A Novel Phytochrome Sequence in *Ceratopteris richardii*

By

Brian Stonaker

Plants rely upon environmental cues such as light in order to acclimatize to constantly changing environmental stressors. Light is a major cue that directs patterns of gene expression in plant development, metabolism, and morphology. Solar tracking, chloroplast movement, stomatal opening, photomorphogenesis, photoperiodism, nyctinasty, and photonasty are all actions taken by plants in response to light conditions. In order for light to induce physiological changes, chromoproteins known as photoreceptors must use light energy to initiate signal transduction pathways that result in changes in gene expression (Castillon *et al.*, 2007). One such photoreceptor class, the phytochromes, respond to red and far-red light. The molecular biology of phytochrome in angiosperms and mosses is well documented however little is still understood of the role of phytochrome in ferns and gymnosperms. Previous studies into the photobiology of ferns using the model *Ceratopteris richardii* have identified five phytochrome sequences: *Cr*PHY1A, *Cr*PHY1B (partial cDNA), *Cr*PHY2, *Cr*PHY4A, and *Cr*PHY4B. This study attempted to confirm the sequence of the previously isolated *Cr*PHY2 and consequently discovered a possible novel phytochrome in *Ceratopteris richardii*. Analysis suggests the sequence in this study is a possible *Cr*PHY1 paralog. The phytochrome family of genes in ferns is the result of several gene duplication events. Previous phylogenetic analysis indicates the divergence between PHY1 and PHY2 in vascular plants predates the advent of seeded plants. A more recent gene duplication event in ferns resulting in homologues PHY2 and PHY4 correspond to the diversification of angiosperms. The novel sequence isolated in this study may have resulted from a more recent gene duplication event.

Phytochromes

The red to far-red light ratio is an important indicator of canopy shade and competition. As light is filtered through the canopy much of the red light is absorbed for use in photosynthesis, while far-red light is not absorbed to the same extent. As canopy density increases, chlorophyll absorption causes the amount of red light to decrease more than the amount of far-red light, thereby lowering the R:FR ratio. Phytochrome-mediated photomorphogenic responses such as seed germination (Shinomura et al., 1996), seedling development (Smith et al., 1997), photoperiodism, flowering (Devlin et al., 1998) and shade avoidance (Devlin et al., 1999) are dependent on the light environment through detection of this R:FR light ratio.

Phytochromes, which are responsible for sensing the R:FR ratio as well as other aspects of the light environment, are a family of cyan pigments that exist in one of two photoreversible isoforms *in vivo*; P_r absorbs primarily red light (650-690nm) and P_{fr} absorbs far-red light (710-750nm). Additionally, phytochromes weakly absorb blue light (Furuya & Song, 1994). P_r photoconverts to P_{fr} upon absorbing a photon, and P_{fr} will revert to P_r after absorbing another photon. Photoconversion of phytochromes is enabled by a bilin chromophore covalently attached through a thioether linkage (Rockwell, 2006) as described below.

Most phytochromes are thermally unstable in the P_{fr} form, and may undergo dark reversion—the process in which P_{fr} gradually reverts back into the P_r form over time, even without absorption of a photon. A number of factors can affect the

rate of dark reversion, including pH, ionic strength, reducing agents, metal ion concentration, and phosphorylation state of select serine residues on the holoprotein. Dark reversion plays a role in the ratio between P_r and P_{fr}, thereby contributing to the overall regulation of phytochrome signaling.

When exposed to red light, most higher plant phytochromes translocate from the cytoplasm to the nucleus, where they interact with transcription factors to regulate the expression of light-responsive genes (Galvao and Fankhauser, 2015). Phytochromes influence gene expression in several other ways; alternative splicing of pre-mRNA resulting in the production of distinct transcripts (Shikata et al., 2014), and distinct transcription start sites (TSSs) from a single locus may be selected in a light-dependent manner, yielding multiple transcripts (Oh & Montgomery, 2017). Phytochrome-dependent alternative promoter selection results in truncated isoforms of some light response gene products. These isoforms translocate to different regions of the cell and vary in function, adding to the complexity of light-regulated responses in plants.

Phytochrome Structure

Phytochrome is a chromoprotein, consisting of an approximately 125-kDa apoprotein covalently linked to an open-chain tetrapyrrole chromophore, phytochromobilin (Lagarias and Rapport, 1980) via a thioether linkage to a cysteine residue in the conserved GAF domain. Photoconversion of phytochromes is triggered primarily by red light and enabled by a Z-E isomerization about the C15-C16 double bond between the C and D porphyrin rings of the linear tetrapyrrole

(Andel, et al., 1996; Kneip et al., 1999), resulting in a conformational change of the apoprotein backbone. P_{fr} can be converted back to P_r slowly by dark reversion or much more quickly by far-red light irradiation (Fankhauser, 2001). The amounts of each phytochrome isoform can be distinguished by difference spectrophotometry.

The chromophore, phytochromobilin (PΦB) is synthesized in plastids and is derived from a heme branch of the chlorophyll biosynthetic pathway (Elich et al., 1989). A heme oxygenase catalyzes the opening of the heme tetrapyrrole ring to convert heme into biliverdin IXα (Davis, 1999). PΦB synthase reduces biliverdin to 3(Z)- PΦB; subsequently PΦB isomerase converts 3(Z)- PΦB to 3(E)- PΦB (the linear phytochromobilin; Li et al., 2011). After being exported from the plastid into the cytosol, phytochromobilin is then autocatalytically attached to the phytochrome apoprotein to form holo-PHY (Verma & Shekhawat, 2013). Mutations *hy1* and *hy2* in *Arabidopsis* dramatically reduce levels of PΦB, consequently inhibiting phytochrome-dependent photomorphogenesis by all five members of this receptor family (Parks & Quail, 1991). HY1 and HY2 genes code for enzymes involved in PΦB synthesis: heme oxygenase and PΦB synthase, respectively (Parks & Quail, 1991; Kohchi et al., 2001).

The canonical higher plant phytochrome apoprotein possesses an NTE-PAS-GAF-PHY (P1-P4) N-terminal region (~70kDa) connected by a flexible hinge to a C-terminal region composed of the PAS –related domains (PRD) and a histidine kinase-related domain (HKRD; ~55 kDa). The N-terminal extension (NTE) domain is unique to plant phytochromes (Fankhauser, 2001), while the PAS, GAF and PHY domains are found in phytochrome homologs in various organisms. Similarly, the C-

terminus PRD is unique to plants whereas HKRD is found in phytochromes of a broad range of organisms (Rockwell, 2006).

As described above, PΦB attaches autocatalytically to the apoprotein GAF domain. The PAS domains can function as protein-protein interaction platforms or co-factor binding domains (Taylor and Zhulin, 1999). The C-terminal PRD (PAS-related domains are required for interaction with phytochrome signaling partners (Fankhauser, 2001; Taylor and Zhulin, 1999). Domain swapping between PHYA and PHYB, along with mutation and deletion analyses indicates the N-terminus is responsible for photosensory functions, while the C-terminus is responsible for most signal transduction functions (Wagner et al., 1996; Quail, et al., 1997).

Fluence, Fluence rates, and 'Modes of action'

Phytochrome-mediated responses are determined largely by the quantity of photons absorbed, a function of total fluence, and by the fluence rate. Fluence is the number of photons falling upon a surface and is measured in micromoles of photons per m^2**,** whereas fluence rate is the number of photons falling upon a surface per unit time and is typically measured in μmol m^{-2} s^{-1}. Phytochrome responses are classified into three 'modes of action' based on the light conditions that elicit a response: Very Low Fluence Responses (VLFR), Low Fluence Responses (LFR) and High Irradiance Responses (HIR).

Both VLFR and LFR are inductive responses, as the effects of an appropriate light pulse continue in subsequent darkness. VLFR can be induced with a minuscule amount of light, approximately 10^{-6} to 10^{-2} μmol m^{-2}, which may convert less than

0.02% of the total phytochrome to P_{fr}. As the P_{fr} is never converted to P_r fully in far-red light, with the maximum conversion ratio being approximately 97% P_r and 3% P_{fr}, VLFR are not photoreversible. Seed germination in *Arabidopsis*, inhibition of mesocotyl growth in oat seedlings, and anthocyanin synthesis are examples of VFLRs (Cone et al., 1985; Clough et al., 1995; Drumm and Mohr, 1974). VLFR are potentiated by the high concentration of type I phytochromes such as phyA in etiolated *Arabidopsis* (Casal et al., 1997; Janoudi et al., 1997).

In general, LFRs require fluences of 1.0 μmol m^{-2} to 1000 μmol m^{-2}, and are photoreversible by subsequent far-red light. In order to elicit a response, a critical threshold of P_{fr} conversion must be met and maintained until the escape period (after which FR light can no longer reverse the response) is exceeded (Li et al., 2011). Both VLFR and LFR follow the law of reciprocity and are dependent on total fluence, regardless of the excitatory fluence rate. Therefore, if the fluence rate is low, longer light exposure is required to elicit a response. Conversely, a response can be induced by higher light intensity with limited exposure time.

High irradiance responses (HIR) require irradiation with relatively high-energy light (>1000 μmol m^{-2}) 100 times higher fluence rate than LFR, for an extended period of time (Schäfer, 1975). Therefore, the law of reciprocity does not apply. HIRs include apical hook opening (Shichijo et al., 2010) in lettuce or slowed hypocotyl growth in *Sinapis alba* (Holmes et al., 1982). HIR are subdivided into two classes: R-HIRs are induced with continuous red light, and continuous far-red light initiates FR-HIRs. In *Arabidopsis*, phyA is chiefly responsible for FR-HIRs, while phyB is primarily responsible for R-HIRs (Quail, 1997).

Localization

Upon photoactivation, the inactive P_r conformation converts into the active P_{fr} form and is translocated from the cytosol into the nucleus, where phytochrome directly interacts with transcription factors to mediate gene expression of light-responsive genes (Kircher et al., 1999; Ni et al., 1999; Zhu et al., 2000). In the fern *Adiantum capillus-veneris*, *Ac*phy2-GUS fusion proteins were shown to translocate from the cytosol to the nucleus in spores after microbeam irradiation with red light (Tsuboi et al., 2012). The same study concluded *Ac*PHY2 is responsible for red-light-dependent spore germination.

Yeast two-hybrid screens have identified phy-interacting nuclear proteins, notably phytochrome-interacting transcription factor 3 (PIF3), a member of the basic helix-loop-helix transcriptional regulator super family (Ni et al., 1998). *In vitro* interaction assays show PIF3 binds to phyB and phyA after photoconversion to the active P_{fr} form (Ni et al., 1999; Zhu et al., 2000). PIF3 is constitutively located in the nucleus and binds to a G-box DNA sequence (CACGTG) commonly found in light-regulated promoters (Martinez-Garcia et al., 2000). PhyB can bind specifically and photoreversibly to a G-box-associated PIF3, indicating PIF3's role in recruiting phytochrome to genes controlled by light-regulated promoters (Martinez-Garcia et al., 2000). Through RNA-blot analysis of PIF3-deficient *Arabidopsis* seedlings, Martinez-Garcia and colleagues (2000) observed a reduced expression of two photoresponsive genes - Circadian Clock-Associated Protein 1 (CCA1) and Late

Elongated Hypocotyl (LHY) - both of which possess G-box sequences within their promoters.

Signal Transduction

The currently accepted model of plant photomorphogenesis suggests that many phytochrome-mediated responses require transduction pathways with secondary signaling factors. In angiosperms, a number of phytochrome signaling factors have been described. A phytochrome-signaling pathway has yet to be ascertained in ferns. To date, several homologues of angiosperm phytochrome signaling factors have been identified in the model *Ceratopteris richardii*, and our laboratory has identified several putative signaling candidates based on yeast two-hybrid analysis, including a PP2A-class protein phosphatase and a COP1 (constitutively photomorphogenic 1) homolog.

PP2A

A common signaling mechanism is reversible protein phosphorylation catalyzed by dueling protein kinases and protein phosphatases. The association between phytochrome and PP2A exemplifies one current model for reversible protein phosphorylation in phytochrome signaling.

Phytochromes act as light-regulated Ser/Thr specific protein kinases (Yeh and Lagarias, 1998; Fankhauser, 2000), which are capable of auto-phosphorylating at distinct serine residues: Ser-7, Ser-17, and Ser-598 in *Arabidopsis* phyA (Yeh and Lagarias, 1998; Lapko et al., 1999), resulting in slight conformational changes

impacting interactions with downstream signaling factors (Park et al., 2000). In particular, phosphorylation at Ser-598 is critical for light regulated auto-phosphorylation and kinase activity of downstream factors (Fankhauser et al., 1999). Ser-598 is selectively phosphorylated in the active P_{fr} form (Park et al., 2000).

In flowering plants, phytochrome-mediated flowering times are modulated by direct phytochrome interaction with FyPP (flower-specific, phytochrome-associated protein phosphatase) a member of the PP2A family (Kim et al., 2002). FyPP in *Arabidopsis* is a serine/threonine specific protein phosphatase and associates with phytochrome to remove a phosphate from Ser/Thr within the phytochrome hinge region in a light dependent manner (Kim et al., 2002).

The PP2A catalytic subunit, FyPP, binds to phytochrome C-terminal domain of PHYA and PHYB in *Pisum, Avena,* and *Arabidopsis,* preferentially binding to P_{fr} and phosphorylated forms (Kim et al., 2002). P_{fr} and phosphorylated phytochrome result from red-light activation, indicating FyPP is regulated by light wavelength via the phytochrome $P_r \leftrightarrow P_{fr}$ photoconversion. FyPP has been shown to regulate flowering time in *Avena* by dephosphorylating PHYA in a light-dependent manner (Kim et al., 2002).

