Cloning and sequencing of the presumptive PHY4B gene from *Ceratopteris richardii*

By

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Abstract

In *Arabidopsis thaliana*, phytochromes are encoded by five genes, PHYA, PHYB, PHYC, PHYD, and PHYE (Majee, *et al.* 2018). Each phytochrome has its own unique set of influences on plant development, acting cooperatively or antagonistically with other phytochromes. To study homologous photoreceptors in homosporous leptosporangiate ferns *Ceratopteris richardii* was used as a model because of its rapid growth and short life cycle (Bartz and Mola, 2018). *C. richardii* has become a popular model system for studying gene regulation, morphology, physiology, and development. Our lab has isolated full-length or nearly full-length cDNAs for six *C. richardii* phytochromes: *Cr*PHY1A/B, *Cr*PHY2A/B, and *Cr*PHY4A/B; two *C. richardii* cryptochromes *CrCRY1*, *CrCRY2*; and *CrPP2A, CrCOP1.* I have cloned the presumptive *Cr*PHY4B gene into the pENTR Gateway and extracted plasmid for sequencing. These verified constructs will be used for future transgenic experiments to analyze the specific functions of the novel Phy4B under various light conditions and in conjunction with genetic over-expressors and knockdowns in other phytochrome and cryptochrome photoreceptors and in the likely signaling components *CrPP2A* and *CrCOP1*.

Introduction

Dating back before the earliest farmers, light has been recognized as one of the major requirements regulating plant development, but not until the early 19th century did scientists discover that different wavelengths of light have a selective effect on plant development and growth. Poggioli (1817) was the first to demonstrate that plants preferentially grow toward broadly defined "violet light", and that plants do not grow well under red light alone. Daubeny (1836) published a monograph describing differential effects of colored light on plants. Daubeny reported red light was causing the appearance of green matter in plants, while blue light yielded more water loss (Kutschera, *et al*., 2013). This report was the beginning of the field of photoreceptor research. Plant survival depends on their responsiveness to the environment, and they have evolved sophisticated systems to monitor and rapidly adapt to the ever-changing environment. The light-sensitive receptor proteins include phytochromes, cryptochromes, phototropins, and UV-B photoreceptors (Kreslavski *et al*., 2018).

Role of light during development

Plant photoreceptors link environmental light cues with physiological responses, measuring the intensity, duration, and wavelengths of light, and the processing of ambient information to modulate physiological responses. A broad range of wavelengths provides the energy for plant growth, and specific spectral regions also serve as important cues for developmental regulation. Light perception and signaling influence plant development, acting in conjunction with those of gravity and temperature to influence overall plant responses to varying growth conditions.

Following germination, the plant follows one of two general contrasting developmental pathways to cope with light conditions. Skotomorphogenesis dominates the pattern of growth in the dark, with characteristics in angiosperms such as elongation of hypocotyls and other tissues, closed and unexpanded cotyledons, and maintenance of an apical hook—all of which allow young seedlings to use the limited energy reserve in the seed to grow rapidly in darkness toward a light source that will subsequently drive photosynthesis. By contrast, photomorphogenesis is a direct negative feedback loop where the seedling uses light as a signal to inhibit the rapid elongation of the hypocotyl, expand the cotyledons, and promote the greening of the plant, allowing the seedling to optimize light harvesting and autotrophic growth.

To suppress skotomorphogenic development and activate photomorphogenesis, plants use multiple photoreceptors for tracking a wide spectrum of light qualities in the environment; these receptors include UVB-RESISTANCE 8 (UVR8) for responding to UV-B light; cryptochromes (CRY), phototropins (PHOT), and the ZEITLUPE/FLAVIN-BINDING, KELCH REPEAT, F BOX 1/LOV KELCH PROTEIN 2 (ZTL/FKF1/LKP2) family of photoreceptors for detecting UV-A/blue light; and phytochromes (PHY) for red/far-red light (Xu, *et al*., 2015). While photoreceptors help govern plant survival in the short term, they also influence the long-term evolutionary direction of plants and have important roles in species diversity. For example, with their migration to land, plants likely used existing photoreceptors to control the synthesis of beneficial biochemicals that would help them adapt to the harsh environment on the land (Kutschera, *et al*., 2013).

Phytochromes perceive ambient red and far-red light signals in the environment, and from that information, regulate the progression of photomorphogenic development by initiating elaborate signaling mechanisms. (Xu, *et al*., 2015). The absorption of photons by the

chromophore of phytochromes produces an isomerization and rotation of the D ring of the linear tetrapyrrole. This rotation generates a corresponding allosteric conformation change in the linked polypeptide, changing phytochromes from the generally inactive Pr form to the active Pfr form. One of the pathways involves inhibition of two classes of repressors of photomorphogenesis called Constitutively Photomorphogenic/Deetiolated/Fusca (COP/DET/FUS) and phytochrome-interacting factors (PIFs; Xu, *et al*., 2015). In darkness, these repressors actively promote skotomorphogenic development by suppressing the default photomorphogenic pathway; therefore, inactivating these repressors by normal light pathways or constitutively through mutations will promote photomorphogenesis.

