A

PHOTOMORPHOGENESIS IN THE MODEL FERN

Ceratopteris richardii

By

Abeer Mohamed

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2005

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

9 September, 2004
Date

Chair of Examining Committee
Dr. Timothy W. Short, Queens College

11/29/04
Date

Richard L. Chappell
Executive Officer
Dr. Richard L. Chappell

Dr. Zahra F. Zakeri, Queens College

Dr. PoKay Ma, Queens College

Miguel Cervantes
Dr. Miguel Cervantes, Lehman College

Anthony L. DePass
Dr. Anthony L. DePass, Long Island University

Supervisory Committee

The City University of New York

ABSTRACT

Photomorphogenesis in The Model Fern *Ceratopteris richardii*

By
Abeer Mohamed

Advisor: Dr. Timothy W. Short

Light is one of the major environmental signals that influence plant growth and development. Plants have evolved a sophisticated system of three major classes of photoreceptors—the red/far-red light-absorbing phytochromes, the blue/UV-A light-absorbing cryptochromes and phototropins, and the UV-B light receptors—to monitor light and regulate plant physiology and morphology.

Although the roles of light in regulating many physiological processes in higher plants have been intensively studied, comparatively little information exists about the role of light on early development such as gametophyte development and embryogenesis. *Ceratopteris richardii*, an increasingly popular fern model, offers unique opportunities for developmental, physiological, genetic, and molecular studies of plant photomorphogenesis.

Information is lacking about the effects of phytochrome over prolonged periods and in more developed mature gametophytes. Allowing spores to germinate and develop under different controlled light conditions has yielded more information about how phytochrome acts on cell division, expansion, morphogenesis, and plastid development of maturing gametophytes and young sporophytes. These characterizations lead to demonstrate that phytochrome and blue light sensory photoreceptors play a major role in regulating germination, the rate and direction of cell division, cell expansion, chlorophyll accumulation, archegonia development and sex determination, and early sporophyte

development in *Ceratopteris*. By comparison with other plant responses to supplementary far-red light or to end-of-day far-red light regimes, *Ceratopteris* responded not only differently but in a manner opposite that of angiosperms, which indicates different mechanisms of phytochrome-regulated morphogenesis.

Until recently, very little was known about the mechanisms of phytochrome signaling in lower plants. To elucidate the signal transduction mechanisms of different phytochromes, it is essential to know the sites of their action within the cell. Furthermore, the molecular biology of *Ceratopteris* is largely unexplored. The addition of transgenic and pharmacological analysis techniques to *Ceratopteris* research would improve the range of possible experiments available.

Studying *At*PhyB and *Cr*Phy1 translocation behavior in *Ceratopteris* has proven suggestive for understanding evolutionary conservation of behavior between higher plant and fern phytochromes. The slow kinetics of *At*PhyB:GFP translocation and lack of *Cr*Phy1:GFP nucleocytoplasmic repartitioning in *Ceratopteris* cells, and their distinct localization patterns, suggest that different mechanisms or signal transduction components may be active in fern and higher plant phytochrome action.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my mentor, Dr. Timothy W. Short, for all his kind help and support and precious advice, for his always being available for help whenever I needed it, for his brilliant suggestions and discussion that brought excitement and led my way to becoming an independent researcher.

I am deeply grateful to my committee members: Dr. Zahra Zakeri, Dr. PoKay Ma, Dr. Miguel Cervantes-Cervantes, and Dr. Anthony DePass, for their advice, discussion, helpful guidance, and their time, which provided a great aid for this work to be achieved.

I would like to give my thanks to my colleagues: Bridgit M. Goldman, Dr. Fang Qi, Dr. Caihong Qiu, Terrence Bissoondial, and Ira Savetsky for their help.

I sincerely appreciate the great assistance of the personnel in Dr. Zakeri's Lab and Dr. Ma's Lab.

I am grateful to my family, for their support, encouragement, and endless help in finishing my dissertation.

Thanks to the Egyptian cultural and Educational Bureau for generous and patient support throughout my study.

Thanks to the Professional Staff Congress and the City University of New York for financial support to this project.

TABLE OF CONTENTS

List of illustrations

List of Tables

CHAPTER I. Introduction

A. Fern as models for photomorphogenesis research

B. Photoreceptors in light signaling

C. Phytochrome mediated responses

D. Physiological responses controlled by photoreceptors in fern

E. Localization and intracellular compartmentalization of phytochromes in plant.

F. Sensory transduction in fern

G. Interaction between phytochrome and blue light receptors

Perception of changes in the natural environment is of the utmost importance for all living organisms, from prokaryotes to higher eukaryotes. This is especially valid in the case of plants—sessile organisms unable to migrate to more favorable locations and forced to adapt to changes in the local environment. Plants have adopted a high degree of developmental plasticity to optimize their growth and reproduction in response to their ambient environments.

Light is one of the major environmental signals that influence plant growth and development. Apart from utilizing light as the primary energy source, plants use it as signal to initiate a complex series of signal transduction events that result in dramatic developmental, physiological, and biochemical changes. In order to accomplish that, plants must be especially plastic in response to their light environment. Therefore, they have evolved a sophisticated system of photoreceptors to monitor all facets of light, including its direction, duration, quantity and wavelengths. The light quality and quantity will be reflected in the morphology and physiology of the plant's development (Kendrick and Kronenberg, 1994).

A. Ferns as models for photomorphogenesis research

Light affects nearly every aspect of plant development, beginning with germination. In higher plants, after germination, the very young seedling must choose between two developmental pathways, according to whether early growth is underway in darkness (D) or in light. In the dark-grown developmental pathway, known as skotomorphogenesis, the etiolated seedling grows heterotrophically, using the seed's resources in an effort to reach and harvest light. This etiolated seedling is characterized by a long hypocotyl, unopened apical hook, and folded and unexpanded cotyledons (figure 1A), features that allow the seedling to grow through a layer of soil and emerge in the light. The shoots are yellowish since no detectable chlorophyll is produced. Once the seedling perceives sufficient light, the transition to a de-etiolated light-grown state initiates a developmental process that optimizes the body plan of the seedling for efficient photosynthetic growth (figure 1A). During de-etiolation, the rate of hypocotyl elongation decreases, the apical hook opens, cotyledons expand, chloroplasts develop, and a new gene expression program is induced. These processes are collectively known as photomorphogenesis.

If a typical fern spore is kept in the dark after being induced to germinate, the prothallus will be etiolated, exhibiting an elongated strap-like shape (Murata et al., 1997). This elongated gametophyte consists of an apical meristem, a subapical elongation zone and a basal growth cessation zone (figure 1B). Unlike in angiosperms, in pteridophytes chlorophyll can be produced in the etiolated prothallus. In light-grown prothalli, induction of symmetric and asymmetric division occurs, rhizoid growth is induced (Murata and Sugai, 2000), cell elongation is inhibited (Murata et al., 1997), and the gametophyte will develop as a heart-shaped prothallus (figure 1B).

By comparing the development of both ferns and higher plants, one can recognize that fern gametophytes possess analogous patterns, although in a simplified form, to many of the basic physiological phenomena found in higher plant sporophytes. Many developmental processes of fern gametophytes depend on light from spore germination to cell elongation, phototropic curvature, cell division, two-dimensional differentiation, and antheridium formation (Wada and Sugai, 1994). This photoregulation of fern development, as well as the simple organization of the fern gametophyte, are quite unique in the plant kingdom and make them an invaluable model system to study development and physiology of plants at the cellular or even subcellular level. Many lower plants have unique characteristics that are valuable for genetic research, including simple growth requirements, diminutive sizes, and simple, independently growing, haploid gametophytes that emerge from a single cell (spore). These free-living organisms can grow autotrophically on an inorganic medium. This fact presents the advantages of easy cultivation and observation (Cooke et al., 1995; Banks, 1999) not offered by the enclosed, sporophyte-dependent gametophytes of higher plants.

B. Photoreceptors in light signaling

Plants utilize various photoreceptors to detect the absence or presence of certain wavelengths of incident light. They have evolved three major classes of photoreceptors with distinct light-absorption characteristics: the red (R)/far-red (FR) light (600-750nm)-perceiving phytochromes (phy), blue (B)/UV-A (320-500nm) absorbing cryptochromes (cry) and phototropins (phot), and one or more uncharacterized UV-B (282-320nm)-absorbing receptors (Kendrick and Kronenberg, 1994; Briggs and Huala, 1999; Briggs, 2001; Briggs and Christie, 2002).

The seedling switch from skotomorphogenesis (development in darkness), to photomorphogenesis (development in light) is induced by signals from the aforementioned three classes of photoreceptors. These photoreceptors continue to be important throughout the life of the plant (Smith, 2000; Lin, 2002; Quail, 2002a, 2002b). Gene duplication and selection events have led to the evolution of multiple cry, phot, and phy receptors in plants (Mathews and Sharrock 1997). that allow efficient detection of the ambient spectrum over a wide range of environmental and developmental conditions. The roles of individual photoreceptors in light perception and in regulation of various physiological responses have been addressed by studying mutations and/or transgenic plants of one or more individual photoreceptors, and by extensive characterization of physiological responses to controlled environmental light.

Blue light receptors

A major group of photoreceptors that plays an important role in plant photomorphogenesis is the B/UV-A photoreceptors, which are present in many different organisms: protists, fungi, green plants, and animals (Senger, 1987; Kumagai, 1988; Cashmore et al., 1999; Brudler et al., 2003). In recent years it has become evident from spectral and genetic evidence that there are at least three groups of B/UV-A photoreceptors: the flavoproteins, which include crys (Cashmore et al., 1999) and phots (Briggs and Christie, 2002) and carotenoids such as xanthophylls (Horwitz and Berrocal, 1997). In addition, phytochromes and light-harvesting complexes also absorb blue wavelengths.

Cryptochromes play multiple important roles in plant photoperception and growth. Phenomena such as inhibition of hypocotyl growth, accumulation of anthocyanin (Ahmad and Cashmore, 1993; Ahmad et al., 1995), and regulation of flowering time (Guo et al., 1998) are mediated by cryptochromes. Most plant cryptochromes are 70-80 kDa proteins with two recognizable domains: an N-terminal domain that shares structural similarity to DNA photolyases enzymes which catalyze B/UV-A light-dependent DNA repair (Cashmore et al., 1999); and a C-terminal domain, with more divergent sequences that are largely unique to the cryptochromes. However, cryptochromes lack the DNA repair activity that is the hallmark of photolyases (Lin et al., 1995b). Each cryptochrome apoprotein can accommodate a pterin or a deazaflavin at one chromophore binding site and a FAD at the second binding site (Briggs and Huala, 1999)

Cryptochromes have been found throughout the plant kingdom, including angiosperms, gymnosperms, ferns, and bryophytes (Ahmad and Cashmore, 1993; Small et al., 1995; Kanegae and Wada, 1998; Imaizumi et al., 2000). Arabidopsis has two cryptochrome genes, designated *CRY1* and *CRY2*, (Ahmad and Cashmore, 1993; Lin et al., 1998). In the fern *Adiantum capillus-veneris* five cryptochrome genes have been isolated. These cryptochromes share a much higher sequence similarity in the N-terminal photolyase-like domain than in the more variable C-terminal region (Lin et al., 1998; Imaizumi et al., 2000).

Numerous blue-light responses have been characterized by focusing on single cells and even on single organelles in lower plants, particularly in mosses and ferns, because of the simple organization of their gametophytes. Blue light receptors mediate induction of phototropic responses and inhibition of spore germination, tip growth, apical swelling and subsequent cell division of *Adiantum* protonemata (Wada et al., 1981b; Hayami et al., 1986; Wada and Kadota, 1989).

The intracellular localization of blue light receptors affecting these responses has been studied with polarized light and microbeam irradiation. Local irradiation with a blue microbeam convincingly showed that only the irradiation of the nuclear envelope region could induce cell division (Wada and Furuya, 1978; Furuya et al., 1997), even after relocation of the nucleus by centrifugation (Kadota et al., 1986a).

The blue light receptors of *Adiantum,* which are involved in phototropism (Hayami et al., 1992), apical cell swelling (Wada et al., 1978), and chloroplast reorientation (Yatsuhashi et al., 1985; Yatsuhashi et al., 1987) are localized on or near the plasma membrane. Data from action dichroism experiments of growth in other ferns under unidirectional polarized light have pointed toward a receptor possibly immobilized on the plasma membrane (Steiner, 1967). Photoreceptor pigments localized throughout the entire cell mediate chloroplast movement; however, those involved in cell swelling and polarotropism are localized only on the apical part of the protonemata.

With the purpose of studying intracellular localization of CRYs, CRY:reporter fusions were transiently expressed in basal and tip cells of *Adiantum* protonemata (Imaizumi et al., 2000). The GUS-CRY3 and GUS-CRY4 chimeric proteins show clear nuclear localization, but GUS-CRY1, GUS-CRY2 and GUS-CRY5 do not. The high degree of conservation in the N-terminal region within *Adiantum* CRYs suggests that the divergent C-terminal regions of these cryptochromes might play important roles in the determination of subcellular distribution. In addition, clusters of small basic amino acids (e.g. KRKAK), which may function as nuclear localization signals, are present in the conserved regions found at the CRY3 and CRY4 C-termini. The fact that localization patterns of CRY3 and CRY4 C-termini are similar to the localization of the full length genes further supports this hypothesis.

As mentioned above, not all CRYs from *Adiantum* localize to the nucleus. CRY1, 2, and 5 are localized in the cytoplasm. Putative membrane-spanning domains are not found in those CRY sequences. However, some localization data indicate that *Arabidopsis* CRY1 protein is enriched in the membrane fractions (Ahmad et al., 1998). Possibly, therefore, CRY1, 2, and 5 proteins may be associated with the plasma membrane to promote the blue-light induced phototropism, apical swelling and chloroplast relocation (Imaizumi et al., 2000).

Phototropins (phot) form another ubiquitous group of blue light receptors. These receptors have not been isolated yet from fern, although partial sequences have been reported for several ferns. In *Arabidopsis*, there are at least two phototropin homologous, designated phot1 and phot2. Both phot1 and phot2 proteins are plasma membrane-associated proteins with two N-terminal LOV domains (for light, oxygen, voltage-regulated PAS-related domains) each of which binds a flavin mononucleotide (FMN) chromophore. The C-terminal half of phot1 and phot2 proteins has serine/threonine kinase activity exhibiting light-activated autophosphorylation. Light activates formation of a flavin C (4a)-cysteinyl adduct, which decays subsequently in the dark (Briggs et al., 2001; Crosson and Moffat, 2001; Christie et al., 2002). The *Arabidopsis PHOT2* gene encodes a slightly smaller protein than *PHOT1*, with 67% sequence similarity to *PHOT1* (Jarillo et al., 1998). Phot1 and phot2 show light-activated conformational changes and subsequent dark recovery similar to that of phytochrome (Salomon et al., 2000; Kasahara et al., 2002). Both phot1 and phot2 play overlapping roles in mediating phototropism, but they are distinct in their responsiveness to different light fluences. Light pulses or low levels of continuous blue light mediate phototropism through phot1, whereas phot2 responds only at high fluence rates of continuous light (Kanegae et al., 2000). Chloroplast movement and accumulation are also mediated by phot1 over a wide fluence range, whereas phot2 modulates chlorophyll accumulation at the cell face under low fluence rates and chloroplast light avoidance at higher fluence rates (Jarillo et al., 2001; Kagawa et al., 2001; Sakai et al., 2001; Kagawa, 2003). Both phot1 and phot2 play role in regulating leaf expansion. (Sakai et al., 2001) and (Sakamoto and Briggs, 2002) illustrate that leaves of the *phot1 phot2* double mutant are sharply curled downward whereas; the presence of either phototropin allows the leaves to appear like those of the wild type. However, from the data available it is unclear how the two phototropins regulate leaf expansion and their relative sensitivity in mediating this response (Briggs, 2004).

Phytochromes

Photochemical and Biochemical properties

Of the photoreceptors in plants, the best understood are the phytochromes. Phytochromes were discovered in 1945 by Borthwick and Hendricks during their studies of the mechanisms plant use to determine day length (Briggs, 1975). Phytochrome photoreceptors are present in higher plants (gymnosperms, angiosperms)(Sharrock and Quail, 1989) and lower plants [ferns(Maucher et al., 1992a; Nozue et al., 1998a), mosses(Thummler et al., 1990) and algae (Lagarias et al., 1995; Winands and Wagner, 1996)]. Phytochromes are apparently ubiquitous in green plants (Mathews and Sharrock, 1997) and recently, have been found in the cyanobacterium *Synechocystis* (Hughes et al., 1997) as well as in fungi and in non-photosynthetic eubacteria and archaebacteria.

The eukaryotic phytochrome molecule is a soluble, dimeric chromoprotein that consists of two ~120-kDa polypeptides, each with a single covalently attached tetrapyrrole chromophore. Each monomer is composed of two discrete structural domains: an N-terminal domain, which perceives light signals, and a C-terminal domain which necessary for dimerization and signal transduction. A flexible hinge region connects the N- and C-terminal domains (Wagner et al., 1996). The C-terminal domain contains several conserved motifs, including two Per-Arnt-Sim (PAS) motifs which play a key role in protein- protein interactions, a so-called Q (Quail) box through which phytochrome binds to phytochrome interacting factor 3 (PIF3)(Ni et al., 1998), at least one dimerization domain which links two monomers, and two histidine kinase-related motifs. The chromophore binding domain–which flanks the invariant Cys residue near amino acid 320—is conserved within the N-terminal region.

After the attachment of chromophore, the photosensory activity of the phytochrome holoprotein results from its capacity to undergo a light-induced, reversible switch between two conformers: the R-absorbing P_r form and the FR-absorbing P_{fr} form. These two forms have distinct light absorption spectra (figure 2) with absorption maxima in the R (P_r) and FR (P_{fr}) regions of the spectrum, respectively. Phytochrome is synthesized in the P_r form in dark-grown seedlings of higher plants. Upon exposure to R (absorption maximum around 667 nm), the P_r form is converted to the P_{fr} form, and exposure to FR light around 730 nm reconverts the P_{fr} form to the P_r form (Neff et al., 2000). Breakdown (or 'destruction') of P_{fr}, and dark reversion also occur but at slower rates than photoconversion. Because even tiny amounts of P_{fr} have major physiological effects, it is generally accepted that this is the active form of phytochrome, although the P_r form has also been shown to be active in a few cases. Intermediates formed during the switch from P_{fr} to P_r, rather than P_r or P_{fr} themselves, may function as short-lived signals induced during the photoconversion (Reed, 1999; Shinomura et al., 2000), or that P_r, which has been cycled through its P_{fr} form behaves differently from the naïve P_r (Shinomura et al., 2000; Schäfer and Bowler, 2002)

Owing to the R/FR reversibility of phytochromes and the distinct absorption spectra of each form,, spectroscopic methods can be used to detect them *in vivo*, and protocols have also been devised to purify the pigment (Borthwick et al., 1952; Butler et al., 1959). Although the two forms of phytochrome are referred to by their absorbance peaks (R and FR), the absorbance spectra of P_r and P_{fr} overlap significantly in the red and blue regions of the spectrum, and the P_r form of phytochrome absorbs a small amount of light in the FR region (Quail, 1997; figure 2). As a consequence, a dynamic equilibrium exists between the two forms under almost all light conditions. When P_r molecules are exposed to R, most of them are converted to P_{fr}, but the P_{fr} also absorbs the R (albeit less efficiently) and is converted back to P_r. Thus, after saturating irradiation by monochromatic 660 nm R, the proportion of phytochrome in the P_{fr} form is about 85%. Similarly, the very small amount of FR absorbed by P_r makes it is nearly impossible to convert P_{fr} entirely to P_r by 730 nm FR, yielding equilibrium of 97% P_r and 3% P_{fr} at 730nm. Both forms can absorb to a variable extent over the entire visible spectrum (Kendrick and Kronenberg, 1994). Therefore, phytochrome effects can be elicited by all visible light (Vierstra and Quail, 1983).

With a few exceptions, studies on spectroscopic and biochemical properties of phytochrome in ferns have as yet received little attention (Grill and Schraudolf, 1981; Tomizawa et al., 1982; Tomizawa et al., 1983). As in the case of angiosperm phytochrome, the level of fern phytochrome decreases under continuous white light (W_c), and the level increases again when de-etiolated tissue was transferred back to darkness as shown by spectrophotometric analysis of fern *Adiantum capillus-veneris* L. phytochrome *in vivo* (Oyama et al., 1990). The rate of decay of the fern phytochrome is considerably slower, with half life of approximately 20 h, than that observed in etiolated tissues of angiosperms (Schafer et al., 1975; Smith et al., 1988)}, in which the half life is usually 1-2 h. The P_{fr} dark reversion to P_r in *Adiantum* takes place with almost no destruction of P_{fr} (Oyama et al., 1990). In other ferns, P_{fr} dark destruction seems to occur simultaneously with dark reversion, as it does in some dicotyledonous plants (Furuya and Hillman, 1964; Tomizawa et al., 1983).

Is P_r an active form of phytochrome?

The proposal that the P_r form of phytochrome may also be a biologically active form has been made several times, and rejected vehemently by others. Since in dark-grown plants phytochrome accumulates in the P_r form, it is presumed to be biologically inactive. At the same time, the realization that several of phyA-mediated responses can be induced by FR seemed inconsistent with the idea that P_{fr} is the only active form, at least for phyA. One possible explanation for the apparent discrepancy has emerged from the finding that the P_r form of phyA, after undergoing photo-cycling between the P_r and

P_{fr} forms, shows greater autophosphorylation activity than naïve P_r (Yeh and Lagarias, 1998b). It is likely that this actively cycled P_r species may be responsible for FR-mediated effects of phyA, and possibly for the R-induced high irradiance response (R-HIR) mediated through phyB. This notion is supported by recent experiments (Shinomura et al., 2000) in which it was observed that brief cyclic pulses of FR with three-minute intervals inhibit hypocotyl elongation to the same extent as continuous FR. Since the response is reversible by R and inducible by FR, it is most likely mediated by a form other than P_{fr}. In all likelihood, this active form is the cycled P_r form that is generated by FR pulses.

The phytochrome gene family

The number and type of phytochrome-encoding genes is different in the few plant species for which comprehensive information is available. In *Arabidopsis thaliana* there are five genes encoding for phytochrome apoproteins and have been designated *PHYA* through *PHYE* (Sharrock and Quail, 1989; Quail et al., 1995). Based on their predicted amino acid sequences, these phytochrome genes can be grouped in three evolutionary lineages in the flowering plants: PHYA, PHYB/D/E, and PHYC/F (Mathews and Sharrock, 1997).

On the basis of their light lability, members of the *PHY* gene family can be classified as either type I (light labile) or type II (light stable) phytochromes. The type I phyA phytochrome is the most abundant phytochrome in etiolated angiosperm seedlings, but under R or white light (W) the amount of phyA will decrease 50- to 100-fold as a result of a combination of factors: transcriptional down regulation caused by P_{fr} form of phyA (P_{fr}A) which inhibits the expression of its own gene, mRNA degradation, and ubiquitin-mediated proteolysis of the P_{fr}A protein (Vierstra, 1994; Clough et al., 1999). phyB through phyE are type II phytochromes. Although detected in green plants, these phytochromes are also present in etiolated plants. Their mRNA expression is not significantly changed by light, and their proteins are much more stable in the P_{fr} form than phyA is (Quail et al., 1995; Clough and Vierstra, 1997).

A family of phytochrome genes has also been identified in ferns. While four genes have been found in *Anemia*, three phytochromes have been described in *Adiantum capillus-veneris*. The deduced amino acid sequences of two of these phytochrome genes (*Ac*PHY1 and *Ac*PHY2) (Nozue et al., 1998a) show a common structure with higher plant phytochromes. However, *Ac*phy3 has a very unique structure; the N terminus has high homology to *Arabidopsis* phyB whereas the C terminus is very similar to the *Arabidopsis* phototropin (nph1) sequence, including a Ser/Thr protein kinase catalytic domain (Nozue et al., 1998b). Partial phytochrome sequences isolated from genomic DNA have been determined and analyzed in other fern plants (Maucher et al., 1992b). This analysis leads, at least for *Dryopteris filix-mas*, to the assumption that the described phytochrome products are more closely related to phyB or phyC than to phyA. This result is consistent with the physiological data pertaining to the stability of the functional phytochromes responsible for the induction of spore germination in *Dryopteris* (Haupt et al., 1988). Similar observations have also been reported in mosses and in other ferns (Thummler et al., 1990; Schneider-Poetsch and Braun, 1991). Short phytochrome cDNA sequence fragments from numerous other ferns and fern allies have since been reported (Schneider-Poetsch et al., 1994) but very few full-length sequences indicating a phyB-like precursor as the more ancient lineage have been published. Two phytochromes have been reported in the moss *Ceratodon purpureus.* One of which is that of a conventional phytochrome(Hughes et al., 1996) but the other one has a protein kinase domain in its C-terminus similar to phy3 from *Adiantum* (Thummler et al., 1992). Complete sequences of algal phytochromes have been reported from the chlorophytes *Mesotaenium* (Lagarias et al., 1995) and *Mougeotia* (Winands and Wagner, 1996), both of which show conventional phytochrome structure.

C. Phytochrome-mediated responses

Plant responses to spectral light quality and quantity are numerous. These responses are characterized by a wide divergence of signal input-output relationships that can be described in terms of their dependence on spectral composition, fluence, and timing. Early physiological and photochemical studies indicated that light responses in angiosperm photomorphogenesis are classified as low- or high-energy reactions according to their irradiance requirements (Mohr, 1962). Subsequently, low-energy reactions induced by short pulses of irradiation with relatively small doses of light were divided into low-fluence responses (LFRs) and very-low-fluence responses (VLFRs) (Mandoli and Briggs, 1981). The high-energy reaction, which required irradiation with relatively high energy light for a comparatively long period of time, was renamed a high-irradiance response (HIR).

Very low fluence responses (VLFRs)

The VLFRs saturate at minute concentration of active phytochrome (P_{fr}) created by 10^{-6} to 10^{-3} μmol m^{-2} of photons. A VLFR is not photoreversible, since even FR light is sufficient to convert enough P_r to P_{fr} to initiate the response. Among theVLFRs that have been characterized in higher plants are changes in the expression of several genes, seed germination, and the control of hypocotyl gravitropic growth. Since phyA accumulates to a relatively high concentration in dark-grown seedlings, it has been proposed that phyA acts as a sensor for small amounts of light. A brief pulse of FR will induce a very small, but sufficient, amount of P_{fr}A to promote the VLFRs. Although a R pulse will induce degradation of $P_{fr}A$, low levels of this form can still induce the VLFR. Studies of *Arabidopsis* photoreceptor mutants confirm that the VLFRs of seed germination (Shinomura et al., 1994; Shinomura et al., 1996) and Cab gene expression (Hamazato et al., 1997) are mediated by PhyA.

Low fluence responses (LFRs)

The LFRs are the most diagnostic, and first described, phytochrome-dependent responses. These responses saturate at 10^{-1} to 10^{2} μmol m^{-2}. The classic example of a LFR is R pulse-induced germination of lettuce seeds. The induction can be inhibited by subsequent FR pulse treatment because of the photoconversion of most of the P_{fr} back to the P_r form. Thus, photoreversibility is one characteristic feature of the LFR. LFRs also obey the law of reciprocity (Furuya and Schafer, 1996), namely, the same number of photons—whether present as a brief, bright pulse or as a longer, dim irradiation—will elicit comparable effects. These responses include most of the classic R/FR photoreversible responses such as shade avoidance and leaf movement. phyB (and, to a lesser extent phyC-E) is the main candidate phytochrome in the induction of these responses. After R exposure, a large proportion of phyB molecules are in the P_{fr} form, which stimulates the LFR. However, under FR or if a R pulse is followed immediately by a FR pulse, the proportion of phyB molecules in the P_{fr} form returns to a level below the threshold for inducing LFRs.

High Irradiance responses (HIRs)

HIRs are saturated at much higher fluences than LFRs—at least 100 times higher—and require continuous light over several hours or days. The HIRs fall into two classes: those induced by R (R-HIR) and those maximized by continuous FR (FR-HIR). As expected, R-HIRs are not photoreversible by FR. The magnitude of the response is a function of the fluence rate and the time of exposure within a certain range. Since neither continuous exposure to dim light nor transient exposure to bright light are effective, HIRs do not obey the law of reciprocity (Mancinelli, 1980).

HIRs are very important because they occur under conditions that most closely resemble those of the natural environment in which plants live. PhyA and phyB are the primary mediators of the most extensively studied HIR, the light-mediated inhibition of hypocotyl elongation in *Arabidopsis* (Nagatani et al., 1991; Quail et al., 1995). The R-HIR responses induced by continuous R are mediated predominantly by phyB (Nagatani et al., 1991; Reed et al., 1993) and to a lesser degree through phyC, phyD and phyE signaling (Whitelam and Harberd, 1994; Whitelam and Devlin, 1997; Franklin et al., 2003). Conversely, the FR-HIR responses induced by continuous FR are mediated predominantly by phyA (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993).

Although there is no doubt about the involvement of phytochrome in the HIRs (Kendrick and Kronenberg, 1994), the mechanisms underlying this group of photomorphogenic responses is still poorly understood. This situation is not from a lack of effort, but is mainly a consequence of the complexity of this group of responses. One of the puzzles faced by researchers during the early years of HIR research was the high effectiveness of FR despite the low values of P_{fr}/P_{tot} established at photoequilibrium. Hartmann was the first to suggest that P_{fr} destruction played an essential role in the FR-HIR (Hartmann, 1966). In etiolated seedlings exposed to continuous irradiation, FR maintained the best balance between the requirement for a P_{fr} level sufficiently high to maintain a signal above threshold and the opposite requirement for a P_{fr} level sufficiently low to keep destruction at a minimum and so sustain the signal over a prolonged period. This hypothesis remains valid based on more recent studies (Parks and Quail, 1993; Whitelam and Harberd, 1994).

At least three groups of theoretical models have been developed to explain phytochrome action in the FR-HIR. All the models are based on the properties of a labile phytochrome and take into consideration the effects of the interaction between photoconversion, dark reversion, destruction, and reaction with an unknown partner or adduct "X" on the state of phytochrome. The basic hypothesis in the first group is that the FR-HIR is induced by two forms of phytochrome, P_{fr} or $P_{fr}X$; one of them is a transient form that decays to the more stable one. The relative abundance of these two forms is dependent on irradiance and wavelength. The calculated amount of $P_{fr}X$ formed correlates well with the inhibition of hypocotyl elongation in etiolated lettuce seedlings (Fukshansky and Schafer, 1983).

The second hypothesis predicts that the irradiance and wavelength dependence of the formation of $P_{fr}X$ is a function of the net rate of cycling between P_r and P_{fr} at photoequilibrium, which in turn is a function of P_{fr}/P_{tot} at equilibrium (φ), a rate constant K and the phytochrome concentration [P] (Fukshansky and Schafer, 1983). The third model is based on the dimeric behavior of labile phytochrome (VanDerWoude, 1987). Each of these models is consistent with at least some of the features of FR-HIR determined in different studies. However, none of them provides a satisfactory match for all the characteristics of the HIR as determined for different combinations of responses, species, and experimental conditions (Mancinelli, 1994)

R:FR ratio perception responses

Another group of phytochrome mediated response was studied because they also respond to the light environment surrounding the plants in nature, and so they might reflect more the real behavior of phytochromes in regulating plant growth. These are responses to the R-to-FR photon flux ratio (R:FR) of incident radiation. Perception of this ratio by phytochrome allows plants to detect nearby or shading vegetation, because chlorophyll-containing tissues reflect and transmit FR while absorbing disproportionately more of the R wavelengths. Plants respond to shade by two extreme strategies. One strategy, that of shade tolerance, involves relatively slow growth rates, the conservation of energy and resources and the development of photosynthetic structures that are especially efficient at low light levels. The opposite extreme is shade avoidance, a response of which internode and petiole extension growth is favored, apical dominance is accentuated, and flowering is accelerated, but leaf development is retarded (Smith and

Whitelam, 1997). As the name suggests, plants responding by shade avoidance grow away from low R:FR and toward higher R:FR light (Smith, 1982). In evolutionary terms, shade avoidance appears to be a relatively recent strategy, since it is predominantly found in the angiosperms, although some gymnosperms show some shade-avoidance characteristics (Smith, 1994).

The most direct way to assess the shade avoidance response is to use carefully controlled light conditions in which varying amounts of supplemental FR is supplied to lower the R:FR ratio while maintaining the amount of photosynthetically-active radiation (PAR; 400-700 nm) constant. Therefore these light conditions adopted the name supplementary FR (Casal and Smith, 1989). The shade-avoidance syndrome is a composite of many different physiological responses all regulated by phytochrome through the perception of the R:FR ratio. Studies using mutants deficient of phytochrome B in *Cucurbita* (*lh*), *Arabidopsis* (*hy*3), and *Brassica rapa* (*ein*) indicate that the absence of phyB largely, but not entirely disables the capacity for R:FR perception (Whitelam and Smith, 1991; Devlin et al., 1992). Although the elongation growth responses to R:FR are essentially lost in *hy3*, the acceleration of flowering is not (Halliday et al., 1994; Whitelam and Harberd, 1994). Leaf area expansion in wild-type *Arabidopsis* is reduced under low R:FR, and this effect is undiminished in *hy3*. At the very least, these data show that not all the components of the shade-avoidance syndrome are mediated by phyB. Therefore it can be concluded that two or more phytochromes cooperate to perceive R: FR ratio and to induce the many integrated responses that together constitute proximity perception and shade avoidance (Robson et al., 1993).

End-of-day (EOD) responses

The P_{fr} form is assumed to be the active form in signaling the plant to respond to the changes in the environment and, therefore, the amount of P_{fr} at the beginning of the dark period is thought to play a critical role in regulating plant morphology such as height. End of the day (EOD) exposure to R or FR light can, therefore, influence plant structure by altering the amount of P_{fr} present at the beginning of the dark period. Plants given short exposures to FR at the end of the daily light period grow faster than those not so treated. These EOD-FR effects are fully reversible by subsequent R, and display normal reciprocity. Consequently, EOD-FR responses appear qualitatively similar to the classic R/FR reversible LFRs. Early evidence showed that EOD-FR responses are mediated by phyB, in which the P_{fr} form is stable and long-lived (Smith and Whitelam, 1990). Reduction of P_{fr} by EOD-FR treatment results in a qualitatively similar response to lowering of the P_{fr} level during the light period by reduction of the R: FR photon ratio with supplementary FR. Retention of a degree of shade avoidance and EOD-FR responsiveness in *Arabidopsis phyB* mutants suggested roles that have now been assigned to phyD and E (Robson et al., 1993; Devlin et al., 1998; Devlin et al., 1999; Franklin et al., 2003).