Bissoondial (2005) identified a PP2A catalytic domain homolog, *Cr*PP2A, sequence using BLAST of the model fern *Ceratopteris richardii* EST library sequences (GenBank) and previously characterized phytochrome-associated PP2A sequences (*Arabidopsis, Oryza, Adiantum*). At the protein level, *Cr*PP2A was found to be more closely related to the phytochrome-associating PP2A/FyPP family rather

than other protein phosphatases, suggesting conservation of PP2A-phytochrome kinase/phosphatases relationship. Yeast two-hybrid analysis determined that *Cr*PP2A interacts with the C- terminal domain of *Cr*PHY1 and *Cr*PHY2— but not with *Cr*PHY4A— *in vitro* (Bissoondial, 2005), consistent with the PP2A-phytochrome interaction observed in flowering plants.

Phytochrome Interacting Factors

PIFs (Phytochrome Interacting Factors) are bHLH domain-containing transcription factors that largely act as negative regulators of photomorphogenesis by repressing light-responsive genes and maintaining skotomorphogenesis in the absence of light-activated phytochrome (Leivar and Quail, 2011; Leivar and Monte, 2014). Photoactivation results in phytochrome translocation to the nucleus where it acts with other kinases (CK2, BIN2, PPK1- PPK4) to phosphorylate PIF1/3. E3 ligases recognize and ubiquitinate PIF, which is then degraded via the COP9 complex/26S proteasome pathway, alleviating repression of light-responsive genes and thereby triggering photomorphogenesis and suppressing skotomorphogenesis (Xu et al., 2015). In the case of PIF1, degradation is induced by the ubiquitin E3 ligase complex CUL4/COP1/SPA in light (Zhu et al., 2015).

PIF3, the first PIF identified using yeast two-hybrid screening, primarily negatively regulates de-etiolation (Ni et al., 1998; Kim et al., 2003; Monte et al., 2004; Leivar et al., 2008). PIF3, like PIF1, represses chlorophyll biosynthesis and photosynthesis in etiolated seedlings (Stephenson et al., 2009; Shin et al., 2009; Kim et al., 2003) and chloroplast greening (Monte et al., 2004). PIF3 also modulates

light-dependent ethylene-mediated hypocotyl elongation (Zhong et al., 2012; Jiang et al, 2017).

HY5

Another transcription factor, HY5, is rapidly transcribed under the influence of activated PHYA in Arabidopsis (Osterlund and Deng, 2000). Elevated levels of HY5 then activate transcription of G-box-containing genes involved in photomorphogenesis (Chattopadhyay et al., 1998). A HY5-like bZIP transcription factor was isolated in *C. richardii* in a PCR amplification using degenerate primers, although it remains uncertain whether this partial sequence corresponds to an actual HY5 functional homolog (Bissoondial, 2005).

COP1

COP1 (constitutively photomorphogenic 1) has been shown to possess E3 ubiquitin-ligase activity over itself and other interacting proteins. In flowering plants, COP1 directly interacts with HY5, HFR1, and LAF1, initiating recognition and degradation by the COP9 signalsome (CSN) proteasome complex (Osterlund et al., 2002; Seo et al., 2003; Sailjo et al., 2003**;** Holm et al., 2002). In darkness, COP1 is found in the nucleus where it mediates ubiquitination of COP1, PHYA, LAF1, HFR1, and HY5 (Osterlund et al., 1999**;** Seo et al., 2004). In continuous light, COP1 is expelled from the nucleus to the cytosol, allowing for the accumulation of target transcription factors in the nucleus to induce photomorphogenesis (Osterlund et al., 1999; Subramanian et al., 2004; Quail, 2002). Osterlund and Deng (2001) showed

that the light-regulated subcellular localization of COP1 is influenced by PHYA, PHYB and CRY1 in white light or by far-red, red, or blue light, respectively. Together, these findings indicate the nuclear/cytoplasmic localization of COP1 is integral to the phytochrome signal transduction pathway and provides a possible intermediate between phytochrome and cryptochrome signaling.

A COP1 homolog designated *Cr*COP1 was isolated in *Ceratopteris* from cDNA using primers specific to previously described COP1 in higher plants, and found to be 77% identical to *At*COP1. In transient RNAi knockdowns of *Cr*COP1, more cell files in the basal and subapical region of etiolated gametophytes were observed. Knockdowns also had elevated levels of chlorophyll and accelerated sexual maturation under exposure of continuous white light (Bissoondial, 2005). These results indicate that much like *At*COP1 in *Arabidopsis*, *Cr*COP1 acts to negatively regulate photomorphogenesis. The exact role of *Cr*COP1 in negative regulation in photomorphogenesis is not yet understood.

Ceratopteris richardii as a model organism

In recent years there has been an increased interest in understanding the developmental genetics of ferns as more commercial and industrial uses are recognized. Studies have looked into the possible pharmacological properties of ferns based on their use in traditional medicines as painkillers, laxatives or antibacterials. Other fern phytochemical secondary metabolites such as steroid and phenolic compounds have been investigated for possible pharmacological applications, as well as those with toxic and carcinogenic properties (Teai, et al.,

2010). Phytoremediation is yet another field in which ferns may be employed. The arsenic hyperaccumulator fern species *Pteris vittata* is currently used to bioconcentrate and thereby reduce arsenic in contaminated soils (Lampis et al., 2015). Additionally, the aquatic fern *Azolla* and its nitrogen-fixing cyanobacteria symbiont, *Anabaena azollae*, are widely used as a natural fertilizer in rice production (Lumpkin & Plucknett, 1980). Academically, ferns are of particular interest to understand the evolutionary link between non-vascular plants and seed-bearing plants.

A foundational methodology of plant developmental genetics involves the study of transgenic lines to test gene function *in planta*. Early attempts using *Agrobacterium tumefaciens* to create transgenics in fern achieved only very low efficiencies (Muthukumar et al., 2013). Studies using particle bombardment on intact developing gametophyte tissue have only proven effective at producing transient transformation. However, a recently published method using microparticle bombardment of undifferentiated sporophyte callus tissue is reportedly highly efficient in producing fern transgenics in both *Ceratopteris richardii* and *Ceratopteris thalictroides* (Plackett et al., 2015).

Ceratopteris richardii has emerged as a model for molecular biology experimentation because of its short lifecycle and the ability to use RNA interference (RNAi) to reduce gene expression of targeted genes during spore imbibition (Renzaglia et al., 1995; Hickok, et al., 1995; Bissoondial, 2005). Previous RNAi studies on *Ceratopteris* suggest that cryptochromes, a family of photoreceptors sensitive to blue light, are involved in the regulation of spore germination and

gametophyte development (Banks, 1999). In flowering plants, cryptochromes are known to mediate various light responses, including entrainment of circadian rhythms, de-etiolation, and flower induction (Cashmore et al., 1999). Several angiosperm cryptochrome signaling pathways exhibit an interconnected relationship with red/far-red sensing phytochromes to mediate photomorphogenesis (Neff & Chory, 1998; Mas et al., 2000; Somers et al., 1998). Prior to our work, full-length phytochromes had yet to be isolated and described in *Ceratopteris richardii*.

Ceratopteris richardii is an annual aquatic fern that grows in tropical and subtropical regions. As a model organism *C. richardii* is easy to cultivate and has a relatively short life cycle. Both the gametophyte and sporophyte generations are typically completed within 4 months. Additionally, *C. richardii* sporophytes can produce over a million spores to be stored as an axenic sample for ongoing cultivation (Salmi et al., 2011).

Fern gametophyte development

Since the Cretaceous, angiosperms have come to predominantly fill the canopy to dominate terrestrial ecosystem. As a result, ferns have evolved and fill diverse understory niches (Schneider et al., 2004). Leptosporangiate ferns, which include *Ceratopteris richardii,* underwent tremendous diversification in response to the rise of the angiosperms (Schuettpelz and Pryer, 2009). In the shadow of the angiosperm-dominated canopy, light perception is crucial to plant survival. The red:far-red light ratio is an indicator of competition, canopy density and available

photosynthetically active radiation (PAR). Therefore, understanding fern phytochromes is critical to understanding fern ecology and evolution.

In a given species of ferns, photomorphogenic responses vary between the gametophyte (an independent photosynthetic and generally haploid sexual generation), and the sporophyte (a usually diploid vegetative generation). While many individuals of the diploid generation possess a fully homozygous diploid version of the same genome as the preceding haploid generation because of the high occurrence of self-fertilization, the development of haploid generation of ferns, and that of many lower plants, appears to be more drastically affected by light than is the sporophyte (Furuya, 1983). Therefore, much of the field of study on fern photobiology focuses on the development of the gametophyte generation.

The timing of spore germination is pivotal in the role of the fern's survival, as it must correspond with suitable seasonal and light conditions**.** Following imbibition, light is the major determining factor in breaking spore dormancy. Light conditions required for spore germination vary among species**.** Fern spore dormancy is broken by imbibition with water, followed by exposure to white or red light. Germination induction with a red light pulse is reversible by a subsequent pulse of far-red light (Cooke et al., 1987). This photoreversibility of spore germination is a defining characteristic of responses mediated by phytochromes, and indicates the presence of functional phytochrome within un-germinated spores. However, imbibed spore germination is not immediately inducible by red light, suggesting a requirement for *de novo* synthesis or activation of phytochrome in newly hydrated spores. Red-light responsive phytochrome, P_r, is

spectrophotometrically evident only after 3 days of dark imbibition in *Lygodium japonicum* (Tomizawa et al., 1983). Additionally, red-light induced germination in *L. japonicum* is inhibited in the presence of gabaculine, an inhibitor of phytochrome chromophore synthesis (Manabe et al., 1987). Collectively, these data suggest that an active collection of phytochrome holoprotein, and thereby phytochrome-mediated germination, is only established following spore imbibition, as opposed to light acting on a pre-existing phytochrome pool in dormant spores.

Germination is photoreversibly inhibited by subsequent far-red light exposure (Sugai & Furuya, 1967). Red light-induced spore germination is also inhibited by subsequent exposure to blue or near-ultraviolet radiation in *Ceratopteris* (Cooke et al., 1987). In fact, blue light radiation prior to or following red light induction inhibits spore germination in *C. richardii* (Cooke et al., 1995; Bissoondial, 2005; Mohamed 2005). These findings suggest spore germination is mediated by both phytochrome and blue light photoreceptors, acting through separate but convergent pathways.

Fern spores have a physiological polarity that is defined by monolete or trilete markings at the spore apex. At germination, the spore ruptures at these markings and the haploid spore cell undergoes an asymmetrical division. Before the first mitotic division, the spore nucleus migrates to one pole of the spore where the apical marking is located. The cell undergoes its first division, and as a result one large cell remains within the spore coat, and a smaller daughter cell forms outside of the spore coat rupture point (Banks, 1999).

The smaller apical cell differentiates into the elongated, single-celled rhizoid, which acts to anchor the gametophyte and absorb nutrients. The larger basal cell develops into the protonemal initial in some ferns or the prothallus initial in other ferns such as *Ceratopteris*. The protonemal initial divides into three cells. The cell most distal to the spore casing divides at a 45-degree angle, while the other two cells undergo periclinal divisions. The mature gametophyte further develops into a one-cell thick, two-dimensional, heart-shaped prothallus with generally fewer than 1000 cells.

Fern gametophyte development is dependent on the quality and quantity of light exposure. For instance, protonemata grow longer and thinner under far-red or very low fluence white light than protonemata grown in blue or high irradiance white light. In *Adiantum* protonemata grow toward a light source, and *Pteris* protonemata elongate under red light (Ito, 1969; Mohr and Miller 1961). Conversely, *Adiantum* protonemal elongation is inhibited under darkness, blue or white light conditions, and instead results in a swollen apical region (Murata and Wada, 1989) whereas *Ceratopteris richardii* undergoes prothallus elongation in both darkness and under far-red light (Mohamed, 2005). Light-mediated protonemal growth in *Adiantum* results from the arrangement of cortical microtubules and cellulose microfibrils in "tip-growing" protonemal cells. A linearly growing protonema under red light exhibits microfibrils perpendicular to the cell's axis in the subapical region and randomly arranged at the apex. Blue light irradiated protonemata exhibit apical swelling and have a random arrangement of microfibrils and microtubules within the protonemal subapical region (Murata and Wada, 1989).

Together these observations indicate the protonemal/prothallar growth is determined by microtubule and microfibril arrangement and is photoreceptor mediated.

Once mature, prothalli develop sex organs. Male prothalli develop antheridia for sperm production. Hermaphrodite prothalli develop both antheridia and egg-producing archegonia. As homosporous ferns, sex is determined by the pheromone antheridiogen produced by early-maturing hermaphrodite prothalli. High concentrations of antheridiogen trigger male gametophyte formation by modifying the gibberellin biosynthesis pathway (Tanaka et al., 2014). Males form under high antheridiogen conditions and hermaphrodites under low concentrations.