In the dark, naïve phytochromes are made and exist in the biologically inactive Pr form, so COP/DET/FUS complexes and PIF proteins function to repress photomorphogenic development. Under any given combination of wavelengths of light, phytochrome perceives ambient light signals and dynamically photoconverts between the Pr and Pfr forms. Activated Pfr suppresses the functions of COP/DET/FUS and PIF, directing seedlings to develop a short hypocotyl and open and expanded green cotyledons for optimal photosynthesis and autotrophic growth (Xu, *et al*., 2015). The ability to photoconvert between two forms is critical for activation of numerous plant pathways, triggering distinct plant development directions depending on the characteristics of environmental light.

Shade Avoidance Syndrome

The shade avoidance syndrome describes changes in plant form and functions in response to neighboring vegetation, potentially reducing the degree and effects of future shading. Vegetation preferentially transmits and reflects far-red light compared with other visible wavelengths; therefore, through high absorption of red light (600-700 nm) and low absorption of

far-red (700-800 nm), the ratio of red: far-red wavelengths is high when plants are growing in open space, and low when in proximity to other plants. In the life cycle of *Arabidopsis thaliana*, germination of seeds will be suppressed as a response to low photosynthetically active radiation (PAR) in the understory. The PAR spectral region encompasses 400-700 nm visible wavelengths and is absorbed by chlorophyll and carotenoid photosynthetic pigments, but FR light is poorly absorbed by the leaf. Therefore, under plant canopy, both the amount of PAR (light quantity) and the R:FR ratio (light quality) are both greatly reduced (Martínez-García, *et al*., 2014). The low R:FR signal is perceived by phytochrome (PHY) photoreceptors that can detect R and FR wavelengths. Phytochromes play major roles in controlling numerous plant responses such as seed germination, stem elongation, leaf expansion and flowering time (Martínez-García, *et al*., 2014). In *Arabidopsis thaliana*, there are a small family of genes encoding the PHY apoproteins: PHYA, PHYB, PHYC, PHYD, and PHYE.

Phytochromes

PHYB is the primary regulator for the shade-avoidance syndrome. Analyses using both genetic and physiological methods have shown that other phytochromes act redundantly with PHYB in controlling different aspects of shade avoidance-driven development. Each of the phytochromes has its own unique contribution and its specialization in plant development, with each of the photoreceptors controlling different stages of morphogenesis programs using the environmental light characteristics as an indicator for flowering (PHYA, PHYB), petiole elongation (PHYD, PHYE), and internode elongation between rosette leaves (PHYE). PHYA has a unique function as a FR-light sensor through the mechanism known as FR-High Irradiance Response (FR-HIR) having a unique negative mediation of the shade avoidance syndrome during hypocotyl photomorphogenic responses. These distinct functions were further elucidated by

comparing wild-type (Col-O) with *phyA* (*phyA*-501) and *phyB* (*phyB-9*) mutant seedlings grown under identical, controlled laboratory conditions. The *phyA*-501 seedlings exhibit long hypocotyl growth under low PAR condition, while PHYB mutant seeding bearing the mutant *phyB-9* allele demonstrated a comparable elongation of hypocotyl growth across a wide range of PAR conditions (Martínez-García,*et al*., 2014).

As stated above, the phytochromes exist in either of two photo interconvertible forms: an inactive red light-absorbing Pr form and an active far-red light-absorbing Pfr form. In a light-grown plant, the state of the phytochrome depends on the ratio of red to far-red wavelengths. Under high R:FR ratios the photo equilibrium will lean towards the active Pfr form and the shade avoidance mechanism is suppressed. Under low R:FR ratios, photo equilibrium will instead lean towards the inactive Pr form and shade avoidance is activated. While many research studies have been conducted, the majority of them are based on the length of the hypocotyl when grown under laboratory conditions providing white light with supplementary FR light to alter the R:FR ratio. The ability of photo transformation is inherent among all phytochromes (PHYA, B, C, D, E) (Kreslavski, *et al*., 2018) with each phytochrome having characteristics that are both unique and complementary to each other.

PHYA is prevalent in etiolated seedlings, while in the light it is degraded rapidly. PHYB is light-stable and plays a major role in modulating biosynthesis of photosynthetic pigments, chloroplast development, and many other processes (Khudyakova, *et al*., 2017). PHYB is one of the most predominent and arguably the most important light-regulated switch in plants, but each type of phytochrome plays a unique role under different environmental circumstances; and that role varies among different plant species. For example, rice (*Oryza sativa*) has only three phytochromes, and PHYC and PHYA take part in the photomorphogenic responses, while in *A.*

thaliana all five phytochromes take part in the photomorphogenic responses, with each functioning over a wide range of light fluences.