D. Physiological responses controlled by fern photoreceptors

Descriptive physiological studies, performed on different plants and selected stages of the life cycle, have revealed considerable information about the range of functions mediated by phytochromes and cryptochromes. Much of our understanding of the role of photoreceptors in higher plant development has come from analysis of mutants and overexpressors of one or more of the photoreceptors. These studies have allowed researchers to identify which photoreceptors control a particular subset of light-regulated developmental responses.

In ferns, on the other hand, very few photomorphogenic mutants have been identified, and cloning the genes responsible for these mutant phenotypes is laborious and inefficient given the very large genome sizes and dearth of genetic information available in ferns compared with other model plants such as *Arabidopsis*, maize, or rice. However, other characteristics of ferns make them an outstanding experimental material for applying newly developed techniques in photobiology and in molecular biology (Chatterjee and Roux, 2000).

Light effects on haploid gametophyte development in ferns

Photomorphogenic responses of haploid and diploid generations are often very different, even in a single species under the same light condition, although diploid cells resulting from self-fertilization contain essentially identical copies of the haploid gametophyte genome. The development of the haploid generation appears to be affected by light more dramatically than that of the comparable diploid form in most lower plants including ferns (Furuya, 1983). Therefore, light effects on developmental processes in ferns will be discussed separately for haploid and diploid generations.

Germination

Spore germination in several species of ferns is phytochrome-dependent (Shropshire and Mohr, 1983). Fern spores require a period of time after imbibition before becoming photosensitive, from 5 min in *Dryopteris* (Haupt, 1985) to 7 days in *Ceratopteris* (Cooke et al., 1987). In *Pteris vittata* (Sugai and Furuya, 1967) and Hn-n strain of *Ceratopteris* (Cooke et al., 1987) spore germination can be induced by brief irradiation with R and inhibited by subsequent FR irradiation. This effect is repeatedly R-FR reversible, indicating the involvement of phytochrome in regulating germination.

Although R-FR reversibility is diagnostic of phytochrome control of germination, further proof lies in the spectrophotometric demonstration of the pigment and its phototransformation from one form to another in the spore. This *in vivo* demonstration was accomplished in spores of *Lygodium japonicum*. Consistent with the physiological data, the concentration of P_r becomes spectrophotometrically measurable after 3 d of dark imbibition (Tomizawa et al., 1982). Leading to ask if this pigment is left over from sporogenesis, or is it synthesized *de novo* upon hydration of the spore. Gabaculine, an inhibitor of chlorophyll and phytochrome chromophore synthesis, prevents R-induced germination of spores, supporting the latter alternative (Manabe et al., 1987). However, gabaculine does not affect spore germination of *Anemia phyllitidis,* making the first alternative also plausible and suggesting a species-specific expression pattern (Schraudolf, 1987a).

The apparent retention of phytochrome from spore development in *Anemia* is further supported by the discovery that the light requirement for germination depends upon light conditions during sporogenesis in parent plants, and germination can be induced in complete darkness from parent plants grown under R, whereas spores from plants grown under ordinary greenhouse conditions require R for germination. Therefore, at least in *Anemia*, P_{fr} accumulates in the developing spores while on the parent plants and is maintained in the dry spores to potentiate germination without additional doses of R (Schraudolf, 1987b). In *Dryopteris*, the onset of photosensitivity within 5 min after sowing (Haupt, 1985) is very rapid for *de novo* phytochrome gene expression to have occurred, suggesting again that phytochrome is present in the dry spores.

These diverse results from different species make it difficult to establish that the increase of detectable phytochrome results from either *de novo* biosynthesis or rehydration of existing photoreceptor protein in the spores. Some studies support the possibility that proteins synthesized during imbibition may be involved in the establishment of photosensitivity (Raghavan, 1992). The correlation of germination and the amount of P_{fr} is also observed in photoinduced spores of *Pteris vittata* (Furuya et al., 1982) and of *L. japonicum* (Tomizawa et al., 1983).

Germination of some ferns is dependent not so much on the quality of light as on its intensity (Marcondes-Ferreira and Felippe, 1984). Generally speaking, at lower light intensities it takes longer for a given lot of spores to attain maximum germination than at higher intensities. Another type of light effect is exhibited by spores whose germination is influenced by the light photoperiod. Spores of *Athyrium niponicum* germinate maximally when they are exposed to continuous light, and interruption of the light regime with dark periods decreases germination (Isikawa and Oohusa, 1954). Although there is a requirement for high intensity light for germination of *Matteuccia struthiopteris* spores, the situation is complicated by the involvement of a photoperiodic effect. This is reflected as a much lower germination rate when spores are exposed to a short period of illumination at high intensity than when the same fluence of light is given for a long period (Pietrykowska, 1962).

In contrast to their role in higher plants, the B and UV-A spectral regions inhibit germination of some fern spores. B administered before or after the inductive R inhibits the normal phytochrome-induced germination in *Ceratopteris*. The available evidence suggests that this inhibition cannot be attributed to B absorption by phytochrome, but rather depends on other B photoreceptors (Sugai and Furuya, 1967; Cooke et al., 1987). As yet, there is little insight regarding the mechanism of the interaction between phytochrome and blue light photoreceptors in fern.

Protonemal growth

In most homosporous ferns, after spore germination, but before two-dimensional differentiation, the early gametophyte undergoes filamentous growth resulting in a tissue known as the protonema (Wada and Murata, 1988). Under R or very low fluence W, protonemata grow longer and thinner than under B or high irradiance W. Under appropriate R conditions, *Adiantum* protonemata grow toward a light source as long single cells, in a true phototropic response mediated through phy3. In contrast, *Pteris* protonemata show linear growth under R but no tropic responses. In *Adiantum,* growth retardation occurs when R-grown protonemata are transferred to darkness, B, or W. Continuous B (or W) induces cell swelling at the apical region within 1-2 h (Murata and Wada, 1989).

Intracellular changes of cytoskeleton components are affected by light and may be involved in these differential growth patterns. During linear growth under R, a band of cortical microtubules and microfilament runs perpendicular to the cell axis around the protonema subapical part. When transferred to B, a disruption of microfilaments starts within 15 min and is complete about 1 h after the onset of B (Murata and Wada, 1989). Consequently, the change of microfibril arrangement at the newly grown area may weaken the apical area, resulting in tip swelling. In *Adiantum* protonemata, phytochrome-mediated growth induced with 5-10 min of R can usually be reversed by subsequent irradiation by FR light. Therefore, the photoreceptor for the effects on protonemal growth is thought to be phytochrome. However, the different photoresponses among other species are difficult to explain on the basis of a simple phytochrome mechanism. For example, in some other ferns such as *Lygodium*, the gametophyte becomes two-dimensional under either R or B, whereas linear growth occurs in FR. A family of phytochrome species has been isolated from *Adiantum*, opening the possibility that each phytochrome may have different transduction chains, as well as shared pathways, as is the case in higher plants. As an initial step to determining whether different phytochromes might function through separate mechanisms, it will be important to determine the intracellular location of the physiologically active phytochrome in one or more fern species.

Cell division and its orientation

The timing, direction, and the site of cell division determine the appropriate development of gametophytes. *Pteris* and *Adiantum* protonema grown under R will grow as very long single cells, and the cell cycle will remain locked at the beginning of the G1 phase (Ito, 1970; Miyata et al., 1979). When transferred to darkness or B, the G1 phase starts to progress, but with different kinetics (Wada and Furuya, 1972). It remains unclear whether this progression occurs *via* the same pathways in B and darkness. The fact that relief of G1 arrest in darkness can be reversed by a R pulse before the cell enters S phase, but becomes irreversible when exposed to B (Wada et al., 1984) suggests that distinct mechanisms are at work. The G2 phase is prolonged by FR given before progression of the G1 phase, and the S and M phases, at least in *Adiantum*, are not influenced by light given before or during these phases of the cell cycle (Wada and Sugai, 1994). In *Adiantum*, R was found to promote the first cell division, whereas B inhibits it. These effects are opposite to those of R and B on the subsequent protonemal cell divisions (Furuya et al., 1997).

Studies on cell division in *Adiantum* are relatively easier to do than studies on *Ceratopteris*, which lacks a true protonemal single cell stage. Even so, *Ceratopteris* can develop into elongated strap-shaped prothalli consisting of 3-6 rows of cells. These different patterns of growth in *Ceratopteris* under darkness have proven to be a good model to study light-induced growth inhibition. As will be discussed in detail in later chapters, continuous irradiation with B inhibits elongation of prothalli cells in *Ceratopteris* (Murata et al., 1997), as is the case in some other ferns (Furuya, 1983). When these dark-grown prothalli are irradiated with continuous W light, marginal cells of the elongation zone divide asymmetrically to give smaller cells which then develop into rhizoids. A pulse of R can initiate this asymmetric division but is not enough to induce subsequent rhizoid growth. Continuous irradiation of W, B, or R is necessary to induce rhizoid growth (Murata and Sugai, 2000).

The direction of the two dimensional growth of the developing gametophyte is controlled in part by light direction. In *Adiantum*, the first division occurs transversely, but the second or third division is longitudinal and parallel to the incident light rays. This transition from transverse to longitudinal division is the turning point from a unidirectional filament growth to the two-dimensional prothallus growth (Wada and Murata, 1988).

Phototropism and polarotropism

Phototropism and polarotropism are induced by directional non-polarized and polarized light, respectively. In the former, protonemata show a tropic response towards the light source, but in the latter, cells grow perpendicular to the vibration plane and the incident ray of the polarized light (Wada and Sugai, 1994)

In many cases, photoreceptors mediating the tropic response of ferns are phytochrome and blue light photoreceptors, but the extent of involvement of both pigment systems differs among species. *Dryopteris* fern and *Physcomitrella* moss protonemata respond to both R and B equally (Mohr, 1956; Etzold, 1965), but *Pteris* lacks the phytochrome-mediated tropic responses (Davis, 1975), exhibiting a tropic response to B but not to R. In another bryophyte, *Ceratodon*, the protonema shows high sensitivity to R light but very low or no sensitivity to B (Hartmann et al., 1983). The polarotropism induced by the B photoreceptor is rather weak in *Adiantum* and can only be detected when one side of the protonema is irradiated with a B microbeam. This response has been detected only in the lower fluence ranges. However, a phytochrome-mediated B response is active at higher fluences (Hayami et al., 1986).

In all the examples studied, it is assumed that an intracellular gradient of activated phytochrome is formed during the light treatment. Consequently, a protonema may grow more at a site with the lowest P_{fr} concentration and thus show a tropic response (Mohr, 1964.; Wada et al., 1981a). Similar results were obtained in centrifuged *Adiantum* protonemata (Wada et al., 1983) in which endoplasm had been spun down to the end of the cell away from the irradiated region. These data indicate that phytochrome must be localized in close proximity to the plasma membrane, with the transition dipole moment at a particular orientation with respect to the cell surface. It is thought that the transition dipole of the P_r form of phytochrome has a prevailing component parallel to the cell surface while the P_{fr} form is perpendicular to the cell surface (Kadota et al., 1982). A heart-shaped prothallus also exhibits a positive phototropic response, but with very different characteristics from that of filamentous protonema. When filamentous protonemata are exposed to unidirectional white light, the resultant prothalli develop in a plane which is at right angles to the direction of incident light and is independent of the direction of the gravity vector (Wada and Furuya, 1971).

Chloroplast photo-orientation:

Under low fluence rate light, chloroplasts move toward the light-irradiated face of the cell presumably to increase efficiency of photosynthesis. To prevent photobleaching and damage to the photosynthetic pigments under high fluence rate light, the chloroplasts will migrate away from irradiated face and toward the lateral walls (Wada and Sugai, 1994). A B-absorbing photoreceptor is the main detector for both the high and low fluence-rate responses in a wide variety of plants. However, in the green alga *Mougeotia*, phytochrome has been determined to be the main photoreceptor that mediates chloroplast movement, whereas in *Adiantum*, phytochrome and a specific B photoreceptor mediate the chloroplast movement. Both high-and low-fluence responses in *Adiantum* are induced by R and B light. Moreover, chloroplasts move toward an area of higher P_{fr} concentration as well as to an area of excited blue photoreceptors under low fluence rate light

(Yatsuhashi et al., 1985). The absorption plane of P_r and another B photoreceptor was predicted to be parallel to the cell surface and that for P_{fr} to be perpendicular (Yatsuhashi et al., 1987).

In most of the studies done on lower plants, it has been suggested that in order for P_{fr} and P_r forms to maintain their respective orientations, phytochrome should be either associated with the cell membrane or with a cylinder of microtubules. The nucleotide sequence of *Adiantum* phytochrome lacks a hydrophobic transmembrane domain (Nozue et al., 1998a). On the other hand, the amino acid sequence at the C-terminal end of *Mougeotia* phytochrome has been found to be similar to that of proteins that bind microtubules (Winands and Wagner, 1996), suggesting that phytochrome is bound to the cytoskeleton adjacent to the plasma membrane.

Light regulation of reproduction

Precise knowledge of light effects on the induction of fern antheridia and archegonia is very limited (Dyer, 1979). Although moderate to high light intensities, which favor vegetative growth, are generally necessary for full sexual differentiation (Millar, 1968), the antheridia of *Polypodium aquilinum* can be formed only under darkness or FR irradiation but not under continuous W. A brief exposure to R inhibits initiation of the antheridium, and this inhibition is totally reversed by FR, indicating an involvement of phytochrome (Schraudolf, 1967). Recent studies on the *dkg1* mutant in *Ceratopteris*, which is impaired in several photoresponses (Cooke et al., 1995; Banks, 1999), demonstrate that archegonia formation is under the control of phytochrome. Fiftytwo percent of dark-grown *dkg1* prothalli develop archegonia close to the lateral meristem, whereas archegonia are totally absent in dark-grown wild-type prothalli. A pulse of R is sufficient to induce archegonia formation in wild-type dark-grown prothalli, and this effect can be canceled by subsequent irradiation by FR. In addition, neither a FR pulse nor a B pulse is capable of inducing archegonia formation (Kamachi et al., 2004). A widely accepted view is that the usual site of archegonia formation on fern gametophytes is in the vicinity of the meristem, and the degree of development is largely dictated by the relatively high metabolic state of the meristematic cells (Raghavan, 1989). The lack of archegonia development without the lateral meristem, supports the idea that the lateral meristem induced by R may simultaneously initiate the program for archegonial differentiation, although the effect could be direct or indirect (Kamachi et al., 2004).

Light effects on diploid sporophyte development in ferns

A phenomenon similar to etiolation in seed plants is found in isolated leaf blades of the fern *Todea barbarsa* (Tavares and Sussex, 1968). After 20 weeks in darkness, the growth of leaf and root is arrested while long petioles and stems are produced. De-etiolation takes place when the plants are returned to light. In the young sporophyte of *Marsilea vestita*, R induces inhibition of petiole and internode elongation, and this inhibition can be reversed by subsequent FR treatment (Laetsch and Briggs, 1962). Sporophyte development is affected under different light regimes, with the longest sporophyte leaves developing under green light. The length of the first true root is also reduced in green light (Mahlberg and Yarus, 1977).

In *Osmunda* sporophytes, progressively shorter photoperiods lead to correspondingly greater induction of fertile fronds. The highest percentage of fertility in *Osmunda* frond cultures is obtained under continuous darkness. Conversely, as light intensity is increased, sporophyll differentiation becomes inhibited (Harvey and Caponetti, 1972). However, the nature of the photoreceptors underlying these effects has not been determined.

E. Localization and Intracellular Compartmentalization of Phytochromes in Plant Cells

Intracellular localization of higher plant phytochromes

Our knowledge about light-controlled plant development has greatly increased since the discovery of phytochrome in 1959. It is well documented that phytochrome plays major roles in mediating many light responses. In higher plants, many of these responses have been shown to be light-dependent modulations of the transcription of specific genes (Mösinger et al., 1985; Tobin and Silverthorne, 1985; Tobin and Kehoe, 1994). To elucidate the signal transduction mechanisms of different phytochromes, it is essential to know the sites of their action within the cell.

In *Arabidopsis*, PIF3 (a nuclear-localized basic helix-loop-helix protein) has been identified as a phytochrome-interacting factor through yeast two-hybrid screening (Ni et al., 1998). Since then, numerous other nuclear-localized phytochrome-interacting proteins have been identified and characterized. Hence, a direct interaction between phytochrome and a transcriptional regulator might be involved in the signaling pathway within the nucleus. On the other hand, several studies have provided evidence for the cytosolic localization of phytochrome. For example, phytochrome appears to be a light-regulated serine/threonine kinase (Yeh and Lagarias, 1998a), and therefore may transmit perception via protein phosphorylation. Moreover, phytochrome kinase substrate 1 (PKS1) was detected in its phosphorylated form in *Arabidopsis* as a PKS1-GFP fusion protein constitutively localized to the cytosol. Immunocytochemical localization assays have demonstrated that the majority of phytochromes, both the P_r and P_{fr} forms, are associated with the cytosol in a light-independent pattern (Pratt, 1994). Red/far-red reversible pelletability of phytochrome (Quail et al., 1973a), reflecting sequestered areas of phytochrome (Speth et al., 1987), indicate that phytochrome is localized in the cytosol and/or plasma membrane. This idea is supported further by microinjection experiments in the chromophore-deficient *aurea* mutant of tomato (Neuhaus et al., 1993; Bowler et al., 1994). In these studies, phytochrome induced activation of a GTP-binding protein—assumed to be associated with the plasma membrane—that subsequently activated two pathways. One of these pathways modulates the cGMP level and leads to induction of anthocyanin formation and chalcone synthase (CHS) expression. The other pathway leads to regulation of calmodulin/Ca^{2+} levels and promotion of chloroplast development.

Based on theses physiological, biochemical, immunocytochemical and molecular analysis, it was generally believed that phytochromes are cytosolic proteins until results obtained using fusion proteins of different phytochromes isolated from higher plants. Sakamoto and colleagues (Sakamoto and Nagatani, 1996) found that in light-grown *Arabidopsis* plants, the nuclear fraction contained a significantly higher amount of phyB than the nuclear fraction isolated from dark-grown seedlings. Furthermore, light-dependent nuclear localization of a phyB: GUS fusion protein was demonstrated by the same laboratory. More recently, the same laboratory used the full length PhyB-GFP fusion protein to complement an *Arabidopsis* mutant deficient in PhyB(Yamaguchi et al., 1999). They demonstrated that the fusion protein is a functional photoreceptor and its nuclear localization can be induced by R and is accompanied by the formation of nuclear spots or "speckles" of phyB:GFP. These findings were further extended when it was shown that tobacco phyB:GFP expression in transgenic plants resulted in complementation of a phyB-deficient mutant, indicating that the chimeric protein is functional. The fusion protein was localized in the cytosol of cells in both etiolated seedlings and dark-adapted plants. Continuous R or multiple R pulses induce nuclear translocation and speckle formation in the nucleus. Subsequent FR treatment reverses the response to R, indicating that the nuclear import of phyB is mediated by a low-fluence response of phytochrome (Kircher et al., 1999). Detailed kinetic studies provided evidence that the nuclear import of phyB:GFP is relatively slow (maximum accumulation in the nuclei is detected within 3 h after the onset of light treatment) and that the import process is strongly fluence-rate dependent (Gil et al., 2000). Furthermore, although the phyB:GFP is abundant in the nuclei, a significant proportion remains in the cytosol.

A similar experimental approach was used to obtain more information about phyA nucleocytoplasmic distribution in transgenic plants grown under different conditions. Intracellular localization of rice phyA:GFP in transgenic tobacco and *Arabidopsis* phyA:GFP in phyA-deficient mutant led to several important conclusions. In etiolated transgenic tobacco or *Arabidopsis* seedlings and in dark-adapted plants, the phyA:GFP fusion protein was localized in the cytosol. Brief irradiation of R, FR, or B induced rapid nuclear import and intracellular speckle formation of phyA:GFP at a rate an order of magnitude faster than that of phyB: GFP. This translocation was preceded by an even faster cytosolic spot formation of the fusion protein, a phenomenon known as SAP formation. Finally, nuclear translocation of phyA was induced by continuous FR, but not by continuous R treatment. Thus, it was concluded that the nuclear translocation of phyA, unlike that of phyB, is mediated by very low fluence and by FR-high irradiance light (Kim et al., 2000), indicating that phyA regulates its own nuclear import independently of any other types of phytochrome. These results are consistent with the fact that phyA regulates specifically VLFRs and FR-HIRs, but not LFRs.

Nuclear import of the same *Arabidopsis* phyA:GFP fusion protein exhibited characteristically different features in transgenic *Arabidopsis* as compared with tobacco. Only FR-HIR-mediated nuclear localization was detected, without any apparent VLFR-type localization in tobacco. It has been hypothesized that molecules responsible for this altered photoregulation are located in the cytosol and affect protein stability or retention in the cytosol rather than the nuclear import mechanism itself (Kim et al., 2000).

On the other hand, analysis of *Arabidopsis* transgenic plants expressing phy:GFP fusion-proteins indicates that the distribution of phyC, D, and E is quite different to that of phyA or phyB, and is dependent on the growth conditions used by the researchers to test translocation. In dark-adapted seedlings germinated under W, the phyC:GFP, phyD:GFP and phyE:GFP fusion proteins were mostly located inside the nuclei, but they were diffusely distributed in the nucleus, suggesting that the nuclear translocation of these phytochromes occurred it the light prior to dark adaptation and that the process is not reversible in darkness. However, with further exposure of the seedling to W or R, these phytochromes exhibit similar nuclear speckle distribution patterns to phyA:GFP or phyB:GFP fusion proteins (Kircher et al., 2002). A pulse of R is sufficient to induce the speckle formation of these phytochromes, and is reversible with a subsequent FR pulse. As the case with phyB:GFP, FR does not induce detectable nuclear localization of phyC, phyD, or phyE (Kircher et al., 2002). The kinetics of nuclear spot formation show subtle differences among phytochromes B, C, D, and E, with phyB and phyE showing the fastest kinetics of speckle formation.

Conformational changes of phytochrome affect nucleocytoplasmic distribution

In etiolated seedlings, when phytochrome exists as a P_r conformer, the phyA: GFP and phyB:GFP fusion proteins are localized outside the nucleus. A phyB mutant protein that is unable to bind its chromophore is localized constitutively in the cytosol (Kircher et al., 1999). These data suggest that the P_r-to-P_{fr} conformational change is required for translocation. However, in a few cases, strong overexpression of phyA:GFP and phyB:GFP has resulted in diffuse GFP fluorescence in the nuclei of dark-grown seedlings. Moreover, it has been shown that a fusion protein containing the C-terminal part of phyB fused to GFP is constitutively localized in the nuclei (Nagy and Schafer, 2000). In addition, the insertion of one or more SV-40 NLS domains into the phyB fusion protein at various positions did not change the translocation pattern. Based on these data, it was predicted that (1) the P_r-P_{fr} conformation change is not absolutely required for initiation of nuclear import; (2) NLS motifs mediating phytochrome binding to the nuclear import receptor importin α are not masked even in the presence of the P_r conformer: and (3) the C- terminal portion of phyB is not able to interact with a factor that ensures cytosolic localization in darkness, whereas overexpressed phyB escapes interaction

These predictions may also explain the nuclear localization of phyC-E:GFP in darkness, especially if the very low endogenous amounts of these phytochromes are considered. This interpretation leads to a model which assumes that P_r conformers of phytochromes are retained in the cytosol, and that excess P_r (when it escapes retention, for example by saturating the retention mechanism) and P_{fr} (which does not interact with the retaining mechanism) are both subject to nuclear import, and that the import process is mainly regulated at the retention level (Nagy and Schafer, 2002).

Localization and intracellular distribution of phytochrome in ferns

The intracellular locations and the dynamics of redistribution of phytochrome in lower vascular plants may differ from that of higher plants. In lower vascular plants, many phytochrome effects show action dichroism, whereas little action dichroism has been observed in the higher plants. The exception is the photometric dichroism in *Avena* (Marme and Schafer, 1972). However, failure of action dichroism in complex higher-plant tissues may simply be the consequence of heavy light scattering and the resulting depolarization of the polarized excitation beam (Wada and Kadota, 1989).

Intracellular localization of phytochrome in filamentous cells of lower plants has been studied by microbeam analysis and responsiveness to polarized light. Partial irradiation with microbeams has shown that photoreceptive sites are located near the cell periphery (Haupt and Bock, 1962; Wada et al., 1981a, 1983). In addition, action dichroism induced by polarized light suggests strongly that phytochrome is localized on or close to the plasma membrane (Etzold, 1965). This localization of phytochrome is likely to be common in filamentous cells (Hartmann et al., 1983). When the growing tip of these cells is irradiated with a R microbeam, the protonema continues to grow without a change in direction. Moreover, when the side of the protonema is irradiated with R light, the cell shows a tropic response. It has been asserted that the high level of P_{fr} produced in the irradiated area promotes this phototropic response. Similar results were obtained in a centrifuged protonema (Wada et al., 1983) in which endoplasm had been spun down, indicating that phytochrome must be localized very close to the plasma membrane.

Microbeam irradiation on one side of the subapical region with a polarized R vibrating through various planes showed that the light with the wave vector parallel to the growing axis (or plasma membrane) is most effective. The results mean that the transition moment of the P_r is more or less parallel to the plasma membrane as has been shown in the green algae *Mougeotia*. In addition, P_{fr} is predicted to be perpendicular to the cell surface (Haupt et al., 1969; Kadota et al., 1982). A similar change in absorption caused by differences in the dipole moments between P_r and P_{fr} was photometrically detected in phytochrome purified from higher plants and immobilized on agarose beads (Sundqvist and Bjorn, 1983).

Characterization of purified phytochrome from higher plants revealed that at least two short-lived intermediates, $I_{692}(I_{700})$ and I_{bi} exist during the phototransformation from P_r to P_{fr}, and that these intermediates can be reconverted by light to P_r (Pratt et al., 1984). These intermediates and their photoreversibility were also detected in fern spores (Scheuerlein and Koller, 1988). Irradiation with polarized double flash photolysis on *Adiantum* protonemata revealed that the orientation of the dipole moment of each intermediate was similar to that of P_r (Kadota et al., 1986b). The reorientation rate of phytochrome was slow in this response, which can be ascribed as a result of a conformational change of the protein or to a change in the interaction between phytochromes and the anchors connecting them to the cell membrane.

Fern phytochrome is not always arranged for dichroic responsiveness. In phytochrome-mediated spore germination of the fern *Dryopteris*, no action dichroism was detectable (Haupt and Bjorn, 1987), although protonema of this species showed typical dichroism in polarotropism (Etzold, 1965). A similar pattern was observed in phytochrome-mediated spore germination of *Pteris* (Sugai and Furuya, 1967). However, no phytochrome response was detected in either polarotropism or phototropism or in chloroplast photo-orientation by means of polarized R. Therefore, it is possible that multiple phytochrome pools exist in ferns, either as different members of a phytochrome family of receptors or as temporal/spatial subpopulations of the same gene product.

F. Sensory transduction in ferns

Even though both photoperception and the ultimate photomorphogenic responses occur within the single cell of a fern protonema, for some phenomena the photoreceptive site may be physically separate from the location of the final response, while for other phenomena both perception and response may occur in one place (Furuya, 1983). In the former case, there must be a signaling system to transduce information between the photoreceptor and the response location. In the latter, signaling intermediates could be involved, though the distance between the two sites cannot be resolved with currently available techniques.

The hypothesis that phytochrome is localized along the plasma membrane in fern (Wada et al., 1983) has led researchers to look for changes in membrane ion permeability as possible primary mechanisms of action for phytochrome. For example, R-induced membrane depolarization has been detected in the fern *Onoclea sensibilis* (Racusen and Cooke, 1982). The membrane potential was re-polarized by FR. The magnitude of these electrical changes depended upon the concentration of Ca^{2+} ions in the growth medium. It has been proposed that phytochrome may transduce their signals through cytosolic messengers such as Ca^{2+}, calmodulin (CaM), and cyclic AMP both in higher plants (Quail et al., 1995) and in lower plants (Serlin and Roux, 1984; Wagner et al., 1984). R stimulates increases of both intracellular Ca^{2+} and a calmodulin-like protein during phytochrome-mediated spore germination in *Onoclea* and *Anemia* (Wayne and Hepler, 1985; Fohr et al., 1987). However, the timing of the Ca^{2+} requirement during phytochrome-dependent fern spore germination does not correlate well with the escape from FR reversibility, indicating that Ca^{2+} transients are not solely responsible for propagating the light signal (Iino et al., 1989; Scheuerlein et al., 1989).

In *Adiantum*, the light effects on the progression of the cell cycle show reciprocal antagonism of phytochrome and a blue light receptors; namely, cell division is synchronously induced by a brief irradiation with B/UV-A light, whereas the induction is prevented under R (Wada and Furuya, 1972; Miyata et al., 1979). A question arises as to what are the molecular events of the antagonistic regulation of the cell cycle progression in spores of *Adiantum*. The expression of a gene encoding an extensin (EXT1) was regulated by phytochrome and blue light receptors antagonistically in *Adiantum* (Uchida et al., 1998). The expression of extensin—a class of proteins involved in cell wall loosening that permits cellular elongation—showed typical R/FR reversibility and no induction by FR or B irradiation alone, indicating LFR regulation by phytochrome. Given the structural similarities of *Ac*EXT1 with dicot and monocot extensins in higher plants, *Ac*EXT1 may play the same roles in cell walls of *Adiantum* spores. Uchida et al. proposed that *Ac*EXT may have a similar function to *Nt*EXT in tobacco pollen tubes, since both germinated spores and pollen grains produce tip-growing structures, a process that may have similar requirements regarding the cell wall mechanical properties, either for protection of the cell or for its progression through intercellular spaces.

G. Interaction between phytochrome and blue light receptors

In plants living under natural conditions with a wide spectral range of visible light, both phytochrome and B photoreceptors are activated. Consequently, various actions mediated by both pigment systems would be maintained at some levels, and various modes of interaction would be expected (Schäfer and Haupt, 1983). Phytochrome also absorbs B light, and in the case of *Lygodium* spores, B raised the concentration of photometrically detectable P_{fr} 16-34% (Tomizawa et al., 1983). Three modes of interaction between the two pigment system has been analyzed and proposed by (Wada and Kadota, 1989).

Interaction through a common transduction pathway

In *Adiantum* protonemata, low fluence rate chloroplast movement is mediated by both B photoreceptor and phytochrome systems. The direction of the responses and pigment localization are the same in both pigment system, suggesting that the same signaling pathway may be shared (Yatsuhashi et al., 1985). Phototropism in *Adiantum* protonemata is also mediated by both phytochrome and B receptors. Both of them are located on or close to the plasma membrane of the subapical part of protonemata. Phytochrome is active in the high fluence range, whereas B photoreceptors are responsible for low-fluence phototropism.

Co-action using different transduction pathways

R and B effects can be recognized as antagonistic responses in some physiological phenomena. For example, R inhibits advancement of the cell cycle and apical swelling, whereas B induces growth retardation and apical swelling but initiates cell division (Furuya et al., 1997). By studying the timing of cell division of fern protonema, it has been determined that B given during the G1 phase causes shortening of this phase, whereas comparable treatment with FR lengthens the G2 phase. If the total length of the cell cycle is observed as one response, it may appear that the two pigments control the response antagonistically. In fact, the results of monochromatic irradiation at individual phases indicate that each pigment controls distinct phases of the cell cycle independently. Moreover, the intracellular sites of the two photoreceptor types are different. The responsible phytochrome was located at the cell periphery, whereas the active B photoreceptors were localized near the nuclear envelope. Therefore, in characterizing complex light functions, it may be necessary to divide a response into its separate components in order to determine whether that response is regulated by interaction or co-action of two (or more) pigments.

Modification of signal transduction pathways

Spore germination of several species of ferns is phytochrome-dependent, being induced by R and reversed by FR pulses (Raghavan, 1992). B applied before or after R inhibits germination in *Ceratopteris*, indicating a level of photosensory interaction with B receptors. Spores of *Cheilanthes* show a similar response, but with further complexities to the interaction in which the FR irradiation does not reverse the effect of R exposure.

Instead, R/FR photoreversibility is observed only when spores are treated with a B pulse before FR irradiation (Raghavan, 1973).

Still very little is known about the mechanisms of phytochrome signaling in lower plants. *Ceratopteris richardii* has several advantages in these studies over other ferns. The plasticity of using *Ceratopteris* make it an excellent model for physiological and genetic approaches toward examining phytochrome function and signal transduction. The addition of transgenic and pharmacological analysis techniques to *Ceratopteris* research would improve the range of possible experiments available for studying lower plants and will provide unique mechanistic and evolutionary insights into photomorphogenesis of higher plants.

CHAPTER II. Photoregulation of prothallus growth and development in the fern *Ceratopteris richardii*

A. Abstract

B. Material and Methods

C. Results

D. Discussion

Abstract

Of the numerous environmental factors that affect plant growth and physiology, light plays one of the most significant roles in plant development. While light provides the ultimate source of biological energy, the light quality, quantity, duration and direction used to modulate many physiological and developmental responses both in higher plants (Quail, 2002b; Sullivan and Deng, 2003)and in lower plants (Wada and Kadota, 1989; Wada and Sugai, 1994).

Although the roles of light in regulating many physiological processes in higher plants have been intensively studied, comparatively little information exists about the role of light on early development such as gametophyte development and embryogenesis (Sullivan and Deng, 2003). *Ceratopteris richardii*, an increasingly popular fern model, offers unique opportunities for developmental, physiological, genetic, and molecular studies of plant photomorphogenesis. (Chasen, 1992; Hickok et al., 1995; Chatterjee and Roux, 2000).

