-beam LED sources with fluence rate of $25\mu\text{mol m}^{-2} \text{ s}^{-1}$. Light intensity and spectral output were measured with a Li-Cor Li-1800 spectroradiometer (Lincoln, NE).

Isolation of total RNA:

Four milligrams of dry pre-sterilized wild type *RNWI* spores (Carolina Biological, Burlington, NC) were suspended in 200 μl of *C-Fern* media (Carolina Biological), placed in darkness for 2 d, then transferred directly in continuous red (R), far-red (FR), blue (B) or white (W) light for 7 to 14 d at 28°C. Some spores were placed in the dark for two days, exposed to W for 1 d, and then placed back in the dark for 6 to 13 days at 28°C. After the light incubation, media was removed from spores by pipetting, and total RNA was isolated using the *RNAqueous-4 PCR kit* (Ambion, Austin, TX). No liquid nitrogen was used in this procedure. The tissue was homogenized in a 1.5 ml microfuge tube in 300 μl of the supplied Lysis Buffer, and total RNA was extracted according to the manufacture's instruction. After the recovery of RNA, the concentration was determined

spectrophotometrically (1 A₂₆₀ unit corresponds to 40 µg/ml of RNA) and the RNA was also examined by agarose gel electrophoresis. Prior to RT-PCR, RNA was DNase treated according to manufacture's instruction for 1-2hrs at 37°C.

Isolation of mRNA:

Spores were imbibed in *C-fern* medium and exposed to the aforementioned light treatments as indicated below. After incubation, the liquid medium was removed and mRNA was isolated from developing gametophytes using *the MicroPoly(A) Pure* small-scale mRNA purification kit (Ambion). The homogenization procedure was the same as described for the total RNA isolation and mRNA was purified and quantified according to manufacture's instruction and stored at -80 °C until needed.

Identifying Fragments of *CrPHY1*, *CrPHY2*, *CrPHY4A* and *CrPHY4B*.

To isolate the phytochrome multigene family in *Ceratopteris*, degenerate oligonucleotide primers were synthesized, designed to amplify the highly conserved sequences of all phytochrome genes surrounding the tetrapyrrole chromophore-binding domain (Kolukisaoglu et al., 1995). These primers

Sense primer

S707:TAT GAY AGG GTK ATG GCT TAT AAG TTC CAT G and

Antisense primers:

A1308:GCW GCT CCA TCA CAT TTC TCW AGR TCC AT or

A1308N:GCR GCT CCA TCA CAT TTC ACM AGR TCC AT

were then used to amplify putative phytochrome genes from the genomic DNA template, resulting in the isolation of a ~620 bp fragment. Upon cloning and sequencing, this fragment showed the highest similarity to *Adiantum PHY4*. The *PHY4* subcloned PCR

fragment was then used as a probe to screen two λ ZAP-based cDNA libraries provided by J.A. Banks. These libraries were made from mRNA extracted from light grown male or hermaphrodite gametophytes. Only one putative phytochrome fragment—which is apparently polyadenylated at a site upstream of the expected position—was identified from these libraries (Mohsin Patwary 1999, unpublished data), designated *CrPHY4A*, based on its sequence similarity to the *AcPHY4* gene. To isolate other fragments homologous to *Adiantum PHY1* and *PHY2*, the following respective primer pairs were used:

PHY1S: CCA TGA GCT TGA CAG GGT CAA AGC T,

PHY1AS: CA TTC CAT CCA TTG ATA AGC CCT TGC,

PHY2S: TGC AGG TGT TTG GTC TTC AGC TGA ACA TGG,

PHY2AS: C ATT CCA TCC ATT GAT AAA CCC ACC TCG.

To determine whether a HKRD is present in *CrPHY4A* and to identify *CrPHY4B*, the following primers were used:

CrPHY4A-S: GAA GAA GCC ATG AAC AGG TCC TTA GTT GG and

PHYAS: CAC CAA AAA TTG GTG GTC TCA AGG GAT.

Determining the Complete cDNA Sequences of *CrPHY1*, *CrPHY2*, *CrPHY4A* and *CrPHY4B*

The ends of *CrPHY1*, *CrPHY2*, *PHY4A* and *PHY4B* were identified as specified by the GeneRacer™ Kit (Invitrogen), which ensures that only transcripts containing full-length cDNA ends are amplified. One microgram (μ g) of the mRNA isolated from gametophytes grown in W was treated according to manufacture's instruction to select for 5'-capped mRNA. After selection and ligation of the anchored RNA oligonucleotide

to the 5' end of the mRNA, 0.5µg of RNA was reverse-transcribed with the oligodeoxynucleotide (dT) primers at 50°C using *Thermo Script RT-PCR* System (Invitrogen). The 5' and 3' ends were identified by PCR using the appropriate gene-specific primers and the provided adapter primers. PCR fragments were subcloned in pGEM T-Easy vector (Promega) and transformed in JM109 competent cells (Promega). Colonies were screened by PCR using primers corresponding to the vector's SP6/T7 promoter sequences flanking the cloned inserts. Plasmids with the appropriate insert size were purified using the SV Miniprep Kits (Promega) and inserts were sequenced by MacroGen, Inc. (South Korea) using SP6, T7, or appropriate internal primers. The complete coding sequence of each gene was then isolated from cDNA that was reverse-transcribed from 1µg of total RNA with oligo (dT) primers at 50°C using the *Thermo Script RT-PCR* System (Invitrogen). First strand cDNA was then amplified by PCR for 35 cycles using gene-specific primers and *LA Taq Polymerase* (Takara) at a denaturing temperature of 94°C for 30 s, an annealing temperature of 55°C for 30 s, an extension temperature of 72°C for 4 min. The gene-specific primers used were:

CrPHY1-S: ATG TCG GAT CCG AAG CAA TCC GTA,

CrPHY1-AS: AGT CAG ATA GGG AAC ATC ATT TTG,

CrPHY2-S: ATG GCG CTT CCC GTT TTT ATT CC,

CrPHY2-AS: GTC TTC TTG AAA AGG GGT AGA TCC,

CrPHY4A-S: ATG CTT AAT CCA ATT ACT GTT TAT TGT CGA AG,

CrPHY4A-AS: GGA AAT ACA AGA GAC CTA AGA ATC CAA CAA ACA,

CrPHY4B-S: ATG CTT AAT CCA ATT ACT GTT TAT TGT CGA AG and

CrPHY4B-AS: TTA TTG AAC AAG AGG CAG CTC TAA ATG CAC

Expression of *CrPHY1*, *CrPHY2* and *CrPHY4A* and *CrPHY4B*

Five hundred nanograms of DNase-treated total RNA was reverse-transcribed with oligo (dT) primers at 50°C using *Thermo Script RT-PCR* System (Invitrogen). First strand cDNA was then amplified by PCR with *Taq Pol* (New England Biolab) for 25 cycles using the following gene specific primers:

CrPHY1-1220S: CAA GCT AGT AAG CTT TGG GGA CTT,

CrPHY1-2134AS: CTA GCA AAG GAA CTT GTA CAC AGG (914bp),

CrPHY2-1177S: TGT TGT TGT GAA TGA CAA TGA TGA,

CrPHY2-2094AS: ATC CAC CAC TAT ATC TCT TAC CAA (917bp),

CrPHY4A-955S: TGG AAA GAA GTT CTG GCT ACT TGG TAT AAC

CrPHY4A-1705AS: GGA AAT ACA AGA GAC CTA AGA ATC CAA CAA

ACA (750 bp),

CrPHY4B-1695S: CAC ATG GCG AAC AGG CTC AGA ATG GTC CTG,

CrPHY4B-2416AS: TTT TTG GTA GCT GTC TCT CAC AAG (721 bp),

CrACT-S: CAA GGC ACA CTG GTG TCA TGG TTG,

CrACT-AS: CAG GAA GTT CGT AAC TCT TCT C (617),

CrUBQ-S: CTT CGT CAA GAC CCT GAC AGG CAA, and

CrUBQ-AS: GTT ATC AAT TGT ATC GGA GCT CTC (526bp).

Isolating the C-termini of Phytochrome Genes from *Adiantum*, *Ceratopteris* and *Pteris* Genomic DNA

Genomic DNA was isolated from *Adiantum pedatum*, *Adiantum tenerum*, *Ceratopteris richardii*, *Ceratopteris thalictroides*, *Pteris cretica*, and *Pteris multifida* as previously described. Using degenerate primer pairs for *PHY1* or *PHY2* (*PHY1-1900S*: GCT TAT

CAA TGG ATG GAA TGG GAA GGT TGC, *PHY1-3240AS*: GA CAG C/ATC CAT TCA TC/GA GGC GTA and *PHY2-2100S*: GGA CCA GTG ATT TTG ATT GTG AAT GCA TGC, *PHY2-2900 AS*: TTG TGA AGA C/TG/TA TCC CAT ACA AAG GAT T), 100ng of genomic DNA was amplified with *LA Taq Polymerase* (Takara) at a denaturation temperature of 94°C for 30 s, an annealing temperature of 55°C for 30 s, an extension temperature of 72°C for 3 min. PCR fragments were purified using the *Qiagen Gel Purification Kit*, subcloned into *pGEM T-Easy*, and sequenced with SP6/T7 primers (Macrogen Inc, South Korea).

Sequence Analysis:

The open reading frame corresponding to the probable deduced translation product of each genomic DNA fragment was determined using ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). DNA or deduced protein sequences were analyzed using CLUSTALW (<http://www.ebi.ac.uk/clustalw/>), or BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST/>). Domains of the phytochrome sequences were determined using CDART: Conserved Domain Architecture Retrieval Tool (NCBI Entrez Protein Database; <http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>) and the Pfam Families Database (<http://www.sanger.ac.uk/Software/Pfam/>). Phosphorylation sites were determined using the The NetPhos 2.0 server (<http://www.cbs.dtu.dk/services/NetPhos/>), PEST motifs were determined using the PESTfind Analysis Webtool (<https://embl.bcc.univie.ac.at/content/view/21/45/>) and hydropathy plots were constructed using the Kyte Doolittle Hydropathy Plot server (http://occawlonline.pearsoned.com/bookbind/pubbooks/bc_mcampbell_genomics_1/me dialib/activities/kd/kyte-doolittle.htm).

Phylogenetic Analysis of the Phytochrome Gene Product

To construct various phylogenetic trees, sequences were first aligned using CLUSTALW implemented in the BioEdit platform (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Indels were manually removed and regions of interest were selected. Phylogenetic trees were constructed using the Neighbor-Joining, Minimum Evolution, Maximum Parsimony methods using MEGA3 software by Kumar S, Tamura K & Nei M (2004), available at <http://www.megasoftware.net/>). Relative rates of divergence were also determined using Tajima's Relative Rate Test provided by MEGA3. Values from 1000 bootstrap replicates are given above branches.

D. RESULTS:

I. *Ceratopteris richardii* Has Homologues of *PHY1* and *PHY2*

To isolate the phytochrome genes in *Ceratopteris richardii* (*Cr*), various degenerate PCR primers based on the *Adiantum capillus-veneris* *AcPHY1* or *AcPHY2* genes were used to amplify the highly conserved chromophore-binding domains of phytochromes. Using cDNA isolated from light grown sporophyte tissue or purified genomic DNA as template, fragments homologous to the *AcPHY1* and *AcPHY2* genes (*CrPHY1* and *CrPHY2*) were identified. The 5' and 3' ends of these *PHY* genes were obtained by RACE using RNA isolated from seven-day-old gametophytes that were grown in continuous white light or in darkness. Deduced translation products of the full-length mRNA sequences using the ORF Finder program (ncbi.nlm.nih.gov) revealed a single continuous reading frame of 1117 amino acid for *CrPHY1*, and another single continuous reading frame of 1140 amino acids for *CrPHY2* if the first start codon is used (**Figure 1**). ClustalW and BLAST analyses of the polypeptide sequences revealed that *CrPHY1* has 78% identity to *AcPHY1*, 64% to *CrPHY2*, 52% to *Arabidopsis thaliana* *AtPHYA*, and 51% to *AtPHYB*. *CrPHY2* has 92% identity to *AcPHY2*, 58% to *AtPHYB* and 57% to *AtPHYA*. Like *AtPHYB*, *CrPHY2* may have an N-terminal extension (NTE), which is absent from *AcPHY2*. *CrPHY2* also has several possible start codons in a single reading frame (**Figure 2**). CDART analysis (ncbi.nlm.nih.gov) shows that both *CrPHY1* and *CrPHY2* have all the architectural and canonical features of *AcPHY1*, *AcPHY2* and *AtPHYA-E*. Both genes contain the P1-P4 domains of the N-terminus, the conserved cysteine residue for the chromophore attachment, and a C-terminus with the 2 PAS-repeats (PRD), the transduction core or Quail Box, and a histidine kinase-related domain

(HKRD) consisting of the histidine-kinase A (HisKA) subdomain found in bacterial sensor protein/histidine kinase and the histidine kinase-like ATPase (HATase) subdomain (**Figure 1**). Unlike the coding sequences, the 3' and 5' untranslated regions of *PHY1* and *PHY2* are highly variable among species and among isoforms of phytochrome of the same species. The 5' and 3' ends of *PHY1* and *PHY2* of *Ceratopteris* are shorter in length than those of *Adiantum* (**Figure 2**). Hydropathy plot analyses of the deduced amino acid sequences show that *CrPHY1* and *CrPHY2* have profiles comparable to those of *AtPHYA* and *AtPHYB*, with no apparent hydrophobic regions that would indicate transmembrane domain motifs (**Figure 3**).

II. *Ceratopteris richardii* Has Two PHY4 Loci

To isolate other phytochrome-related sequences, two cDNA libraries (provided by Dr. JoAnn Banks)—generated with RNA isolated from light-grown male or hermaphrodite gametophytes—were screened with a 602 bp fragment corresponding to the region flanking the chromophore-binding domain of *CrPHY4*. The probe was first isolated by PCR amplification of genomic DNA using degenerate primers (Kolukisaoglu et al., 1995). Only one positive plaque was identified from the libraries, containing a fragment (approximately 1100 bp) that had a 92% identity to *AcPHY4* (Mohsin Patwary and Jeanette Kew-Chao, unpublished studies). Subsequent 5' and 3' RACE of RNA isolated from light-grown gametophyte tissue resulted in the identification of a full-length transcript for *CrPHY4* (1750 bp). However, unlike *CrPHY1* or *CrPHY2*, *CrPHY4* has only the chromophore-binding GAF domain, the PHY domain, and one of the two PAS domains (**Figure1**). A poly(A) tail was found directly after the first PAS domain of

CrPHY4, resulting in a polypeptides that terminated at the start of the Quail Box transduction core. In addition, the 5' end of this phytochrome sequence was truncated by approximately 200 amino acids (relative to *CrPHY1* and *CrPHY2*), missing the complete P1 and part of the P2 domains. Comparison of the 3' end of the mRNA of *CrPHY4* at the nucleotide level revealed that *CrPHY4* diverges from other phytochromes at a conserved splice site, which is retained in the mRNA of *CrPHY4* (**Figure 4**). Cloning of the 3' end of *CrPHY4* from genomic DNA showed that the mRNA and the DNA are identical at this region and there is no intervening sequence between the splice site and the poly(A) tail (**Figure 5**). The “intron” on the genomic DNA was not removed from the processed mRNA. This suggests the possibility of an alternative-splicing event in which failure to remove an “intron” results in premature termination (a stop codon is located shortly after the splice site). To determine whether *CrPHY4* possesses a histidine kinase-related domain, or if there are other loci of *CrPHY4*, various antisense primers were designed complementary to the HKRD of both *PHY1* and *PHY2*. Using a specific sense primer for the *CrPHY4* (but not for the *CrPHY1* or *CrPHY2*) variable hinge region between the PHY and first PAS domain, and an anti-sense primer to the conserved HKRD sequences of *PHY1* and *PHY2*, a fragment was amplified from genomic DNA of *Ceratopteris*. Sub-cloning and sequencing of this fragment revealed that another locus of *CrPHY4* with a HKRD exists. The gene lacking the HKRD was designated *CrPHY4A* and the other locus with the HKRD, *CrPHY4B* (**Figure 5**). Attempts to isolate the 3' end of *CrPHY4A* (beyond the poly-adenylation tail) from genomic DNA using sense PCR primers specific for the 3' untranslated region of *CrPHY4A* and antisense specific primers the PRD or the HKRD region of *CrPHY4B*, proved fruitless. In *Adiantum*, *AcPHY4* has a large intron

(~10 kb), which contains an ARET1 retro-transposable element (**Figure 2**). No HKRD has yet been identified for *AcPHY4* nor have there been any reports of duplicated loci. Although the intron sequences between the two PAS domains of *CrPHY4A* and *CrPHY4B* differ, the coding sequences of this region are identical (**Figure 2**). *CrPHY4B* full-length sequences were determined by 5' and 3' RACE of pre-selected full length mRNA. Similar to *CrPHY4A*, *CrPHY4B* is missing the 5' P1 domain and a portion of the P2 domain. Unlike *CrPHY4A*, it has both PAS domains and the complete HKRD. The nucleotide sequence of *CrPHY4A* specifies a single continuous reading frame of 558 amino acids and *CrPHY4B* specifies a deduced polypeptide consisting of 968 amino acids (**Figure 1**).

III. Conserved Serines Among *CrPHYs*

Similar to *AtPHYA* and *AtPHYB*, *CrPHY1* and *CrPHY2* have low complexity regions at the N-termini. Low complexity regions are defined as regions of biased compositions that are composed of mosaics of a small subset of amino acids. Although the functions of these regions are not very well understood, they are believed to be functionally important for phytochromes (Pfam database). Within this low complexity region of the N-terminus are several serine residues that have been demonstrated to be phosphorylated *in vivo* and *in vitro* such as Ser-7 (**Ser-8** in this alignment), and Ser-17 (**Ser-18**) in oat PHYA. Unlike *AtPHYA* and *AtPHYB*, *CrPHY1* (Ser-7) and *CrPHY2* (Ser-51) have a serine residue that aligned with Ser-8 of oat PHYA (**Figure 1**). When these two serines were analyzed to determine if they could be possible phosphorylation sites using NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos>), both residues showed a

high score (>0.9), suggesting a high probability for phosphorylation. With the exception of *AtPHYB*, none of the *CrPHY*s sequences showed a Ser residue corresponding to Ser-18 of oat *PHYA*. Although the serine residues from other phytochromes do not align with the supposed critical serines of oat *PHYA*, there are many serine residues with high theoretical phosphorylation scores that may be functionally equivalent to those in oat *PHYA*. Ser-599 located in the hinge region of oat *PHYA* is critical in regulating protein-protein interaction between *PHYA* and its signal transducer proteins, however there is no serine that aligns with this residue in *AtPHYA* nor *AtPHYB*. *AtPHYA* does have one serine in the hinge region but this residue has a low phosphorylation score (0.256), suggesting that it is not phosphorylated. All of the phytochromes of *Ceratopteris* have two serines in the hinge region but only the Ser-608 in *CrPHY2* has a high phosphorylation score (0.908). Thus, based on these algorithms, it seems that a phosphorylated serine in hinge region may not be critical for functioning in all phytochromes. If *CrPHY4A* and *CrPHY4B* are functional phytochromes, then phosphorylation of serine residues at the N-terminus may not be necessary for all phytochromes.

IV. PEST Sequences in *CrPHY*s

Besides phosphorylation on serine residues, another demonstrated mechanism for posttranslational regulation of phytochrome is its degradation when photoconverted from the Pr to the Pfr form. When dark-grown *Arabidopsis* seedlings are exposed to R or W light, the half-life of phyA decreases more than 100-fold. Kinetic and biochemical studies have shown that the ubiquitin/26S proteasome pathway mediates Pfr-specific

degradation of phyA. It was proposed that both the N- and C-termini are required to provide specificity for ubiquitination and degradation. Within the N-terminal half (323-360 in oat PHYA) lays a PEST domain, which may be phosphorylated and recognized by the ubiquitin/26S proteasome pathway. This PEST sequence is, however, defined by the PESTfind server (<https://embl.bcc.univie.ac.at/cgi-bin/EMBnet/PESTfind>) as a poor PEST sequence and was given a value of +2.78 for *At*PHYA and -2.66 for oat (As) PHYA. Any value greater than positive 5.0 is considered a good PEST sequence. In none of the phytochromes from *Ceratopteris* or *At*PHYB is this region recognized as a PEST sequence by the PESTfind algorithm, nor is there any alternative sequence of comparable value located in the N-terminus.

At the C-terminal end, it was demonstrated that the last five residues (1118-1123) affect the degradation of the holoprotein of oat PfrA (Clough et al., 1999). These residues, however, are poorly conserved among PHYAs of different species and between other phytochromes of the same species. In CrPHY1, a PEST sequence was identified by PESTfind in the C-terminus (position 1046-1063) and was given a value of +4.54 (**Figure 1**). Although this score is below the threshold for an expected functional PEST domain, it represents the highest PEST value assigned and the best-defined PEST sequence from a survey of twenty full-length phytochromes (data not shown). Unlike the PHYAs where the PEST sequence is relatively conserved among species in the N-terminus, this C-terminal PEST is not conserved in PHY1s. Neither *Adiantum* nor *Selaginella* has a PHY1 with a PEST motif of significant value.

V. A *PHOT2*-like protein, But Not a *PHY3*, Was Found in *Ceratopteris*

It has been suggested that polypod ferns would contain a homologue of *PHY3*, the chimeric PHY-PHOT photoreceptor originally identified in *Adiantum*. However, numerous attempts to isolate the unconventional *PHY3* in *Ceratopteris* by genomic PCR or RT-PCR resulted only in a gene fragment that has more identity with *AcPHOT2* (80%) and *AcPHOT1* (58%) than with *AcPHY3* (45%; **Figure 6**). The inability to isolate *PHY3* in *Ceratopteris* is consistent with the finding that blue light but not red light affects the chloroplast relocation of light grown and dark-adapted gametophytes (Kamachi et al., 2004). Moreover, unlike *Adiantum*, *Ceratopteris* does not display any red light-mediated phototropism (Kamachi et al, 2004; Short, unpublished).

VI. *Ceratopteris richardii* Contains a Small Gene Family of Phytochromes.

Using a 2600 bp fragment of *CrPHY2* as probe, Southern hybridization analysis was performed on *Ceratopteris* genomic DNA digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III or *Pst*I (**Figure 7**). Consistent with the number of different fragments isolated by PCR and the cDNA library screening, *Ceratopteris richardii* seems to possess a small phytochrome gene family consisting of three to four members. This result is also consistent with the number of phytochromes that have been previously isolated from various ferns, including *Adiantum capillus-veneris* and *Anemia phyllitidis*.

VII. *PHY1* and *PHY2* Evolved Before the Divergence of Seed-Bearing Plants From Ferns.

To clarify the evolutionary relationships among the members of *Ceratopteris richardii* phytochrome multigene family and other groups of plants, phylogenetic

analyses were conducted using the amino acid sequences of the GAF (P3) and PHY (P4) domains of phytochromes. Analyses were limited to this region since many of the reported phytochrome sequences available are incomplete and confined to this region. Using several different methods for computing the evolutionary distances between phytochrome amino acid sequences, phylogenetic trees were constructed from these distance values. Regardless of the method (Neighbor-Joining, Minimum Evolution, or Maximum Parsimony), the topology of the tree is essentially the same. Rooting the trees with the bryophytes (*Physcomitrella* and *Ceratodon*), it can be demonstrated that *PHY1* and *PHY2* genes of ferns (*Ceratopteris* and *Adiantum*) evolved before the separation of ferns from seed-bearing plants (**Figure 8**). The phytochromes of bryophytes form a distinct, slowly evolving clade, and those of higher plants, a rapidly evolving clade. Consistent with previous findings (Mathews and Sharrock, 1997), duplication of the PHYA-like and PHYB-like phytochromes preceded the separation of gymnosperms (e.g. *Pinus sylvestris*) from angiosperms (e.g. *Arabidopsis thaliana*). After the divergence of ferns from seed plants, a duplication of a PHY2-like phytochrome occurred, producing the present PHY2 and PHY4 (**Figure 9**). This duplication event preceded the separation of *Ceratopteris* from *Adiantum*.

VIII. *PHY3* Shares Ancestry With *PHYs* of Seed Plants

Although few previous studies have focused on the relationship between phytochrome sequences of ferns and those of seed-bearing plants, the chromophore-binding region of PHY3 seems to have been derived from an ancestral phytochrome with direct lineage to the phytochromes of seed plants (**Figure 8**). Despite the evolution of PHY3 into a novel

chimeric protein with very different functions from those of seed-bearing plant phytochromes, the phytochrome-derived domains of PHY3 are apparently more closely related to the gymnosperm and angiosperm phytochromes than are PHY1, PHY2, or PHY4. This branching pattern of PHY3 with the phytochrome of seeded plants is supported using different tree-building methods and high bootstrap values that always resulted for all major nodes. It should be noted that the chromophore-binding domain of PHY3 is diverging relatively rapidly.

IX. *PHY2* and *PHY4* Resulted From a Gene Duplication That Occurred During the Rapid Angiosperm Radiation.

To determine the relative period when the duplication of PHY2/4 occurred, all available orthologues of PHY1, PHY2 or PHY4 were used to construct phylogenetic trees. To simplify the tree and to better show relationships between phytochromes, ferns with more than one reported phytochrome sequence were preferentially used. Using the Neighbor-Joining or Minimum Evolution Method, various phylogenetic trees were constructed. Since the phytochrome sequences used to construct these trees were relatively short in length (182 amino acids), the bootstrap values at several nodes were low. Despite low bootstrap scores, however, different tree-constructing methods yield the same result. The first sign of a duplication event to produce orthologues PHY2 and PHY4 occurred some time before the origin of polypod ferns (**Figure 9**). From the limited sequences available, this duplication would have occurred during the diversification of the osmundaceous ferns (*Osmunda*) and the water ferns (*Salvinia*), between 280-220 Mya, approximately the time the origin and radiation of angiosperm began (Schneider et al., 2004).

X. *PHY1* is Diverging More Rapidly Than *PHY2* or *PHY4*

As is evident from the branch length, the rate of change for *PHY1* is much greater than that of *PHY2* and *PHY4*. Using the Tajima's relative rate test (Tajima, 1993), which compares two sequences (*Adiantum* and *Ceratopteris*) to an outgroup (*Physcomitrella* or *Ceratodon*), the number of unique differences between *PHY1* and *PHY2* can be determined. As seen in **Table 1**, more unique differences have accumulated in *PHY1* since the duplication of *PHY1* and *PHY2* and their divergence from the phytochromes of bryophytes (various typical chlorophyta phytochromes such as CP2, CP3, PP1-4 were used as outgroups, producing the same results). Statistically, the most significant changes occurred in the P1-P2 domains and in the HKRD of *PHY1*, suggesting that these regions may not be vital for full functionality as they are also missing from Cr*PHY4A* and Cr*PHY4B*. Alternatively, a co-evolutionary process with signaling components that interact with the P1 and P2 domains may actively drive the changes in these domains.

XI. Gene Duplication by Retro-Transposition in Ferns.

To determine if the relative rate of change for *PHY1* is also greater than the rate of change for *PHY2* in different species within the same genus, fragments of *PHY1* and *PHY2* corresponding to the PAS₂ and HisKA domains of phytochromes were isolated by PCR from three species of *Adiantum* (*Adiantum capillus veneris*, *Adiantum pedatum*, and *Adiantum tenerum*), two species of *Pteris* (*Pteris cretica* and *Pteris multifida*) and two species of *Ceratopteris* (*Ceratopteris richardii* and *Ceratopteris thalictroides*). This region of the phytochrome was chosen because it is highly variable among species and

contains two introns, making it possible to easily discern between closely related sequences and to identify possible contamination. When various fragments from the same species were compared, it was found that *PHY1* recently duplicated within *C. richardii* and *PHY2* also independently duplicated within *P. cretica* and *C. thalictroides* (**Figure 10**). Analysis of the duplicated *PHY2* sequences revealed that one fragment corresponds to a *PHY2* with introns and the other, without introns, suggesting the duplication occurred by means of retro-transposition (**Figure 10**). Analysis of the *PHY1* sequences from *C. richardii* showed that one fragment has the expected two introns and the other fragment has only one intron (**Figure 10**). This analysis suggests that *PHY1* may have duplicated, and that retro-transposition followed by homologous recombination of retrotransposed DNA with the genomic copy maintained one intron while effectively deleting the remaining two introns. Alternatively, a retro-transposition followed by gene conversion may have reintroduced an intron-containing fragment into the otherwise fully processed cDNA. It cannot be determined from the incomplete sequences if both forms of the recently duplicated genes are functional. Trees constructed from the translated amino acid sequences of these fragments show that *PHY1* and *PHY2* are highly conserved within a genus (**Figure 11**). Divergence occurred between genera. As expected, in *Pteris*, *PHY1* is also evolving at a faster rate than *PHY2*.

XII. Decrease of Transcript Level of *CrPHYs* in *Rc*

To determine the expression of the various phytochromes under different light conditions, pre-sterilized RNW1 dry spores were placed in liquid media for two days in the dark and transferred directly to R, FR, B or W light for 7 d. Some spores were placed in liquid culture for 2 d, exposed to 1 d of continuous W to initiate germination, and then

placed in the dark for 6 d. After the light treatment, total RNA was isolated, DNase treated, and subjected to RT-PCR to measure the relative concentrations of *CrPHY1*, *CrPHY2*, *CrPHY4A*, and *CrPHY4B*. To verify that equal amounts of RNA were used and the amplifications were within the linear range, the *Ceratopteris* ubiquitin gene was amplified for 15, 20 and 25 cycles (**Figure 12**). Within this range of amplification, the increase is still logarithmic and the amounts of RNA from plants subjected to various light treatments was apparently equivalent, as determined spectrophotometrically and by agarose gel electrophoresis. Analysis of the amplified products of *CrPHY1*, *CrPHY2*, *CrPHY4A* and *CrPHY4B* under the same conditions used for the ubiquitin amplification showed that the level of transcripts of these genes decreases with prolonged exposure to R. The transcript levels, however, are relatively static in all other light treatment (FRc, Bc, Wc), suggesting that the CrPHYs may be light stable Type II phytochromes if the mRNA levels reflect protein content. Presently, it is not possible to differentiate between the two isoforms of *CrPHY1* by RT-PCR because of their identical coding sequence. This differentiation may be possible once the 5' and 3' UTR from *CrPHY1B* mRNA are determined.

E. DISCUSSION:

I. Phytochrome is Represented by a Multi-Gene Family in Ferns

Previous studies have shown that ferns have a multigene family of phytochromes. In *Anemia phyllitidis*, four phytochrome gene fragments (*ApPHY1*-*ApPHY4*) have been reported, though the sequences for only two fragments have been made publicly available. In *Adiantum capillus-veneris* (*Ac*), three complete phytochromes (*AcPHY1*-

AcPHY3) and a partial *AcPHY4* have been identified. In *Ceratopteris*, a multigene family of phytochromes has been isolated, with homology to *AcPHY1*, *AcPHY2* and *AcPHY4*. Analyses of available vascular pteridophyte phytochrome sequences have yielded definitive homologues of *CrPHY1* in *Anemia phyllitidis*, *Angiopteris evecta*, *Blechnum spicant*, *Marattia laevis*, *Ophioglossum vulgatum*, *Osmunda regalis* (2 homologues from a gene duplication), and *Polypodium vulgare*. Definitive homologues of *CrPHY2* have also been found in *Anemia phyllitidis*, *Azolla filiculoides*, *Botrychium lunaria*, *Dryopteris felix-mas*, *Osmunda regalis*, *Platycerium angolense*, *Polypodium vulgare*, and *Salvinia natans*. Although labeled as *PHY2*, homologues of *CrPHY4* can be also be found in *Azolla filiculoides*, *Blechnum spicant*, *Dicksonia antarctica*, *Marsilea quadrifolia*, *Pilularia globulifera*, *Polypodium vulgare*, *Salvinia natans*, and *Vittaria lineate*.

Consistent with the phylogenic analyses, a precursor *PHY* duplicated to produce ancestral *PHY1* and *PHY2*. This duplication event occurred very early in vascular plant evolution, before the separation of seed plants from cryptogams. The presence of *PHY1-like* and *PHY2-like* genes in lycopods (*Huperzia selago*) indicates that the duplication occurred before the separation of *Lycophyta* from *Pteridophyta*. Duplication of a *PHY2-like* predecessor gene to produce *PHY2* and *PHY4* occurred during the diversification of the leptosporangiate ferns (280-220 Mya). The first sign of definitive homologues of *PHY2* and *PHY4* in fern evolution can be found in *Azolla* and *Salvinia* (perhaps also in *Anemia*). Since polypods (e.g. *Ceratopteris* and *Adiantum*) diverged later than these water ferns, and these three gene members are found in many of the polypods examined, it seems that all polypods would have at least three types of typical phytochromes corresponding to *PHY1*, *PHY2*, and *PHY4*. At the genomic DNA level, preliminary

characterization shows that the intron positions—but not the intron sequences—have been maintained, except in those phytochromes duplicated or converted by retro-transposition.

II. Phytochrome Duplication and Species Divergence

During speciation among ferns, duplication of one or more phytochromes occurred, as suggested by the presence of multiple *PHY1* genes in *Osmunda regalis* and *Ceratopteris richardii*, *PHY2* genes in *Angiopteris evecta*, *Pteris cretica* and *Ceratopteris thalictroides*, and two *PHY4* genes in *Ceratopteris richardii*. During diversification, some phytochrome copies may also have lost functionality, as most likely occurred in *PHY4* in *Adiantum* as a result of damage to a duplicate gene or insertion of a gene fragment or pseudogene into the genome. It has been shown that several polypods also contain a fourth, atypical, type of phytochrome designated *PHY3*. This phytochrome has been found to confer R control of several functions normally mediated through B, such as phototropism and chloroplast redistribution. However, *Ceratopteris* and *Pteris* show no R-mediated phototropism or chloroplast photorelocation, and *PHY3* could not be amplified by PCR from *Ceratopteris* genomic DNA or cDNA with the primers Kawai *et al* (2003) used to isolate *PHY3* fragments from other pteridophytes. In the inferred phylogenetic tree of fern sequences, the phytochrome-derived domains of *PHY3* are apparently more closely related to the gymnosperm and angiosperm phytochromes than are to the phytochromes of ferns, despite the absence of a *PHY3*-like chimera in seed-bearing plants. Moreover, the branch length of *Adiantum PHY3* indicates that within the GAF/PHY domains the rate of divergence is comparable to those of corresponding

sequences from angiosperm phytochromes (**Figure 8**). The phytochrome-derived domains of PHY3 may have a direct ancestral lineage to the phytochromes of seed plants. If there were a direct lineage, this would require an early gene duplication of the progenitor phytochrome prior to separation of ferns from seed plants. The *PHY3* like phytochrome would have been evolving under relaxed constraints and most likely have been silenced in ferns until the recombinant event with a phototropin gene. Since phototropism and chloroplast relocation are mediated exclusively by blue light (phototropins) in *Ceratopteris* and *Pteris*—as they are in angiosperms— the N-terminus of PHY3 may have become lost in these ferns. *PHY3* may have conferred a selective advantage in a small group of polypods. Survival under the low-light canopy of the angiosperm forests may have been aided by duplication and selective maintenance/divergence of photoreceptors as exemplified by the angiosperm *Arabidopsis*, which has five phytochromes, two cryptochromes, two phototropins and FRK1, a possible green light photoreceptor. In parallel, *Ceratopteris* has at least five phytochromes, and five cryptochromes have been identified in *Adiantum*. As seen with the high number of phytochromes and cryptochromes and coupling of phytochrome/phototropin to form the chimaeric *PHY3*, ferns have evolved tightly coupled mechanisms to perceive red and blue light, both light are necessary for such processes (besides photosynthesis) as germination, protonemata and prothalli development, rhizoid formation and elongation, chlorophyll production, archegonia maturation, sporophyte development, chloroplast photorelocation and phototropism.

III. PHY1 is Evolving Faster in Ferns

Comparison of the branch lengths in inferred phylogenetic trees for *PHY1* and *PHY2* in several species of ferns has shown that *PHY1* is evolving at a greater rate than *PHY2* or *PHY4* (**Figure 9**). The maintenance of *PHY2* and *PHY4* sequences suggests that at least one of these genes must be critical for the fern's basal life functions such as germination, development, and ultimately, reproductive success. *PHY1* appears to be evolving under less selective constraints (or subject to diversifying selection) than *PHY2* and, like *PHYA* of the angiosperms, may confer competitive advantages. The duplication and separation of *PHY1* and *PHY2* before the divergence of seed plants suggests that these phytochromes may have limited functional similarities to those of higher plants, although the strong conservation of phytochrome structure and sequence indicates that at least some functions are also conserved, and under strong purifying selection. In angiosperms, *PHYA* promotes germination of seeds in the VLFR mode. Through the FR-HIR, *PHYA* suppresses hypocotyl growth, promotes cotyledon unfolding, inhibits chlorophyll synthesis, and promotes anthocyanin accumulation. When *Arabidopsis* seedlings are grown under high fluence rate FR_c for 4 days, they fail to green upon subsequent transfer to W_c because of the *PHYA*-mediated block of chlorophyll biosynthesis. Through the LFR and R-HIR, *PHYB* has been shown to be primarily responsible for hypocotyl growth inhibition, chloroplast maturation, regulation of flowering time, shade avoidance, stem and leaf expansion, and circadian rhythmicity. *Ceratopteris* does not show any *PHYA*-like VLF germination response (Cooke et al., 1987), and like many other ferns, does not de-etiolate in FR_c or exhibit a block of chlorophyll synthesis after young tissues are grown in FR for 4 d and transferred to W, responses mediated through *PHYA* in

angiosperms (Bissoondial et al, unpublished data). Unlike seedlings of angiosperms, etiolated gametophytes of ferns are constitutively green, as are many etiolated gymnosperms seedlings. The lack of detailed physiological characterization of the germinating spores under different wavelengths of light for prolonged period limits any further comparison between processes mediated by PHYA and PHYB of angiosperms to those mediated by the phytochromes of ferns.

IV. *CrPHY4A* and *CrPHY4B*

Like the cyanobacteria *Cph2* family, *CrPHY4A* and *CrPHY4B* lack the P1 and P2 domains found in plant phytochromes but contain the conserved cysteine present in the P3 domain. Both *CrPHY4A* and *CrPHY4B* contain the minimum region necessary in both phytochromes for autocatalytic chromophore attachment (Deforce et al. 1991; Boylan & Quail 1991; Cherry et al 1993; Vierstra 1993; Quail, 1997). However, the lack of the P1 and P2 domains suggests that the maintenance of photoreversibility and absorption spectra in both Pr and Pfr form would be altered from those characteristics displayed by phytochromes containing the complete NH₂-terminal structural domains. Deletion of segments within the first 115 positions (P1 and P2 sub-domains) of the NH₂ terminus of oat phyA altered the stability and spectral integrity of the Pfr and Pr forms (Quail, 1997). *CrPHY4A* and *CrPHY4B* may display a blue-shifted absorption maxima of both Pr and Pfr forms and an increased rate of dark reversion of Pfr to the Pr form, reiterating the importance of blue light in ferns' development and the importance of continuous light for fern development. Deletions within the serine-rich region in oat phyA result in a phytochrome that, depending on the species of transgenic host plant, was

normal, hypersensitive for VLFR, or showed a dominant-negative suppression of the FR-HIR (Casal et al., 2002). If, as in angiosperms, the phytochromes of ferns were also nuclear localized, the lack of the serine-rich region and much of the C-terminus and their putative nuclear localization sequences would restrict *CrPHY4A* activity to the cytosol. The lack of the C-terminus of *CrPHY4A* may also affect the degradation of the protein, as deletion of the C-terminus of oat phyA yields a protein with a longer half-life (Clough et al., 1999).

Recently it was demonstrated that the C-terminus, specifically the histidine kinase-related domain (HKRD), is dispensable for biological activity in PHYB and is not required for signal transduction as long as there is a dimerization domain and a nuclear localization signal. The C-terminal domain of PHYB may be necessary only for attenuation of the activity of PHYB rather than positive transduction of signals to other factors (Matsushita et al, 2003, Krall and Reed, 2000). *CrPHY4A* may play a role in attenuating the signals (e.g. dominant negative suppression) of other phytochromes by forming nonproductive heterodimers, effectively titrating out the active phytochromes, or by forming homodimers that associate with phytochrome reaction partners and thus compete with the normally productive downstream signaling components. The presence of the PAS_I motif suggests that dimerization may be possible. Moreover, transcripts of *CrPHY4A* are expressed at low levels and cannot be detected in dry spores or during imbibition, indicating that its role may be to regulate prothallus development rather than spore germination. It is of interest to determine how *CrPHY4A* and *CrPHY4B* are regulated at the protein level and to determine if the P1-P2 domains were lost early in fern evolution (strengthening the case for a functional role of the modification) or if this

truncation is a species-specific adaptation or possibly a recently arisen pseudogene.

Besides *CrPHY4A* and *CrPHY4B*, no complete homologues of *PHY4* have been reported in the literature.

V. Expression Pattern of the Phytochrome Gene Family in *Ceratopteris richardii*

In *Adiantum capillus veneris* (*Ac*), two complete conventional phytochromes have been isolated, *AcPHY1* and *AcPHY2*. *AcPHY1* was first reported by Okamoto *et al* (1983) and was from isolated only from dark-grown gametophytes and sporophytes. *AcPHY2* cDNA is similar in sequence to previously cloned cryptogam phytochromes, and is less similar to phytochromes from seed plants (Nozue *et al.* 1998a). RT-PCR analysis showed that the *AcPHY2* gene is expressed in all developmental stages examined (imbibed spore, protonema, dark or light grown young leaf), suggesting that *AcPHY2* might function constitutively (Nozue *et al.* 1998a). In the liverwort *Marchantia paleacea* var. *diptera* (*Mpd*), *MpdPHY1* transcript accumulates in the dark and is suppressed in the light. A R irradiation results in degradation of the *MpdPHY1* transcript and subsequent FR suppresses this degradation (Suzuki *et al* 2001). In *Anemia*, it was reported that the mRNA level of *PHY1* and *PHY4* accumulated during spore imbibition in the dark, and that that level decreased after spores were transferred to continuous R. *Anemia PHY3* (a standard phytochrome not closely related to the chimeric *AcPHY3*, based on sequence and on the evidence that the family containing *Anemia* diverged before the origin of the pteridaceae to which *Adiantum* belongs) mRNA could not be detected in spores during the initial 48 h of darkness, but increased when spores were transferred to R (Maucher, Schraudolf and Scheuerlein, unpublished result). *Ceratopteris*

richardii is similar to *Adiantum*, *Marchantia*, and *Anemia* in that it contains a phytochrome multigene family and some members of that family are that constitutively expressed in all developmental stages (data not shown). Moreover, R_c results in the degradation of the *CrPHY* transcripts. *Ceratopteris* differs from the other ferns characterized in that transcripts for all the phytochromes are expressed in W at levels comparable to those in D or in FR. None of its phytochromes are upregulated after 7 d in R. It will be interesting to characterize the development of gametophytes in continuous R and FR—as well as in other light conditions—to determine whether changes of phytochrome levels correlate with responsiveness to light during development.

VI. Conserved Serine Residues and PEST Motif

At this stage it is not possible to determine how individual phytochromes are regulated at the protein level. The presence of a putative PEST motif in its C-terminus suggests that *CrPHY1* may have a shorter half-life than the *CrPHY2*, and by analogy to angiosperm phytochromes, this lability may be light dependent. The presence of numerous serine residues in the N-terminal end and in the hinge region suggest that phytochromes may also be regulated by phosphorylation and dephosphorylation, and suggests conservation in regulatory elements such as FyPP (or Protein Phosphatase 6), which has been shown to bind and dephosphorylate oat PHYA. BLAST analyses of FyPP to the *Ceratopteris* EST cDNA library has identified a putative protein phosphatase that has high homology to the FyPP that has been shown to bind to the C-terminal of PHYA or PHYB (Kim et al., 2002) in pea and *Arabidopsis*. FyPP dephosphorylates oat PHYA in a light-dependent manner (possibly on S599), and affects PHYA-mediated light

signaling in the control of flowering time (Kim et al., 2002). It is believed that the phosphorylation of S598/S599 in the hinge region attenuates phytochrome activity (prevents interaction between signal transducers such as NDPK2 and PIF3). In *Ceratopteris*, the mechanism of regulation phytochrome activity by dephosphorylation may exist but the serine residues that are affected may vary. It remains to be determined if the homologue of FyPP found in *Ceratopteris* interacts to CrPHY1 or CrPHY2.