The formation of archegonia is known to be partially under phytochrome control. Archegonia formation is favored by a pulse of red light, and is photoreversible with subsequent far-red light irradiation (Kamachi et al., 2004), but sexual development is not strictly light-regulated. In the *C. richardii dkg1* mutant, characterized by germination in darkness, archegonia develop at an occurrence of 52% in prothalli grown in darkness, while wild-type prothalli are absent of archegonia when grown in the dark (Kamachi et al., 2004; Mohamed, 2005), although this may be an indirect result of overall lower levels of cellular and tissue development in darkness.

Many parallels can be inferred between the photobiology of angiosperms and ferns, particularly regarding red and far-red light-mediated photomorphogenesis. While the influences of light on fern germination and gametophyte development are well document, a comprehensive model for understanding the molecular pathways

of fern photomorphogenesis has yet to be determined. In order to gain a better understanding of light-mediated gametophyte development and phytochrome signal transduction in *C. richardii,* the following studies into the function of *Cr*PHY2A—and a possibly novel phytochrome detected in the course of these studies—were conducted in the context of addressing the roles of all the *Ceratopteris richardii* phytochromes, as well as the other photoreceptors and the signal transduction mechanisms involved in their control of morphogenesis, physiology, and gene expression in this model fern.

Materials and Methods

Spore sterilization and germination

One bulk tube (approximately 40,000-50,000) wild-type (RNWT1) *Ceratopteris richardii* (C-Fern®**)** spores (Carolina Biological Supply Company, NC) were sterilized with 20% commercial bleach (v/v, for a final sodium hypochlorite concentration of 1.05%) for 3 minutes followed by three one-minute rinses using sterile deionized water. Spores were then suspended in 1ml dH_2O, then 150µl aliquots of the suspension were distributed into each of seven 50 ml Falcon® tubes containing 30 ml of Parker-Thompson *C-Fern* ® medium (Carolina Biological), prepared according to manufacturer's instructions and brought to pH 6.0 using 1.0 N NaOH. The spores were placed in absolute darkness for at least one week, followed by incubation in white light for one day to induce germination, and then transferred to far-red light (far-red LED lights in a Percival LED30 incubator filtered

through a far-red Plexiglas cutoff filter, yielding approximately 50 μmol m^{-2} s^{-1}) for 6 days of growth at 28°C.

Isolation of mRNA

RNA was isolated from gametophyte prothalli using solid-phase extraction with the RNeasy® Plant Mini Kit (Qiagen, Valencia, CA) using the following protocol: *C. richardii* prothalli were filtered from the Parker-Thompson medium using a sterile 250μm nylon mesh filter. Gametophytes were weighed, flash-frozen, and pulverized with a mortar and pestle chilled in liquid nitrogen. While frozen, 100 mg of powdered plant tissue was transferred to 1.5 ml Eppendorf tubes. Then, 450 μl of extraction buffer RLT (proprietary, Qiagen) per 100 mg tissue was added to the sample and vortexed. The lysate was then transferred to the QIAshedder spin column and centrifuged for 2 minutes at maximum speed (approximately 13,000 x *g*). Next, 0.5 volume of ethanol (100%) was added to and mixed with the lysate. The lysate, including precipitate, was then added to an RNeasy mini spin column and centrifuged for 15 seconds at 13,000 x *g*. Next, 700 μl of Buffer RW1 (proprietary, Qiagen) was added to the column which was then centrifuged at 10,000x *g* for 15 seconds. Then, 500 μl of Buffer RPE (proprietary, Qiagen) was added and the column was centrifuged at 13,000 x *g* for 2 minutes. The column was placed in a new collection tube and 30 μl of RNase-free water was added to elute RNA from the column by centrifugation for 1 minute at 8,000 x *g*.

After RNA recovery, concentration was measured using a Nanodrop™ spectrophotometer (with one A_{260} optical density unit corresponding to 40 μg/ml of

RNA and A_{260}/A_{280} ratio of 1.94) and qualified via gel electrophoresis on a 1%agarose/37% formaldehyde gel containing 0.5 μg/ml ethidium bromide at 60 volts and compared to Hyperladder1 DNA ladder (Bioline). RNA isolate was aliquoted and stored frozen at -80°C until needed.

cDNA synthesis

Total cDNA corresponding to the purified RNA template was synthesized using the Superscript™ First-Strand Synthesis System for reverse transcription (Invitrogen). First, each reagent was mixed and briefly centrifuged. The thermal cycler was preheated to 65°C. At 4°C, the following components were mixed in a thin-walled 0.2 ml PCR tube: 5μl of the purified RNA sample corresponding to between 1 and 5 μg total RNA, 1μl 50μM oligo $(dT)_{20}$ primer, 1μl annealing buffer, and 1μl DEPC-treated H_2O. The reaction was incubated in the thermal cycler at 65°C for 5 minutes, then immediately placed on ice for no less than 1 minute. The contents of the tube were collected by brief centrifugation, as some of the reaction condensed on the tube walls. In a separate tube the following components were combined in this order in to prepare a 2X reaction mixture; 2μl 10X RT Buffer, 4μl 25mM $MgCl_2$, 2μl 0.1M DTT, 1μl RNaseOUT™ (40U/μl). Then, 9μl of the 2X reaction were gently mixed with the RNA/Primer reaction. The reaction was incubated at 42°C for 2 minutes. Then, 1μl SuperScript II RT™ was added. The reaction was then left to proceed at 42°C for 50 minutes. The reaction was terminated by placing the tube in a 70°C water bath for 15 minutes. The reaction was then chilled on ice, collected by brief centrifugation, and incubated with 1μl of RNase H for 20 minutes

at 37°C. The reaction was immediately used for gene-specific PCR and unused portions were frozen at -20°C for short-term storage or -80°C for longer storage.

PCR

A double-stranded PCR product of *Cr*PHY2 was synthesized using the total cDNA as template. Primers were designed using the ApE program (A Plasmid Editor, Utah) based on a previous *Cr*PHY2 sequence (Bissoondial, 2005). In a thin walled PCR tube the following components were combined in the following order in a 50 µl reaction: 32 µl nuclease-free water, 10 µl 5x PrimeSTAR® GXL buffer (TaKaRa), 4 µl dNTP mixture, 0.2 µM forward *Cr*PHY2 coding region primer (including a leading CACC sequence for use with the topoisomerase-based cloning vector pENTR D-TOPO): CACC ATG TCC TCC AAA ACC ATG ACG TAC TC, 0.2 µM reverse *Cr*PHY2 coding region primer: GGA ATC AGT CTT CTT GAA AAG GGG TAG ATC, 1µl *Cr*PHY2 cDNA template and 1µl TaKaRa/Clontech PrimeSTAR® GXL DNA Polymerase (Figure 1). The reaction took place in a Bio-Rad T100™ Thermal Cycler under the following conditions: Denaturation at 95°C for 3 minutes, followed by 35 cycles of 95°C denaturation for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 3.5 minutes, and finished with a final 5 minute 68°C extension step. The reaction was immediately used for cloning and/or stored at -20°C.

Cloning

The *Cr*PHY2 PCR product was directionally cloned into Gateway pENTR™ Directional TOPO® vector (Invitrogen) and transformed into MACH1™ competent

cells (Invitrogen) according to the manufacturer's instructions. Putative transformants were grown on LB plates with 1% agar supplemented with 10mg/ml of kanamycin. Colonies were screened by PCR using the same forward and reverse *Cr*PHY2 coding region primers used to amplify *Cr*PHY2 from cDNA. Colonies with the appropriate amplified insert size were grown in liquid LB-kanamycin at 37°C 18-24 hours and the plasmids isolated using PureYield™ Plasmid Miniprep System (Promega) and inserts were sequenced by Macrogen, Inc. (Rockville, MD) using universal M13 primers corresponding to vector sequences flanking the insert and internal coding region primers. Sequence alignments and analyses were determined using Basic Local Alignment Search Tool (BLAST) https://blast.ncbi.nlm.nih.gov/Blast.cgi and ApE, A plasmid Editor.

Results

To date, six phytochrome cDNAs, designated *Cr*PHY1A, *Cr*PHY1B, *Cr*PHY2A, *Cr*PHY2B, *Cr*PHY4A, and *Cr*PHY4B, have been identified in *Ceratopteris richardii*. In this study, an effort was made to confirm the sequence for *Cr*PHY2A. Ultimately, the isolation and confirmation of a definitive *Cr*PHY2A proved elusive. However the resulting phytochrome sequence isolated may indicate the presence of a previously uncharacterized phytochrome in *Ceratopteris*.

Initially, RNA isolation from *Ceratopteris* prothalli proved difficult. After numerous attempts using various methods, using the RNeasy® Plant Mini Kit (Qiagen) provided sufficient quality and quantity of RNA isolate for cDNA synthesis.

Multiple confirmed PCR products were isolated from *Ceratopteris*-derived cDNA using coding region primers based on Bissoondial (2005) *Cr*PHY2A sequence. These findings confirm that *Cr*PHY2A is expressed as mRNA in 7-day-old (1 day in white light, 6 day in far-red light) *Ceratopteris* gametophyte prothalli. Each PCR product matched the expected size for *Cr*PHY2A as determined by 1% agarose gel electrophoresis as a single band, approximately 3,500 bp. (Figure 2)

Cloning

Three separately produced *Cr*PHY2 PCR products (labeled A, V, and X) were selected for independent verification of the *Cr*PHY2 gene sequence. Each PCR product was inserted into a pENTR/D-TOPO vector (Invitrogen) (Figure 3). Bacterial transformation was conducted using MACH1™ competent cells (Invitrogen). Early trials using Top10™ competent cells resulted in very low numbers of colonies. MACH1™ competent cells provided numerous colonies to select colonies with the proper *Cr*PHY2 vector insert.

Plasmid isolation

Although a number of plasmid isolation methods and manufactured kits were used, none resulted in the desired concentration or purity of plasmid isolate. Ultimately, the multiple minipreps isolated using the Promega PureYield™ plasmid miniprep system were combined, lyophilized and re-eluted to yield sufficient concentrations for sequencing.

For each colony chosen for sequencing the vector insert was confirmed by gel electrophoresis analysis of PCR products obtained using the *Cr*PHY2 coding region primers and plasmid isolate as a template (Figure 4).

Successful transformations were determined by colony growth on Luria-Bertani (LB) medium containing 50µg/ml kanamycin, thereby confirming the transformation of vector containing the kanamycin resistance gene KanR2.

Sequences

Most samples sent for sequencing (Macrogen, Maryland) resulted in low quality sequences. A single sample provided a high fidelity sequence, *Cr*PHY2_A4 (Figure 5). However, This sample's sequence showed high sequence alignment with *Ceratopteris richardii* PHY1 (97% identity for 959bp) (Bissoondial, 2005) and with *Ceratopteris thalictroides* PHY1 (97% identity for 959bp) (Figures 6 & 7). To rule out the possibility I had isolated a previously isolated *C. richardii* PHY1, I ran PCR using *Cr*PHY1 coding region primers (provided by May Moe, Short Lab) on the isolated plasmid with insert *Cr*PHY2_A4. No product appeared on a 1% agarose gel. Furthermore, the *Cr*PHY2 primers used in this study did not match any sequence found in *Cr*PHY1 sequence.

When aligned with the previous isolated *Cr*PHY2 sequence (Bissoondial, 2005), the isolated partial sequence (1144bp) for this study, designated *Cr*PHY2_A4 (sequenced from M13R primer) aligns with only a 69% identity match (Figure 8). This may suggest that this sequence is indeed isolated from a unique phytochrome

gene within *C. richardii.* This would indicate that the sequence isolated in this study might be a third paralog of *Cr*PHY1.

In contrast, the previous isolated *Cr*PHY2 sequence (Bissoondial, 2005) does not align with any PHY1 sequence with as much fidelity as the sequence isolated in this study. In fact, the previously isolated *Cr*PHY2 sequence (Bissoondial, 2005) only received a 69% identity match when aligned with the sequence for *C. richardii* PHY1. Additionally this sequence for *Cr*PHY2 (Bissoondial, 2005) did not match with the aforementioned PHY1 homologs from either *Adiantum capillus-veneris* or *Ceratopteris thalictroides*. Instead, the Bissoondial *Cr*PHY2A/B sequences align with high similarity with PHY2 mRNA (complete/partial cds) for *Ceratopteris thalictroides* (Accession KT071864.1) and *Adiantum capillus-veneris* (Accession ABO016232.1) sequences reported in Genbank, with identity matches of 99% and 83% respectively. This further suggests that the isolated sequence in this study is not in fact the previously isolated *Cr*PHY2 (Bissoondial, 2005), and unlikely a PHY2 homolog of other ferns species.

Discussion

Isolated sequence

Based on previous isolation of both *Cr*PHY2 and *Cr*PHY1 (Bissoondial, 2005), both genes are approximately the same length (~3,500bp), making discrimination of PCR products from mRNA impossible. Furthermore, the primers used to isolate *Cr*PHY2 (*Cr*PHY2_A4) in this study were not found to match any sequence found

within the *Cr*PHY1 sequence. The question remains as to how the sequence isolated in this study matches the sequence for *Cr*PHY1 with 97% identity.

One possible explanation is the primers, while showing low affinity for any particular region within the *Cr*PHY1 sequence, were still able to amplify *Cr*PHY1 from the *C. richardii* mRNA. However, this does not explain why the PHY2 primers designed for this study were unable to amplify *Cr*PHY1 PCR product from *C. richardii* RNA.

Another possibility is contamination of either the primers used or the assumed *Cr*PHY2 PCR product itself. At the time in which this study was taking place a parallel study investigating the sequence of *Cr*PHY1 also took place. The close spatial and temporal relationship of the two studies may have provided the opportunity of cross contamination of phytochrome sequence even though all reasonable precautions were taken to ensure no such contamination would occur. However, this does not explain why the PHY2 primers were then able to produce a PCR product from the pENTR vector insert.