Phytochrome Response Genes

COP1 (Constitutively photomorphogenic 1) is a repressor of photomorphogenesis. In darkness, COP1 accumulates in the nucleus and targets degradation of positive regulators of photomorphogenesis, whereas in the light, COP1 function is largely inactivated by translocation to the cytoplasm (Swain, *et al*., 2017). COP1 is also required for activation of the shade avoidance response. In experiments using *A. thaliana*, *cop1* loss of function mutants have a reduction in to vegetation shade sensitivity, continues to concomitantly increase in hypocotyl growth, While COP1 overexpression shows exaggerated shade avoidance phenotypes. COP1 rapidly accumulates in the nucleus under low R:FR ratios and this buildup is essential for shade-avoidance syndrome responses. Nuclear-localized COP1, by degrading negative regulators of transcription activators, also inhibits other regulatory genes which then lead to increases in PIF-regulated genes, higher auxin levels, and greater stem growth. (Swain, *et al*., 2017).

Phytohormones such as jasmonate – whose accumulation is influenced by R:FR ratio – play an important role in light control of plant development. The exact mechanism and components of jasmonate signal transduction are still unknown, but recent evidence has shown the interplay between jasmonate and shade responses. This process may take part in resource distribution during plant growth and pathogen interactions (Swain, *et al*., 2017). The *jar1* mutant shows reduced sensitivity to jasmonate and enhanced susceptibility to soil fungus (Swain, *et al*., 2017). Subsequently, *JAR1* was found to be an allele of the FIN219 locus, which was isolated as a suppressor of the *cop1* mutation under darkness. FIN219 belongs to the GH3 family of phytohormone response proteins, and its expression is greatly increased by the introduction of

auxin. *FIN219/JAR1* encodes JA-amino-synthetase, which conjugates isoleucine (Ile) with jasmonic acid (JA) to form the bioactive form jasmonoyl-L-isoleucine (JA-Ile). JA-Ile is an oxylipin that profoundly affects plant developmental and stress responses (Swain, *et al*., 2017). Loss-of-function *fin219/jar1* mutants show a constant growth rate under far-red light, suggesting its likely role in PHYA-mediated signaling that leads to a rapid decrease in hypocotyl growth in wild-type plants. The FIN219/JAR1 protein physically interacts with COP1 in darkness and far-red light, and negatively regulates COP1 translation under far-red light. In addition, FIN219 may also help in signaling by other hormones such as auxin, ethylene, gibberellin, and abscisic acid by regulating many basic helix-loop-helix (bHLH) transcription factors (Swain, *et al*., 2017). The double mutant *phyA-211 fin219-2* had a synergistic effect in response to the simulated shade, which suggested that PHYA and FIN219/JAR1 work in parallel to regulate shade signaling. FIN219/JAR1 and PHYA are regulated by shade; interestingly. in the *fin219* mutant background, PHYA and COP1 levels were affected in artificial shade, suggesting FIN219/JAR1 overexpression under shade can change COP1 subcellular localization from the nucleus to the cytoplasm. Further studies showed that FIN219/JAR1, PHYA and COP1 interact with each other. These data show FIN219/JAR1 a vital role in regulating shade responses, likely by modulating the expression and subcellular location of shade-signaling components (Swain, *et al*., 2017).

Phytochromes in cyanobacteria and lower plants

There are many phototrophic photosynthetic organisms that perceive light signals from different photoreceptors, including cryptochromes, carotenoids, and phytochromes (Khudyakova, *et al*., 2017). Cyanobacteria are photosynthetic prokaryotes that incorporate histidine kinases and histidyl/aspartyl phosphotransferases into the cyanophytochrome

photoreceptor as part of the signal transduction chain, resembling the phosphorelays of plant phytochromes (Khudyakova, *et al*., 2017). As with their eukaryotic cousins, cyanophytochromes consist of homodimers, with each polypeptide having a PAS (Per, ARNT, Sim) and GAF chromophore-binding domains. As with plants, red light converts cyanophytochrome into its physiologically active form, and far-red photon absorption isomerizes the phytochromobilin back to the inactive form.

Even now there are many aspects of the cyanobacterial phytochrome-like protein that are not fully understood. These receptors are known to control carotenoid synthesis, photoprotection and complementary chromatic adaptation—the regulation of photosynthetic machinery components to maximize light harvesting under different ambient light qualities (Khudyakova, *et al*., 2017). The processes are controlled by bacterial two-component systems, which regulate the ability of cyanobacteria to change the pigment composition. The first component contains the histidine kinase, is part of a sensor protein that auto-phosphorylates after absorption of light. The phosphate group is then transferred to an aspartate on the second component, a response regulator protein. The phosphorylated response regulator will then interact with a gene promoter, which in turn will alter the pigment composition and color of the cyanobacterium (Khudyakova, *et al*., 2017).