F. CONCLUSION

Ceratopteris richardii is a homosporous fern that is emerging as a model organism to study photomorphogenesis at a cellular level. Although extensive studies have elucidated photobiological responses of *Ceratopteris richardii*, no photoreceptors have been isolated until now. Phylogenetic analyses made in this study suggest that the phytochromes of ferns would have few functional similarities to the phytochromes of angiosperms. Moreover, in addition to evolving the chimeric *PHY3*, gene duplication of specific phytochromes may have allowed some ferns to cope with the low-light canopy conditions created by the angiosperm radiation. It remains to be discovered how having multiple copies of *PHY* genes provides a competitive advantage for these ferns. Extensive physiological studies on the prolonged effect of different wavelengths of continuous light (e.g. R and FR-HIR) on germinating spores may provide further insight on the role of these phytochromes in gametophyte development, and may further elucidate the relationship between the phytochromes of ferns and those of other plant groups. Furthermore, determining the copy number of phytochromes in various ferns and more detailed phylogenetic analyses of isolated phytochrome sequences may better show

how species of ferns diversified as well as provide more data on the adaptive evolution of phytochromes in plants. Identification of the phytochrome multigene family in *Ceratopteris richardii*—and the ability to alter expression of targeted genes by RNA interference (RNAi)—makes possible the characterization of specific roles for individual phytochrome paralogues (*PHY1*, *PHY2* and *PHY4*) in a fern during the early stages of gametophyte development. Further analyses may elucidate the evolutionary significance of multiple *PHY* gene duplications that have occurred within *Ceratopteris* and in other fern species.

CHAPTER 3: THE ROLE OF PHYTOCHROMES IN *CERATOPTERIS*

A. ABSTRACT

B. INTRODUCTION

C. MATERIALS AND METHODS

D. RESULTS

- I. *CrPHY1*, *CrPHY2* and *CrPHY4B* Transcripts Increase with Imbibition
- II. High Temperature and R-HIR Delay Germination in *Ceratopteris richardii*
- III. Gametophytes of Ferns Do Not Show Typical PHYA and PHYB-Like Responses
- IV. B_c or W_c Inhibits the Elongation of the Primary Rhizoids
- V. Incubation of Spores With dsRNA of *CrPHY4B* Stimulates Gene-Specific Expression
- VI. High Level of Phytochromes Stimulate Cell division and Chlorophyll Production
- VII. Spores Are Sensitive to B After Germination
- VIII. Knock-Down of *CRY4* in *Ceratopteris* Increases Germination in
- IX. Increased Level of *CrPHY1*, *CrPHY2* and *CrPHY4B* in the *dkg1* Background

E. DISCUSSION

- I. RNAi in *Ceratopteris***
- II. Spore Germination**
- III. Rhizoid Initiation and Elongation**
- IV. Prothallus Development and Chlorophyll Accumulation**

A. ABSTRACT

Although the early photobiology of *Ceratopteris richardii* has been extensively characterized, there is little quantitative data on the prolonged effects of D, FR_c, R_c, B_c or W_c on cell division, rhizoid development and chlorophyll production in the gametophyte prothalli and the underlying molecular mechanism of photomorphogenesis in ferns. In this study it was found that the absolute dosage of the Pfr form of phytochromes affects cell division, which is integrally linked to rhizoid formation and chlorophyll production in gametophytes. In conditions that allow for high expression of phytochrome transcripts and more photoconversion of the Pr form to Pfr form (B_c and W_c) there is more cell division, rhizoid production, and chlorophyll accumulation in the prothalli. Although the R_c-grown gametophytes are more developed at 7-14 d than D- or FR_c-grown plants, prolonged exposure to R_c reduces the level of phytochrome transcripts and inhibits further development. This effect is irreversible and leads to atrophy of the gametophytes without any sporophyte development. Unlike angiosperms, *Ceratopteris* shows a very limited de-etiolated response in FR_c, as gametophytes grown in FR_c are only slightly longer, broader, and greener than those grown in D. Moreover, there is no inhibition of chlorophyll synthesis under FR_c in gametophytes of *Ceratopteris*. These physiological results are consistent with previous phylogenetic analyses that demonstrated that the phytochromes of ferns are very divergent from the PHYA and PHYB of angiosperms. Attempts to knock down the levels of phytochromes by incubation of dry spores with short homologous dsRNA resulted in the decrease of *CrPHY1* and *CrPHY2*; the latter is more strongly affected because of its constitutively low level of expression. Attempts to

knock down of *CrPHY4B* by RNAi produced an unexpected gene-specific stimulation of transcription. No change in the level of *CrPHY4A* could be detected from any RNAi treatment since the expression of this gene is barely detected in imbibed spores. When spores were incubated with a mixture of short homologous dsRNA of *CrPHY1*, *CrPHY2* and *CrPHY4*, the levels of transcripts for all of these genes were increased.

Phenotypically, spores that were incubated with dsRNA for *CrPHY4B* or a mixture of dsRNA for *CrPHY1*, *CrPHY2* and *CrPHY4B* produced prothalli with more cell division, rhizoid production, and chlorophyll accumulation than with other treatments, consistent with other physiological data. The latter treatment produced the most dramatic results in W_c or R_c . No effect of the triple-dsRNA treatment was observed in D , or FR_c . B_c

inhibited spore germination regardless of the dsRNA treatment. Although the gametophytes of *dsPHY4A*-treated spores were always slightly smaller than the controls, no significant effect could be detected from the knock-down of *CrPHY1* and *CrPHY2*, suggesting that several members of the phytochrome multigene family of *Ceratopteris richardii* may act redundantly to regulate germination and the ensuing prothallus development. Since the dsRNA of phytochromes—regardless of type or combination—could not produce any significant change in germination especially in B_c , attempts were made to isolate and characterize other photoreceptors in *Ceratopteris*. Knock-down of *CrCRY4* resulted in a three-fold increase of germination in B_c , further linking *CRY4* to the B-inhibition of germination in ferns. Under W_c , *CrCRY4* knock-down also resulted in larger prothalli with more chlorophyll than those not dsRNA-treated. These results suggest that specific cryptochromes (e.g. *CrCRY4*) may act antagonistically to

phytochromes to regulate germination and prothallar development, while others may act synergistically with phytochromes to allow for greater development in W_c or B_c .

B. INTRODUCTION

In *Ceratopteris richardii*, physiological studies have demonstrated that phytochromes stimulate spore germination in a characteristic *low fluence response* (LFR; (Cooke et al., 1987). In addition, spores require at least 7-10 d of dark incubation after imbibition to achieve maximum light sensitivity, a period during which the level of phytochromes has been shown spectrophotometrically to increase (Cooke, 1993). Similar to many other ferns, blue light (B) can inhibit spore germination even after brief exposure to red light (R) in *Ceratopteris richardii*. The inhibitory and antagonistic effects of B suggest that cryptochromes may also be involved in regulating germination and act farther downstream of phytochrome signaling pathway, accounting for the inability of R to completely overcome the inhibitory effect of cryptochromes. CRY3 or CRY4 (or both) of ferns are believed to be responsible for this inhibition because of their translocation to the nucleus (Imaizumi et al., 2000). Other physiological studies on the development of the prothalli of *Ceratopteris* have shown that exposure of the dark-grown gametophytes to R or B alter the prothallar morphology, increasing cell division and differentiation, resulting in a broadened notch meristem and the transition from filamentous to prothallial growth. Cryptochromes and phytochromes are believed to act synergistically to inhibit unidirectional cell elongation, and to promote two-dimensional cell expansion and cell division. Through a LFR, phytochromes have been shown to induce marginal cells of the elongation zone of etiolated gametophytes to divide asymmetrically, resulting in cells that develop into secondary rhizoids (Murata and Sugai, 2000). Elongation of these rhizoid initials, however, requires continuous irradiation with

W, B or R; a high fluence response suggesting that secondary rhizoid formation and rhizoid elongation involve separate signal transduction pathways.

The myriad varieties and photosensitivities of phytochrome-mediated responses, as well as the presence of multiple phytochromes in most plants, suggest that several phytochromes may regulate gametophyte development. Recently, five phytochrome sequences have been identified in *Ceratopteris richardii*: *CrPHY1A*, *CrPHY1B* (partial cDNA), *CrPHY2*, *CrPHY4A* and *CrPHY4B*. *CrPHY1B* and *CrPHY4A* have resulted from recent duplications, possibly by retro-transposition and gene conversion or homologous recombination. It remains to be determined whether *CrPHY1B* encodes a canonical phytochrome. *CrPHY1A* and *CrPHY2* encode full-length canonical plant phytochromes. *CrPHY4A* is an unusual phytochrome in that it has lost the PAS₂ domain and the HKRD at the C-terminus. Furthermore, both *CrPHY4A* and *CrPHY4B* are missing the P1 and P2 domains of the N-terminus and are otherwise identical in their coding sequences except for the *CrPHY4A* C-terminal truncation. Semi-quantitative RT-PCR demonstrated that *CrPHY1*, *CrPHY2*, *CrPHY4A* and *CrPHY4B* are expressed at the mRNA level, and that they are regulated such that R_c decreases transcripts for all of the phytochromes. Phylogenetic analyses show that *PHY1* and *PHY2* evolved very early in the history of vascular plant, and that their divergence preceded the separation of ferns from seed plants. In ferns, with the exception of chimeric *PHY3*, the role of specific phytochrome at a molecular level is unknown. This study relates the previous phylogenetic analyses with specific phytochrome-mediated responses (PHYA-like FR-HIR or PHYB-like R-HIR), characterizes the expression the specific phytochromes and cryptochromes, and assesses their role in germination and development by RNA silencing or RNAi.

Moreover, this study provides quantitative data on the prolonged effect of different wavelengths of light and other environmental factors on cell division, rhizoid growth and chlorophyll production during the development of prothalli.

C. MATERIALS AND METHODS:

Isolation of total RNA from dry or imbibed spores:

Four milligrams of dry pre-sterilized wild type *RNWI* spores (Carolina Biological) were suspended in 200 µl of *C-Fern* media (Carolina Biological), and then placed dark for 1-5 d. After the appropriate incubation, media was removed from spores by pipetting, and total RNA was isolated from the fresh tissue using the *RNAqueous-4 PCR kit* (Ambion). The tissue was homogenized in a 1.5 ml eppendorf tube in 300 µl of the supplied *Lysis Buffer* and total RNA was extracted according to manufacture's instruction. The purified RNA was *DNase* (1U/ug) treated for 1-2h at 37 °C prior to RT-PCR. RNA was stored at -80°C.

Double Stranded RNA (dsRNA) Synthesis:

PCR fragments of 3' ends of *CrPHY1*, *CrPHY2*, *CrPHY4A* and *CrPHY4B* were amplified from previously subcloned genes using paired primers, each primer containing a 5' T7 promoter TAATACGACTCACTATAGGGAGACCAC. The primers used were
CrPHY1 T7F: TAATACGACTCACTATAGGGAGACCACGTAACATGTCCTTCATCGTTAAG,
CrPHY1T7R: TAATACGACTCACTATAGGGAGACCACCAATACAGAAGGTTGATCAC,
CrPHY2 T7F: TAATACGACTCACTATAGGGAGACCACCAGAAAGCTTGTAATAATTAATG,
CrPHY2 T7R: TAATACGACTCACTATAGGGAGACCACGCTCACCATTATTTATTGATG,
CrPHY4A T7F:
 TAATACGACTCACTATAGGGAGACCACCAATGAGATGGTCCGTTTGATTGAGAC,
CrPHY4AT7R: TAATACGACTCACTATAGGGAGACCACCTAAAGACAGCAACCGCTCCACAA,

CrPHY4B T7F: TAATACGACTCACTATAGGGAGACCACGCTTGTGAAATTGATGAATGGAG,

CrPHY4B T7R: TAATACGACTCACTATAGGGAGACCACCTTCAAACACAGCCAGCGCCAG.

PCR fragments for the control were isolated from *Myosin IIA* cDNA from chicken using the primers

MyoIIA-T7F: TAATACGACTCACTATAGGGAGACCACGACCTGCCCTTTGTCGTCACAC,

MyoIIIT7R: TAATACGACTCACTATAGGGAGACCACGGTCATTCCATCATGTGTAAC.

A fragment of *CrCRY4* was amplified from genomic DNA using the following primers:

CrCRY4F: TCAGTGGCTTCCTGAACTTGTTAGACT and

CrCRY4R: TGTCGGAACCTTTACCTGTTAGGTGAG.

The PCR fragment was subcloned into pGEMT (Promega) vector and sequenced. Using this fragment, internal PCR fragments with the T7 promoters for *CrCRY4* were amplified using the primers

CRY4-T7F: TAATACGACTCACTATAGGGAGACCACCATCATCCTTGGGATGCTCCTCCTGCTGT, and

CRY4-T7R: TAATACGACTCACTATAGGGAGACCACCAACTTCATTCCCTGCTTAAACGCCGATG.

PCR-amplified fragments were purified using the QIAGEN Gel Purification Kit and eluted in 30 µl of DEPC-treated water. 0.5 µg of the purified fragment was used as the template for RNA synthesis using the RiboMAX Large Scale RNA Production System (Promega). Sense and anti-sense RNA were synthesized simultaneously using the T7 RNA Polymerase as specified by the manufacturer's protocol. dsRNA was purified by phenol/chloroform partitioning and ethanol precipitation. The integrity of transcripts was analyzed on a 1.0% agarose gel with ethidium bromide and the RNA concentration was determined spectrophotometrically. dsRNA was stored at -80 °C until needed.

RNAi

Four milligrams of dry pre-sterilized wild type *RNWI* spores (Carolina Biological) were suspended in 200 µl of *C-Fern* media (Carolina Biological) containing 0.2 mg/ml of double stranded RNA (dsRNA). Spores were incubated with dsRNA for 48 h in darkness with continuous shaking, and then placed in W for 24 h. Spores were plated on 1% agar-solidified *C-fern* medium plates in W, then exposed to the appropriate light conditions (R, FR, B, D or W). Alternatively, knock down of specific genes was assessed by semi-quantitative RT-PCR after the 24 h white light exposure. Media was removed from spores by pipetting, and total RNA was isolated using the *RNAqueous-4 PCR kit* (Ambion) as previously described.

RT-PCR

Five hundred nanograms of DNase-treated total RNA was reversed transcribed with oligo (dT) primers at 50°C using the *Thermo Script RT-PCR* system (Invitrogen). First-strand cDNA was then amplified by PCR with *Taq Pol* (New England Biolabs) for 20 or 25 cycles using the following gene specific primers:

CrPHY1-1220S: CAAGCTAGTAAGCTTTGGGGACTT,

CrPHY1-2134AS: CTAGCAAAGGAACTTGACACAGG (914bp),

CrPHY2-1177S: TGTTGTTGTGAATGACAATGATGA,

CrPHY2-2094AS: ATCCACCACTATATCTCTTACCAA (917bp);

CrPHY4A-955S: TGGAAAGAAGTTCTGGCTACTTGGTATAAC

CrPHY4A-1705AS: GGAAATACAAGAGACCTAAGAATCCAACAAACA (750 bp);

CrPHY4B-1695S: CACATGGCGAACAGGCTCAGAATGGTCCTG,

CrPHY4B-2416AS: TTTTGGTAGCTGTCTCTCACAAG (721 bp),

CrACT-S: CAAGGCACACTGGTGTTCATGGTTG,

CrACT-AS: CAGGAAGTTCGTAACCTCTCTC (617),

CrUBQ-S: CTTCGTCAAGACCCTGACAGGCAA

CrUBQ-AS: GTT ATCAATTGTATCGGAGCTCTC (526bp).

The primers used to assess the expression of *CrCRY4* were those previously described.

Light Sources

General W light was emitted from cool white fluorescent bulbs at a fluence rate of $100\mu\text{mol m}^{-2} \text{ s}^{-1}$. FR_c light was supplied at a fluence rate of $4\mu\text{mol m}^{-2} \text{ s}^{-1}$ by FR LED light sources (Quantum Devices Inc., Barneveld, WI) with λ_{max} at 735 nm and filtered through far-red plastic resin (FRF700, Westlake plastics, Lenni mills, PA) to eliminate minor red-light emissions from the LED source. R_c was obtained from 667-nm Q-beam LED sources (Quantum Devices Inc., Barneveld, WI) at a fluence rate of $100\mu\text{mol m}^{-2} \text{ s}^{-1}$ and B_c was supplied from 464 nm Q-beam LED sources with fluence rate of $25\mu\text{mol m}^{-2} \text{ s}^{-1}$. Light intensity and spectral output were measured with a Li-Cor Li-1800 spectroradiometer (Lincoln, NE).

Chlorophyll Determination

Fifteen gametophytes were removed from the agar plates after 14 d of the appropriate light treatment. Gametophytes were placed in 1 ml of 80% acetone, placed in darkness at 4°C for 24 h. Chlorophyll a and b levels were calibrated using the equation for chlorophyll $a=12.7(A_{663})-2.69(A_{645})$ and chlorophyll $b=22.9(A_{647})-4.48(A_{645})$.

Determining Gametophyte Areas

Using a Wild dissecting stereomicroscope with a SPOT RT camera, pictures of the gametophytes grown under different light conditions were captured and quantitatively analyzed using SigmaScan Pro software (SPSS, Chicago, IL).

D. RESULTS:

I. *CrPHY1*, *CrPHY2* and *CrPHY4B* Transcripts Increase With Imbibition

Earlier, Cooke *et al* (1987) demonstrated that 7-10 d D imbibition of *Ceratopteris richardii* spores prior to light stimulation displayed an increased competency for germination to a subsequent inducing R or W exposure. The competency of spores has been associated with a spectrophotometrically detectable increase in the amount of phytochrome during imbibition (Cooke, 1993). To determine specific transcript changes in *Ceratopteris* that may occur during imbibition, wild type *RNWI* pre-sterilized dry spores were incubated in liquid medium for 1 d or 3 d in D. After the incubation period, total RNA was extracted from the non-germinated spores and the relative levels of phytochrome transcripts were determined by semi-quantitative RT-PCR (**Figure 13**). Spores with a 3-d D incubation time showed a dramatic increase level of *CrPHY1* mRNA (it is not yet possible to differentiate between *CrPHY1A* and *CrPHY1B* transcripts). Although the levels of *CrPHY2* and *CrPHY4B* also increased, their levels were not comparable to that of *CrPHY1*. *CrPHY4A* could not be detected under the PCR conditions used to measure other phytochrome levels. However, if the number of amplification cycles was increased from 25 cycles to 35 cycles, *CrPHY4A* could be detected at a trace level (data not shown). When spores are incubated in liquid *C-fern* medium for 3 d in the dark, then exposed to W for 1 d and placed back into the dark for 3 d, spores with higher level of phytochrome transcripts showed a higher level of germination than spores with only a 1-d dark imbibition treatment (**Figure 14**). The increased germination competency of spores after 1 d or 3 d of imbibition is also apparent if spores are placed in FR. In comparison to their response to R or W, few spores

germinated when exposed to 1 d of FR immediately after imbibition. After 3 d of imbibition in liquid media, approximately 90% of spores germinated when exposed to 1 d of FR (**Figure 14**). Less than 1% of spores displayed germination after 7 d of dark imbibition and exposure to a VLFR-range light pulse (data not shown), suggesting that there is no PHYA-like VLFR response for germination as is seen in angiosperms. The competency of spores to respond to light is not only dependent on the length of time for dark imbibition but also on the method of imbibition. Spores allowed to imbibe in liquid media require a shorter period (3 d) to display 90-95% germination in W, R or FR than spores imbibed on agar media (7-10 d).

II. High Temperature and R-HIR Delay Germination in *Ceratopteris richardii*

Similar to other filicales such as *Rumora adiantumformis*, *Dicksonia sellowiana*, *Onoclea sensibilis*, *Dryopteris paleacea* and *Dryopteris felix-mas*, high temperature (32°C or greater) and saturating R fluence rates inhibits spore germination in *Ceratopteris richardii* (**Figure 15**). This inhibition is neither total nor permanent, as 80-90% of spores will eventually germinate after 7 d of R_c exposure. Moreover, this R-HIR delay in germination is not detected at lower temperatures (28°C).

III. Gametophytes of Ferns Do Not Show Typical PHYA and PHYB-Like Responses

Previously, it was demonstrated that the phytochromes of ferns are phylogenetically distinct from those of higher plants and diverged before the separation of seed plants from ferns. To determine if any *Ceratopteris* phytochrome mediates any

PHYA-like FR-HIR or PHYB-like R-HIR during prothalli development, imbibed spores were exposed to W to induce germination, and then placed in the D, R_c, B_c or W_c for 13 d (**Figure 16**). Under FR_c, gametophytes display only a very limited de-etiolation response in that they are slightly broader (from a slight increase in cell division and cell elongation) than the gametophytes grown in darkness (**Figure 17**). Unlike angiosperm seedlings, R_c is not sufficient for normal gametophyte development, and requires either B_c or W_c. Gametophytes grown under R_c are diminished in size and in cell number. Moreover, when the amount of chlorophyll was measured, the relative amount of chlorophyll per gametophyte correlates directly to the number of cells per gametophyte (**Figure 18**). Gametophytes grown in D, FR_c or R_c had fewer cells and less chlorophyll than those grown in B_c or W_c. Gametophytes grown in B_c were smaller than those grown in W_c but had a comparable level of chlorophyll, suggesting that increased activation of specific cryptochromes or phototropins stimulates chlorophyll production (**Figure 18**).

In angiosperms, seedlings grown under FR_c are unable to produce chlorophyll after 4 d of FR_c due to a PHYA-mediated block of protochlorophyllide oxidoreductase transcription. These seedlings are unable to recover from this FR block, even upon subsequent W exposure, and will eventually die. When gametophytes of *Ceratopteris* are grown in FR_c or R_c, both light treatments restrict sporophyte development. Moreover, less than 5% of the hermaphrodites from the R_c treatment are able to recover in subsequent W exposure and produce sporophytes. Most gametophytes after 14 d of R_c treatment are unable to develop because of a cessation in cell division and the inability to produce additional chlorophyll (**Figure 19**). In contrast, gametophytes grown under FR_c

or D show dramatically increased cell division and chlorophyll production upon subsequent transfer to R_c , B_c or W_c and can produce sporophytes in W_c or B_c .

IV. B_c or W_c Inhibits the Elongation of the Primary Rhizoids

In *Ceratopteris*, R induces secondary rhizoid formation, and continuous W, B, or R stimulates the elongation of these rhizoids (Murata and Sugai, 2000). However, the number of rhizoids that developed in D, FR_c , R_c , B_c or W_c and the degree of elongation in these light treatments was never quantitatively determined. In this study, when spores are induced to germinate with 1 d W and are allowed to develop in D, R_c , FR_c , B_c or W_c for 13 d, the gametophytes grown under B_c or W_c have the greatest number of rhizoids but these rhizoids are short in length when compared with those exposed to R_c , FR_c or D (**Figure 18**). The number of rhizoids per gametophyte is directly proportional to the total cell number per gametophyte, and as with germination, B_c yielded the greatest inhibition of rhizoid emergence and elongation. In *Ceratopteris*, the elongation of rhizoids is not only dependent on light conditions but also on the substratum for spore development. If spores are grown in liquid culture or on agar plates covered with liquid medium, the inhibition of rhizoid elongation by B_c or W_c is not observed (**Figure 20**). In addition, rhizoid elongation can be inhibited if spores are grown on a higher percentage agar medium regardless of the light conditions. It is not clear from these experiments whether this effect is the result of solidity of the substrate acting, for example, through touch receptors, or a specific inhibitory effect of the agar.

V. Incubation of Spores With dsRNA of *CrPHY4B* Stimulates Gene-Specific Expression

As demonstrated by Stout *et al* (2003), incubation of sterilized dry spores with specific dsRNA can effectively knock down the targeted transcript. Using ~200bp of dsRNA targeting 3' OFR-UTR borders of *CrPHY*, *CrPHY2*, *CrPHY4A* or *CrPHY4B*, attempts were made to knock down *CrPHY* transcripts in non-germinating and germinating spores. All of the 3'ends used for the RNAi contained portions of the coding and untranslated regions and, with the exception of *CrPHY4A* and *CrPHY4B*, are highly divergent and have little similarity to each other. When spores were incubated with the dsRNA for *CrPHY1* or *CrPHY2* for 2 d in the dark followed by 1 d in W, it was possible to lower the amount of transcripts of these genes in the spore cells. This knock-down was particularly apparent for *CrPHY2*, which is expressed at low level (**Figure 21**). The levels of *CrPHY1* were not significantly reduced either because there are two *CrPHY1* loci or because the RNAi-mediated knock down is not effective for genes that are transcribed at high level.

Since the *CrPHY4A* gene was not detectable in spores by RT-PCR, it was not possible to observe a knock down *CrPHY4A* (**Figure 21**). Although the coding sequences of *CrPHY4A* and *CrPHY4B* are identical, spores incubated with the divergent 3' UTR and a small part of the coding region of *CrPHY4A* did not knock down *CrPHY4B* (**Figure 21**). When spores were incubated with dsRNA for the 3' end of *CrPHY4B* (which has no sequence homology to *CrPHY4A* since this gene lacks the PAS₂ and HKRD), these spores showed an unexpected increase in the transcript level of *CrPHY4B* rather than a knocked down. Moreover, when spores were incubated with a mixture of

dsRNA for *CrPHY1*, *CrPHY2*, and *CrPHY4B*, the levels of transcripts for all these phytochromes increased (**Figure 22**). No change in the level of *CrPHY4A* could be detected, regardless of the RNAi treatment. Control spores that were incubated with dsRNA of the chicken *Myosin IIA* gene (the highly variable non-helical tail region which has no homology to any plant gene), showed no change in any of the phytochrome levels regardless of the dosage, duration, or light treatment and produce no discernable phenotypic effect when compared with spores untreated with any dsRNA (data not shown). Thus, dsRNA for *MyoIIA* was used as the control for all subsequent experiments.

VI. High Level of Phytochromes Stimulate Cell division and Chlorophyll Production

To assess how a knock down or a stimulation of phytochrome transcripts would affect germination or gametophyte development, pre-sterilized spores were incubated with dsRNA for 2 d in the dark followed by 1 d in W, removed from the dsRNA media, plated on agar medium, and then allowed in to develop in W_c, FR_c, R_c, or D. Although the effect of RNAi is very transient when spores are removed from the dsRNA medium (Stout *et al*, 2003 and this study), removal from liquid culture was necessary to study the development of individual spores. During development in W_c, the spore germination was indistinguishable among those treated with the control dsRNA or those treated with dsRNA of any of the phytochromes (*dsPHY*) (**Figure 23**). Likewise, no significant difference in germination could be detected between *MyoIIA*-treated and any *PHY*-treated spores that were exposed to 1 d of W followed by 3 d in D, or if spores were treated with dsRNA continuously during light exposure and germination. Moreover, spores that were

incubated in dsRNA for 2 d in the D, plated quickly with brief exposure to W, and placed in B for 3 d, yielded less than 10% germination in all dsRNA treatments (**Figure 23**).

Therefore, the inhibition of germination by B was still maintained. When spores were kept in the dark in dsRNA without any exposure to light, no germination was detected.

However, as germination and development continued in W_c, dramatic changes were detected in the gametophytes that developed from spores treated with *dsPHY* RNA.

Spores treated with the *dsPHY4B* RNA or with a mixture of *dsPHY1*, *dsPHY2* and *dsPHY4B* RNA were significantly larger than those treated with *dsMyoIIA* (Control), *dsPHY1*, or *dsPHY2* RNA (**Figure 24**). Spores treated with *dsPHY4A* RNA developed gametophytes that were smaller than those subjected to any other treatment. When the cell number and the amount of chlorophyll per gametophyte were determined, a direct correlation could be found with the size of the gametophytes, as those with larger size had more cells and chlorophyll accumulation (**Figure 25 and Figure 26**). If dsRNA-treated spores were allowed to develop in D or FR_c, no significant change could be detected for any treatment group. In R_c, only spores treated with *dsPHY4B* RNA or the mixture of *dsPHY* displayed a distinct phenotype. These gametophytes were larger and greener, yet they were still limited in their development and unable to produce the normal heart-shaped prothalli (**Figure 27**).

VII. Spores Are Sensitive to B After Germination

If spores are exposed to W prior to germination, and then placed in R_c for 13 d, the resulting gametophytes are small and are unable to synthesize chlorophyll. If spores are placed in R for 3 d to germinate, exposed to B light for 5h then returned to R_c for 11

d, the gametophytes that developed are larger and greener than those untreated with B. Exposure to B during the pre-germination period is insufficient to overcome the R inhibition of cell division and chlorophyll production. Only after the prothalli emerge is gametophyte development sensitive to W or B (**Figure 28**).

VIII. Knock-Down of *CRY4* in *Ceratopteris* Increases Germination in B

In *Ceratopteris richardii*, lowering or increasing the level of any *CrPHY* did not increase germination in B_c. Previous studies with *Adiantum capillus-veneris* suggested that cryptochromes (*AcCRY4* or *AcCRY3*) might be responsible for the B block of germination in ferns. To determine if this mechanism is conserved in *Ceratopteris*, a fragment of *CrCRY4* was first isolated from the *Ceratopteris* EST library in GenBank by BLAST analysis. RT-PCR of RNA isolated from 7-day-old gametophytes grown in D, FR_c, R_c, B_c or W_c revealed that this expression of this gene is upregulated only in FR_c and is maintained at a relatively constant level in all the other condition tested (**Figure 29**). To knock down *CrCRY4*, ~200bp of dsRNA of *CrCRY4* was synthesized and incubated with sterilized dry spores in the dark for 2 d. Total RNA was then isolated from a set of treated spores, and RT-PCR was used to determine if there is a reduction of the respective transcripts (**Figure 30**). Alternatively, another set of treated spores was quickly plated on agar plates with minimal exposure to W, and then placed in B_c for 7 d. When germination was measured, approximately 33% of the spores treated with *dsCRY4* germinated, in contrast to 10% of the spores that germinated when treated with the control *dsMyoIIA* RNA (**Figure 31**). Moreover, spores incubated with *dsCRY4* RNA

showed reduced *CrCRY4* transcript levels, however spores incubated with the *dsMyoIIA* showed no change in the levels of *CrCRY4* (**Figure 30**).

Since only 10% of the spores from the control group germinated and developed prothalli, and most of these were males, it was difficult to compare and assess statistically the effect of *CrCRY4* knock-down on cell division and chlorophyll production in B. Although there is no noticeable effect on germination in W, the gametophytes that developed from a *CrCRY4* knock-down had larger prothalli, more cells, and higher chlorophyll accumulation than the controls (**Figure 32**).

IX. Increased Level of *CrPHY1*, *CrPHY2* and *CrPHY4B* in the *dkg1* Background

The *Ceratopteris richardii* mutant, *dark germinator 1* (*dkg1*) displays the phenotype of germination in complete darkness when placed on appropriate medium (Cooke et al, 1993) and cell division and cell expansion that occur independent of light stimulation (Kamachi et al, 2004). To determine if the *dkg1* mutation had any effect on the expression of phytochromes, RT-PCR was performed on RNA isolated from spores imbibed in the dark for 5 d. In the *dkg1* spores, there is higher level of *CrPHY1*, *CrPHY2* and *CrPHY4B* than in wild-type spores (**Figure 33**).

E. DISCUSSION

I. RNAi in *Ceratopteris*

In *Ceratopteris richardii*, the ability to lower specific targeted transcript levels in spores by incubation with short homologous dsRNA is dependent not only upon the dosage of dsRNA or the type of RNA (antisense ssRNA or dsRNA), but also on the

nature of the gene. Based on the small sample size of genes tested in our studies, the RNAi technique seems most effective for genes that are expressed at low level (e.g. *CrPHY2*, *CrCRY4*), and does not seem to affect genes whose expression increases strongly with imbibition or development, such as *CrPHY1*. Regardless of the regions used to make the homologous dsRNA or the type of RNA (-ssRNA or dsRNA), the level of *CrPHY1* could not be significantly lowered (data not shown). Although there are two loci for *CrPHY1*, since these genes are identical at the coding sequence, the transcript levels of both genes should be simultaneously affected by the homologous dsRNA. Furthermore, as the spores develop into the multicellular gametophytes and the level of phytochrome increases, it becomes difficult to detect any knock down effect by RT-PCR. Knock down of a specific phytochrome cannot be detected in an individual gametophyte by this technique if spores are removed from the RNAi solution and allowed to develop on agar media. For better post-transcriptional gene silencing (PTGS) of the phytochromes, other RNAi approaches should be explored, such as transforming gametophytes with DNA gene silencing vectors via particle bombardment (Rutherford et al, 2004) or microinjection (Mohamed and Short, unpublished data), or with more stable morpholino RNA.

Other than that for *CrPHY4*, no single photoreceptor-targeted dsRNA stimulated the expression of their homologous genes. The ability to increase the level of *CrPHY4B* by incubating spores with homologous dsRNA may have resulted from a feedback effect. An initial lowering of the level of *CrPHY4B* by RNAi may result in the stimulation of its own transcription to a degree that cannot be overcome by the dsRNA. Also, the dsRNA for *CrPHY4B* was synthesized from the 3' ORF-UTR border of the gene. Therefore, if

CrPHY4B transcripts were cleaved at the 3' end by the RNA induced silencing complex (RISC; Hannon 2002), the resulting truncated transcripts would be similar to *CrPHY4A*, which is missing the HKRD. An increase in the level of “*CrPHY4A*” may produce the unstable effect of increasing level of *CrPHY4B*. The combination of dsRNA of *CrPHY1*, *CrPHY2* and *CrPHY4B* may amplify the effect produced by dsPHY4B, resulting in the increase in transcription of all these phytochromes. The specificity by which the dsRNA induces the transcription of its target gene suggests that specific phytochromes of *Ceratopteris* may regulate their own transcription through a common factor or mechanism. The simultaneous increase in transcription of *CrPHY1*, *CrPHY2* and *CrPHY4B* during imbibition or in the *dkg1* mutant, and the decrease of all these phytochromes in R_c (Chapter 2), lends additional credibility to this interpretation.

The recent discovery that plants rely more heavily than other eukaryotes on micro-RNA (miRNA) to regulate specific transcript abundance (Zhang, 2005) suggests another possible mechanism for the anomalous upregulation of the targeted *CrPHY4B* gene. If the dsRNA construct serves to degrade the miRNA, the two inhibitory RNA groups may effectively cancel out one another, allowing increased accumulation of the *CrPHY4B* mRNA.

II. Spore Germination

Like most of the ferns studied, including *Lygodium japonicum*, *Pteris vittata*, *Anemia phyllitidis*, *Adiantum capillus-veneris*, germination in *Ceratopteris richardii* is induced by phytochromes via a LFR. The increased sensitivity of spores to light after a period of dark imbibition has now been associated with a transcriptional change in the level of specific phytochromes in *Ceratopteris*. The collective germination response of

spores to low fluence R or to high irradiance FR, the increase in the transcription of *CrPHY1*, *CrPHY2* and *CrPHY4B* during imbibition and the inability to lower germination by lowering *CrPHY1* or *CrPHY2*, suggest multiple phytochromes may regulate this process in *Ceratopteris richardii* or that a minimum threshold level of the Pfr form of *CrPHY1*, *CrPHY2* and/or *CrPHY4B* can stimulate germination. These results suggest that phytochromes of *Ceratopteris* act redundantly to induce germination. The dramatic increase in the level of *CrPHY1* suggests that this phytochrome may be the primary phytochrome inducing germination in R or FR; other phytochromes may have accessory functions or exhibit their effects only in the absence of *Crphy1*. Since there are two isoforms of *CrPHY1* that are identical to each other in the coding region, it is difficult to determine at this stage if the transcript level represents the expression from a single locus or from multiple loci. The inability to detect any change in germination by lowering the *CrPHY1* level by RNAi may be a result of light levels that saturate even the lowered quantities of expressed phytochromes. The effect of the RNAi on germination may become apparent at near-threshold fluences of W or R. These experiments await complete fluence-response curves for the responses.

As with *Rumora adiantumtformis* and *Dicksonia sellowiana*, high irradiance R and high temperatures delay spore germination. It has been proposed that the high temperature uncouples the P_{fr}-dependent signal from the processes leading to germination (Raghavan, 1989). In our experiments, it was sometimes observed that spores treated with a mixture of dsRNA for *CrPHY1*, *CrPHY2* and *CrPHY4B*, which resulted in the stimulation of transcripts level rather than suppression, showed higher germination rates in R_c and high temperature than did the control dsRNA-treated spores (not shown). If

this effect proves repeatable, it may indicate that higher levels of phytochromes can counter the effect of high temperature on germination and development. Because there are no antibodies available for *Ceratopteris* phytochromes, the link between mRNA levels and holoprotein abundance remains uncertain.

Cooke and colleagues (1987) demonstrated that B alone (without R pre-treatment) does not induce germination and inhibits R-induced germination in *Ceratopteris*. As demonstrated by RNAi, knock-down of the *CrCRY4* transcript level increases germination of spores in B_c. The elevated but still incomplete germination level, however, may result from the transient effect of the RNAi, differential uptake by different spores, or partial functional redundancy mediated by another cryptochrome in addition to *CRY4*, although other *CRY* genes remain to be identified in *Ceratopteris richardii*.

In *Ceratopteris richardii*, the DKG1 protein is believed to couple the phytochrome signaling process to the germination process (DNA synthesis, nuclear migration, asymmetric cell division, and rhizoid emergence). According to this model, the DKG1 protein blocks the germination processes unless the appropriate phytochrome photostationary state (P_{fr}/P_{total} or $[P_{fr}]$) is achieved (Cooke et al, 1987). As seen from the semi-quantitative RT-PCR data in this study, DKG1 may directly or indirectly affect the transcription of phytochromes in *Ceratopteris richardii*. From these collective data, the following working model for germination is proposed: Imbibition stimulates the expression of DKG1 protein and, through a common transcription factor (which may also be regulated by the DKG1 protein), stimulates the transcription of *CrPHY1*, *CrPHY2* and *CrPHY4B*. In darkness, germination is blocked by the DKG1 protein. With increasing

fluences of R or FR, the level of active P_{fr} increases, which may interact directly or indirectly with the DKG1 protein to relieve inhibition. The activity of the P_{fr} form is also regulated by *CrCRY4*, which is stimulated by B. For germination to occur, a sufficient level of the P_{fr} form from one or several phytochromes is needed to overcome the block by DKG1 and *CrCRY4* proteins.

III. Rhizoid Initiation and Elongation

In this study, it was demonstrated that the primary rhizoids that resulted from the initial light induction are not only dependent upon quality and quantity of light, but also on the substratum on which spores are grown. While germination is measured by the emergence of the primary rhizoids and B represses germination through at least one cryptochrome, it seems that cryptochromes also repress the elongation of rhizoids. However, elongation of the rhizoids is not reflective of cell elongation in the prothalli of gametophytes. The inhibition of primary rhizoid elongation (perhaps by cryptochromes) can be overcome if spores are grown in liquid media or on lower percentage agar media, or simulated if grown on higher percentage agar media, regardless of the light conditions. Thus, the primary rhizoids may elongate in search of a solid substratum and may be inhibited by specific touch receptors on contact with the agar environment. In contrast to the primary rhizoids, the formation of secondary rhizoids is indicative of cell division in etiolated or de-etiolated gametophytes. Since secondary rhizoids develop from cells of the subapical region (in contrast to the primary rhizoids that developed from basal cells), which are affected by light, the mechanism for the elongation of these rhizoids may be primarily light regulated. It remains to be discovered if an aqueous media also affects the

elongation of these rhizoids. If the elongation of secondary rhizoids were only affected by high fluence light, their formation and elongation would involve different factors (i.e. different phytochromes or amounts of P_{fr}) of the light signal transduction pathway as previously proposed by Maruta and Sugai (2000).

IV. Prothallus Development and Chlorophyll Accumulation

In *Adiantum*, the first cell division is stimulated by a pulse of R—presumably acting through phytochromes—which can be blocked by brief irradiation of B that activates cryptochromes as well as phototropins. However, upon germination, as measured by the emergence of the primary rhizoids, B exhibits a promotive effect on subsequent cell divisions and development of the protonema than R (Furuya, 1997). B can induce apical swelling of the protonema and can re-arrange the cortical cytoskeleton of the apical protonemal cell, thus affecting subsequent patterning and cell division.

Although *Ceratopteris* does not have a visible protonemal stage, the effect of R and B on cell division seems to be conserved. Earlier, Murata demonstrated that exposure of the dark-grown gametophyte to R and B can alter the meristem morphology, increasing cell division and differentiation, and resulting in a broadened notch meristem and inducing the transition from filamentous to prothallial growth. This study reveals that cell division in *Ceratopteris* is dependent on the absolute concentration of P_{fr} present, such that the greater the level of P_{fr} , the more cell division occurs in prothalli. In D or FR_c , although there is a high level of phytochrome transcripts, the amount of active P_{fr} is low, resulting in less cell division. Since the Pr form can also absorb FR (albeit at low efficiency) and photoconvert to Pfr, gametophytes grown under FR would be expected to display more

cell division than those grown in D, but less than those in R. In R_c, the high level of Pfr stimulates early cell division but the level of phytochrome transcripts (and presumably protein) decreases. With less phytochrome synthesis, the [P_{fr}] decreases and cell division slows down. In B or W, the level of phytochrome transcripts is high and the amount of Pfr also remains high; hence, there is greater cell division in these conditions. Similar to the photoreceptors in angiosperms, some cryptochromes and the phytochromes of ferns seem to act redundantly and even synergistically to inhibit unidirectional cell elongation, and to promote cell division (Lin, 1995). Other cryptochromes (such as CRY4) may have antagonistic effects on the phytochrome-mediated responses thus blocking germination, cell division, and chlorophyll production. Consistent with the phylogenetic analyses, the phytochromes of *Ceratopteris richardii* show limited PHYA- or PHYB-like responses of the angiosperms. Germination of spores and the development of the prothalli of gametophytes require the synergistic activity of specific cryptochromes and phytochromes at specific developmental times. Since high dosages of Pfr increase germination, cell division, and development, multiple functional phytochromes from gene duplication—eventually yielding redundant and/or more specialized forms of phytochromes—may be favored by natural selection and seems to favor a genome with many functional phytochromes in *Ceratopteris*.

F. CONCLUSIONS

Physiological and molecular data demonstrate that the phytochromes of *Ceratopteris* regulate a plethora of processes during early gametophyte development, such as germination, rhizoid formation, cell division, chlorophyll production, and

archegonia development and maturation. Although not definitively shown, germination of spores may be mediated predominantly through the promoting and repressing actions of phy1 and cry4, respectively. The wavelengths and fluences of light determine the overall effect of each photoreceptor on germination. The development of the emerging prothallus requires the synergistic effect of several phytochromes, acting as positive regulators of photomorphogenesis. It remains to be determined which phytochromes are required, although phy2 and phy4B are strong candidates based on preliminary data. Although phytochromes are necessary for the stimulation of germination via a low-fluence response, development of normal prothalli requires the combined effects of phytochromes and cryptochromes. The total number of cryptochrome loci in *Ceratopteris* is yet to be determined, as well as the mode of action for each individual member. Preliminary data suggest that some cryptochromes might act antagonistically to each other (as well as to the phytochromes) to regulate prothalli development. The sequential and temporal requirement for specific photoreceptors, the complex interplay among them, and the seeming redundancy of function suggest that the roles of these photoreceptors in gametophyte development are adaptively different in ferns than in other groups of plants.