Finally, it is likely that the isolated sequence in this study represents a previously unidentified phytochrome found in *Ceratopteris richardii*. The implications of this would suggest this sequence, (based on its high percentage alignment with *Cr*PHY1) may represent a previously undiscovered paralog to *Cr*PHY1. If the isolated sequence were indeed a paralog, this would imply the two *Cr*PHY1 genes could have resulted from a duplication event. In order to test this hypothesis, conclusive sequences of this possible novel *Cr*PHY1 paralog must be isolated and identified. If it were to be determined that *Cr*PHY2_A4 (the 1144bp

partial sequence isolated in this study) represents an independent sequence paralogous to *Cr*PHY1, this gene would be designated as *Cr*PHY1C. The next steps would be to determine the function of *Cr*PHY1C by conducting overexpression and knockdown trials.

Furthermore, BLAST (NCBI) analysis of the isolated sequence (partial) in this study most highly aligns with PHY1 homologues in *Adiantum capillus-veneris* (mRNA, complete cds) (77% identity) and *Ceratopteris thalictroides* (mRNA, partial cds) (97% identity) as well as aligning with other partial Polypodiopsida fern PHY1 mRNA homologs to a lesser extent (*Lonchitis hirsuta* 67%, *Vittaria appalachiana* 70%, *Asplenium platyneuron* 70%, *Plagiogyria japonica*, 68%, *Blechnum spicant* 68%, *Pilularia globulifera* 68%). Providing further evidence that the sequence isolated in this study may be a paralog to *Cr*PHY1.

Recently, an independent *Cr*PHY2 sequence with 5 nucleotide mismatches from the Bissoondial (2005) *Cr*PHY2 sequence has been isolated. This sequence has been isolated using a difference set of primers than those used in this experiment.

Phytochrome Localization

It has yet to be determined whether *Ceratopteris* phytochromes undergo nuclear translocation upon exposure to red and/or far-red. In *Adiantum*, imbibed spores were subjected to red and/or far-red microbeam irradiation to determine localization of PHY:GUS fusion proteins. It was determined that *Ac*PHY2 translocates to the nucleus much like in flowering plants, however *Ac*PHY1 does not (Tsuboi et

al., 2012). Given that *Ac*PHY1 and *Cr*PHY1, and that *Ac*PHY2 and *Cr*PHY2 are homologous gene pairs, respectively, it is hypothesized that *Cr*PHY2 and *Cr*PHY1 will behave in a similar manner to their *Adiantum* orthologs. *Cr*PHY2 is expected to translocate to the nucleus under red light irradiation and *Cr*PHY1 will not. As the isolated sequence in this experiment is believed to be a paralog to *Cr*PHY1A, tentatively designated *Cr*PHY1C, it would not be expected to translocate. In order to study phytochrome localization in *Ceratopteris* the isolated phytochrome genes will be recombined into destination vectors pMDC43 and pMDC83 (Marks Gateway™ Vectors), resulting in PHY:GFP fusion proteins with GFP fused at the 3' or 5', respectively, after recombination. GFP fluorescence microscopy will indicate location of phytochrome before and after red and/or far-red light exposure. Localization studies of *Cr*PHY1 are currently underway in the Short lab.

Light-Mediated Expression: Germination

Once *Cr*PHY2 localization is determined, the future research efforts will explore the light-mediated effects of *Cr*PHY1A, *Cr*PHY1C, *Cr*PHY2A, *Cr*PHY4A and *Cr*PHY4B overexpression in *C. richardii*. Currently, sequences for *Cr*PHY1 and *Cr*PHY4A have been confirmed and are being inserted into destination vectors (pMDC83 and pMDC43) for particle bombardment.

A previous study in the Short lab examined RNAi phytochrome knockdowns and found altered phenotype expressions in phytochrome mRNA levels, germination rates, cell division, rhizoid production and chlorophyll accumulation. Spores were incubated with dsRNA of either *Cr*PHY1, *Cr*PHY2, *Cr*PHY4B or a

mixture of the three to reduce expression levels of corresponding mRNA *in vivo* (Bissoondial, 2005). This study found *Cr*PHY2 expression was reduced to the highest degree while *Cr*PHY1 was reduced to a lesser extent. The difference in expression reduction is believed to be a consequence of the constitutively low level of *Cr*PHY2 expression.

Interestingly, spores incubated with dsRNA *Cr*PHY4B or the dsRNA combination of *Cr*PHY1, *Cr*PHY2, and *Cr*PHY4B showed elevated levels of mRNA expression for these phytochromes. It is possible that *Cr*PHY4B plays a role in negatively regulating *Cr*PHY1, *Cr*PHY2 and *Cr*PHY4B itself. Alternatively, it is possible the lower concentration of these phytochrome proteins via post-transcriptional downregulation leads to increased phytochrome transcription. A similar elevated expression in *Cr*PHY1C would be expected if this experiment were to be repeated.

None of the aforementioned knockdowns lowered *Ceratopteris* germination rates in white or blue light (Bissoondial, 2005). Bissoondial (2005) concluded that *Ceratopteris* phytochromes act redundantly in germination induction or that a very low threshold of a given phytochrome is required for spore germination, and that RNAi is insufficient to reduce the specified phy transcript below the threshold. At this time it is unclear whether *Cr*PHY1C plays a redundant role in germination or other phytochrome-mediated responses, to those of *Cr*PHY1. Further investigation into the gene expression of *Cr*PHY1C is required.

Ceratoptersis richardii wild-type spore germination rates increase after 7-10 day imbibition in darkness followed by red or white light exposure (Cook et al.,

1987). Action spectra indicating the amount of phytochrome increases during imbibition and dark incubation (Cooke, 1993). These findings were supported by Bissoondial's observations that found increases in *Cr*PHY1, *Cr*PHY2, and *Cr*PHY4B transcripts in imbibed spores. Similar findings were observed in the fern *Anemia phyllitidis (*Wada et al., 1997*).* It would appear that phytochrome transcription is initially activated by imbibition of spores independent of light.

The *Ceratopteris* single-nuclear gene mutant *dkg1* exhibits germination in individuals grown in darkness, but germination is inhibited by white light (Cooke et al., 1993). Bissoondial (2005) showed elevated expression levels of *Cr*PHY1, *Cr*PHY2, and *Cr*PHY4B in *dkg1* spores imbibed for 5 days of darkness compared to wild-type spores. DKG1 protein is believed to act pleiotropically in phytochrome signaling, including inhibition of dark germination (Kamachi et al., 2004)**.** Presumably, overexpression of phytochrome will only further amplify germination rates in dark-incubated *dkg1* mutants if there is any change in rate at all. However it is yet to be determined if overexpression of *Cr*PHY2, *Cr*PHY1, *Cr*PHY4B or a combination of the three in a wild-type individual would result in a similar phenotype as a *dkg1* mutant, providing further evidence for phytochrome-mediated germination in *Ceratopteris*.

In *Adiantum* spore germination, specifically the first mitotic division can be initiated by brief irradiation with red light and is photoreversible with far-red light and photo-irreversibly with blue light (Furuya et al., 1997). The same study found *Adiantum* spore germination can also be induced with blue light micro-beam irradiation only when directed at the nucleus. These findings suggest both

phytochrome and probably cryptochrome are involved in regulation of fern spore germination.

RNAi treatments of phytochromes proved ineffective in manipulating spore germination in blue light. Earlier studies in *Adiantum* indicated cryptochromes might play a role in blue light inhibition of germination in ferns. Bissoondial (2005) conducted dsRNA *Cr*CRY4 knockdown treatments resulting in blue-light germination increasing three-fold compared to controls. These observations suggest that in *Ceratopteris Cr*CRY4 plays an antagonistic role to phytochrome in spore germination through a yet to be defined pathway.

Prothalli development

Spores treated with either dsRNA of *Cr*PHY4B or the dsRNA combination of *Cr*PHY1 *Cr*PHY2 and *Cr*PHY4B resulted in prothalli with more cell division and rhizoid production (Bissondial, 2005). These results may be the consequence of elevated levels of phytochrome mRNA (and presumably phytochrome proteins) seen in these RNAi trials as discussed earlier. No significant effect was observed in the dsRNA *Cr*PHY1 or *Cr*PHY2 treatments, reinforcing the hypothesis that the phytochrome family in *Ceratopteris richardii* may act redundantly, not only to regulate germination, but subsequent prothalli development as well.

In *Adiantum,* germination (first mitotic division) is induced by red light and inhibited by blue and far-red light. However, blue light promotes subsequent cell divisions and development of the protonema, while red light inhibits development (Furuya, 1997). While inconclusive, there are parallel reports of red-light limiting

gametophyte growth in *Ceratopteris* and far-red light promoting elongation of subapical cells. In *Ceratopteris*, white and blue light produce the typical heart-shaped prothallus. It appears phytochrome and cryptochrome regulate prothalli growth patterns. Adequate light conditions for photosynthesis following germination and subsequent prothalli growth in order to seek appropriate light conditions would be expected to provide a selective advantage for survival. In other words, sub-optimal photosynthetic conditions (far-red, dark) induce growth that would bring tissues into more photosynthetically appropriate light, while adequate photosynthetic conditions (red, blue, white light) shift limiting resources from energy-costly cell elongation to increased light-capturing photosynthetic structures.

Chlorophyll Production

Angiosperm chlorophyll synthesis is induced by red light. Seedlings grown in far-red light fail to produce chlorophyll due to a PHYA-mediated block of protochlorophyllide oxidoreductase transcription (Schoefs, 2000). Ferns and gymnosperms on the other hand are capable of producing chlorophyll in the dark. Bissoondial found that chlorophyll production was markedly lower, but not absent, in *Ceratopteris richardii* gametophytes grown under far-red, red light and dark conditions. In *Ceratopteris richardii*, chlorophyll accumulation and rhizoid production corresponds to cell division. Cell division occurs at higher rates under conditions in which photoconversion favors higher P_{fr} concentrations, i.e. red, blue and white light. In angiosperm seedlings the inability to produce chlorophyll in far-red light is irreversible and results in death. While far-red light or dark grown *C.*

richardii undergo increased cell division and chlorophyll production once transferred to red, blue, or white light and can produce sporophytes. The alternate light-mediated pathway for chlorophyll production in ferns provides a select advantage for life in the light-filtered environment of the forest floor where many ferns grow.

Incubation of spores with a combination of dsRNA for *Cr*PHY1, *Cr*PHY2 and *Cr*PHY4B (yielding an unexpected increase in phytochrome transcription) resulted in an increase in chlorophyll accumulation (Bissoondial, 2005), reinforcing the evidence for phytochrome-dependent positive regulation of chlorophyll production within prothalli. While chlorophyll in ferns is present in the absence of light, light-mediated production of additional chlorophyll would provide a select advantage in allocating resources for photosynthesis. Once *Cr*PHY1C has been confirmed and the gene product isolated, it will be imperative to determine its possible role in chlorophyll production in *C. richardii*. Additionally, overexpression trials of *C. richardii* phytochrome, including *Cr*PHY1C, may determine if each member of the gene family acts redundantly to increase chlorophyll accumulation or if there are nuances among the phytochromes.

In *Ceratopteris,* chlorophyll biosynthesis is also regulated by blue light. *Cr*CRY4 RNAi knockdowns result in larger prothalli and increased chlorophyll when compared with controls. Additionally, gametophytes of *Cr*COP1 RNAi knockdowns resulted in three-fold higher chlorophyll accumulation over control trials (Bissoondial, 2005). Together these results may indicate *Ceratopteris* chlorophyll biosynthesis could be regulated by both phytochrome and cryptochrome via COP1

interactions. A possible method of testing this would be to observe overexpression of *Cr*COP1 in *Ceratopteris* to determine whether there is a reduction in chlorophyll and then introduce an inducible *Cr*CRY4 gene to see if it results in rescue of the control phenotype.

Chlorophyll a/b-binding (CAB) protein is an essential component in chlorophyll function. In *Adiantum*, CAB is transcriptionally activated by red light and suppressed with blue light irradiation (Christensen & Silverthrone, 2001). The *Ceratopteris* CAB protein has a 69% identity to the *Arabidopsis* CAB protein (Bissoondial, 2005). In *Arabidopsis*, absolute levels of CAB mRNA are repressed by COP1 in darkness (Deng et al., 1991; Kim et al., 2002). It would be of interest to determine if light-mediated chlorophyll accumulation in *Ceratopteris* is regulated by a possible conserved pathway involving phytochrome, cryptochrome, COP1 and CAB, similar to that of *Arabidopsis.* Further work into *C. richardii* phytochrome, including the phytochrome sequence isolated in this study, is required to determine the phytochrome-mediated cellular pathways that regulate chlorophyll production in ferns. Protein-protein interaction assays can be undergone to determine possible relationships between phytochrome, cryptochrome, CAB and COP1 regarding chlorophyll production regulation.