Several light-mediated responses have been described for *Ceratopteris* but few quantitative or long-term studies have been reported (Murata and Sugai, 2000; Kamachi et al., 2004). Information is lacking about the effects of phytochrome over prolonged periods and in more developed mature gametophytes. Therefore allowing spores to germinate and developed under different spectra of continuous light would be expected to

be very informative about how phytochrome acts. This work reveals more information on cell division, expansion, and chlorophyll development of maturing gametophytes and young sporophytes.

Most of the isolated phytochromes from ferns and fern allies show more similarity to phyB sequences than to phyA from higher plants (Thummler et al., 1990; Schneider-Poetsch and Braun, 1991; Maucher et al., 1992b). One way to study whether the native phytochromes of *Ceratopteris* will behave similar to phyB in *Arabidopsis* is to study the shade avoidance response, which is mediated predominantly through phyB (Robson et al., 1993) in *Arabidopsis*. Shade avoidance is the most dramatic response induced by FR-enriched light, which characterized by stimulation of elongation growth, often associated with reduced leaf development, increased apical dominance, and a reduction in branching. Moreover, the shade avoidance syndrome may include acceleration of flowering and a reduction of seed set and germination capacity (Smith and Whitelam, 1997). Whether a comparable series of responses occurs in ferns is largely unknown. Supplementary FR is an experimental simulation of a shade environment. Supplementing W_c with FR_c shows effects on *Ceratopteris* that are different to those in higher plants.

A. Material and Methods

Plant culture and growth conditions:

Spores of *Ceratopteris richardii* (strain RN3) were originally a gift from Dr.Thomas R. Warne (Department of Botany, University of Tennessee). Mature spores were soaked in sterile distilled water for 10 min, surface sterilized with a 20% (v/v) solution of commercial bleach (final sodium hypochlorite concentration of 0.05% for 3 minutes, then rinsed three times with sterile deionized water and suspended in Parker-Thompson nutrient medium (Klekowski, 1969) without agar. The concentration of spores was adjusted to 100-200 per mL. For continuous examination of gametophyte growth, the spores were sown on 1% agar-solidified Parker-Thompson medium. Following sowing of the spores, the plates were sealed with parafilm and placed in darkness for 7-10d, after which they were exposed to various light treatments described below. The plates were placed in constant temperature incubators at 28°C. To grow sporophytes for longer observation, at least 20 sporophytes were transplanted at the 2-3 leaf stage from each treatment onto floating supports in liquid Parker & Thompson medium to allow sufficient space and gas exchange for growth and continuous observation.

Light sources and conditions

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

Germination efficiency

To ensure synchronization of spore germination, spores were imbibed in darkness for 7-10 days before inducing germination. Light treatments were given to dark-cultured spores at various times after sowing, as indicated for each experiment. Germination rates were determined under a stereomicroscope by monitoring the emergence of rhizoids and their apical growth in each population tested. Each data point represents the mean of at least three independent experiments, from which 100-200 spores per plate were counted.

Area and length measurements

Samples were collected at the times under the controlled light conditions indicated below, and the tissues digitally photographed under a Wild microscope with a SPOT RT CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI). These pictures were analyzed using SigmaScan Pro software (SPSS, Chicago). For each experiment at least 3 replicates were analyzed with a minimum of 30 individuals per replicate.

Chlorophyll determinations

Chlorophyll determinations were performed 14 d after inducing germination. Spores were grown as described above and plants collected from the agar plates. Subsequently, all plants were counted and harvested onto filter paper to remove excess water before measuring the fresh weight. The chlorophyll from the tissue was then extracted in 80% (v/v) acetone in darkness at 4°C for 24 h. Parallel determinations of chlorophyll content per unit fresh weight and per individual gametophyte was done concurrently. Chlorophyll *a* and *b* concentrations were calibrated using the equation for chlorophyll $a = 12.7(A_{663}) - 2.69(A_{645})$ and chlorophyll $b = 22.9(A_{647}) - 4.48(A_{645})$ as described (Chory et al., 1991).

Archegonial development and cell division

After 14 d of light treatment, prothalli growing on agar medium were observed under a Wild dissecting microscope. The number of archegonia clustered at the meristematic notch was scored visually. The completeness of archegonial maturation was determined by the presence of an opening within the collar of four neck cells resulting from breakdown of the neck canal cell. The archegonial maturation was confirmed by the presence of pigmentation of the canal cells.

Results

Germination efficiency is dependent on light quality

In confirmation of previous work (Cooke et al., 1987), a dark interval of 7 to 10d was found to be necessary before the induction of maximum germination by a brief irradiation with red light. This dark interval was used in all experiments to ensure maximum germination and synchronization, and the dark period was followed by the specific light conditions indicated for each experiment. Cook and colleagues (1987) showed that the effect of a red light (R) pulse could be reversed with a subsequent pulse of FR or blue light (B). In the current study, the effects of continuous red light (R_c), far-red (FR_c), red given simultaneously with far-red (R_c+FR_c), white light (W_c), or blue light (B_c) on germination were studied in greater detail. After 7-10 d in darkness, spores were grown for 2, 3, 4, 7 or 14 d (figure 3) in continuous light of different wavelengths. The proportion of spores that had germinated was measured after the indicated time period. At 2 d from initiating light treatments, very few spores had germinated in any light condition. After 3 and 4 d of light exposure, a delay in germination was apparent in R_c-grown compared with W_c-grown spores. At 3 d, germination of W_c-treated spores approached 80% and reached its maximum germination of 98% at 7 d. On the other hand, only 15% of spores treated with R_c had germinated at 3 d, and germination was limited to 80% even after 7 d. There was very little effect on germination of extending the exposure from 7 d to 14 d, regardless of the light regime. B_c inhibited germination, with less than 15% of spores germinating after 14 d of continuous exposure, whereas nearly all the W_c-

treated spores germinated (figure 3). Although germination under Rc was very low, supplementing the R_c with FR_c increased the germination level to 60% at 3 d, lower than that obtained under W_c but comparable to that in FR_c alone. Spores treated 7 d under their respective illumination conditions and then transferred to W_c, yielded slightly higher germination rates than if left in their respective light treatments. Those transferred from the B_c or dark conditions produced high germination levels, although none of the pretreated spores reached the germination levels of 14 d W_c (data not shown).

Prothallus morphology, cell division, and cell expansion are affected by light

Not only does the proportion of spores germinating depend on the light regime, but the shape of the emerging tissues varies widely according to the wavelengths used. The R and B effects on *Adiantum* development were found to be promotion and inhibition of the first cell division, respectively, whereas the roles of these wavelengths were largely reversed in the subsequent protonemal cell divisions (Furuya et al., 1997; Murata et al., 1997). A comparable effect of R_c and B_c light on cell division in *Ceratopteris* has not been described because *Ceratopteris* does not develop into the protonemata stage under Rc (Wada and Sugai, 1994). In etiolated *Ceratopteris* gametophytes, continuous irradiation with B inhibited cell growth of the elongation zone, although R or FR had no effect on elongation (Murata et al., 1997). This effect of B was described only in the elongated prothalli, but was not explored in the heart-shaped prothalli.

In the experiments described here, after the dark period spores were left to germinate under W_c for 1 d and then grown under either R_c, B_c, R_c+FR_c, or FR_c for 13 d.

Figure 4 shows typical morphologies of the gametophytes developing under each light treatment. To understand the causes underlying different morphologies, the areas and the cell numbers for gametophytes from all light treatments were measured (figures 5 and 6). Under R_c, the gametophyte developed was severely reduced in size, although male and hermaphrodites could be distinguished by antheridia development (figure 4A). Although the forms of the gametophytes are quite distinct between R_c- and dark-grown individuals (figure 4A, C), the cell number in each was indistinguishable (figure 6).

Under FR_c (figure 4B), the gametophyte developed as an elongated prothallus similar to the dark-grown gametophyte (figure 4C) but it is larger and broader than those maintained in darkness, as measured by total area (figure 5), width/length ratios (figure 7) and the number of cell files that constitute the subapical region (Table 1), indicating more cell division of the meristematic cells on their apical tips. In B_c, the germinated prothalli were more completely developed with increased cell division (figure 4E) and a typical heart-shaped prothallus. The prothalli grown under R_c+FR_c (figure4D) were similar to those grown under Bc, but the shape of the prothallus was distinct, at least in part because of increased cell elongation in the subapical region of the R_c+FR_c-treated gametophytes (figure 4D). In turn, both R_c+FR_c- and B_c-grown prothalli are significantly smaller than those grown under W_c (figure 5,6). On the other hand, the cell numbers making up B_c- and R_c+FR_c-treated prothalli are nearly double that of dark-grown tissues (figure 6).

Chlorophyll accumulation is controlled by light

Unlike most flowering plants, ferns are able to produce chlorophyll in the dark.

Very few studies have been reported concerning the content of chlorophyll *a/b*-binding proteins (Lhcb) in lower plants. In *Adiantum*, Lhcb mRNA accumulates in response to phytochrome activation and is inhibited by B (Christensen et al., 1998). In *Ceratopteris*, it is not clear which photoreceptors, if any, affect chlorophyll accumulation in the haploid gametophyte. Therefore, chlorophyll *a* and *b* content were assayed after gametophyte growth under different light regimes. Chlorophyll accumulation is very low in R-grown prothalli compared with that in plants grown under other light conditions, and even lower than that in dark-grown plants (figure 8). FR_c increases the chlorophyll content considerably over R_c, to a level marginally higher than that in unexposed gametophytes. However, the combined R_c+FR_c treatment causes a further augmentation of the chlorophyll content, generating considerably more pigment than either wavelength alone and comparable to the level observed in W_c-grown tissues. B_c causes still greater accumulations of chlorophyll, even surpassing the levels induced by W_c.

Rhizoid length and number are regulated differentially by light

In the fern *Onoclea sensibilis*, the growth and development of the rhizoids is completely dependent on the internal delivery of photosynthates from green cells, whereas the conversion of the one-dimensional filament into a two-dimensional prothallus requires rhizoid uptake of monovalent cations (Racusen, 2002). Because of differences observed in chlorophyll accumulation—and presumably in photosynthates available to the developing gametophytes as well—rhizoid development in *Ceratopteris* was quantified under various light conditions. At 14 d following induction of germination, gametophytes were scored for the length and number of rhizoids presents (figure 9A and B). Rhizoid lengths under W_c and R_c are similar, whereas dark, FR_c, and combined R_c+FR_c treatments yield generally longer rhizoids than those of W_c- or R_c-treated plants. The elongation of rhizoids is considerably reduced in B_c.

Rhizoid number is also affected by exposure to different wavelengths. W_c-grown gametophytes produce significantly more rhizoids than those grown under other wavelengths tested. R_c- and FR_c-grown tissues generate similarly low numbers of rhizoids to dark-grown gametophytes. R_c+FR_c-treated plants produce larger numbers of rhizoids than either R_c- or FR_c-treated gametophytes. On the other hand, plants grown under B_c produce intermediate numbers of rhizoids between those of W_c-treated and R_c+FR_c-treated gametophytes.

The male:hermaphrodite ratio is affected by light

The role of antheridiogens in sex determination in *Ceratopteris* has been extensively studied (Banks, 1994), but little is known about the effect of light on this process. The *dkg1* mutant, which is altered in many responses to light, does not exhibit obvious differences from wild-type gametophytes in sex determination (Cooke et al., 1993). To determine whether controlled light conditions might regulate the ratio of male to hermaphrodite prothalli, the numbers of each morphotype were scored after growth under different wavelengths of light for 14 d. FR_c did not affect the proportion of male plants compared with darkness, whereas all other light treatments, including W_c, R_c, R_c+FR_c, and B_c decreased the relative number of gametophytes developing as males by approximately half (figure 10).

Archegonia development is altered under different light conditions

The number of archegonia on individual gametophytes after 14 d growth under different light conditions was scored. As shown in figure 11 and table 3, R_c treatment strongly affects archegonial development, with 24% of the hermaphrodites producing a maximum of two archegonia. Under FR_c, 99% of the hermaphrodites produce no archegonia, while the remaining 1% produces only one archegonium. However, combined R_c+FR_c induced production of multiple archegonia, averaging about 8 per gametophyte and in some cases producing as many as 11 archegonia on a single individual. W_c induced comparably high numbers of archegonia, with a maximum of 16 recorded. Under B_c, an average of 4 archegonia, ranging from 1 to 6, were found per individual hermaphrodite.

Among the archegonia produced under all light conditions, only those from W_c were mature with visually detectable pigment at 14 d. Even in W_c, the population of archegonia on a given individual consisted of mature and immature organs.

Embryogenesis is delayed by supplementing W_c with FR_c

When sterilized spores were maintained in darkness for 7-10d, then exposed to 5h R and transferred to W_c or W_c+FR_c, the supplementary FR (W_c+FR_c) resulted in differences in fertilization timing and embryo development, which was perpetuated throughout the development of sporophyte leaves. The plants under W_c developed embryos and grew sporophyte leaves earlier and faster than those with supplementary FRc, as shown in figure 13. At any given time, the number of sporophyte leaves that developed from each gametophyte was higher in W_c than under W_c+FR_c (figure 14). The supplementary FR_c did not significantly affect either germination (figure 15A) or the proportion of gametophytes developing as males (figure 15B). However, Archegonia total number after 7d of light treatment (figure 16A) and the proportion of mature archegonia (opened with pigment) to immature (closed without pigment) archegonia after 14d of light exposure (figure 16B) indicate that the FR_c supplement reduces the rate or capacity of archegonia to mature, as measured by the proportion of mature archegonia under each light regime.

End of day far-red light (EOD-FR) does not affect the growth of gametophytes and sporophytes

When wild type higher plants are exposed to FR at the end of the daily light period, they grow faster than those not so treated (Downs et al., 1957). These EOD-FR treatments are fully reversible by subsequent R. These responses have been suggested to be mediated primarily by phytochrome, in which P_{fr} is stable, and long-lived (Smith and Whitelam, 1990). Since most of the phytochromes isolated and characterized from ferns are from light-grown plants, it has been suggested that they are light-stable phytochromes, and that they might be expected to elicit responses to EOD-FR. This in turn would reveal considerable information about the physiological function of *Ceratopteris* phytochromes.

In this work, many aspects of growth that previously were shown to be affected by phytochrome in *Ceratopteris* or are altered by EOD-FR in higher plants were measured, namely germination, growth rates, hermaphrodite to male gametophyte ratios, timing and extent of sporophyte initiation, chlorophyll content, number and extent of fertile and infertile frond growth, among various other parameters.

Germination rates are apparently unaffected by EOD-FR, compared with spores grown under 16 h light/8 h dark and assayed after 4 d (Figures 17A). The ratio of male gametophytes at 12 d was not significantly different from that of controls, although under EOD-FR a slightly smaller percentage of males were consistently observed (Figure 17B). The timing and extent of sporophyte initiation was also similar in both treatments (Figure 17C). After transfer to liquid medium, sporophyte growth was followed until maturation. No differences in the number of leaves after 10, 20 or 30 d post-transfer were detected, nor were variations in the ratios of fertile to infertile fronds after 40 and 50 d observed. The total chlorophyll content and the chlorophyll *a/b* ratios were also determined at 20, 30, and 70 d after transplanting to liquid medium, and again, no significant differences were found (data not shown)

Discussion

As it has been found in higher plants, no single set of growth conditions will provide a complete picture of the numerous phytochrome functions in *Ceratopteris*. To begin defining the photobiological parameters, responses, and mechanisms affected by phytochromes, a variety of physiological experiments were performed under different light conditions. Until recently, no phytochrome genes from *Ceratopteris* had yet been isolated (Bissoondial and Short, unpublished), although their presence and importance in mediating development can be inferred from physiological experiments and comparison of the results with those from higher plant studies.

Spore germination is controlled by at least one phytochrome

It is well known that the germination of *Ceratopteris* spores requires light, in which the effect of light is mediated in part by phytochrome, such that germination can be induced by a brief irradiation of R and reversed by a subsequent pulse of FR. Moreover, the R induction can be prevented by brief irradiation with B (Cooke et al., 1987). The results shown here (figure 3) indicate a phytochrome-dependent germination response distinct from the R-HIR observed in *Adiantum capillus-veneris* (Furuya et al., 1997). The very high level of germination under FR_c compared with that under R_c—especially after 3 and 4 days of light treatment—could be explained if at least one phytochrome is controlling germination in *Ceratopteris*. In higher plants, multiple phytochromes share numerous roles during development, such as phytochrome A- and B-specific induction of seed germination in *Arabidopsis*. In this case, a R pulse induces germination in a FR-reversible manner via phyB, but if the seeds are allowed to develop in darkness following imbibition, phyA accumulates and the seeds acquire a FR-HIR and a VLFR germination response (Reed et al., 1994; Shinomura et al., 1994). The germination of wild type *Arabidopsis* under FR-HIR was lower than that in a *phyB* mutant, indicating an antagonistic effect between phyA and phyB in this higher plant model. Under R-HIR high germination occurs because of the inductive effect of phyB. According to this hypothesis, one phytochrome is responsible for the R/FR reversible response and the R-HIR and another is necessary for the FR-HIR and VLFR induction of germination. If there is similar scenario in *Ceratopteris*, the level of germination would be expected to be very high in Rc, which is not the case. At least one of these phytochromes, a necessary component in the signaling pathway, or a mechanism for competence to respond to light is likely produced *de novo*, as *Ceratopteris* spores require a dark period of at least 7 d to yield maximum germination. Consistent with this hypothesis, a correlation between P_r content and the dark period needed for imbibition was shown in another fern, *Lygodium japonicum* (Tomizawa et al., 1982).

Several hypotheses have been advanced to explain the R-and FR-HIRs in higher plants, including measurements of the cycling rate, including. an active intermediate in the $P_r \leftrightarrows P_{fr}$ transitions and of the transient association of phytochromes with a reaction partner; or modification of the phytochrome, e.g. through phosphorylation to distinguish between unirradiated "naïve" and cycled phytochromes or by selective degradation of one form of P_{fr}, but the actual mechanism(s) have not yet been elucidated. The P_{fr}/P_{tot} ratio may be involved in the regulation of germination, but it is insufficient to explain all of the germination data. When the steady-state P_{fr}/P_{tot} ratio is high, as it is under R, germination is delayed for at least one day. On the other hand, a low but detectable P_{fr}/P_{tot} ratio under FR_c could be responsible for the increased germination rate. As predicted by this model, R_c+FR_c produces an intermediate rate of germination (figure 3). However, if this ratio were sufficient to induce high germination levels, the unirradiated spores should germinate well. If naïve P_r in unexposed spores is unable to induce germination and requires "cycling" through the Pfr form, a pulse of R followed by a pulse of FR would be expected to induce germination, but this effect has not been observed in previous experiments (Cooke et al., 1987); Kew-Chou and Short, unpublished). Furthermore, if simple cycling rates between P_r and P_{fr} were responsible for the effect, the R_c+FR_c treatment would be expected to cause the highest germination rates, rather than the levels intermediate between R_c and FR_c levels that were observed. The so-called "high energy phenomenon"—an early designation for the HIR in higher plants—can be explained if a light-labile receptor that is present in large excess over its reaction partner (Hartmann, 1966). If sufficient P_{fr} is present to exceed the threshold of activity, the response will be induced. However, if most or all the phytochrome is converted into the active form, it will be degraded and lost, yielding a short-term transient saturation of the response but not a long term signal. If the threshold for the response is very low, the estimated 3% of phytochrome that will be maintained in the P_{fr} form under FR_c will be sufficient to propagate the response over a much longer period than would R_c. Intermediate levels of P_{fr}, as would be essentially determined by the R:FR ratio of the light source, would yield a higher P_{fr}/P_{tot} although with a concomitant decrease in the lifetime of the phytochrome pool. Hartmann's hypothesis could explain the results observed for spore germination, with the assumption that the FR_c produces a more effective compromise between P_{fr}/P_{tot} and P_{fr} lifetime than does R_c+FR_c or R_c alone. Although it has been shown that the main pool of phytochrome in dark-grown higher plants meets these criteria, it is unclear whether this hypothesis reflects the cellular mechanisms underlying R- and FR-HIRs in either angiosperms or *Ceratopteris*.

The high level of germination induced under W_c compared with that under R_c+FR_c at 3 d is also difficult to reconcile from the monochromatic irradiation data. The W light from fluorescent lamps contains very little light in the FR wavelengths, yet the R wavelengths are less effective than the full W despite having comparable photosynthetically active radiation (PAR). Moreover, B alone induces only very low germination rates. The implication from these data is that either R and B act synergistically to induce maximal germination, or another wavelength acting through a novel photoreceptor could induce germination more effectively than R or B. If the former hypothesis is correct, this is the first study to show a positive effect of B_c in conjunction with R_c on *Ceratopteris* spore germination, and to reveal initiation—albeit at a very low level—of germination. All previous studies of the B effect on fern germination (Cooke et al., 1987; Furuya et al., 1997) have shown that brief irradiation with B actively inhibits germination regardless of R treatment before or after B, but the Bc treatment used in this study induces a low but significant level of germination. Whether this germination is mediated by phytochrome, cryptochrome, or another receptor cannot be definitively shown from these experiments. Regardless of the receptors involved, B pulses that inhibit the low-fluence phytochrome-mediated germination response described previously (Cooke et al., 1987) act in a manner distinct from the inductive effect of Bc, provided either alone or combination with Rc. If the Hartmann hypothesis is correct, W_c should convert the majority of phytochrome to the P_{fr} form and lead to its rapid degradation, yielding a reduced level of germination. Perhaps the simplest explanation for these data is that the activation of a B receptor stabilizes the P_{fr} itself or allows the phytochrome signal to be propagated, even after the loss of the unstable phytochrome pool.

Phytochrome regulates cell division and chlorophyll accumulation under similar conditions

Light treatment of dark-grown seedlings *Arabidopsis* will reduce the cell elongation rate in the hypocotyls while inducing cell division in the cotyledons and in the shoot apex (von Arnim and Deng, 1996). Phytochromes and cryptochromes appear to act additively to inhibit cell elongation in *Arabidopsis* and to induce cell division (Goto et al., 1993; Lin et al., 1995a). Similar light effects have been observed in *Ceratopteris*; dark grown gametophytes are elongated, strap-shaped prothalli (Banks, 1999). The extreme cell elongation has been shown to be inhibited by B, but the effect of R or FR is unclear, suggesting that phytochrome may not play a major role in elongation (Murata et al., 1997). Furthermore, it is not clear from the previous studies whether cell division is regulated through the same pathway as cell elongation. The results shown here demonstrate clearly that phytochrome is involved in cell division during early development of *Ceratopteris*, although in an unexpected manner. In order for plant cells to initiate division, the cell must first to expand enough to accommodate appropriate contents within each daughter cell (Wada and Murata, 1988). Our findings suggest that the processes of cell expansion and cell division are regulated coordinately, but through different pathways than unidirectional cell elongation, that has been observed in the subapical region. As indicated by counts of the cell number (figure 6), R alone does not induce cell expansion nor cell division; the cell number in R-grown prothalli was similar to that in the elongated dark-grown prothalli. However, the doubling of cell number in FRc-grown compared with dark-grown prothalli and of the four-fold increase of cell number under R_c+FR_c can be explained by the action of at least one phytochrome. Not only is one or more phytochrome involved in regulating cell division, but B alone and as a component of W increases cell division above that of even R_c+FR_c, presumably acting through a cryptochrome or an undescribed B-absorbing photoreceptor other than phytochrome. Again, the cell number in W indicates a synergistic effect that is greater than expected from additive R_c and B_c cell division rates. When put into the context of the average cell size over the growing gametophyte tissue (table 2), Cell expansion was in consistency with the cell division results

Angiosperms require R light for the enzyme protochlorophyllide oxidoreductase (POR) to be active and for chlorophyll to be synthesized. Lower plants and gymnosperms contain both the light-dependent POR pathway and a light-independent pathway (Schoefs, 1999). The result of the light-independent pathway is that dark-grown tissues produce chlorophyll and appear green. However, in the *Ceratopteris* gametophytes, R apparently represses chlorophyll accumulation actively, whether comparing R_c- with dark-grown gametophytes or W_c- with B_c-grown tissue (figure 8). The data that are most difficult to reconcile with higher plant phytochrome function are that FR_c increases chlorophyll accumulation over that in dark-grown tissues, and that R_c+FR_c-grown tissues produce considerably higher levels of chlorophyll than either R_c or FR_c alone, whether calculated on a per-gram-fresh weight basis or per individual.

There are several hypotheses that might explain this combination of responses One is that at least one phytochrome (in addition to one or more B receptors) is affecting both the cell division and chlorophyll synthesis pathways, and that a modified form of the Hartmann model (Hartmann, 1966) for HIRs is at work in lower plants. Based on this model, the implication is that high levels of P_{fr}, as found in R_c-grown tissue, suppresses chlorophyll production and cell expansion and, at the very least, does not promote cell division. On the other hand, the lower levels of P_{fr} produced by R_c+FR_c and the very low levels produced in FR_c alone promote the responses. Unlike the germination response, the small amount of P_{fr} produced by FR_c is insufficient to promote the full response, and only the increased P_{fr}/P_{tot} ratio produced under R_c+FR_c can saturate the growth response. If that is the case, it further implies that there are different mechanisms of signaling—or even different phytochromes—mediating the growth and chlorophyll responses versus the germination response.

A second hypothesis that can explain the unique set of responses is through the cycling of a stable phytochrome. It has been demonstrated that phytochromes in higher plants, most notably *Avena* phyA, undergo autophosphorylation in a light-dependent manner, and that the phosphorylated form exhibits different properties from the naïve form of the molecule, even when reconverted to the P_r form. Assuming a phosphatase that can return the phosphorylated form to its original state (i.e., the P_r is photoconverted to P_{fr}, which becomes phosphorylated to $P_{fr}*$, photoreversed to P_r*, and finally dephosphorylated back to P_r), the relative amounts of each form will be a function of the kinetics of each step. Therefore, the rate of cycling, which corresponds to the absorbance characteristics of each form and the spectral output and fluence rate of the light source, can be translated into different relative concentrations of each of the phytochrome forms according to the relative quantum efficiencies of photoconversion and the kinetics of phosphorylation and dephosphorylation. Additional flexibility comes from the physical sequestration of one form over another, as with the nuclear translocation of phytochromes from the cytosol under R, and in some cases back to the cytoplasm under FR, and from the differential association of P_r versus P_{fr} with reaction partners (e.g. Pif3). If the kinetics of translocation into and out of the nucleus or of association/dissociation with a signaling partner are substantially different, it would be possible to have a rapidly cycling phytochrome exhibit much stronger activity than either the R_c- or FR_c-treated individuals where most of the pigment is in one form or the other but is cycled relatively slowly.

Phytochrome regulates cell expansion and elongation in fern

In *Adiantum* protonemata, a B pulse will inhibit cell elongation and induce more expansion at the protonema tip and so allow for cell division of the protonemal cell (Murata and Wada, 1989) indicating different regulation of each response. In elongated prothalli of *Ceratopteris*, a B pulse inhibits cell elongation of strapped-shape prothalli but was not clear whether it induces two-dimensional expansion. In our studies, R_c actively inhibits cell elongation (figure 4A), but apparently does not affect cell expansion (table 2) and cell division in young gametophytes, resulting in reduced gametophytes of very small cells, but an equal number of cells to the dark controls (figure 6). FRc promotes elongation of subapical cells to produce the strap-shaped prothalli (figure 4B) and promotes two dimensional cell expansion in apical cells, which allows for increased cell division reflected as greater cell numbers (figure 6) and greater average cell size than in R_c-grown prothalli. Under R_c+FR_c, cell elongation is intermediate between that of plants grown in R_c or FR_c, especially in the subapical region. However, the cell expansion increased more in R_c+FR_c than in FR_c and is directionally varied across the gametophyte such that prothalli take on the "heart" shape rather than the strap shape of FR_c-grown gametophytes (figure 4D, table 2). On the other hand, B_c inhibits cell elongation but induces more cell division than R_c+FR_c reflected by a greater cell number (figure 6). The average cell size under B_c (table2) is smaller than the cell size under R_c+FR_c, perhaps because the increased cell elongation of subapical cells under R_c+FR_c disproportionally contributes to the overall effect of cell expansion. Under W_c, B photoreceptors and phytochrome seems to act synergistically to inhibit unidirectional cell elongation, and to a promote more cell expansion and cell division.

These data suggest that the direction of cell expansion is regulated by light, and that both a blue-light receptor and phytochrome system interact to mediate gametophyte morphology. Unidirectional cell elongation is primarily dependent on the P_{fr}/P_{tot} ratio of at least one phytochrome, such that under R_c when P_{fr}/P_{tot} is high, cell elongation will decrease. Under FR_c and darkness, the ratio will be at its lowest and so induce very long cells. Under R_c+FR_c, an intermediate level of elongation is observed as a result of the intermediate P_{fr}/P_{tot} ratio.

This dependence of elongation on P_{fr}/P_{tot} ratio is superficially similar to the germination responses, but differs from germination in that the Hartmann model or a P_r/P_{fr} cycling mechanism is not required to explain the response. Although B_c could cause significant cycling of phytochrome, as both P_r and P_{fr} have small and partially overlapping absorbance in the blue wavelengths, this effect is insufficient to explain the morphological differences observed among the B_c, R_c, and R_c+FR_c. The fact that dark-grown prothalli are as long as FR_c-treated gametophytes, despite the higher cycling rate under FR_c than D, argues for a specific R_c and B_c interactions that can not be induced by the small amount of P_{fr} or the low cycling rate produced by FR_c.

Phytochrome and cryptochrome affect rhizoid development:

This study demonstrates that both rhizoid length and number are affected by the spectral quality of the light. The number of rhizoids is regulated through a different process than is rhizoid elongation. In darkness, rhizoid number is very low, whereas in W_c, the opposite phenotype is displayed. The rhizoid number correlates well with the cell number and chlorophyll content in all treatments, as was reported for gametophyte growth of *Onoclea sensibilis* L. previously (Racusen, 2002). Those studies indicated that there is a strict interdependence between the photosynthate-producing prothallus cells and the zone of osmotic ion uptake (rhizoid). Both K^+ and Na^+ were shown to be essential for the normal development of prothalli (Racusen, 2002). According to our data, both cryptochrome and phytochrome affect rhizoid number. This effect can be explained through the same hypothesis as for cell division and chlorophyll synthesis. Whether this effect occurs indirectly through chlorophyll accumulation and photosynthate production or directly through increased asymmetric cell division or the bidirectional transport of photosynthates and nutrient ions requires further study.

Rhizoid length is also affected by the light wavelength, but the effect of light quality on length correlates more closely with the pattern of prothallus cell elongation, so it may be dependent on the same regulatory pathways. B_c—presumably through cryptochrome—inhibits rhizoid elongation compared with that of unirradiated rhizoids (figure 9A). R_c inhibited elongation, but less effectively than did B_c. This B response seems to act additively with phytochrome to reduce rhizoid elongation as shown by the differences in length between W_c- and B_c-grown rhizoids (figure 9A). The rhizoid elongation can be explained by the level of P_{fr}/P_{tot} hypothesis described above, which explains why under R_c, with high P_{fr}, elongation is inhibited, whereas under FR_c, elongation was promoted by the low P_{fr} level. As the P_{fr} level reaches an intermediate dynamic equilibrium under R_c+FR_c, a corresponding intermediate to high degree of elongation is also achieved. Alternatively, the high cycling rate under R_c+FR_c may decrease the efficiency of the P_{fr} form and induce more elongation consistent with the results shown. We hypothesize that B_c inhibits the elongation by itself to produce short rhizoids, and when combined with R wavelength present in W_c, it acts additively with the higher P_{fr} concentration to produce intermediate lengths similar to those measured from R_c-grown plants. The reason for that is under these conditions, R_c will produce more P_{fr} which will act in concert with the B receptor to inhibit elongation. The small fraction of

FR_c in W_c will initiate an intermediate cycling rate which in turn would reduce the efficiency of P_{fr} and promote more elongation. Therefore, the length in W_c will not be as great as in FR_c, nor as short as in B_c. This hypothesis predicts that not only is P_{fr} required, but also the cycling rate of phytochrome affects rhizoid elongation by a separate mechanism from that regulating prothallus cell elongation.

The hermaphrodite versus male developmental decision is affected by light

Detailed knowledge of how light affects development of antheridia and archegonia is limited to a few fern species and light conditions (Dyer, 1979; Banks, 1999). In general, at least two different modes control the induction of antheridia; one, through naturally produced pheromones, termed antheridiogens, as described for *Ceratopteris richardii* (Banks et al., 1993);and another one, through light as in the case of *Polypodium aquilinum* in which antheridia can be formed only under darkness or FR_c irradiation but not under W_c. A pulse of R inhibits initiation of the antheridia, and this inhibition is totally reversed by FR, indicating the involvement of phytochrome (Schraudolf, 1967). In *Pteris vittata* the male sex is produced more frequently when spores are germinated in darkness. A pulse of R or B reduces the formation of antheridia, whereas a FR pulse increases the number of antheridia. In darkness, more antheridia develop spontaneously without apparent antheridiogen secretion, but under light antheridiogenesis are induced by antheridiogen, so FR-grown gametophytes will produce fewer antheridia than will dark-grown prothalli. This effect implies that the P_{fr} form inhibits the generative tendency and promotes the development of vegetative tissue. On the other hand, in light-grown gametophytes of *Pteris*, antheridiogen is the main factor in determining sexual differentiation (Gemmrich, 1986). Based on the previous studies in other ferns, it would not be unexpected to find direct or indirect effects of light on the proportion of gametophytes developing as males versus hermaphrodites in *Ceratopteris*. Unlike the other responses described here, all light regimes except FR_c produced comparable decreases in the relative numbers of males in the population (figure 10). Since both dark- and FRc-grown gametophytes produce elongated prothalli, it is possible that the same pathways are involved in growth habit and in sexual development, or that the poorly defined meristem observed in both D and FR conditions is inadequate for producing hermaphrodites. Light may decrease production or sensitivity to antheridiogen as well. Regardless of the mechanism, one might speculate that an embryo growing from a hermaphrodite in conditions lacking PAR wavelengths would be unlikely to survive, and that the best strategy for reproduction would be to produce only male gametes that may reach an archegonium under more favorable light conditions.