CHAPTER 4: OTHER LIGHT SIGNALING FACTORS IN CERATOPTERIS

A. ABSTRACT

B. INTRODUCTION

C. MATERIALS AND METHODS

D. RESULTS

I. Putative Photomorphogenic Genes in the *Ceratopteris* EST Library

II. Identification of Putative Photomorphogenic Genes by PCR

III. CrCRY1 and CrCRY2

IV. PIF3-like

V. HY5-like

VI. CrNDPK

VII. COP1

VIII. COP9 (FUS7)

IX. Preliminary Data on the RT-PCR of Putative Light Signaling Factors

X. Yeast Two Hybrid Screen

XI. RT-PCR and RNAi of PP2A and NDPK (preliminary data)

XII. Preliminary Data from the RNAi of CrCOP1

E. DISCUSSION

I. Conserved Regulation by PP2A

II. Cryptochromes and COP1

III. GT-2 Transcription Factor

IV. Other Putative Light Signaling Factors

F. CONCLUSIONS

A. ABSTRACT

In *Ceratopteris richardii*, no chromosomal markers are currently available to map photomorphogenic mutants such as *germ1-germ4* or *dkg1*. To isolate putative light signaling components, three strategies were taken: 1) BLAST analyses of well characterized photomorphogenic signaling factors from other organisms to the *Ceratopteris* EST cDNA library, 2) PCR amplification of genomic DNA or cDNA using primers specific for signaling factors previously characterized in angiosperms, 3) constructing and screening libraries for interacting factors of PHY1, PHY2 or PHY4 using the yeast two-hybrid system. Using the first strategy, homologues of *GT-2*, *PP2A* (*FyPP*), *NPR* and *CAB* were isolated in *Ceratopteris*. Using the second strategy, homologues of *CRY1*, *CRY2*, *COP1*, *COP9* (*FUS7*), and an *NDPK* were isolated. Attempts to isolate *HY5* and *PIF3* by PCR resulted in the identification of a putative uncharacterized bZIP transcription factor and helix loop helix protein, respectively. The yeast two-hybrid screen isolated no putative phytochrome interacting factors. Using the full-length sequences of two of the previous identified genes (*CrNDPK* and *CrPP2A*), it was demonstrated that *CrPP2A* (but not *CrNDPK*) interacts with the C-termini of PHY1 or PHY2 *in vivo*, consistent with phylogenetic analysis that showed *CrPP2A* closely related to the family of protein phosphatase (PP2A) that binds to phytochromes in higher plants. RT-PCR shows that *CrPP2A* is down regulated in conditions of low Pr levels (darkness) or low phytochrome transcript levels (continuous red). Preliminary results on the RNAi of *CrPP2A* revealed that incubation of spores with dsRNA of *CrPP2A* has no effect on germination but reduces the sizes of gametophytes. Incubation of spores with dsRNA of *CrNDPK* had no effect on germination or gametophyte development. RT-

PCR of *CrNDPK* demonstrated that this gene is not light-regulated at the transcript level. Preliminary analysis of *CrCOP1* shows that this gene may have a significant role in gametophyte development, as knock-downs of *CrCOP1* resulted in etiolated gametophytes with more cell files in the basal and subapical regions. These gametophytes also had three times more chlorophyll than the control and quickly matured to produce sporophytes when exposed to continuous white light. These findings are the first to isolate downstream light signaling components (e.g. *CrCOP1*, *CrPP2A*) and to assert their roles in fern gametophyte development.

B. INTRODUCTION

Although the amount of phytochrome in plant cells is small, the photoreception of the molecule has a great effect on the whole plant. Such pronounced responsiveness implies that the photoconversion of phytochrome holoprotein becomes amplified through a signal transduction pathway. To understand the mechanism of action of phytochrome, two general experimental approaches in *Arabidopsis thaliana* have been taken: 1) the screening for mutants defective in phytochrome signaling, 2) the biochemical or molecular assaying for immediate reaction partners with the photoreceptors (e.g. co-immunoprecipitation, yeast two-hybrid screens).

To isolate signal transduction mutants, plants are grown under appropriate light conditions to find those with light response defective phenotypes (e.g. the *hy* mutants), or grown in the dark to identify mutants with light-grown phenotypes (e.g. *cop/det/fus* mutants). Genetic interactions or crosses between the mutants and known phytochrome mutants can then determine whether one gene is epistatic to another. For instance, the

COP/DET/FUS gene products were all determined to be downstream of the photoreceptors and act as repressors of photomorphogenesis. In *Ceratopteris richardii*, using a selection where spores were first induced with low fluence R and then exposed to high fluence B, three mutant loci were identified: *germ1-germ3*. All three mutants show suppressed germination responses to R and do not exhibit R/FR photo-reversibility. *germ1* and *germ2* are completely inhibited by B but not when the B follows R irradiation (Cooke et al., 1995). Both mutants produce elongated prothalli in blue light, suggesting that the mutations may be in the cryptochrome-signaling pathway. *germ3* is also inhibited by B regardless of the previous light treatment and display de-etiolated growth in the dark, a phenotype shared by a fourth mutant, *germ 4* (Cooke et al., 1995).

Some biochemical approaches used to identify downstream signal transduction factors include characterizing the pool of proteins phosphorylated by phytochromes, inhibition/activation studies of heterotrimeric G proteins using compounds such as pertussis toxin and cholera toxin, and microinjection studies using purified phytochromes, G-proteins, cGTP, Ca^{2+} , or calmodulin (Chory, 1994; Barnes et al., 1995; Quail et al., 1995). Molecular assay approaches include the yeast two-hybrid system used to identify phytochrome-interacting factors. Using the C-terminal domain of *Arabidopsis* PHYA or PHYB as the “bait,” several factors have been shown to interact with phytochromes: phytochrome kinase substrate 1, PKS1, (Fankhauser et al., 1999), nucleotide diphosphate kinase 2, NDPK2, (Choi et al., 1999), cryptochrome 1, CRY1, (Eide et al., 2002; Lin and Shalitin, 2003), cryptochrome 2, CRY2, (Ahmad et al., 1998), phytochrome interacting factors 1, 2, and 3, PIF1-3, (Ni et al., 1999), early flowering 3, ELF3, (Covington et al., 2001; Hicks et al., 2001), *ZTL/ADO1*, auxin/indole-3-acetic

acid, Aux/IAA, (Jarillo et al., 2001) proteins, *Arabidopsis thaliana* response regulator 4, ARR4, (Imamura et al., 1999; Shen et al., 2003).

In lower plants, no downstream signaling factors in the photomorphogenesis pathway have been isolated nor characterized. In this study, putative downstream signaling factors were identified using BLAST analyses, PCR, and yeast two-hybrid assays.

C. MATERIALS AND METHODS

Isolation of Putative Light Signaling Factors in *Ceratopteris* by PCR

To identify putative light signal factors in *Ceratopteris richardii*, well-characterized photomorphogenesis-related genes from *Arabidopsis* and other organisms were compared by BLAST search analysis (www.ncbi.nlm.nih.gov) to the *Ceratopteris* EST library to determine whether homologues of these genes have been identified. Because the EST library is incomplete, if no homologues were found in the *Ceratopteris* library but have been identified in multiple other organisms, sense and antisense primers were designed from the conserved regions in these genes. Only genes with reported homologues in several different species were sought. Using genomic DNA or cDNA from light-grown gametophytes, the following primer pairs were used to identify *CrCRY1*, *CrPHY2*, *CrCOP1*, *CrCOP9 (FUS7)*, *CrNDPK*, *CrPP2A*, *CrGT-2*, *CrbZIP*, *CrH1H*, *CrCAB*, and *CrNRP*;

CRY1F: TTC TTC AAC CAT CTA TAT GAT CCT ATC,

CRY1R: CAA TGA AAG ATT TTG CGG ACA CTA AGC,

CRY2F: GGC GCA GAG AAT GGT TTC CCA TTG AGT,

CRY2R: G ATC AAC ACT GTT CTT CTT GAC AGG AGC,

CRY4F: TCA GTG GCT TCC TGA ACT TGT TAG ACT,
CRY4R: TGT CGG AAC CTT TAC CTG TTA GGT GAG,
COP1F: CTA CTG CTG GTG TAT CAA GG,
COP1R: TTG ATC TCG TGG GCA TCT CAG CAA,
COP9F: CTG CTC ACC AAG AAG CCA TGG AGA AGA,
COP9R1: ACT TGG CTG CAT CAG TAA CAC TAA TTG,
NDPK-F: TGA AGT TTC TTC AAA TCG ATC GCT CGT,
NDPK-R: CGC CAA AAA TTC ACC CTT ACT TTC ACT,
HLH-F1: CTC GAG GAT GAT CTG CTG GAT GAT GCT,
HLH-R1: TGC ATT GCA AAT GCA GTC ATT GCA GCA,
bZIPF1: GGG ATG AGG TCC AAA GGT TGA AAG TAG,
bZIPR1: CAT CAT GTA CAT CCA TTC TTC GTA TAA GGC,
GT-F1: TCA AGT GCT CTC ATA TCT GTA CAA CCA,
GT-R1: AAT TGC AGT TGG CAA GTC CCT CAC GTC,
CAB-F1: GCC AAG CCT GCC CCC AAG TCC GCG AAA,
CAB-R1: TGC AAA CTG ATC AGG ATC TTT TGC AAG,
NRP-F1: TGA TCA GAA ACC CAA GCT GTT GGC CGA,
NRP-R1: AGC TTG TAT CAT ACA ACA TGT CCT GCT,
CrPP2AF: ATT CAT GGC CAA TTC CAT GAT TTA ATG,
CrPP2AR: GC GAC AGC AGT AAT AAG GGG CAG ACC

Isolation of mRNA:

Four milligrams of dry pre-sterilized wild type *RNWI* spores (Carolina Biological) were suspended in 200 µl of *C-Fern* media (Carolina Biological), placed in darkness for 2 d, then transferred directly in continuous white (W) light for 7 d at 28°C. After the light incubation, the medium was removed from the spores by pipetting, and mRNA was isolated using the *MicroPoly(A) Pure* small scale mRNA purification kit (Ambion).

Tissue was not frozen in liquid nitrogen for this procedure. The tissue was homogenized

in a 1.5 ml Eppendorf tube in 300 μ l of the supplied *Lysis Buffer* and RNA was extracted according to the manufacturer's instructions. After elution, mRNA samples were pooled from several isolations and concentrated by first precipitating the RNA using 0.1 volume of supplied 5M ammonium acetate and 2.5 volumes ethanol. After the recovery of RNA by centrifugation (12,000 x g for 20 min at 4°C), the pellet was resuspended in 20 μ l of DEPC-treated water and the concentration was determined spectrophotometrically (1 A_{260} unit corresponds to 40 μ g/ml of RNA). RNA was also examined by agarose gel electrophoresis.

Complementary DNA (cDNA) Library Construction

A cDNA library was constructed using the HybriZAP-2.1 XR Library Construction Kit (Stratagene). Using 2.5 μ g of mRNA isolated from light grown gametophytes, first and second strand cDNA were synthesized exactly as specified by manufacturer's instructions. After synthesis, double stranded cDNA was blunt-ended and the supplied EcoRI adapters were ligated to cDNA ends (4°C for 2 d). Following heat inactivation of the ligase, EcoRI ends were phosphorylated and the cDNA was digested with XhoI (the oligo dT linker primer contained an *XhoI* site) at 37°C for 1.5 h. Digested cDNA was then purified using the QIAGEN PCR Purification Kit and size fractionated using the SIGMA Spin Column 400 (recovered cDNA of 872 bp or larger). Size-fractionated cDNA was ethanol precipitated and dissolved in 5 μ l of distilled water. After concentration was determined spectrophotometrically, 100 ng of cDNA was ligated into 1.0 μ g of predigested (EcoRI/XhoI) HybriZAP-2.1 vector (4°C for 2 d).

Transfecting Cells and Amplifying Library

To package the recombinant lambda phage, the *Gigapack III Gold Packaging Extract* (Stratagene) was used to which either 2 μ l or 3 μ l of the ligated cDNA was added. The packaged ligation product was then transfected into the supplied XL1-Blue MRF' host *E.coli* bacteria that was prepared according to manufacturer's instructions. The titer of the primary library was $2.5\text{--}4 \times 10^6$ pfu/ μ g of lambda arms. The primary library was then amplified according to manufacturer's instructions to 10^{10} pfu/ml.

Mass Excision of pAD-GAL4-2.1 Phagemid Library

To mass excise the entire library, 10^8 pfu from the amplified library stock was combined with XL1-Blue MRF' cells at a MOI of 1:10 lambda phage-to-cell ratio in a 250 ml flask. ExAssist helper phage at a 10:1 helper phage-to-cells ratio was added to ensure that every cell is coinfecting with lambda phage and helper phage. The excised pAD-GAL4-2.1 phagemid vectors were recovered according to manufacture's instruction and the titer of Amp^r colonies per milliliter was determined. The ratio of the number of colony-forming units was equal to the number of input lambda phage.

Amplification of the Excised Phagemid Library

Supernatant containing the excised phagemid library (10^8 pfu) was incubated with XL0LR cells at cells-to-phage ratio of 10:1. The excised phagemid library was then amplified according to manufacture's instruction and large-scale pAD-GAL4-2.1 plasmids were isolated and purified from 200 ml of LB broth containing 50 ug/ml of ampicillin using the Promega Midi-Plasmid Isolation Kit.

Construction of the Bait Proteins

To construct the phytochrome bait constructs, the C-termini of *CrPHY1*, *CrPHY2* and *CrPHY4B* was amplified from full-length cloned genes using the following primers:

CrPHY1-Y2HF: AGCCCGGGCG GAT GCT GTC TGT GGA ATG GC,

CrPHY1-Y2HR: AGTCGAC TCA AGT CAG ATA GGG AAC ATC,

CrPHY2-Y2HF: AGCCCGGGCA GAT GCT GTG TGT GGA ATG GC,

CrPHY2-Y2HR: AGTCGAC CTA GGA ATC AGT CTT CTT GAA AAG,

CrPHY4B-Y2HF: AGCCCGGGCG ATG GAA GTT GAG CTA GCA GCA CAG ATG,

CrPHY4B-Y2HR: AGTCGAC TTA TTG AAC AAG AGG CAG CTC TAA ATG.

The forward primer contains a 5' *SrfI* restriction site and the reverse primer has a 5' *SalI* restriction site. PCR fragments were amplified using high fidelity *LA Taq* polymerase (Takara), subcloned in pGEM-T plasmids (Promega), and transformed into JM109 competent cells. Recombinant plasmids were recovered using the Promega SV Mini-prep isolation kit from colonies that tested positive by PCR for the presence of the inserts. One microgram of plasmid was then double-digested with *SrfI* and *SalI* restriction enzymes and the inserts were purified from the plasmids using the Qiagen gel isolation kit. 200 ng of insert DNA was then ligated into 100 ng of the pBD-GAL4 Cam vector that had been double-digested with *SrfI* and *SalI*. Fragments were ligated at 4°C for 2 d before recombinant plasmids were transformed into the supplied XL1-Blue competent cells. Transformed colonies were randomly screened by PCR for the presence of inserts and plasmids were recovered as previously described. To verify that the GAL4 activation domain (AD) and the phytochrome C-terminus was in one continuous reading frame, the junctions between domains were sequenced using the primer *pBD-S*: TTC CTC GAG AAG ACC TTG ACA.

Isolation of Full-Length *CrPP2A* and *CrNDPK*

To clone *CrPP2A* and *CrNDPK* into the pAD-GAL4 2.1 target vector, full length genes were amplified from cDNA using the following primers;

CrNDPK-S: AAAGGA TCCATG GAG ACC ACG TTC ATC ATG ATC,

CrNDPK-AS: AAAGTCGACTCA CTC GTA GAT CCA ATC ATG TGC,

CrPP2A-S: AAAGGATCCATG GAT CTG GAC GGA TGG ATC GAG,

CrPP2A-AS: AAAGTCGACTCA GGC TGC GCA ACT GTT GGG AAG.

The PCR products were then subcloned into pGEM-T vectors, transformed into JM109 cells, and screened by PCR using the same primers used to isolate the fragments. Mini-preps of plasmids were prepared from positive colonies. Plasmids were sequenced to verify that the complete coding sequence was present (Macrogen Inc). Inserts were removed from the plasmids by double restriction digestion with *EcoRI* and *SalI*.

Fragments were purified using the Qiagen gel purification kit. The target vector was prepared by digesting pAD-GAL2.1 vector with *EcoRI* and *SalI*. The fragments and digested vectors were ligated together, and then transformed into the supplied XL1-Blue competent cells. Since there is no selection for insert, colonies were screened randomly by PCR to determine the presence of insert. Plasmids with inserts were then purified using a mini-prep isolation kit and sequenced using the T7 primers to confirm that the genes were in frame. To swap domains between *CrNDPK*, *CrPP2A* and *CrPHY2*, the *CrNDPK* and *CrPP2A* sequences were excised from pAD plasmid by double restriction digestion using *EcoRI* and *SalI*. Purified gene fragments were then ligated to pBD that had also been digested with *EcoRI* and *SalI*. *CrPHY2* was excised from the pAD plasmid with *EcoRI* and *SalI* and ligated to pBD predigested with the same enzymes.

Yeast Transformation and Selection

To detect protein-protein interactions *in vivo*, the bait and target vectors were sequentially transformed into the supplied YRG-2 yeast strain and plated onto the appropriate drop-out media as specified by manufacturer's instructions. This strain carries the auxotrophic markers leucine (*leu2*) and tryptophan (*trp1*) for selection of yeast, which was transformed with the pAD or pBD plasmids, respectively. YRG-2 also carries the auxotrophic marker histidine (*his3*) and a *lacZ* reporter system to assay for protein-protein specific interactions. Yeast that has been transformed with plasmids encoding interacting proteins that together activate transcription of the *HIS* and *LacZ* reporter genes. All media for the growth, maintenance and transformation of yeast was made according to manufacturer's instructions except for the synthetic minimal (SD) dropout medium in which the D-sorbitol was omitted.

Double Stranded (ds) RNA Synthesis:

A fragment of the *CrNDPK* and *CrPP2A* was amplified using the primers

NDPK-T7F: TAATACGACTCACTATAGGGAGACCACGACGTGGTGCCGTTCTTACT,

NDPK-T7R: TAATACGACTCACTATAGGGAGACCACTCGCCAAAAATTCACCCTTA,

CrPP2A-T7F: TAATACGACTCACTATAGGGAGACCACGGATGGATCGAGAAGGTCAA,

CrPP2A-T7R: TAATACGACTCACTATAGGGAGACCACTGTTATAACCGCGATCCACA.

A fragment of the *CrCOPI* gene was amplified using the primers;

COPI-T7F: TAATACGACTCACTATAGGGAGACCACCTGTTTGGGATGTAAACACTCG,

COPI-T7R:

TAATACGACTCACTATAGGGAGACCACGAAAATGTGCACTGGAGAGAGTG,

which also contain T7 promoter sequence. PCR-amplified fragments were purified using the Qiagen Gel Purification Kit and eluted in DEPC-treated water. 0.5 µg of the purified

fragment was used as template for RNA synthesis using the RiboMAX Large Scale RNA Production System (Promega). Sense and anti-sense RNA were synthesized simultaneously using the T7 RNA Polymerase as specified by the protocol. The integrity of transcripts was analyzed on a 1.0% Agarose gel with ethidium bromide and the concentration was determined spectrophotometrically.

RNAi:

Four microgram of pre-sterilized dry *RNWI* spores (Carolina Biological) were resuspended 200 µl sterilized C-Fern Media (Carolina Biological) containing 0.2 mg/ml of double stranded RNA (dsRNA). Spores were incubated with RNA for 72 h in W with continuous shaking, plated, and then placed in the dark or exposed to W for 7 d.

Chlorophyll Determination

Fifteen gametophytes were removed from the agar plates after 14 d of the appropriate light treatment. Gametophytes were placed in 1 ml of 80% acetone, placed in darkness at 4°C for 24 h. Chlorophyll *a* and *b* levels were calibrated using the equations for chlorophyll *a*=12.7(A_{663})-2.69(A_{645}) and chlorophyll *b*=22.9(A_{647})-4.48(A_{645}).

Determining Area of Gametophytes

Using a Wild microscope with a SPOT RT camera, gametophytes grown under different light conditions were first imaged, and the images quantitatively analyzed using the SigmaScan Pro software (SPSS, Chicago, IL).

D. RESULTS

I. Putative Photomorphogenic Genes in the *Ceratopteris* EST Library

To identify photomorphogenic signaling components in *Ceratopteris* homologous to those identified in other plants, well characterized genes from other organisms (e.g. *Arabidopsis*, *Oryza*, *Adiantum*) were compared by BLAST to the *Ceratopteris* EST library sequences (GenBank). Using this strategy, only four fragments homologous to the genes of serine/threonine protein phosphatase *PP2A* (BE642714), a *GT-2* transcription factor (BE642174), chlorophyll a/b binding protein or *CAB* (BE643223), and negatively phytochrome regulated or *NPR* (BE643282) were identified (**Figure 34**).

The catalytic subunit of a PP6 subclass of PP2A, designated FyPP (flower-specific phytochrome-associated protein phosphatase), has been shown to bind to the C-termini of PHYA and PHYB (Kim et al., 2002) in pea and *Arabidopsis*. FyPP dephosphorylates oat PHYA in a light-dependent manner (possibly on S599), and affects PHYA-mediated light signaling in the control of flowering time (Kim et al., 2002). At the protein level, the *Ceratopteris* PP2A identified is more closely related to *Arabidopsis* or *Pisum* FyPP—sharing 91% identity to either (**Figure 34A**)—than to other PP2A catalytic subunits.

In rice, a trihelix DNA-binding protein, GT-2, was isolated that binds to the GT-motif in rice PHYA gene promoter to regulate transcription (Dehesh et al., 1992). Previously we demonstrated that the *dkg1* protein might affect the transcription of phytochromes in *Ceratopteris*, and that the same light conditions affect multiple phytochrome messages similarly. This suggests that a common transcription factor may be involved in regulating these genes. Searching for factors that could bind to

phytochrome promoters resulted in the identification of a putative trihelix transcription factor with 50% identity at the protein level to the *GT-2* of *Oryza sativa* (**Figure 34B**). Preliminary analysis by RT-PCR shows that *GT-2* is down-regulated in R and upregulated by D or FR (data not shown). To search for the existence of genes transcriptionally up-regulated or repressed by light, the *Ceratopteris* EST library was searched by BLAST for various light-regulated targets. Of those tested, only *CAB* and *NPR* were identified from among the limited EST subset. Chlorophyll a/b-binding protein (*CAB*) is a crucial component necessary for chloroplast differentiation and photosynthesis, and is regulated in angiosperms by phytochromes via expression of transcription factors *CCA1* and *LHY*. *Ceratopteris* *CAB* protein (*CrCAB*) has 69% identity to *Arabidopsis* *CAB* protein (**Figure 34C**). In the aquatic monocot, *Lemna gibba*, and in *Veronica fondii*, several genes have been shown to be negatively regulated transcriptionally by phytochromes (Okubara et al., 1993). These genes are poorly defined and have been designated as *NPR* (negatively phytochrome regulated) genes. *Ceratopteris* *NPR* protein has a 63% identity to the only characterized *NPR* of *Vernicia fondii* but has no homology to the *NPRs* of *Lemna gibba* (**Figure 34D**).

II. Identification of Putative Photomorphogenic Genes by PCR

Since only four putative factors could be identified from the EST library by direct BLAST analyses, PCR amplification using degenerate primers was attempted to identify other light signaling components in *Ceratopteris*. Factors such as cryptochromes (*CRY*), constitutively photomorphogenic 1 (*COP1*), phytochrome-interacting factor 3 (*PIF3*) and *HY5* were chosen because they contained highly conserved motifs e.g. WD-40, Ring

Finger, Helix Loop Helix (HLH) and basic Leucine Zipper (bZIP). Moreover, isoforms or paralogues of these genes have been isolated within the same organism (e.g. *Arabidopsis*) or orthologues found in different organisms, providing a means of comparison to design gene-specific primers for PCR. With the exception of *Arabidopsis*, little is known about the light-signaling pathways in other plants. Factors in *Arabidopsis* where no homology could be found (e.g. *PKSI*, *PAT1*, *SUB1*) in other sequenced genomes were not pursued; nor were those factors that were specific to a single phytochrome pathway e.g. *FAR1*, *FIN219*, *PAT1*, etc in the *PHYA* pathway. Using PCR, definitive homologues of CRY1, CRY2, an NDPK, COP1, and COP9 (CSN8) were identified. Two fragments with a bZIP and HLH motif were also identified (**Figure 35**).

III. *CrCRY1* and *CrCRY2*

Since *Arabidopsis* CRY1 and CRY2 have been shown to be substrates for phosphorylation by a purified recombinant PHYA (Ahmad et al., 1998) and cryptochromes can interact with PHYA *in vitro* and in the yeast two-hybrid assay, attempts were made to isolate homologues of these cryptochrome genes in *Ceratopteris* by PCR. In the fern *Adiantum capillus veneris*, five cryptochromes have been identified (*AcCRY1*- *AcCRY5*). Using primers specific to *AcCRY1* and *AcCRY2*, fragments of two isoforms of the cryptochrome genes have been isolated, *CrCRY1* and *CrCRY2*. *CrCRY1* protein has an 83% identity to *AcCRY1* (**Figure 35A**) and *CrCRY2* protein has a 54% identity to *AcCRY2* (**Figure 35B**). Earlier, it was demonstrated that a homologue of *Adiantum CRY4* could also be found in *Ceratopteris*, and *CrPHY4* plays a role in the blue light inhibition of germination and chlorophyll production (Chapter3). Homologues of

other cryptochromes genes found in *Adiantum* (*CRY3* and *CRY5*) were not detected by PCR. However, since some of the cryptochromes are very closely related to each other, it is difficult to match the sequences of one orthologue from another. Besides a fragment of *CrCRY4*, no cryptochrome sequences could be identified from the *Ceratopteris* EST library by BLAST analysis.

IV. PIF3-like

Using the C-terminal of PHYB of *Arabidopsis* as bait, phytochrome-interacting factor 3 (PIF3), a basic helix-loop-helix (HLH) transcription factor, was identified. Besides PHYB, PIF3 has been shown to also bind PHYA with lesser affinity and interacts with both phytochromes in their photoactivated Pfr form (Zhu et al., 2000). PIF3 plays a major role in regulating several light-dependent responses including seedling hypocotyl elongation, cotyledon opening and expansion, the accumulation of anthocyanins and chlorophyll (Halliday et al., 1999; Kim et al., 2003).

Using a primer specific for the PIF3 gene with a general primer for the HLH domain, a gene fragment with a putative HLH domain was isolated (**Figure 35C**). It is yet to be determined if this gene is the functional equivalent of PIF3. BLAST analysis of this sequence with the *Ceratopteris* EST library found a larger uncharacterized EST fragment (BE641987). Although the HLH motif is conserved, the *CrHLH*-related fragment has little or no similarity to *AtPIF3* at the nucleotide or protein levels.

V. HY5-like

HY5 is a bZIP nuclear-localized transcription activator. Upon photoactivation of PHYA, *HY5* transcription is rapidly induced, while proteasome-mediated degradation of the protein via COP1 targeting is blocked by photoactivated cryptochromes (Osterlund and Deng, 1998). The resulting increased level of HY5 in the nucleus allows for the activation of downstream, G-box-containing genes involved in photomorphogenesis. Using a degenerate primer specific for the bZIP motif and another specific for the HY5 gene in *Arabidopsis*, a putative bZIP transcription factor with weak identity to HY5 was isolated in *Ceratopteris*. This putative bZIP transcriptional activator has greater similarity (56% identity) to a bZIP transcriptional activator found in *Nicotiana tabacum* that regulates cell elongation by controlling the level of gibberellins than to HY5 (**Figure 35D**). Moreover, it also has homology (48%) to PKS1, which is associated with adventitious shoot regeneration in *Paulownia kawakami*, and homology to bZIP transcription factors of the *R2R3-MYB* gene family in *Arabidopsis*. A subset of these MYB-like transcription factors are believed to be involved in regulating light and circadian rhythm-related responses. It is yet to be determined if this putative transcription factor plays a role in *Ceratopteris* photomorphogenesis. BLAST analysis of this sequence with the *Ceratopteris* EST library identified a fragment with the complete coding sequence (BE643232).

VI. CrNDPK

In the presence of PHYA, NDPK2 is catalytically activated and plays a role in the cotyledon unfolding and greening responses in *Arabidopsis*. It has been shown to bind to preferentially to the Pfr form of PHYA in the yeast two-hybrid system and by *in vitro* binding assays. NDPK2 plays a role as a transcription factor in the gene expression

response to ultraviolet radiation (Choi et al., 1999). In *Arabidopsis*, there are at least three isoforms of the NDPK gene but only NDPK2 has been shown to interact with phytochromes. Using degenerate primers for the *NDPK2* gene, an isoform was isolated that shows 68% identity to *AtNDPK1* and 55% similarity to *AtNDPK2* amino acid sequence when translated (**Figure 35E**). BLAST analysis of the *CrNDPK* fragment identified the complete coding sequence of the *NDPK* gene in the *Ceratopteris* EST library (BE643276), though the submitted sequence is uncharacterized. Like many other genes, the nucleotide sequence of *CrNDPK* could not be identified from the EST library by direct BLAST because of the little or no homology it has with any of the *NDPKs* of *Arabidopsis*. These sequences could only be identified by first translating the nucleotide sequences then analyzing the protein sequences by BLAST.

VII. *CrCOP1*

Since Constitutively Photomorphogenic 1 (COP1) protein in *Arabidopsis* is important for integrating light signals and negatively regulates HY5, attempts were made to isolate *CrCOP1*. COP1 is a protein with both a zinc-binding “ring finger” motif and the WD-40 repeat motif of G beta proteins. Using a degenerate primer specific for the WD-40 domain and gene specific primer, a putative *COP1* gene (77% identity to *AtCOP1*) was isolated from *Ceratopteris* (**Figure 35F**). It is believed that COP1 interacts directly with HY5 in the nucleus, facilitating its ubiquitination, hence targeting HY5 for degradation in the dark. Exposure to light causes translocation of *AtCOP1* from the nucleus to the cytosol, resulting in increased levels of HY5 in the nucleus (Quail, 2002).

VIII. *CrCOP9 (FUS7)*

Degradation of HY5 is mediated by the COP9 signalsome. COP9 is closely related to the lid sub-complex of the 26S proteasome and is an essential complex that represses light-regulated development. The COP9 signalsome consists of eight distinct components (CSN1-CSN8) and CSN8 is the smallest subunit (Karniol and Chamovitz, 2000). Using primers specific to the CSN8/FUS7 subunit, which is well conserved among plants and animals, a putative homologue of the CSN8 was identified in *Ceratopteris* by PCR. *CrCSN8* has 51% identity to *AtCSN8* (**Figure 35G**). Little is known about individual subunits of the COP9 signalsome in plants.

IX. Preliminary Data on the RT-PCR of Putative Light Signaling Factors

To quickly assess the expression of these putative genes, RNA from 7 day-old light-grown gametophytes was extracted, DNase-treated, and reverse-transcribed into cDNA. Using gene-specific primers for the *Ceratopteris* homologues isolated, the cDNA was amplified for 35 cycles. A PCR product was amplified for all of the genes tested (**Figure 36**). No attempts, however, were made to determine the expression of these genes relative to each other or to determine changes in expression of these genes when gametophytes are grown in darkness.

X. Yeast Two Hybrid Screen

To isolate potential phytochrome-interacting factors, a yeast two-hybrid screen was performed. Since many of the putative light-signaling genes identified by PCR were expressed in light grown gametophytes, a cDNA library was constructed from RNA

isolated from 7-d-old light-grown gametophytes. The cDNA library was cloned into the HybriZAP-2.1 lambda vector (Stratagene), amplified and converted by mass excision into pAD-GAL4-2.1 phagemid vector, containing the upstream GAL4 transcriptional activator domain (AD). Various bait domains were then constructed by fusing the complete C-terminus of *CrPHY1*, *CrPHY2*, or *CrPHY4B* into the pBD-GAL4 Cam phagemid vector, containing the upstream GAL4 DNA-binding domain (BD). To determine if each bait sequence was in frame with the GAL4 DNA binding domain, the junctions between fusion proteins were sequenced. Sequential transformation of yeast with first the bait plasmids, and then the target plasmids resulted in approximately 100 colonies/ μ g of target vectors when selected for the expression of *HIS3* reporter gene. However, when these colonies were further screened for the expression of the *lacZ* reporter gene, no colonies showed a positive interaction. The screen allowed for identification of only strong interactions (pBD-WT and pAD-WT). *LacZ* expression could not be detected for the control plasmids (pBD-MUT and pAD-MUT) that would indicate weak interactions.

Since the yeast two-hybrid screen did not identify putative phytochrome-interacting factors, attempts were made to determine if any of the factors isolated by screening the *Ceratopteris* EST library and PCR amplification could interact with the phytochrome bait. Of the genes identified, the complete coding sequence for *CrNDPK* was available from the *Ceratopteris* EST database and the sequence for a *CrPP2A* was missing approximately 200 bp at the 3' end. Using various sense primers specific for *CrPP2A* and an anchored oligo (dT), the 3' end of *CrPP2A* was quickly identified by RT-PCR of cDNA from light-grown gametophytes. Full length *CrNDPK* or *CrPP2A* was

then subcloned in the pAD-GAL4-2.1 phagemid vector plasmid and the fusion proteins were sequenced to determine if the activator domain and target genes were in frame. The *pAD-CrNDPK* or *pAD-CrPP2A* phagemid vectors were then transformed into yeast containing *pBD-CrPHY1*, *pBD-CrPHY2* or *pBD-CrPHY4* as bait. When selected for expressed of *HIS3* reported gene, both CrPP2A and CrNDPK showed a positive interaction with either CrPHY1 or CrPHY2 bait but not with CrPHY4 bait (**Figure 37 and 38**). None of these bait polypeptides showed a positive interaction with the pAD-Laminin control. However, when these colonies were assayed for lacZ expression, no positive interactions were detected besides that of the control (pAD-WT/pBD-WT). Again, no activity could be detected for the control weak interaction (pAD-MUT/pBD-MUT).

To verify a possible weak interaction, the GAL4 activation domain and binding domain was swapped between fusion proteins. CrNDPK and CrPP2A became the bait and were fused to the DNA binding domain (BD) and the C-terminus of CrPHY2 was then fused to the GAL4 activator domain (AD). After sequential transformation of yeast with first the bait (*pBD-CrPP2A* or *pBD-NDPK*) then the target vector (*pAD-CrPHY2*), only cotransformant with pBD-CrPP2A/pAD-CrPHY2 showed a positive interaction for the expression of the *HIS3* reporter gene (**Figure 39**). No interactions were detected for CrNDPK, suggesting that the interactions between CrNDPK and CrPHY1/2 were most likely nonspecific. No lacZ expression was detected for the *HIS*⁺ transformants.

XI. RT-PCR and RNAi of PP2A and NDPK (preliminary data)

Although no strong interaction (LacZ expression) was detected between for *CrPP2A* and *CrPHY2*, phylogenetic analysis of *CrPP2A* showed that this protein phosphatase is more closely related to PP2A family that binds to phytochromes in higher plants than any other type of protein phosphatase, suggesting that the interaction between *CrPP2A* and *CrPHY2* may indeed be specific (**Figure 40**). To determine if *CrPP2A* was light regulated at the transcription level, RT-PCR was performed on RNA isolated from 7-d-old gametophytes grown under various light conditions. R_c and darkness down-regulate the level of *CrPP2A*. *CrNDPK* expression was unaffected by these light conditions (**Figure 41**). To determine whether either gene might encode a regulator of photomorphogenesis, dsRNA of *CrPP2A* or *CrNDPK* was incubated with dry spores, and the spores were allowed to develop in W for 7 d. Neither the dsRNA of *CrPP2A* nor *CrNDPK* had any effect on spore germination, however the dsRNA of *CrPP2A* reduced the area of the gametophytes (**Figure 42**).

XII. Preliminary Data from the RNAi of *CrCOP1*

In higher plants, COP1 is essential to the integrated control of photomorphogenesis. COP1 acts as a convergence point in the light-signaling pathway, and therefore has great significance in plant light responses (Quail, 2002). To determine if *CrCOP1* plays a role in photomorphogenesis in gametophytes, *CrCOP1* was knocked down in spores (**Figure 43**). Dry spores were incubated with approximately 200 bp of dsRNA for *CrCOP1* for 3 d in continuous W light, plated, and placed in D for 7 d. Knockdown of *CrCOP1* had no measurable effect on germination. However, the dsRNA treatment increased the number of cell files in the basal region of the etiolated

gametophytes and decreased cell elongation (**Figure 44**). This effect has not yet been quantified. Following the 7 d of D treatment, if gametophytes were then exposed to continuous W for 7 d, it was found that spores treated with dsRNA of *CrCOP1* yielded gametophytes with more chlorophyll than the control (**Figure 45**). Moreover, if the etiolated gametophytes are allowed to develop in continuous W for 10-20 d, those that were treated with *CrCOP1* dsRNA developed sporophyll leaves within 10 d, in contrast to the control gametophytes, which took approximately 20 days (data not shown).

E. DISCUSSION

I. Conserved Regulation by PP2A

Although no definitive phytochrome-interacting factors were identified in *Ceratopteris* from the constructed cDNA library of light-grown gametophytes, several DNA fragments of putative signaling factors have been identified by BLAST analyses and PCR. The availability of full-length sequences of *CrPP2A* and *CrNDPK* made it possible to determine if their gene products could interact *in vivo* with the C-terminal of *Ceratopteris* phytochromes using the yeast two-hybrid system. Consistent with its phylogenetic analyses, *CrPP2A* is not only closely related to the PP2A/*FyPP* that binds to phytochromes in higher plants, but the protein also binds to *CrPHY1* and *CrPHY2*. This interaction suggests that phytochrome regulation by protein phosphatase may be conserved in other groups of plants and that this form of regulation is not specific to higher plants and their phytochromes. It was not possible to further examine the *Ceratopteris* PP2A-phytochrome interaction by co-immunoprecipitation or *in vitro* pulldown assays because of a lack of specific antibodies to the proteins or genetic

transformation that would allow epitope-tagged proteins to be expressed *in vivo*. In angiosperms, FyPP (PP2A) over-expression in transgenic plants resulted in phytochrome hypersensitivity of flowering and inhibition of hypocotyl elongation. RNAi of *FyPP* in transgenic plants resulted in decreased phytochrome activity (Kim et al., 2002). It is believed that the phosphorylation of S598/S599 in the hinge region attenuates phytochrome activity and inhibits interaction with signal transducers such as NDPK2 and PIF3. Although it was not verified whether *CrPP2A* message was indeed knocked down in spores, incubation of dsRNA for *CrPP2A* in *Ceratopteris* resulted in decreased expansion of the gametophytes. If gametophytes are grown in R_c, which reduces the level of *CrPP2A* transcripts, the small size of the gametophytes can be partially phenocopied. Moreover, unlike the *FyPP* in angiosperms, *CrPP2A* seems to be light-regulated and is down-regulated in conditions where there is a low level of Pr by lack of photoconversion (in D) or from a probable overall low level of phytochrome because of reduced transcript abundance. A threshold level of Pr activity may stimulate expression of *CrPP2A*, which in turn may regulate phytochrome activity by dephosphorylation. The conservation of serines in the N-termini and in the hinge region of CrPHY1 and CrPHY2 suggest that these residues may also be the dephosphorylation substrates for CrPP2A (Chapter 2). For more precise conclusions to be drawn, it must be determined whether CrPP2A definitively binds to CrPHY1 or CrPHY2, if there is indeed a knock down of *CrPP2A*, as well as if *Ceratopteris* phytochromes autophosphorylate at the conserved residues and are dephosphorylated by *CrPP2A*. It is of interest to determine if there is differential level of phytochrome phosphorylation/dephosphorylation in various light conditions, and if knockdown of *CrPP2A* affects those changes. Does continuous R

result in a higher degree of autophosphorylation during photoconversion, which attenuates phytochrome activity? Does dephosphorylation by *CrPP2A* of some residues affect the stability and activity of phytochromes in R_c ? How does phosphorylation affect the conformational change and localization of fern phytochromes? Is there a negative feedback regulation between the level of CrPHY1 and CrPHY2 and the level of CrPP2A?

In *Arabidopsis*, the *AtFyPP* genes are expressed predominantly in floral organs and antisense suppression of these genes result in early flowering (Kim et al., 2002). In *Ceratopteris*, phytochromes have been implicated in regulating archegonia formation and development (Kamachi et al., 2004). Detailed analysis of RNAi of *CrPP2A* in *Ceratopteris* would reveal whether *CrPP2A* affects archegonia development. Does knock down of *CrPP2A* lead to faster or delayed archegonia development, maturation and subsequent sporophyte development? Moreover, *in situ* hybridization studies would help determine if there is differential spatial expression of *CrPP2A* and specific phytochromes in the notch meristem or other regions of the gametophytes. If PP2A and phytochromes regulate archegonial development in ferns, understanding the expression and interaction between PP2A and phytochromes in *Ceratopteris* would provide insight into the evolutionary mechanism of how the structure and function of PP2A coevolved with phytochromes to regulate the photoperiodic control of flowering time in angiosperms.

II. Cryptochromes and COP1

In *Arabidopsis*, it was demonstrated that the WD-40 domain of COP1 interacts with the PAS domain of PHYA. This interaction can lead to the ubiquitination of PHYA

by COP1 *in vitro*, targeting the PHYA photoreceptor for destruction (Seo et al., 2004). COP1 also interacts with *Arabidopsis* photoreceptors PHYB, CRY1 and CRY2 (Wang et al., 2001; Yang et al., 2001) as well as with photomorphogenesis regulatory proteins such as SPA1, SPA2 (Hoecker and Quail, 2001), and HY5 (Saijo et al., 2003). CRY1 and CRY2 repressed COP1 activity through direct protein-protein interactions, resulting in the cryptochrome mediated blue light regulation of seedling photomorphogenesis (Wang et al., 2001). In *Ceratopteris* and other ferns, blue light regulates many physiological processes such as germination, gametophyte-tip growth, apical swelling, cell division, chlorophyll biosynthesis, phototropism, and chloroplasts photorelocation (Sugai and Furuya, 1967; Furuya et al., 1997; Murata and Sugai, 2000). In *Adiantum capillus-veneris* (*Ac*), five cryptochromes (*AcCRY1*-*AcCRY5*) and two phototropins (*AcPHOT1* and *AcPHOT2*) have been identified in the B-signaling pathway but no downstream signaling factor such as *COP1* as ever been identified in a lower plant. The identification of *CrCOP1* and the isolation of several cryptochromes in *Ceratopteris* suggests that the link or the integration between the phytochrome- and cryptochrome-signaling pathways have been established very early in plant evolutionary history. Moreover, it also suggests a candidate gene for the *germ4* mutation that displays a non-etiolated phenotype when grown in the dark.

It remains to be discovered whether *CrCOP1* binds to any of the phytochromes or cryptochromes of *Ceratopteris*. This would require the identification of full-length sequences of *COP1* and the cryptochromes from *Ceratopteris* and testing the interaction using the already available yeast two-hybrid system and other assays. The stimulation of cell division and chlorophyll production by B (after induction of germination by R)

suggest that one or more cryptochromes may bind and inhibit *CrCOP1* from repressing cell division and chlorophyll production in *Ceratopteris* gametophytes also. It remains to be determined if *Ceratopteris* has the five homologues of cryptochromes found in *Adiantum*, as only three were detected by PCR. Gene duplication of the cryptochromes may have occurred in *Adiantum* since its divergence from *Ceratopteris*, or sufficient divergence may have occurred to prevent binding of even degenerate primers.

Preliminary phylogenetic analysis shows that *AcCRY3* is related to *AcCRY4*, and *AcCRY5* is related to *AcCRY1* and *AcCRY2*. Moreover, it may be of interest to sequence the cryptochrome genes of the *germ1* and *germ2*. The mutations of these mutants are believed to be in the cryptochrome-signaling pathway as these mutants produce elongated prothalli in blue light.