Interacting factors

*Cr*PP2A

The parallels between the biology of *Cr*PP2A in *Ceratopteris* and FyPP in higher plants suggest parallels in the function of *Cr*PP2A and FyPP in phytochrome-

mediated photomorphogenesis. *Cr*PP2A protein more closely resembles FyPP than other PP2A homologs that do not interact with phytochrome (Bissoondial, 2005). Additionally, *Cr*PP2A and FyPP interact with the C-terminal region of *Cr*PHY1/2 and PHYA/B, respectively (Bissoondial, 2005; Kim et al., 2002). Future studies will investigate if there is a parallel interaction between *Cr*PP2A and *Cr*PHY1C . Similarities between *Cr*PP2A to FyPP indicate a possibly conserved mode of phytochrome regulation through dephosphorylation by *Cr*PP2A. Future work must be conducted in order to determine if *Cr*PP2A functions to dephosphorylate phytochrome (including *Cr*PHY1C) in *Ceratopteris*, or whether interaction with phytochromes regulates activity of *Cr*PP2A in dephosphorylating other targets that have not yet been identified.

In *Arabidopsis*, FyPP is primary expressed in the floral organs and acts to mediate flowering initiation in a phytochrome dependent manner. In *Ceratopteris*, archegonia formation is also known to be regulated by phytochrome. By subjecting *Ceratopteris* gametophytes to RNAi or overexpression of *Cr*PP2A and *Cr*PHY1A/1C/2/4A/4B by way of particle bombardment and observing possible archegonia formation acceleration or delay may provide insight into the possible role of the PP2A family in sex organ development in plant evolution.

*Cr*COP1

In *Arabidopsis*, COP1 interacts with PHYA, PHYB, CRY1 and CRY2 (Osterlund and Deng, 2001) as well as with photomorphogenesis regulatory transcription factors including HY5 (Saijo et al., 2003). COP1 has been shown to regulate hypocotyl length in a light-dependent manner (Von Arnim & Deng, 1994). In

darkness, the presence of COP1 in the nucleus corresponds with elongated hypocotyl length, a trait of etiolation. Conversely, under conditions of white, or blue, and to a lesser degree far-red, or red light COP1 is expelled from the nucleus. Under these conditions *Arabidopsis* seedlings express shorter hypocotyl lengths, a trait of de-etiolation (Osterlund and Deng, 2001). Consistent with these findings Osterlund and Deng (2001) also determined that in far-red and blue light phyA is capable for COP1 nuclear exclusion, primarily in red light (also in white and far-red) phyB is capable for COP1 nuclear exclusion, and in blue light CRY1 is capable for COP1 nuclear exclusion. COP1 maintains skotomorphogenesis via negatively regulating photomorphogenesis through proteasome degradation of components in phytochrome and cryptochrome signaling pathways (Ling et al., 2017). In turn COP1 is regulated by light-activated photoreceptors.

RNAi trials of *Cr*COP1 result in *Ceratopteris* gametophytes with increased cell files in the basal and subapical region, de-etiolated traits in etiolated gametophytes. As determined by RNAi, *Cr*COP1 is involved with the regulation of chlorophyll accumulation and prothalli maturation; traits that Bissoondial (2005) showed are at least partly light-mediated. These results indicate that much like COP1 in *Arabidopsis*, *Cr*COP1 may act to negatively regulate photomorphogenesis in *Ceratopteris*.

Given the isolation of *Cr*COP1, *Cr*CRY1, and *Cr*CRY2 in *Ceratopteris*, the question remains whether the phytochrome-cryptochrome-COP1 interrelationship modeled in higher plants is evolutionarily conserved in ferns. In order to elucidate the interconnected roles of red/far-red and blue light photoregulation in

Ceratopteris, interactions between *Cr*COP1 and *Cr*CRY1/2 or *Cr*PHY1/1A/2/4A/4B must be determined. Yeast two-hybrid assays or other protein-fragment complementation assays can be used to detect interaction between *Cr*COP1 and phytochromes or cryptochromes in *Ceratopteris*. Alternatively, mutant lines of *Cr*CRY1/2 or *Cr*PHY1/1C/2/4A/4B can be tested for abundance and localization of *Cr*COP1 under varying light conditions. Additionally, transgenic lines for overexpression of *Cr*COP1 can provide further insight in to the light mediated responses mediated by *Cr*COP1.

As *Ceratopteris richardii* is conducive for molecular studies such as RNAi and transformation via particle bombardment, there is a lot of potential for using *C. richardii* as a model for understanding the molecular pathways in ferns**.** Using the well-defined physical aspects of fern photobiology and what is known about the photobiology at the molecular level in angiosperms and non-vascular plants, future investigations using *Ceratopteris* may illuminate the molecular mechanisms involved in fern photobiology The discovery of *Cr*PHY1C adds insight into the greater evolutionary history of phytochrome-mediated photomorphogenesis in ferns.

Figures

Figure 1. Using primers designed based on the *Cr*PHY2 Bisssondial (2005) sequence a PCR fragment was isolated from *Ceratopteris richardii* cDNA. This isolated PCR product measured approximately 3,500 bp on a 1% agarose gel electrophoresis gel.

Sequence Name	Sequence	Start	Length	Tm (°C)	GC%
CrPHY2 Forward Primer	caccATGTCCTCCAAAACCATGACGTACTC	1	26	60	46
CrPHY2 Reverse Primer	GGAATCAGTCTTCTTGAAAAGGGGTAGATC	3248	30	61	43
M13F	GTAAAACGACGGCCAGT		17	53	53
M13R-pUC	CAGGAAACAGCTATGAC		17	47	47
Internal F27	tcctcagccctctaacctctcgcag	27	25	65	60
Internal F744	AGGCGCTCCCTGTTGGCGATATTG	744	24	65	58
Internal F1627	TGCGGCCAAGATAACCACCAGAGAC	1627	25	65	56
Internal F2306	ACAGGGCAGAAGGTGGTGATGGAC	2306	24	65	58
Internal F2582	GCGATGGGTGGACAGGATTCTGAG	2582	24	63	58
Internal F3009	TGGGTAGAGTGATGGATGCTGTG	3009	23	60	52
Internal R378	CCCTCCTCGACTGCAAGCATACATC	378	25	63	56
Internal R999	GGAGCTGCACGGCAATCACAAATC	999	24	63	54
Internal R1749	GGGTGCATCTTCCTGCCATCGTC	1749	23	64	61
Internal R2528	AGCAGGCTAACTCGGAACCAAAGAC	2528	25	63	52
Internal R3320	ACATCTGCTGAACAAGGGCTTCTGG	3320	25	63	52

Figure 2. PCR of *Cr*phy2 isolated from *Ceratopteris richardii* derived cDNA. Measuring approximately 3,500bp (comparison with Hyperladder I).

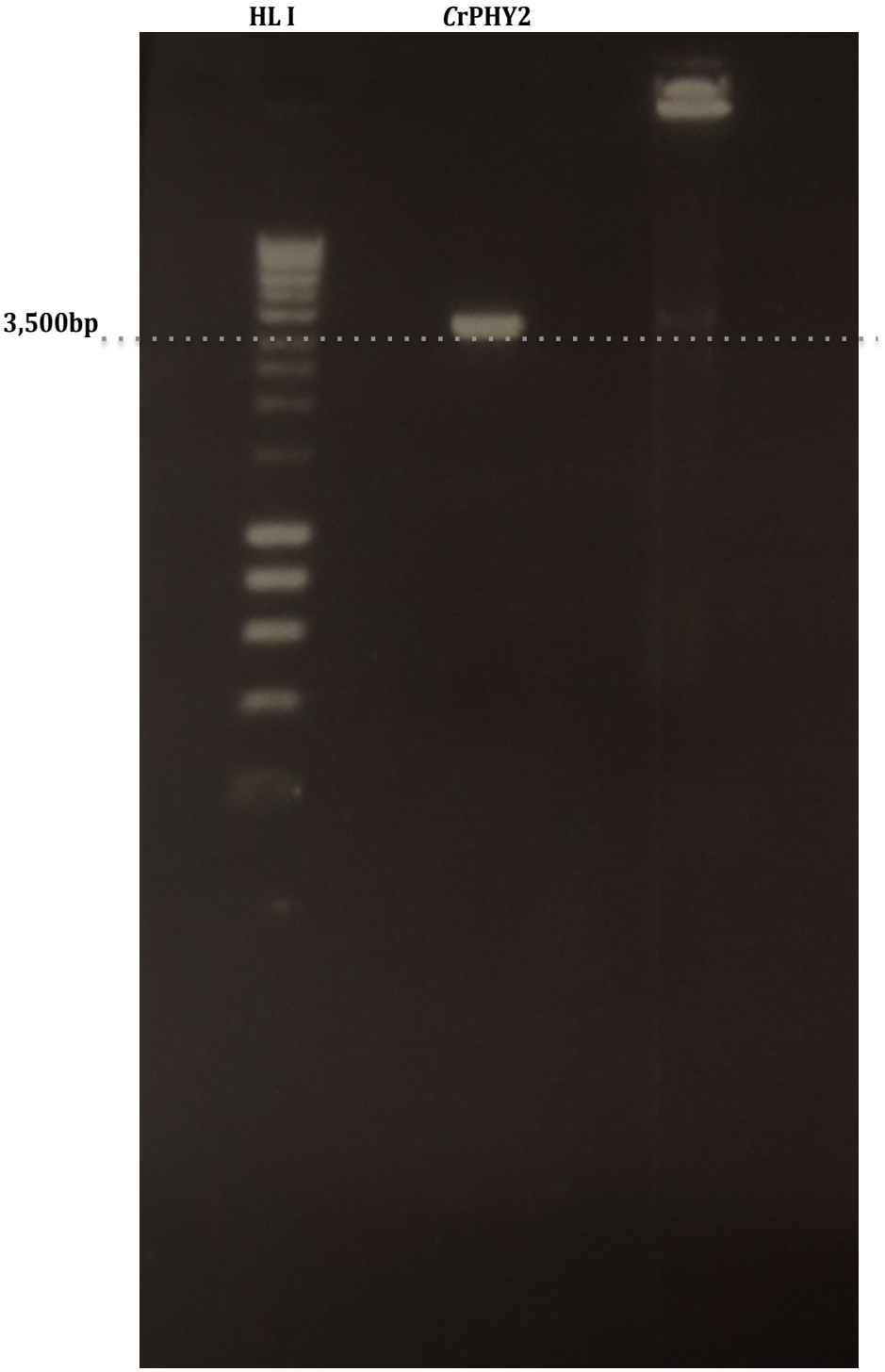

Figure 3. pENTR™/D-TOPO® vector map with *Cr*PHY_A4 insert.

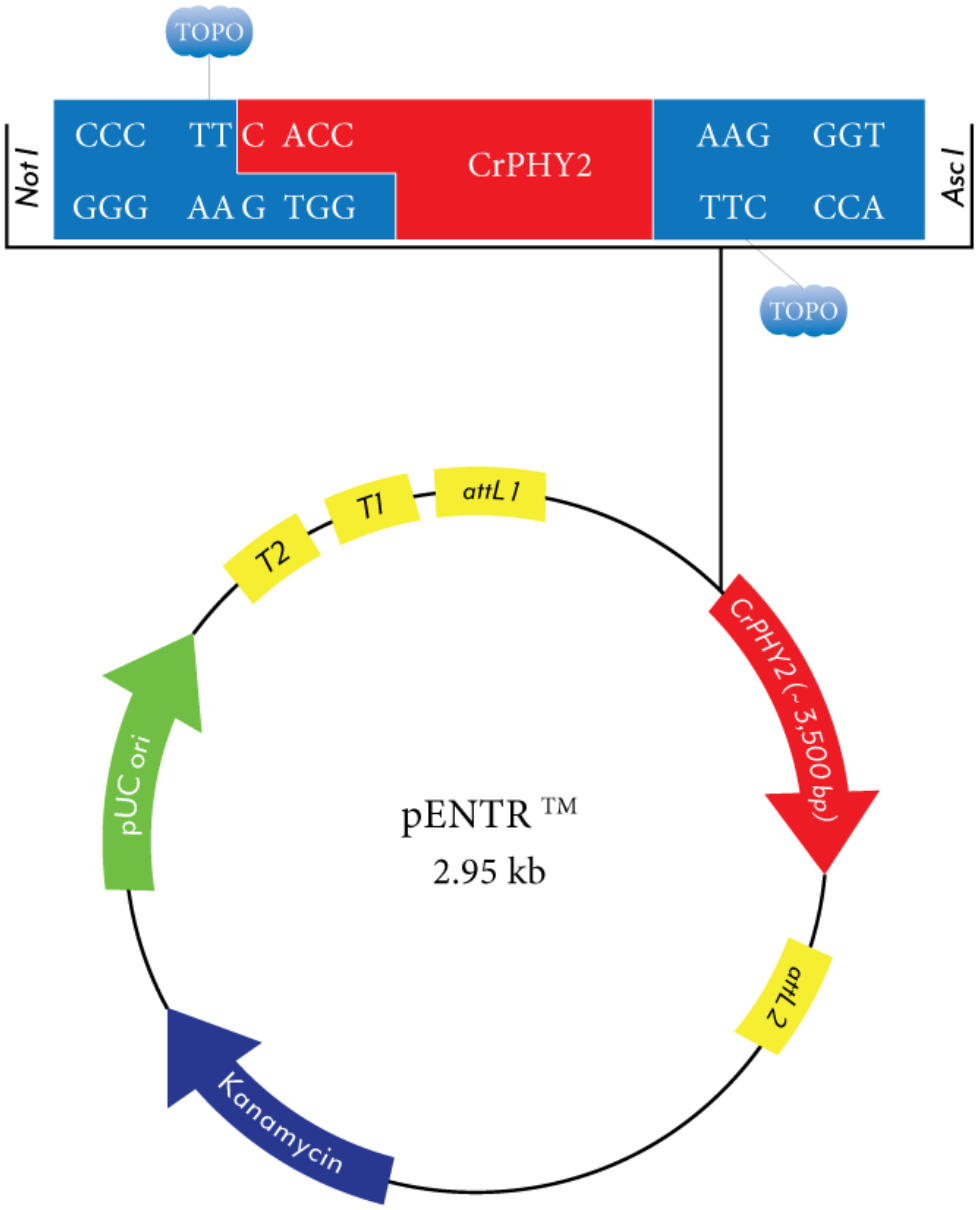

Figure 4. PCR of *Cr*PHY2_A4 isolated from pENTR Vector. Measuring approximately 3,500bp (comparison with Hyperladder I). *Cr*PHY2_A4 selected for sequencing.