Archegonia development is affected by light quality:

The literature has only a few studies reporting effects of light on archegonia. A recent paper from Kamachi and colleagues suggested an effect of phytochrome on the induction of archegonia formation in dark-grown *Ceratopteris* prothalli by a pulse of R. The effect of R was cancelled by subsequent irradiation by FR. In addition, neither a FR pulse nor a B pulse was able to induce archegonia in the dark-grown prothalli (Kamachi et al., 2004). In our observation of archegonia formation in continuous light, we found that FR_c does not induce formation of archegonia (Table 3, figure 11) as above the basal dark levels. Despite the R_c-mediated reduction in cell division and chlorophyll synthesis, and the concomitant diminutive size of the gametophyte, the prothalli still produce 23% more archegonia than those grown in FR_c. This increase in archegonia under R_c may result from the more highly differentiated lateral meristem on R_c-grown prothalli than on FR_c-grown plants. The result is not entirely consistent with the hypothesis that archegonia formation is largely dictated by the relatively high metabolic state of the meristematic cells (Raghavan, 1989; Kamachi et al., 2004), but it is more likely a function of the age of the cells and their level of differentiation into an active meristematic notch. The earlier the meristem develops, the more archegonia will be produced. This idea is supported by the fact that different light conditions strongly affect the maximum number of archegonia formed. Under W_c many more archegonia are produced than in R_c+FR_c or B_c (Table 3), which correlates with the time of lateral meristem development.

Archegonia and sporophyte development are slowed by supplementary FR

Supplementary FR (W_c+FR_c) produces well characterized responses in higher plants and are predominantly controlled by phyB in Arabidopsis (Robson et al., 1993). Most of the isolated sequences of phytochromes from ferns (Schneider-Poetsch and Braun, 1991; Schneider-Poetsch et al., 1994; Nozue et al., 1998a) and especially those from *Ceratopteris* (Bissoondial and Short, unpublished) show more similarity to phyB than to phyA. The effect of W_c+FR_c on *Ceratopteris* did not enhance elongation growth or accelerate development as it does to higher plants, but rather yielded the opposite effect. This retardation of *Ceratopteris* growth has been observed at the level of archegonial development and maturation (figure 16A, B) and is particularly obvious in its effect on slowing sporophyte development (figure 13, 14).

Elicited primarily through phyB in higher plants, supplementary FR converts a higher proportion of the phytochrome to the P_r form and it also increases the theoretical cycling rate, throughout the light period; while EOD-FR reduces the influence of P_{fr} during the night period by converting most of the phytochrome to P_r at the end of the light period. Because of the similarities in phenotypes observed under these two conditions—the so-called "shade avoidance responses", it has been assumed that supplementary FR acts through a similar mechanism to EOD-FR (Franklin et al., 2003).

EOD-FR responses can be used to gain information about the effect of stable P_{fr} reversion to P_r in darkness and the importance of the P_{fr} level at the transition from light to dark. However, in *Ceratopteris* EOD-FR seems much less effective than supplementary FRc in altering growth and development. (Figure 17A, B, C). One explanation is that there is naturally a very fast dark reversion of P_{fr} to P_r so the EOD-FR essentially mimics the normal phytochrome. If the responsible phytochrome is somewhat labile, it is also possible that there are few photoreceptors remaining after an extended light period, such that EOD-FR has only a minor effect on the abundance of P_{fr}. According to this alternative explanation, one might expect to find a higher concentration of the labile phytochrome at the end of the supplementary FR_c day than after the standard W_c day. By maintaining a pool of light-labile phytochrome in its stable P_r form, the steady-state level of that phytochrome will be higher and potentially more effective in the dark portion of the day/night cycle. Testing of these hypotheses awaits accurate measurements of phytochrome in each tissue and more detailed physiological measurements.

CHAPTER III. Developing methods for *Ceratopteris* transformation to study intracellular localization of phytochromes

Abstract

To elucidate the signal transduction mechanisms of different phytochromes, it is essential to know the sites of their action within the cell. Based on many physiological, biochemical, immuno-cytochemical and cellular analyses of higher plants, it was generally believed that phytochromes were largely cytosolic proteins. However, transgene expression of phytochrome::reporter fusions showed that the intracellular distribution of phytochromes, is more complex than was previously thought and is critical to their mechanism of action (Kircher et al., 1999; Gil et al., 2000; Kim et al., 2000; Kircher et al., 2002).

Action dichroism studies in fern and fern allies induced by polarized light suggests strongly that phytochrome is associated with a fixed structure on or close to the plasma membrane in some species (Etzold, 1965). Most of these studies have shown that membrane association of phytochrome is likely to be common in filamentous cells (Hartmann et al., 1983). Reporter fusion proteins with heterologous and native phytochrome may be useful in determining whether this association is consistent in the case of meristematic and other prothallus cells.

Because full length *Ceratopteris* phytochrome cDNAs have only been isolated very recently, one of the most closely related phytochromes from higher plants, phyB from *Arabidopsis*, was used to test chimeric photoreceptor localization. These constructs could also be used to examine the ubiquitous nature of the translocation response and likely

evolutionary conservation of mechanism in phytochrome signaling. *At*PhyB::GFP phytochrome undergoes slower nuclear translocation kinetics in *Ceratopteris* than in *Arabidopsis*. With native *Arabidopsis* phyB, a pulse of R is sufficient to induce maximal accumulation in the nucleus within 3 hr, but in *Ceratopteris*, noticeable redistribution requires 1 d of R. Therefore, phytochromes can relocalize to the nucleus upon R treatment in *Ceratopteris*, and the apparently slow kinetics of heterologous phyB redistribution may indicate a more effective retention mechanism in fern cells than in *Arabidopsis*, a less efficient nuclear import system for at least this non-native protein, or a comparatively efficient nuclear export process, all resulting in a more cytoplasmic concentration of phytochrome than is the case in higher plants.

Consistent with earlier studies in ferns, in which polarized light and action dichroism results indicated an association with membranes or cytoskeletal components near the plasmalemma (Kraml, 1994), *Ceratopteris* Phy1::GFP localization experiments suggest that Phy1 remains primarily in the cytosol both in darkness and in light. In consistency with the physiological data (table 4), it is likely that very different mechanisms of phytochrome action have evolved in ferns and in angiosperms.

A. Materials and Methods

Plant culture

Plant materials and growth conditions are as described in the Materials and Methods of the previous chapter, except the dark period following spore imbibition was reduced to 3 d and Wc was used to induce germination. Spores were kept under Wc until ready for transformation. Before injection, 300 mM mannitol as osmoticum was added on the medium surrounding the prothallus to be injected. After injection, prothalli were rinsed with autoclaved distilled deionized water 3 times to eliminate the excess mannitol.

For electroporation experiments, spores were sterilized and grown in liquid Parker & Thompson (PT) medium with shaking at 100 rpm in darkness for 3 d and then exposed to Wc for 3 d until germination. The germinated spores were then subjected to electroporation as described below. The transformed gametophytes were kept in darkness for 16-24 h before observation by fluorescence microscopy.

Microinjection

Several parameters were optimized for microinjection, including the developmental stage of the gametophyte at time of microinjection, the capillary needle size and shape, the concentration of DNA, time of injection, and pressure of injection. Injection capillaries were pulled from borosilicate glass using a P-97 puller (Sutter Ins. Co.) to an inner diameter of 0.5-0.3μm (estimated as described by (Schnorf et al., 1994). The capillary holder was coupled to an Eppendorf 5172 piezo stepper which in turn was mounted on top of an Eppendorf 5171 micromanipulator. Pressure and injection conditions were controlled by an Eppendorf 5246 transjector.

Manipulations were monitored through a Nikon TE-200 inverted microscope. After placement of the needle and puncturing of the cell wall, injections were initiated by increasing the pressure to 2000-3000 hPa for 1-2 s. After a 1 min delay, the needle was slowly removed. The volume of the injected solution ranged between 2 to 5 pL as estimated under cell-free conditions in low-viscosity oil according to (Minaschek et al., 1989). This estimated volume could vary from cell to cell depending on cell turgor. Transformation was attempted on gametophyte cells at various stages of development. Slightly elongated or early-stage germinating spores required immobilization to facilitate injection. To hold the tissue in a place during injections, a closed capillary on a Narashige micromanipulator was used. Young heart-shaped gametophytes (~100 cells) do not need any immobilization. The pipette tip reached no more than 3 μm into the cytoplasm of the gametophyte cells, and the injection solution was loaded into the cytoplasm.

The plasmids for injection were dissolved at a concentration of 0.1-0.5 μg/μL in deionized distilled water. The water was filtered through a 0.2 μm disposable filter unit to sterilize the solution and to avoid particle contamination. All injections were done under near sterile conditions. Microinjected gametophytes were then incubated at 25°C in darkness for 24 h before initial microscopic observation and photography.

Particle bombardment

1 μg of plasmid DNA was adsorbed onto 0.5 mg of 1 μm gold particles according to the instructions of the manufacturer (Bio-Rad, CA) and bombarded into one-week-old prothalli with 1100 psi burst disks using a PDS-1000/He helium gene gun system (Bio-Rad, CA). After incubation for 24 h at 25°C to allow expression of the transforming DNA, the prothalli were observed under a Nikon TE-200 inverted fluorescence microscope.

Electroporation

After 3-4 d of light treatment with agitation, the germinated spores were washed and suspended immediately prior to electroporation in 500 μl of MES electroporation buffer (70 mM KCl, 5 mM $CaCl_2$, 0.4 mannitol, 5 mM MES, PH 6.5). After 15-20 min, 25 μg of supercoiled plasmid DNA and 4 mg germinating spores were combined. Then 300 μL of the mixed DNA and spores were added to an ice-cold 4-mm-gap electroporation cuvette. Each sample was pulsed twice at 1800 V/cm and capacitance at 200Ω in a Bio-Rad Gene Pulser II with a Capacitance Extender Plus module (Hercules, CA). The tissues were then incubated on ice for 15 min, washed with fresh PT liquid medium, and incubated for 24 h in PT medium for recovery. Spores were then transferred to agar-solidified PT medium containing 150μg/ml hygromycin B for selection of transformed prothalli.

DNA plasmid constructs

The vector pBASGFP control construct (courtesy of Dr. T. Lamparter, Freie Universität Berlin, Germany) is based on the pBluescript II KS+ vector, containing a slightly modified rice actin1-promoter (Zhang et al 1991) and the 35S polyadenylation signal of pGL2 (Pietrzak et al., 1986) flanking a modified GFP (TYG-GFP) coding region (Sheen et al., 1995). This construct was used in initial microinjection studies to determine starting parameters and feasibility of the promoter and reporter in ferns.

A second control vector, pGFP-2, contains a monomer 35S promoter driving the modified GFP (Haseloff et al., 1997) with F64L and S65T for enhanced fluorescence; a Nos terminator is inserted after GFP. For preparation of the Phy1::GFP construct, the Phy1 gene was first amplified using PCR to add XhoI restriction sites on both 5' and 3' ends. The amplified fragments from PCR reactions were ligated into the pGEM T-Easy vector (Promega, Madison, WI), and the ligation products were used to transform JM109 competent cells by heat shock (Sambrook et al., 1989). For identifying positive colonies, the transformed JM109 cells were grown overnight at 37°C on sterile LB medium (10g tryptone, 5g yeast extract, 5g NaCl per liter) containing 1% agar, ampicillin (50μg/mL), X-gal (50μg/mL) and IPTG (20ug/mL) for loss of β-galactosidase activity for further screening. Only those white colonies shown to be positive by PCR were picked for growth in liquid culture overnight. Plasmids were extracted from overnight cultures with a Wizard Plus SV miniprep DNA purification system (Promega, Madison, WI). The inserted fragments were excised with XhoI for insertion into the pGFP-2 vector (Haseloff et al., 1997) the ligated construct was used to transform by JM109 cells. Positive colonies were screened by PCR and plasmids were extracted as above. The pBI-Hyg/35S-PHYB-sGFP-NosT construct (a generous gift of Dr. A. Nagatani, Kyoto University, Japan) contains the *Arabidopsis* phyB gene inserted at the N-terminus of synthesized GFP (sGFP)(Chiu et al., 1996). The construct contains *At*PhyB:GFP inserted between the 35S promoter and a Nos terminator from the *Agrobacterium* Ti plasmid

Plasmid pRTL2-GFP-VIP1, pRTL2-GFP-VirE2, and pRTL2-GFP-VirD2 (generous gifts from Drs. V. Citovsky and T. Tzfira, Stony Brook, State University of New York, USA) contain the VIP1, VirE2, and VirD2 ORF, respectively, fused to the C-terminus of GFPmut1 (from pEGFP-C1, Clontech) and cloned as *Sal*I-*Bam*HI, *Xho*I-*Bam*HI, and *Sal*I-*Bam*HI fragments. Transient expression of these GFP fusion proteins compared with expression of GFP alone serve to show that GFP localization in *Ceratopteris* can be directed to nuclei by the protein fused to it. Furthermore, the initial data on expression and localization of these proteins involved in *Agrobacterium*-mediated transformation suggest that stable transformation using T-DNA complexes may be feasible, even in the absence of a live pathogen or recognition of *Ceratopteris* as a host.

Fluorescence microscopy

Epifluorescence was observed with a Nikon TE-300 with inverted optics and 4x, 10x, and 40x objectives. For monitoring fluorescence, fluorescein-isothiocyanate-dextran (FITC-dextran, Sigma), or GFP, an excitation wavelength of 480±20nm and emission at 535±25nm were controlled with a filter cube. Under these conditions, the fluorescent dyes appear yellowish to greenish; chlorophyll autofluorescence appears red in the absence of emission filtering. Nomarski images were taken on the same field with a 40X objective. Digital images were captured with a Zeiss Axiocam.

Results

By combining the transformation techniques (e.g. microinjection, particle bombardement) with the advantages of the *Ceratopteris* gametophyte system, we established protocols that allow for transient expression of many promoter::reporter gene constructs as a step toward understanding phytochrome localization and signaling mechanisms in *Ceratopteris* prothalli, and may provide the basis for stable transformation in the future.

I: Seven-day-old prothalli are more feasible targets for microinjection than germinating spores

As the haploid spores divide into two cells, the basal cell gives rise to rhizoids and the apical cell divides transversely into two cells. The middle cell undergoes several divisions to form the lateral meristem, which will eventually differentiate into archegonia or antheridia, whereas the apical cell contributes to the terminal non-meristematic part of prothallus (Banks, 1999). Thus, spores in early stages of germination (2-3 cells) represent excellent candidates for uniform transformation of the precursor to a hermaphrodite gametophyte.

For visual optimization of injection parameters, fluorescein was initially used in the injection solution and subsequently monitored by fluorescence microscopy. For reproducible delivery, precise control of the pressure applied to the capillary is important and can be achieved with the precision transjector eppendorf 5246 in which two pressure ranges are set before starting microinjection: the holding pressure (P_c), which prevents internal cellular pressure from forcing cell material or medium into entering the capillary tip, and the injection pressure (P_i) required for injection against a high internal turgor pressure. A P_c range of 100-200 hPa was determined to be sufficient under the medium osmotic conditions used in these experiments. The optimal P_i was found to vary more from cell to cell, depending on growth conditions of the cells and the characteristics of the capillary tip. A range of 2000- 3000 hPa has been effective at delivering visible dyes into the cells (not shown). Finally, the time of injection (t) is also critical to injection of appropriate amounts of genetic material into the cells. Injections were more successfully performed using 1 s or less under the P_i values used. Another parameter that could influence the efficiency of injection is the turgor pressure of the injected cells, which can be monitored generally by plasmolysis of the cells. The plasmolysis of prothalli cells occurred at a concentrations of 350 mM mannitol and above. The estimated high turgor pressure in cells grown on medium without supplementary mannitol could not be overcome by the limit pressure of the transjector (5000 hPa), and the internal pressure tended to cause excessive loss of cytosolic content into the medium and the injection needle. For this reason, 300 mM mannitol was added as osmoticum shortly before injection. This treatment effectively doubled the yield such that approximately 20% of injected germinated spores fluoresced. Although the three-cell stage presents a theoretically more appealing target because the central cell is a precursor to most of the prothallus and all of the gametes, it is technically more demanding and more laborious to inject because it requires a micromanipulator to hold each gametophyte in place and so it is less efficient than injecting multicellular prothalli, in which retain fluorescein in up to 53% of injection (table 5).

The 35S promoter is more efficient than the rice actin 1 promoter at driving transgene expression in *Ceratopteris*

To test the effectiveness of various transformation or transient expression methods, an enhanced green fluorescent protein (GFP) reporter was used. While it is effective alone for measuring promoter activity and for marking cells *in vivo*, GFP retains fluorescence when fused in frame to other proteins at either the N- or C-terminal end. This characteristic makes it an attractive fluorescent tag to monitor transgene expression, protein- protein interactions, and protein trafficking and localization (Chaffee et al., 1994; Sheen et al., 1995).

The expression of GFP was driven by either rice (*Oryza sativa*) actin 1 (Act-1) promoter or cauliflower mosaic virus (CaMV) 35S constitutive promoters. The rice Act-1 promoter was chosen to drive expression of the coding sequence since it has proven to be superior to the widely used 35S promoter, especially in monocots (McElroy et al., 1990) and in non-vascular plants (e.g. *Ceratodon*; Brücker et al., 2000). Therefore, the first attempts to inject germinating *Ceratopteris* spores were done using the vector pBAS which contain a slightly modified rice actin 1 promoter (Zhang et al., 1991). A modified GFP (TYG-GFP) was inserted in this plasmid downstream of the constitutive promoter as a reporter gene, resulting in the screenable vector pBASGFP. After 24 h of incubation, the injected gametophytes were analyzed for altered cellular responses and for GFP expression. Although approximately 15% of surviving gametophytes injected with this construct show GFP expression, the longer-term accumulation after several rounds of cell division is far lower (1-3%). Although these studies demonstrate the utility of the rice actin promoter for driving expression of a transgene in *Ceratopteris*, cells injected with actin::GFP, promoter::reporter construct, frequently stopped dividing. In the most successful example, the transformed gametophyte seemed to be stably transformed (based on the extended period over which GFP continued to accumulate), but its growth stopped after reaching the approximately 200-cell-stage, well before maturation. GFP appeared to aggregate at or near the plastids, and this gametophyte lacked chlorophyll, suggesting that GFP may be toxic to the cells when expressed at high levels. Some evidence exists that high levels of GFP expression in certain locations can be detrimental to the cell. For example, (Haseloff et al., 1997) found it difficult to obtain highly fluorescent transgenic plant lines unless the GFP was sequestered in the ER. Also, some toxic effects of GFP expression have been noted in animal cells (Hanazono et al., 1997; Liu et al., 1999). This finding is consistent with that of the Wada group (M. Wada, personal communication) in their attempts to express GFP in *Adiantum,* and with our observations that many of the individual germinated-spores cells that were injected and began to express GFP died before dividing. Therefore, the use of the 35S promoter, which is constitutive but predicted to have less activity, was judged to be more effective for driving long-term GFP transformation.

Even with improvements that come from the use of the 35S promoter to direct GFP expression in germinating spores, the difficulties of low transformation efficiency and the lack of stable transformation still persist. By using young prothalli (7-14d old), transformation efficiency improved to 3-7% with the use of 35S promoter and 2-4% with the *Act1* promoter driving transient expression (table 5). Based on these data, 35S-mediated transcription is a more effective tool for studying the transient expression and localization of proteins in *Ceratopteris* prothalli cells (figure18). However, the efficiency remains fairly low.

Alternate methods of introducing DNA simultaneously into larger numbers of plants were also used, in the hope of generating larger numbers of transformants. Electroporation of constructs containing the *HPT* gene for hygromycin resistance driven by the 35S promoter yielded spore survival rates of 7-10% on hygromycin-containing agar medium. As the cells divided during gametophyte growth, the resistance decreased and the transient expression did not allow the gametophytes to maintain resistance more than two weeks. Furthermore, electroporation of 3-cell germinating spores was technically difficult, as individuals tended to clump in liquid medium and became nearly impossible to separate for placement on agar medium, exposure to hygromycin, and microscopic observation.

Biolistic bombardment of young prothalli was more efficient than electroporation, insofar as observation of GFP and handling of the samples is much less time consuming. Because the number of cells penetrated by particles could not be determined accurately, the relative efficiency could not be precisely calculated. However, each bombardment resulted in over one hundred cells expressing the GFP reporter.

Localization of GFP-fusion proteins is feasible by transient expression in *Ceratopteris*

Because GFP is detectable in live cells without the complication of a histochemical assay, this reporter has numerous advantages over the GUS reporter enzymatic method. However, because of its small size, GFP can enter nuclear pores and so has been observed in both the cytoplasm and nucleus of plant cells, animal and yeast cells even in the absence of an added nuclear localization signal (Grebenok et al., 1997; Kohler et al., 1997; von Arnim et al., 1998). A similar pattern of GFP has been detected in *Ceratopteris* prothalli (figure 18), both in D and R. Although GFP can be taken up spontaneously into the nucleus, it has proven to be a valuable tool for studying cellular localization of fusion proteins. GFP fusions with several plant gene sequences results in GFP fluorescence in the nucleus only, leading to the conclusion that the fused sequence codes for a nuclear-localized protein. Among these are two *Agrobacterium* virulence (Vir) proteins, VirD2 and VirE2, and the plant protein Vip1 (VirE2-interacting protein). These proteins are known to facilitate T-DNA transport into the plant cell nucleus during infection (Tzfira and Citovsky, 2000). GFP fused to these proteins is observed exclusively in the nuclei of plant, mammalian, and yeast cells (Tzfira and Citovsky, 2001a; Tzfira et al., 2001b; Tzfira and Citovsky, 2002). Their localization in *Ceratopteris* is very similar to that in higher plants (figure 20) which indicates that in *Ceratopteris* GFP can be targeted by fusion proteins as it is in other eukaryotic cells, and that the *Agrobacterium* Vir and *Arabidopsis* Vip proteins can serve as positive controls for nuclear localization studies.

*At*PhyB: GFP localization displays slower kinetics in *Ceratopteris* than its pattern of localization in *Arabidopsis*

Although in ferns a few phytochrome responses have been investigated at the cellular level, the organization of the phytochrome gene family in these plants is not well understood. Analysis of partial sequences of two phytochrome genes isolated from *Anemia phyllitidis* and one from *Dryopteris* have yielded 62-72% deduced amino acid identity to those of higher plant PHYB, with more congruence to PHYB and PHYC than to PHYA (Maucher et al., 1992b). In *Adiantum Capillus-veneris*, *PHY1* cDNA isolated from dark-grown sporophytes was found to be 50-55% identical to phytochromes in seed plants and most closely related to PHYB (Okamoto et al., 1993). Phy1 and phy2 cDNAs from *Ceratopteris* are more similar to phyB than to phyA and were isolated from light grown gametophyte tissue (Bissoondial and Short, unpublished) indicating that they are transcribed in the light, as is *Arabidopsis* phyB (*At*phyB).

In higher plants, phyB:GFP fusion protein is localized in the cytosol of dark-adapted plants. Nuclear translocation of this fusion protein is induced by continuous R treatment, or multiple R pulses. This nuclear import of phyB: GFP reaches maximum accumulation after about 3h following the onset of light treatment (Kircher et al., 1999; Gil et al., 2000). By comparison, the injected or bombarded *Ceratopteris* cells only show high *At*phyB: GFP accumulation in the nucleus after 24 h R, but not after 1 or 3 h (figure 21). In darkness, *At*phyB: GFP accumulates in the cytosol and does not preferentially enter nuclei.

Nucleocytoplasmic distribution of *Cr*phy1 is light-dependent

With the isolation of native phytochromes from *Ceratopteris* (Bissoondial and Short, unpublished), predictions of intracellular location from DNA sequence are suggestive but not conclusive. Furthermore, some plant proteins have been observed to be targeted to more than one location (Small et al., 1998). Cell fractionation and purification of a protein for verification of intracellular location is often technically challenging, and antibody production for immunodetection of a protein in sectioned tissues can be time-consuming and laborious, and has not proven sufficiently sensitive for phytochrome localization in higher plants (Quail et al., 1973b; Pratt et al., 1991). Fusion of GFP coding sequences to coding regions of phytochromes of unknown location has therefore become an extremely valuable tool for determining where a protein, and thus a biochemical or regulatory process, resides within the plant cell. Initial studies with *Cr*Phy1:GFP in injected or bombarded prothalli and left in darkness for 24 h indicate that the fusion protein is distributed in the cytosol (figure 22) as predicted from higher plant phytochromes in the P_r form. However, unlike higher plant phytochrome fusions expressed in *Arabidopsis* and *Nicotiana*, or even the *Arabidopsis At*PhyB:GFP in *Ceratopteris*, Rc exposure for 1 d does not induce translocation of phy1:GFP to the nucleus. The fluorescence remains in the cytoplasm and no speckle were noticed in the cytosol as has been noticed in higher plant phytochromes (figure 22). Speckles were seen even when light-independent GFP-fusion proteins were used, especially in the cells that have very bright GFP fluorescence, suggesting that they may be a nonspecific aggregate rather than light-regulated functional complexes.

Discussion

As a model for photomorphogenesis, *Ceratopteris richardii* provides several advantages over other ferns, and it has become a valuable model systems for studying developmental and cellular processes (Chasen, 1992; Hickok et al., 1995; Banks, 1999; Short et al., 2003). Despite the strengths of *Ceratopteris* for genetic and cellular manipulation, many processes concerning developmental flexibility in response to light remain relatively uninvestigated, and so studies of photoreceptor function in ferns has lagged behind the intensive study of phytochromes in more complex plant system (e.g. *Arabidopsis*). There are currently at least two primary limitations of the *Ceratopteris* system, and overcoming them will undoubtedly encourage more plant scientists to use it to solve basic problems in genetics, cell biology, and development.

The lack of reliable transformation system is one of these limitations. The capacity to introduce and express specific genes in *Ceratopteris* can provide a powerful new experimental tool, allowing direct genetic testing of hypotheses in plant physiology that have been exceedingly difficult to resolve using other biochemical approaches (Kendrick and Kronenberg, 1994). Although a few genes have been isolated from *Ceratopteris* (Bissoondial and Short, unpublished) and approximately 2,100 EST sequences have been entered in the NIH Genbank database(Chatterjee et al., 2000), large scale mapping, sequencing, and high throughput genomic studies are still impaired without the capacity for genetic manipulation by transgenesis. Despite many attempts at transforming

Ceratopteris by several laboratories (Chatterjee et al., 2000), there is, as yet, no established method for fern transformation. A recent publication using biolistics to silence genes through short hairpin RNA transcripts (Rutherfo et al., 2004) and RNAi in *Marsillea* (Klink and Wolniak, 2000) had suggested that transient expression at the RNA level are possible, but *in vivo* expression of proteins—much less stable transformation—of *Ceratopteris* has not been reported. Therefore we aimed to develop techniques to improve the possibility of transgene expression and transformation in *Ceratopteris*. These techniques should contribute not only to expanding the likelihood of integrating simple gene constructs into *Ceratopteris*, but for eventual transformation with reporter gene fusions, gene trapping constructs, promoter trapping sequences, gene silencing constructs, and many other genetic tools to advance experimentation on this unique fern.

Establishing microinjection as a method for *Ceratopteris* transformation

Many methods that have been instrumental for studying the mechanisms of cellular processes depend on the capacity to identify, express, and analyze specific genes in plant cells (Birch, 1997). Vector-less gene transfer methods, such as polyethylene glycol (PEG)-mediated direct gene transfer or electroporation depend strongly on efficient protoplast regeneration systems. However, one of the biggest problems associated with PEG-mediated gene transfer is protoplast fusion (Griesbach, 1985). Biological vectors, such as *Agrobacterium tumefaciens* are routinely used to transform intact cells or tissues in higher plants, but the use of *A. tumefaciens* is restricted to its natural hosts, dicotyledonous plants, with only very limited success in monocots and gymnosperms. In addition, all these methods depend on selection systems for recovery of transformed cells, so a cell culture selection system must be established first for the plant species. Biolistic methods bypass these problems by shooting metal particles coated with DNA through the cell wall into the cell. The disadvantages of this method, even in higher plants, include the damaging of tissues, the inability to target particular cells, and the low yield of stable transformation (1-5%) (Finer and McMullen, 1990). Moreover, in ferns the single-celled protonemata (desired because of their subsequent growth into the entire hermaphrodite gametophyte) presents an extremely small target for the random spread pattern of biolistics.

Microinjection is a direct physical approach that offers many advantages. It is host-range independent, and allows substances to be introduced under precise microscopic control into defined cells without permanently damaging them. It does not require protoplasting, and allows transfer of controlled amounts of DNA in forms from plasmids to intact chromosomes or even entire organelles (Neuhaus and Spangenberg, 1990). Furthermore, techniques have been developed to inject DNA into protoplasts (Crossway et al., 1986) cultured embryonic cell suspensions (Nomura and Komamine, 1986), and intact multicellular structures (Neuhaus et al., 1987) (Lusardi et al., 1994).

Although the newly germinated spore with 2-3 apical cells emerging is a desirable target for microinjection because it will divide and give rise to the entire gametophyte, the multicellular young prothallus presents a larger and more tractable target for initial studies. The likely reasons for reduced efficiency of germinated spore transformation are the small size of the cells at this stage and the extremely high turgor pressures exhibited at this early stage that lead to cell bursting and resultant loss of contents upon injection. The injection of germinated spores is less efficient and more laborious than injection of bigger prothalli. Therefore, young prothalli were used throughout this study, in the hope that successfully developed techniques could be applied with minor modifications to the more desirable newly germinated spores. Microinjection and electroporation of constructs harboring 35S::GFP versus actin::GFP indicate that in the tissues and under the conditions used, the 35S promoter drives more vital and efficient expression than does the *ACT1* promoter (figure 18). Ironically, this finding may be attributable to the very high expression of actin promoter-driven GFP, which leads to more toxic protein levels in *Ceratopteris* cells.

Nuclear localized GFP-fusion proteins provide positive controls and information about Vip1 in *Ceratopteris*

Because of its small size, GFP may be able to enter nuclear pores and so can be found in both cytoplasm and nucleus of diverse plant cells (Grebenok et al., 1997; Haseloff et al., 1997; von Arnim et al., 1998). GFP fusion proteins that are known to be localized to certain compartments in plant cells are indispensable as a positive control for GFP localization in *Ceratopteris* cells. For this purpose, three nuclear-localized proteins were used in GFP fusions as potential positive controls. Vip1, a nuclear envelope import protein found ubiquitously in eukaryotes, and VirE2 and VirD2, *Agrobacterium* proteins that interact with Vip1 to bring Vir-associated DNA into the host nucleus, were used because of their demonstrated nuclear localization and because of their potential as mediators of pathogen-free, stable T-DNA-based transformation of *Ceratopteris* for future studies.

T-DNA nuclear import is a central event in genetic transformation of plant cells by *Agrobacterium*. This event is thought to be mediated by two bacterial proteins: VirD2 and VirE2 (Zupan and Zambryski, 1997). VirD2 is imported into nuclei of plant, animal and yeast cells, where it may serve to protect the 5' end of the single-stranded T-strand of the T-DNA, and its nuclear localization signal aids in efficient nuclear import. The VirE2 protein coats the length of the T-strand and is more efficiently translocated to the nuclei of plant cells. Nuclear import of the T-complex—the T-strand with associated proteins—lacking VirD2 still occurs through the VirE2 protein, and can be increased significantly by expression of the VirE2-interacting proteinVIP1 encoded by the host plant (Zupan et al., 1996; Tzfira et al., 2002). To determine whether *Ceratopteris* cells produce Vip1 as part of their own machinery is important for understanding whether *Ceratopteris* cells might be susceptible to nuclear import and integration of T-DNA. The successful localization of VirE2::GFP to the nucleus, which is known to require Vip1, indicates that that a Vip1-like protein exists in *Ceratopteris* prothalli cells, and functions to aid in transport. VirD2::GFP and Vip1::GFP fusion protein translocation to the nucleus provides further supportive evidence for Vip1 availability and similar behavior of these fusion proteins to their counterpart in higher plants. This information will be instrumental in planning future studies for stable transformation of *Ceratopteris*.

*At*PhyB and *Cr*Phy1 exhibit different translocation kinetics in *Ceratopteris* than in higher plants

Despite the growing wealth of data about the physical characteristics of signaling pathways controlled by phytochrome in higher plants, the primary molecular function of phytochromes remained largely obscure until recently. The competing views as to whether phytochromes are membrane- associated receptor proteins (Hendricks and Borthwick, 1967), light-regulated enzymes (Yeh et al., 1997; Yeh and Lagarias, 1998a), or direct regulators of gene transcription (Hock and Mohr, 1964) coexisted during the last several decades. Very recent data indicate that all of these competing views are partially correct. Notably, there is an increasing body of evidence showing that phyA and phyB have at least two different modes of function: On one hand, they directly interact with transcription factors and act as transcriptional modulators in the nucleus (Martinez-Garcia et al., 2000), and on the other hand, they function as light-regulated kinases in the cytoplasm (Yeh and Lagarias, 1998a). It should be noted, however, that despite recent progress, most aspects of the phytochrome signaling network are still not understood.

It is evident that the subcellular distribution of phytochromes is a key to this problem, and not too surprisingly, the results describing the intracellular localization of phytochromes have been as heterogeneous as the methods used. *In situ* immunocytochemical localization assays and light-dependent pelletability of phytochrome provided some compelling evidence for the cytosolic localization of phytochromes (Speth et al., 1987; Pratt, 1994) In addition, microbeam irradiation experiments in green algae and fern gametophytes have indicated that phytochrome, which mediates various cellular responses in these system, resides in the cytoplasm (Wada et al., 1993).

Recent results have changed dramatically views regarding the molecular nature of the signaling cascade required for phyB-controlled responses. PhyB::GFP fusion protein is localized to the nucleus upon exposure to a R pulse or R_c. Thus, the nuclear import of phyB:GFP show the basic characteristics of the well-described phyB modes of function, and these correlations suggest that the nuclear import of phytochromes is a major regulatory step in light-induced signaling (Nagy et al., 2000).

Given these observations of PhyB localization in higher plants, it is possible that similar activities occur in fern, especially since most of the isolated fern phytochrome genes are most closely related to phyB. Until the *Ceratopteris* phytochrome genes were isolated (Bissoondial and Short, unpublished), the localization of higher plant phytochromes expressed in the fern was a logical first step in determining whether the mechanisms of translocation are conserved. Understanding phyB localization in fern could provide considerable evolutionary insight into phytochrome signaling mechanisms beyond simple sequence comparisons. Also, because of the lack of information about how fern phytochromes actuate their signaling pathways, conserved translocation events may indicate common pathway constituents as well.