III. GT-2 Transcription Factor

Imbibition in the dark increases the levels of the different phytochrome transcripts in *Ceratopteris*, whereas continuous R exposure decreases these levels. The existence of a putative GT-2 transcription factor and its light-mediated transcript abundance provides a mechanism that could explain this observation. Imbibition may result in an increase of GT-2, which then binds to promoters of phytochromes to enhance their transcription. The level of Pfr then regulates its own transcription by regulating the GT-2 level in a feedback loop. Ongoing high levels of Pfr (as in the R-HIR) inhibit the transcription of GT-2. A low level of GT-2 may then result in the decreased synthesis of phytochromes. Identifying the promoters of *CrPHY1*, *CrPHY2* and *CrPHY4B* and identifying canonical GT-2-binding motifs within these promoters, and performing DNA/protein binding

assays would provide evidence for or against a role of this putative transcription factor. In addition, if the GT-2 does regulate the transcription of phytochromes in *Ceratopteris*, a knock down of *GT-2* by RNAi should not only lower the level of *GT-2* transcripts but would also be expected to lower transcript levels of all the *CrPHY* genes. Knockdown of *GT-2* may also affect phytochrome-mediated phenotypes such as germination, cell division, and chlorophyll accumulation. To study the long-term effects of knock down or over-expression of all phytochromes in ferns, it might be of interest to identify *gt-2* mutants.

IV. Other Putative Light Signaling Factors

The activation of various photoreceptors by light is accompanied by the regulatory expression of a large array of photomorphogenic genes. In *Arabidopsis*, activation of PHYA and PHYB leads to the transcription of light-induced genes by transcription factors such as PIF3 and HY5. In *Ceratopteris*, the tissue-specific expression of phytochromes has yet to be determined, and the intracellular localization of phytochromes is unknown. If the phytochromes of ferns are constitutively cytosolic, one of the mechanisms by which they might stimulate the expression of photomorphogenic genes might be to block the degradation of transcription factors by interacting with negative regulating factors (e.g. COP1) or directly regulating the translocation of transcription factors from the cytosol to the nucleus through interactions or modifications such as phosphorylation. The vast divergence between the phytochromes of ferns and angiosperms suggests that the mechanisms of phytochrome action may also be divergent between the two groups of plants. This notion is somewhat supported by the inability to

identify well-conserved genes of angiosperms in *Ceratopteris* by PCR using even degenerate primers and by the infrequent identification of sequence matches by BLAST analysis of the EST library. However, the presence of several apparently conserved components (*CrPP2A*, *CrCOP1*) suggests that more ancient mechanisms have been retained. Also, the existing EST library represents only a fraction of expected genes expressed in *Ceratopteris*.

Since it has not yet been determined if the putative HLH and bZIP transcription factors are components in the photomorphogenesis signaling pathway, it is difficult to speculate on the mechanism of light-induced gene regulation. Are the HLH and bZIP transcription factors the functional equivalence of a PIF3 and a HY5, respectively? When complete coding sequences of the HLH and bZIP genes are isolated, these sequences can be used with the already constructed phytochrome bait to determine if any interaction occurs in the yeast two-hybrid system. These factors could also be tested with *CrCOP1* bait in the event that they are light regulated transcription factors but have no direct interactions with phytochromes.

F. CONCLUSIONS

The isolation of several phytochromes, cryptochromes, a phototropin, COP1 and other putative downstream light signaling factors in *Ceratopteris*, the availability of a partial EST library, the construction of several cDNA libraries (including the yeast two-hybrid library), the development of techniques for transiently knocking down a gene or to transiently overexpress a gene in the gametophyte by the microinjection or the ballistic approach, make *Ceratopteris richardii* a model fern ripe for further study of photomorphogenesis. Should it become possible to stably transform this organism or if

molecular markers become available to map genes, fern studies would know few boundaries and would not lay fallow in unsupported speculations. Moreover, the availability of multiple mutants in *Ceratopteris* and the discernable phenotypes of these mutants make it possible to determine the epistatic effect of wild-type genes expressed in the gametophytes.

TABLES AND FIGURES

Figure 1

CrPHY4B	-----	
CrPHY4A	-----	
CrPHY2	MARRRMLPAAMAAGFLEWRFPFLFLSPLTSRRSMSSKTMITYSSSAAGPRS S GSSAVRSKH	60
CrPHY1	-----MSDPK S VSSTASDMS	16
AtPHYB	----- MVSGVGGSGGGGRGGGRGEEEPSSSHTPNNRRGGEQA QSSSGTKSLRPRSNTE	52
AsPHYA	-----MSSSRPA S SSSSRNRS	17
AtPHYA	-----MSGSRPTQSSSEGRSR	17
CrPHY4B	-----	
CrPHY4A	-----	
CrPHY2	NRRVVVAQTADARLHAVFEQAQSEGDMGGSSFDYMRSIEDARDSVLSEKVPQAQVTA-Y	119
CrPHY1	KRNQKIAQTSPDAKLYRAYEESTDSG-----SFDYSQSVNAN-----NEKLPGELIVA-Y	65
AtPHYB	S MSKAIQQYTVDARLHAVFEQSGESG---KSFYDYSQSLKTT---TYGSSVPEQQITA-Y	104
AsPHYA	S QARVLAQTTLDAELNAEYEEES-----GDSFDYSKLVEAQRDGPVPVQQGRSEK VIA-Y	69
AtPHYA	HSARI IAQTTVDAKLHADFEES-----GSSFDYSTSVRVTPGVVENQPPRS DKVTTY	70
CrPHY4B	-----	
CrPHY4A	-----	
CrPHY2	LQRMQRGGLIQPFGCMLAVEEGSFRVIAYSENAEMLDLLPHSVPTVGMQSSILGIGTDA	179
CrPHY1	LQRMQRGGLIQPFGCLVAVEEESFKVIAYSENAREMLDVSQAVPTMG-KYSRLDIGVDF	124
AtPHYB	LSRIQRGGYIQPFGCMIAVDESSFRIGYSENAREMLGIMPQSVPTLE-KPEILAMGTDV	163
AsPHYA	LQHIQKGKLIQTFGCLLALDEKSFNVIASFENAPEMLTTVSHAVPSVD-DPRLGIGTNV	128
AtPHYA	LHHIQKGKLIQPFGCLLALDEKTFKVIAYSENASELLTMASHAVPSVG-EHPVLGIGTDI	129
CrPHY4B	-----	
CrPHY4A	-----	
CrPHY2	RTLFTPASAAALEKASGAVDVSM LNPIHVHCRSSNKPFNAIVHRIDVGLVIDFEP IRPSD	239
CrPHY1	RTLLSPASAAALDRVIGVVDVSMFNPI TVQSRSSAKPFYAILHRNDVALVIDLEPIRPDD	184
AtPHYB	RSLFTSSSSIL LERAFVAREITLLNPVWIHSKNTGKPFYAILHRIDVGVIDLEPARTED	223
AsPHYA	RSLFSDQGATALHKALGFADVSL LNPI LVQCKTSGKPFYAI VHRATGCLVDFEPVKPTE	188
AtPHYA	RSLFTAPSASALQKALGFGDVSL LNPI LVHCR TSAKPFYAI IHRVTGSI IIDFEPVKPYE	189
	:*: *: .*: ** *:** *:** *: :	
CrPHY4B	IAVSA-AGALQSHKLAAKAISRLQSLPVG DIGLLCDSVVEEVRELTGYDRVMAYKFHEDE	97
CrPHY4A	IAVSA-AGALQSHKLAAKAISRLQSLPVG DIGLLCDSVVEEVRELTGYDRVMAYKFHEDE	97
CrPHY2	VALWATAGALQSHKLAAKAISRLQALPVG DIDLLCDSVVEEVRELTGYDRVMAYKFHEDE	299
CrPHY1	TSIAG--GAVQSHKLAAKAIARLQSLPGG DIGLLCDAVVEEVHGLTGCDRVMAYKFHDDE	242
AtPHYB	PALSI-AGAVQSQKLAVRAISQLQALPGG DIKLLCDTVVESVRDLTGDRVMVYKFHEDE	282
AsPHYA	FPATA-AGALQSYKLAAKAISIKIQLSPGG SMEVLCNTVVKEVFDLTGYDRVMAYKFHEDD	247
AtPHYA	VPMTA-AGALQSYKLAAKAITRLQSLPSG SMERLCDTMVQEVFELTGDRVMAYKFHEDD	248
	. **: * ** :*:** *: : ** :*:** *: ** ** *:** *: :	
GAF DOMAIN		
CrPHY4B	HGEVVAEIRKSDLEPYLGLHYPATDIPQASRFLFMKNRVRMICDCRATPIKVIQDRELRO	157
CrPHY4A	HGEVVAEIRKSDLEPYLGLHYPATDIPQASRFLFMKNRVRMICDCRATPIKVIQDRELRO	157
CrPHY2	HGEVLAEIRRSDEPYLGLHYPATDIPQASRFLFMKNRVRMICDCRAAPVRVVIQDKELRO	359
CrPHY1	HGEVIAEIRRSDEPYLGLHYPATDIPQAARFLFMKNRVRMICDCRVSPVKLVQNTALTQ	302
AtPHYB	HGEVVAESKRDDLEPYLGLHYPATDIPQASRFLFKQNRVRMIVDCNATPVLVVQDDRLTQ	342
AsPHYA	HGEVFSEITKPGLEPYLGLHYPATDIPQAARFLFMKNRVRMICDCRARSIKVIEAEALPF	307
AtPHYA	HGEVVSEVTKPGLEPYLGLHYPATDIPQAARFLFMKNRVRMIVDCNAKHARVLQDEKLSE	308
	****. : * : .****:*****:***** :*:***** **.. : : : *	
PΦB		
CrPHY4B	PLSLAGSTLRAPHG CH SQYMANMGSAASLVMAVIVNDNEEE---LTNRPHQPKPRKLWG	213
CrPHY4A	PLSLAGSTLRAPHG CH SQYMANMGSAASLVMAVIVNDNEEE---LTNRPHQPKPRKLWG	213
CrPHY2	PLSLAGSTLRAPHG CH SQYMANMGSIASLVMAVVVNDNED---VSNRAHHPKMRRLWG	415
CrPHY1	PMSLTGSTLRAPHG CH TKYMSNMGSISSLVMAVTVNESIES---KMGTAQAAS--KLWG	356
AtPHYB	SMCLVGSTLRAPHG CH SQYMANMGSIASLAMAVIINGNEDD---GSNVASGRSSMRLLWG	398
AsPHYA	DISLCGSALRAPH SCH LQYMENMNSIASLVMAVVVNENEDEAESEQPAQQQKKKKLWG	367
AtPHYA	DLTLCGSTLRAPH SCH LQYMANMNSIASLVMAVVVNEDGEDGAP-DATTQPOKRRKLWG	367
	: * ** :*****. * : ** * :*:***** :* :***	
PEST-MOTIF		

Figure 1 continued

CPHY4B	LVVCHHTTPRAVFPPLRSACEFLMQVFGQLQNMEEVLAQAQMRKHI	273
CrPHY4A	LVVCHHTTPRAVFPPLRSACEFLMQVFGQLQNMEEVLAQAQMRKHI	273
CrPHY2	LVVCHHTVPRAVFPFALRSACEFLMQVFGQLQNMEEVLAQAQMRKHI	475
CrPHY1	LIVCHHTSPRYIPFPVRSACQFLMQVFSQLQNMEEVMAAQLEKHI	416
AtPHYB	LVVCHHTSSRCIPFPLRYACEFLMQAFGLQNMELQALQMSSEKRV	458
AsPHYA	LLVCHHESPRYVFPFLRYACEFLAQVFAVHVNRFEFELEKQLREKN	427
AtPHYA	LVVCHNTTPRFVFPFLRYACEFLAQVFAIHVNKEVELDNQMVKEKN	427
	*:***: . * :*:*: * *:*** * .:***: * :*: * *:***:***:*	
CrPHY4B	A-PIGIVSESPNIMDLVKCDGAALYYGKFWLLGITPTEAQIKDLAEWLLDVHRDSTGLS	332
CrPHY4A	A-PIGIVSESPNIMDLVKCDGAALYYGKFWLLGITPTEAQIKDLAEWLLDVHRDSTGLS	332
CrPHY2	A-PIGIVSESPNIMDLVKCDGAALYYGKFWLGVTPTEAQIKDLADWLLTVHKDSTGLS	534
CrPHY1	A-PIGIVIQEPNIMDLVSCDGAVALYYGRKCWCLGTTPTESQIADIAAWLLESHKSTGIS	475
AtPHYB	S-PAGIVTQSPSIMDLVKCDGAALFYHGKYYPLGVAPSEVQIKDVVEWLLANHADSTGLS	517
AsPHYA	ASPLTIIVSGTPNIMDLVKCDGAALYYGKFWRLRNAPTESQIHDIADFWSLVHRDSTGLS	487
AtPHYA	A-PLGIVSQSPNIMDLVKCDGAALYYKDKIKWLGITPSEFHLQEIASWLCEYHMDSTGLS	486
	: * * * * .*****.***: * * : * :*: * : : . . * * * :***:*	
PHY DOMAIN		
CrPHY4B	TDSLADAGYPGAAALGDAVCGMAAARITSKDFLFWFRSHTAKEIKWGGARHDPEDRDDGR	392
CrPHY4A	TDSLADAGYPGAAALGDAVCGMAAARITSKDFLFWFRSHTAKEIKWGGARHDPEDRDDGR	392
CrPHY2	TDSLADAGYPGAAALGDAVCGMAAAKITTRDFLFWFRSHTAKEIKWGGAKHDPDEKDDGR	594
CrPHY1	TDSLAKAGYPGAASL-DAVCGMAAARITATDFLFWFRSHTASEVVKWGGAKHDPDEDEDGR	534
AtPHYB	TDSLGDAGYPGAAALGDAVCGMAVAYITKRDFLFWFRSHTAKEIKWGGAKHHPEDKDDGQ	577
AsPHYA	TDSLHDAGYPGAAALGDMICGMAVAKINSKDLFWFRSHTAAEIRWGGAKNDPDDMDSR	547
AtPHYA	TDSLHDAGFPRALSLGDSVCGMAAVRISSKDMIFWFRSHTAGEVVRWGGAKHDPDRDDAR	546
	*** .*: * * : * * :****. . * . *:***** * *:*****: . . : * :	
	Hinge Region	
CrPHY4B	KMHPRSSFKAFLEVVKRRSLPWEDVEMDAIHSLQLILRGSFQDIDD	451
CrPHY4A	KMHPRSSFKAFLEVVKRRSLPWEDVEMDAIHSLQLILRGSFQDIDD	451
CrPHY2	KMHPRSSFNAFLEVVKRRSLPWEDMEMDAIHSLQLILRGSFQDIDD	653
CrPHY1	RMHPRSSFKAFLRVVKRRSLPWEDVEMDAIHSLQLILRGSFQDIDD	593
AtPHYB	RMHPRSSFQAFLEVVKRSRSPWETAEMDAIHSLQLILRDSFKESEAA	636
AsPHYA	RMHPRLSFKAFLEVVKMKSLPWSDYEMDAIHSLQLILRGTLNDASK	606
AtPHYA	RMHPRSSFKAFLEVVKTRSLPWKDYEMDAIHSLQLILRNAFKDSET	606
	:*** *:***.*** : * *. *****.***.***. * :*** * ***** : : *	
PAS₁		
CrPHY4B	LK----LQGMDELSTVANEMVRLIETATAPIFAVDAGGFINGWNVKVAELTGLSVEEAMN	507
CrPHY4A	LK----LQGMDELSTVANEMVRLIETATAPIFAVDAGGFINGWNVKVAELTGLSVEEAMN	507
CrPHY2	LK----LHGMDELSTVANEMVRLIETATAPIFAVDAGGFINGWNAKVAELTGLSVEEAMN	709
CrPHY1	LR----LQGLDELSTVASEMVRLIETATAPILAVDAEGLINGWNGKVAELTGLPFETAMG	649
AtPHYB	CRDMAGEQGIDELGAVAREMVRLIETATVPIFAVDAGGICINGWNAKIAELTGLSVEEAMG	696
AsPHYA	LK----LDGLAELQAVTSEMVRIMETATVPILAVDGNGLVNGWNAKAAELTGLRVDDAIG	662
AtPHYA	LK----IDGIQLEAVTSEMVRLIETATVPIAVDSDGLVNGWNTKIAELTGLSVDEAIG	662
	: .*: * * :*: *****.***.***. * :*** * ***** : : *	
Transduction Core		
CrPHY4B	RSLVGDIVDESAEVVERLLSLALQGEKRNVEIKLKTHTGEQAQNGPVILVNTCSSRDF	567
CrPHY4A	RSLVGDIVDESAEVVERLLSLALQGTGDIVCWILRSLVFPSTSNLKEIEG-----	535
CrPHY2	RSLVRDIVVDASMETVEKVLDLALQGEQNVKIKLTYGEQAEKGPVILVNTACSSRDF	769
CrPHY1	KSLAKELVHRDSKATVERVLQALALDQGEQDIEIHLRTYNKQKGERGVVILVNTCCSRDV	709
AtPHYB	KSLVSDLIYKENEATVNKLLSRALRGDEEKNVEVKLTFSPQLQKAVFVVNTACSSKDY	756
AsPHYA	RHILT-LVEDSSVPVQRMVLRALQGEKEEVRFEVKTHTGPKRDDGPVILVNTACSRDL	721
AtPHYA	KHFLT-LVEDSSVEIVKRMLENALGTEEQNVQFEIKTHLSRADAGPISLVNTACSRDL	721
	: : : : . *::* * *	
PAS₂		
CrPHY4B	TENVVGVCFFQDVTROKQVMDKFTRIQGDYKAIVQNPNPPLIPPIFGADEFGYCTEWNPA	627
CrPHY4A	-----	
CrPHY2	TDNVVGVCFFQDVTGQKQVMDKFTRIQGDYKAIVQNPNPPLIPPIFGADEFGYCSSEWNP	829
CrPHY1	SNNVVGVCFFQDVTGQKLVLDRIYRMQGDYKAIVQSPNPPLIPPIFGADEYGFCSSEWNSA	769
AtPHYB	LNNIVGVCFFQDVTQSQKIVMDKFINIQGDYKAIVHSPNPPLIPPIFAADENTCCLEWNMA	816
AsPHYA	HDHVVGVCFFQDMTVHKLVMKFTTRVEGDYKAIHNPNPPLIPPIFGADEFGWCSEWNAA	781
AtPHYA	HENVVGVCFFHDLTGQKTVMKFTTRIEGDYKAIQNPNPPLIPPIFGTDEFGWCTEWNPA	781

Figure 1 continued

CrPHY4B	MEELSGWKRRDDVMGRMMIGEVFGTDLTCCRLRNC	DDLTkFMIVLNTAMGGSDSDKFPFGF	687
CrPHY4A	-----	-----	552
CrPHY2	MEKLSGWKREDVVGKMLVGEVFGSELACCKLRGQ	DSITkFMIILNSAMGGQDSERFPFGF	889
CrPHY1	MEKLFKLEKGRSHRKELIGEIPGLEMVCCRLDGQ	DVVTkLMIILNDAVNGHDSEQYPLVF	829
AtPHYB	MEKLTGWSRSEVIGKMIVGEVFG--SCCMLKGP	DALTKFMIVLHNAIGGQDTDKFPFPF	873
AsPHYA	MTKLTGWNRDEVLDKMLLGEVFDSSNASCPLKNR	DAFVSLCVLINSALAGEETEKAFFGF	841
AtPHYA	MSKLTGLKREEVIDKMLLGEVFGTQKSCCRLKNC	EAFVNLGIVLNNAVTSQDPEKVSFAF	841
	. * .	:::	
CrPHY4B	FDRNGRYVEAILIAHKRTDGDGAVTGVFCFLRTTS	PELQQALQVQKQSARTIAVERLKEEA	747
CrPHY4A	-----	-----	558
CrPHY2	FDRHGKYEALLIANKRSDSDGAITGVFCFLHTAC	PELQQALQVQKRSARTALDRLEKVA	949
CrPHY1	YDRMGHRIEALLIASKRTDADGHITGVFCFLHTAG	SELLQAIVIRKAKEKVE---KELS	885
AtPHYB	FDRNGKFVQALLTANKRVSLEQKVGAFCFLQIPS	PELQQALAVQRQDTECFCTKAKELA	933
AsPHYA	FDRSGKYIECLLSANRKENEGGLITGVFCFIHVAS	HELQHALQVQQASEQTSKRLKAFS	901
AtPHYA	FTRGGKYVECLLCVSKKLDREGVTVGVFCFLQ	LASHELQQALHVQRLAERTAVKRLKALA	901
	:: .	H-box	
HisKA			
CrPHY4B	YMKQEIIRNPLYGIVFTRKLLQSMDLSDQQKQILETSAPCERQLPKILDEDNFEKLDQG--		805
CrPHY4A	-----	-----	
CrPHY2	YMKQEIKNPLYGIIFTRKLLDNTNLTDQQRQIMETSALCEKQLQNLLEEDSFEKLDQG--		1007
CrPHY1	YVRTEARKSLDGLTFTRMVMEGTDLSEAEQRQLIKTNALCEKQLRKILD-DELNNIEEG--		942
AtPHYB	YICQVIKNPLSGMRFANSLLLEATDLNEDQKQLLETSSVCEKQISRIVGDMDLSEIEDG--		991
AsPHYA	YMRHAINNPLSGMLYSRKALKNTDLNEEQMKQIHVGDNCHHQINKILADLDQDSITEKSS		961
AtPHYA	YIKRQIRNPLSGIMFTRKMIETGELGPEORRILOTSALCQKQLSKILDDSDLESIIEG--		959
CrPHY4B	TLELDTLEFSMGAVMDAVVSQGMIRSREKGLQFVRETPVDIKNTRLFGDQLRL	LQQVLADF	865
CrPHY4A	-----	-----	
CrPHY2	NVELDTLGFTMGRVMDAVISQGMIRSREKGLQLIRETHAELKNTRLFGDQYRL	LQQVLADF	1067
CrPHY1	YMELDMMQFLMETVMDVVISQGMVAARLKEVQILTEVPDEVKYLFLYGDQVRL	LQQALGDL	1002
AtPHYB	SFVLKREEFFLGSVINAIVSQAMFLLRDRGLQLIRDIPEEIKSIEVFGDQIRI	QQLLAEF	1051
AsPHYA	CLDLEMAEFLLDQDVVVAASVQLITCQGGKIRISCNLPERFMKQSVYGDGVR	LQQILSDF	1021
AtPHYA	CLDLEMKEFTLNEVLTASTSQVMMKSNKSVRITNETGEEVMSDTLYGDSIR	LQQVLADF	1019
HATase			
CrPHY4B	LTTAVRFTASSDG--WVGIVVPTVKQIGDSLQVVHFEFRISHPGCGIPEDLVQQMHD-		921
CrPHY4A	-----	-----	
CrPHY2	LTTAIRFTSSSDG--WVGIVVATIKNIRDGLHIAHFEFRVSHPGSGIPEALVQQMYD-		1123
CrPHY1	LFFTINYTNAPHGQRKWVAIKVSYMKNQLDDGVHVVNLEFRISHSGNSIPETLVSEMSD-		1061
AtPHYB	LLSIIRYAP-SQE--WVEIHLSQLSKQADGFAAIRTEFRMACPGEGLPPELVDRMFH-		1106
AsPHYA	LFISVKFSPVGGG----VEISSKLTKNSIGENLHLIDLELRIKHQGLGVPAELMAQMFEF		1077
AtPHYA	MLMAVNFTPSGGQ----LTVSASLRKQDLGRSVHLANLEIRLTHTGAGIPEFLNQMFQ		1074
		PEST-Motif	
CrPHY4B	RSPEITQEGMGLSVSRKLVKLMNGDVSYVRDG-	GLCYFLVHLELPLVQ-----	968
CrPHY4A	-----	-----	
CrPHY2	RSQEIITQEGMGLSVSRKLVKLMNGDVRYIREF-	RVCYFLVTADLPLFKKTD-----	1174
CrPHY1	ERAWTPEGLGMSVSSALVRLMNGSVEYTSDD	EGNMSFIVKIEFPLAQNDVPYLT-----	1116
AtPHYB	SSRWTSPEGLGLSVCRKILKLMNGEVQYIRES-	ERSYFLIILELPVPRKRPLSTASGSGD	1165
AsPHYA	DNKEQSEEGSLSLVSRNLLRLMNGDVRHLREA-	GVSTFIITAELASAPTAMGQ-----	1129
AtPHYA	TEEDVSEEGSLSMVSRKLVKLMNGDVQYLRQA-	GKSSFIIITAEALAAANK-----	1122
CrPHY4B	-----		
CrPHY4A	-----		
CrPHY2	-----		
CrPHY1	-----		
AtPHYB	MMLMPY	1172	
AsPHYA	-----		
AtPHYA	-----		

Figure 1. Sequence comparison of the phytochrome multigene Family in *Ceratopteris richardii* (Cr) with *Arabidopsis thaliana* (At) PHYA, PHYB and *Avena sativa* (As) PHYA using CLUSTALW. Various domains are indicated, including the GAF domain, (present in phytochromes and in vertebrate *cGMP-specific phosphodiesterases*, in cyanobacterial *adenylate cyclases* and in the *formate hydrogем lyase* transcriptional activator FhlA and contain the bilin lyase domain (BLD) capable of autocatalytic assembly of the chromophore to the apoprotein (Aravind and Ponting, 1997; Wu and Lagarias, 2000), the PHY domain or the Phytochrome region is specific to phytochrome proteins and is required for the stabilization of the Pfr chromophore (Wu and Lagarias, 2000), the PAS-related domains (PRD) contain two PAS repeats (PAS₁ and PAS₂), and the HKRD consisting of the histidine-kinase A (HisKA) subdomain found in bacterial sensor protein/histidine kinase and the histidine kinase-like ATPase (HATase) subdomains. PHYA has a PEST motif after the chromophore, PΦB. A PEST motif can also be detected in CrPHY1 in the HATase domain (position 1046-1063) of the C-terminus. Conserved serine residues of oat PHYA can be detected in the P1 domain of the N-terminal but not in the hinge region. CrPHY4A lacks the P1-P2 domains and all other domains directly downstream of the Quail Box transduction core.

Figure 2

AcPHY1	GCAGTTTCAAATTCAAGGTTTCCCATGGTTTCCCCTGTTCTGTAGCTCCCTTGTGTGGG	60
CrPHY1		
AcPHY2	-----ACCATCCACCACCGCTCTCCGACTCCCGCCGGCCTCTCTCCTT	43
CrPHY2	-----	
CrPHY4B	-----	
CrPHY4A	-----	
AcPHY4	-----	
AcPHY1	CATTGCCTACGCCTCTCGTCTCCAAAATCTCCCATCTCTATCTTTCTTTGAAGGTCCATC	120
CrPHY1	-----TCCCCTTAGGGAACACTT	18
AcPHY2	ACTGTGTTGCCAAGTAGACGCGATCTCCGGCCCTTATCTGCGCACACATACAGGTAAAGT	103
CrPHY2	-----TGGTGC-TGTTTACTGATATGCG	22
CrPHY4B	-----	
CrPHY4A	-----	
AcPHY4	-----	
AcPHY1	ATCAGGCTCGATGCAATGGGCAGGAGCAGCGAATTGAGAGCCATTAGTAACCAATAAC	180
CrPHY1	CTCACTTTTCA-----AGGTGGACTCTACGATCTGAAAAAGTTCTGTCATCGACGTCAC	72
AcPHY2	AGGAATTTTCA-GCT-CCCATGGAAGCTTCAA-TCACCCCTTCTCTCTTCCCCCCCCAC	160
CrPHY2	CCGACGTCGCATGCT-CCC--GGCCGCCATG-CCGCGGGATTCTAGAAATGGCGCTTCC	78
CrPHY4B	-----	
CrPHY4A	-----	
AcPHY4	-----	
AcPHY1	---TGAGCTGAGAGCAGCAGTCTTTGCTGAGGAAGAGGCACACACACACACACGCACT	237
CrPHY1	---TGT-CTGCTTTTCA--GTTTACTTTGAGGACTTAAGTCTAACCTA-AAGCAGTTCTC	125
AcPHY2	TAACATCCTAATTCCATCAACAATTGAGGCCCTTTCCCTCTTGCTGCAACATGTCCTCC	220
CrPHY2	---CGTTTTTATTCC-TCA-----GCCCTTAACCTCTCGCAGAAGCATGTCCTCC	125
CrPHY4B	-----	
CrPHY4A	-----	
AcPHY4	-----	
AcPHY1	ACCCTT---CATACTACGCAAGCATGTCGAGTACAAGACACAGCTACTCTTCTGGAGGC	293
CrPHY1	AGTCTC---TGC-TTTCGAGACATGTCGGATCCGAAGCAATCCGTATCATCAACGGCT	180
AcPHY2	AAAACCATGACCTACTCTCTCAGCTGCCGAACCTCGCAGCAGCAGCTCCTCCGTGGTT	280
CrPHY2	AAAACCATGACGTACTCTCTCATCAGCTGCAGGTCCACGCAGCAGCGGCTCTTCTGCCGTC	185
CrPHY4B	-----	
CrPHY4A	-----	
AcPHY4	-----	
AcPHY1	TCTGGCAAAATCCAAGCATGGCAGGAGAATCGCACAAACATCAGCAGATGCTAAGCTTTAT	353
CrPHY1	TCGGACATGTCGAAACGTAACCAGAAAAATTGCTCAAACATCACCAGATGCGAAACTATAT	240
AcPHY2	GGCTCCAAGCACAATCGCCGGTGTGTTGTTGCACAGCAACTGCAGATGCCAAATTGCAT	340
CrPHY2	CGCTCCAAGCACAATCGCCGGGTGTTGTTGCCAGACAACCTGCAGATGCCAGGTTGCAC	245
CrPHY4B	-----	
CrPHY4A	-----	
AcPHY4	-----	
AcPHY1	GCCGCATATGAAGAGTC---GTCCGAG-----TCTGGG-----TCTTTCGACTACAGT	398
CrPHY1	AGAGCGTACGAAGAGTC---AACCGAC-----TCTGGG-----TCTTTCGATTACAGC	285
AcPHY2	GCAGTTTTTGTAGCAAGCGCAGTCCGAGGGCGATACTGGGGGCGAGTCCCTTTGACTACATG	400
CrPHY2	GCAGTTTTTCGAGCAAGCCCAGTCCGAGGGGGACATGGGTGGCAGTCCCTTTGACTACATG	305
CrPHY4B	-----	
CrPHY4A	-----	
AcPHY4	-----	
AcPHY1	CAATCTGTTAG---TGCAAGG-----AAAGAAGGCATTTCTTCCCAACTGGTT	443
CrPHY1	CAGTCTGTCAA---TGCAAAAC-----AATGAAAAGTTGCCCGGGGAGCTAATT	330
AcPHY2	CGTTCCATTGAGGATGCCCGCGGCTCAGTTTTTGAGCGAAAGGTTGCCAGCACAGGCAGTG	460
CrPHY2	CGCTCAATTGAGGATGCACGCGATTGAGTGTGAGTGTGAGGAGTTGCCAGCCAGGCAGTG	365
CrPHY4B	-----	
CrPHY4A	-----	
AcPHY4	-----	

AcPHY1	ACTGCCTACCTTCAGCGTATGCAACGCGGAGGTCTTGTTCAGCAATTGGGTGCTTGATT	503
CrPHY1	GTGGCTTATCTTCAGCGCATGCAGCGTGGAGGTCTCATTTCAGCCATTCGGGTGTTTGGTT	390
AcPHY2	ACGGCATACCTTCAAAGAATGCAGAGAGGCGCCTCATCCAACCATTTGGATGCGATGCTT	520
CrPHY2	ACGGCGTACCTTCAGCGTATGCAGCGAGGAGGCCTCATCCAGCCCTTTGGATGTATGCTT	425
CrPHY4B	-----	
CrPHY4A	-----	
AcPHY4	-----	
AcPHY1	GCAGTGGAGGAAGAGACCTTCAGAGTCCCTTGCATATGGTGCGAATGCACCTGAGATGCTA	563
CrPHY1	GCAGTGGAGAAGAATCATTCAAGGTGATTGCATATAGTGAAAATGCACGCGAGATGTTG	450
AcPHY2	GCACTGGAGGAGGCGAGCTTTAGGGTAATTGCCTACAGTGAAAATGCTGCAGAAATGCTT	580
CrPHY2	GCAGTCGAGGAGGGAAGCTTCAGGGTAATTGCCTATAGTGAAAATGCCGAGAGATGCTC	485
CrPHY4B	-----	
CrPHY4A	-----	
AcPHY4	-----	
AcPHY1	GATGTAGCCACTCAAGCCGTGCCAACAATGGGGCAATATAGCC--GT-CTCTGTATAGGT	620
CrPHY1	GATGTATCAGTGCAAGCGGTGCCTACGATGGGGAAGTATAGTC--GT-TTAGATATTGGA	507
AcPHY2	GACCTCATGCCTCAATCTGTACCTAGTGTAGGGGTGCAAGTCTGGTGCTTGGCATCGGC	640
CrPHY2	GACCTCCTTCCTCATTTCAGTGCCTACCGTGGGGATGCAGAGCTCAATACTTGGCATAGGT	545
CrPHY4B	-----	
CrPHY4A	-----	
AcPHY4	-----	
AcPHY1	GCTGATGTAAGAACTCTGTTATCTCCGGCGAGTGCATCAGCATTGGATCGAGTCATCGGT	680
CrPHY1	GTTGATTTTAGGACTCTCTTATCCCGCGCAAGTGCTGCAGCATTGGACCGAGTCATTGGT	567
AcPHY2	ACTGACGCGCGCAGCCTTTTACATATGCAAGTGCAAGTGCAGCTGCCCTGGAGAAGGCCCTCAGG	700
CrPHY2	ACTGATGCTCGTACTCTCTTTACTCTGCTAGTGTGCTGCAGCACTTGAGAAAAGCCTCAGGT	605
CrPHY4B	-----TTATTTTCACGCCTGCGAGTGCCCATTCCTTCGAGAAGGCCGTGGGT	47
CrPHY4A	-----TTATTTTCACGCCTGCGAGTGCCCATTCCTTCGAGAAGGCCGTGGGT	47
AcPHY4	-----	
AcPHY1	GTTGTCGATGTGAGCATGTTCAATCCCATAAACCGTGCAAAGCCGAAGCTCTGGAAAGCCC	740
CrPHY1	GTTGTTGATGTGAGCATGTTCAATCCCTATAACTGTTCAAAGCAGGAGTTTCAGCAAAACCA	627
AcPHY2	GCTGTTGATGTCTCCATGCTCAATCCCATTAAGTGTGCACTGCCGAGCTCGAGCAAGCCT	760
CrPHY2	GCTGTTGATGTGTCATGCTGCTGAATCCTATTACGTACACTGCCGAGCTCTAATAAGCCT	665
CrPHY4B	GCTGTGGATGTCTCAATGCTTAATCCAATTACTGTTTATTGTCGAAGCTCTGGAAAACCA	107
CrPHY4A	GCTGTGGATGTCTCAATGCTTAATCCAATTACTGTTTATTGTCGAAGCTCTGGAAAACCA	107
AcPHY4	-----	
AcPHY1	TTCTATGCAATTTTGCACAGAAATGATGTGGGGTTGGTAATTGACCTTGAGCCTATCAGG	800
CrPHY1	TTTTATGCAATTTTGCACAGGAATGACGTGGCATTGGTGATTGACCTTGAGCCAATCAGG	687
AcPHY2	TTCAATGCAATTTGTGCACCGCATTGATGTGGGCTTAGTTATTGACTTTGAACAGTCCGG	820
CrPHY2	TTCAATGCCATCGTTTACCGCATTGATGTTGGTTTAGTCATTGATTTCGAACCAATTAGG	725
CrPHY4B	TTCAATGCCATTTTGCACCGCATAGATGTTGGTTTAGTGATGGATTAGAACCGATACGA	167
CrPHY4A	TTCAATGCCATTTTGCACCGCATAGATGTTGGTTTAGTGATGGATTAGAACCGATACGA	167
AcPHY4	-----	
AcPHY1	CCTGATGATGCATCTAT-----CACTGGCGGTGCCTTGCAATCAGTACATAAGCTGGCAGCG	854
CrPHY1	CCCGACGATACATCTAT-----TGCAGGTGGTGCTGTGCAGTCCCATAAGTTGGCTGCA	741
AcPHY2	CCTGCTGATGTGTCTGTATGGGCTGCTGCCGAGCCTTGCAATCACACAAGCTAGCTGCC	880
CrPHY2	CCTTCTGACGTAGCTTTATGGGCTACTGCTGGAGCCTTGCAATCACACAAGCTAGCTGCC	785
CrPHY4B	GGTTCTGATATAGCTGTCT---CAGCTGCTGGTGCCCTCCAATCCCACAAGCTAGCTGCG	224
CrPHY4A	GGTTCTGATATAGCTGTCT---CAGCTGCTGGTGCCCTCCAATCCCACAAGCTAGCTGCG	224
AcPHY4	-----	
AcPHY1	AAGGCCATCGCTCGGTTGCAGTCTTTGCCAGGAGGTGATATTGGCCTCTTATGTGACTCA	914
CrPHY1	AAAGCCATTGCTCGCTTACAATCTTTACCTGGAGGGGACATTGGTCTCTTGTGTGATGCA	801
AcPHY2	AAGGCCATATCCAGATTACAGGCCTTCCAGTAGGGGATATTGATCTCTTATGTGATTTCT	940
CrPHY2	AAAGCCATATCCAGATTACAGGCCTCCCTGTTGGCGATATTGATCTTTTGTGTGATTTCT	845
CrPHY4B	AAGGCCATTTCTAGGCTACAGTCTCTTCCAGTAGGTGATATCGGCTTGCTATGTGATTTCT	284
CrPHY4A	AAGGCCATTTCTAGGCTACAGTCTCTTCCAGTAGGTGATATCGGCTTGCTATGTGATTTCT	284
AcPHY4	-----	

Figure 2 continued

AcPHY1	GTAGTGGAGGAGGTACATGAGCTTACGGGTTTCGACAGAGTGATGGCATATAAGTTTCAT	974
CrPHY1	GTAGTAGAGGAGGTCCATGGGCTGACTGGCTGTGACAGAGTAATGGCTTATAAATTCAC	861
AcPHY2	GTTGTGGAAGAAGTCAGAGAGCTGACAGGGTATGATCGTGTCTATGGCTTATAAATCCAC	1000
CrPHY2	TTGTGCGAAGAAGTGAGAGGCTTAAGTGGCTATGTAGATGAGTATGGCTTACAAATTCAT	905
CrPHY4B	GTTGTAGAAGAAGTTTCGTGAACCTACAGGATATGATCGAGTCATGGCTTACAAATTCAT	344
CrPHY4A	GTTGTAGAAGAAGTTTCGTGAACCTACAGGATATGATCGAGTCATGGCTTACAAATTCAT	344
AcPHY4	-----GATCTTGAGCCCTACCTG	18
	* * * * *	
AcPHY1	GAAGATGAGCATGGAGAGGTGGTTGCTGAGATAAGGCGTACGGATCTTGAGCCTTATATT	1034
CrPHY1	GATGATGAACATGGAGAGGTGATTGCTGAGATAAGGCGATCCGATCTTGAGCCTTACATT	921
AcPHY2	GAAGATGAACACGGTGAAGCTTAGTCTGAGATTGCAAGATCTGACTTGAACCGGTACTTG	106
CrPHY2	GAAGACGAACACGGTGAAGTTTGGCCGAAATAAGAAGGTCCGACCTGGAGCCATATTTG	965
CrPHY4B	GAGGACGAACATGGGAGGTTGTTGCAGAAATTCGAAAGTCCGATCTCGAGCCTTACTTG	404
CrPHY4A	GAGGACGAACATGGGAGGTTGTTGCAGAAATTCGAAAGTCCGATCTCGAGCCTTACTTG	404
AcPHY4	-----GATCTTGAGCCCTACCTG	18
	* * * * *	
AcPHY1	GGGCTGCATTACCCAGTCTACTGATATCCACAAAGCTGCTCGTTTTCTCTTCATGAAAAAC	1094
CrPHY1	GGTCTACATTACCCAGTCTACTGATATCCACAGGCTGCAGGTTTCCTTTTCATGAAGAAC	981
AcPHY2	GGGTTGCATTATCCTGCAACTGACATTCCACAGGCATCGCGTTTCCCTTTTCATGAAGAAC	1120
CrPHY2	GGGCTGCATTATCTGCTACAGATATCCCTCAGCGTCCAGGCTCCAGGTTCTTTTCATGAAGAAC	1025
CrPHY4B	GGTTTGCATTACCTGCAACTGACATACCTCAGGCATCTCGGTTTCTCTTCATGAAAAAT	464
CrPHY4A	GGTTTGCATTACCTGCAACTGACATACCTCAGGCATCTCGGTTTCTCTTCATGAAAAAT	464
AcPHY4	GGGTTGCATTATCCTGCAACTGATATACCAAGGCATCTCGATTCTTTTATGAAAAAC	78
	* * * * *	
AcPHY1	AGAGTGAGGATGATATGTGATTGCAGGTGGCCGCCAGTTAAGCTTATTCAGGACAAGACG	1154
CrPHY1	AGAGTGAGAATGATCTGTGACTGCAGAGTATCACTGTTAAGCTGGTTCAGAACACGGCA	1041
AcPHY2	AGGGTTAGGATGATTTGTGATTGCCGGGCACTTCCAGTAAGAGTTATTCAGGACAAAGAG	1180
CrPHY2	AGGGTTAGGATGATTTGTGATTGCCGGGCACTTCCAGTCCAGTCCAGGTTCTTTTCATGAAGAAC	1085
CrPHY4B	AGAGTGAGAATGATTTGCGACTGCCGAGCTACTCCCATCAAAGTTATCCAAGATAGGGAA	524
CrPHY4A	AGAGTGAGAATGATTTGCGACTGCCGAGCTACTCCCATCAAAGTTATCCAAGATAGGGAA	524
AcPHY4	AGAGTGAGAATGATATGTGATTGCCGAGCTATTCAGTCAAAGTATCCAGGATAGAGAA	138
	* * * * *	
AcPHY1	CTTAGCCAGCCCATGAGCTTGACAGGGTCAAAGCTCCGCGCGCCGCATGGATGCCACACC	1214
CrPHY1	TTGACACAGCCCATGAGCTTGACAGGCTCAACACTTCGTGCACACATGGATGCCATACA	1101
AcPHY2	CTAAGGCAGCCTTTGAGTTTAGCGGGTCTACGCTACGGGCTCCATGGGTGTCACAGT	1240
CrPHY2	TTAAGGCACCTTTGAGCTTGCGCATTCGATCGCATTCGAGGCCCCATGGATGTCATAGT	1145
CrPHY4B	TTAAGGCAGCCTTTGAGCTTAGCTGGCTCTACACTGAGAGCTCCTCATGGTTGCCATAGT	584
CrPHY4A	TTAAGGCAGCCTTTGAGCTTAGCTGGCTCTACACTGAGAGCTCCTCATGGTTGCCATAGT	584
AcPHY4	CTGAGGCAGCCCTTAAGTCTGGCGGCTCCACATTACGAGCTCCCCATGGGTGCCATAGT	198
	* * * * *	
AcPHY1	CAATACATGGCCAAACATGAATTCAATCTCCTCCTTGGTGATGGCTGTAATTGTGAATGAT	1274
CrPHY1	AAGTATATGAGTAACATGGGTTCCATATCTTCTTTAGTAATGGCTGTTACTGTAATGAA	1161
AcPHY2	CAGTACATGGCTTAACATGGGATCGATTGGCTTCATTGGTGATGGCTGTTGTTGTCAATGAT	130
CrPHY2	CAATACATGGCCAAACATGGGCTGATTCGATCTTTGGTATGGCTGTTGTTGTGAATGAC	1205
CrPHY4B	CAGTATATGGCCAAACATGGGCTCTGCTGCATCTCTTGTGATGGCAGTCATTGTGAATGAC	644
CrPHY4A	CAGTATATGGCCAAACATGGGCTCTGCTGCATCTCTTGTGATGGCAGTCATTGTGAATGAC	644
AcPHY4	CAGTACATGGCGAACATGGGCTCTGGCGCTTCTTTGGTAAATGGCAGTGCTCGTGAATGAC	258
	* * * * *	
AcPHY1	AGTGATGACGATA-----GTCCCGGCCATTCTTCGCAAGGC-ATTAAACTTTGGGGCTTG	1328
CrPHY1	AGCATCGAAAGCA-----AGATGGGTACTGCTGCTCAAGCT-AGTAAGCTTTGGGGACTT	1215
AcPHY2	AACGATGAAGATGTGAGTAACCGATCTCAACAACCAAAGATGAGGAGGCTATGGGGACTT	1360
CrPHY2	AATGATGAAGATGTGAGTAACCGAGCTCATCACCCAAAGATGAGGAGGCTGTGGGGCTTG	1265
CrPHY4B	AATGAAGAAGAAATTGACTAACACAGACCTCATACGCCAAAGCCAAGGAAACTCTGGGGCTT	704
CrPHY4A	AATGAAGAAGAAATTGACTAACACAGACCTCATACGCCAAAGCCAAGGAAACTCTGGGGCTT	704
AcPHY4	AATGAAGAAGGATCTAGCAGAGATCTCATAAAGCCAAAGCCAAGGAAACTTTGGGGCTC	318
	* * * * *	
AcPHY1	GTCGTATGCCACCATACGCTCACCACGCTATGTACCTTTCCCCGTAAGGTGGCTTGTGAG	1388
CrPHY1	ATAGTGTGTCATCATACATCACCACGGTACATACCTTTCCAGTTAGATCAGCTTGTGAG	1275
AcPHY2	GTCGTTTGCCACCACATCTACCTAGAGCAGTACCATTGACATTAGGTGAGTTGCGAG	1420
CrPHY2	GTAGTATGCCACCACACTGTGCCCTAGAGCAGTCCACTTTGCACTTAGGTGAGCTTGTGAG	1325
CrPHY4B	GTTGTATGTCACCATACCAACCAAGGGCGTGCCATTTCCTTCCACTTAGGTGAGCTTGTGAG	764
CrPHY4A	GTTGTATGTCACCATACCAACCAAGGGCGTGCCATTTCCTTCCACTTAGGTGAGCTTGTGAG	764
AcPHY4	GTAGTATGTCACCATACCAACCAAGGAGCTGTGACATTTTCCCTTAGGTGAGCTTGTGAG	378
	* * * * *	