Among the most critical functions of phytochromes is the regulation of photosynthetic processes under various stressors. In particular, the photosystem II reaction center is easily damaged under stressors such as high fluence rate light and UV wavelengths. Phytochromes can detect light intensity to regulate mechanisms for dissipating excess light energy and thereby improve resistance to these natural stress conditions. Functioning in way to change the plant morphology and physiology to adapt to the presence of various stress related to Pr/Pfr ratio and

fluence rate. Experiments reported in Khudyakova, *et al*. (2017) demonstrated that wheat plants grown under UV irradiation showed a measurable loss of chlorophyll, but minimal exposure to low intensity red light increased resistance to UV stress largely preventing the decrease in chlorophylls. Also, lipid peroxidation was accelerated by the UV light resulting from high levels of reactive oxygen species (ROS) generated, but when UV was supplemented with brief pulses of red-light illumination, the lipid peroxidation as well as protein oxidation were reduced.

Under saturating light, the Pr/Pfr dynamic photo equilibrium will be primarily dependent on the ambient R:FR light ratio. The total amount of type II phytochromes such as PHYB (Pr + Pfr) remains largely constant. Arabidopsis PHYB can undergo this photo conversion from Pfr to Pr (Casal, *et al.,* 2012) but under dark or very low light conditions, dark reversion of Pfr back to inactive Pr also occurs, reducing cellular allocations to photosynthesis in favor of hypocotyl growth. The dark reversion is relatively slow, as the Pfr form is stable into the dark for several hours, such that only long unirradiated periods return most phytochrome to the Pr form, and a red-light pulse or daylight reestablishes photo equilibrium.

In mature angiosperms, light cues also provide information that can regulate the position of the leaf and is used to sense circadian as well as seasonal rhythms. This property allows appropriate measurement for light induction and breaking of leaf bud dormancy, diurnal reorientation of leaves, seasonal initiation of flowering, and changing stomatal aperture. It also regulates many other aspects of development and reproductive growth throughout the plant kingdom (Kutschera, *et al*., 2013).

Ceratopteris richardii

The model we chose for our experiments – *Ceratopteris richardii* – is a representative of the leptosporangiate fern clade. They have a biphasic life cycle with in both free-living haploid gametophytes and diploid sporophyte generations (Bartz, *et al*., 2018). *C. richardii* is homosporous, and therefore the gametophyte from any spore can develop as either a male producing only antheridia to generate abundant sperm, or as a hermaphrodite that produces both antheridia and egg-generating archegonia. This dimorphism can be manipulated through plant density; as hermaphrodites develop, they release the pheromone antheridiogen, which in sufficient concentrations will suppress subsequently germinating spores from developing into hermaphrodites and yield primarily male gametophytes. *C. richardii* is known for its rapid life cycle compared with other fern species and its development is strongly influenced by ambient light conditions. Therefore, this species serves as a valuable model for studying the regulation of numerous developmental processes, in part because the free-living gametophytes exhibit rapid growth and plastic morphology that can be easily manipulated through their environment.

Ceratopteris gametophytes are easily cultured on agar plates or in liquid media. Following sterilization, the spores can be maintained in a dormant state in darkness, only germinating after an obligate light induction. Gametangial development occurs 6-8 days after germination, making this organism an excellent model for our studies. Our laboratory has previously isolated six phytochrome genes from *C. richardii*, designated *Cr*PHY1A/B, *Cr*PHY2A/B, and *Cr*PHY4A/B by a former graduate student (Bissoondial, 2005). Phy4B – the subject of this study – is largely conserved and is transcribed and polyadenylated, but the product is truncated at the amino terminus and its normal function remains unknown. PHY4A and PHY4B may display a blue-shifted absorption of both Pr and Pfr forms and increased rate of

dark reversion of Pfr to the Pr form, suggesting possible roles in fern development in both skotomorphogenesis and photomorphogenesis (Bissoondial, 2005). To understand the functions and behavior of PHY4B *in vivo,* we used the reference sequence from Dr. Bissoondial (2005) as a starting point for obtaining cDNA clones to verify the sequence and to produce Gateway clones. These constructs may be used for transformation of *C. richardii* callus tissue and generation of genetic plant lines with integrated GFP fusion-tagged PHY4B genes to assess light-induced nuclear translocation and to measure overexpression phenotypes of red light-regulated processes. This thesis describes the isolation and cloning of PHY4B into the pENTR vector and its sequence verification, in preparation for subsequent recombination into destination vectors.

Methods

Tissue Growth

For RNA isolation, spores of *Ceratopteris richardii* (strain RNWT1) were sterilized with a 20% (v/v) solution of commercial bleach with a final sodium hypochlorite concentration of 0.05%. After an initial wash with sterile deionized water (sdH_2O), 100 mg spores were sterilized in bleach solution for 3 min, then rinsed three times with sdH_2O. Sterilized spores were then resuspended with 2 ml sdH_2O, aliquoted to seven 50 ml Falcon tubes with 30 ml of Parker-Thompson C-Fern medium (Carolina Biological) with approximately 250 µl in each tube. The tubes were placed at room temperature in complete darkness for at least 7 days before incubating at 29°C under continuous white light for 24 hours to induce germination and then transferred to continuous far-red light (735 nm filtered light-emitting diodes at approximately 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for an additional 6 days.