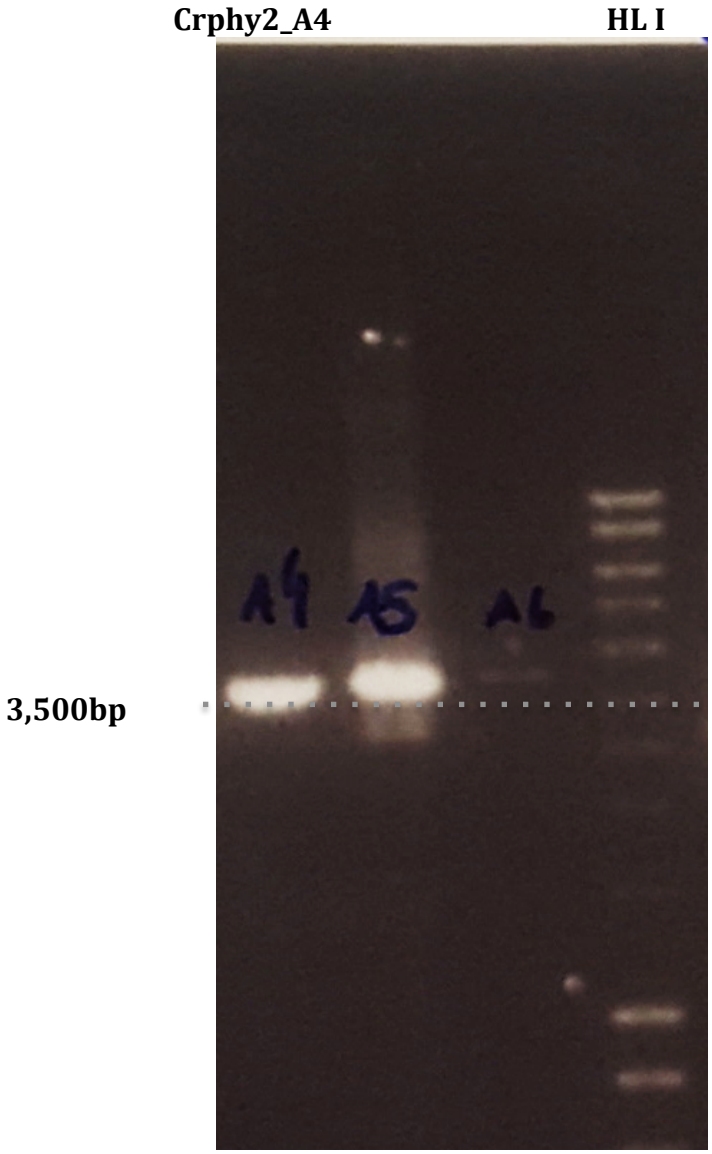

Figure 5. Isolated sequence *Cr*PHY2_A4. Sequenced by Macrogen, Maryland using M13R sequencing primer and lyophilized plasmid isolate.

TCAGCTGGATGGCAATAATGGATTTTATTTTGACNNATAGTGACCTGTTCGTTGCAACAAATTGATAAGC
AATNGCTTTCTTATAATNGCCAACTTTGTACAAGAAAGCTGGGTCGGCGCGCCCACCCTTCACCATGTCGG
ATCCGAAGCAATCCGTATCATCAACGGCTTCGAACATGTCGAAACGTAACCAGAAAATTGCTCAAACATCA
GCAGATGCGAAACTATATAGAGCGTACGAAGAGTCAACCGACTCTGGGTCTTTCGATTACAGCCAGTCTGT
CAATGCAAACAATGAAAAGTTGCCCGGGGAGCTAATTGTGGCTTATCTTCAGCGCATGCAGCGTGGAGGT
CTCATTCAGCCATTCGGGTGTTTGGTTGCAGTGGAAGAAGAATCATTCAAGGTGATTGCATATAGTGAAA
ATGCACGCGAGATGTTGGATGTATCAGTGCAAGCGGTGCCTACGATGGGGAAGTATAGTCGTTTAGATAT
TGGAGTTGATTTTAGGACTCTCTTATCCCCGGCAAGTGCTGCAGCATTGGACCGAGTCATTGGTGTTGTTG
ATGTCAGCATGTTCAATCCTATAACTGTTCAAAGCAGGAGTTCAGCAAAACCATTTTATGCAATTTTGCAC
AGGAATGACGTGGCATTGGTGATTGACCTTGAGCCAATCAGGCCCGACGATACATCTATTGCAGGTGGTGC
TGTGCAGTCCCATAAGTTGGCTGCAAAAGCCATTGCTCGCTTACAATCTTTACCTGGAGGGGACATTGGTC
TCTTGTGTGATGCAGTAGTAAAGGAGGTCCAGGGGCTGACTGGCTGTGACAGAGTAATGGCTTATAAATT
TCACGATGATGAACATGGAAGAGGTGATTGCTGAGATAAGGCNAATCCGATCTTGAGCCTTACATTGGGT
CTACATTACCCAGCTACAGATATCCCACAGGGCTGCACGTTTCCTTTTCATGAAGAACAGANTNNNGAAT
GATCTGTGAACTGCAGAGTATCACCTGGTTAAGCTGGGTTCAGAAACNCGGCATTGAACACAGCCCATGA
AGCTTGACAGGNTNCAACACTTCCTGCACCNACATGGATGCCNTACAAANGNNNNTGANNTAANANGGG
GNTTCCNNANCCTTCTTT

Figure 6. Sequence comparison of *Cr*PHY2_A4 and *Cr*PHY1 coding region conducted by BLAST.

Score	Expect	Identities	Gaps	Strand
1600 bits(1774)	0.0	947/972(97%)	13/972(1%)	Plus/Plus

```
PHY2_A4 135 ATGTCGGATCCGAAGCAATCCGTATCATCAACGGCTTCGAACATGTCGAAACGTAACCAG  194
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PHY1   1    ATGTCGGATCCGAAGCAATCCGTATCATCAACGGCTTCGAACATGTCGAAACGTAACCAG   60

PHY2_A4 195 AAAATTGCTCAAACATCAGCAGATGCGAAACTATATAGAGCGTACGAAGAGTCAACCGAC  254
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
PHY1     61 AAAATTGCTCAAACATCACCAGATGCGAAACTATATAGAGCGTACGAAGAGTCAACCGAC  120

PHY2_A4 255 TCTGGGTCTTTCGATTACAGCCAGTCTGTCAATGCAAACAATGAAAAGTTGCCCGGGGAG  314
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PHY1    121 TCTGGGTCTTTCGATTACAGCCAGTCTGTCAATGCAAACAATGAAAAGTTGCCCGGGGAG  180

PHY2_A4 315 CTAATTGTGGCTTATCTTCAGCGCATGCAGCGTGGAGGTCTCATTCAGCCATTCGGGTGT  374
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PHY1    181 CTAATTGTGGCTTATCTTCAGCGCATGCAGCGTGGAGGTCTCATTCAGCCATTCGGGTGT  240

PHY2_A4 375 TTGGTTGCAGTGGAAGAAGAATCATTCAAGGTGATTGCATATAGTGAAAATGCACGCGAG  434
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PHY1    241 TTGGTTGCAGTGGAAGAAGAATCATTCAAGGTGATTGCATATAGTGAAAATGCACGCGAG  300

PHY2_A4 435 ATGTTGGATGTATCAGTGCAAGCGGTGCCTACGATGGGGAAGTATAGTCGTTTAGATATT  494
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PHY1    301 ATGTTGGATGTATCAGTGCAAGCGGTGCCTACGATGGGGAAGTATAGTCGTTTAGATATT  360

PHY2_A4 495 GGAGTTGATTTTAGGACTCTCTTATCCCCGGCAAGTGCTGCAGCATTGGACCGAGTCATT  554
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PHY1    361 GGAGTTGATTTTAGGACTCTCTTATCCCCGGCAAGTGCTGCAGCATTGGACCGAGTCATT  420

PHY2_A4 555 GGTGTTGTTGATGTCAGCATGTTCAATCCTATAACTGTTCAAAGCAGGAGTTCAGCAAAA  614
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PHY1    421 GGTGTTGTTGATGTCAGCATGTTCAATCCTATAACTGTTCAAAGCAGGAGTTCAGCAAAA  480

PHY2_A4 615 CCATTTTATGCAATTTTGCACAGGAATGACGTGGCATTGGTGATTGACCTTGAGCCAATC  674
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PHY1    481 CCATTTTATGCAATTTTGCACAGGAATGACGTGGCATTGGTGATTGACCTTGAGCCAATC  540

PHY2_A4 675 AGGCCCGACGATACATCTATTGCAGGTGGTGCTGTGCAGTCCCATAAGTTGGCTGCAAAA  734
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PHY1    541 AGGCCCGACGATACATCTATTGCAGGTGGTGCTGTGCAGTCCCATAAGTTGGCTGCAAAA  600

PHY2_A4 735 GCCATTGCTCGCTTACAATCTTTACCTGGAGGGGACATTGGTCTCTTGTGTGATGCAGTA  794
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PHY1    601 GCCATTGCTCGCTTACAATCTTTACCTGGAGGGGACATTGGTCTCTTGTGTGATGCAGTA  660

PHY2_A4 795 GTAAAGGAGGTCCAGGGGCTGACTGGCTGTGACAGAGTAATGGCTTATAAATTTCACGAT  854
            ||| ||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
PHY1    661 GTAGAGGAGGTCCATGGGCTGACTGGCTGTGACAGAGTAATGGCTTATAAATTTCACGAT  720

PHY2_A4 855 GATGAACATGGAAGAGGTGATTGCTGAGATAAGGCNAATCCGATCTTGAGCCTTACATTG  914
            ||||||||||| ||||||||||||||||||||||  ||||||||||||||||||||||
PHY1    721 GATGAACATGG-AGAGGTGATTGCTGAGATAAGGCG-ATCCGATCTTGAGCCTTACATT-  777
```

```
PHY2_A4 915 GGTCTACATTACCCAGCTACAGATATCCCACAGGGCTGCACGTTTCCTTTTCATGAAGAA  974
            |||||||||||||||||||||||||||||| |||||||||||||| ||||||||||||
PHY1    778 GGTCTACATTACCCAGCTACAGATATCCCACA-GGCTGCACGTTTCCCTTTCATGAAGAA  836

PHY2_A4 975 CAGANTNNNGAATGATCTGTGAACTGCAGAGTATCACCTGGTTAAGCTGGGTTCAGAAAC 1034
            |||| |   |||||||||||| |||||||||||||||| |||||||| ||||||| |||
PHY1    837 CAGAGT-GAGAATGATCTGTG-ACTGCAGAGTATCACCT-GTTAAGCT-GGTTCAG-AAC  891

PHY2_A41035 NCGGCATTGAACACAGCCCATGAAGCTTGACAGGNTNCAACACTTCCTGCACCNACATGG 1094
             |||||||| |||||||||||| |||||||||| | ||||||||| |||||| ||||||
PHY1    892 ACGGCATTG-ACACAGCCCATG-AGCTTGACAGGCT-CAACACTTCGTGCACC-ACATGG  947

PHY2_A41095   ATGCCNTACAAA  1106
              ||||| ||||||
PHY1    948   ATGCCATACAAA  959
```

Figure 7. Sequence comparison of *Cr*PHY2_A4 and *Ceratopteris thalictroides* isolate PIVW phytochrome (PHY1) mRNA, partial cds conducted by BLAST.