Figure 2 continued

AcPHY1	GTACACGAGGAGTCAAAAACGATTGTGGAAGAGTTCTCCATCTTGCAATTGGAAGGTGAA	2288
CrPHY1	GTACACAGGGATTCGAAGGCTACTGTTGAAAGAGTATTGCAACTTGCTTTGGACGGTCAA	2175
AcPHY2	GTGGTGAATGCATCCATGGAAACAGCTGAAAGAGTGTAGATTGGCACTACAAGGACAA	2320
CrPHY2	GTGGTGGATGCATCTATGGAACACTGTGGAAGAGTGTGGATCTGGCATTGCAAGGGCAA	2225
CrPHY4B	ATCGTAGATGAGTCTGCAGAGGTTGTGGAGCGGTTGCTGTCTTTAGCTCTTCAAGGTGAA	1664
CrPHY4A	ATCGTAGATGAGTCTGCAGAGGTTGTGGAGCGGTTGCTGTCTTTAGCTCTTCAAGGTACT	1664
AcPHY4	GTCTGGAGGATTCGCAGAAATTGTAGAACGACTTTTGTGTTTAGCCCTGCAAGGTGAA	1278
	* * * * *	
AcPHY1	GAAGAACAAGATATAGAAATACACTTGAGAACTTATGACCAGCATAAGCAGAAAGGGGTA	2348
CrPHY1	GAAGAACAGGACATAGAGATACATTTGAGAACTTATAACAAGCAAAAAGAGAGAGGTGTT	2235
AcPHY2	GAAGAGCAGAAATGTGGAAATTAAGCTCAAGACGTATGGTGACCAAGCTATAAAGGGACCA	2380
CrPHY2	GAAGAACAGAAATGTAGAAATCAAACCTAAGACTTATGGTGAACAAGCTGAAAAGGACCA	2285
CrPHY4B	GAAAAACGAAATGTGGAAATAAAGTTGAAACACATGGCGAACAGGCTCAGAAATGGTCCT	1724
CrPHY4A	GGAGA-CATTGTTTG-----TTGGATTCTTAGGTC-----TCTTGTATTTCAT	1707
AcPHY4	T-ATATTTTGTGAGAGTTGTGCTCTTTGTAGCTTCTATC-----ACATGTTAATCTTTTC	1332
	* * * * *	
AcPHY1	GTCATTCTAATTGTTAACACTTGCTGCAGCAGGGACGTTTCCAACAATGTTGTGGGCGTT	2408
CrPHY1	GTCACTCCTAATAGTGAACACCTGCTGCAGCAGGGATGTCTCCAACACGTAAGTTGTTGTT	2295
AcPHY2	GTGATATTAATTGTGAATGCATGCTCGAGCAGGGATTTTACGGACAATGTGGTTGGAGTT	2440
CrPHY2	GTGATTTTGATTGTGAATGCATGCTCCAGTAGGGATTTTACAGACAAATGTTGTTGGGGTA	2345
CrPHY4B	GTTATCCTGGTTGTTAATACATGTTCAAGCAGGGATTTTACAGAGAATGTTGT-GGAGTC	1783
CrPHY4A	CTACTTCTAATTTAAAAGAGATAGAAGGCTAGGGGAAAAAAAAAAAAAAAAAAAAA-----	1759
AcPHY4	AATGTGTTGATGGTTGATGTGTGT---GGTGTGTGTGTGAGAGAGAGAGAGA--GAGAGA	1387
	* * * * *	
AcPHY1	TGTTTCGTGGGCGAGGATGTGACGGGTCAAAAGTTGGTGCTGGACAGGTTTATACGCATC	2468
CrPHY1	TGCTTTGTTGGACAGGATGTAACAGGTCAAAAGTTGGTATTAGATAGGTATATACGCATG	2355
AcPHY2	TGTTTCGTTGGTCAGGATGTAACAGGTCAAAAGGTGGTGATGGACAAATTCACTCGTATT	2500
CrPHY2	TGCTTTGTTGGTCAAGATGTGACAGGCGAGAAGGTGGTGATGGACAAATTTACACGCATT	2405
CrPHY4B	TGCTTTGTTGGTCAGGATGTTACAAGACAAAAAGTGGTTATGGATAAATTTACACGCATT	1843
CrPHY4A	-----	
AcPHY4	GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGCTGAATGTTGTGGGCCCATCCTTGTC	1447
AcPHY1	CAAGGGGACTATAAAGCAATTGTGCAGAGTCTGAATCCGCTTATTCCCCCATTTTTTGGT	2528
CrPHY1	CAAGGAGACTATAAGGCAATTGTTTCAGAGCCCGAATCCGCTCATCCCTCCAATCTTTGGT	2415
AcPHY2	CAGGGTGATTACAAGACTATAGTACAGAACCCAAATCCCTTGATACCACCTATTTTTTGGT	2560
CrPHY2	CAGGGTGATTATAAGGCTATAGTACAGAACCCAAATCCCTTGATACCACCAATTTTTTGGT	2465
CrPHY4B	CAAGGTGACTACAAGGCCATTGTACAGAATCCAAACCCCTGATACCGCCAATCTTTGGT	1903
CrPHY4A	-----	
AcPHY4	TGCTCTGAATTTCTGCCGGTGCCTGAAATCCC--CTCGCTTGCTAGCTCTGTTCCTCTGC	1505
AcPHY1	GCAGATGAATATGGGTTTTGCTCAGAGTGGAATGCGGCCATGGAAGTTATCCAA--TTG	2587
CrPHY1	GCTGATGAGTATGGATTTTGTTCAGAGTGGAATTCAGCTATGGAAAAATTATTCAGCTG	2475
AcPHY2	GCAGATGAGTTTGGTTATTGTTCCGAGTGGAACCCAGCAATGGAAAAATTTT-CAGGCTG	2619
CrPHY2	GCTGATGAATTTGGATATTGTTCTGAGTGGAATCCAGCAATGGAGAAATTAT-CGGGCTG	2524
CrPHY4B	GCTGATGAGTTTGGGTACTG-TCGGAGTGGAACCTGCCATGGAGGAGCTTT-CAGGCTG	1961
CrPHY4A	-----	
AcPHY4	CCTGCAGCCCTCTTATATCCCCCT--TCCCATTGCTTTAATGCCATAGCCTT--ATATTG	1561
AcPHY1	GAGAAGGGAGGAGGCTCTGGGGAAATGCTCGTGGGTGAGATATTTGGATTGCAAAATGGT	2647
CrPHY1	GAAAAGGGAAGAAGTCATAGGAAAGA-GCTAATTGGAGAGATATTTGGGCTTGAAATGGT	2534
AcPHY2	GAAGCGTGAGGATGTGATCGGTAAATGCTAATAGGGGAAGTATTTGGCTCTGATTTGGC	2679
CrPHY2	GAAGCGTGAAGATGTTGTTGGTAAATGCTTGTAGGGGAAGTCTTTGGTTCCGAGTTAGC	2584
CrPHY4B	GAAAAGGGATGATGTTATAGGACGAATGATGATTGGAGAGGTGTTTGAACGGATCTGAC	2021
CrPHY4A	-----	
AcPHY4	CTCCGTGTAGCACATTGCCCTCGCAGATCATAGCAGCCTTACATTACGCCGCTGCAACC	1621

Figure 2 continued

AcPHY1	TTGCTGCCGCTTGCAGGGGCAAGATGTCGTTACCAAATTGATGA----TAGTGCTGAATG	2703
CrPHY1	CTGTTGCCGCTCTGGACGGGCAAGACGTAGTCACAAAGTTGATGA----TAATTCTCAACG	2590
AcPHY2	ATGCTGCAAGTTGAGAGGTCAGGACTCAATGACCAAGTTCATGA----TAATTTTGAATG	2735
CrPHY2	CTGCTGTAAGTTGAGAGGTCAGGACTCCATCACCAAGTTCATGA----TAATTCTTAACT	2640
CrPHY4B	GTGTTGTAGGCTACGGAATCAGGATGATTTGACAAAGTTTATGA----TAGTCTTAAATA	2077
CrPHY4A	-----	
AcPHY4	CCGTAGCCG-TCATTCTTTCAGCACTCAGCCGCCACCTTTTATAGCCCTGACCACCAGCA	1680
AcPHY1	ATGCTGTCAATGGCCA--AGAATCGGAAAAATTTCTCTAGTGTTTTATGACCGCAATGG	2761
CrPHY1	ATGCCGTGAATGGGCA--TGATTCTGAACAGTACCCCTCTAGTATTCTATGACCGCATGGG	2648
AcPHY2	CTGCAATGGGGGGACG--AGACTCTGATAGGTTCCCTTTTCGGTTTCTTTGACCGATATGG	2793
CrPHY2	CAGCGATGGGTGGACA--GGATTCTGAGAGATTTCCCTTTGGTTTCTTTGATCGCCATGG	2698
CrPHY4B	CAGCAATGGGTGGCAG--TGACTCAGACAAGTTTCCTTTTGGGTTTTCGACCGAAATGG	2135
CrPHY4A	-----	
AcPHY4	CTCCCATCGCTTGCAACCTTATCCGCTTGGGCACACCTTTCGGCTTTCT--CATAATGC	1737
AcPHY1	GCGCCGTGTCGAAGCTCTACTCATCGCAAGCAAGAGAAGTATGCTGACGGGCGAATCAC	2821
CrPHY1	ACATCGTATTGAAGCCCTACTAATAGCAAGCAAAAGGACTGATGCAGATGGGCACATTAC	2708
AcPHY2	TAAATATGCAGAGGCGCTTCTGATCGCCAATAAGAGAAGTATAGTATGGAGCAATCAC	2853
CrPHY2	TAAATACATGGAGGCACTTTTGATTGCAAAATAAGAGATCTGATAGTATGGAGCTATCAC	2758
CrPHY4B	CAGATATGTGGAAGCGATATTGATAGCTCACAAGAGAACAGATGGTATGGGGCAGTGAC	2195
CrPHY4A	-----	
AcPHY4	ACAACCTT-CAGTGATGTTACTG--CATTATAGGGAGAATCTTCACCAGTGGTGGTGTCTT	1794
AcPHY1	CGGTGTCTTCTGCTTTTCTTACACTGCAAGCCCCGAGCTGCTTCAGGCGTTGATCATAAA	2881
CrPHY1	AGGGGTGTTCTGTTTCTTCTTACACTGCAGGTTCTGAGCTCCTTCAGGCTATTGTCTAAAG	2768
AcPHY2	TGGCGTGTCTGTTTCTTCTTACACAGCGAGTCCGGAGTTGCAGCAAGCTTTGCAAGTTCA	2913
CrPHY2	AGGAGTTTTTTGTTTTCTGCATACAGCGTGTCCGGAGTTGCAGCAAGCCTTGCAAGTTCA	2818
CrPHY4B	AGGAGTCTTTTGTGTTTTTACGAACCACCACTCCAGAGCTGCAACAAGCGTTGCAGGTTCA	2255
CrPHY4A	-----	
AcPHY4	CTATGTGTCGGTGGTCATAAGCCTTTTAGGTTCTAGACTCCTGTAAGC--TGCTCTATTG	1852
AcPHY1	GAGAGCCAAGGAAAAGGTTGAC-----AAAGAGTTGAGTTATGTGAAGGAGGA	2929
CrPHY1	GAAGGCAAAAGAAAAGTTGAA-----AAGGAGCTGAGCTATGTGAGGACTGA	2816
AcPHY2	GAAGCGATCTGCAAGGACTGCCCTAGATAGACTAAAAGAGGTTGCTTACATGAAACAAGA	2973
CrPHY2	GAAACGATCTGCAAGGACTGCCCTTGATAGATTGAAAGAGGTAGCGTACATGAAGCAGGA	2878
CrPHY4B	GAAGCAGTCAGCAAGGATTGCTGTAGAAAGGCTGAAAGAGGAAGCTTATATGAAGCAAGA	2315
CrPHY4A	-----	
AcPHY4	GTGGAAAATCCCATTTGGTTCAC-----CAACTTGTA-GCACTCAAA	1892
AcPHY1	GTTGAAGAAGCCTTTGGAAGGACTTGCAATTCACACGGACGGTGCTAGAAGGTACAAATCT	2989
CrPHY1	AGCGAGAAAGTCATTGGATGGCCTTACATTCACTCGGATGGTGATGGAGGGTACAGATTT	2876
AcPHY2	GATTAGGAATCCTTTGTATGGGATCGTCTTCAAGGAAGCTGCTGGATAATACAAATCT	3033
CrPHY2	GATAAAAAATCCTTTGTATGGGATAATCTTCAAGGAAATTAAGTAGATAACACAAATCT	2938
CrPHY4B	GATCAGAAATCCTTTGTATGGTATTGTTTTTACACGGAAGCTGTTGCAGAGCATGGATCT	2375
CrPHY4A	-----	
AcPHY4	TGTTACTCATTTTCTCTGTGGTGGTGTCTTTACTGTGTGCGGTGGTCATGAGCCTTTTAGG	1952
AcPHY1	AACTATAGAGCAG-AGGCAGCTCATCAAGACAAATGCTTGGTGCGAAAGGCAGCTGCGAA	3048
CrPHY1	GTCTGCAGAACAG-AGGCAGCTGATAAAGACAAATGCTTTGTGCGAAAAACAGCTGCGAA	2935
AcPHY2	TACAGATGAGCAG-AAGCAAATATGGAGACCAGTTCTTTGTGTGAAAAACAGCTGCAAA	3092
CrPHY2	TACCGATGAGCAA-AGACAAATAATGGAGACAAGTGCACTTTGTGAGAAGCAGTTGCAAA	2997
CrPHY4B	TTCCGATCAGCAG-AAGCAGATCTTGGAGACAAGTGCGCCTTGTGAGAGACAGCTACCAA	2434
CrPHY4A	-----	
AcPHY4	TTCTAGACTCTGGTAAGCTGCTC-TATTGGTGGG-ACGTACGTGCACATCTAACCTTAT	2010

Figure 2 continued

AcPHY1	AGATATTGGAAGATGAC---CTGAACAATATAGAAGAAGGGTACATGGATTGGAGATGT	3105
CrPHY1	AGATACTGGATGATGAA---TTGAACAATATTGAAGAGGGGTACATGGAGCTAGATATGA	2992
AcPHY2	ATATCCTGGATGAGGACAACCTCGAAAAGCTTGATCAAGGAAATGTGGATTGGATACGT	3152
CrPHY2	ACATTTTGGAGAGGACAGCTTTGAGAAGCTTGATCAGGGGAATGTGGAATTGGATACAC	3057
CrPHY4B	AAATATTGGATGAGGACAATTTTGAGAACTTGATCAAGGAACATTAGAGCTCGACACAC	2494
CrPHY4A	-----	
AcPHY4	GTGTACGTGGACAAGCTA--CTTCATGGCATGGGTTCAAGTTGAGTTAGATTAGGAAGTGA	2068
AcPHY1	CCGAATTTTTCATGGGATCAGTTATTGATGCTGTGATAAGTCAAGGAATGGCTGCCTCAA	3165
CrPHY1	TGCAGTTTAAATGGAACAGTTATGGATGTTGTTATCAGTCAAGGAATGGTTCAGCCC	3052
AcPHY2	TAGAGTTCAACCATGGGCACAGTGATGGATGCTGTGATCAGCCAAGGCATGATCAGATCAA	3212
CrPHY2	TGGGGTTTACCATGGGTAGAGTGATGGATGCTGTGATTAGTCAAGGCATGATAAGGTCAA	3117
CrPHY4B	TTGAATTTTCAATGGGCGCAGTCATGGATGTCAGTGGTAAAGTCAAGGGATGATCAGATCAA	2554
CrPHY4A	-----	
AcPHY4	TCTGGCACTTTTGTGAGTCTGTT-TGCAGACCGTG-CATGTCCAGACAC--TTAAAGTAG	2124
AcPHY1	GAGGGAAGGGAGTGCAGATTCTAACTGAGATTCCAAATGATGTGAAGTTGATGTGTTTGT	3225
CrPHY1	GTTTGAAGGAAGTACAGATTCTTACTGAGGTTCCAGATGAGGTAAAGTACCTGTTCTTAT	3112
AcPHY2	GAGAGAAAGGACTACAACCTTATAAGAGAACTCATGTTGAGATTAACACACGCGCTTT	3272
CrPHY2	GGGAAAAGGGTCTACAATAATCAGAGAACTCATGCTGAGTTGAAGAATACCCGGCTCT	3177
CrPHY4B	GAGAGAAGGGCCTGCAATTCGTTCCGGAAACACCTGTGGACATAAAGAACACCCGTTTAT	2614
CrPHY4A	-----	
AcPHY4	ATGCATA--TCCTCTGGTTGCACATAGGATGATCATCAGAACCATTAGGAGCATTTTT	2181
AcPHY1	TTGGCGACCAAGGCACGGCTGCAGCAAGTGCTGGCAGATTTGTTGTTTGTGCCATAAATC	3285
CrPHY1	ACGGTGACCAAGTGCGATTGCAGCAAGCATTGGGTGATCTCTTGTCTTTACTATCAATT	3172
AcPHY2	TTGGAGATCAATACCGATTGCAGCAAGTGTTAGCTGACTTTCTCACCACAGCAATACGAT	3332
CrPHY2	TTGGAGATCAATACAGACTACAGCAGGTTTGGCGGATTTTCTCACAACAGCCATAAGGT	3237
CrPHY4B	TTGGTGATCAACTAAGACTTCAGCAAGTGCTGCTGATTTTCTTACCACGGTGTAAGAT	2674
CrPHY4A	-----	
AcPHY4	ATAGTGCATAGACCTATTTTCCTCA--TTTTAGCCAATTTTCAATTATTATTTTAAAGTG	2239
AcPHY1	ATGCAACAACGACGAATGAGGATGAGAAG--GATTGGGTCACAATCAAGGTGTCACGTAC	3343
CrPHY1	ACACAAATGCACCTCATGG---TCAGAGG--AAATGGGTGCAATCAAAGTATCTTATAT	3227
AcPHY2	TCACCTCTTCTTCAGATGG-----GTGGGTGGGCATCAAAGTTGTGCCGAC	3378
CrPHY2	TCACATCATCATCTGATGG-----TTGGGTGGGCATCAAAGTTGTGGCTAC	3283
CrPHY4B	TCACCGCGTCTCGGATGG-----TTGGGTGGGAATAAAAGTGGTGCCTAC	2720
CrPHY4A	-----	
AcPHY4	ACTTTTGTGGGAACTGGGTAGGGAAAACGACCAAGCAAGTATACAAGTAAACCTAGC	2299
AcPHY1	CAAAACCAGACTTGATGATGGTGATACATTTGATGCACTTCGAGTTTCGGATTTCAT--CATT	3402
CrPHY1	GAAGAATCAACTGGATGATGGAGTGATGTTGTTGAAGTTGAGTTTCGAATTTCAT--CATT	3286
AcPHY2	TATCAAGAACATGAAGGATGGACTGCATATAGTTTCATTTTGTGAGTTTCAGGGTATCC--CATC	3437
CrPHY2	AATAAAGAACATAAGAGATGGACTACATATTGCTCATTTTGTGAGTTTCAGGGTGTCA--CATC	3342
CrPHY4B	TGTGAAGCAAAATGGAGACTCACTTCAAGTTGTTTCATTTTGTGAGTTTCAGGATATCT--CATC	2779
CrPHY4A	-----	
AcPHY4	GCTAGCACTTTTGGGCAAAAGAGGCAGGAGACCGCCCATCAAGTGCACCTTCTCAGCAAG	2359
AcPHY1	CAGGGCAAGGGATTTCTGAAGCCTTGGTGGAGGAAATGACCAACAAG--TCGCAGAAATGG	3461
CrPHY1	CTGGAAATTCATTTCCAGAGACCCTTGTGAGTGAGATGTCAGATGAA--AAACGAGCATGG	3345
AcPHY2	CTGGTTCTGGAATACCAAGAAGACCTTGTCCAGCAGATGTATGACCGC--TCTCAAGAAATA	3496
CrPHY2	CAGGATCTGGGATACCAAGAAGCCCTTGTTCAGCAGATGTATGACCGC--TCTCAGGAAATC	3401
CrPHY4B	CAGGATGTGGAATCCCTGAGGATTTGGTGCAGCAGATGTCATGATCGC--TCCCCAGAAAT	2838
CrPHY4A	-----	
AcPHY4	AACAACAGGAGAACCCTAGCATTTTCAAGCAAGCAAAATCAGCCAGCGTTTTTGGAGCAG	2419
AcPHY1	ACACCAGAAGGTTTAGCCATATCAATAAGCTGCACGCTAATACGCCTCATGAATGGAGAT	3521
CrPHY1	ACACCTGAGGGTCTTGAATGTCTGTGAGCTCTGCTCTGGTACGCCTGATGAATGGATCT	3405
AcPHY2	ACACAAGAAGGAATGGGACTGAGTGTTTCAGGAAGCTTGTAAAGTTGATGAATGGTGAT	3556
CrPHY2	ACACAAGAAGGATGGGACTCAGTGTTTCCAGAAAGCTTGTAAATTAATGAATGGAGAT	3461
CrPHY4B	ACACAGGAAGGAATGGGTCTCAGTGCTCTCGTAAGCTTGTGAAATGATGAATGGAGAT	2898
CrPHY4A	-----	
AcPHY4	ATACCTAGCGTTTT---TTGCTGCACCTTTTGGATGCGGTCCTCAATGAGGATTGATG--	2472

Figure 2 continued

AcPHY1	GTCAATTAATACACCCACCGATGCCGGA---AACAAATGCTTTCTTGACTATCCAGTTCCTT	3578
CrPHY1	GTTCGAGTACACAAAGTGTATGATGAGGGTAACATGTCTTCATCGTTAAGATCGAGTTCCTT	3465
AcPHY2	GTAAATACACTCGTGAAGCTGGA---GTTTGCTATTTTCTAGTTACGGTGGAGCTTCCC	3613
CrPHY2	GTGAGTATCATTCGAGAACCTAGG---GTTTGCTACTTTCTAGTTACGGCGGATCTACCC	3518
CrPHY4B	GTGAGCTATGTGCGGGATGGTGA---TTGTGCTATTTTCTCGTGCATTTAGAGCTGCCT	2955
CrPHY4A	-----	
AcPHY4	GTCAGATGACCACAGGGAGACCAACGGAATCATTTTCCTAAAATTTAGGGAAGTCTAT-T	2531
AcPHY1	TTGGCTCACAGAGA-TGATGCTACTAGTGTAAAGTGCACAAGGCAGCTTTACATAGGGAAA	3637
CrPHY1	TTGGCTCA---AAA-TGATGTTTCCCCTATCTGACTTGAGAAAGATGTATTGCC---ACAT	351
AcPHY2	CTTGTTCAAGAAGA-TGACTGAT-GTTGAGAACATGA-AGATCTGCAGTGGTGAGAA-AT	3669
CrPHY2	CTT-TTCAAGAAGACTGATTCCTAGTTACTAACATCA-ATA--AATAATGGTGAGCTTAT	3574
CrPHY4B	CTTGTTCAAATAAGA-TGAAAGCAAACAGCCAAGTACCAAAGCTGCATGGTTCTCTGTAG	3014
CrPHY4A	-----	
AcPHY4	TTGGGCCACGAAATGTGTTTCGCGCTATTACCTCCGGTATGACTAGGGGAACACTTTACAA	2591
AcPHY1	TTTGGTGGAAGTGTTT-----CCAAATTTTGAGTAAATAAAATTTTCCTTATGTAT	3688
CrPHY1	CTTAAAAAATGCGG-----AAAAGCTGTAAAGAAATGAGGGGTGATCAACCTT	3568
AcPHY2	TCCAACCTAACAGTTTC-----AAACTGCTTCGGACTTCTTCAGAACCCCAAGAAC	3720
CrPHY2	TATCATTTAAAAA-----AAAAAAAAAAAAAAAAA-----	3608
CrPHY4B	CTTGAATAGGTTTTTGTGTTTCTGGCGCTGGCTGTGTTTGAAGAAATTAATGATAGAGAGT	3074
CrPHY4A	-----	
AcPHY4	CTCTGCCCGAAGTCAACT---GAGCGACCGGTCGGGAGATTTTAAAGCGGC--ATACC	2645
AcPHY1	CTAGGTTAACCCCACTATTTTAAAGATATGTGGGATTAGTATGTAGTACACCTTT-CACCT	3747
CrPHY1	CTGTATTGCAGTAGCTACTTATGGATA---GCCATCTCTATGTAATGGATTTTCATAGAA	3625
AcPHY2	ACATGCACACATGCTCTTGCCCTTCTC---CTTTTCATGAAGCGGTCAAGTCTAAGTTT	3777
CrPHY2	-----	
CrPHY4B	AAAAATCAGCAGGGGTTTTTTGAGCTCTAGAGGCAGGCTAATGGGTGAGGAGCTCAGCTTT	3134
CrPHY4A	-----	
AcPHY4	CGAAACGGGAAATGGTTTGTAAATGCGCCAAGTGGCACACTCTTGTGCACTTTCTCTCATG	2705
AcPHY1	TCACCTGCCCGTGCTCTTCTACACAGGCGACTTTTTGAAGCTGAATTTGGTGGAAGTGTGT	3807
CrPHY1	TAGTTTGATCAATGCAAAATCCTTCGGATATTACAAAGC--AATTTCCCAAAAAAAAAA	3683
AcPHY2	GGGTTCATATAAGTAACATTTTTTGTGAGTTCTAGGGCTACTTCTCTTAGTGATACAT	3837
CrPHY2	-----	
CrPHY4B	CTTTTGTAGTTGCATGTAGCTACCTCACGAACTTCCAAGCT--CATGCCCATCAGTATGA	3192
CrPHY4A	-----	
AcPHY4	CAAGAAGAATTGACGTTGAAT--TTCATGCTGGGAGAGGCCTCATTTGCAAACTGTCTCA	2763
AcPHY1	GCAATTTTGAACAAATAGATCTTCCCTTTTGTAGGGCAAACCCCACTATTTTCAGACTTG	3867
CrPHY1	AAAAAAAAAAAAAAAAA-----	3698
AcPHY2	TACTTCGTGTCCTTATTAGGGACACACAAATTTGCTTTTCTTACTCCCCTTAGCATTG	3897
CrPHY2	-----	
CrPHY4B	AGTGTGGGCCACCTAATGCAGATTTGTATACATGTACTTTGTGGCTTCCTTTGGATGGTG	3252
CrPHY4A	-----	
AcPHY4	CATGTTAAGAGCTATGGAGTGCTTCCTTTCCCTCTTATGTACAGTCTGGTGCCAGGAGTG	2823
AcPHY1	TTGGGAGTAGTATCTAGTACACCTTTACCTTTACCCACCCGGCAACCCGGCCATGTTTTT	3927
CrPHY1	-----	
AcPHY2	TTTGTGATGGAAGGTAGAAGAAGTTTGTGTGTGCCTAAGCGCTTCTTACGTTTTGACAGA	3957
CrPHY2	-----	
CrPHY4B	GGTGCTTACTTAACAGAAATGATTGTGTGTCTCTTGAAGGTGAAACCTTCATGATTTTTA	3312
CrPHY4A	-----	
AcPHY4	ATGCCCGGTACGCTATGCAGTGCTCTCTCTCTCTCTCCCTATGAGCAGTATGTTATG	2883

Figure 2 continued

AcPHY1	TACATAGGCGACTTTTTGCAGCTCAATG-CACATAACCAGAGGGCCATCTCATCCCCTGG	3986
CrPHY1	-----	
AcPHY2	AGAACCAACAATTGCCCTCAAAACCTC-AATCACTTCCAAGATTTCCTCTGTTTCAGGA	4016
CrPHY2	-----	
CrPHY4B	GCTTCCCATAACATCAGGTAAATAAGCA-TATAGGTAAACAGGGGTATTTCTAAACTAG	3371
CrPHY4A	-----	
AcPHY4	CCCCTCTAGTGGGCTTTTCATTGACCAATGTACAGTTATGTGGGTTTCATGCGCGATTGC	2943
AcPHY1	AAATGGA--GACTTTGTGCAAAGGCTCCTGGTATTGGAGCCCTTGGGACTCCATTACTT	4043
CrPHY1	-----	
AcPHY2	GGTAGAATTGAGTGATGGTTTCAGGATCGTGTGATTGAGAGGGGAGAGCTTACGAGCCT	4076
CrPHY2	-----	
CrPHY4B	CATCCTTTGGGTAGTGGATTTCTATGTAACCTCACTTATGGTTTACCCTTCCTCACAAT	3431
CrPHY4A	-----	
AcPHY4	CGAGGCAAGAGCCTCTTGCACAGGTATTGGATTAGCTAGTGGCTAATCCCCTGTAGGTAC	3003
AcPHY1	TTGTACTTTTCTGGTTTAATAATGGAGTAGTAGT-----	4077
CrPHY1	-----	
AcPHY2	GTAGTGGTATTGTATAAATTACGTAAAAATGTTTCTGTTCCCCTCAGTTGTAAGCTGTA	4136
CrPHY2	-----	
CrPHY4B	ACATCATCCATTCAATTTCGTTCCCAAAAAAAAAAAAAAAAAAAAAA-----	3473
CrPHY4A	-----	
AcPHY4	AGGTGAA-----	3010

Figure 2. Nucleotide sequence comparison of *PHY1*, *PHY2* and *PHY4* from *Adiantum capillus-veneris* (Ac) and *Ceratopteris richardii* (Cr) using CLUSTALW.

Only the complete mRNA for *AcPHY1* (AB016168) and *AcPHY2* (AB016231) are available. *AcPHY4* (AB003364) was isolated from genomic DNA and is incomplete. At position 1270 of *AcPHY4* is an intron splice site. All noncoding regions are highlighted. The four possible start codons in a single reading frame of *CrPHY2* are highlighted. A conserved splice site is still maintained in the cDNA.

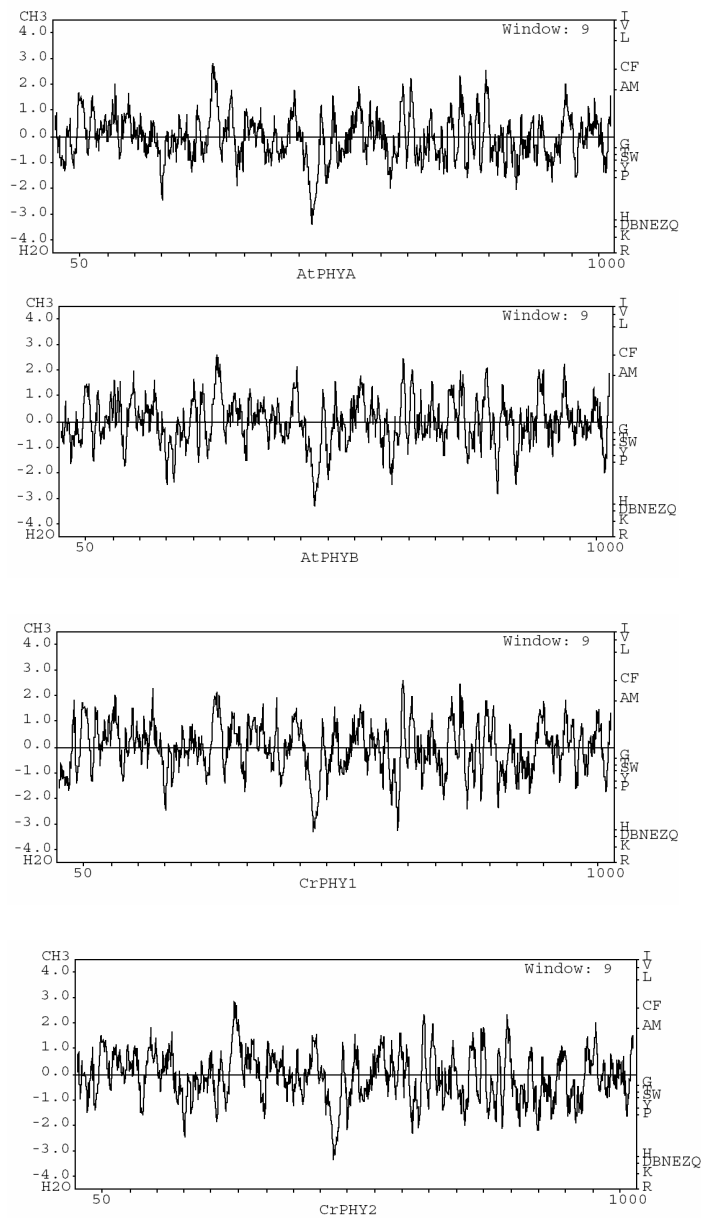


Figure 3. Comparison of the hydropathy plots of *Ceratopteris richardii* (Cr) PHY1 and PHY2 with those of *Arabidopsis thaliana* (At) PHYA and PHYB. *Ceratopteris* PHY1 and PHY2 do not show significantly different profiles from those of *Arabidopsis* phytochromes, suggesting that, like their angiosperm counterparts, the fern phytochromes are not integral membrane proteins.

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CrPHY4      AGAAGCCATGAACAGGTCCTTAGTTGGGGATTTAATCGTAGATGAGTCTGCAGAGGTTGT 1630
AcPHY2      GGAGGCCATGAGTAGGTCGCTGGTCAGAGATGTGGTGGTGAATGCATCCATGGAAACAGC 3481
AcPHY1      AACGGCGATGGGAAATCACTAGCCAAAGAGCTTGACACGAGGAGTCAAAACGATTGT 3832
AcPHY4      AGAAGCTATGAACAGGTCCTTGGTTGGGGACCTAGTCGTGGAGGATTCTGCAGAAATTGT 1244
          ** *** * ** * * ** * * * * *

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Conserved Splice site

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CrPHY4      GGAGCGGTTGCTGTCTTTAGCTCTTCAAGGTACTGGAGACATTGT----TTGTTGGATTC 1686
AcPHY2      TGAAAGAGTGTTAGATTGGCACTACAAGGTTATAGTCTGTTTCTAACTCTTTGAGCTC 3541
AcPHY1      GGAAAGAGTTCTCCATCTTGCAATTGGAAGGTTG----- 3865
AcPHY4      AGAACGACTTTTGTGTTTAGCCCTGCAAGGTGAATAT----- 1281
          ** * * * * * * * * * *

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STOP

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CrPHY4      TAGGTCTCTTGT-ATTTCCATCTACTTCTAATTAAAAAGAGATAGAA----GGCTAGGG 1741
AcPHY2      ATTGTTTAAAGTGATTGGAAATTGATGATCGCGAAATAGACCTGAACCTCCGGTTAGGT 3601
AcPHY1      ---GTTTGAAT-----T 3876
AcPHY4      ---ATTTGTCAG----- 1291
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Poly(A) tail

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CrPHY4      AAAAAAAAAAAAAAAAAA----- 1759
AcPHY2      TATTAGTAGCTTGAATTAATCCTGTCAAGGGTGCAAATTCGTAAGAAATACCTGTGCCTT 3661
AcPHY1      CATTGATGG---GTGTTTTTCTTTATGGGT--AACTTATGGTACG----- 3919
AcPHY4      -AGTTGTGCTCTTGTAGCTTCTATCACATGTTAATCTTTCAATGTGT-----T 1340
          *

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Figure 4: CLUSTALW nucleotide alignment of the 3' end of the mRNA of *CrPHY4* and the phytochrome genomic DNA sequences of *PHY1* (AB01651), *PHY2* (AB016231) and *PHY4* (AB003364) *Adiantum capillus-veneris*. The 3' end of mRNA of *CrPHY4* contains the conserved splice site found in cryptogams and higher plants, a stop codon and a poly (A) tail.

Figure 5

A

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CrPHY4Ac      TCTCTGTTGAAGAAGCCATGAACAGGTCCTTAGTTGGGGATTTAATCGTAGATGAGTCTG 1620
CrPHY4Ag      TCTCTGTTGAAGAAGCCATGAACAGGTCCTTAGTTGGGGATTTAATCGTAGATGAGTCTG 1558
*****

CrPHY4Ac      CAGAGGTTGTGGAGCGGTTGCTGTCTTTAGCTCTTCAAGGTACTGGAGACATTGTTTGTT 1680
CrPHY4Ag      CAGAGGTTGTGGAGCGGTTGCTGTCTTTAGCTCTTCAAGGTACTGGAGACATTGTTTGTT 1618
*****

CrPHY4Ac      GGATTCTTAGGTCTCTTGATTTCCATCTACTTCTAATTTAAAAGAGATAGAAGGCTAGG 1740
CrPHY4Ag      GGATTCTTAGGTCTCTTGATTTCCATCTACTTCTAATTTAAAAGAGATAAATC----- 1672
*****

CrPHY4Ac      GAAAAAAAAAAAAAAAAAAAA 1759
CrPHY4Ag      -----

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B

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CrPHY4Bg      GTTTATCAATGGATGGAATGTAAAGGTTGCAGAACTCACTGGACTCTCTGTTGAAGAAGC 780
CrPHY4Bc      GTTTATCAATGGATGGAATGTAAAGGTTGCAGAACTCACTGGACTCTCTGTTGAAGAAGC 780
*****

CrPHY4Bg      CATGAACAGGTCCTTAGTTGGGGATTTAATCGTAGATGAGTCTGCAGAGGTTGTGGAGCG 840
CrPHY4Bc      CATGAACAGGTCCTTAGTTGGGGATTTAATCGTAGATGAGTCTGCAGAGGTTGTGGAGCG 840
*****

CrPHY4Bg      GTTGCTGTCTTTAGCTCTTCAAGGGGTGTTGATACTTGCAACATTGGAAATGAAAGGCTA 900
CrPHY4Bc      GTTGCTGTCTTTAGCTCTTCAAGG----- 864
*****

CrPHY4Bg      CACAATGCAATTAACCTCATGATTCAGTCTGGAATATTGCGAAGAAATGCTGATTATCTTAG 960
CrPHY4Bc      -----

CrPHY4Bg      GTGACCATGCAATAATTTTGCGTCTAAGTTGGTGAAGAAAAACGAAATGTGGAAATAAAG 1020
CrPHY4Bc      -----TGAAGAAAAACGAAATGTGGAAATAAAG 892
*****

CrPHY4Bg      TTGAAAACACATGGCGAACAGGCTCAGAATGGTCCTGTTATCCTGGTTGTTAATACATGT 1080
CrPHY4Bc      TTGAAAACACATGGCGAACAGGCTCAGAATGGTCCTGTTATCCTGGTTGTTAATACATGT 952
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C

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CrPHY4Ag      AGTACTGTTGCCAATGAGATGGTCCGTTTGATTGAGACTGCTACTGCTCCTATATTTGCC 1440
CrPHY4Bg      AGTACTGTTGCCAATGAGATGGTCCGTTTGATTGAGACTGCTACTGCTCCTATATTTGCC 706
*****

CrPHY4Ag      GTAGATGCTGGAGGGTTTATCAATGGATGGAATGTAAAGGTTGCAGAACTCACTGGACTC 1500
CrPHY4Bg      GTAGATGCTGGAGGGTTTATCAATGGATGGAATGTAAAGGTTGCAGAACTCACTGGACTC 766
*****

CrPHY4Ag      TCTGTTGAAGAAGCCATGAACAGGTCCTTAGTTGGGGATTTAATCGTAGATGAGTCTGCA 1560
CrPHY4Bg      TCTGTTGAAGAAGCCATGAACAGGTCCTTAGTTGGGGATTTAATCGTAGATGAGTCTGCA 826
*****

CrPHY4Ag      GAGGTTGTGGAGCGGTTGCTGTCTTTAGCTCTTCAAGGTAC-TGGAGACATTGTTTGTTG 1619
CrPHY4Bg      GAGGTTGTGGAGCGGTTGCTGTCTTTAGCTCTTCAAGGGGTGTTGATACTTGCAACATTG 886
*****

CrPHY4Ag      GATTCTTAGGTCTCTTGATTTCCATCTACTTC-TAATTTAAAAGAGATAAATC----- 1672
CrPHY4Bg      GAAATGAAAGGCTAC-ACAATGCAATTAACCTCATGATTCAGTCTGGAATATTGCGAAGAAAT 945
**      * * * *      * * * *      * * * *      * * * *      * * * *

```

Figure 5. Comparison of *CrPHY4A* and *CrPHY4B* using CLUSTALW. (A) The 3' end of the cDNA of *CrPHY4A* (*CrPHY4Ac*) is identical to the genomic DNA (*CrPHY4Ag*). (B and C) Identification of the second locus of *PHY4* in *Ceratopteris richardii* (*CrPHY4B*) with a kinase domain beyond a short intron.