As shown in figure 21, *At*phyB::GFP remains in the cytosol in dark-adapted *Ceratopteris* cells, similar to its position in higher plant cells. Although continuous exposure of R for 3 h was not sufficient to induce maximum nuclear localization as it is in higher plants, nuclear localization of phyB does occur with more extended 24 h irradiation of R. Also, a pulse of R was not effective for inducing translocation of *At*phyB: GFP in the *Ceratopteris* nucleus. Comparable to the situation in higher plants, the phyB nucleocytoplasmic portioning mechanism in fern is dependent on retention of P_r in the cytosol in the dark, and light-mediated photoconversion induces its nuclear import as the P_{fr} conformer. The very slow kinetics of *Arabidopsis* phyB movement in *Ceratopteris* might be expected if phytochromes in fern play a larger role in the cytosol and so the retention mechanisms prevent their translocation even in the light. Perhaps more likely, the translocation mechanisms have diverged sufficiently that transport proteins associating with the heterologous phytochrome are simply less effective at moving the phytochrome. It is also possible that a second rate-limiting modification of the receptor or a protein(s) interacting with it is required to reach transport competence.

Surprisingly, in higher plants after cell fractionation only approximately 30% of phyB or phyB::GFP was recovered in purified nuclei from tobacco. This finding suggests a possible role of phyB P_{fr} in the cytoplasm of higher plants. Neither the function of these cytosolic phytochromes nor the mechanism(s) preventing their transport into the nucleus after transformation to P_{fr} are known. In this respect we note that ever since the discovery of the kinase activity of phyA (Yeh and Lagarias, 1998a) and the identification of PKS1 (Fankhauser and Chory, 1999) it has been asserted that the kinase activity of phytochromes could be involved in regulating the nucleocytoplasmic distribution of the phytochrome photoreceptors. Alternatively, the kinase activity of phytochrome has been proposed to regulate the cellular localization of other components required for light-regulated transcription of plant genes.

Although the localization of heterologous phyB in *Ceratopteris* suggests some conservation of functional mechanisms over evolutionary time, comparable studies with native fern phytochrome sequences may provide a more direct indication of whether nucleocytoplasmic partitioning is involved in photoreceptor function in pteridophytes. Localization of at least one of native phytochrome is essential to test the prediction of a more predominant cytoplasmic role of phytochrome in ferns, and recently became possible following the isolation of Phy1 from *Ceratopteris*. *Cr*Phy1:GFP localization was studied after different periods of R exposure. Prothallus injected or bombarded with these chimeric constructs and left in darkness or Rc for 1 d was examined. Since most of the injected prothalli cells were immature, and therefore lack a large central vacuole, the cytoplasm comprised most of the cell volume. Therefore, in young cells the cytosolic GFP fluorescence appeared to cover the entire transformed cells on all focal planes. Phy1::GFP in dark-incubated prothalli was localized throughout the cytoplasm. Surprisingly, exposure of the gametophytes to 1 d R does not induce obvious translocation to the nuclei, but the GFP fluorescence stayed in the cytosol. These findings may support the hypothesis that phytochromes are predominantly cytosolic in either form, and that the *At*PhyB does not reflect the functional mechanism of native Phy1. If the translocation mechanism is not of primary importance in the fern, it may explain why *At*phyB moved very slowly from the cytoplasm. According to these observations, phytochromes in fern may play a more major role in the cytoplasm than in the nucleus, consistent with results of light polarization studies in moss and other ferns and fern allies (Kraml, 1994).

If this difference in translocation proves consistent in studies of other fern phytochromes and other fusions, it may help to explain the contradictory physiological effects of phytochromes between *Ceratopteris* and higher plants (table 4). Confirmation of these data and further understanding of phytochrome action in *Ceratopteris* will require stable transformation to become routine. Techniques allowing constitutive or regulated phytochrome overexpression, antisense and RNAi knockdown of select phytochromes and study of possible phytochrome interacting factors will be possible. Eventually, we hope that our studies will make feasible the elucidation of phytochrome signaling pathways and determinations of the evolutionary conservation of these ancient photoreceptors.

Figures and Figure Legends

Figure1. Phenotypes of dark- vs. light-grown seedlings of *Arabidopsis* and prothalli of *Ceratopteris* fern.

(A) Dark-grown (D) seedlings undergo a skotomorphogenic development program (etiolation), which is characterized by elongated hypocotyls, closed cotyledons, and an apical hook. Light-grown (L) seedling undergo photomorphogenesis and are characterized by shorter hypocotyls and open, expanded, green cotyledons (http://www.molbio.gu.se).

(B) Dark-grown (D) gametophytes undergo etiolation, which is characterized by elongated strap-shaped prothalli and an undeveloped lateral meristem. Light-grown (L) gametophytes are characterized by typical heart shape prothalli the absence of elongation in the subapical region, and a well-developed active meristem.

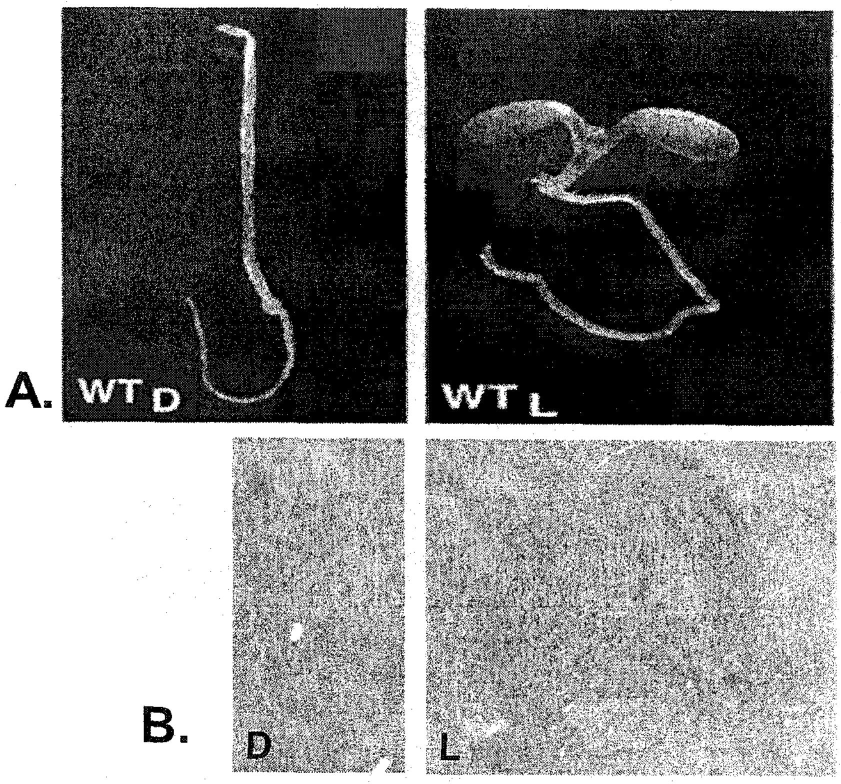


Figure 1

Figure 2. The absorption spectra of the P_r and P_{fr} forms of phytochrome overlap.

Phytochrome is synthesized as the red-light-absorbing form (P_r) in dark-grown plants. The P_r form is converted by red light to a far-red light-absorbing form called P_{fr}, which in turn can be converted back to P_r by far-red light. The P_{fr} is known to be the form which activates most of the phytochrome-mediated physiological responses and, in Type I phytochromes, is much less stable than the P_r form. (Modified from http://www.life.uiuc.edu/bioseries2/lec19m15.jpeg)

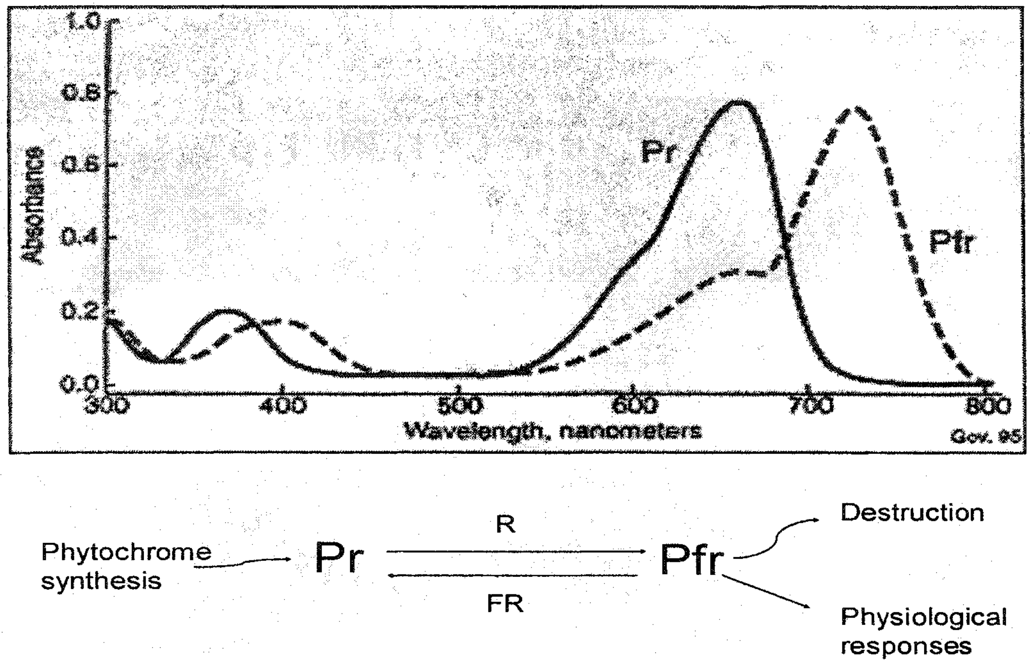

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
300
400
500
600
700
800
Wavelength, nanometers
Gov. 95
Pr
Pfr
Phytochrome synthesis
Pr
R
FR
Pfr
Destruction
Physiological responses

Figure 2

Figure 3. Germination after continuous treatment with different wavelengths.

Spores were imbibed in darkness for 7-10 days. Light treatments were given to dark-cultured spores at various times after sowing. Germination rates were determined under a standard microscope by monitoring the emergence and apical growth of rhizoids in each population tested.

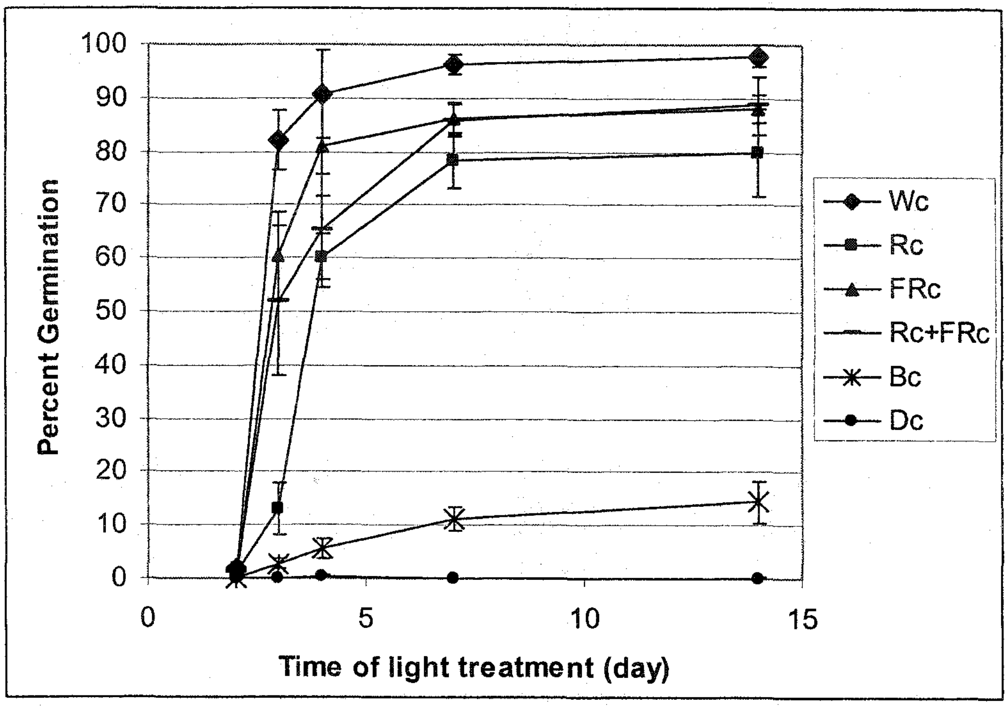

Percent Germination
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
Time of light treatment (day)
Wc
Rc
FRc
Rc+FRc
Bc
Dc

Figure 3

Figure 4. Typical morphologies of gametophytes developing under different light conditions.

Spores were prepared as described in the text and grown under W_c, R_c, FR_c, R_c+FR_c, B_c, or D for 13 d. The smaller figures show the overall relative sizes and shapes of the gametophytes, and the higher magnification figures, with standard bar 100μm, permit comparison of the cell morphologies. Under D (C,C1) and FR_c (B,B1), gametophytes grow as elongated prothalli with a poorly differentiated meristematic notch, whereas, R_c- (A,A1), R_c+FR_c- (D,D1), and B_c-grown (E,E1) gametophytes are more like typical W_c-grown (F,F1) heart-shaped prothalli producing well-differentiated meristematic notches except under R_c. In R_c, the size of prothalli is reduced severely in comparison those of prothalli under other light conditions. R_c+FR_c induces more elongation in the subapical region than do B_c, W_c, or R_c.

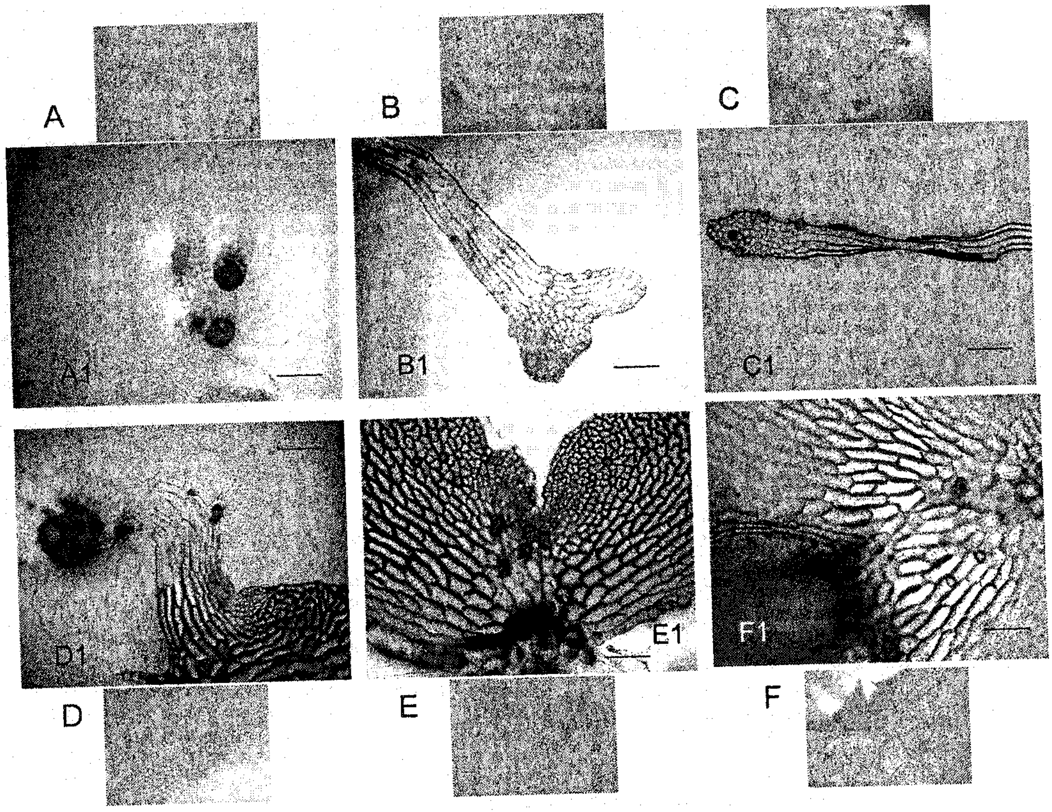


Figure 4

Figure 5. Prothalli area varies under different light conditions

Area measurements of prothalli grown under W_c, R_c, FR_c, R_c+FR_c, B_c, and D were made 14 d following initiation of light treatment. Spores plated on Parker & Thompson agar were induced to germinate in 1 d W_c, then transferred to the corresponding light regimes for 13 d. W_c, R_c, FR_c was supplied with fluence rate of 100 µmol $m^{-2}sec^{-1}$, whereas R_c+FR_c was supplied with 100 µmol $m^{-2}sec^{-1}$ R simultaneously with 100 µmol $m^{-2}sec^{-1}$ FR. The fluence rate of B_c was 25 µmol $m^{-2}s^{-1}$.

Prothalli grown under D were slightly larger than R_c and slightly smaller than FR_c. In comparison to R_c+FR_c and B_c, D-grown prothalli were significantly smaller. W_c-grown gametophytes were the largest in area. At least 45 prothalli were measured for each treatment in each experiment. Three replicate experiments were performed and the results combined.

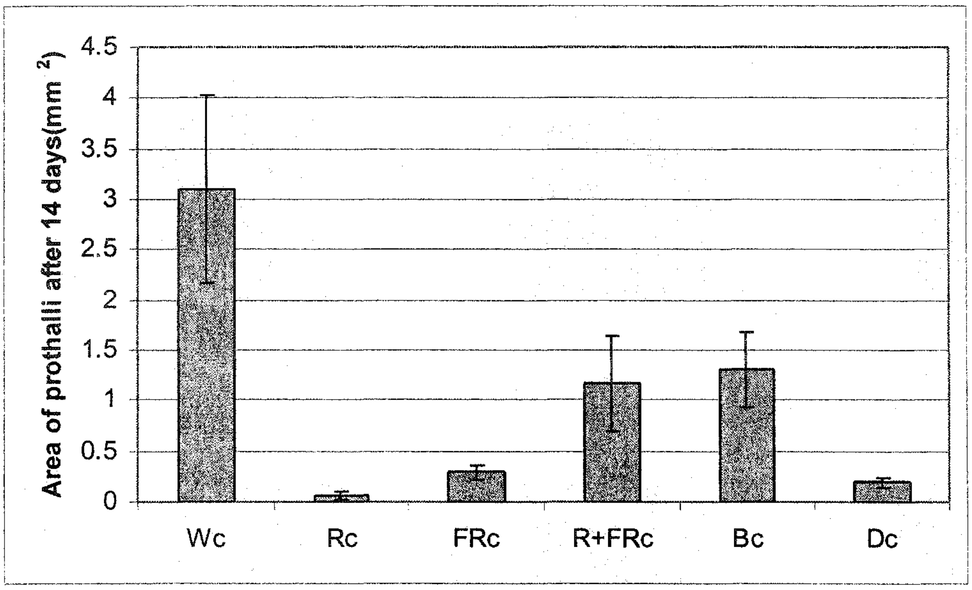

Area of prothalli after 14 days(mm ^{2})
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Wc
Rc
FRc
R+FRc
Bc
Dc

Figure 5

Figure 6. Cell numbers in prothalli varies under different light treatments

Spores plated on Parker & Thompson agar were induced to germinate in 1 d W_c, then transferred to the corresponding light regimes for 13 days. Cell numbers in 14-d-old prothalli grown under W_c, R_c, FR_c, R_c+FR_c, B_c, or D were counted. W_c, R_c, and FR_c were supplied at a fluence rate of 100 μmol $m^{-2}sec^{-1}$, whereas R_c+FR_c was supplied with 100 μmol $m^{-2}sec^{-1}$ R simultaneously with 100 μmol $m^{-2}sec^{-1}$ FR. The fluence rate of B_c was 25 μmol $m^{-2}s^{-1}$.

Prothalli grown under D or R_c contained the fewest cells, and FR_c-grown prothalli have slightly more cells. R_c+FR_c- and B_c-grown prothalli were significantly larger, and consisted of double the numbers of cells in D-grown gametophytes. W_c-grown gametophytes produced the highest number of cells. At least 30 prothalli were measured for each treatment in each experiment. Three replicate experiments were performed and the results pooled.

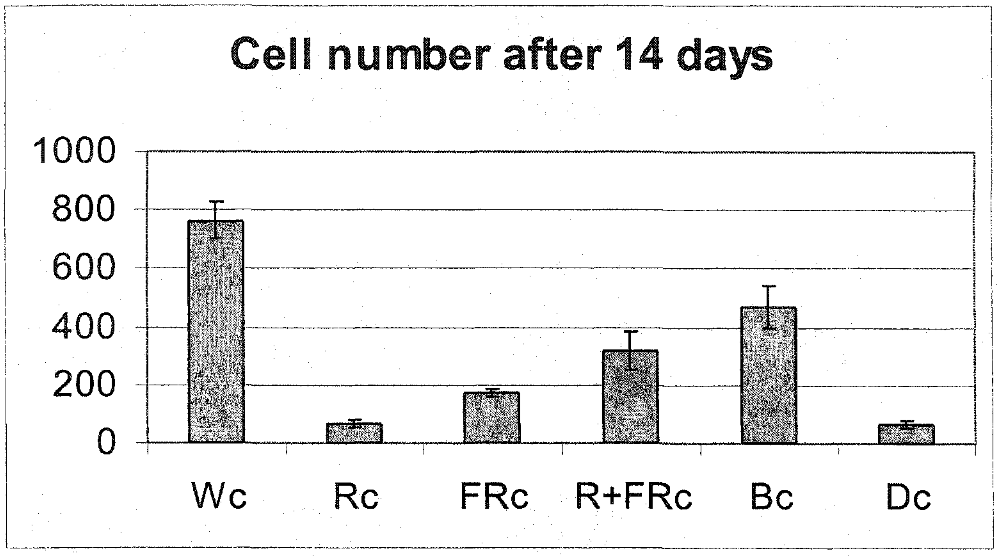

Cell number after 14 days
1000
800
600
400
200
0
Wc
Rc
FRc
R+FRc
Bc
Dc

Figure 6

Figure7. FR$_c$ induces more lateral cell division than D

Spores plated on Parker & Thompson agar were induced to germinate in 1 d W$_c$, then transferred to D or FR$_c$ for 13 days. Width and length of elongated prothalli was measured using the tissues digitally photographed under a Wild microscope with a SPOT RT CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI). These pictures were analyzed using SigmaScan Pro software (SPSS, Chicago). For each experiment at least 3 replicates were analyzed with a minimum of 30 individuals per replicate.

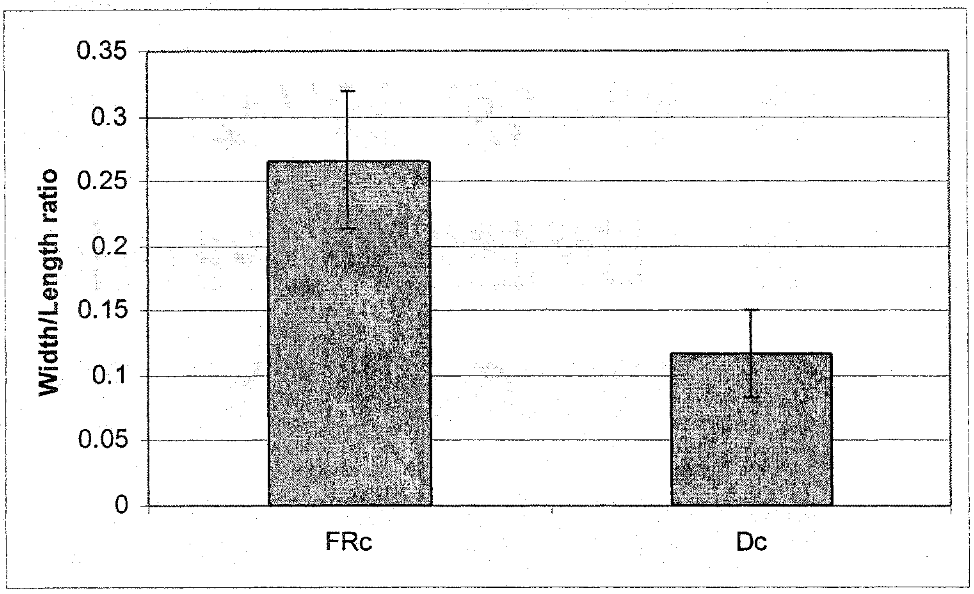


Figure 7

Figure 8. Chlorophyll accumulation varies under different light exposures

Spores were sterilized and plated on Parker & Thompson agar medium and synchronized by 7 d D. They were induced to germinate in 1 d W, transferred to different light conditions as indicated in the text. After 14 d of light exposure, pooled gametophytes of approximately 4 mg tissue were weighed, total chlorophyll was extracted in 80% acetone, and chlorophyll *a* and *b* were measured spectrophotometrically. Despite having very small vacuoles to increase cell mass, chlorophyll accumulation in R-grown prothalli was very low compared with that in plants grown under other light conditions, and even lower than that in D-grown plants. FR_c increases the chlorophyll content considerably over R_c, to a level marginally higher than that in unexposed gametophytes. However, the combined R_c+FR_c treatment causes a further augmentation of the chlorophyll content, generating considerably more pigment than either wavelength alone and comparable to the level observed in W_c-grown tissues. B_c causes still greater accumulations of chlorophyll, even surpassing the levels induced by W_c. At least 4 replicates of each experiment were performed.

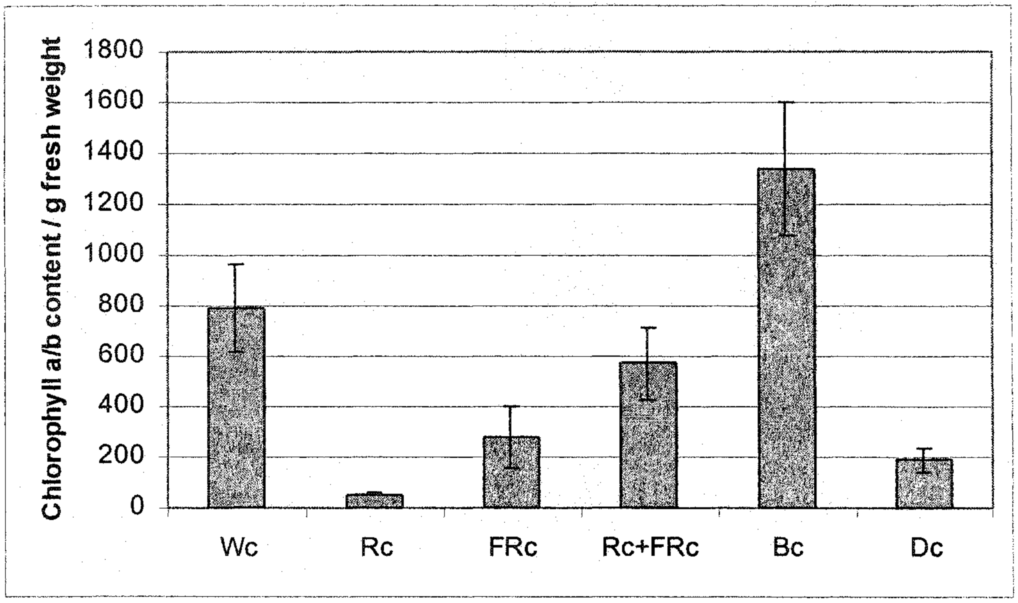
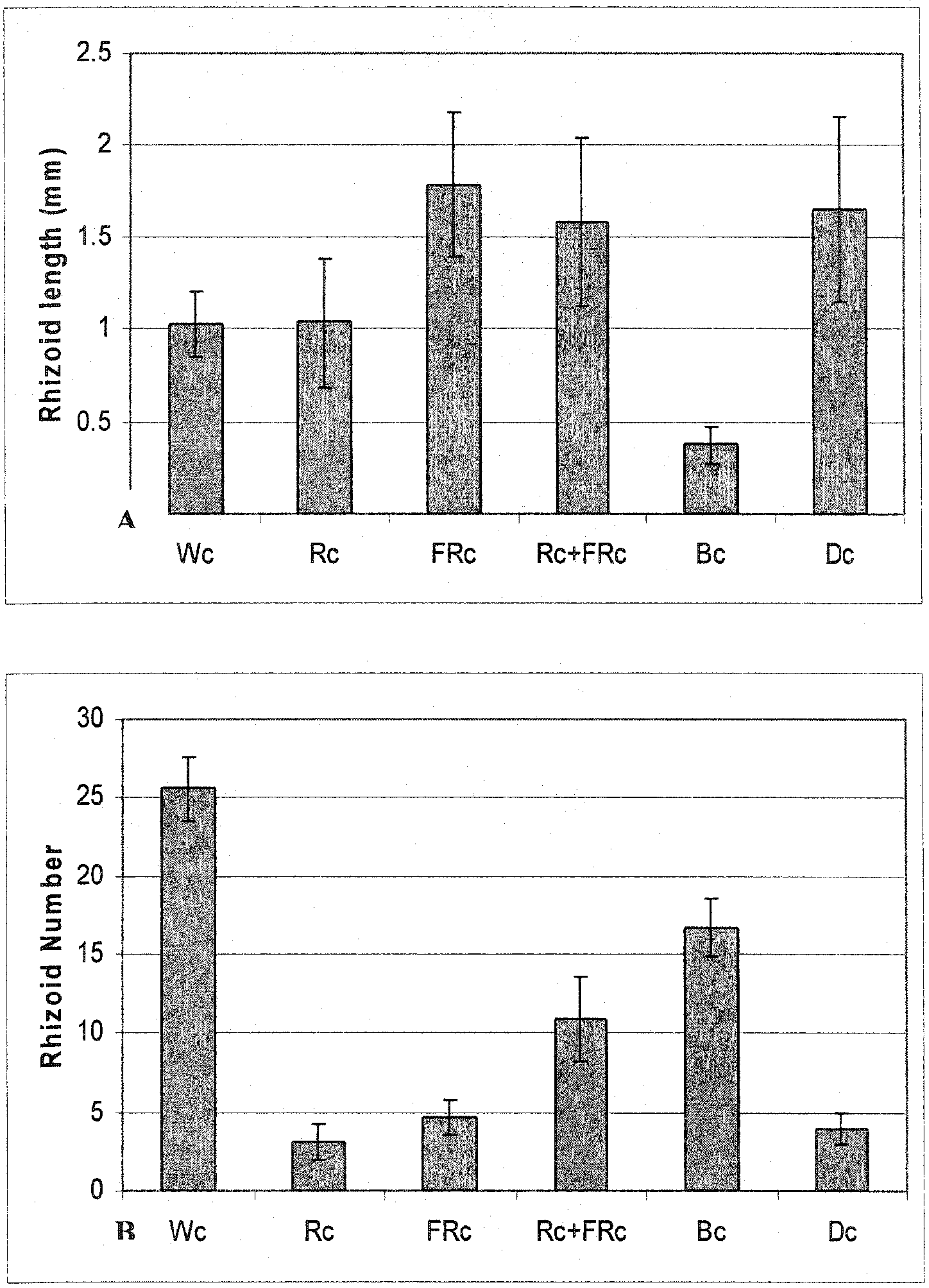

Chlorophyll a/b content / g fresh weight
1800
1600
1400
1200
1000
800
600
400
200
0
Wc
Rc
FRc
Rc+FRc
Bc
Dc

Figure 8

Figure 9. Rhizoid length and number are regulated differentially by light.

At 14 d following induction of germination, gametophytes were scored for the length (A) and number (B) of rhizoids present. (A) Rhizoid lengths were measured using the tissues digitally photographed under a Wild microscope with a SPOT RT CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI). These pictures were analyzed using SigmaScan Pro software (SPSS, Chicago). Rhizoid length under W_c and R_c are similar, whereas D, FR_c, and combined R_c+FR_c treatments yield generally longer rhizoids than those of W_c- or R_c-treated plants. The elongation of rhizoids is considerably reduced in B_c. For each experiment at least 3 replicates were analyzed with a minimum of 50 individuals per replicate.

(B) Rhizoid number under W_c, R_c, FR_c, R_c+FR_c, B_c, or D was measured using the tissues digitally photographed under a Wild microscope with a SPOT RT CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI). W_c-grown gametophytes produce significantly more rhizoids than those grown under other wavelengths tested. R_c- and FR_c-grown tissues generate similarly low numbers of rhizoids to D-grown gametophytes. R_c+FR_c-treated plants produce larger numbers of rhizoids than either R_c- or FR_c-treated gametophytes. On the other hand, plants grown under B_c produce intermediate numbers of rhizoids between those of W_c-treated and R_c+FR_c-treated gametophytes. For each experiment at least 3 replicates were analyzed with a minimum of 50 individuals per replicate.



2.5
2
1.5
1
0.5
Rhizoid length (mm)
A
Wc
Rc
FRc
Rc+FRc
Bc
Dc
30
25
20
15
10
5
0
Rhizoid Number
B
Wc
Rc
FRc
Rc+FRc
Bc
Dc

Figure 9

Figure 10. The male: hermaphrodite ratio is affected by light

Imbibed sterilized spores were plated at comparable densities on Parker & Thompson agar medium, synchronized by 7 d darkness, and induced to germinate in 1 d W. They were then transferred to different light conditions as indicated in text. After 14 d of light exposure, the numbers of each morphotype, males and hermaphrodites, were scored. FR_c did not affect the proportion of male plants compared with D, whereas all other light treatments, including W_c, R_c, R_c+FR_c, and B_c decreased the relative number of gametophytes developing as males by approximately half. At least 4 replicates of each experiment were performed with 100-200 spores per replicate.