RNA extraction and RT-PCR Preparation

Contents of each tube was drained on a sterile 100 µm screen, fern tissue weight recorded, and flash frozen in liquid N_2. Tissue was ground in a mortar and pestle while maintaining the material in a frozen state. Approximately 1g of the ground tissue was then added to 4.5ml of the denaturing solution RLT (Qiagen) and 450µl of lysate was pipetted onto Qiagen shredder columns to remove plant debris. The flow-through was then transferred to a microcentrifuge tube and 225µl of 100% ethanol was added. The lysates were then transferred to the RNeasy pink RNA binding columns and centrifuged for 15 sec at 10,000 rpm. Remaining contaminants were removed by washing with 700µl RW1 (Qiagen) solution and centrifuging for 15 sec at 10,000 rpm, followed by two more washes of 500µl RPE solution wash at 15 sec and 2

min at 10,000, respectively. The flow-through was then discarded, and the total RNA eluted with 20µl of DEPC-treated sdH_2O into a new 2 ml microcentrifuge tube by centrifuging 1 min at 10,000 rpm.

The extracted RNA run on a formaldehyde gel (**Figure 1)** for confirmation, then synthesized into cDNA using Superscript III according to the manufacturer's instructions. 5 µl of RNA with addition of 1µl of oligo d(T) primer, 1µl of annealing buffer, and 1µl of DEPC H_2O to a small PCR tube. It was then placed at 65°C for 5 min, followed by an immediate ice treatment for 1 min. Then 10 µl of 2x First Strand reaction mix and 2 µl of enzyme mix was added to the PCR tube, which then continued to incubate for 50 min at 50°C, and the reaction terminated at 85°C for 5 min. Finished cDNA products were then stored in -20°C until further use. PCR was performed using Platinum Taq polymerase and gene-specific primer with sequences listed below. Fully synthesized PCR product **(Figure 2)** was cloned into the pENTR D-TOPO vector (Invitrogen) and One Shot TOP 10 chemically competent *E. coli* cells (Invitrogen) according to the instructions of the manufacturer.

Cell Transformation and Plasmid extraction

During the initial construct transformation, we encountered various problem, including impurities from PCR. Therefore, the additional step of gel purification (**Figure 3)** was performed by running the full 50 µl of PCR product on an agarose gel and excising the appropriate DNA product. The DNA was inserted into a microcentrifuge tube with 1 ml of 3M KOAc and incubated at 37°C for 10-20 min depending on the size of the gel band. This step was repeated for thicker gel bands. The supernatants were discarded, and the gel slices containing the PHY4B PCR product were repeatedly flash frozen in liquid nitrogen and thawed at room temperature for a total of four freeze-thaw cycles. The excised band was transferred to a clean 0.65ml

microcentrifuge tube with a hole bored in the bottom and a plug of sterile glass wool covering the puncture. This apparatus was placed into a 1.5ml microcentrifuge tube and spun at 10,000 rpm for 10 min. The upper tube with gel was discarded and the eluted DNA at the bottom of the lower Eppendorf tube was collected, dried in a speed vac, and resuspended in 6 µl of DEPC-treated H_2O. Samples of the purified DNA were run on an analytical agarose gels to confirm that the desired product was retained. Confirmed PCR product was then used for ligation into the pENTR/D-TOPO entry vector. A pENTR vector mix was assembled in a 0.2 ml PCR tube using 1 µl of the purified PCR product, 1 µl of provided Salt Solution, 3 µl of DEPC-treated H_2O, and 1 µl of the vector were added and incubated at room temperature for 5 mins and retained on ice until use. One Shot TOP 10 chemically competent *E. coli* cells at -80°C were thawed on ice and immediately combined with 2 µl of the vector mixture by tapping the *E. coli* tube 4-5 times gently to minimize cell disruption and then incubated on ice for 30 mins. Cells are then heat shocked at 42°C for 30 sec and immediately placed back on ice. A 250µl aliquot of SOC medium was added to the transformation mixture and incubated at 37°C with rotation for 1 hour. Transformed cells were selected on LB agar plates supplemented with 50ug/µl kanamycin.

Miniprep

Cells from the surviving colonies were inoculated into 3 ml liquid LB medium with kanamycin to culture 18-20 hours at 37 °C for mini-prep isolation of plasmid DNA with the Qiagen QIAprep miniprep kit. The overnight cultures were centrifuged to collect the cells, and each pellet was resuspended in 250µl of provided P1 buffer. An equal volume of buffer P2 was added to each set of cells, and the tubes were inverted 4-6 times and allowed to incubate for 5 min to lyse the cells. An additional 350 µl of buffer N3 was added and mixed by inverting the