```

CrPHOT      ---IAEVAN-----AAVVPPLSDRRSSLGRSSLGTSKTD--QTS DGM MKRHRSSGFRS 48
AcPHOT2     KSTIAEVPS-----VATVPMTDRRS-----SLGPGKSD--SMSDGI PKRHRSSGFRS 431
AcPHOT1     VRPVAPSGPRKGPDLRELLTAQQYTPRSATSVQPKGSNLTEPGSHRESISKRNRS SGFFS 503
AcPHY3      ---VETPR-----LSSAMRFSLTGQTIESLPQPTAIP-REGGGRTRRPRSSSFLS 873
           .               .           :.   ..      :           . : * ** . * *

CrPHOT      FMGFDKFG--KSAQQEPIEFIEPEILMTRDEENTDGLDDFDDKERLQEIRRGIDLATTL 105
AcPHOT2     LIGLDKFG--KSAQQEPIEFIEPEILMTRDEE-TDSLDELDDKERLQEIRRGIDLATTL 487
AcPHOT1     FLGLDKLAGKPGNQHDAAEFIEPEILMTKDEDSSEASFELD-KARLKEIRRGIDLATTL 562
AcPHY3      LLGMEKEK----DIPEEDELQELEVIMLEDAS-VGRPGSLDDPERT--RRGIDLATTL 924
           ::*::*          .      :  * : * *::* . * ..      .:*.  * .. *****

CrPHOT      ERIEKNFVITDPRLPDNPIIFASDSFLELLEYTREEIIGRNCRFLQGPDTDQKTVQKIR 165
AcPHOT2     ERIEKNFVITDPRLPDNPIIFASDSFLELLEYTREEIIGRNCRFLQGQDQDQKTVQKIR 547
AcPHOT1     ERIEKNFVITDPRLPDNPIIFASDNFLELLEYTREEIIGRNCRFLQGPDTNRET VKLIRD 622
AcPHY3      ERIGKSFVITDPRLPDNPIIFASDRFLELLEYTREEVLGNCRFLQGRGTRDRKAVQLIRD 984
           ***  * .*****  ***** :***::*.*****  .*::*:  ***

CrPHOT      AIKEQREITVQLLNYTKSGKRFWNLFHLQPMRDQKGELQYFIGVQLDGSQ-----QIEPI 220
AcPHOT2     AIREQREITVQLLNYTKTKGRFWNLFHLQPMRDQKGELQYFIGVQLDGSQ-----QLEPI 602
AcPHOT1     AIDNEKEVTVQLLNYTKGRTFWNLFHLQPMRDHKGELQYFTGVQLDGE-----YLEPL 677
AcPHY3      AVKEQRDVTVQVLNYTKGGRAFWNLFHLQVMRDENGDVQYFIGVQQEMVAPRPVHQPP EL 1044
           * : ::::*:***.*****  * : *****  ***.:*::***  ***  :

CrPHOT      QKRLXEKTEKESAKIVRATAFNNGEGGCEESPRCKL----- 255
AcPHOT2     QKRLSEKTEKEGAKIVRATALNVEEAVGELPDANLTPDDLWANHSKSVSAKPH-KVHSDL 661
AcPHOT1     TKRLSQQIASEGAKI IRETAANVNEALRELPDANLKVEDLWRIHSRLVLPKPH-KLNHDS 736
AcPHY3      PDILPDRVEQEKAEVVRATAQRVDAAARELPDANLVPDHLFAPHSKVVTPLPKSKTNSSS 1104
           . * ::  . * *::*: * * . : .  * * . : *

CrPHOT      -KRLDDLX----- 263
AcPHOT2     WKALQKIRE---RGEKIGLKHFRPVKPLGFGDTGSVHLVELRGSGELFAIKAMEKSVMLN 718
AcPHOT1     WGVIRKIHA---SGEKVKLKHFRPLRPLGYGDTGSVHLVELRG TGKLFAMKAMEKNVMVK 793
AcPHY3      WFAIRRVQRRLRGERLGLKHFRPIKPLGSGDTGSVHLVELRG TGQVFALKAMDKSMMLQ 1164
           :  ::

```

Figure 6. Comparison of a phototropin-related fragment isolated from *Ceratopteris richardii* (CrPHOT) with *Adiantum capillus veneris* (Ac) PHOT1 (BAA95669), AcPHOT2 (BAD16729) and AcPHY3 (BAA36192) using CLUSTALW. The CrPHOT1 fragment has an 80% and a 58% identity to AcPHOT2 and AcPHOT1, respectively. The CrPHOT1 fragment has only a 45% identity to AcPHY3.

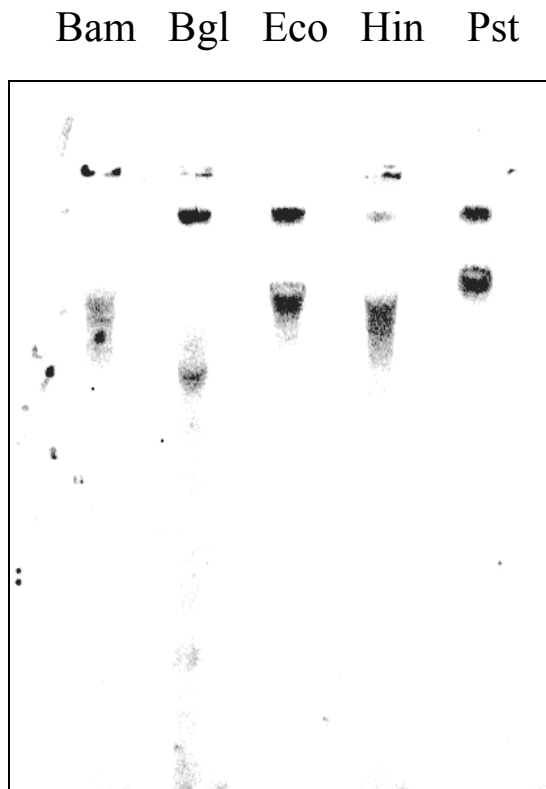


Figure 7. Southern hybridization analysis of *Ceratopteris* genomic DNA. Digested DNA was probed with a 2488 bp fragment (between positions 184 and 2672) of *CrPHY2* containing the chromophore-binding domain and the PAS-repeat domains. Based upon the cDNA sequences of *CrPHY1*, *CrPHY2* and *CrPHY4*, *Bam*HI cuts *CrPHY1* once at position 150 from the 5' end, but does not cut *CrPHY2* nor *CrPHY4*. *Bgl*I does not cut *CrPHY4* but cuts *CrPHY1* at position 1923 and *CrPHY2* at position 48 from the 5' ends. *Eco*RI does not cut *CrPHY2* and *CrPHY4* but cuts *CrPHY1* at position 2445. Unlike *Bam*HI, *Bgl*I and *Eco*RI, both *Hind*III and *Pst*I cut *CrPHY1*, *CrPHY2* and *CrPHY4* multiple times within the probing region. Thus, if there are only three copies of phytochrome sequences in the *Ceratopteris* genome, three bands should be seen in each of the *Bam*HI, *Bgl*I and the *Eco*RI digests.

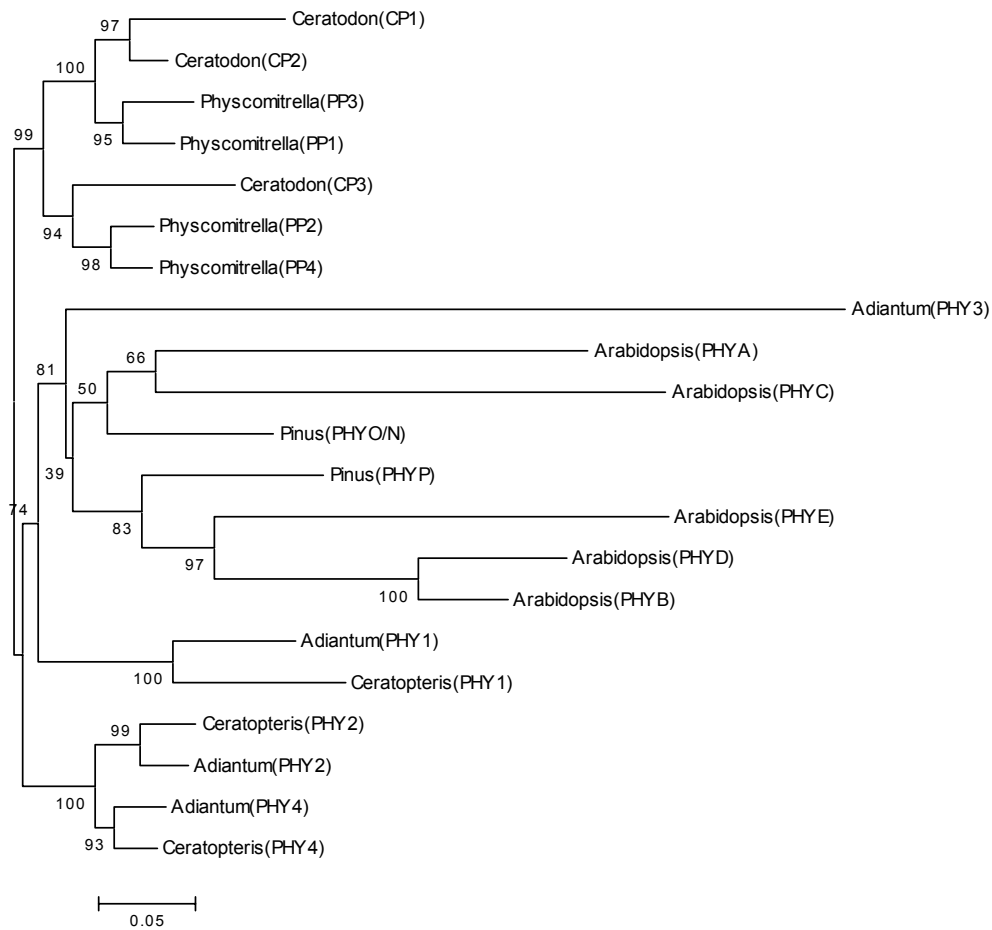


Figure 8. Phylogenetic analysis of phytochromes isolated from *Ceratopteris richardii* using the Neighbor Joining Method. Phytochromes sequences were aligned, indels were removed and 399 amino acids corresponding the chromophore-binding domain were selected using BIOEDIT. Trees were constructed using MEGA3 and were rooted using the phytochromes from bryophytes (*Physcomitrella* and *Ceratodon*). The sequences used were *Physcomitrella patens* PP1 (AAM94955), PP2 (AAM94954), PP3 (AAM94953), PP4 (AAM94952), *Ceratodon purpureus* CP1 (S27396), CP2 (Q39557), CP3 (AAM94956), *Adiantum capillus veneris* PHY1 (P42496), PHY2 (BAA33775), PHY3 (BAA36192), PHY4 (BAA24885), *Pinus sylvestris* PHYP (Q40762), PHY0/N (CAA65510) and *Arabidopsis thaliana* PHYA (P14712), PHYB (P14713), PHYC (P14714), PHYD (P42497), and PHYE (P42498). Values from 1000 bootstrap replicates are given next to branches.

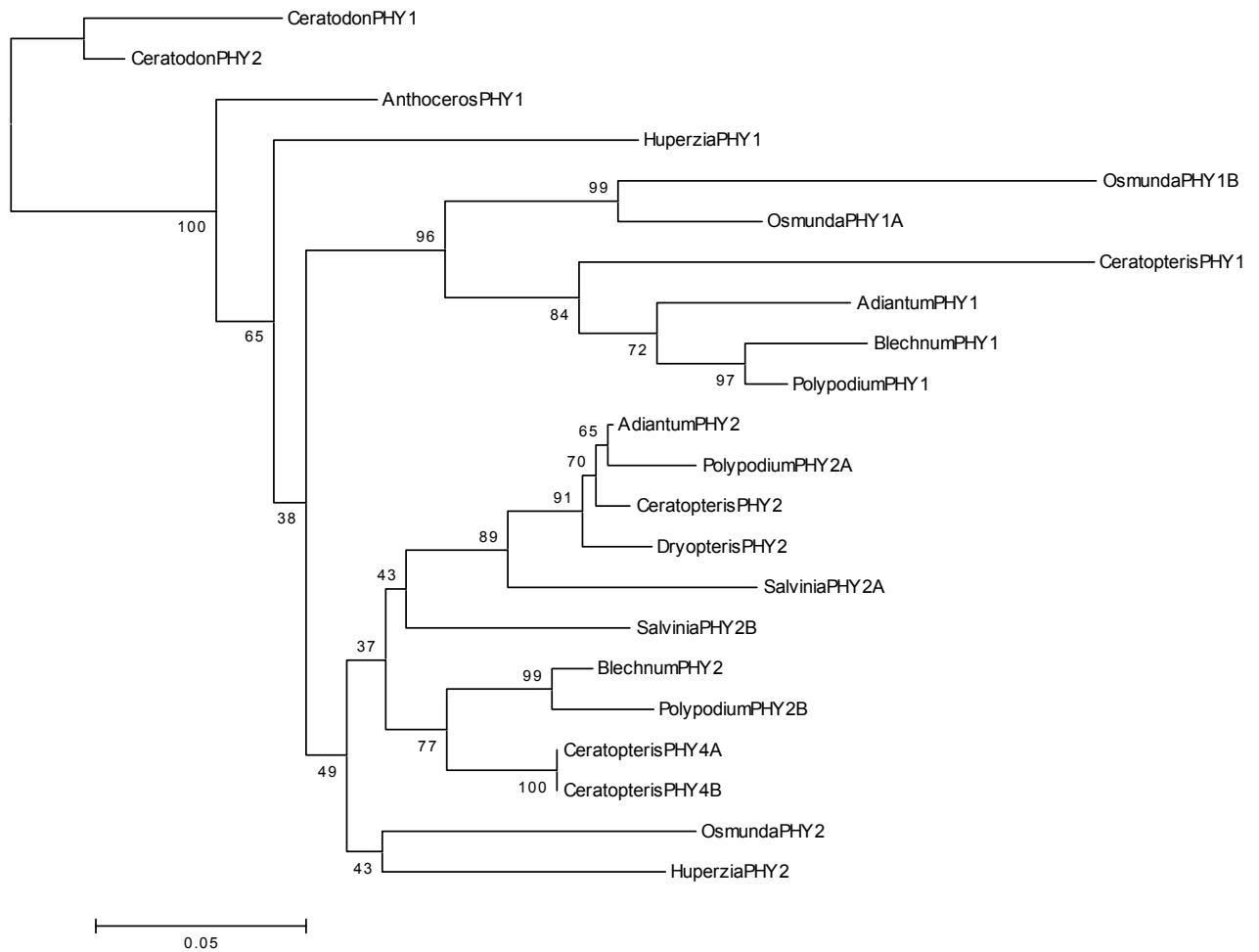


Figure 9. Phylogenetic analysis of cryptogam phytochromes using the Neighbor Joining Method. Phytochromes sequences were aligned and edited using BIOEDIT. Using MEGA3, trees were constructed using 182 amino acids of the GAF domain and rooted with the phytochromes from a bryophyte. The sequences used were *Ceratodon purpureus* CP1 (S27396) and CP2 (Q39557), *Anthoceros punctatus* PHY1 (CAC10618), *Huperzia selago* PHY1 (CAC10989) and PHY2 (CAC10990), *Osmunda regalis* PHY1A (CAC10999), PHY1B (CAC11000) and PHY2 (CAC11001), *Salvinia natans* PHY2A (CAA74126) and PHY2B (CAA74127), *Adiantum capillus veneris* PHY1 (P42496), and PHY2 (BAA33775), *Blechnum spicant* PHY1 (CAC10638) and PHY2 (CAC10637), *Polypodium vulgare* PHY1 (CAC11017), PHY2A (CAC11016) and PHY2B (CAC11015) and *Dryopteris-felix mas* PHY2 (AAB24768). Values from 1000 bootstrap replicated are given next to branches.

Figure 10**A.**

```

PcPHY1      CTAGCCAAAGAGCTGGTACATGAGGAGTCTAGGGCTACTGTTGAAAGAGTCCTGCATCTT 134
PmPHY1      CTAGCCAAAGAGCTCGTACATGAGGAGTCTAGGGCTACTGTTGAAAGAGTCCTGCATCTT 134
ApPHY1      CTAGCCAAAGAGCTTGTACATGAGGAGTCTAGGGCTATTGTTGAAAGAGTACTACTTCTT 134
AtPHY1      CTAGCCAAAGAGCTTGTACACGAGGAGTCAAAAACGATTGTGGAAAGAGTTCTCCATCTT 134
CrPHY1c     CTAGCAAAGGAACCTTGTACACAAGGATTCAGGGCTACTGTTGAAAGAGTATTGCAACTT 2160
CrPHY1Bg    CTAGCAAAGGAACCTTGTACACAAGGATTCAGGGCTACTGTTGAAAGAGTATTGCAACTT 322
CrPHY1Ag    CTAGCAAAGGAACCTTGTACACAAGGATTCAGGGCTACTGTTGAAAGAGTATTGCAACTT 93
            *****
            * * * * *

PcPHY1      GCATTAGAAGGTAGCTC-----TTAAAATCTACCTATCC 168
PmPHY1      GCATTAGAAGGTAGCTCCCGCATCTTGCAATTAGAAGGTAGCTCTTAAAATCTACCTATCC 194
ApPHY1      GCATTGGAAGGTGTGTTTGAATTTAT-----CAATGGGTGTTATTATTTTA--- 182
AtPHY1      GCATTGGAAGGTGGTTTGAATTCAT-----TGATGGGTGTTTTTCTTTATTG 185
CrPHY1c     GCTTTGGACGCT----- 2172
CrPHY1Bg    GCTTTGGACGTTGGTTTCAAGAACTTCG-----ACATGTTTTTTCGTTTCTG 370
CrPHY1Ag    GCTTTGGACGTTGGTTTCAAGAACTTCG-----ACATGTTTTTTCGTTTCTG 141
            ** * * * *

PcPHY1      CGATGATCTTTGCACCTTGTTTAAGGTTTTACTGTCATGAGTCTTTCTTTCCACAGGTGA 228
PmPHY1      CGATAATCTTTGCACCTTGTTTAAGGTTTTACTGTCATGGGCTTTCTTTCCACAGGTGA 254
ApPHY1      ---AAAATTGTGG---TCTTGACATTGCTAAT-TGATTGGTCTTCTCTGTGCACAGGTGA 234
AtPHY1      GGTAAACTTATGG---TACGCACAATTTGAATATATTTGGTCTTCTCTTGA--GGTGA 239
CrPHY1c     -----CA 2174
CrPHY1Bg    CTTCTATGCTTGT---TTTAACATTATTTTAGGACGTTCTTTCTGCTCT-TATAGGTCA 426
CrPHY1Ag    CTTCTATGCTTGT---TTTAACATTATTTTAGGACGTTCTTTCTGCTCT-TATAGGTCA 197
            *

PcPHY1      AGAAGAGCAAGACATAGAGATCCACCTGAGGACTTATGGCGAGCATAAGCAAAAGGGGGC 288
PmPHY1      AGAAGGGCAAGACATAGAGATCCACCTGAGGACTTATGGCGAGCATAAGCAAAAGGGGGC 314
ApPHY1      AGAAGAGCAAGATATAGAAATACACTTGAGAACTTACGACCAGCATAAGCAGAAAGGGGT 294
AtPHY1      AGAAGAACAAGATATAGAAATACACTTGAGAACTTATGACCAGCATAAGCAGAAAGGGGT 299
CrPHY1c     AGAAGAACAGGACATAGAGATACATTTGAGAACTTATAACAAGCAAAAGGAGAGAGGTGT 2234
CrPHY1Bg    AGAAGAACAGGACATAGAGATACATTTGAGAACTTATAACAAGCAAAAGGAGAGAGGTGT 486
CrPHY1Ag    AGAAGAACAGGACATAGAGATACATTTGAGAACTTATAACAAGCAAAAGGAGAGAGGTGT 257
            *****
            * * * * *

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B.

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CrPHY1c     CGGATGGTGATGGAGGGTACAGATTTGTCTGCAGAACAGAGGCAGCTGATAAAGACAAAT 2910
CrPHY1Bg    CGGATGGTGATGGAGGGTACAGATTTGTCTGCAGAACAGAGGCAGCTGATAAAGACAAAT 1162
CrPHY1Ag    CGGATGGTGATGGAGGGTACAGATTTGTCTGCAGAACAGAGGCAGCTGATAAAGACAAAT 933
            *****

CrPHY1c     GCTTTGTGCGAAAAACAGCTGCGAAAGATACTGGATGATGAATTGAACAATATTGAAGAG 2970
CrPHY1Bg    GCTTTGTGCGAAAAACAGCTGCGAAAGATACTGGATGATGAATTGAACAATATTGAAGAG 1222
CrPHY1Ag    GCTTTGTGCGAAAAACAGCTGCGAAAGATACTGGATGATGAATTGAACAATATTGAAGAG 993
            *****

CrPHY1c     GGGTACATG-----GAGCTAGATATGATGCAGTTTTTAATGGAAACAGTTATGGATGTTG 3025
CrPHY1Bg    GGGTACATG-----GAGCTAGATATGATGCAGTTTTTAATGGAAACAGTTATGGATGTTG 1277
CrPHY1Ag    GGGTGTGTGCTTGTGTCCCGATTTTTGTGTAATATGAAA-GGATCAAGTCAAGACTCTTA 1052
            *****
            * * * * *

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Figure 10. CLUSTALW alignment showing conserved intron position of fragments of *PHY1* and *PHY2* from the genomic DNA (g) of *Adiantum pedatum* (Ap), *Adiantum tenerum* (At), *Ceratopteris richardii* (Cr), *Ceratopteris thalictroides* (Ct), *Pteris cretica* and *Pteris multifida* (Pm). A The first intron position (of three) in the C-terminus is conserved in all the *PHY1* sequences examined, however the latter two introns are missing from *CrPHY1B*, which is identical to the cDNA (*CrPHY1c*) downstream of the first intron (B). *CrPHY1Ag* has all three introns. (C) *PHY2* has the same intron position as *PHY1*, however two fragments (*CtPHY2Bg* and *PcPHY2Bg*) are missing introns. *CtPHY2Ag* and *PcPHY2Ag* have introns. The sequences highlighted are introns however because of the limited sequences available for some, it is not possible to show the gaps in cases where no introns exist using CLUSTALW.

Domains (amino acids)	AcPHY1:AcPHY2 (p-value)	CrPHY1:CrPHY2 (p-value)	AcPHY1:CrPHY1 (p-value)	AcPHY2:CrPHY2 (p-value)
P1-P2 (207)	43:23 (0.014)	40:15 (.001)	8:18 (0.050)	2:7 (0.096)
P3 or GAF (173)	17:10 (0.178)	23:7 (0.003)	4:14 (0.018)	0:1 (0.317)
P4 or PHY (201)	18:4 (.003)	22:7 (0.005)	10:11 (0.827)	4:4 (1.000)
PRD (198)	25:21 (0.555)	26:14 (.058)	5:9 (.285)	1:5 (0.102)
HKRD (327)	67:40 (0.009)	70:39 (.003)	22:32 (0.174)	8:14 (0.201)
Full- Length (1102)	161:83 (0.000)	170:83 (0.000)	48:69 (0.052)	16:28 (0.070)

Table 1. Comparisons of unique amino acid differences between PHY1 and PHY2 of *Adiantum capillus veneris* (Ac) and *Ceratopteris richardii* (Cr). PHY1 and PHY2 were aligned with several phytochromes from bryophytes using BIOEDIT. Using the Tajima's relative rate test (MEGA3), full length or specific domains were selected and two sequences were compared to an outgroup (*Physcomitrella* or *Ceratodon*). The ratio of unique differences accumulated since the divergence of ferns from mosses is shown with the p-value for these differences. Regardless of the species or the bryophyte outgroup, PHY1 accumulated more unique amino acids than PHY2 and these differences can be found mostly in the P1-P2 domains and the HKRD. A *p-value* less than 5% is considered statistically significant.

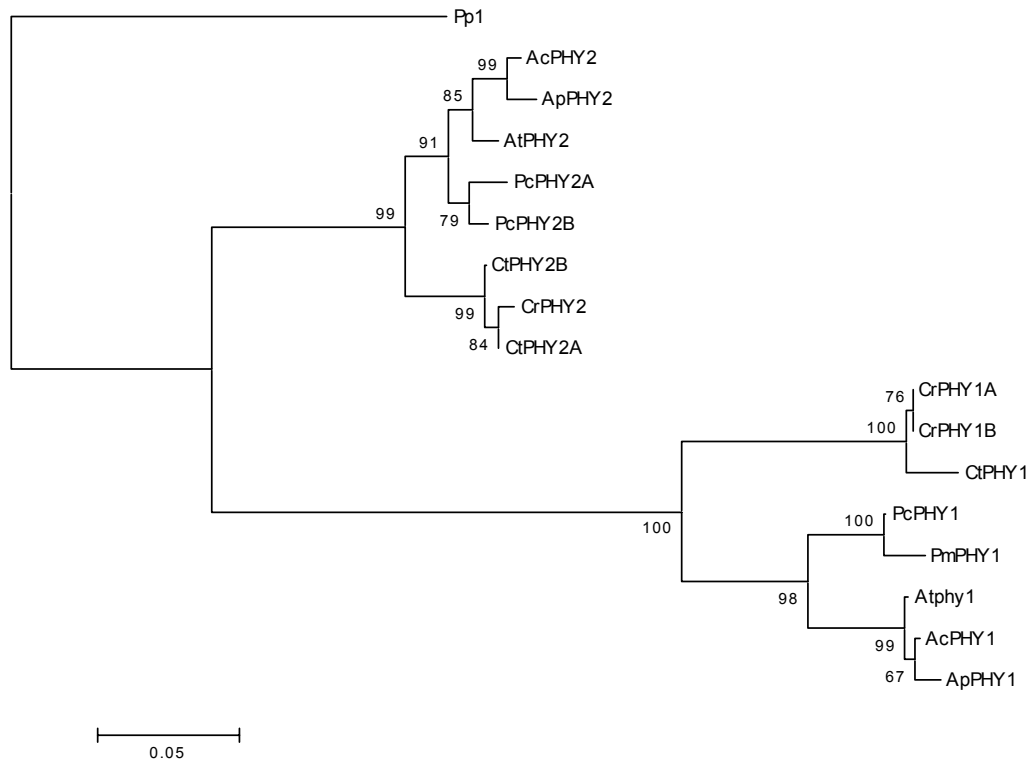
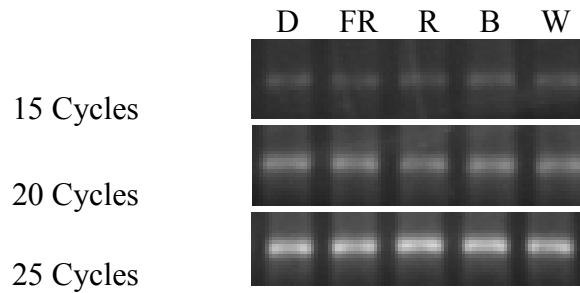


Figure 11. Phylogenetic analysis of the C-terminal end of *PHY1* and *PHY2* from *Adiantum capillus veneris* (Ac), *Adiantum pedatum* (Ap), *Adiantum tenerum* (At), *Ceratopteris richardii* (Cr), *Ceratopteris thalictroides* (Ct), *Pteris cretica* (Pc) and *Pteris multifida* (Pm) using the Neighbor Joining Method. The coding regions from various phytochrome-related fragments of the aforementioned species were aligned and edited using BIOEDIT. Using MEGA3, various trees were constructed and rooted with a phytochrome from *Physcomitrella patens* (Pp1). Regardless of the method used, the branch lengths are consistent with the relative rate test that show PHY1 is evolving at a greater rate than PHY2. In *Ceratopteris richardii*, a recent duplication has occurred, producing *CrPHY1A* and *CrPHY1B*. In *Ceratopteris thalictroides* and *Pteris cretica*, independent recent duplication events have produced *CtPHY2A*, *CtPHY2B*, *PcPHY2A* and *PcPHY2B*. Values from 1000 bootstrap replicates are given next to branches.

A.



B.

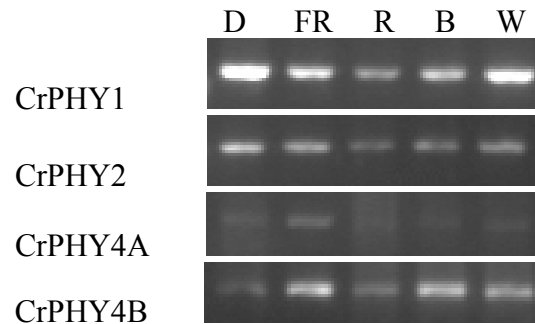


Figure 12. RT-PCR of RNA isolated from 7 day-old gametophytes grown in continuous white (W), red (R), far red (FR), blue (B) light or from gametophytes that were exposed to white light for 1 day then grown in the dark (D) for 6 days. A. Ubiquitin was amplified at 15, 20 and 25 cycles to determine that equal concentration of cDNA template was used and the amplified products was still within linear detected range. **B.** CrPHY1, CrPHY2, CrPHY4A and CrPHY4B were then amplified (25 cycles) from the same cDNA using the same conditions.

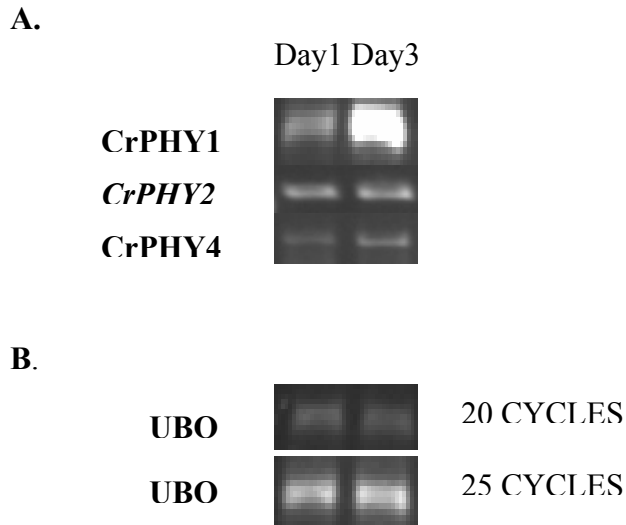


Figure 13. Semi-quantitative RT-PCR of *CrPHY1*, *CrPHY2* and *CrPHY4B* from spores that were dark imbibed in *C-fern* media for 1 or 3 days. Total RNA was extracted from spores, DNase-treated, reversed transcribed and amplified for 20 or 25 cycles with gene specific primers as described in the text. **A.** At 25 cycles, change in the relative level of phytochromes between 1 or 3 days can be seen for *CrPHY1*, *CrPHY2* and *CrPHY4B* but not for *CrPHY4A*, which cannot be detected under these conditions. The level of *CrPHY1* may represent combined mRNA expression from two loci (*CrPHY1A* and *CrPHY1B*) that are identical in coding sequences. **B.** Amplification of the ubiquitin (*UBQ*) control gene at 20 and 25 cycles shows the relative amount of template used and that amplifications are within the linear range.

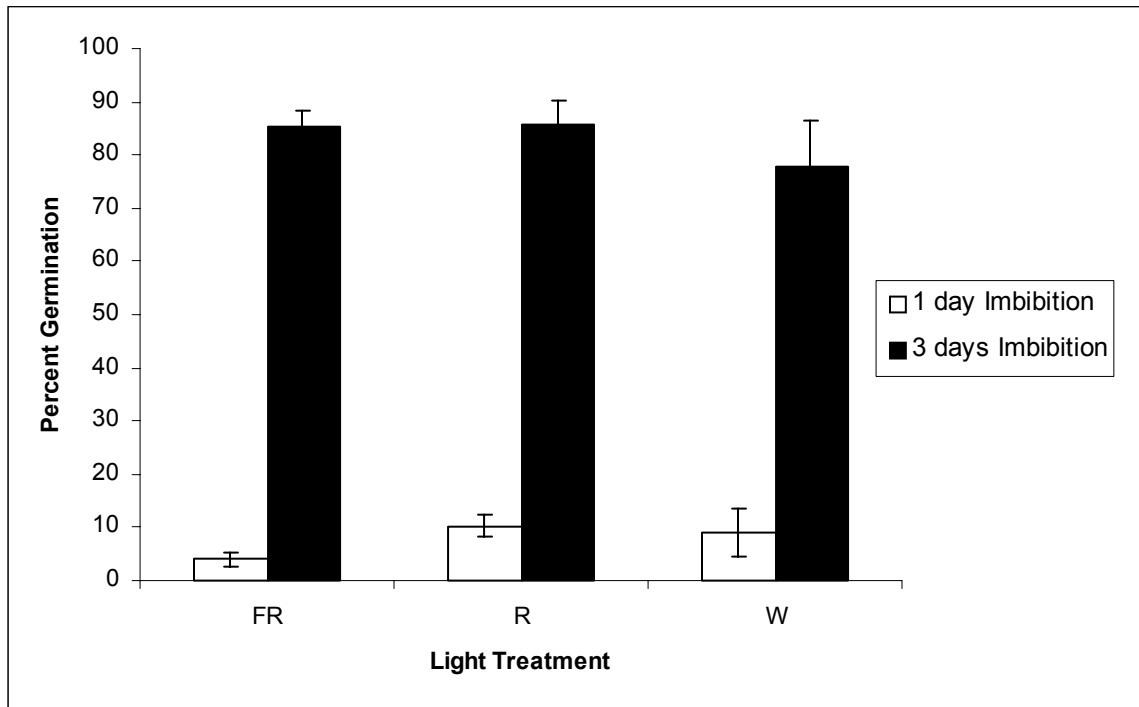


Figure 14. Percent germination of spores after dark imbibed for 1 or 3 days. Pre-sterilized *RNWI* dry spores were dark imbibed in liquid *C-fern* media, exposed to far-red (FR), red (R) or white (W) light for 1 day, then placed in the dark for 3 days. Germination was scored by the emergence of the rhizoid through the exine of the spore coat. Error bars represent +/- S.D. Experiments were repeated at least 3 times.

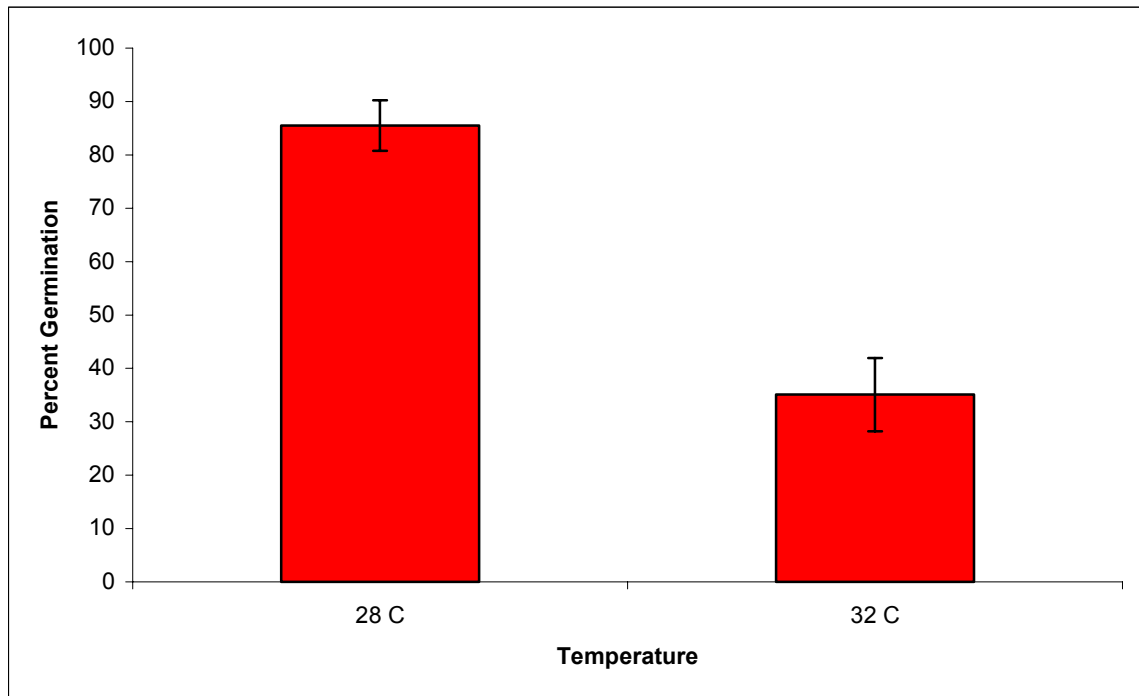


Figure 15. High temperature and high fluence red (R) light inhibit germination of spores in *Ceratopteris richardii*. Pre-sterilized RNW1 dry spores were imbibed in distilled water, plated onto *C-fern* agar media, incubated in the dark for 7 days, then exposed to continuous R for 3 days at 28°C or 32°C. Germination was measured by the emergence of the rhizoid through the exine of the spore coat. Error bars represent +/- S.D. Experiments were repeated twice and for each experiment, and in each experiment approximately 300 spores were plated on at least three plates, each treated as a separate population.

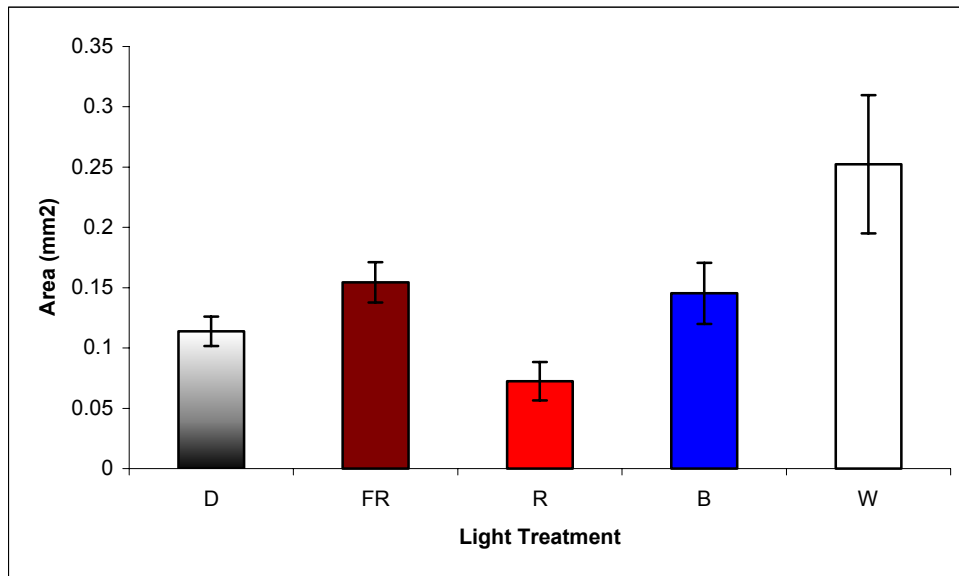
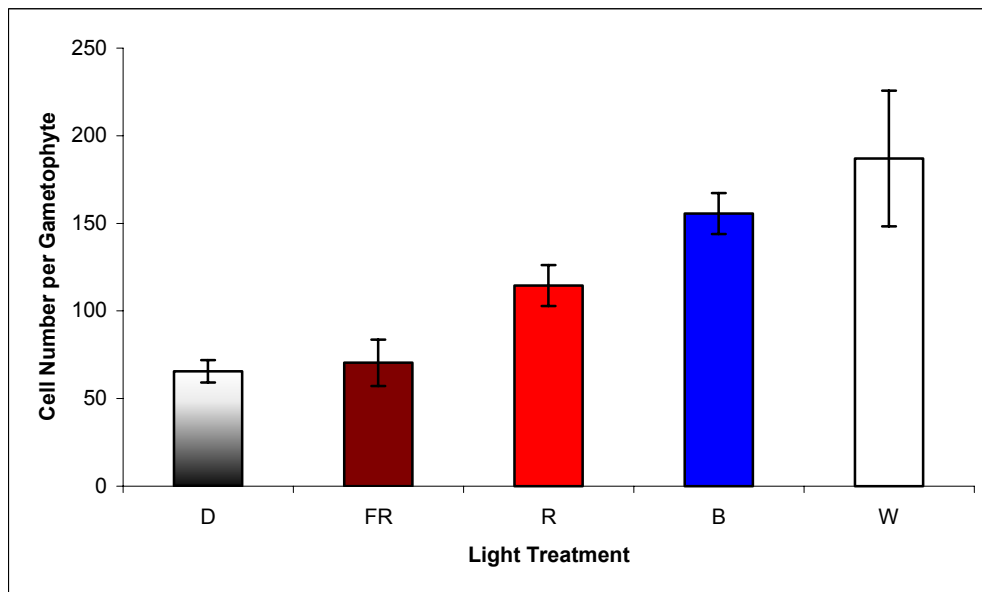
A.**B.**

Figure 16. Prothallus development in darkness (D) or under continuous far-red (FR), red (R), blue (B) or white (W) light. Dark imbibed spores were exposed to W for 1 day then placed in darkness or exposed to continuous light for 6 days. **A.** The area of 30 gametophytes was measured and **(B)** the number of cells per gametophyte were determined. Error bars represent $\pm$ S.D. Experiments were repeated three times.

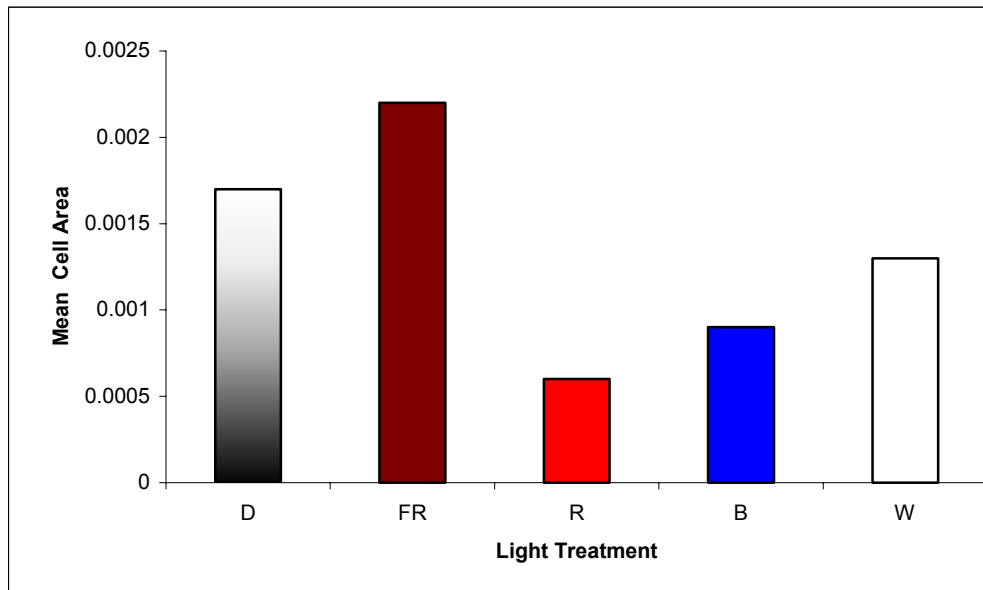
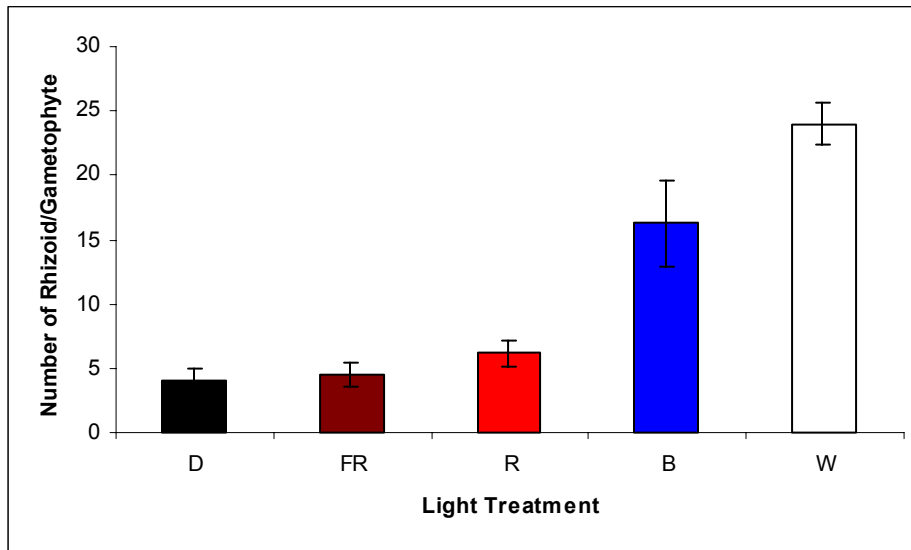


Figure 17. Mean cell size of prothalli grown under different light regimes. Using the average prothallus area and cell number from three experiments, the ratio of area to cell number was determined. Far-red light (FR) stimulates overall cell expansion and red light (R), blue light (B), or white light (W) inhibit cell expansion.