Score	Expect	Identities	Gaps	Strand
1579 bits(1750)	0.0	941/970(97%)	13/970(1%)	Plus/Plus

```
PHY2_A4 135 ATGTCGGATCCGAAGCAATCCGTATCATCAACGGCTTCGAACATGTCGAAACGTAACCAG  194
            |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
CtPHY1    1 ATGTCGGATCCGAAGCAATCCGTATCATCAACGGCTTCGAACATGTCGAAACGTAGCCAG  60

PHY2_A4 195 AAAATTGCTCAAACATCAGCAGATGCGAAACTATATAGAGCGTACGAAGAGTCAACCGAC  254
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CtPHY1   61 AAAATTGCTCAAACATCAGCAGATGCGAAACTATATAGAGCGTACGAAGAGTCAACCGAC  120

PHY2_A4 255 TCTGGGTCTTTCGATTACAGCCAGTCTGTCAATGCAAACAATGAAAAGTTGCCCGGGGAG  314
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CtPHY1  121 TCTGGGTCTTTCGATTACAGCCAGTCTGTCAATGCAAACAATGAAAAGTTGCCCGGGGAG  180

PHY2_A4 315 CTAATTGTGGCTTATCTTCAGCGCATGCAGCGTGGAGGTCTCATTCAGCCATTCGGGTGT  374
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CtPHY1  181 CTAATTGTGGCTTATCTTCAGCGCATGCAGCGTGGAGGTCTCATTCAGCCATTCGGGTGT  240

PHY2_A4 375 TTGGTTGCAGTGGAAGAAGAATCATTCAAGGTGATTGCATATAGTGAAAATGCACGCGAG  434
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||| ||||
CtPHY1  241 TTGGTTGCAGTGGAAGAAGAATCATTCAAGGTGATTGCATATAGTAAAAATGCACACGAG  300

PHY2_A4 435 ATGTTGGATGTATCAGTGCAAGCGGTGCCTACGATGGGGAAGTATAGTCGTTTAGATATT  494
            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
CtPHY1  301 ATGTTGGATGTATCAGTGCAAGCGGTGCCTACGATGGCGAAGTATAGTCGTTTAGATATT  360

PHY2_A4 495 GGAGTTGATTTTAGGACTCTCTTATCCCCGGCAAGTGCTGCAGCATTGGACCGAGTCATT  554
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CtPHY1  361 GGAGTTGATTTTAGGACTCTCTTATCCCCGGCAAGTGCTGCAGCATTGGACCGAGTCATT  420

PHY2_A4 555 GGTGTTGTTGATGTCAGCATGTTCAATCCTATAACTGTTCAAAGCAGGAGTTCAGCAAAA  614
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CtPHY1  421 GGTGTTGTTGATGTCAGCATGTTCAATCCTATAACTGTTCAAAGCAGGAGTTCAGCAAAA  480

PHY2_A4 615 CCATTTTATGCAATTTTGCACAGGAATGACGTGGCATTGGTGATTGACCTTGAGCCAATC  674
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CtPHY1  481 CCATTTTATGCAATTTTGCACAGGAATGACGTGGCATTGGTGATTGACCTTGAGCCAATC  540

PHY2_A4 675 AGGCCCGACGATACATCTATTGCAGGTGGTGCTGTGCAGTCCCATAAGTTGGCTGCAAAA  734
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CtPHY1  541 AGGCCCGACGATACATCTATTGCAGGTGGTGCTGTGCAGTCCCATAAGTTGGCTGCAAAA  600

PHY2_A4 735 GCCATTGCTCGCTTACAATCTTTACCTGGAGGGGACATTGGTCTCTTGTGTGATGCAGTA  794
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CtPHY1  601 GCCATTGCTCGCTTACAATCTTTACCTGGAGGGGACATTGGTCTCTTGTGTGATGCAGTA  660

PHY2_A4 795 GTAAAGGAGGTCCAGGGGCTGACTGGCTGTGACAGAGTAATGGCTTATAAATTTCACGAT  854
            ||| |||||||||| ||||||||||||||||||||||||||||||||||||||||||||
CtPHY1  661 GTAGAGGAGGTCCATGGGCTGACTGGCTGTGACAGAGTAATGGCTTATAAATTTCACGAT  720
```

```
PHY2_A4 855 GATGAACATGGAAGAGGTGATTGCTGAGATAAGGCNAATCCGATCTTGAGCCTTACATTG  914
            |||||||||||| ||||||||||||||||||||||  ||||||||||||||||||||||
CtPHY1  721 GATGAACATGG-AGAGGTGATTGCTGAGATAAGGCG-ATCCGATCTTGAGCCTTACATT-  777

PHY2_A4 915 GGTCTACATTACCCAGCTACAGATATCCCACAGGGCTGCACGTTTCCTTTTCATGAAGAA  974
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
CtPHY1  778 GGTCTACATTACCCAGCTACAGATATCCCACA-GGCTGCACGTTTCCTTTTCATGAAGAA  836

PHY2_A4 975 CAGANTNNNGAATGATCTGTGAACTGCAGAGTATCACCTGGTTAAGCTGGGTTCAGAAAC 1034
            |||| |   |||||||||||| |||||||||||||||| |||||||| ||||||| |||
CtPHY1  837 CAGAGT-GAGAATGATCTGTG-ACTGCAGAGTATCACCT-GTTAAGCT-GGTTCAG-AAC  891

PHY2_A41035 NCGGCATTGAACACAGCCCATGAAGCTTGACAGGNTNCAACACTTCCTGCACCNACATGG 1094
             | |||||| |||||||||||| |||||||||| | ||||||||| |||||| ||||||
CtPHY1  892 ACAGCATTG-ACACAGCCCATG-AGCTTGACAGGCT-CAACACTTCGTGCACC-ACATGG  947

PHY2_A41095 ATGCCNTACA  1104
            ||||| | ||
CtPHY1  948 ATGCCATGCA  957
```

Figure 8. Sequence comparison of *Cr*PHY2_A4 and *Cr*PHY2 Bissoondial (2005) full sequence conducted by BLAST.

Score	Expect	Identities	Gaps	Strand
253 bits(280)	2e-70	476/687(69%)	22/687(3%)	Plus/Plus

```
CrPHY2   323 GGCTTATCTTCAGCGCATGCAGCGTGGAGGTCTCATTCAGCCATTCGGGTGTTTGGTTGC  382
             ||| || |||||||| |||||||| ||||| ||||| ||||| || || ||| || ||||
PHY2_A4  307 GGCGTACCTTCAGCGTATGCAGCGAGGAGGCCTCATCCAGCCCTTTGGATGTATGCTTGC  366

CrPHY2   383 AGTGGAAGAAGAATCATTCAAGGTGATTGCATATAGTGAAAATGCACGC-GAGATGTTGG  441
             ||| || || | |   |||| ||| ||||| |||||||||||||| ||| |||||| | |
PHY2_A4  367 AGTCGAGGAGGGAAGCTTCAGGGTAATTGCCTATAGTGAAAATGC-CGCAGAGATGCTCG  425

CrPHY2   442 ATGTATCAGTGCAAGCGGTGCCTACGATGGGGAAGTATAG-TCGTT--TAGATATTGGAG  498
             |  |        ||  | ||||||||  |||||| | | || ||  |  | |  || ||
PHY2_A4  426 ACCTCCTTCCTCATTCAGTGCCTACCGTGGGGATGCAGAGCTCAATACTTGGCATAGGTA  485

CrPHY2   499 TTGATTTTAGGACTCTCTTATCCCCGGCAAGTGCTGCAGCATTGGACCGAGTCATTGGTG  558
              ||||  | | ||||||||  | || || |||||||||||| | ||   || |   ||||
PHY2_A4  486 CTGATGCTCGTACTCTCTTTACTCCTGCTAGTGCTGCAGCACTTGAGAAAGCCTCAGGTG  545

CrPHY2   559 TTGTTGATGTCAGCATGTTCAATCCTATAACTGTTCAAAGCAGGAGTTCAGCAAAACCAT  618
             |||||||||   |||| | ||||||||    || ||  || | || ||    || || |
PHY2_A4  546 CTGTTGATGTGTCCATGCTGAATCCTATTCACGTACACTGCCGCAGCTCTAATAAGCCTT  605

CrPHY2   619 TTTATGCAATTTTGCACAGGAATGACGTGGCATTGGTGATTGACCTTGAGCCAATCAGGC  678
             |  |||| ||  | ||| | | ||| || |  || || |||||  | || ||||| ||||
PHY2_A4  606 TCAATGCCATCGTTCACCGCATTGATGTTGGTTTAGTCATTGATTTCGAACCAATTAGGC  665

CrPHY2   679 ---CCGACGATACATCTAT----TGCAGGTGGTGCTGTGCAGTCCCATAAGTTGGCTGCA  731
                | |||| ||  | |||    | | | ||| ||  ||||||| || ||| | |||||
PHY2_A4  666 CTTCTGACG-TAGCTTTATGGGCTACTGCTGGAGCCTTGCAGTCACACAAGCTAGCTGCC  724

CrPHY2   732 AAAGCCATTGCTCGCTTACAATCTTTACCTGGAGGGGACATTGGTCTCTTGTGTGATGCA  791
             ||||||||  |  | |||||  |  | ||||  || || |||| ||| |||||||||  |
PHY2_A4  725 AAAGCCATATCCAGATTACAGGCGCTCCCTGTTGGCGATATTGATCTTTTGTGTGATTCT  784

CrPHY2   792 GTAGTAAAGGAGGTCCAGGGGCTGACTGGCTGTGACAGAGTAATGGCTTATAAATTTCAC  851
             || ||  | || ||    | | | ||||||| ||| ||||| |||||||||||||| ||
PHY2_A4  785 GTTGTCGAAGAAGTGAGAGAGTTAACTGGCTATGATAGAGTCATGGCTTATAAATTCCAT  844

CrPHY2   852 GATGATGAACATGGAAGAGGTGATTGCTGAGATAAGGCNAA--TCCGATCTTGAGCCTTA  909
             || || ||||| ||  || ||  | || || |||||   ||  ||||| || ||||| ||
PHY2_A4  845 GAAGACGAACACGG-TGAAGTTTTGGCCGAAATAAG---AAGGTCCGACCTGGAGCCATA  900

CrPHY2   910 CATTGGGTCTACATTACCCAGCTACAGATATCCCACAGGGCTGCACGTTTCCTTTTCATG  969
              ||||| || ||||| || |||||||||||||| ||||  | || |||| |||||||||
PHY2_A4  901 -TTTGGGCCTGCATTATCCTGCTACAGATATCCCTCAGGCGTCCAGGTTT-CTTTTCATG  958

CrPHY2   970 AAGAACAGANTNNNGAATGATCTGTGA  996
             ||||| ||  |   | ||||| |||||
PHY2_A4  959 AAGAATAGGGTTAGG-ATGATTTGTGA  984
```

References

Andel, F., Lagarias, J. C., & Mathies, R. A. (1996). Resonance Raman analysis of chromophore structure in the lumi-R photoproduct of phytochrome. *Biochemistry*, *35*(50), 15997-16008.

Casal, J. J., Sanchez, R. A., & Yanovsky, M. J. (1997). The function of phytochrome A. *Plant, Cell & Environment*, *20*(6), 813-819.

Cashmore, A. R., Jarillo, J. A., Wu, Y. J., & Liu, D. (1999). Cryptochromes: blue light receptors for plants and animals. *Science*, *284*(5415), 760-765.

Chattopadhyay, S., Ang, L. H., Puente, P., Deng, X. W., & Wei, N. (1998). Arabidopsis bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. *The Plant Cell*, *10*(5), 673-683.

Christensen, S., & Silverthorne, J. (2001). Origins of phytochrome-modulated Lhcb mRNA expression in seed plants. *Plant physiology*, *126*(4), 1609-1618.

Cooke, T. J., Hickok, L. G., VANDERWOUDE, W. J., Banks, J. A., & Scott, R. J. (1993). Photobiological characterization of a spore germination mutant dkgl with reversed photoregulation in the fern Ceratopteris richardii. *Photochemistry and photobiology*, *57*, 1032-1041.

Cooke, T. J., Hickok, L. G., & Sugai, M. (1995). The fern Ceratopteris richardii as a lower plant model system for studying the genetic regulation of plant photomorphogenesis. *International Journal of Plant Sciences*, *156*(3), 367-373.

Cooke, T. J., Racusen, R. H., Hickok, L. G., & Warne, T. R. (1987). The photocontrol of spore germination in the fern Ceratopteris richardii. *Plant and cell physiology*, *28*(5), 753-759.

Deng, X. W., Caspar, T., & Quail, P. H. (1991). cop1: a regulatory locus involved in light-controlled development and gene expression in Arabidopsis. *Genes & development*, *5*(7), 1172-1182.

Devlin, P. F., Robson, P. R., Patel, S. R., Goosey, L., Sharrock, R. A., & Whitelam, G. C. (1999). Phytochrome D acts in the shade-avoidance syndrome in Arabidopsis by controlling elongation growth and flowering time. *Plant Physiology*, *119*(3), 909-916.

Elich, T. D., McDonagh, A. F., Palma, L. A., & Lagarias, J. C. (1989). Phytochrome chromophore biosynthesis. Treatment of tetrapyrrole-deficient Avena explants with natural and non-natural bilatrienes leads to formation of spectrally active holoproteins. *Journal of Biological Chemistry*, *264*(1), 183-189.

Fankhauser, C. (2001). The phytochromes, a family of red/far-red absorbing photoreceptors. *Journal of Biological Chemistry*, *276*(15), 11453-11456.

Fankhauser, C. (2000, December). Phytochromes as light-modulated protein kinases. In *Seminars in cell & developmental biology* (Vol. 11, No. 6, pp. 467-473). Academic Press.

Furuya, M. (1983). Photomorphogenesis in ferns. In *Photomorphogenesis* (pp. 569-600). Springer, Berlin, Heidelberg.

Furuya, M., & Song, P. S. (1994). Assembly and properties of holophytochrome. In *Photomorphogenesis in plants* (pp. 105-140). Springer, Dordrecht.

Furuya, M., Kanno, M., Okamoto, H., Fukuda, S., & Wada, M. (1997). Control of mitosis by phytochrome and a blue-light receptor in fern spores. *Plant Physiology*, *113*(3), 677-683.

Hickok, L. G., Warne, T. R., & Fribourg, R. S. (1995). The biology of the fern Ceratopteris and its use as a model system. *International Journal of Plant Sciences*, *156*(3), 332-345.

Holmes, M. G., Beggs, C. J., Jabben, M., & Schäfer, E. (1982). Hypocotyl growth in Sinapis alba L: the roles of light quality and quantity. *Plant, Cell & Environment*, *5*(1), 45-51.