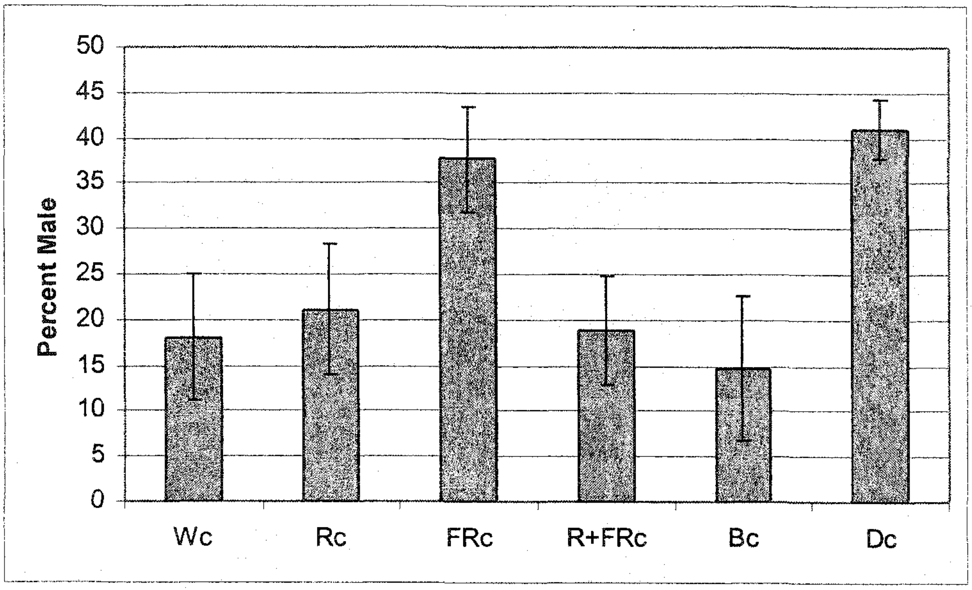


Figure 10

Figure 11. Numbers of archegonia under different light conditions.

The number of archegonia on individual gametophytes after 14 d growth under different light conditions was scored and represented as a distribution for each light treatment tested. R_c treatment strongly affects archegonial development, with 24% of the hermaphrodites producing archegonia. Under FR_c, only 1% of the hermaphrodites produce archegonia, whereas combined R_c+FR_c induced production of multiple archegonia, averaging about 8 per gametophyte and in some cases producing as many as 11 archegonia on a single individual. W_c induced comparably high numbers of archegonia, with a maximum of 16 recorded. Under B_c, an average of 4 archegonia, ranging from 1 to 6, were found per individual hermaphrodite. At least 3 replicates of each light condition were performed with at least 100 individuals scored in each replica.

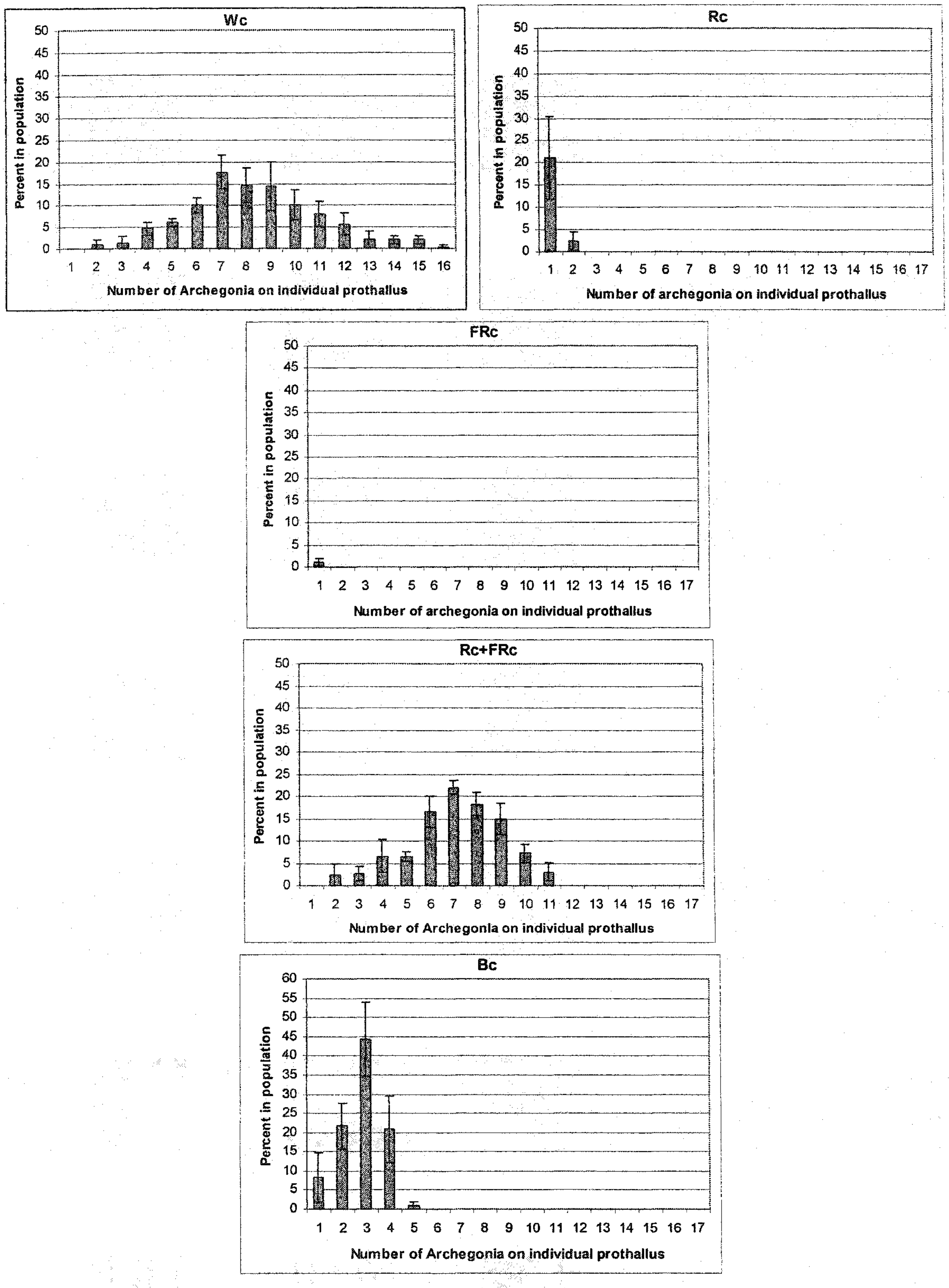

Wc
Percent in population
0 5 10 15 20 25 30 35 40 45 50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Number of Archegonia on individual prothallus
Rc
Percent in population
0 5 10 15 20 25 30 35 40 45 50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Number of archegonia on individual prothallus
FRc
Percent in population
0 5 10 15 20 25 30 35 40 45 50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Number of archegonia on individual prothallus
Rc+FRc
Percent in population
0 5 10 15 20 25 30 35 40 45 50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Number of Archegonia on individual prothallus
Bc
Percent in population
0 5 10 15 20 25 30 35 40 45 50 55 60
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Number of Archegonia on individual prothallus

Figure 11

Figure 12. Generalized model of [Pfr] under Rc, FRc, or Rc+FRc.

This model suggests how predicted [Pfr] would change over time under Rc, FRc, or Rc+FRc assuming a light labile phytochrome. The precise effect would be dependent on the threshold and saturation levels of the response, the inactivation kinetics of the Pfr form, and the time necessary to elicit the response.

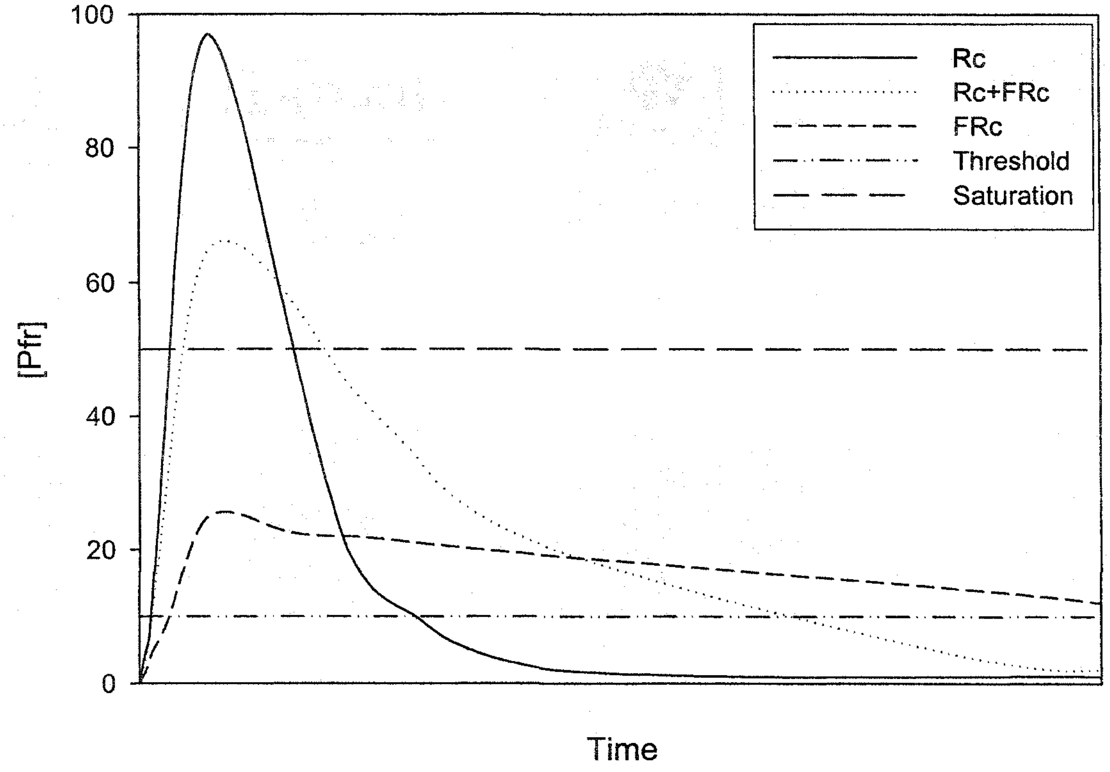


Figure 12

Figure 13. Supplementary FR delays sporophyte development

When sterilized spores were maintained in darkness for 7-10 d, then exposed to 5 h R and transferred to W_c or supplementary FR (W_c+FR_c), the W_c+FR_c resulted in differences in fertilization timing and embryo development. Prothalli growing on agar medium were observed under a Wild dissecting microscope. Only hermaphrodites were scored for the presence or absence of sporophyte tissue. The plants under W_c developed embryos and grew sporophyte leaves earlier and faster than those with supplementary FR_c. At least 5 replicates were performed with 200-300 spores each.

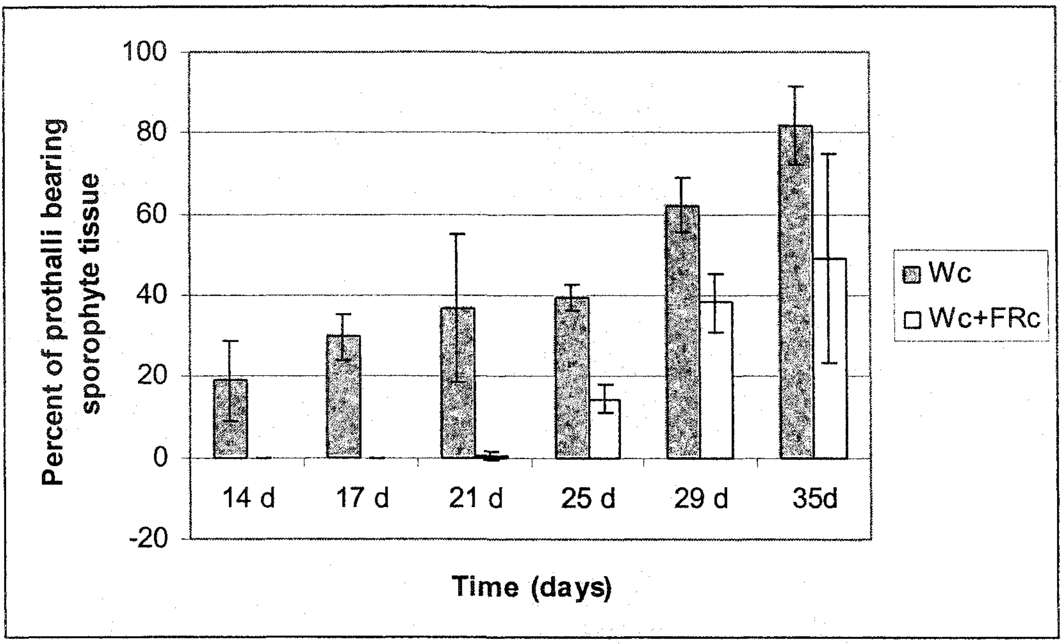

100
80
60
40
20
0
-20
Percent of prothalli bearing sporophyte tissue
14 d
17 d
21 d
25 d
29 d
35d
Time (days)
▨ Wc
☐ Wc+FRc

Figure13

Figure 14. Supplementary FR slows the production of sporophyte leaves.

Spores were synchronized by dark treatment for 7 d, followed by 5 h R to induce germination, then treated with either W_c only or supplementary FR (W_c+FR_c). Prothalli growing on agar medium were observed under a Wild dissecting microscope. The number of leaves developing from each individual sporophyte was counted at the times indicated. At any given time, the number of sporophyte leaves that developed from each gametophyte was consistently higher in W_c than under W_c+FR_c. At least 5 replicates were performed with 200-300 spores each.

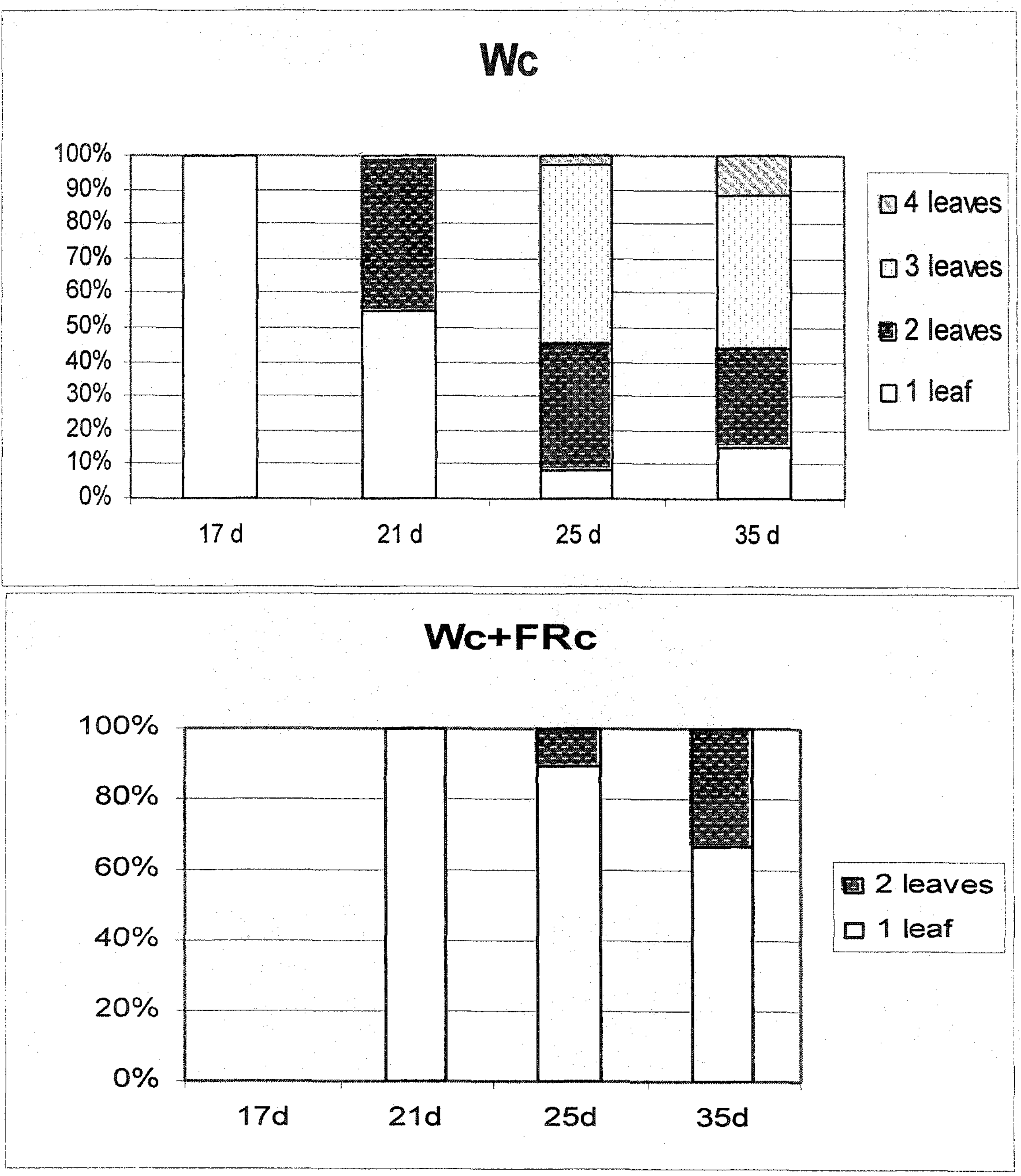
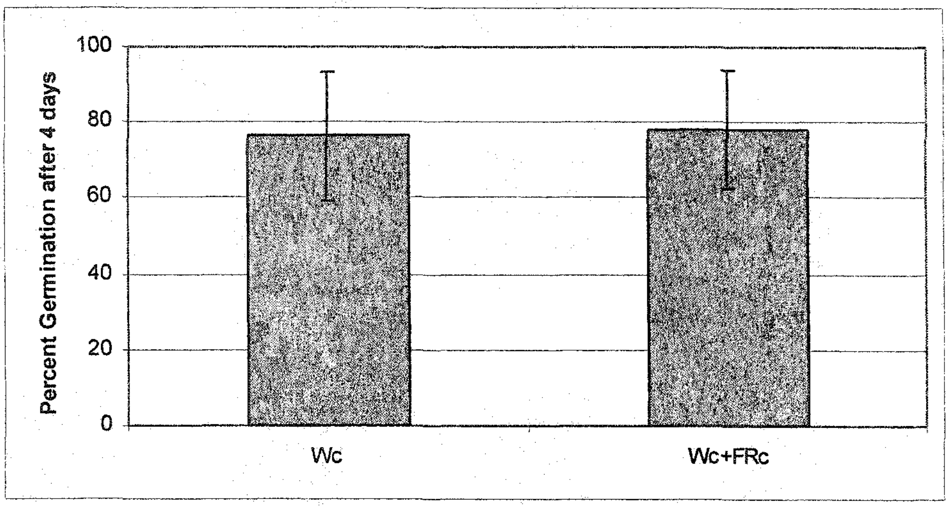

Wc
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
17 d
21 d
25 d
35 d
4 leaves
3 leaves
2 leaves
1 leaf
Wc+FRc
100%
80%
60%
40%
20%
0%
17d
21d
25d
35d
2 leaves
1 leaf

Figure 14

Figure 15. Supplementary FR did not alter germination and sex determination.

Spores were synchronized by dark treatment for 7 d, followed by 5 h R to induce germination, then treated with either W_c only or supplementary FR (W_c+FR_c). Prothalli growing on agar medium were observed under a Wild dissecting microscope. (A) germination percentage was monitored after 4 d of light exposure. Germination rates were determined by monitoring the emergence and apical growth of rhizoids in each population tested. (B) Proportions of male versus hermaphrodite gametophytes was monitored after 10 d in W_c or in W_c+FR_c.

The supplementary FR_c did not significantly affect either germination (A) or the proportion of gametophytes developing as males (B). At least 5 replicates were performed with 200-300 individual in each.


Percent Germination after 4 days
100
80
60
40
20
0
Wc
Wc+FRc

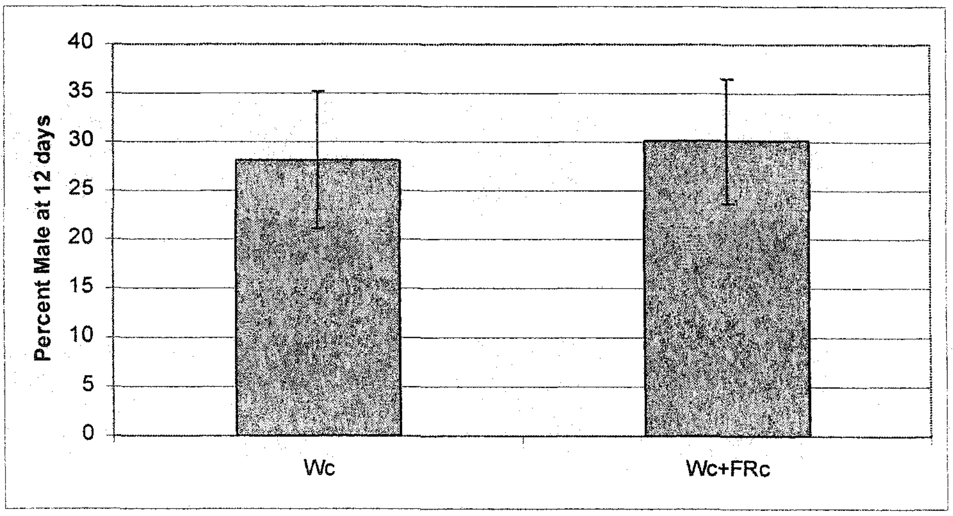
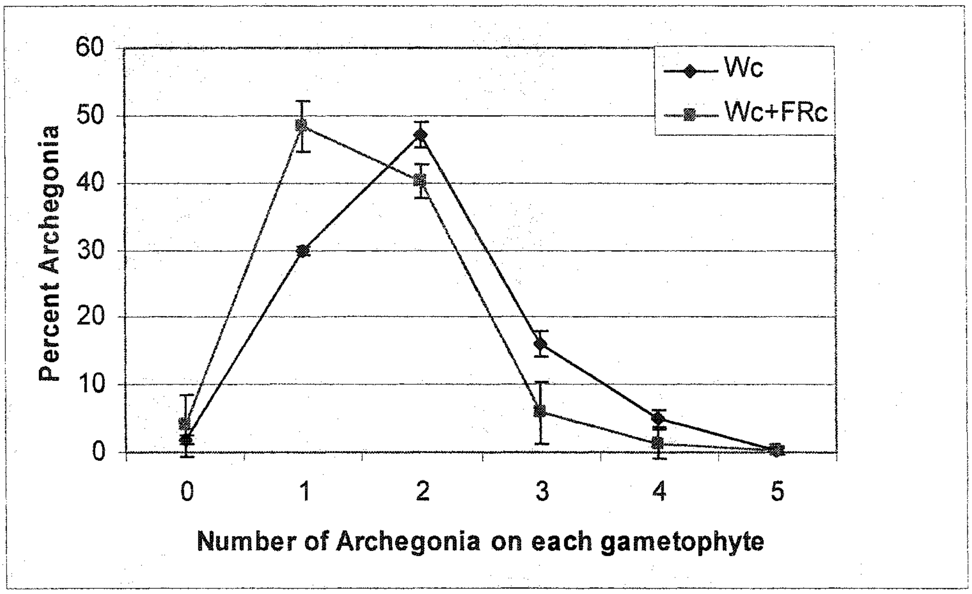

Percent Male at 12 days
40
35
30
25
20
15
10
5
0
Wc
Wc+FRc

Figure 15

Figure 16. Number of Archegonia developed under Wc or Wc+FRc light regimes

(A) After 7 d of light treatment, prothalli growing on agar medium were observed under a Wild dissecting microscope. The number of archegonia clustered at the meristematic notch was scored visually. At least 3 replicates were performed with minimum of 100 prothalli in each replica. Higher numbers of archegonia were observed under W_c compared with those under Supplementary FR (W_c+FR_c).

(B) After 14 d growth in their respective light conditions, prothalli growing on agar medium were observed under a Wild dissecting microscope. The degree of archegonial maturation was determined by the presence of an opening within the collar of four neck cells resulting from breakdown of the neck canal cell. The archegonial maturation was confirmed by the presence of pigmentation of the canal cells. At least 3 replicates were performed with a minimum of a 100 prothalli in each experiment. The proportion of mature archegonia (opened with pigment) to immature (closed without pigment) archegonia indicate that the FR_c supplement reduces the rate or capacity of archegonia to mature.


Percent Archegonia
60
50
40
30
20
10
0
Wc
Wc+FRc
0
1
2
3
4
5
Number of Archegonia on each gametophyte

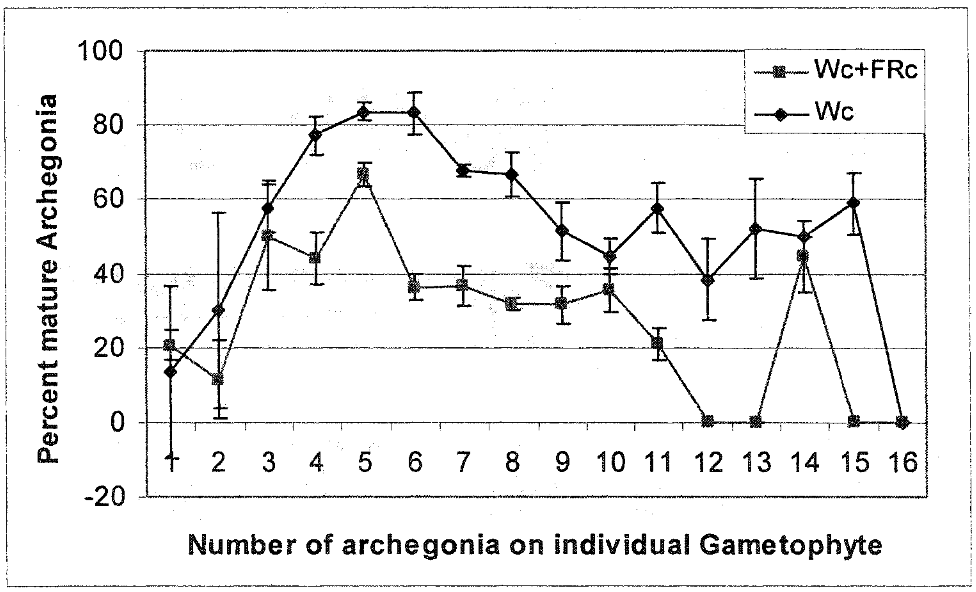

Percent mature Archegonia
100
80
60
40
20
0
-20
Wc+FRc
Wc
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Number of archegonia on individual Gametophyte

Figure 16

Figure 17. Percent germination, male, and sporophyte initiation under EOD-FR are not altered.

(A) Spores were synchronized by dark treatment for 7 d, then treated with 5 h R to induce germination, followed by either treatments of 16 h W light/8 h dark or supplemented with a 10 min FR pulse immediately after the end of light period. Prothalli growing on agar medium were observed under a Wild dissecting microscope. Germination percentage was monitored after 4 d of light exposure. Germination rates were determined by monitoring the emergence and apical growth of rhizoids in each population tested. At least 3 replicates were pooled with 100-300 prothalli per each replicate. Germination rates are apparently unaffected by EOD-FR, compared with plants grown under 16 h light/8 h dark for 4 d.

(B) The percentage of gametophytes developing as males was monitored after 10 d of light treatment with or without EOD-FR. Males were scored by the prolific development of antheridia without archegonia and the small size of male prothalli. A least 3 replicates were collected with a minimum of 100-300 gametophytes each. The ratio of hermaphrodite to male gametophytes at 10 d was not significantly different from that of controls, although under EOD-FR slightly more hermaphrodites were consistently observed.

(C) Over time, sporophyte initiation was monitored with or without EOD-FR. Prothalli growing on agar medium were observed under a Wild dissecting microscope. The timing and extent of sporophyte initiation was also similar in both treatments (Figure 16C). At least 3 replicates contributed to each point of data with 100-300 prothalli each.

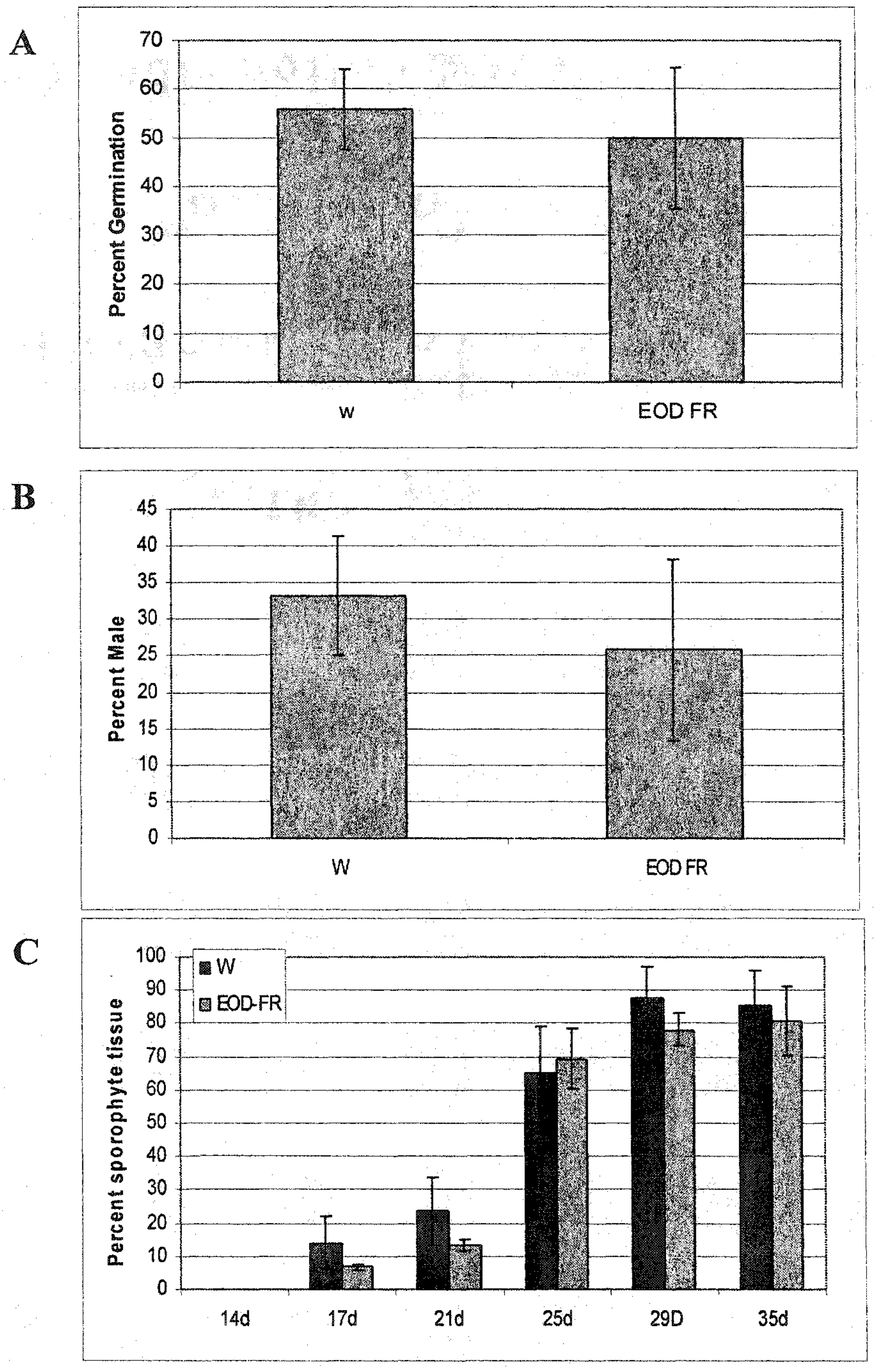

A
70
60
50
40
30
20
10
0
Percent Germination
w
EOD FR
B
45
40
35
30
25
20
15
10
5
0
Percent Male
W
EOD FR
C
100
90
80
70
60
50
40
30
20
10
0
Percent sporophyte tissue
W
EOD-FR
14d
17d
21d
25d
29D
35d

Figure17

Figure 18. 35S promoter and Actin promoter expression

Prothalli cells were injected with the plasmid DNA constructs as indicated in the text, left in D for 24 h, then observed for the expression of GFP. For monitoring fluorescence of or GFP, a blue excitation (480±20nm) and green emission (535±25nm) cube was used on a Nikon TE-300 with inverted optics and a 40x objective. (A) Showing Actin::GFP fluorescent with its corresponding to the phase contrast image. (B) Showing 35S::GFP fluorescent with its corresponding to the phase contrast image. The arrow is pointing at the nucleus of the corresponding cells. (standard bar showing 50µm)

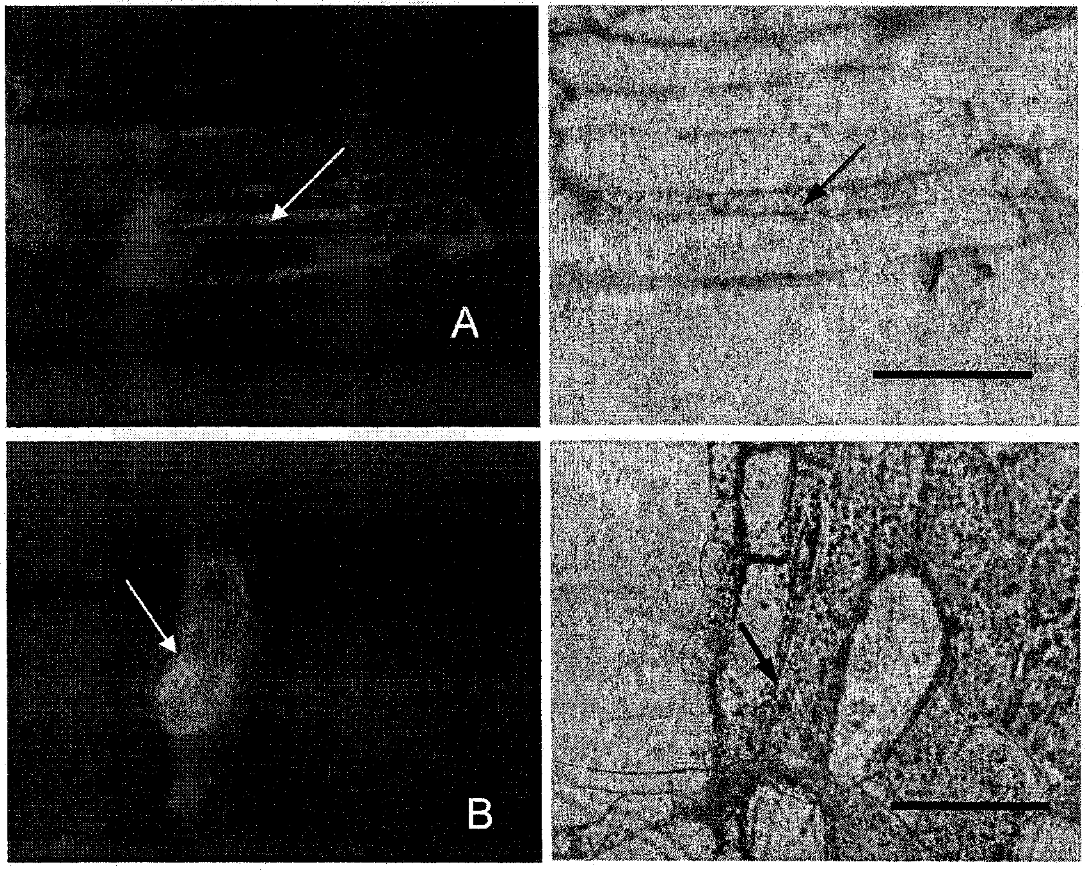


Figure 18

Figure 19. Intracellular localization of 35S-GFP under red (R) and dark (D).