A.



B.

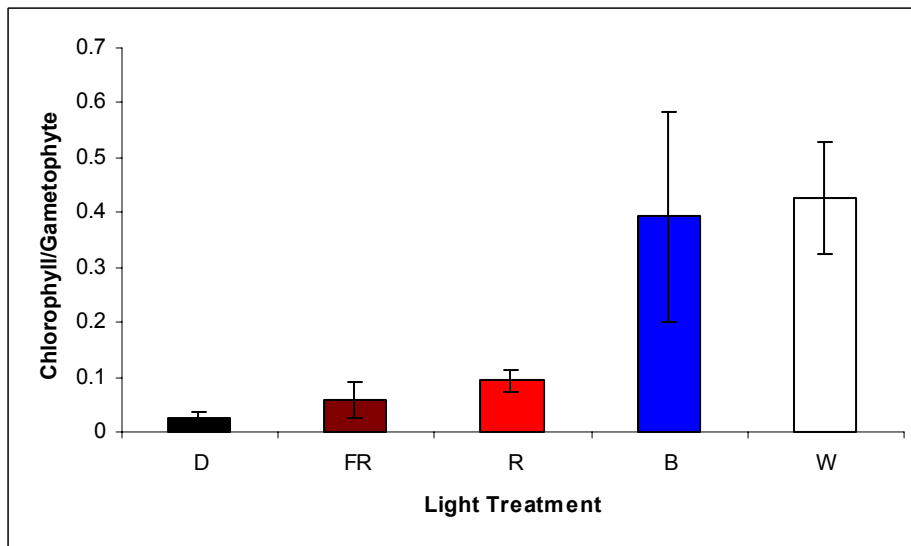


Figure 18. Number of rhizoids and the relative amount of chlorophyll accumulation in gametophytes grown in continuous far-red (FR), red (R), blue (B), or white (W), or in darkness (D). Dark imbibed spores were exposed to 1 day of W to induce germination, and then placed in darkness or in continuous light for 13 days to develop. The number of rhizoids (A) and the relative amount of chlorophyll (B) for 30 gametophytes was determined. Error bars represent $\pm$ S.D. Experiments were repeated 3 times.

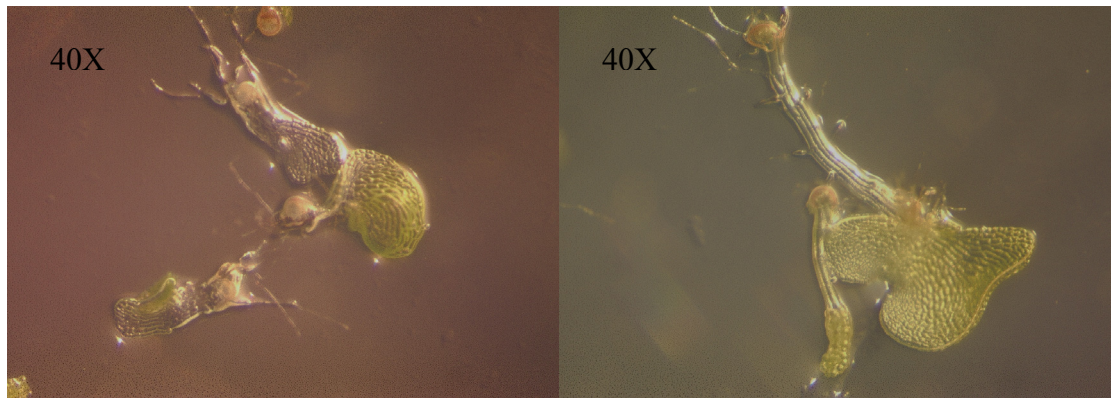


Figure 19. Development of gametophytes in white light (5 days) after 14 days of continuous exposure to R or FR. Most hermaphrodites that developed in R_c are unable to continue development when placed in subsequent W, in contrast to the FR_c -hermaphrodite that will continue to develop and produce sporophytes.

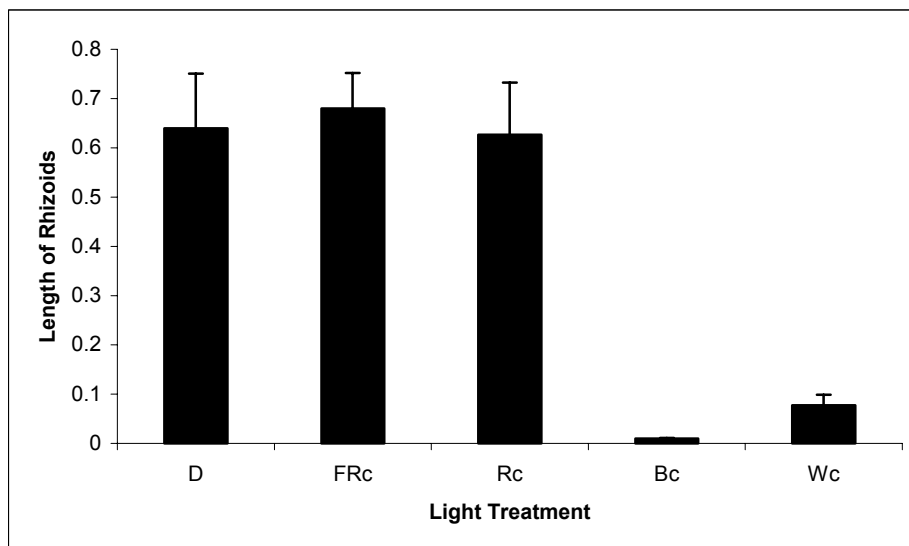
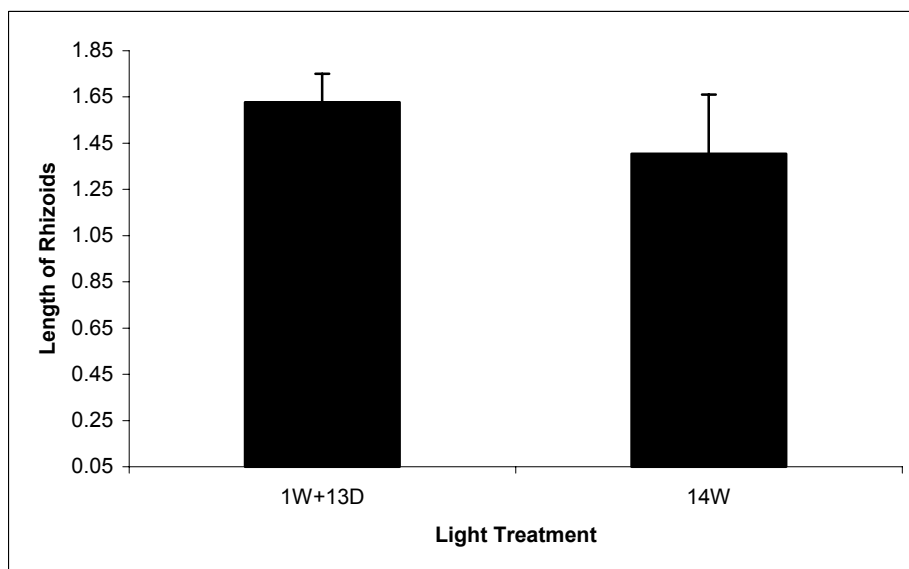
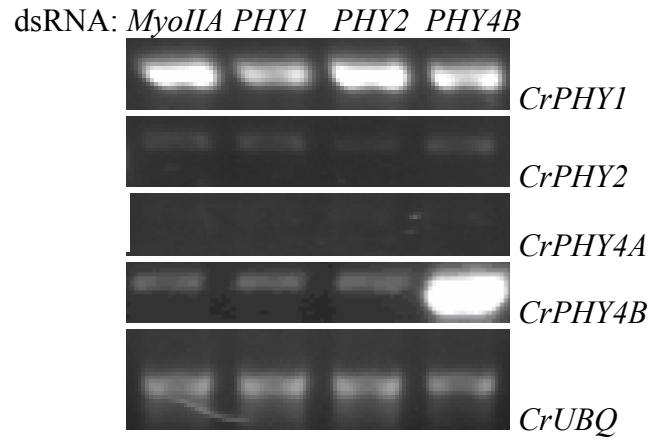
A.**B.**

Figure 20. The length of the primary rhizoids in darkness (D) or under continuous far-red (FR), red (R), blue (B) or white (W) light. **A.** Dark imbibed spores sown on agar medium were exposed to W for 1 day then placed in darkness or exposed to light for 6 days. The length of the longest rhizoid was measured from each of 30 gametophytes. Error bars represent $\pm$ S.D. Experiments were repeated three times. **B.** Experiments were repeated as in A, but spores were plated on agar covered with a layer of liquid medium and allowed to develop for 14 days. Spores were allowed to develop in D or in W.

A.



B.

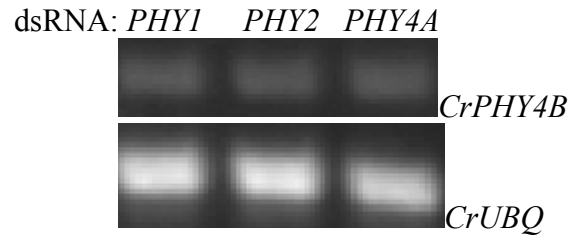


Figure 21. RNAi of *CrPHY* in spores. **A.** Pre-sterilized spores were incubated with double stranded (ds) RNA of chicken *Myosin IIA* (*MyoIIA*), *PHY1*, *PHY2*, or *PHY4B* in *Ceratomyxa*. After three days of incubation, total RNA was extracted from treated spores and knock down was assessed by semi-quantitative RT-PCR using gene-specific primers for *CrPHY1*, *CrPHY2*, *CrPHY4A*, *CrPHY4B* and ubiquitin (*CrUBQ*). *CrPHY4A* can only be detected at trace levels in spores. **B.** Spores incubated with dsRNA of *PHY4A* showed no change in the level of *CrPHY4B*.

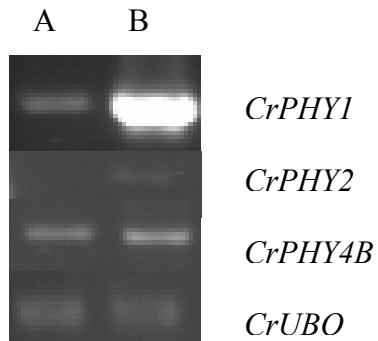


Figure 22. RNAi stimulates of the transcript accumulation of CrPHY1, CrPHY2 and CrPHY4B. Dry spores were incubated with dsRNA of *MyoIIA* (A) as a negative control or a mixture of dsRNA for *PHY1*, *PHY2* and *PHY4B* (B). After 48 hrs of incubation, total RNA was extracted from spores and the level of *CrPHY1*, *CrPHY2*, *CrPHY4B* and ubiquitin (*CrUBQ*) message was assessed by semi-quantitative RT-PCR as described in the text.

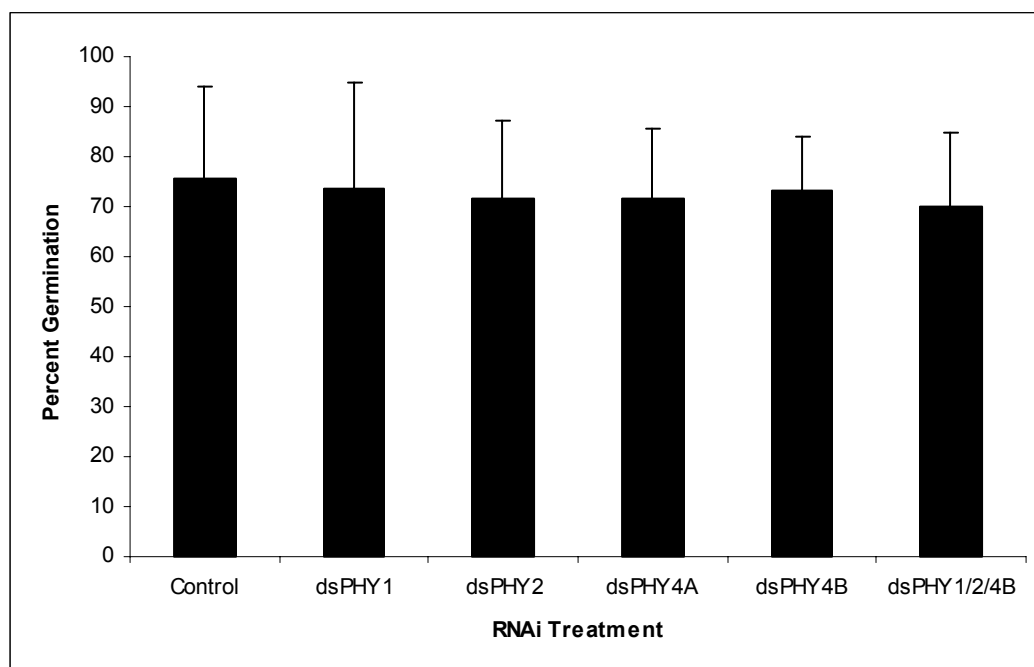
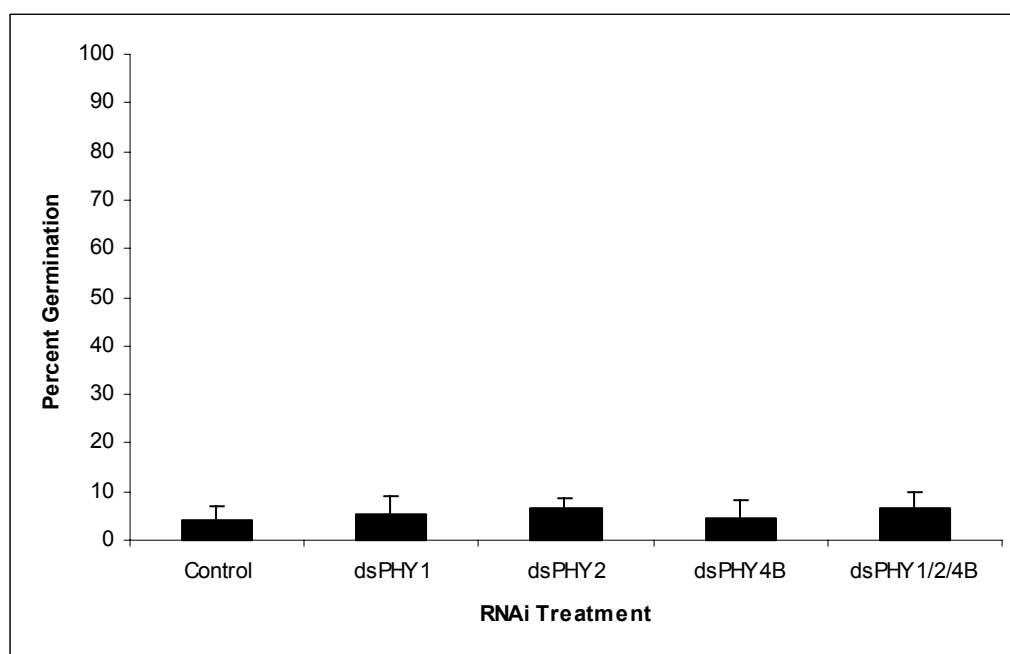
A.**B.**

Figure 23. The effect of dsRNA on the germination of spores in W_c or B_c. Dry spores were incubated with homologous dsRNA of *PHY1*, *PHY2*, *PHY4A* or *PHY4B* or a mixture of dsRNA for 2 days in the dark, removed from the RNAi media, plated on agar media then exposed to continuous W (**A**) or B (**B**) light. No change in germination can be detected between the untreated controls and the RNAi-treated spores.

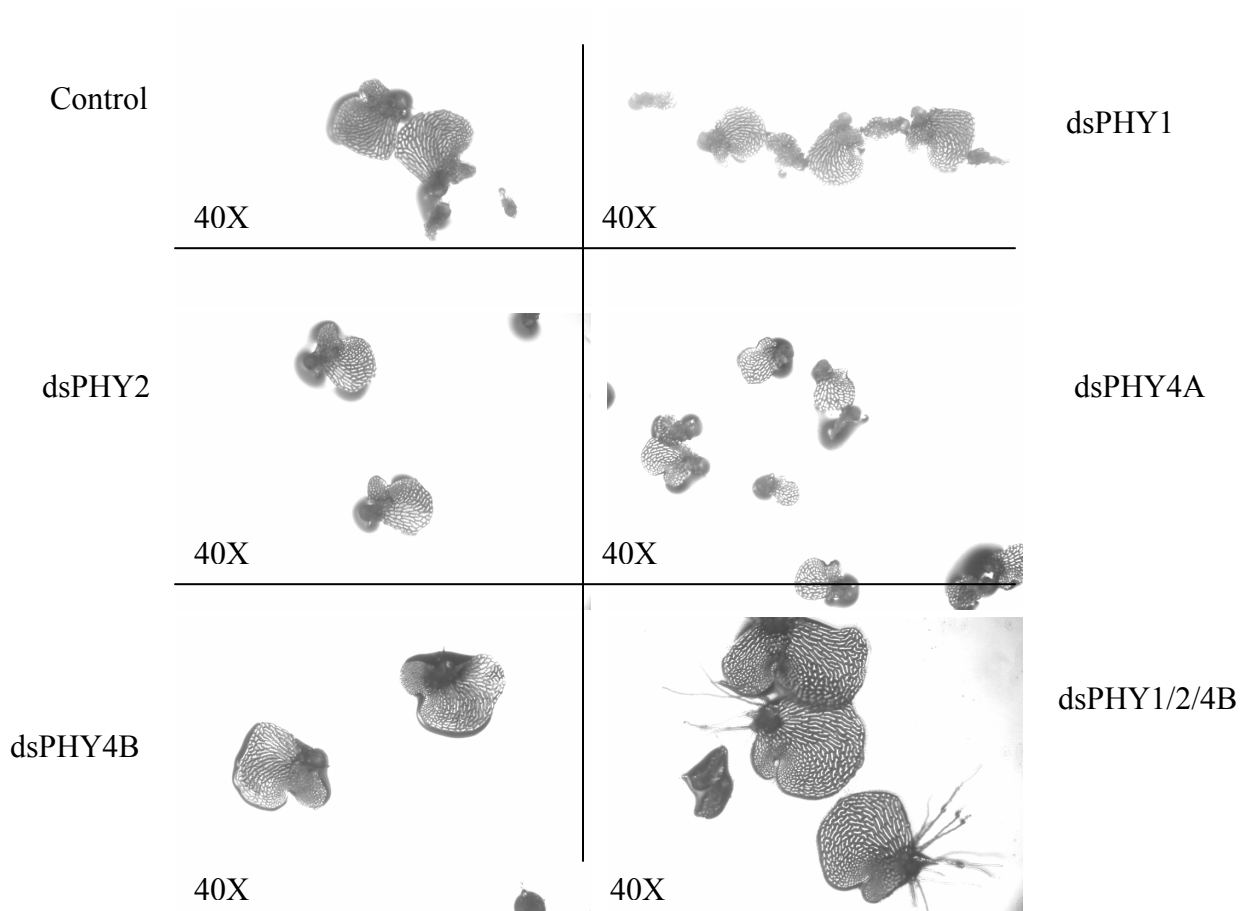


Figure 24. Development of the prothalli from spores treated with dsRNA in continuous white light. Dry spores were incubated for 48 h with dsRNA for *PHY1*, *PHY2*, *PHY4A*, *PHY4B* or a mixture of *PHY1*, *PHY2*, *PHY4A* and *PHY4B* in the dark, and then exposed to W for 24 h. Spores were removed from RNAi media, plated on agar media and allowed to develop in W_c (A) 7 d. The controls were spores that were incubated with dsRNA of chicken *Myosin IIA* (MyoIIA)

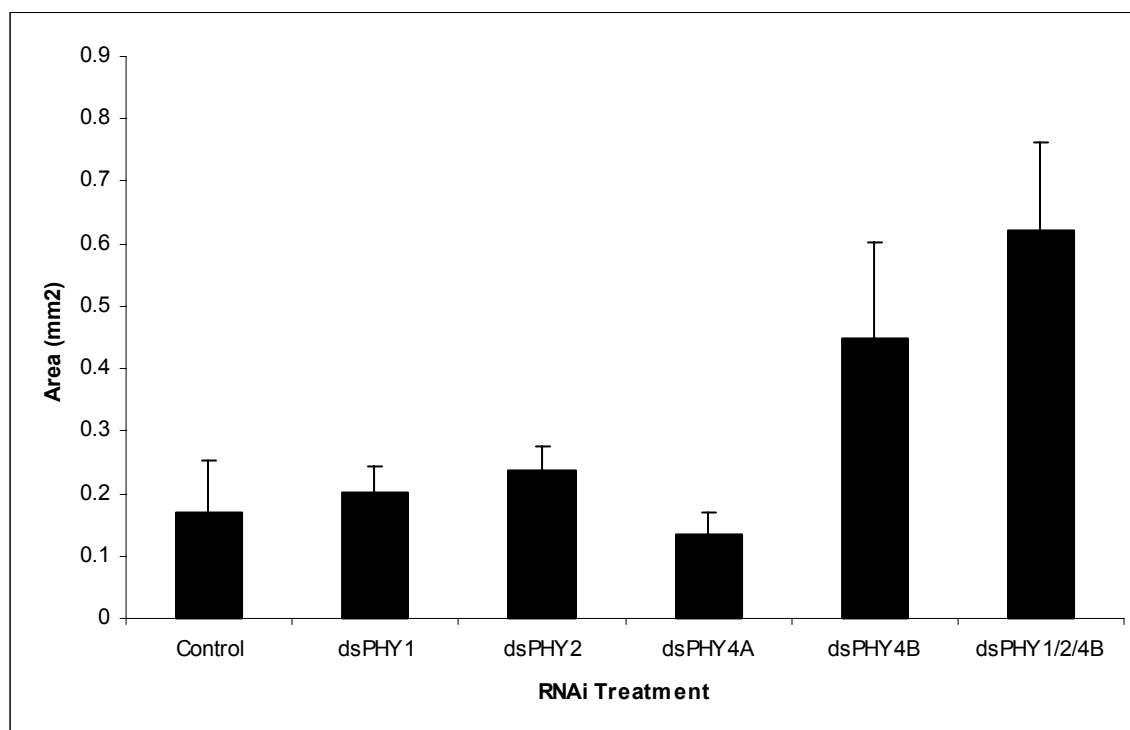
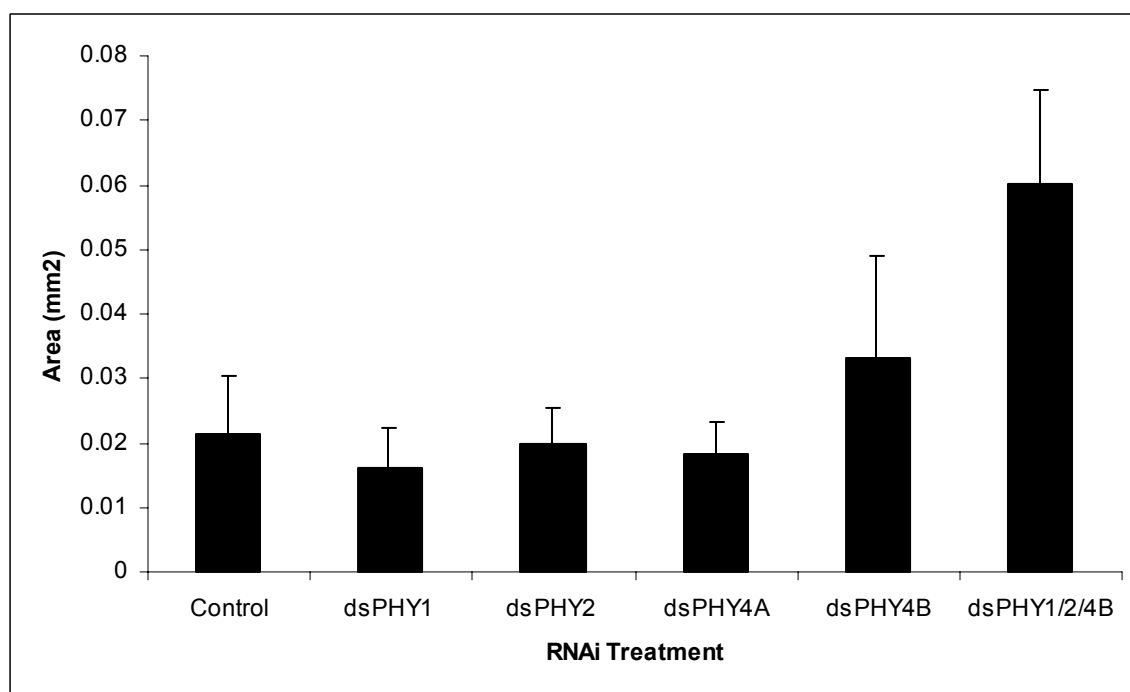
A.**B.**

Figure 25. Development of the prothalli from spores treated with dsRNA in continuous white or red light. Dry spores were incubated for 48 h with dsRNA for *PHY1*, *PHY2*, *PHY4A*, *PHY4B* or a mixture of *PHY1*, *PHY2*, *PHY4A* and *PHY4B* in the dark, and then exposed to W for 24 h. Spores were removed from RNAi media, plated on agar media and allowed to develop in W_c (A) or R_c (B) for 7 d. The areas and cell numbers of 30 gametophyte prothalli were measured. The prothallus area directly correlates with the number of cells per gametophyte (not shown). Experiments were repeated three times. Error bars represent +/- S.D.

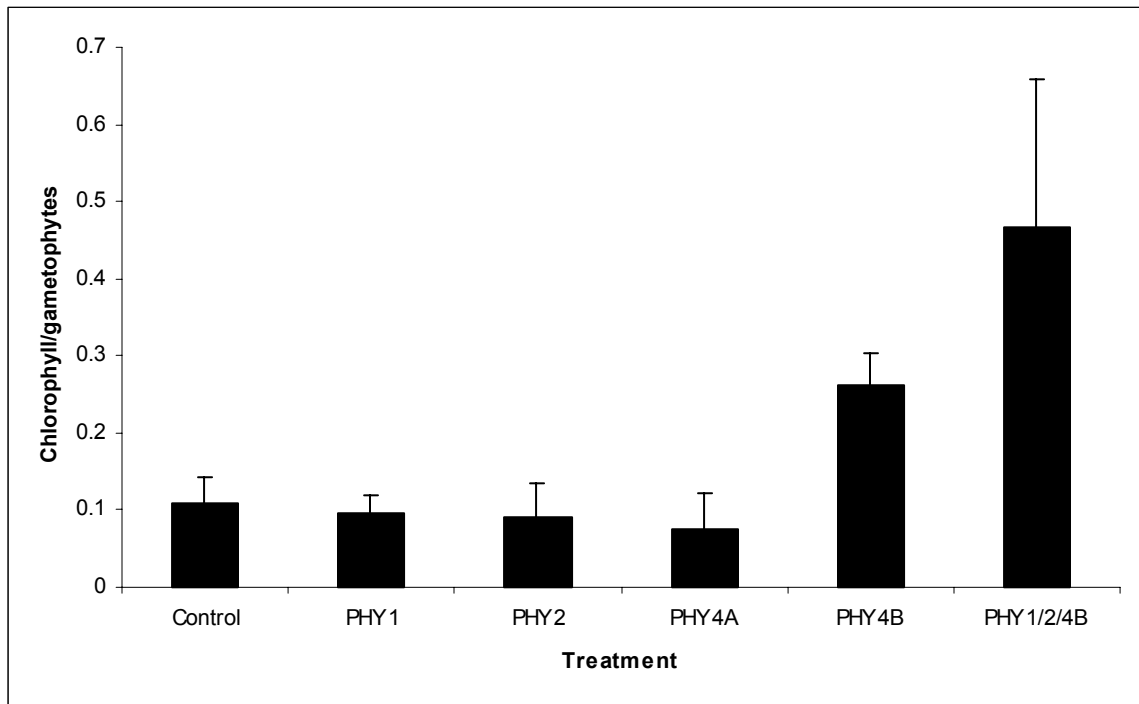
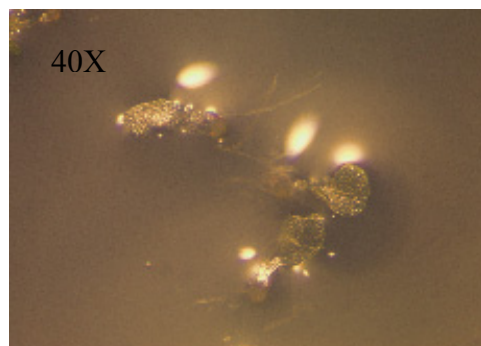
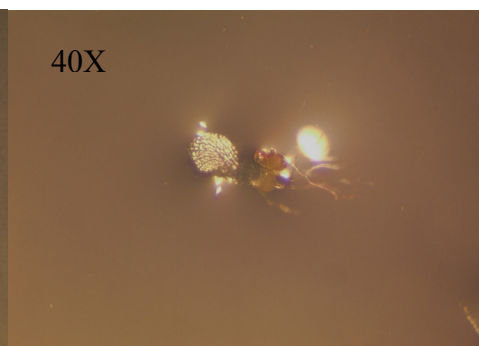


Figure 26. Chlorophyll accumulation in W_c -grown RNAi-treated prothalli. Dry spores were incubated for 48 h with dsRNA for *PHY1*, *PHY2*, *PHY4A*, *PHY4B* or a mixture of *PHY1*, *PHY2*, *PHY4A* and *PHY4B* in the dark, and then exposed to W for 24 h. Spores were removed from RNAi media, plated on agar media and allowed to develop in W_c for 14 days. The chlorophyll content for 30 gametophytes was measured. Error bars represent $\pm$ S.D. Experiments were repeated three times.

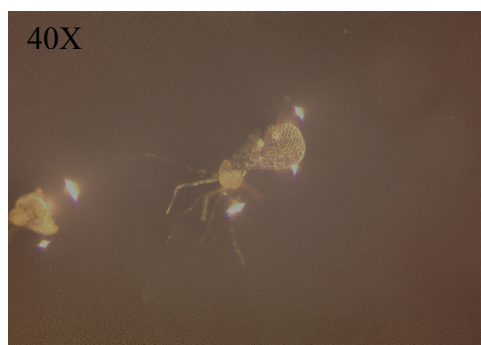
Control



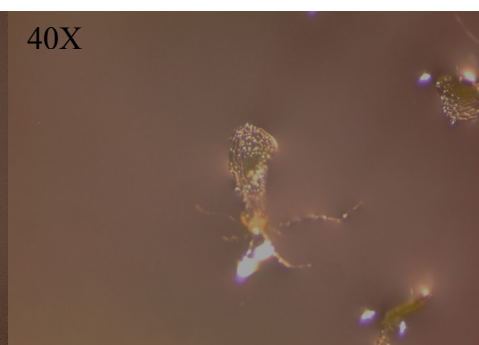
dsPHY1



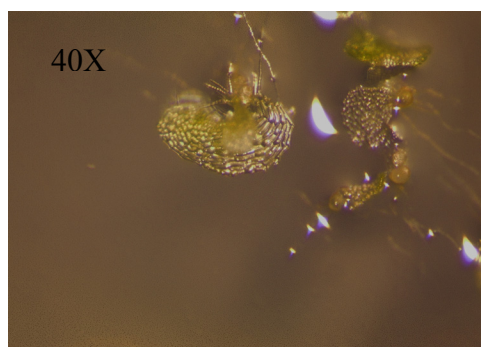
dsPHY2



dsPHY4A



dsPHY4B



dsPHY1/2/4B

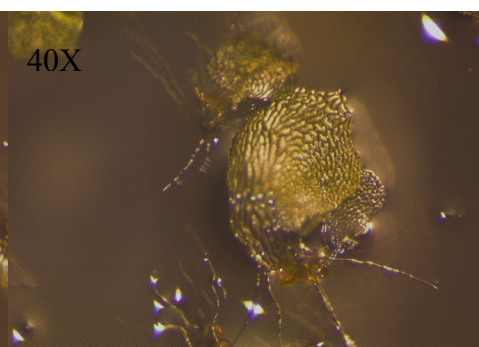


Figure 27. Development of the prothalli from spores treated with dsRNA in continuous red light. Dry spores were incubated for 48 h with dsRNA for *PHY1*, *PHY2*, *PHY4A*, *PHY4B* or a mixture of *PHY1*, *PHY2*, *PHY4A* and *PHY4B* in the dark, and then exposed to W for 24 h. Spores were removed from RNAi media, plated on agar media and allowed to develop R_c (B) for 7 d. The controls were spores that were incubated with dsRNA of chicken *Myosin IIA* (MyoIIA)

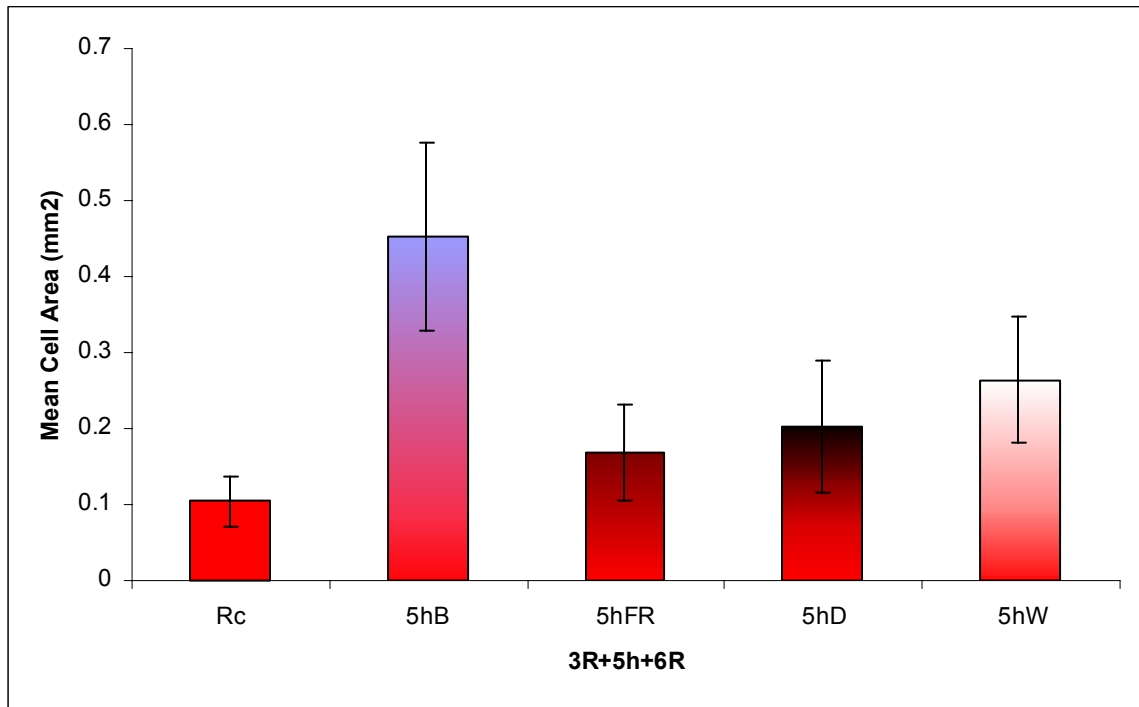


Figure 28. Blue light exposure after germination stimulates cell division and development. Dark-imbibed spores were exposed to R for 3 d, placed in blue (B), far-red (FR), dark (D) or white (W) light for 5 h, and then returned to continuous R for 6 d. The control group remained in Rc without any light interruption. After 9 d of development, the areas and cell numbers for 30 prothalli were determined and used to calculate the mean cell size. The prothallus area directly correlates with the number of cells per gametophyte. Error bars represent +/- S.D. Experiments were repeated twice.

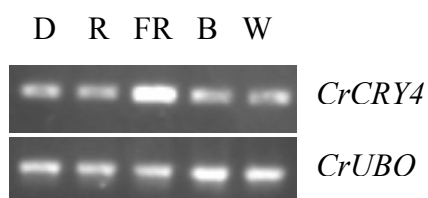


Figure 29. Semi-quantitative RT-PCR of *CrCRY4* from 7-day-old gametophytes grown in continuous white (W), red (R), far-red (FR), or blue (B) light, or from gametophytes that were exposed to white light for 1 day then grown in the dark (D) for 6 days. Ubiquitin was amplified at 15, 20 and 25 cycles to determine that equal concentration of cDNA template was used and the amplified products was still within the linear detection range. *CrCRY4* was then amplified under identical conditions.

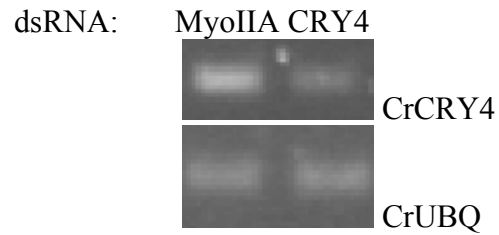


Figure 30. RNAi of *CrCRY4* in spores. Pre-sterilized spores were incubated with double stranded (ds) RNA of chicken *Myosin IIA* (MyoIIA), or *CrCRY4*. After three days of incubation, total RNA was extracted from treated spores and knock down was assessed by semi-quantitative RT-PCR using gene specific primers for *CrCRY4* and *ubiquitin* (*CrUBQ*).

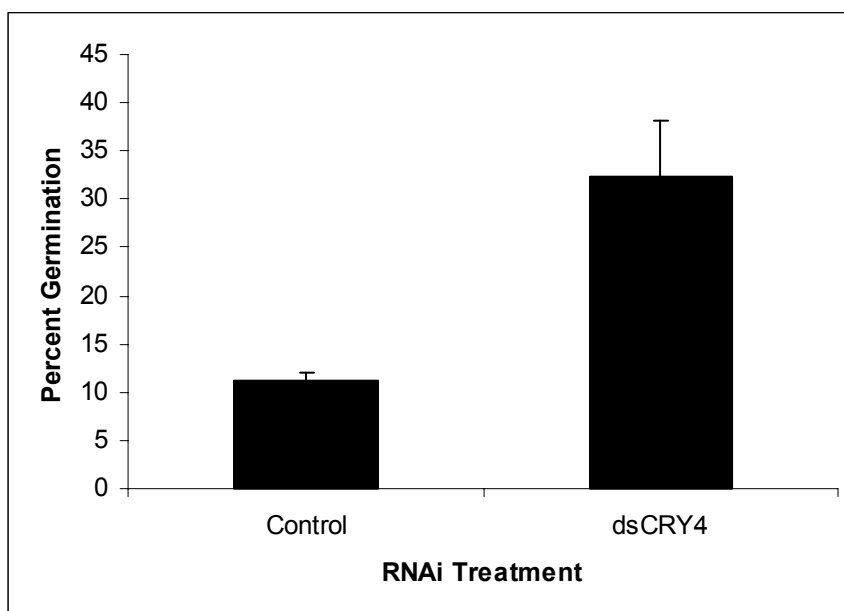


Figure 31. dsRNA for *CrCRY4* stimulates germination of spores in *B_c*. Pre-sterilized dry spores were incubated with dsRNA for 48 h in the dark, plated onto agar media with minimal exposure W, and then exposed to *B_c* for 7 d. The controls were spores that were incubated with dsRNA of chicken *Myosin IIA* (*MyoIIA*). After three days, germination was measured by the emergence of the rhizoid through the exine of the spore coat. Error bars represent +/- S.D. Experiments were repeated 3 times.

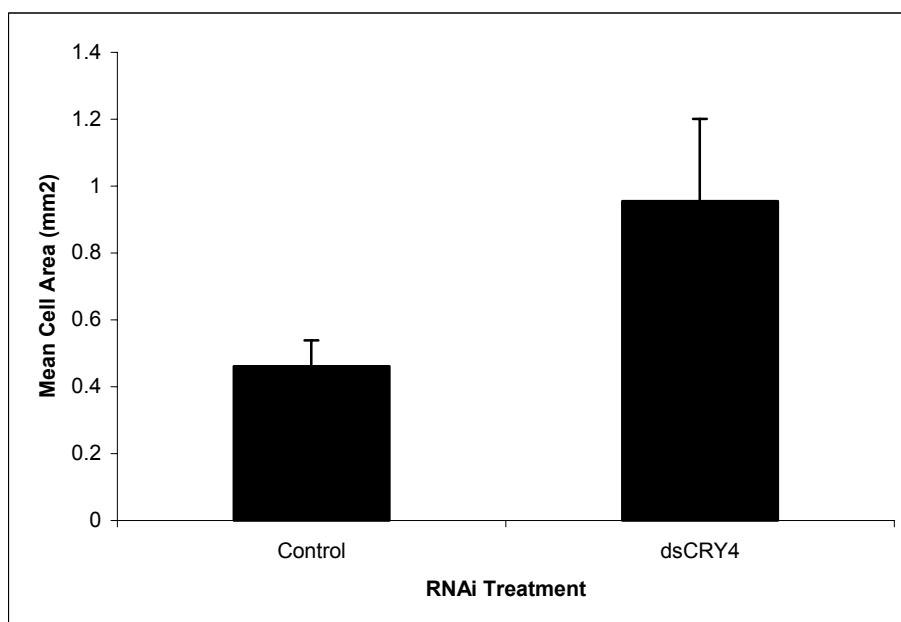
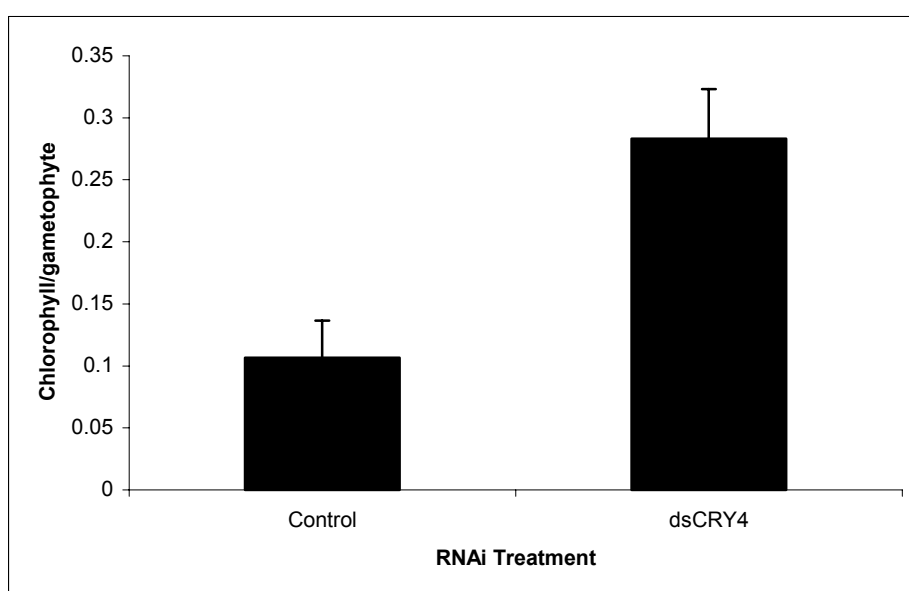
A.**B.**

Figure 32. dsRNA for *CrCRY4* stimulates prothalli development and chlorophyll production in W_c . Pre-sterilized dry spores were incubated with dsRNA for 48 h in the dark, plated onto agar media and then exposed to W_c for 14 d. The areas and chlorophyll accumulation for 30 prothalli were determined. The prothallus area directly correlates with the number of cells per gametophyte. Error bars represent $\pm$ S.D. Experiments were repeated 3 times. The controls were spores that were incubated with dsRNA of chicken *Myosin IIA* (MyoIIA)

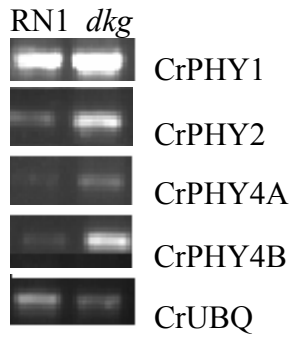


Figure 33. Semi-quantitative RT-PCR of *CrPHY1*, *CrPHY2*, *CrPHY4A* and *CrPHY4B* from wild type *RNW1* and *dkg1* spores that were dark imbibed in *C-fern* media 5 d. Total RNA was extracted from spores, DNase-treated, reversed transcribed and amplified for 25 cycles with gene specific primers as detailed in the text. Ubiquitin primers (CrUBQ) were used as positive and loading controls.