tube 4-6 times, then centrifuged for 10 min at 13,000 rpm. The 800 µl supernatant was applied to a QIAprep 2.0 spin column and spun for 30 sec. The flow through was discarded and the column was washed four times with 750 µl of buffer PE for the 30s with the final wash spun for 1 min to remove residual washing buffer. After transferring th spin column to a clean microcentrifuge tube, 50 µl of DEPC-treated H_2O was added to the column and allowed to sit for 1 min before spinning for 1 min at 10,000 rpm to elute the bound DNA. To confirm the successful isolation of the plasmid (**Figure 4)**, purified DNA product was subjected to PCR and verified by gel electrophoresis. The plasmid was quantified on the Nanodrop spectrophotometer and 0.5 µg aliquots were dried with appropriate sequencing primers and sent to Macrogen for DNA sequencing. Sequence data were aligned and compared with the reference PHY4B sequence initially reported by Dr. Terrence Bissoondial. Two individual clones were sent for bi-directional sequencing of the insert using internal primers as shown. For each clone, ten sample tubes containing dry plasmid samples with a final concentration of 500ngplasmid with 25pmol of PHY4B gene-specific primers to give full coverage of both strands. The following sense strand ("Forward") and antisense strand (Reverse) sequencing primers were designed to allow both strands of each clone to be fully determined with overlapping products by standard Sanger cycle sequencing.

Coding region Primers

PHY4B Forward: CACCATGCTTAATCCAATTACTGTTTATTGTCGAAG

PHY4B Reverse: TTGAACAAGAGGCAGCTCTAAATG

Internal Primers

PHY4B Internal Forward Primer 1: GGTCTGCTGCATCTCTTGTGAT

PHY4B Internal Forward Primer 2: GCACGGTTGAATGATTTGAAG

PHY4B Internal Forward Primer 3: GCTCACAAGAGAACAGATGGTG

PHY4B Internal Forward Primer 4: GTCTCAGTGTCTCTCGTAAGCT

PHY4B Internal Reverse Primer 1: GTCTCTCACAAGGCGCACTT

PHY4B Internal Reverse Primer 2: GAAGAGCTAAAGACAGCAACCG

PHY4B Internal Reverse Primer 3: CTCAGACCCGTTGAATCTCT

PHY4B Internal Reverse Primer 4: CTCGGCAGTCGCAAATCATT

Results

The far-red growth method produced sufficient yield of plant tissue for total RNA extraction, and the incubation in darkness for 7 days prior to white light induction synchronized the development and growth of the spores. The 24 hours in white light ensures the spore receives the signals need for inducing germination, while the 6 days of far-red light gave the highest yield in RNA need for cDNA synthesis for PHY4B according to the methods developed by Dr. Bissoondial. The RNA quality was confirmed with a formaldehyde agarose gel, and the quantity and quality checked by measuring the 260/280 nm absorbance ratio with a Nanodrop spectrophotometer. The successful RNA preparation provided sufficient RNA to generate cDNA templates by reverse transcription, as confirmed by results from multiple PCR syntheses using various positive controls.

The initial cell transformation yielded 3 independent colonies that survived selection on the LB/kan plate, and the cloned inserts confirmed by colony/plasmid PCR and the sequences analyzed following commercial sequencing (Macrogen). Sequence alignments allowed identification of six individual base differences from Dr. Bissoondial's preliminary original reference sequences of the coding region; the differences included adenine 2237 to thymine, thymine 2244 to cytosine, cytosine 2354 to thymine, cytosine 2372 to adenine, cytosine 2451 to thymine, and guanine 2551 to adenine shown in the appendix.

To determine which of the constructs were the correct sequence, production of a third independent set of constructs was undertaken, but in this case the second cell transformation yielded 16 colonies, and each of the plasmids harvested from every colony generated a PCR product band at the expected size by gel electrophoresis. However, the sequencing results from

these plasmids were inconsistent and generally of very poor quality, containing high levels of background with most primers. In some cases, for example, the same template would yield excellent sequence from the two end primers, yet the same internal primers that yielded very clear reads on the template in the prior round of sequencing produced negligible useful sequence data; in some cases, the same template and the same primers sequenced at different times produced very different qualities of data. The cleanest sequences obtained were those using the M13 universal primer in the vector, which verified that the 3' end of PHY4B, where the deviations in sequence between the reference and the first set of verification clones appeared. These results suggest the newer sequences are correct, and that there are errors in the reference sequences. However, because only the 5' and 3' ends of the gene sequence were confirmed, the clones are not yet ready for recombination into destination vectors, pending verification of the internal region of the phy4B gene.

Discussion

In order to understand the role of the unique truncated phytochrome PHY4B, the corresponding coding region cDNA was amplified using PCR, cloned successfully into the pENTR directional Gateway entry vector, and transformed into commercial DH5α cells using heat shock followed by selection on kanamycin-supplemented LB agar plates. To resolve the multiple differences between Dr. Bissoondial's original reference sequence and the sequence of the newly isolated clone, another independent set of cDNAs was constructed, the PHYB coding region amplified, ligated, and transformed as a second set of *E. coli* subclones; with the combination of experiments, we now have three separate copies of PHY4B DNA sequence. The two most recent sequences indicate that the discrepancies are the result of errors in the reference sequence, however the most recent clone yielded only 700 bases of readable sequences from both the 5' end of the gene and 3' end of the gene, and internal sequences were not of sufficient quality to allow a high level of confidence, so the results of that portion must be considered incomplete until further sequencing is performed.