Prothalli cells were injected, then left in R (A) or D (B) for 24 h and observed for the expression of GFP. For monitoring epifluorescence of GFP, a blue excitation (480±20nm) and green emission (535±25nm) cube was used on Nikon TE-300 with inverted optics and a 40x objective. GFP localization was similar in R and D, with GFP apparently located both in the cytoplasm and the nucleus. The arrow is pointing at the nucleus of the corresponding cells. (standard bar showing 50µm)

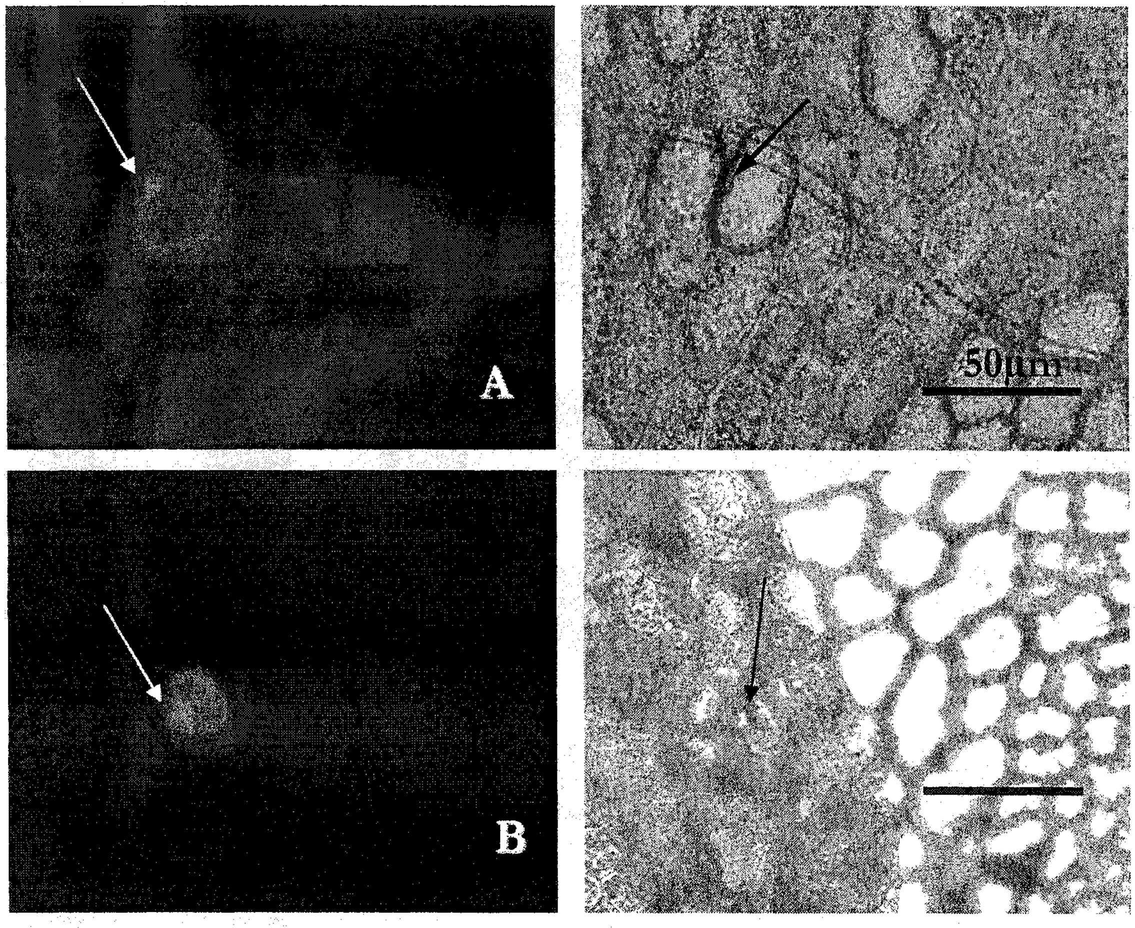


Figure 19

Figure 20. Localization of Vip1:GFP, VirE2:GFP, and VirD2:GFP fusion proteins in *Ceratopteris.*

At 24 h after injection, prothalli were observed for GFP expression as described in the previous figure legend. (A) Intracellular compartmentalization of Vip1: GFP fusion protein, (B) VirE2:GFP, and (C) VirD2::GFP show that all three are localized exclusively in the nuclei (pointed by arrow). For monitoring epifluorescence of GFP, a blue excitation (480±20nm) cube was used. (appropriate standard bar shown)

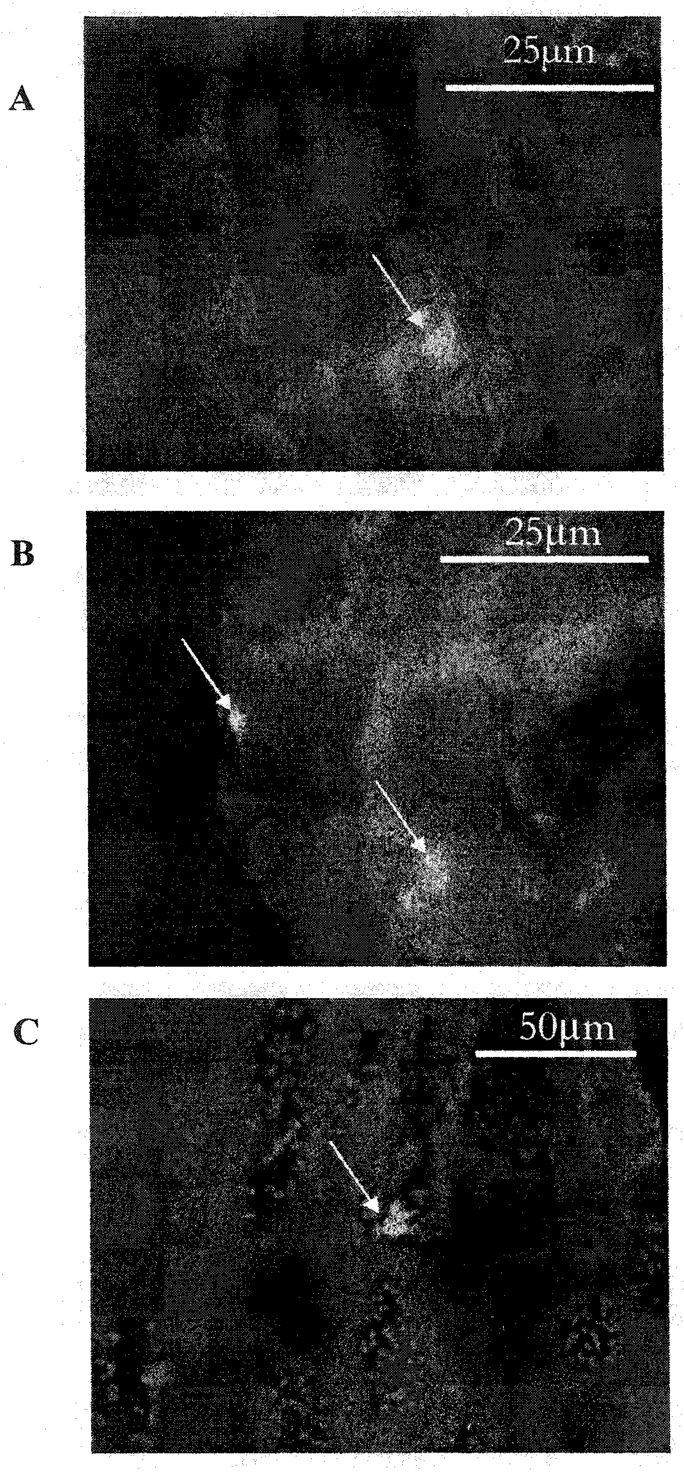


Figure 20

Figure 21. Representative images of *At*PhyB intracellular distribution in prothalli cells.

Intracellular distribution of PhyB: GFP fusion protein under different light conditions is shown. (A) Showing the phyB::GFP fluorescent after 24h in D with the corresponding phase contrast image. (B) Showing the phyB::GFP fluorescent after 24h in D followed by 3 h R with its corresponding phase contrast image. (C) PhyB::GFP fluorescent after 1 d D treatment followed by 1 d R then immediately observed for GFP fluorescence with the corresponding phase contrast image. Cells showing GFP expression were photographed using a Nikon TE-300 with inverted optics (standard bar of 50μm shown). Arrows point to the cells corresponding to the fluorescent images.

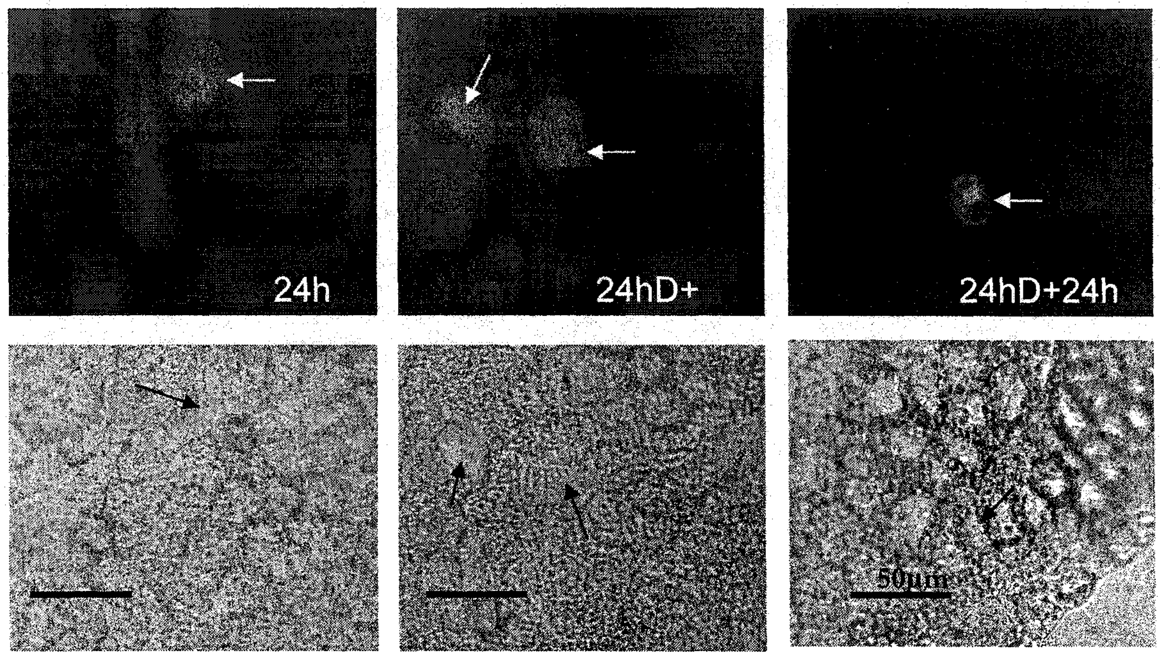


Figure 21

Figure 22. Representative images of *Cr*Phy1: GFP intracellular distribution in prothalli cells.

Intracellular distribution of *Cr*Phy1:GFP fusion protein under different light conditions is shown. (A1,2) Prothalli were left for 24 h in D, followed by phy1::GFP observation. The phase contrast images of the corresponding fluorescent cells are shown below A1 and A2. (B1,2) After 24h in D followed by 1 h R phy1::GFP fluorescent was observed. The corresponding phase contrast image is shown. (C1,2) showing the phy1::GFP fluorescent after 24h in D followed by 3h R with its corresponding phase contrast image. (D1,2) phy1::GFP expression after 1d D followed by 1dR. Arrows point to the cells corresponding to the fluorescent images. Cells showing GFP expression were photographed using a Nikon TE-300 with inverted optics (standard bar showing 50µm).

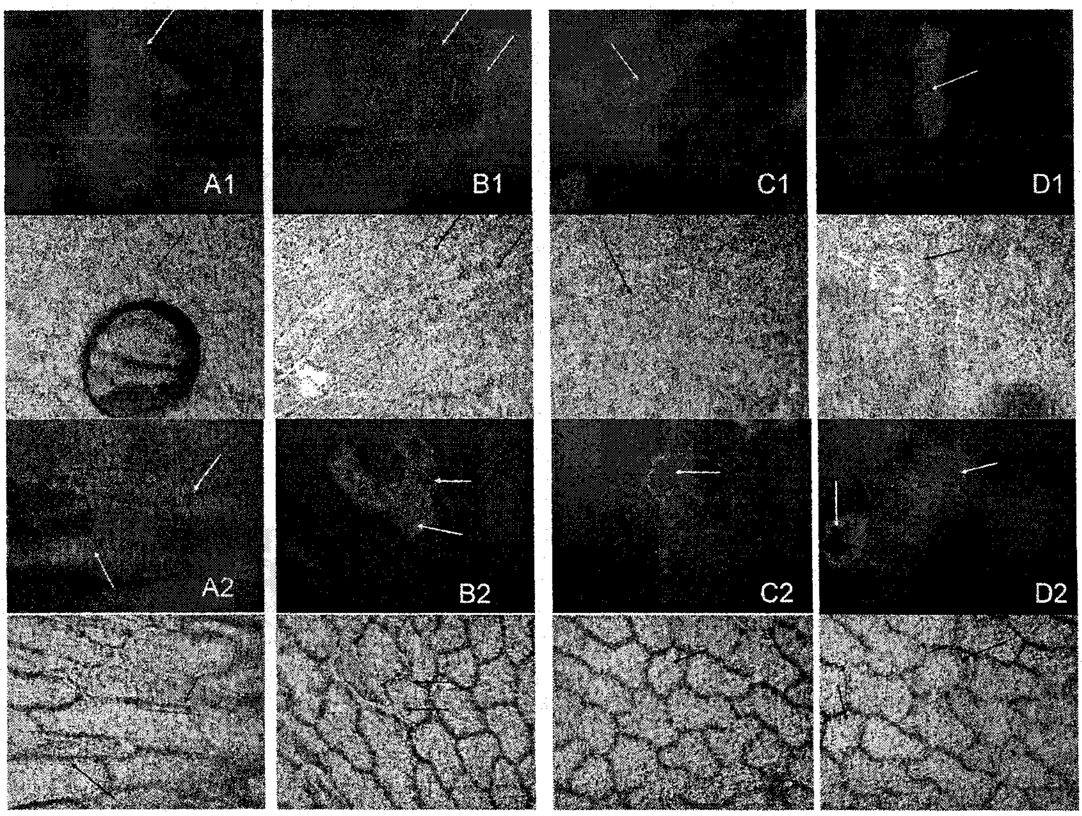


Figure 22

Tables

Table 1. Number of cell files in the subapical area of the elongated prothalli

Spores were plated on agar medium as indicated in the text. Samples were collected after 14 d of light exposure. The number of cell files was counted using digital photographs of the tissues taken with a SPOT RT CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI) on a Wild dissecting microscope. These pictures were analyzed using SigmaScan Pro software (SPSS, Chicago). For each experiment at least 3 replicates were analyzed with a minimum of 30 individuals per replicate.

	Number of cell files in the subapical region
Far-red	**7.2±0.97**
Dark	**4.86±0.80**

Table 1

Table 2. Average cell size of prothalli after 14d of light treatment.

Cell size was calculated using the average cell count divided by average prothallus size.

	Cell Count	**Prothallus Area(mm2)**	**Average area/Cell**
Wc	764± 61	3.10±0.9	0.003816
Rc	66± 9.5	0.056±0.04	0.000876
Rc+FRc	318±65	1.16±0.47	0.003688
Bc	469±72	1.29±0.36	0.002711
FRc (tip)	116±20	0.11±0.02	0.000972
Dc (tip)	59±15	0.04±0.02	0.000712

Table 2

Table 3. Percent archegonia and maximum number available in individual prothalli.

The proportion of hermaphrodites with at least one archegonium and the maximum number of mature and immature archegonia on individual gametophytes was scored after 14 d growth under W_c, R_c, FR_c, R_c+FR_c, and B_c light conditions. The number of archegonia clustered at the meristematic notch was scored visually. At least 3 replicates were performed with minimum of 100 prothalli in each replica.
Among the archegonia produced under all light conditions, only those from Wc were mature with visually detectable pigment at 14 d. Even in Wc, the population of archegonia on a given individual consisted of mature and immature organs.

	Archegonia formation	Maximum No. of Archegonia
W_c	98.8%	17
R_c	23.4%	2
FR_c	1%	1
R_c+FR_c	97.7%	11
B_c	96.03%	6

Table 3

Table 4. Photoresponses in *Ceratopteris* are different from those in higher plants.

This table provides a comparison between relevant light-mediated responses in *Ceratopteris* as fern plant and higher plants. As summarized in the table, some of the observed effects of light on *Ceratopteris* are inconsistent with those reported for angiosperms.

Light-Induced Reactions under Continuous light	***Ceratopteris*, haploid and diploid phases**	**Higher plants, diploid phase**
Germination **Under Rc.** **Under FRc**	 **Inhibition** **Promotion**	 **Promotion (1,2)** **Inhibition (1,2)**
Cell elongation **Under Rc** **Under FRc**	 **Inhibition** **Promotion**	 **Inhibition (3)** **Inhibition (3)**
Cell division **Under Rc.** **Under FRc**	 **Inhibition** **Promotion**	 **Promotion (4,3)** **Inhibition (4,3)**
Chlorophyll Synthesis **Under Rc** **Under FRc**	 **Inhibition** **Promotion**	 **Promotion (5)** **Inhibition (5)**
Supplementary FR responses	**No effect on haploid germination, cell division, male %.** **Retardation of archegonia maturation and sporophyte development.**	**Unknown effect on the haploid phase.** **Increase of elongation and acceleration of flowering. (6)**
EOD-FR responses	**No effect on either gametophyte or sporophyte**	**Acceleration of growth. (7)**

Table 4

1-(Reed et al., 1994)
2-(Shinomura et al., 1994)
3-(Whitelam and Harberd, 1994)
4-(Liscum and Hangarter, 1993)
5-(von Arnim and Deng, 1996)
6-(Whitelam and Smith, 1991)
7-(Smith and Whitelam, 1990)

Table 5. Transient expression efficiency using microinjection

Spores were plated on agar medium as indicated in the text. Prothalli were observed 24-48 h after injection, using a Nikon TE-200 with blue excitation (480±20nm) and green emission (535±25nm) cube. The number of cells showing GFP expression was counted. At least 3 replicates were performed, each with 200-400 prothalli cells or germinated spores as indicated in the table.

Stage injected	Injected material	Concentration	Number of injections	Fluorescent Cells (%)
Germinating spores	Fluorescein	0.03%	435	20%
	Actin::GFP	0.3μg/μl	860	1%
	35S::GFP	0.3μg/μl	Not tested	Not tested
Prothalli	Fluorescein	0.03%	800	53%
	Actin::GFP	0.3μg/μl	1625	2-4%
	35S::GFP	0.3μg/μl	1210	3-7%

Table 5

References

Ahmad, M., and Cashmore, A.R. (1993). The *HY4* gene involved in blue light sensing in *Arabidopsis thaliana* encodes a protein with the characteristics of a blue light photoreceptor. Nature **366,** 162-166.

Ahmad, M., Lin, C., and Cashmore, A.R. (1995). Mutations throughout an Arabidopsis blue light photoreceptor impair blue-light-responsive anthocyanin accumulation and inhibition of hypocotyls elongation. Plant J. **8,** 653-658.

Ahmad, M., Jarillo, J.A., Smirnova, O., and Cashmore, A.R. (1998). Cryptochrome blue-light photoreceptors of Arabidopsis implicated in phototropism. Nature **392,** 720-723.

Banks, J.A. (1994). Sex-determining genes in the homosporous fern *Ceratopteris*. Development **120,** 1949-1958.

Banks, J.A. (1999). Gametophyte development in ferns. Annu Rev of Plant Physiol Plant Mol Biol **50,** 163-186.

Banks, J.A., Hickok, L., and Webb, M.A. (1993). The programming of sexual phenotype in the homosporous fern *Ceratopteris richardii*. Int J Plant Sci **154,** 522-534.

Birch, R.G. (1997). PLANT TRANSFORMATION: Problems and Strategies for Practical Application. Annu Rev Plant Physiol Plant Mol Biol **48,** 297-326.

Bissoondial, T., and Short, T.W. (unpublished).

Borthwick, H.A., Hendricks, S.B., Parker, M.W., Toole, E.H., and Toole, V.K. (1952). A reversible photoreaction controlling seed germination. Proc. Natl. Acad. Sci. USA **38,** 662-666.

Bowler, C., Neuhaus, G., Yamagata, H., and Chua, N.-H. (1994). Cyclic GMP and calcium mediate phytochrome phototransduction. Cell **77,** 73-81.

Briggs, W.R. (1975). H. A. Borthwick and S. B. Hendricks - Pioneers of Photomorphogenesis. In Light and Plant Development - Proceedings of the 22nd Nottingham Easter School in Agricultural Sciences, H. Smith, ed (London: Butterworth and Co.), pp. 1-4.

Briggs, W.R. (2004). BLUE/UV-A RECEPTORS: Historical overview, Unpublished.

Briggs, W.R., and Huala, E. (1999). Blue-light photoreceptors in higher plants. Ann Rev Cell Dev Biol **15,** 33-62.

Briggs, W.R., and Christie, J.M. (2002). Phototropins 1 and 2:versatile plant blue-light receptors. Trends Plant Sci **7**, 204-210.

Briggs, W.R., Beck, C.F., Cashmore, A.R., Christie, J.M., Hughes, J., Jarillo, J.A., Kagawa, T., Kanegae, H., Liscum, E., Nagatani, A., Okada, K., Salomon, M., Rudiger, W., Sakai, T., Takano, M., Wada, M., and Watson, J.C. (2001). The phototropin family of photoreceptors. Plant Cell **13,** 993-997.

Briggs, W.R., and Olney, M.A. (2001). Photoreceptors in plant photomorphogenesis to date. Five phytochromes, two cryptochromes, one phototropin, and one superchrome. Plant Physiol **125,** 85-88.

Brudler, P., Hitomi, K., Daiyasu, H., Toh, H., Kucho, K.-I., Ishiura, M., Kaneshisa, M., Roberts, V.A., and Getzoff, E.D. (2003). Identification of a new cryptochrome class: structure, function, and evolution. Mol. Cell **11,** 59-67.

Butler, W.L., Norris, K.H., Siegelman, H.W., and Hendricks, S.B. (1959). Detection, assay, and preliminary purification of the pigment controlling photoresponsive development of plants. Proc. Natl. Acad. Sci. USA **45,** 1703-1708.

Casal, J.J., and Smith, H. (1989). The function, action and adaptive significance of phytochrome in light-grown plants. Plant Cell Environ. **12,** 855-862.

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

Chasen, R. (1992). *Ceratopteris*: a model plant for the 90s. Plant Cell **4,** 113-115.

Chatterjee, A., and Roux, S.J. (2000). *Ceratopteris richardii*: a productive model for revealing secrets of signaling and development. J Plant Growth Regul **19,** 284-289.

Chatterjee, A., San Miguel, P., Stout, S.C., Banks, J.A., and Roux, S.J. (2000). Expressed sequence tags of cDNA clones from a C. richardii library (GenBank).

Chiu, W.-I., Niwa, Y., Zeng, W., Hirano, H., Kobayashi, H., and Sheen, J. (1996). Engineered GFP as a vital reporter in plants. Curr Biol **6,** 325-330.

Chory, J., Aguilar, N., and Peto, C.A. (1991). The phenotype of Arabidopsis thaliana det1 mutants suggests a role for cytokinins in greening. Symp Soc Exp Biol **45,** 21-29.

Christensen, S., Tokuoka, Y., Silverthorne, J., and Wada, M. (1998). Phytochrome regulation of expression of mRNA encoding the major light-harvesting Chlorophyll a/b-binding proteins of photosystem II in the haploid phase of *Adiantum capillus-veneris*. Plant Cell Physiol **39,** 647-654.

Christie, J.M., Swartz, T.E., Bogomolni, R.A., and Briggs, W.R. (2002). Phototropin LOV domains exhibit distinct roles in regulating photoreceptors function. Plant J **32,** 205-219.

Clough, R.C., and Vierstra, R.D. (1997). Phytochrome degradation. Plant Cell Environ **20,** 713-721.

Clough, R.C., Jordan-Beebe, E.T., Lohman, K.N., Marita, J.M., Walker, J.M., Gatz, C., and Vierstra, R.D. (1999). Sequences within both the N- and C-terminal domains of phytochrome A are required for PFR ubiquitination and degradation. Plant J **17,** 155-167.

Cooke, T.J., Hickok, L., and VanDerWoude, W.J. (1993). Photobiological characterization of a spore germination mutant *dkg1* with reversed photoregulation in the fern *Ceratopteris richardii*. Photochem Photobiol **57,** 1032-1041.

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

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

Crosson, S., and Moffat, K. (2001). Structure of a flavin-binding plant photoreceptor domain: insights into light-mediated signal transduction. Proc Natl Acad Sci USA **98,** 2995-3000.

Crossway, A., Oakes, J.V., Irvine, J.M., Ward, B., Knaurf, V.C., and Shewmaker, C.K. (1986). Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts. Mol Gen Genet **202,** 179-185.

Davis, B.D. (1975). Bending growth in fern gametophyte protonema. Plant Cell Physiol **16,** 537-541.

Devlin, P.F., Patel, S.R., and Whitelam, G.C. (1998). Phytochrome E influences internode elongation and flowering time in Arabidopsis. Plant Cell **10,** 1479-1487.

Devlin, P.F., Rood, S.B., Somers, D.E., Quail, P.H., and Whitelam, G.C. (1992). Photophysiology of the elongated internode (*ein*) mutant of *Brassica rapa. ein* mutant lacks a detectable phytochrome B-like polypeptide. Plant Physiol **100,** 1442-1447.

Devlin, P.F., Robson, P.R., Patel, S.R., Goosey, L., Sharrock, R.A., and Whitelam, G.C. (1999). Phytochrome D acts in the shade-avoidance syndrome in Arabidopsis by controlling elongation growth and flowering time. Plant Physiol **119**, 909-915.

Dyer, A.F. (1979). The experimental biology of ferns. (Academic Press, London New york).

Etzold, H. (1965). Der polarotropismus und phototropismus der Chloroneen von *Dryopteris filix mas* L. Schott. Planta **64,** 254-280.

Fankhauser, C., and Chory, J. (1999). Light receptor kinases in plants! Curr Biol **9,** R123-126.

Finer, J.J., and McMullen, M.D. (1990). Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. Plant Cell Rep **8,** 586-589.

Fohr, K.J., Enssle, M., and Schraudolf, H. (1987). Calmodulin-like protein from the fern *Anemia phyllitidis* L. Sw. Planta **171,** 127-129.

Franklin, K.A., Praekelt, U., Stoddart, W.M., Billingham, O.E., Halliday, K.J., and Whitelam, G.C. (2003). Phytochromes B, D, and E act redundantly to control multiple physiological responses in Arabidopsis. Plant Physiol **131,** 1340-1346.

Fukshansky, L., and Schafer, E. (1983). Models in photomorphogenesis. In Encyclopedia of plant physiology, W. Shropshire, Jr. and H. Mohr, eds (Springer-Verlag, Berlin.), pp. 69-95.

Furuya, M. (1983). Photomorphogenesis in ferns. In Encyclopedia of plant physiology. pp. 569-600. (Berlin).

Furuya, M., and Hillman, W.S. (1964). Observation on spectrophotometrically assayable phytochrome in vivo in etiolated *Pisum* seedling. Planta **63,** 31-42.

Furuya, M., and Schafer, E. (1996). Photoperception and signalling of induction reactions by different phytochromes. Trends in Plant Science **1,** 301-307.

Furuya, M., Kadota, A., and Uematsu, H. (1982). Percent Pfr-dependent germination of spores in *Pteris vittata.* Plant Cell Physiol **23,** 1213-1217.

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

Gemmrich, A.R. (1986). Antheridiogenesis in the fern *Peteris Vittata.* I. Photocontrol of Antheridium formation. Plant Science **43,** 135-140.

Gil, P., Kircher, S., Adam, E., Bury, E., Kozma-Bognar, L., Schafer, E., and Nagy, F. (2000). Photocontrol of subcellular partitioning of phytochrome-B:GFP fusion protein in tobacco seedlings. Plant J **22,** 135-145.

Goto, N., Yamamoto, K.T., and Watanabe, M. (1993). Action spectra for inhibition of hypocotyl growth of wild-type plants and of the hy2 long-hypocotyl mutant of *Arabidopsis thaliana* L. Photochem Photobiol **57,** 867-871.

Grebenok, R.J., Pierson, E.A., Lambert, G.M., Gong, F.-C., Afonso, C.L., Haldemann-Cahill, R., Carrington, J.C., and Galbraith, D.W. (1997). Green-fluorescent protein fusion for efficient characterization of nuclear localization signals. The Plant Journal **11,** 573-586.

Griesbach, R.J. (1985). Advances in the microinjection of higher plant cells Biotechniques **3,** 348-350.

Grill, R., and Schraudolf, H. (1981). In vivo phytochrome differences spectrum from dark grown gametophytes of *Anemia phyllitidis* L. Sw. treated with Norflurazon. Plant Physiol **68,** 1-4.

Guo, H., Yang, H., Mockler, T.C., and Lin, C. (1998). Regulation of flowering time by Arabidopsis photoreceptors. Science **279,** 1360-1363.

Halliday, K., Koornneef, M., and Whitelam, G. (1994). Phytochrome B and at least one other phytochrome mediate the accelerated flowering response of *Arabidopsis thaliana* to low red/far-red ratio. Plant Physiol **104,** 1311-1315.

Hamazato, F., Shinomura, T., Hanzawa, H., Chory, J., and Furuya, M. (1997). Fluence and wavelength requirements for Arabidopsis CAB gene induction by different phytochromes. Plant Physiol **115,** 1533-1540.

Hanazono, Y., Yu, J.M., Dunbar, C.E., and Emmons, R.V. (1997). Green fluorescent protein retroviral vectors: low titer and high recombination frequency suggest a selective disadvantage. Human Gene Therapy **8,** 1313-1319.

Hartmann, E., Klingenberg, B., and Bauer, L. (1983). Phytochrome-mediated phototropism in protonemata of the moss *Ceratodon purpures* Bird. Photochem Photobiol **38,** 599-603.

Hartmann, K.M. (1966). A general hypothesis to interpret 'high energy phenomena' of photomorphogenesis on the basis of phytochrome. Photochem. Photobiol. **5,** 349-366.

Harvey, W.H., and Caponetti, J.D. (1972). In vitro studies on the induction of sporogenous tissue on leaves of cinnamon fern. I. Environmental factors. Can J Bot. **50,** 2673-2682.

Haseloff, J., Siemering, K.R., Prasher, D.C., and Hodge, S. (1997). Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc Natl Acad Sci USA **94,** 2122-2127.

Haupt, W. (1985). Effects of nutrients and light pretreatment on phytochrome-mediated fern-spore germination. Planta **164,** 63-68.

Haupt, W., and Bock, G. (1962). Die Chloroplast endrehung bei *Mougeotia.* IV. Die Orientierung der Phytochrom-Molekule im Cytoplasma. Planta **59,** 38-48.

Haupt, W., and Bjorn, L.O. (1987). No action dichroism for light-controlled fern spore germination. J Plant Physiol **129,** 119-128.

Haupt, W., Mortel, G., and Winkelnkemper, I. (1969). Demonstration of different dichroic orientation of phytochrome Pr and Pfr. Planta **88,** 183-186.

Haupt, W., Leopold, K., and Scheuerlein, R. (1988). Light-induced fern-spore germination: effect of spore age on responsitivity to light. Photochem Photobiol **1,** 415-427.

Hayami, J., Kadota, A., and Wada, M. (1986). Blue light-induced phototropic response and the intracellular photoreceptive site in *Adiantum* protonemata. Plant Cell Physiol **27,** 1571-1577.

Hayami, J., Kadota, A., and Wada, M. (1992). Intracellular dichroic orientation of the blue light-absorbing pigment and the blue-absorption band of the red-absorbing form of phytochrome responsible for phototropism of the fern *Adiantum* protonemata. Photochem Photobiol **56,** 661-666.

Hendricks, S.B., and Borthwick, H.A. (1967). The function of phytochrome in regulation of plant growth. Proc Natl Acad Sci USA **58,** 2125-2130.

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

Hock, B., and Mohr, H. (1964). Die Regulation der O_2-Aufnahme von Senfkeimlingen (*Sinapis alba* L.) durch licht. Planta **61,** 209-227.

Horwitz, B.A., and Berrocal, T.G.M. (1997). A spectroscopic view of some recent advancs in the study of blue light photoreception. Botanica Acta **110,** 360-368.

Hughes, J., Lamparter, T., and Mittmann, F. (1996). CERPU; PHY0;2, a 'Normal' phytochrome in Ceratodon (Accession No. U56698)(PGR96-067). Plant Physiol **112,** 446.

Hughes, J., Lamparter, T., Mittmann, F., Hartmann, E., Gartner, W., Wilde, A., and Borner, T. (1997). A prokaryotic phytochrome. Nature **386,** 663.

Iino, M., Endo, M., and Wada, M. (1989). The occurrence of a Ca 2+- dependent period in the red light-induced late G1 phase of germinating Adiantun spores. Plant Physiol **91,** 610-616.

Imaizumi, T., Kanegae, T., and Wada, M. (2000). Cryptochrome nucleocytoplasmic distribution and gene expression are regulated by light quality in the fern *Adiantum capillus-veneris*. Plant Cell **12,** 81-96.

Isikawa, S., and Oohusa, T. (1954). Effects of light upon the germination of spores of ferns. I. The relation of daily illuminating period to germination rate. Bot. Mag. tokyo. **67,** 193-197.