Figure 34. The isolation of putative light signaling factors from the EST library of *Ceratopteris richardii*. Two-way BLASTP alignments of the deduced translation sequences of DNA isolated from *Ceratopteris richardii* (Query) with characterized genes in other organisms (Sbjct). Sequences shown between the Query and Subject represent identity (amino acid letter symbols) and similarity (+) between the two sequences.

A. CrPP2A

```

Query: 1 MDLDGWIEKVKDQGHLLEDDLKRLCEYVKEILIEESNVQPVNSPVTVCGDIHGQFHDLMK 60
        MDLD WI KVKDQGH ED+L+ LCEYVKEILIEESNVQPVNSPVTVCGDIHGQFHDLMK
Sbjct: 1 MDLDQWISKVKDQGHLSEDELQLLCEYVKEILIEESNVQPVNSPVTVCGDIHGQFHDLMK 60

Query: 61 LFQTGGHVPETNYIFMGDFVDRGYSLEVFTILLLLKARYPANMTLLRGNHESRQITQVY 120
        LFQTGGHVP+TNYIFMGDFVDRGYSLEVFTILLLLKARYPAN+TLLRGNHESRQ+TQVY
Sbjct: 61 LFQTGGHVPDTNYIFMGDFVDRGYSLEVFTILLLLKARYPANITLLRGNHESRQLTQVY 120

Query: 121 GFYDECQRKYGNSNAWRYCTDVFDTLSAIDGRVLCVHGGLSPDVPTIDQITLIERQC 180
        GFYDECQRKYGN+NAWRYCTDVFDTLSAIDG VLCVHGGLSPDV TIDQI LIER C
Sbjct: 121 GFYDECQRKYGNANAWRYCTDVFDTLSAIDGTVLCVHGGLSPDVRTIDQIRLIERNK 180

Query: 181 EIPHEGPFCDLMWSDPEDJETWAVSPRGAGWPFGARVTSEFNHINGLELVCRAHQQLVQEG 240
        EIPHEGPFCDLMWSDPEDJETWAVSPRGAGW FG+RVT+EFNHIN L+LVCRAHQQLVQEG
Sbjct: 181 EIPHEGPFCDLMWSDPEDJETWAVSPRGAGWLFGRVTTTEFNHINKLDLVCRAHQQLVQEG 240

Query: 241 LKYMFPDKGLTVVWSAPNYCYRCGNVASILSFDEKMERQVKYFTET 286
        LKYMF DKGLTVVWSAPNYCYRCGNVASILSF++ MER+VK+FTET
Sbjct: 241 LKYMFDKGLTVVWSAPNYCYRCGNVASILSFNDNMEREVKFFTET 286

```

Using PP2A protein phosphatase (catalytic subunit) of *Arabidopsis thaliana* (*AtFyPPI*) as a presumptive homologue, a putative *CrPP2A* was identified from the *Ceratopteris richardii* EST library sequences deposited in GenBank. *CrPP2A* has a 91% identity to *AtFyPPI* at the protein level. The *CrPP2A* EST (accession number BE642714) contains only partial nucleotide sequence. Full-length sequence is provided in the Appendix.

B. CrGT-2

```

Query: 29  QPLQSQYSLQTIVPIAAQPVESPYPSQSSALISVQPVQSAPPLYEDEISHQYEDHEFSDS 208
          QP Q Q+ +TI  A  P  +P P+  S+L  V          P  E  +          E  +
Sbjct: 435  QPPQPQHK-ETIHHEAVTPRRAP-PTSGSSLELV-----PAAEQHVESGLGGGEGGSA 485

Query: 209  NSKRWLKPEVLHLIELHNSLRAKFSEAGPKGPLWEAIANGMAQKGYPSRTAKRCKEKWEN 388
          +S RW K EV  LI+L  L  ++ E GPKGPLWE I++GM + GY +R++KRCKEKWEN
Sbjct: 486  SSSRWPKTEVQALIQLRMELDMRYQETGPKGPLWEEISSGMRRLLGY-NRSSKRCKEKWEN 544

Query: 389  INKYFKRAKESNKKRPENSKTCPYFHELDALYSRK 493
          INKYFK+ KESNKKRPE+SKTCPYFH+LD +Y RK
Sbjct: 545  INKYFKVKESNKKRPEDSKTCPYFHQLDVIYRRK 579

```

BLAST comparisons of the *GT-2* transcription factor of *Oryza sativa* (*OsGT-2*) with the *Ceratopteris richardii* EST library yielded putative homologue *CrGT-2*. *CrGT-2* has 50% identity to *OsGT-2* at the protein level. Partial nucleotide sequence of *CrGT-2* is in the EST Library (accession number BE642174).

C. CrCAB

```

Query: 262  NEELAKWYGPSRRIYLP EGLLLDRSDVPEYLSGEVPGDYGFDPFGLSKKPADFEKYQAYEL 441
          ++ELAKWYGP RRI+LP+GLLDRS++PEYL+GEV GDYG+DPFGL KKP +F KYQA+EL
Sbjct: 56   SDELAKWYGPDRIIFLPDGLLRSEIPEYLNGEVAGDYGYPFGLGKKPENFAKYQAFEL 115

Query: 442  IHARWAMLGAAGFVLPEAFNKYGAVCGPEAVWFKTGALLLDGGSLEYFGATVPXXXXXXX 621
          IHARWAMLGAAGF++PEA NKYGA CGPEAVWFKTGALLLDG +L YFG  +P
Sbjct: 116  IHARWAMLGAAGFIIPEALNKYGANCPEAVWFKTGALLLDGNTLNYFGKNIPINLVLA 175

Query: 622  XXXXXXXXXXXXXYRASNLXGF-DRLHPGGPFDPYSLAKDPDQFALLKVKEIKNGRLAMXS 798
          YYR +N   F D+LHPGGPFDP LAKDP+Q ALLKVKEIKNGRLAM +
Sbjct: 176  VAEVVLLGGSEYYRITNGLDFEDKLHPGGPFDPGLGLAKDPEQGALLKVKEIKNGRLAMFA 235

Query: 799  MPGFFLQAYVT 831
          M GFF+QAYVT
Sbjct: 236  MLGFFIQAYVT 246

```

BLAST comparisons of the *chlorophyll a/b binding protein* of *Arabidopsis thaliana* (*AtCAB*) with the *Ceratopteris richardii* EST library yielded putative homologue *CrCAB*. *CrCAB* has 69% identity to *AtCAB* at the protein level. Partial nucleotide sequence of *CrCAB* is in the EST Library (accession number BE643223).

D. CrNPR

```

Query: 1  GGIMPKKPPLISKDHERAYFDSADWALGKGQPDQKPKLLAEVLRPKLQPTPH-QPLPSR 177
          GG++ KK PLISKDHERA+FDSADWALGK G  QKPK  E LRPKLQP+PH  + SR
Sbjct: 6  GGMLLKKKPLISKDHERAFFDSADWALGKQGA--QKPKGLEALRPKLQPSPHLHQVRSR 63

Query: 178 RASYENAENED 210
          R++Y  A  + D
Sbjct: 64  RSAYAPAGDCD 74

```

BLAST comparisons using the negatively light-regulated protein of *Vernicia fordii* (VfNRP), against the *Ceratopteris richardii* EST library yielded putative homologue CrNPR. CrNPR has 63% identity to VfNRP. Partial nucleotide sequence of CrNPR is in the EST Library (accession number BE643282).

Figure 35. The isolation of putative light signaling factors by PCR amplification. BLASTP alignment of the deduced translation of DNA sequences isolated from *Ceratopteris richardii* (Query) with characterized genes in other organisms (Sbjct). Sequences shown between the Query and Subject represent identity (amino acid letter symbols) and similarity (+) between the two sequences.

A. CrCRY1

```

Query: 17  LGSPLILCRSRSTTALLHIIHQTNATQVFFNHLYDPISLVRDHRVKQELVQAGISVTTF 76
          LGSPLI+ R++S + L  II+QT ATQVFFNHLYDP+SLVRDH+VKQEL+QAG++VTTF
Sbjct: 67  LGSPLIMRRAQSAISVLSQIIKQTGATQVFFNHLYDPVSLVRDHKVKQELLQAGVTVTTF 126

Query: 77  NADLLYEPWEINDEHGQPYTTFGSFWSHCMDRAMEPDAPILPPKRLVSWPGTITSLPIEE 136
          NADLLYEPWEINDE G+P+TTF FWS CMD A+EP+AP+LPPKR+ WPG + PIE+
Sbjct: 127 NADLLYEPWEINDEQGRPFTTFAGFWSLCMDMAIEPEAPLLPPKRITKWPGIAATSPIED 186

Query: 137 LELENESEKGSNDLLARACFPWANADRALEAFVNGPLMDYASNSQKVDSXAXSLLSPHL 196
          LELENESEKGSNDLLARA PGWANADRALEAFV+GPLM+YASN QKVDS SLLSPHL
Sbjct: 187 LELENESEKGSNDLLARAWAPGWANADRALEAFVSGPLMEYASNRQKVDSATTSLLSPHL 246

Query: 197 XYGELSVRKIFHCIRMKQVLWVKEGNIKAEE SVNLYLRAIGFREYSRYXSFNFPFTHERS 256
          YGELSVRK+FH +RMKQVLWVKEGN AEESVNL+LR+IG REYSRY FNFPPFTHERS
Sbjct: 247 HYGELSVRKVFHSVRMKQVLWVKEGNADAEE SVNLFRLSIGLREYSRYLCFNFPFTHERS 306

Query: 257 LLSNLKYFPWRNDEGYFKVWRQGRTGYPLVDAGMRELWATGWLHNRI RVIVSNFFVKFLQ 316
          LLSNLK+FPWRNDEGYFKVWRQGRTGYPLVDAGMRELWATGWLHNRI RVIVS+FFVKFLQ
Sbjct: 307 LLSNLKFFPWRNDEGYFKVWRQGRTGYPLVDAGMRELWATGWLHNRI RVIVSSFFVKFLQ 366

Query: 317 LPWRWGMKYFWDTLDDADLES DVLGWQYISGSLPDGH 353
          LPWRWGMKYFWDTLDDADLES DVLGWQYISGSLPDGH
Sbjct: 367 LPWRWGMKYFWDTLDDADLES DVLGWQYISGSLPDGH 403

```

Using gene-specific primers to the *CRY1* gene of *Adiantum capillus veneris* (*AcCRY1*), a putative CRY1 PCR fragment from *Ceratopteris* (*CrCRY1*) was isolated. *CrCRY1* has 83% identity to *AcCRY1* at the protein level. The nucleotide sequence of *CrCRY1* can be located in the Appendix.

B. CrCRY2

```

Query: 2   DAPPSVLHAAGIELGSNYPRPIVEITAARERLQRALSMDWANEAAANKATLANGLEEGLGD 61
          DAPPSVL AAGIELGSNYPRPIVEI AARERL++AL++MWANEAA+KATLANGLEEGLGD
Sbjct: 451 DAPPSVLRAAGIELGSNYPRPIVEIAAARERLEQALAEMWANEAADKATLANGLEEGLGD 510

Query: 62   TIEVQGAENGFLSMDVDIGPETVHRGNQVSFWAKSPHDQMVPMTMANGVTRSASGAF--- 118
          T+EVQG NGFP +MD+D GP      + +   +SPHDQMVP +   GV RS +GA
Sbjct: 511 TVEVQGMNRNGFPANMDIDRGP-----GEKALLVRSPHDQMVPNV--GVRRTTGAVQGG 562

Query: 119  -----PDAEHEN--DDMDSTAESSARDTLA 143
          P      +N DD STAES+SAR +LA
Sbjct: 563 AANQGSALQAVLREVPANGHVQAVQAQSQRMAAPPMAQNGYDDTHSTAESTSARHWSLA 622

Query: 144   LGLPVCQYVPV-FPVRQSGSQVPLVPDLHPVLISRQHSMLPVMKLEASKTNSLS 198
          +GLPV SQYVPV +RQ SQVPLVPDLH ++SR+ H P++ EA+KT+SLS
Sbjct: 623 IGLPVWSQYVPVCLHMRQDDSQVPLVPDLHHGVMSRRLPHAPPMVNFEATKTSSLS 678

```

Using gene-specific primers to the *CRY2* gene of *Adiantum capillus veneris* (*AcCRY2*), a putative CRY2 PCR fragment was isolated from *Ceratopteris* (*CrCRY2*). *CrCRY2* has 54% identity to *AcCRY2* (and 48% to *AcCRY1*) at the protein level. The complete nucleotide sequence of *CrCRY2* is located in the Appendix.

C. CrbHLH Putative Transcription Factor

```

Query: 41   GLNEQADISVDNTCCVANLDELEKTSSKKRTRDDGGS-TSSKACREKMRRDRLNDRFLEL 99
          G++ +   VD +   N + ++ SKKR R + S TSSKACREK RRDRLND+F+EL
Sbjct: 37   GVSSNSSAGVDGSA--GNSEASKEPGSKKRGRCESSATSSKACREKQRRDRLNDKFEMEL 94

Query: 100  STLLDPGRAPKSDKIVILSDALRTLNLQRAEAQQLNESNERLHNSIKKLKTEKSELRDEK 159
          +L+PG PK+DK IL DA+R + QLR EAQ+L +SN L + IK+LKTEK+ELRDEK
Sbjct: 95   GAILEPGNPPKTDKAAILVDVAVRMVTQLRGEAQKLKDSNSSLQDKIKELKTEKNELRDEK 154

Query: 160   QRLKSEKEKLEQQLKMSMP-TGYMPHXAXLHAAMTAFAMQSVSAGGKPASVPXYPSMG 218
          QRLK+EKEKLEQQLK ++ P   + P   +   TAFA   + G K   + YP G
Sbjct: 155   QRLKTEKEKLEQQLKAINAPQPSFFPAPPM---PTAFASAQQAAGNKMVPIISYP--G 209

Query: 219   MGLWEWMPPAALDPFQNHLLRPPIA 243
          + +W++MPPA++D Q+H+LRPP+A
Sbjct: 210   VAMWQFMPPASVDTSQDHVLRPPVA 234

```

Using gene-specific primers to the *PIF3* gene of *Arabidopsis thaliana* (*AtPIF3*), a PCR fragment was isolated from *Ceratopteris* that has a 49% identity to an uncharacterized bHLH protein in *A. thaliana*. Partial nucleotide sequence of *CrbHLH* is in the EST Library (accession number BE641987).

D. *CrbZIP* Putative Transcription Factor

```

Query: 2   QRDSTGLTNENNELKLRLQSMEQQAQLREALHEALRDEVQRLKVATGQIQGSFNEHFFHM 181
          QRD++GLTNEN ELKLRLQ++EQ A LR+AL+EALR+E+QRLK+A GQI G+ N +
Sbjct: 242 QRDNSGLTNENKELKLRLQALEQGAHLRDLALNEALREELQRLKIAAGQILGA-NGNRGPR 300

Query: 182 MHQNPQHSLNQIDQQCPQVHLQQRQ 256
          H PQ Q QC H QQ+Q
Sbjct: 301 PHFPPQ---PQSFVQCGSHHAQQQQ 322

```

Using primers for the light-regulated *HY5* transcription factor of *Arabidopsis thaliana* (*AtHY5*), a PCR fragment was isolated from *Ceratopteris* that has 56% identity to the RSG transcriptional activator in *Nicotiana tabacum*. Partial nucleotide sequence of *CrbZIP* can be found in the EST Library (accession number BE643232).

E. *CrNDPK*

```

Query: 37  METTFIMIKPDGVQRLVGKIISRFEKGFILKALKFLQIDRSFASQHYADLAGKPFDDG 216
          +E T+IM+KPDG+QRLVG+IISRFE+KGF L LK Q + A +HY +L+ K FF
Sbjct: 83  VEETYIMVKPDGIQRLVGEEIISRFEKKGFKLIGLKMFCPKELAEHYKELSAKSFFLT 142

Query: 217 LIDYVTSGPVVAMVWYGRGAVLTGRKMLGATKPADSNPGTIRGDYAVDVGRNVIHGSDSV 396
          LI+Y+TSGPVV M W G G V + RK++G T P + PGTIRGD AV GRN++HGSDS
Sbjct: 143 LIEYITSGPVVCMWEGVGVVASARKLIGKTDPLQAEFGTIRGDLAVQTGRNIVHGSDSP 202

Query: 397 ESAQKEIALWFPPEG-ITEWRSCAHDWIYE 480
          E+ ++EI LWF EG + +W S W+ E
Sbjct: 203 ENGKREIGLWFKGELCKWDSALATWLRE 231

```

Using primers for *NDPK2* of *Arabidopsis thaliana* (*AtNDPK2*), a PCR fragment was isolated from *Ceratopteris* that has a deduced 55% amino acid identity to *AtNDPK2*. The complete nucleotide sequence of *CrNDPK* can be found in the EST Library (accession number BE643276)

F. CrCOP1

```

Query: 2    DELFATAGVSRRIKIFEFSTVVNVLEEVEHFPVAEMPTRSKLSCLSWNKSVMKQIASSDYE 181
           DELFATAGVSR IK+F+FS+VVN ++ P+ EM TRSKLSCLSWNK KN IASSDYE
Sbjct: 383 DELFATAGVSRCIKVFDFSSVVNEPADMQCPIVEMSTRSKLSCLSWNKHEKNHIASSDYE 442

Query: 182  GIITVWDVNTRQSIMYEYEEHEKRAWSVDFSRDLDPMSLVSGSDDCKVKVWCTRQETSVLDI 361
           GI+TVWDV TRQS+MEYEEHEKRAWSVDFSR +PSMLVSGSDDCKVKVWCTRQE SV++I
Sbjct: 443  GIVTVWDVTTTRQSLMEYEEHEKRAWSVDFSRTEPSMLVSGSDDCKVKVWCTRQEASVINI 502

Query: 362  DMKANICCVKYNPGSSSHHLAVGSADHHIHYDLRNTLSPVHIFSGHRKAVSYVKFISSTE 541
           DMKANICCVKYNPGSS+++AVGSADHHIHYDLRN P+H+FSGH+KAVSYVKF+S+ E
Sbjct: 503  DMKANICCVKYNPGSSNYIAVGSADHHIHYDLRNISQPLHVFSGHKKAVSYVKFLSNNE 562

Query: 542  LASASTDSTLRLWDVRENTHIRTFTKGTNEKNFVGLSVNSEYIACGSETNEVCVYHKAIS 721
           LASASTDSTLRLWDV++N +RTF+GHTNEKNFVGL+VNSEY+ACGSETNEV VYHK I+
Sbjct: 563  LASASTDSTLRLWDVKNLPLVTRFRGHTNEKNFVGLTVNSEYLACGSETNEVVYVYHKEIT 622

Query: 722  RPAAWHKFGPTDLDDSDDEF-SHFISAVCWKNESPTMLAANSAGTIK 859
           RP H+FG D+DD+++E S+FISAVCWK++SPTML ANS GTIK
Sbjct: 623  RPVTSHRFGSPDMDDAEEEAAGSYFISAVCWKSDSPTMLTANSQGTIK 669

```

Using primers for *COP1* of *Arabidopsis thaliana* (*AtCOP1*), a PCR fragment was isolated from *Ceratopteris* that has a deduced 77% amino acid identity to *AtCOP1*. Partial sequence of *CrCOP1* can be found in the Appendix.

G. CrCOP9

```

Query: 121  LQEAINARRFGELASMCETFELEMAAKGLEKPSDWPYALHLLGHIYISDLNGARFLWKRV 300
           ++EA+ A+ F ++A +C+T L++A++G+E DWPYA+HLLG+ Y+ D + ARFLWKRV+
Sbjct: 6    VKEALAAKSFDKIADICDTLMLQVASEGIEYHDDWPYAIHLLGYFYVDDCDSARFLWKRI 65

Query: 301  PSAVKQGQPELVAAWKIGQCLWTYDYAGVHEAFKGYSWSPAVQQIVAAIKDEYSKKNMIL 480
           P+A+K+ +PE+VAAW IGQ LWT+DYAGV+EA +GY WS + +VAA D Y+K+ L
Sbjct: 66   PTAIKERKPEVVAAWGIGQKLWTHDYAGVYEAIRGYDWSQEAKDMVAAFSDLYTKRMFQL 125

Query: 481  LSTAYSTISVTDAAKFLGLTESETIXSTTQNGWTLTSSSNMLTAVPMVTATAQKLDSSKL 660
           L +AYSTI++ D A FLG+TE + +NGWT+D++S M + QK+DSSKL
Sbjct: 126  LLSAYSTITIHDALFLGMTEDDATTYVVENGWTVDAASQMASVKKQAVKREKQKVDSSKL 185

Query: 661  QDLTEYVF 684
           Q LTEYVF

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Using primers for *COP9* (signalsome complex subunit 8) of *Arabidopsis thaliana* (*AtCSN8*), a PCR fragment was isolated from *Ceratopteris* that has a deduced 51% amino acid identity to *AtCSN8*. Partial sequence of *CrCSN8* can be found in the Appendix.

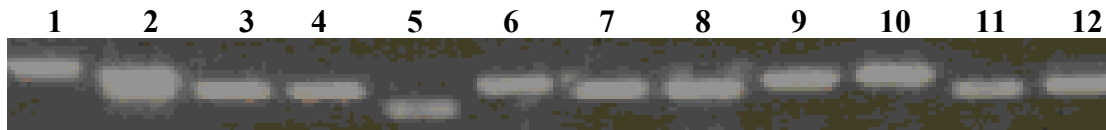


Figure 36. RT-PCR of RNA isolated from 7-day-old gametophytes grown in continuous white light. Total RNA was extracted from spores, DNase-treated, reverse-transcribed, and amplified for 30 cycles with the following gene specific primers described in the text: 1: *CRY1*, 2: *CRY2*, 3: *CRY4*, 4: *COP1*, 5: *COP9*, 6: *GT-2*, 7: *CAB*, 8: *NDPK*, 9: *PP2A*, 10: *bHLH*, 11: *bZIP*, 12: *NPR*.

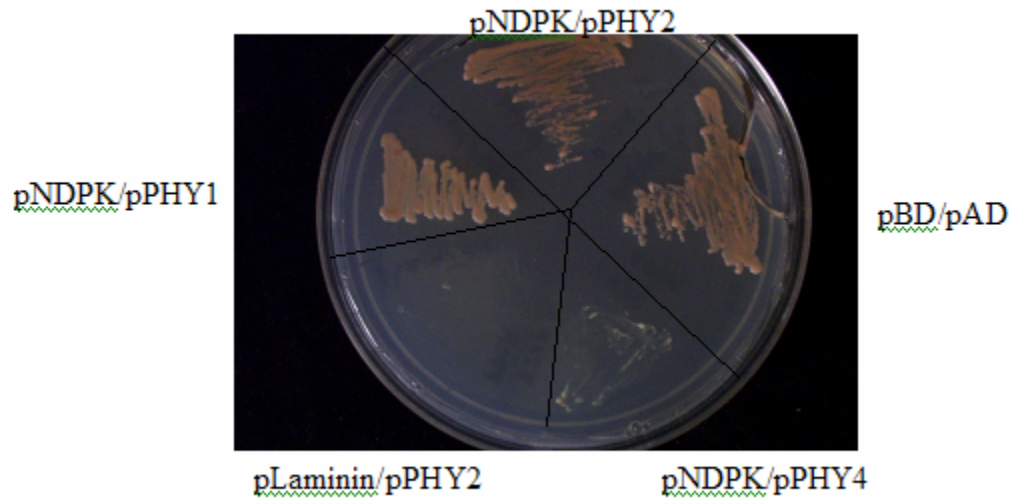


Figure 37. Putative physical interaction of *CrNDPK* with the C-termini of *CrPHY1* and *CrPHY2*. YRG-2 yeast cells were transformed with the full length *CrNDPK* fused to the GAL4 activator domain (AD) in the pAD-GAL4-2.1 phagemid vector and selected on *leu*⁻ dropout plates. The pBD-GAL4 Cam phagemid bait vector containing the C-termini (approximately 600 aa comprising the PRD and the complete HKRD) of *CrPHY1*, *CrPHY2* or *CrPHY4B* fused to the upstream GAL4 DNA-binding domain (BD) was then co-transformed into cells and selected on *trp*⁻*leu*⁻ double dropout plates. Cotransformed yeast cells were then selected for positive interaction between target and bait via the expression of *HIS3* reported gene on *-his-trp-leu* triple dropout plates shown above. Controls for strong positive interactions were co-transformed with the pBD/pAD plasmids. Negative interaction controls were co-transformed with the pLaminin/pPHY2 plasmids.

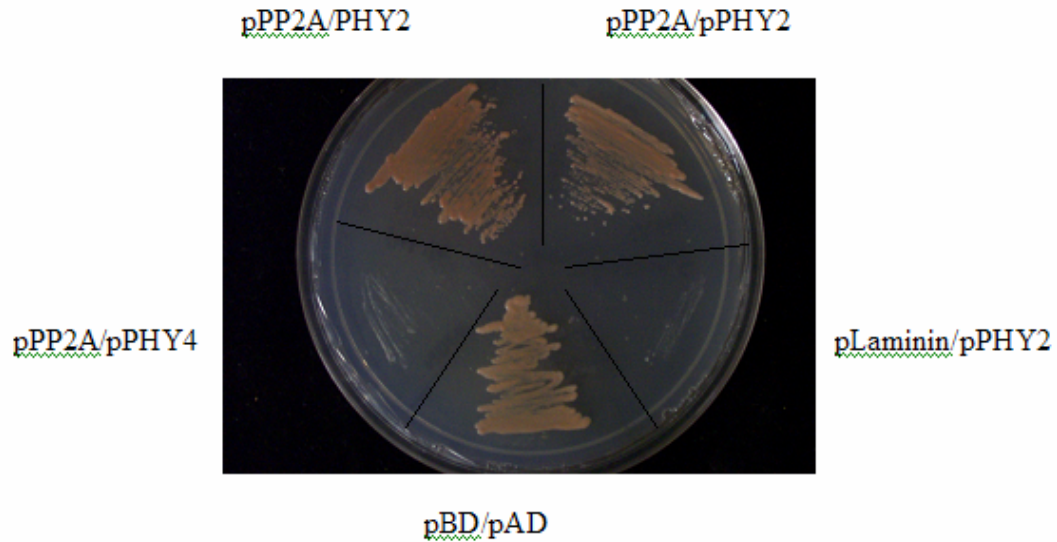


Figure 38. Putative physical interaction of CrPP2A with the C-termini of CrPHY1 and CrPHY2. YRG-2 yeast cells were transformed with the full-length *CrPP2A* fused to the GAL4 activator domain (AD) in the pAD-GAL4-2.1 phagemid vector and selected on *leu*- dropout plates. The pBD-GAL4 Cam phagemid bait vector containing the C-terminus of CrPHY1, CrPHY2 or CrPHY4B as described above fused to the upstream GAL4 DNA binding domain (BD) was then co-transformed into cells and selected on - *trp-leu* double dropout plates. Cotransformed yeast cells were then selected for positive interaction between target and bait via the expression of *HIS3* reported gene on -*his-trp-leu* triple dropout plates. Controls for strong positive interaction were cells co-transformed with pBD/pAD plasmids. Controls for no interaction were cells co-transformed with the pLaminin/pPHY2 plasmids

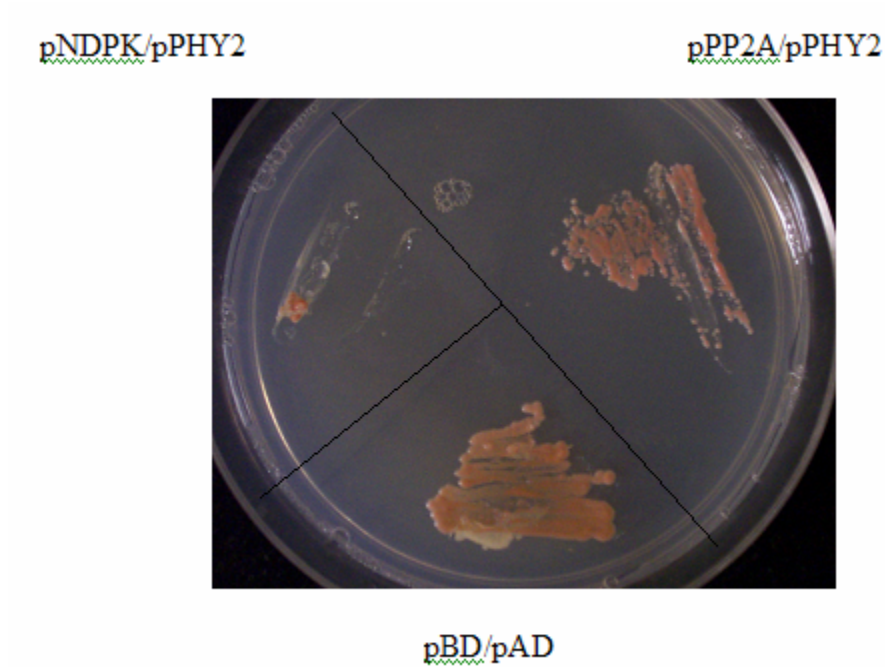


Figure 39. Verification of yeast two-hybrid interaction. *CrNDPK* and *CrFyPP* were fused to the DNA binding domain (BD) in the pBD-GAL4 Cam phagemid bait vector and the C-terminus of *CrPHY2* was then fused the GAL4 activation domain in the pAD-GAL4-2.1 phagemid vector. After sequential transformation of yeast with first the bait (*pBD-CrFyPP* or *pBD-NDPK*) then the target vector (*pAD-CrPHY2*), cotransformed yeast cells were then selected for positive interaction between target and bait via the expression of *HIS3* reported gene on *-his-trp-leu* triple dropout plates. Controls for strong positive interaction were cells cotransformed with pBD/pAD plasmids.

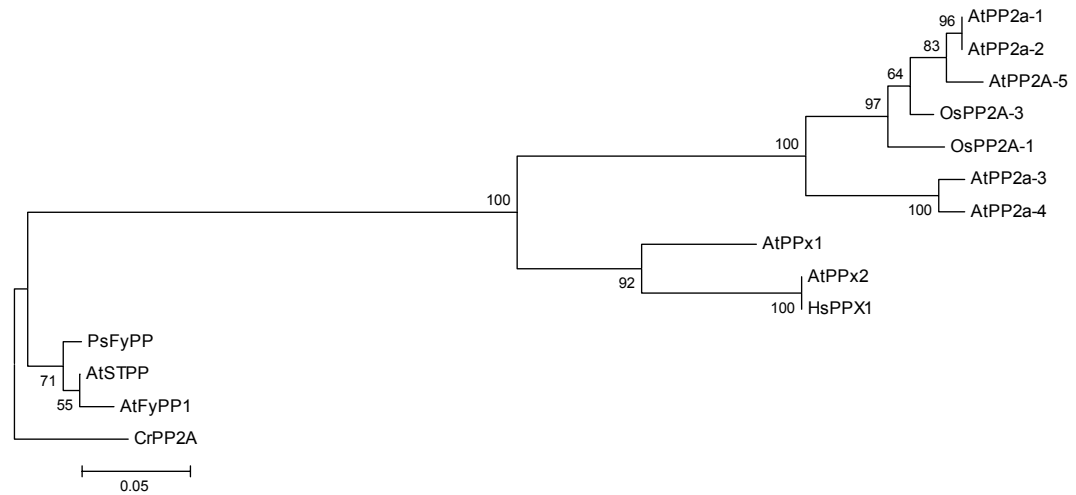


Figure 40. Presumptive phylogenetic analyses of *CrPP2A* isolated from *Ceratopteris richardii* using the Neighbor Joining Method. The complete protein sequences were aligned, and the indels were removed using BIOEDIT. Trees were constructed using MEGA3 and were rooted with *Ceratopteris* (*CrPP2A*). The sequences and GenBank accession numbers used were those referenced by Kim *et al*, 2002. Values from 1000 bootstrap replicates are given adjacent to branch nodes.

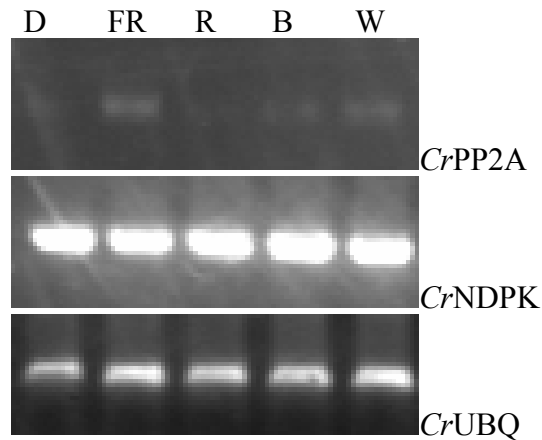


Figure 41. Semi-quantitative RT-PCR of *CrNDPK* and *CrPP2A* from 7 day-old gametophytes grown in continuous white (W), red (R), far red (FR), blue (B) light or from gametophytes that were exposed to white light for 1 day then grown in the dark (D) for 6 days. Identical reactions of Ubiquitin were amplified for 15, 20 and 25 cycles to confirm that equal concentrations of cDNA template were used and the amplified products were still within linear detection range. *CrNDPK* and *CrPP2A* were amplified at 25 cycles.

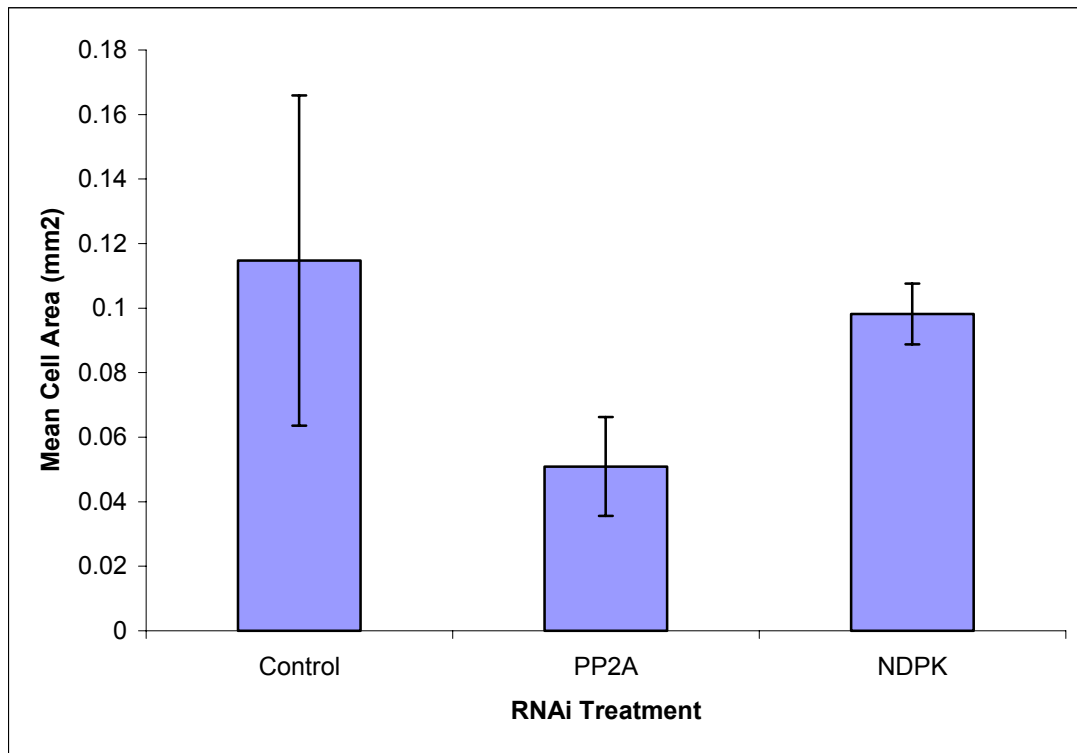


Figure 42. Development of the prothalli from spores treated with dsRNA in continuous white. Dry spores were incubated for 48 h with dsRNA for *MyoIIA*, *CrPP2A* and *CrNDPK* in the dark, and then exposed to W for 24 h. Spores were removed from RNAi media, plated on agar media and allowed to develop in W_c for 7 d. The areas and cell numbers of the prothalli for 30 gametophytes were measured. The area directly correlates with the number of cells per gametophyte. Error bars represent $\pm$ S.D. Experiments were repeated twice.

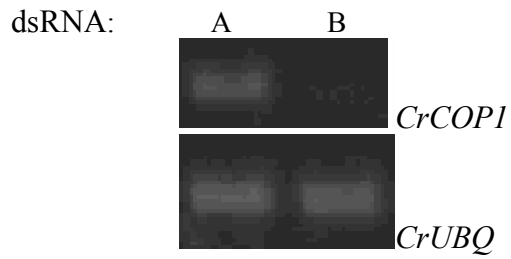


Figure 43. RNAi of *CrCOPI*. Dry spores were incubated with dsRNA of *MyoIIA* (A) or dsRNA of *CrCOPI* (B). After 48 h incubation, total RNA was extracted from spores and the level of *CrCOPI* and ubiquitin (*CrUBQ*) message was assessed by semi-quantitative RT-PCR.

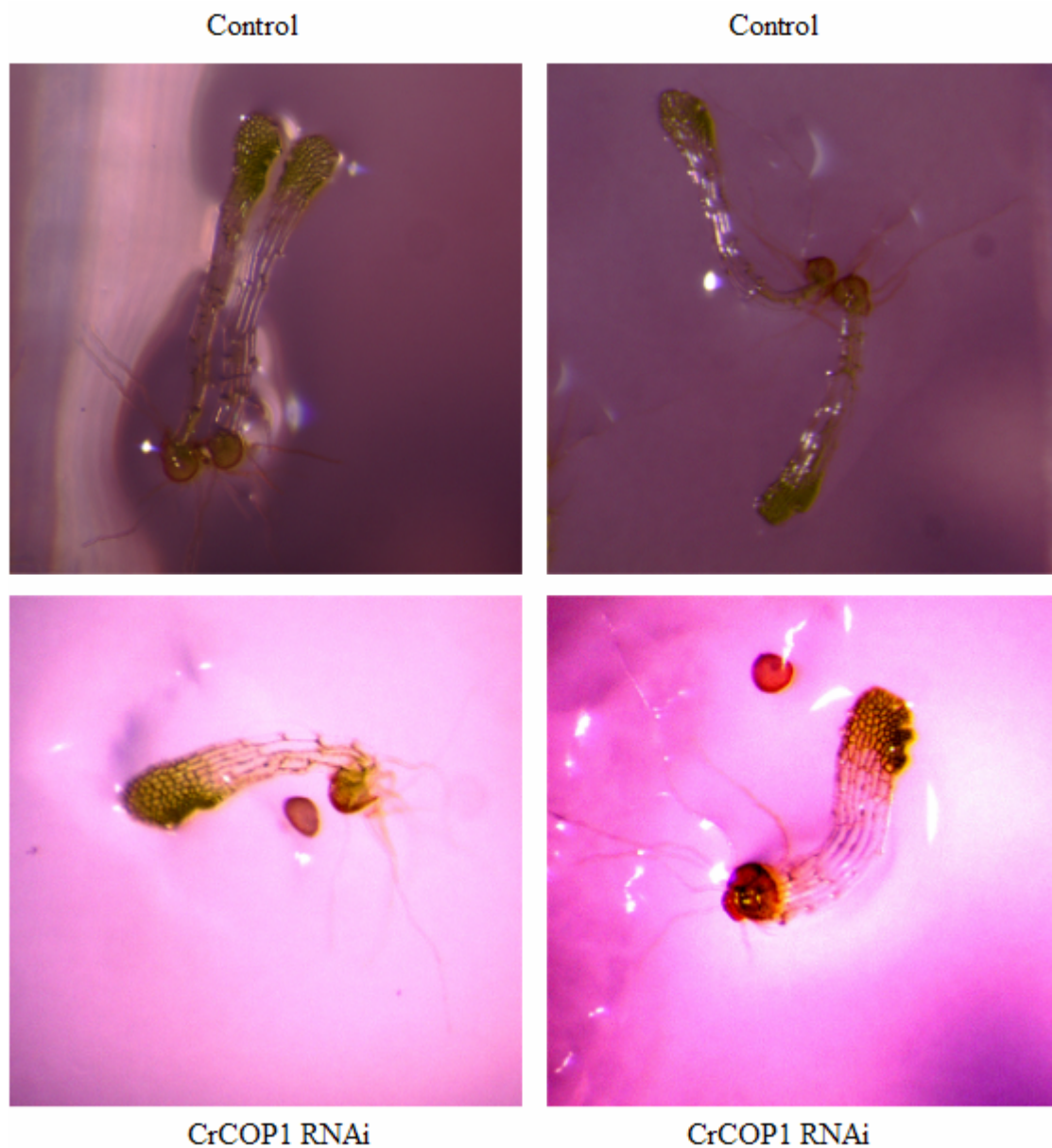


Figure 44. Development of the etiolated prothalli from spores treated with dsRNA.

Dry spores were incubated for 48 hrs with dsRNA of *MyoIIA* (negative control) or *CrCOP1* in the dark, and then exposed to W for 24 h. Spores were removed from RNAi media, plated on agar media and allowed to develop in the dark for 7 d. Spores treated with dsRNA of *CrCOP1* developed partially de-etiolated gametophytes with more cell files at the bases of the gametophytes. These cells are less elongated. Data were not quantified, but photographs are representative of prothalli observed on the plates.

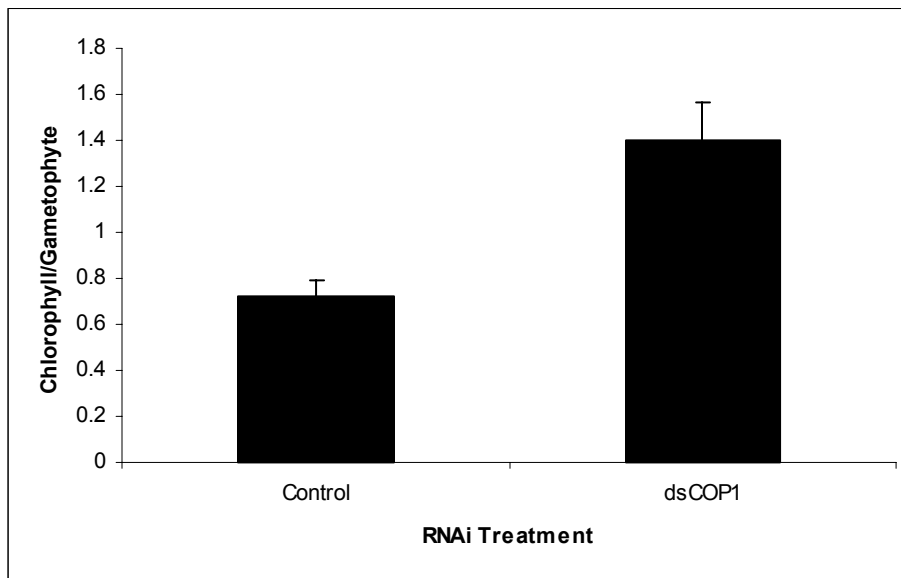


Figure 45. dsRNA for *CrCOP1* stimulates chlorophyll production in *W_c*. Pre-sterilized dry spores were incubated with dsRNA for 48 h in the dark, plated onto agar media, and then exposed to *W_c* for 14 d. The chlorophyll accumulation for 30 prothalli was determined. Error bars represent +/- S.D. Experiments were repeated twice. Controls were treated with chicken *MyoIIA* dsRNA.

APPENDIX

CrPHY1

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CrPHY2

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CrPHY4A

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1751 aaaaaaaaa

CrPHY4B

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CrCRY1

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CrCOP9

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