A clear explanation of this high background interference in the third clone sequences; despite producing the correct insert size by PCR and subjecting the construct to additional purification steps, only two of the eight sequencing primers produced high quality reads on this template. If there were contamination from a second plasmid or multiple inserts, the correct template would still be expected to serve as a functional template; in the event of an incorrect insert only then none of the primers would be expected to bind and produce sequence. As these primers functioned on the first confirming construct template, the oligonucleotides are known to bind and support sequence reactions correctly, so the most likely flaw is in the second confirming construct insert. Therefore, the best solution is to create additional clones of

*Cr*PHY4B for sequencing, providing the second confirmation sequence and presumably constructs containing a full-length coding region inserted in the Gateway entry vector, ready for recombination into any of several destination vectors described below.

Future Directions

Obtaining clones containing the full confirmed Phy4B coding region shown in the appendix in pENTR will be the most immediate goal. Upon verification, this entry clone can be used for numerous experiments to understand the roles of *Cr*PHY4B *in planta*, by recombination into destination vectors to generate RNAi knock down or overexpression transgenic fern plants. These methods involve a biolistic particle delivery system. DNA particle bombardment consists of coating gold microcarrier particles with plasmid DNA and delivering the bound particles into plant callus tissues. *Ceratopteris* and other ferns have been resistant to many traditional methods of genetic transformation, these largely undifferentiated have recently been shown to effectively incorporate transgenes delivered into sporophyte callus for stable transformation (Plackett, *et al.*, 2014).

The function of Phy4B is currently unknown, but it appears to be a novel photoreceptor lacking the histidine kinase-related domain present in other members of this phytochrome family. RNAi experiments from our lab suggest it may perform regulatory functions on gametophyte morphogenesis, but without more robust genetic evidence, this hypothesis is difficult to address empirically. With these clones, it should be possible to generate knockdowns, overexpressors, and site-directed mutations to study more precisely the nature of *Cr*PHY4B. Preparation and maintenance of the target callus tissue has been ongoing since 2015, and preliminary Biolistic transformation has been performed with *Cr*PHY1A and *Cr*PHY2A constructs. If these are successful, we expect to generate multiple lines of each set of genetic

constructs, and attempt parallel experiments with *Cr*PHY4B when they become available. Initial studies will focus phytochrome:GFP fusion proteins to examine whether each phytochrome translocates to the nuclei upon activation to Pfr by red light exposure, as occurs in seed-bearing plants. Alternatively, phytochromes in the moss *Physcomitrella patens* do not appreciably move to the nuclei in the Pfr form, so at least some of the *C. richardii* phytochromes may function more comparably to those of the non-vascular plants.

In conjunction with genes encoding other phytochromes including *Cr*PHY1A, *Cr*PHY1B, *Cr*PHY2A, *Cr*PHY2B, and *Cr*PHY4A, cryptochrome photoreceptors CRY1-CRY4, and putative signaling components PP2A and NDPK1, all of which were identified and partially or fully sequenced in our lab previously, loss of function and gain of function combinations in transgenic *C. richardii* will be instrumental in determining mechanisms underlying photomorphogenesis in ferns, and provide evidence for evolutionary relationships to extant higher plant light regulated processes. Each individual gene would have its unique effect on plant development and elucidating the functions of each may provide insights into agriculture and forestry.

Figures

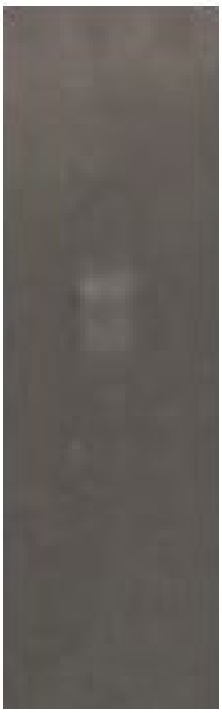

Figure 1. Total RNA separated on a 1% agarose formaldehyde gel. RNA was extracted from spores that were sterilized, sown on 1% agarose plates containing Parker-Thompson media, maintained in darkness for 7 days, then induced to germinate under white light for 24 hours and transferred to far-red light for an additional 6 days of growth.