Ito, M. (1969). Light-induced synchrony of cell division in the protonema of the fern, Pteris vittata. *Planta*, *90*(1), 22-31.

Janoudi, A. K., Gordon, W. R., Wagner, D., Quail, P., & Poff, K. L. (1997). Multiple phytochromes are involved in red-light-induced enhancement of first-positive phototropism in Arabidopsis thaliana. *Plant Physiology*, *113*(3), 975-979.

Kamachi, H., Matsunaga, E., Noguchi, M., & Inoue, H. (2004). Novel mutant phenotypes of a dark-germinating mutant dkg1 in the fern Ceratopteris richardii. *Journal of plant research*, *117*(2), 163-170.

Kim, D. H., Kang, J. G., Yang, S. S., Chung, K. S., Song, P. S., & Park, C. M. (2002). A phytochrome-associated protein phosphatase 2A modulates light signals in flowering time control in Arabidopsis. *The Plant Cell*, *14*(12), 3043-3056.

Kim, J., Yi, H., Choi, G., Shin, B., Song, P. S., & Choi, G. (2003). Functional characterization of phytochrome interacting factor 3 in phytochrome-mediated light signal transduction. *The Plant Cell*, *15*(10), 2399-2407.

Kim, Y. M., Woo, J. C., Song, P. S., & Soh, M. S. (2002). HFR1, a phytochrome A-signalling component, acts in a separate pathway from HY5, downstream of COP1 in Arabidopsis thaliana. *The Plant Journal*, *30*(6), 711-719.

Kircher, S., Kozma-Bognar, L., Kim, L., Adam, E., Harter, K., Schäfer, E., & Nagy, F. (1999). Light quality–dependent nuclear import of the plant photoreceptors phytochrome A and B. *The Plant Cell*, *11*(8), 1445-1456.

Kneip, C., Hildebrandt, P., Schlamann, W., Braslavsky, S. E., Mark, F., & Schaffner, K. (1999). Protonation state and structural changes of the tetrapyrrole chromophore during the Pr→ Pfr phototransformation of phytochrome: a resonance Raman spectroscopic study. *Biochemistry*, *38*(46), 15185-15192.

Kohchi, T., Mukougawa, K., Frankenberg, N., Masuda, M., Yokota, A., & Lagarias, J. C. (2001). The Arabidopsis HY2 gene encodes phytochromobilin synthase, a ferredoxin-dependent biliverdin reductase. *The Plant Cell*, *13*(2), 425-436.

Lagarias, J. C., & Rapoport, H. (1980). Chromopeptides from phytochrome. The structure and linkage of the Pr form of the phytochrome chromophore. *Journal of the American Chemical Society*, *102*(14), 4821-4828.

Lampis, S., Santi, C., Ciurli, A., Andreolli, M., & Vallini, G. (2015). Promotion of arsenic phytoextraction efficiency in the fern Pteris vittata by the inoculation of As-resistant bacteria: a soil bioremediation perspective. *Frontiers in plant science*, *6*, 80.

Lapko, V. N., Jiang, X. Y., Smith, D. L., & Song, P. S. (1999). Mass spectrometric characterization of oat phytochrome A: isoforms and posttranslational modifications. *Protein Science*, *8*(5), 1032-1044.

Leivar, P., & Quail, P. H. (2011). PIFs: pivotal components in a cellular signaling hub. *Trends in plant science*, *16*(1), 19-28.

Li, J., Li, G., Wang, H., & Wang Deng, X. (2011). Phytochrome Signaling Mechanisms. The Arabidopsis Book / American Society of Plant Biologists, 9**,** e0148.

Ling, J. J., Li, J., Zhu, D., & Deng, X. W. (2017). Noncanonical role of Arabidopsis COP1/SPA complex in repressing BIN2-mediated PIF3 phosphorylation and degradation in darkness. *Proceedings of the National Academy of Sciences*, *114*(13), 3539-3544.

Lumpkin, T. A., & Plucknett, D. L. (1980). Azolla: botany, physiology, and use as a green manure. *Economic Botany*, *34*(2), 111-153.

Más, P., Devlin, P. F., Panda, S., & Kay, S. A. (2000). Functional interaction of phytochrome B and cryptochrome 2. *Nature*, *408*(6809), 207.

Martínez-García, J. F., Huq, E., & Quail, P. H. (2000). Direct targeting of light signals to a promoter element-bound transcription factor. *Science*, *288*(5467), 859-863.

Möglich, A., Yang, X., Ayers, R. A., & Moffat, K. (2010). Structure and function of plant photoreceptors. *Annual review of plant biology*, *61*, 21-47.

Mohamed, A. (2005). *Photomorphogenesis in the model fern Ceratopteris richardii*. City University of New York.

Monte, E., Tepperman, J. M., Al-Sady, B., Kaczorowski, K. A., Alonso, J. M., Ecker, J. R., ... & Quail, P. H. (2004). The phytochrome-interacting transcription factor, PIF3, acts early, selectively, and positively in light-induced chloroplast development. *Proceedings of the National Academy of Sciences*, *101*(46), 16091-16098.

Murata, T., & Sugai, M. (2000). Photoregulation of asymmetric cell division followed by rhizoid development in the fern Ceratopteris prothalli. *Plant and Cell Physiology*, *41*(12), 1313-1320.

Murata, T., & Wada, M. (1989). Organization of cortical microtubules and microfibril deposition in response to blue-light-induced apical swelling in a tip-growing Adiantum protonema cell. *Planta*, *178*(3), 334-341.

Muthukumar, B., Joyce, B. L., Elless, M. P., & Stewart, C. N. (2013). Stable transformation of ferns using spores as targets: Pteris vittata and Ceratopteris thalictroides. *Plant physiology*, *163*(2), 648-658.

Neff, M. M., & Chory, J. (1998). Genetic interactions between phytochrome A, phytochrome B, and cryptochrome 1 during Arabidopsis development. *Plant physiology*, *118*(1), 27-35.

Ni, M., Tepperman, J. M., & Quail, P. H. (1999). Binding of phytochrome B to its nuclear signalling partner PIF3 is reversibly induced by light. *Nature*, *400*(6746), 781.

Ni, M., Tepperman, J. M., & Quail, P. H. (1998). PIF3, a phytochrome-interacting factor necessary for normal photoinduced signal transduction, is a novel basic helix-loop-helix protein. *Cell*, *95*(5), 657-667.

Oh, S., Montgomery, B.L., (2017) Phytochromes: Where to Start? Cell 171:1254-1255.

Osterlund, M. T., Ang, L. H., & Deng, X. W. (1999). The role of COP1 in repression of Arabidopsis photomorphogenic development. *Trends in cell biology*, *9*(3), 113-118.

Osterlund, M. T., Hardtke, C. S., Wei, N., & Deng, X. W. (2000). Targeted destabilization of HY5 during light-regulated development of Arabidopsis. *Nature*, *405*(6785), 462.

Park, C. M., Bhoo, S. H., & Song, P. S. (2000, December). Inter-domain crosstalk in the phytochrome molecules. In *Seminars in cell & developmental biology* (Vol. 11, No. 6, pp. 449-456). Academic Press.

Parks, B. M., & Quail, P. H. (1991). Phytochrome-deficient hy1 and hy2 long hypocotyl mutants of Arabidopsis are defective in phytochrome chromophore biosynthesis. *The Plant Cell*, *3*(11), 1177-1186.

Plackett, A. R., Rabbinowitsch, E. H., & Langdale, J. A. (2015). Protocol: genetic transformation of the fern Ceratopteris richardii through microparticle bombardment. *Plant Methods*, *11*(1), 37.

Quail, P. H. (1997). An emerging molecular map of the phytochromes. *Plant, Cell & Environment*, *20*(6), 657-665.

Quail, P. H. (2002). Phytochrome photosensory signalling networks. *Nature reviews Molecular cell biology*, *3*(2), 85.

Renzaglia, K. S., & Warne, T. R. (1995). Ceratopteris: an ideal model system for teaching plant biology. *International Journal of Plant Sciences*, *156*(3), 385-392.

Saijo, Y., Sullivan, J. A., Wang, H., Yang, J., Shen, Y., Rubio, V., ... & Deng, X. W. (2003). The COP1–SPA1 interaction defines a critical step in phytochrome A-mediated regulation of HY5 activity. *Genes & Development*, *17*(21), 2642-2647.

Salmi, M. L., Bushart, T., & Roux, S. J. (2011). Cellular, molecular, and genetic changes during the development of Ceratopteris richardii gametophytes. In *Working with Ferns*(pp. 11-24). Springer, New York, NY.

Schäfer, E. (1975). A new approach to explain the "high irradiance responses" of photomorphogenesis on the basis of phytochrome. *Journal of Mathematical Biology*, *2*(1), 41-56.

Schneider, H., Schuettpelz, E., Pryer, K. M., Cranfill, R., Magallón, S., & Lupia, R. (2004). Ferns diversified in the shadow of angiosperms. *Nature*, *428*(6982), 553.

Schoefs, B. (2000). The light-dependent and light-independent reduction of protochlorophyllide a to chlorophyllide a. *Photosynthetica*, *36*(4), 481-496.

Schuettpelz, E., & Pryer, K. M. (2009). Evidence for a Cenozoic radiation of ferns in an angiosperm-dominated canopy. *Proceedings of the National Academy of Sciences*, *106*(27), 11200-11205.

Seo, H. S., Yang, J. Y., Ishikawa, M., Bolle, C., Ballesteros, M. L., & Chua, N. H. (2003). LAF1 ubiquitination by COP1 controls photomorphogenesis and is stimulated by SPA1. *Nature*, *423*(6943), 995.

Seo, H. S., Watanabe, E., Tokutomi, S., Nagatani, A., & Chua, N. H. (2004). Photoreceptor ubiquitination by COP1 E3 ligase desensitizes phytochrome A signaling. *Genes & development*, *18*(6), 617-622.

Sharrock, R. A., & Clack, T. (2004). Heterodimerization of type II phytochromes in Arabidopsis. *Proceedings of the National Academy of Sciences*, *101*(31), 11500-11505.

Shichijo, C., Ohuchi, H., Iwata, N., Nagatoshi, Y., Takahashi, M., Nakatani, E., ... & Hashimoto, T. (2010). Light exaggerates apical hook curvature through phytochrome actions in tomato seedlings. *Planta*, *231*(3), 665-675.

Shikata, H., Hanada, K., Ushijima, T., Nakashima, M., Suzuki, Y., & Matsushita, T. (2014). Phytochrome controls alternative splicing to mediate light responses in Arabidopsis. *Proceedings of the National Academy of Sciences*, *111*(52), 18781-18786.

Shinomura, T., Nagatani, A., Hanzawa, H., Kubota, M., Watanabe, M., & Furuya, M. (1996). Action spectra for phytochrome A-and B-specific photoinduction of seed germination in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, *93*(15), 8129-8133.

Smith, H. (2000). Phytochromes and light signal perception by plants—an emerging synthesis. *Nature*, *407*(6804), 585.

Smith, H., Xu, Y., & Quail, P. H. (1997). Antagonistic but complementary actions of phytochromes A and B allow optimum seedling de-etiolation. *Plant Physiology*, *114*(2), 637-641.

Somers, D. E., Devlin, P. F., & Kay, S. A. (1998). Phytochromes and cryptochromes in the entrainment of the Arabidopsis circadian clock. *Science*, *282*(5393), 1488-1490.

Tanaka, J., Yano, K., Aya, K., Hirano, K., Takehara, S., Koketsu, E., ... & Matsuoka, M. (2014). Antheridiogen determines sex in ferns via a spatiotemporally split gibberellin synthesis pathway. *Science*, *346*(6208), 469-473.

Taylor, B. L., & Zhulin, I. B. (1999). PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol. Mol. Biol. Rev.*, *63*(2), 479-506.

Tsuboi, H., Nakamura, S., Schäfer, E., & Wada, M. (2012). Red light-induced phytochrome relocation into the nucleus in Adiantum capillus-veneris. *Molecular Plant*, *5*(3), 611-618.

Verma, K., & Shekhawat, G. S. (2013). PHYTOCHROME-CHROMOPHORE BIOSYNTHESIS AND CHLOROPLAST DEVELOPMENT: POSSIBLE ROLE AND REGULATION OF HO (HAEMOXYGENASE). *New developments in chromophore research*, 267.

Wada, M., Kanegae, T., Nozue, K., & Fukuda, S. (1997). Cryptogam phytochromes. *Plant, Cell & Environment*, *20*(6), 685-690.

Yeh, K. C., & Lagarias, J. C. (1998). Eukaryotic phytochromes: light-regulated serine/threonine protein kinases with histidine kinase ancestry. *Proceedings of the National Academy of Sciences*, *95*(23), 13976-13981.

Zhu, L., Bu, Q., Xu, X., Paik, I., Huang, X., Hoecker, U., ... & Huq, E. (2015). CUL4 forms an E3 ligase with COP1 and SPA to promote light-induced degradation of PIF1. *Nature communications*, *6*, 7245.

Zhu, Y., Tepperman, J. M., Fairchild, C. D., & Quail, P. H. (2000). Phytochrome B binds with greater apparent affinity than phytochrome A to the basic helix–loop–helix factor PIF3 in a reaction requiring the PAS domain of PIF3. *Proceedings of the National Academy of Sciences*, *97*(24), 13419-13424.