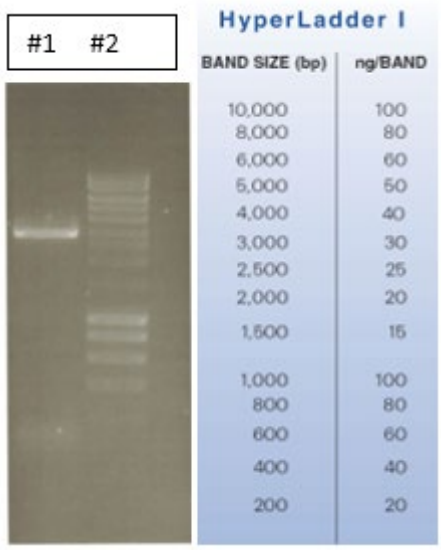

Figure 2. Non-quantitative RT-PCR of *Cr*PHY4B. cDNA produced using an oligo d(T) primer on total RNA and SuperScript reverse transcriptase was amplified with gene-specific primers for 35cycles described in the text. The single major band corresponds to the expected size of 2.9 kb. Hyperladder I was used as the standard marker.

Lane #1: PHY4B coding region Lane #2: Hyperladder II

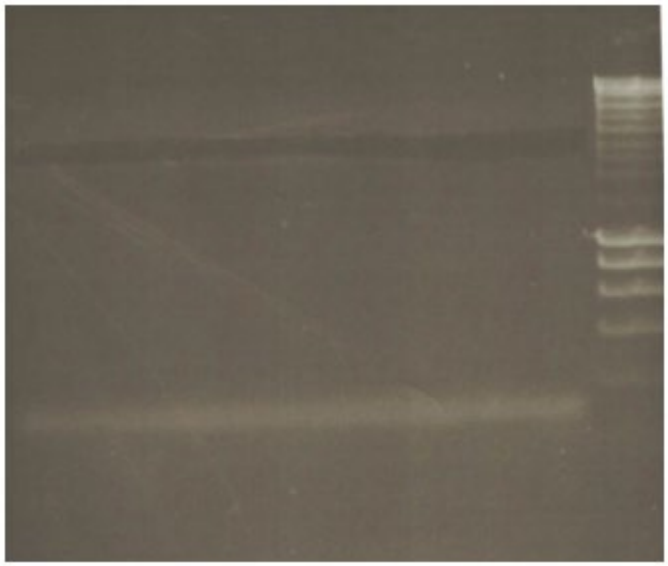

Figure 3 Agarose gel from preparative electrophoresis after 2.9 kb PCR product excised for purification and cloning as described in Materials and Methods.

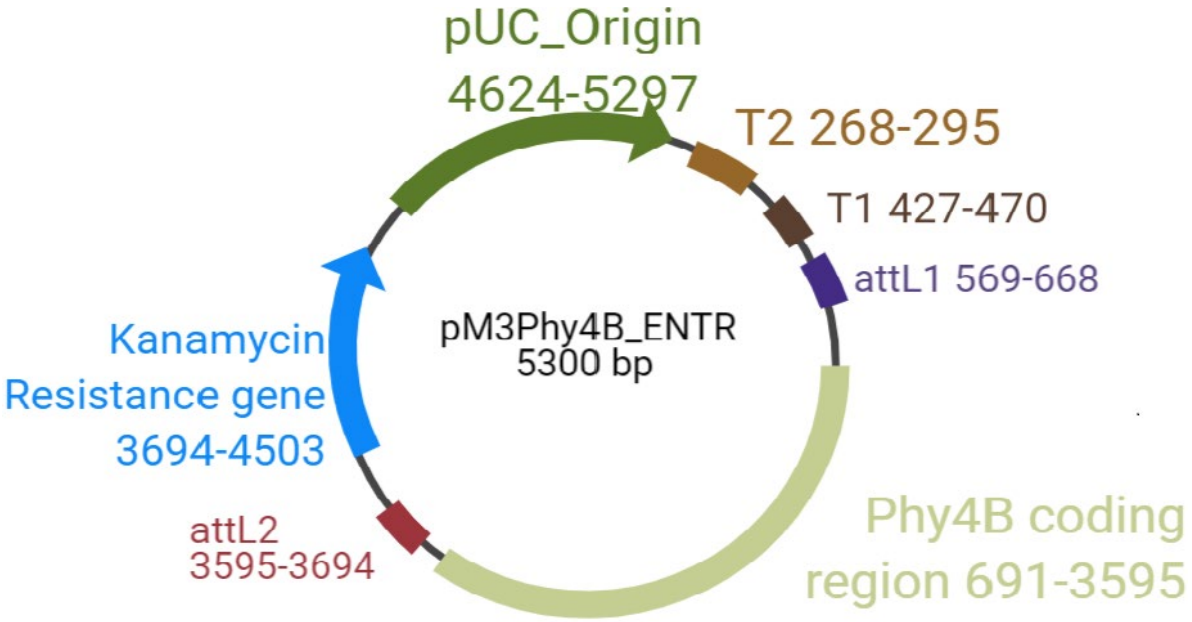

Figure 4

The pM3Phy4B construct with the inserted *Cr*PHY4B using the pENTR vector after successful transformation. pM3Phy4B contains the gene of interest *Cr*PHY4B, the pUC origin, kanamycin resistance gene; and the attL1 and attL2 recombination sites flanking the Phy4B gene.

Appendix

Original reference PHY4B sequence

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