Ito, J. (1970). Light-induced synchrony of cell division in the protonemata of the fern, *Pteris vittata*. Planta **90,** 22-31.

Jarillo, J.A., Ahmad, M., and Cashmore, A.R. (1998). NPL1: A second member of NPH serine/threonine kinase family for Arabidopsis. Plant Physiol **117,** 719.

Jarillo, J.A., Gabrys, H., Capel, J., Alonso, J.M., Ecker, J.R., and Cashmore, A.R. (2001). Phototropin-related NPL1 controls chloroplast relocation induced by blue light. Nature **410,** 952-954.

Kadota, A., Wada, M., and Furuya, M. (1982). Phytochrome-mediated phototropism and different dichroic orientation of Pr and Pfr in protonemata of the fern *Adiantum capillus-veneris* L. Photochem Photobiol **35,** 533-536.

Kadota, A., Fushimi, Y., and Wada, M. (1986a). Intracellular photoreceptive site for blue light-induced cell division in protonemata of the fern *Adiantum*- Further analysis by polarized light irradiation and cell centrifugation. Plant Cell Physiol **27,** 989-995.

Kadota, A., Inoue, Y., and Furuya, M. (1986b). Dichroic orientation of phytochrome intermediates in the pathway from Pr to Pfr as analyzed by double laser flash irradiation in polarotropism of *Adiantum* protonemata. Plant Cell Physiol **27,** 867-873.

Kagawa, T. (2003). The phototropin family as photoreceptors for blue light-induced chloroplast relocation. J Plant Res **116,** 77-82.

Kagawa, T., Sakai, T., Suetsugu, N., Oikawa, K., Ishiguro, S., Kato, T., Tabata, S., Okada, K., and Wada, M. (2001). Arabidopsis NPL1: a phototropin homolog controlling the chloroplast high-light avoidance response. Science **291,** 2138-

2141.

Kamachi, H., Matsunaga, E., Noguchi, M., and Inoue, H. (2004). Novel mutant phenotypes of a dark-germinating mutant dkg1 in the fern *Ceratopteris richardii.* J Plant Res **117,** 163-170.

Kanegae, H., Tahir, M., Savazzini, F., Yamamoto, K., Yano, M., Sasaki, T., Kanegae, T., Wada, M., and Takano, M. (2000). Rice NPH1 homologues, OsNPH1a and OsNPH1b, are differently photoregulated. Plant Cell Physiol **41,** 415-423.

Kanegae, T., and Wada, M. (1998). Isolation and characterization of homologues of plant blue-light photoreceptor (cryptochrome) genes from the fern *Adiantum capillus-veneris.* Mol Gen Genet **259,** 345-353.

Kasahara, M., Swartz, T.E., Olney, M.A., Onodera, A., Mochizuki, N., Fukuzawa, H., Asamizu, E., Tabata, S., Kanegae, H., Takano, M., Christie, J.M., Nagatani, A., and Briggs, W.R. (2002). Photochemical properties of the flavin mononucleotide-binding domains of the phototropins from *Arabidopsis,* rice, and *Chlamydomonas reinhardtii.* Plant Physiol **129,** 762-773.

Kendrick, R.E., and Kronenberg, G.H.M. (1994). Photomorphogenesis in plants. (Dordrecht: Kluwer).

Kim, L., Kircher, S., Toth, R., Adam, E., Schafer, E., and Nagy, F. (2000). Light-induced nuclear import of phytochrome-A:GFP fusion proteins is differentially regulated in transgenic tobacco and Arabidopsis. Plant J **22,** 125-133.

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

Kircher, S., Gil, P., Kozma-Bognar, L., Fejes, E., Speth, V., Husselstein-Muller, T., Bauer, D., Adam, E., Schafer, E., and Nagy, F. (2002). Nucleocytoplasmic partitioning of the plant photoreceptors phytochrome A, B, C, D, and E is regulated differentially by light and exhibits a diurnal rhythm. Plant Cell **14,** 1541-1555.

Klekowski, E.J., Jr. (1969). Reproductive biology of the pteridophyta. III. A study of the blechnaceae. Bot. J. Linn. Soc. **62,** 361-377.

Klink, V.P., and Wolniak, S.M. (2000). The efficacy of RNAi in the study of the plant cytoskeleton. J Plant Growth Regul **19,** 371-384.

Kohler, R.H., Zipfel, W.R., Webb, W.W., and Hanson, M.R. (1997). The green fluorescent protein as a marker to visualize plant mitochondria *in vivo.* The Plant Journal **11,** 613-621.

Kraml, M. (1994). Light Direction and Polarization. In Photomorphogenesis in Plants, R.E. Kendrick and G.H.M. Kronenberg, eds (Dordrecht: kluwer Academic Publishing), pp. 417-443.

Kumagai, T. (1988). Photocontrol of fungal development. Photochem Photobiol **47,** 889-896.

Laetsch, W.M., and Briggs, W.R. (1962). Photomorphogenic responses of sporeling of *Marsilea vestita*. Plant Physiol **37,** 142-148.

Lagarias, D.M., Wu, S.H., and Lagarias, J.C. (1995). A typical phytochrome gene structure in the green alga *Mesotaenium caldariorum*. Plant Mol Biol **29,** 1127-1142.

Lin, C. (2002). Blue light receptors and signal transduction. Plant Cell **14 Suppl,** S207-225.

Lin, C., Ahmad, M., Gordon, D., and Cashmore, A.R. (1995a). Expression of an Arabidopsis cryptochrome gene in transgenic tobacco results in hypersensitivity to blue, UV-A, and green light. Proc Natl Acad Sci U S A **92,** 8423-8427.

Lin, C., Yang, H., Guo, H., Mockler, T., Chen, J., and Cashmore, A.R. (1998). Enhancement of blue-light sensitivity of Arabidopsis seedling by a blue light receptor cryptochrome 2. Proc Natl Acad Sci USA **95,** 2686-2690.

Lin, C., Robertson, D.E., Ahmad, M., Raibekas, A.A., Jorns, M.S., Dutton, P.L., and Cashmore, A.R. (1995b). Association of flavin adenine dinucleotide with the *Arabidopsis* blue light receptor CRY1. Science **269,** 968-970.

Liscum, E., and Hangarter, R.P. (1993). Light stimulated apical hook opening in wild type *Arabidopsis thaliana* seedlings. Plant Physiol **101**,567-572

Liu, H.S., Jan, M.S., Chou, C.K., Chen, P.H., and Ke, N.J. (1999). Is green fluorescent protein toxic to the living cells? Biochem Biophys Res Commun **260,** 712-717.

Lusardi, M.C., Neuhaus-Url, G., Potrykus, I., and Neuhaus, G. (1994). An approach towards genetically engineered cell fate mapping in maize using the Lc gene as a visible marker. Transactivation capacity of Lc vectors in differentiated maize cells and microinjection of Lc vectors into meristematic. Plant J **5,** 571-582.

Mahlberg, P.G., and Yarus, S. (1977). Effects of light, pH, temperature, and crowing on megaspore germination and sporophyte formation in *Marsilea*. J Exp Bot **28,** 1137-1146.

Manabe, K., Ibushi, N., Nakayama, A., Takaya, S., and Sugai, M. (1987). Spore germination and phytochrome biosynthesis in the fern *Lygodium japonicum* as affected by gabaculine and cycloheximide. Physiol Plant **70,** 571-576.

Mancinelli, A. (1980). The photoreceptors of the high irradiance responses of plant photomorphogenesis. Photochem. Photobiol. **32,** 853-857.

Mancinelli, A.L. (1994). The physiology of phytochrome action. In Photomorphogenesis in plants, 2nd ed., R.E. Kendrick and G.H.M. Kronenberg, eds (Dordrecht: Kluwer), pp. 211-269.

Mandoli, D.F., and Briggs, W.R. (1981). Phytochrome control of two low-irradiance responses to etiolated oat seedlings. Plant Physiology **67,** 733-739.

Marcondes-Ferreira, W., and Felippe, G.M. (1984). Effects of light and temperature on the germination of spores of *Cyathea delgadii*. Revis. brasil. Bot. **7,** 53-56.

Marme, D., and Schafer, E. (1972). On the localization and oreintation of phytochrome molecules in corn coleoptiles (*Zea mays* L.). Z. Pflanzenphysiol. **67,** 192-194.

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

Mathews, S., and Sharrock, R.A. (1997). Phytochrome gene diversity. Plant, Cell and Environment **20,** 666-671.

Maucher, H.P., Scheuerlein, R., and Schraudolf, H. (1992a). Detection and partial sequence of phytochrome genes in the ferns *Anemia phyllitidis* (L.)Sw (Schizaeaceae) and Dryopteris filix-mas L. (Polypodiaceae) by using polymerase-chain reaction technology. Photochem Photobiol **56,** 759-763.

Maucher, H.P., Scheuerlein, R., and Schraudolf, H. (1992b). Detection and partial sequence of phytochrome genes in the ferns *Anemia phyllitidis* (L.)Sw (SCHIZAEACEAE) and *Dryopteris filix-mas* L. (POLYPOIACEAE) by using polymerase-chain reaction technology. Photochem Photobiol **56,** 759-763.

McElroy, D., Zhang, W., Cao, J., and Wu, R. (1990). Isolation of an efficient actin promoter for use in rice transformation. The Plant Cell **2,** 163-171.

Millar, J.H. (1968). Fern gametophytes as experimental material. Bot. Rev **34,** 361-440.

Minaschek, G., Bereiter-Hahn, J., and Bertholdt, G. (1989). Quantitation of the volume of liquid injected into cells by means of pressure. Exp Cell Res **183,** 434-442.

Miyata, M., Wada, M., and Furuya, M. (1979). Effects of phytochrome and blue-near ultraviolet light-absorbing pigment on duration of component phases of the cell cycle in *Adiantum* gametophyte. Develop. Growth Differ. **21,** 577-584.

Mohr, H. (1956). Die Beeinflussung der Keimung von Farnsporen durch Light und andere Faktoren. Planta **46,** 534-551.

Mohr, H. (1962). Primary effects of light on growth. Annu Rev Plant Physiol **13,** 465-488.

Mohr, H. (1964.). The control of plant growth and development by light. Biol Rev **39,** 87-112.

Mösinger, E., Batschauer, A., Schafer, E., and Apel, K. (1985). Phytochrome control of in vitro transcription of specific genes in isolated nuclei from barley (Hordeum vulgare). Eur J Biochem **147,** 137-142.

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

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

Murata, T., Kadota, A., and Wada, M. (1997). Effects of blue light on cell elongation and microtubule orientation in dark-grown gametophytes of *Ceratopteris richardii.* Plant Cell Physiol **38,** 201-209.

Nagatani, A., Chory, J., and Furuya, M. (1991). Phytochrome B is not detectable in the *hy3* mutant of *Arabidopsis*, which is deficient in responding to end-of-day far-red light treatments. Plant Cell Physiol. **32,** 1119-1122.

Nagatani, A., Reed, J.W., and Chory, J. (1993). Isolation and characterization of *Arabidopsis* mutants deficient in phytochrome A. Plant Physiol. **102,** 269-277.

Nagy, F., and Schafer, E. (2000). Nuclear and cytosolic events of light-induced, phytochrome-regulated signaling in higher plants. Embo J **19,** 157-163.

Nagy, F., and Schafer, E. (2002). Phytochromes control photomorphogenesis by differentially regulated, interacting signaling pathways in higher plants. Annu Rev Plant Biol **53,** 329-355.

Nagy, F., Kircher, S., and Schafer, E. (2000). Nucleo-cytoplasmic partitioning of the plant photoreceptors phytochromes. Semin Cell Dev Biol **11,** 505-510.

Neff, M.M., Fankhauser, C., and Chory, J. (2000). Light: an indicator of time and place. Genes Dev **14,** 257-271.

Neuhaus, G., and Spangenberg, G. (1990). Plant transformation by microinjection techniques. Physiol. Plant **79,** 213-217.

Neuhaus, G., Spangenberg, G., Scheid, O.M., and Schweiger, H.-G. (1987). Transgenic rapeseed plants obtained by the microinjection of DNA into microspore-derived embryos. Theor. Appl. Genet. **75,** 30-36.

Neuhaus, G., Bowler, C., Kern, R., and Chua, N.-H. (1993). Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. Cell **73,** 937-952.

Ni, M., Tepperman, J.M., and 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,** 657-667.

Nomura, K., and Komamine, A. (1986). Embryogenesis from microinjected single cells in a carrot cell suspension. Plant Science **44,** 53-58.

Nozue, K., Fukuda, S., Kanegae, T., and Wada, M. (1998a). Isolation of a second phytochrome cDNA from *Adiantum capillus-veneris*. Plant Physiol **118,** 711.

Nozue, K., Kanegae, T., Imaizumi, T., Fukuda, S., Okamoto, H., Yeh, K.C., Lagarias, J.C., and Wada, M. (1998b). A phytochrome from the fern *Adiantum* with features of the putative photoreceptor NPH1. Proc Natl Acad Sci USA **95,** 15826-15830.

Okamoto, H., Hirano, Y., Abe, H., Tomizawa, K., Furuya, M., and Wada, M. (1993). The deduced amino-acid-sequence of Phytochrome from Adiantum included consensus motifs present in phytochrome-B from seed plants Plant Cell Physiol **34,** 1329-1334.

Oyama, H., Yamamoto, K.T., and Wada, M. (1990). Phytochrome in the fern, *Adiantum capillus-veneris* L.: spectrophotometric detection in vivo and partial purification. Plant Cell Physiol **31,** 1229-1238.

Parks, B.M., and Quail, P.H. (1993). Hy8, a new class of Arabidopsis long hypocotyl mutants deficient in functional phytochrome A. Plant Cell **5,** 39-48.

Pietrykowska, J. (1962). Investigations on the germination of spores of the fern *Matteuccia struthiopteris* L. Tod. ActaSoc. Bot. Polon. **31,** 437-447.

Pietrzak, M., Schilito, R.D., Hohn, T., and Potrykus, I. (1986). Expression in plants of two antibiotica resistance genes after protoplast transformation with a new plant expression vector. Nucleic Acids Res **14,** 5857-5868.

Pratt, L.H. (1994). Distribution and localization of phytochrome within the plant. In Photomorphogenesis in plants, 2nd ed., R.E. Kendrick and G.H.M. Kronenberg, eds (Dordrecht: Kluwer), pp. 163-185.

Pratt, L.H., Inoue, Y., and Furuya, M. (1984). Photoactivity of transient intermediates in the pathway from the red-absorbing to the far-red-absorbing form of *Avena* phytochrome as observed by a double-flash transient-spectrum analyzer. Photochem Photobiol **39,** 241-246.

Pratt, L.H., Stewart, S.J., Shimazaki, Y., Wang, Y.-C., and Cordonnier, M.-M. (1991). Monoclonal antibodies directed to phytochrome from green leaves of Avena sativa L. cross-react weakly or not at all with the phytochrome that is most abundant in etiolated shoots of the same species. Planta **184,** 87-95.

Quail, P., Schafer, E., and Marme, D. (1973a). Turnover of phytochrome in pumpkin cotyledons. Plant Physiol **52,** 128-131.

Quail, P.H. (2002a). Photosensory perception and signalling in plant cells: new paradigms? Curr Opin Cell Biol **14,** 180-188.

Quail, P.H. (2002b). Phytochrome photosensory signalling networks. Nat Rev Mol Cell Biol **3,** 85-93.

Quail, P.H., Marme, D., and Schafer, E. (1973b). Particle-bound phytochrome from maize and pumpkin. Nat New Biol **245,** 189-191.

Quail, P.H., Boylan, M.T., Parks, B.M., Short, T.W., Xu, Y., and Wagner, D. (1995). Phytochromes: photosensory perception and signal transduction. Science **268,** 675-680.

Racusen, R. (2002). Early development in fern gametophyte:interpreting the transition to prothallial architecture in terms of coordinated photosynthate production and osmotic ion uptake. Annals of Botany **89,** 227-240.

Racusen, R.H., and Cooke, T.J. (1982). Electrical changes in the apical cell of the fern gametophyte during irradiation with photomorphogenetically active light. Plant Physiol **70,** 331-334.

Raghavan, V. (1973). Blue light interference in the phytochrome-controlled germination of the spores of *Cheilanthes farinosa*. Plant Physiol **51,** 306-311.

Raghavan, V. (1989). Developmental Biology of fern gametophytes. (Cambridge.).

Raghavan, V. (1992). Germination of fern spores. Am Sci **80,** 176-185.

Reed, J.W. (1999). Phytochromes are Pr-ipatetic kinases. Curr Opin Plant Biol **2,** 393-397.

Reed, J.W., Nagpal, P., Poole, D.S., Furuya, M., and Chory, J. (1993). Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development. The Plant Cell **5,** 147-157.

Reed, J.W., Nagatani, A., Elich, T.D., Fagan, M., and Chory, J. (1994). Phytochrome A and phytochrome B have overlapping but distinct functions in *Arabidopsis* development. Plant Physiol. **104,** 1139-1149.

Robson, P.R.H., Whitelam, G.C., and Smith, H. (1993). Selected components of the shade-avoidance syndrome are displayed in a normal manner in mutants of *Arabidopsis thaliana* and *Brassica rapa* deficient in phytochrome B. Plant Physiol. **102,** 1179-1184.

Rutherfo, G., Tanurdzic, M., Hasebe, M., and Banks, J.A. (2004). A systemic gene silencing method suitable for high throughput, reverse genetic analyses of gene function in fern gametophytes. BMC Plant Biol. **16; 4,** 6-16.

Sakai, T., Kagawa, T., Kasahara, M., Swartz, T.E., Christie, J.M., Briggs, W.R., Wada, M., and Okada, K. (2001). Arabidopsis nphl and npL1: blue light receptors that mediate both phototropism and chloroplast relocation. Proc Natl Acad Sci USA **98,** 6969-6974.

Sakamoto, K., and Nagatani, A. (1996). Nuclear localization activity of phytochrome B. Plant J **10,** 859-868.

Sakamoto, K., and Briggs, W.R. (2002). Cellular and subcellular localization of phototropin 1. Plant Cell **14,** 1723-1735.

Salomon, M., Christie, J.M., Knieb, E., Lempert, U., and Briggs, W.R. (2000). Photochemical and mutational analysis of the FMN-binding domains of the plant blue light receptor, phototropin. Biochemistry **39,** 9401-9410.

Sambrook, J., Frisch, E.F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual. (Cold Spring Harbor, NY: Cold Spring Harbor Publications).

Schafer, E., Lassig, T.U., and Schopfer, P. (1975). Photocontrol of phytochrome destruction in grass seedlings. The influence of wavelength and irradiance. Photochem Photobiol **22,** 193-202.

Schäfer, E., and Haupt, W. (1983). Blue-light effects in phytochrome-mediated responses. In Photomorphogenesis, W. Shropshire Jr. and H. Mohr, eds (Berlin: Springer-Verlag), pp. 723-744.

Schäfer, E., and Bowler, C. (2002). Phytochrome-mediated photoperception and signal transduction in higher plants. EMBO Rep **3,** 1042-1048.

Scheuerlein, R., and Koller, D. (1988). Intermediates in the photoconversion of functional phytochrome in fern spores of *Dryopteris*: I. Demonstration and quantitative characterization of the photochromic system Pr----I 700 using nanosecond-laser pulses. Photochem Photobiol **48,** 511-518.

Scheuerlein, R., Wayne, R., and Roux, S.J. (1989). Calcium requirement of phytochrome-mediated fern-spore germination: no direct phytochrome-calcium interaction in the phytochrome-initiated transduction chain. Planta **178,** 25-30.

Schneider-Poetsch, H.A., and Braun, B. (1991). Proposal on the nature of phytochrome action based on the C-terminal sequences of phytochrome. J. Plant Physiol **137,** 576-580.

Schneider-Poetsch, H.A., Marx, S., Kolukisaoglu, H.U., Hanelt, S., and Braun, B. (1994). Phytochrome evolution: Phytochrome genes in fern and mosses. Physiol Plant **91,** 241-250.

Schnorf, M., Potrykus, I., and Neuhaus, G. (1994). Routine system for characterization of microcapillaries by bubble pressure measurement. Exp Cell Res **210,** 260-267.

Schoefs, B. (1999). The light-dependent and light-independent reduction of protochlorophyllide a to chlorophyllide a. Photosynthetica **36,** 481-496.

Schraudolf, H. (1967). Die Steuerung der Antheridienbildung in *Polypodium crassifolium* L. (*Pessopteris crassifolia* Underw. and Maxon) durch Licht. Planta **76,** 37-46.

Schraudolf, H. (1987a). The effect of gabaculin on germination and gametophyte morphogenesis of *Anemia phyllitidis* L. Sw. Plant Cell Physiol **28,** 53-60.

Schraudolf, H. (1987b). Parental predetermination of dark germination in spores of *Anemia phyllitidis*. Naturwissenschaften **74,** 138.

Senger, H. (1987). Blue Light Responses: Phenomena and Occurrence in Plants and Microorganisms. (Boca Raton, FL: CRC Press).

Serlin, B.S., and Roux, S.J. (1984). Modulation of chloroplast movement in the green alga *Mougeotia* by the Ca2+ ionophore A23187 and by calmodulin antagonists. Proc Natl Acad Sci USA **81,** 6368-6372.

Sharrock, R.A., and Quail, P.H. (1989). Novel phytochrome sequences in *Arabidopsis thaliana*: structure, evolution, and differential expression of a plant regulatory photoreceptor family. Genes Dev **3,** 1745-1757.

Sheen, J., Hwang, S., Niwa, Y., Kobayaski, H., and Galbraith, D.W. (1995). Green-fluorescent protein as a new vital marker in plant cells. Plant J **8,** 777-784.

Shinomura, T., Uchida, K., and Furuya, M. (2000). Elementary processes of photoperception by phytochrome A for high-irradiance response of hypocotyl elongation in Arabidopsis. Plant Physiol **122,** 147-156.

Shinomura, T., Nagatani, A., Hanzawa, H., Kubota, M., Watanabe, M., and Furuya, M. (1996). Action spectra for phytochrome A- and B-specific photoinduction of seed germination in Arabidopsis thaliana. Proc Natl Acad Sci USA **93,** 8129-8133.

Shinomura, Y., Nagatani, A., Chory, J., and Furuya, M. (1994). The induction of seed germination in *Arabidopsis thaliana* is regulated principally by phytochrome B and secondarily by phytochrome A. Plant Physiol. **104,** 363-371.

Short, T.W., Mohamed, A., and Bissoondial, T.Y. (2003). RNAi and light alter growth and development in the fern *Ceratopteris richardii.* In International Plant Photobiology Meeting (Marburg: Philipps-Universität), pp. 68.

Shropshire, W.J., and Mohr, H. (1983). Photomorphogenesis: Encyclopedia of Plant Physiology. (Berlin: Springer).

Small, D.G., Min, B., and Lefebvre, P.A. (1995). Characterization of a Chlamydomonas reinhardtii gene encoding a protein of the DNA photolyase/blue light photoreceptor family. Plant Mol Biol **28,** 443-454.

Small, I., Wintz, H., kinya, A., Akashi, H., and Mireau, H. (1998). Two birds with one stone: genes that encode products targeted to two or more compartments. Plant Mol Biol **38,** 265-277.

Smith, H. (1982). Light quality, photoperception and plant strategy. Ann. Rev. Plant physiol. **33,** 481-518.

Smith, H. (1994). Sensing the light environment: the functions of the phytochrome family. In Photomorphogenesis in plants, 2nd ed., R.E. Kendrick and G.H.M. Kronenberg, eds (Dordrecht: Kluwer), pp. 377-416.

Smith, H. (2000). Phytochromes and light signal perception by plants--an emerging synthesis. Nature **407,** 585-591.

Smith, H., and Whitelam, G.C. (1990). Phytochrome, a family of photoreceptors with multiple physiological roles. Plant Cell Env. **13,** 695-707.

Smith, H., and Whitelam, G.C. (1997). The shade avoidance syndrome: multiple responses mediated by multiple phytochromes. Plant Cell Environ. **20,** 840-844.

Smith, H., Jackson, G.M., and Whitelam, G.C. (1988). photoprotection of phytochrome. Planta **175,** 471-477.

Speth, V., Otto, V., and Schafer, E. (1987). Intracellular localization of phytochrome and ubiquitin in red-light irradiated oat coleoptiles by electron microscopy. Planta **171,** 332-338.

Steiner, A.M. (1967). Action spectra for polarotropism in germlings of a fern and a liverwort. Naturwissenschaften **18,** 497-498.

Sugai, M., and Furuya, M. (1967). Photomorphogenesis in *Pteris vittata* I. Phytochrome-mediated spore germination and blue light interaction. Plant Cell Physiol. **8,** 737-748.

Sullivan, J.A., and Deng, X.W. (2003). From seed to seed: the role of photoreceptors in Arabidopsis development. Developmental Biology **260,** 289-297.

Sundqvist, C., and Bjorn, L.O. (1983). Light-induced linear dichroism in photoreversibly photochromic sensor pigments. II. Chromophore rotation in immobilized phytochrome. Photochem Photobiol **37,** 69-75.

Tavares, J.E., and Sussex, I.M. (1968). Expansion growth of isolated leaf blades of *Todea barbara.* Planta **80,** 113-128.

Thummler, F., Beetz, A., and Rudiger, W. (1990). Phytochrome in lower plants. Detection and partial sequence of a phytochrome gene in the moss *Ceratodon purpureus* using the polymerase chain reaction. Febs Lett **275,** 125-129.

Thummler, F., Dufner, M., Kreisl, P., and Dittrich, P. (1992). Molecular cloning of a novel phytochrome gene of the moss *Ceratodon purpureus* which encodes a putative light-regulated protein kinase. Plant Mol Biol **20,** 1003-1017.

Tobin, E.M., and Silverthorne, J. (1985). Light regulation of gene expression in higher plants. Annu. Rev. Plant Physiol. **36,** 569-593.

Tobin, E.M., and Kehoe, D.M. (1994). Phytochrome regulated gene expression. Semin Cell Biol **5,** 335-346.

Tomizawa, K., Manabe, K., and Sugai, M. (1982). Changes in phytochrome content during imbibition in spores of the fern *Lygodium japonicum.* Plant Cell Physiol **23,** 1305-1308.

Tomizawa, K., Sugai, M., and Manabe, K. (1983). Relationship between germination and Pfr level in spores of the fern *Lygodium japonicum.* Plant Cell Physiol **24,** 1043-1048.

Tzfira, T., and Citovsky, V. (2000). From host recognition to T-DNA integration: the function of bacterial and plant genes in the *Agrobacterium*-plant cell interaction. Molec Plant Pathol **1,** 201-212.

Tzfira, T., and Citovsky, V. (2001a). Comparison between nuclear localization of nopaline- and octopine-specific *Agrobacterium* VirE2 proteins in plant, yeast and mammalian cells. Molec Plant Pathol **2,** 171-176.

Tzfira, T., and Citovsky, V. (2002). Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. Trends Cell Biol **12,** 121-129.

Tzfira, T., Vaidya, M., and Citovsky, V. (2001b). VIP1, an Arabidopsis protein that interacts with Agrobacterium VirE2, is involved in VirE2 nuclear import and Agrobacterium infectivity. Embo J **20,** 3596-3607.

Tzfira, T., Vaidya, M., and Citovsky, V. (2002). Increasing plant susceptibility to *Agrobacterium* infection by overexpression of the *Arabidopsis* nuclear protein VIP1. Proc Natl Acad Sci USA **99,** 10435-10440.

Uchida, K., Muramatsu, T., Jamet, E., and Furuya, M. (1998). Control of expression of a gene encoding an extensin by phytochrome and a blue light receptor in spores of *Adiantum capillus-veneris* L. Plant J **15,** 813-819.

VanDerWoude, W.J. (1987). Application of dimeric model of phytochrome action to high irradiance responses. In phytochrome and Photoregulation in plants, M. Furuya, ed ((ed.)academic press, Tokyo), pp. 249-258.

Vierstra, R.D. (1994). Phytochrome degradation. In Photomorphogenesis in plants, 2nd ed., R.E. Kendrick and G.H.M. Kronenberg, eds (Dordrecht: Kluwer), pp. 141-162.

Vierstra, R.D., and Quail, P. (1983). Purification and initial characterization of 124-kilodalton phytochrome from *Avena*. Biochemistry **22,** 2498-2505.

von Arnim, A., and Deng, X.-W. (1996). Light control of seedling development. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47,** 215-243.

von Arnim, A.G., Deng, X.W., and Stacey, M.G. (1998). Cloning vectors for the expression of green fluorescent protein fusion proteins in transgenic plants. Gene **221,** 35-43.

Wada, M., and Furuya, M. (1971). Photocontrol of the orientation of cell division in *Adiantum*. II. Effects of the direction of white light on the apical cell of gametophytes. Planta **98,** 177-185.

Wada, M., and Furuya, M. (1972). Phytochrome action on the timing of cell division in

Adiantum gametophytes. Plant Physiol **49,** 110-113.

Wada, M., and Furuya, M. (1978). Effects of narrow-beam irradiations with blue and far-red light on the timing of cell division in *Adiantum* gametophyte. Planta **138,** 85-90.

Wada, M., and Murata, T. (1988). photocontrol of the orientation of cell division in *Adiantum.* IV. light-induced cell flattening preceding two-dimensional growth. Bot. Mag. tokyo. **101,** 111-120.

Wada, M., and Kadota, A. (1989). Photomorphogenesis in lower green plants. Ann Rev Plant Biol **40,** 169-191.

Wada, M., and Sugai, M. (1994). Photobiology of ferns. In Photomorphogenesis in plants, 2nd ed., R.E. Kendrick and G.H.M. Kronenberg, eds (Dordrecht: Kluwer), pp. 783-802.

Wada, M., Kadota, A., and Furuya, M. (1978). Apical growth of protonemata in *Adiantum capillus-veneris.* II. Action spectra for the induction of apical swelling and the intracellular photoreceptive site. Bot. Mag. tokyo. **91,** 113-120.

Wada, M., Kadota, A., and Furuya, M. (1981a). Intracellular photoreceptive site for polarotropism in protonema of the fern *Adiantum capillus-veneris* L. Plant Cell Physiol **22,** 1481-1488.

Wada, M., Kadota, A., and Furuya, M. (1981b). Apical growth of protonemata in *Adiantum capillus-veneris.* II. Action spectra for the induction of apical swelling and the intracellular photoreceptive site. Bot. Mag. tokyo. **91,** 113-120.

Wada, M., Kadota, A., and Furuya, M. (1983). Intracellular localization and dichroic orientation of phytochrome in plasma membrane and/or ectoplasm of a centrifuged protonema of fern *Adiantum capillus-veneris* L. Plant Cell Physiol **24,** 1441-1447.

Wada, M., Hayami, J., and Kadota, A. (1984). Returning dark-induced cell cycle to the beginning of G1 phase by red light irradiation in fern *Adiantum* protonemata. Plant Cell Physiol **25,** 1053-1058.

Wada, M., Grolig, F., and Haupt, W. (1993). Light-oriented chloroplast positioning:contribution to progress in photobiology. J. Photochem. Photobiol. B. Biol. **17,** 3-25.

Wagner, D., Fairchild, C.D., Kuhn, R.M., and Quail, P.H. (1996). Chromophore-bearing NH2-terminal domains of phytochromes A and B determine their photosensory specificity and differential light lability. Proc. Natl. Acad. Sci. USA **93,** 4011-4015.

Wagner, G., Valentin, P., Dieter, P., and Marme, D. (1984). Identification of calmodulin in the green alga *Mougeotia* and its possible function in chloroplast reorientational movement. Planta **162,** 62-67.

Wayne, R., and Hepler, P.K. (1985). Red light stimulates an increase in intracellular calcium in the spores of *Onoclea sensibilis*. Plant Physiol **77,** 8-11.

Whitelam, G.C., and Smith, H. (1991). Retention of phytochrome-mediated shade avoidance responses in phytochrome-deficient mutants of *Arabidopsis*, cucumber and tomato. J. Plant Physiol. **139,** 119-125.

Whitelam, G.C., and Harberd, N.P. (1994). Action and function of phytochrome family members revealed through the study of mutant and transgenic plants. Plant Cell Environ. **17,** 615-625.

Whitelam, G.C., and Devlin, P.F. (1997). Roles of different phytochromes in Arabidopsis photomorphogenesis. Plant Cell Environ **20,** 752-758.

Whitelam, G.C., Johnson, E., Peng, J., Carol, P., Anderson, M.L., Cowl, J.S., and Harberd, N.P. (1993). Phytochrome A null mutants of Arabidopsis display a wild-type phenotype in white light. Plant Cell **5,** 757-768.

Winands, A., and Wagner, G. (1996). Phytochrome of the green alga Mougeotia: cDNA sequence, autoregulation and phylogenetic position. Plant Mol Biol **32,** 589-597.

Yamaguchi, R., Nakamura, M., Mochizuki, N., Kay, S.A., and Nagatani, A. (1999). Light-dependent translocation of a phytochrome B-GFP fusion protein to the nucleus in transgenic *Arabidopsis*. J. Cell Biol. **145,** 437-445.

Yatsuhashi, H., Kadota, A., and Wada, M. (1985). Blue- and red-light action in photoorientation of chloroplasts in *Adiantum* protonemata. Planta **165,** 43-50.

Yatsuhashi, H., Hashimoto, T., and Wada, M. (1987). Dichroic orientation of photoreceptors for chloroplast movement in *Adiantum* protonemata. Non-helical orientation. Plant Science **51,** 165-170.

Yeh, K.C., and Lagarias, J.C. (1998a). Eukaryotic phytochromes: light-regulated serine/threonine protein kinases with histidine kinase ancestry. Proc Natl Acad Sci USA **95,** 13976-13981.

Yeh, K.C., Wu, S.H., Murphy, J.T., and Lagarias, J.C. (1997). A cyanobacterial phytochrome two-component light sensory system. Science **277,** 1505-1508.

Yeh, K.-C., and Lagarias, J.C. (1998b). Eukaryotic phytochromes: light-regulated serine/threonine protein kinases with histidine kinase ancestry. Proc. Natl. Acad. Sci. USA **95,** 13976-13981.

Zhang, W., McElroy, D., and Wu, R. (1991). Analysis of rice Act 1 5' region activity in transgenic rice plants. Plant Cell **3,** 1155-1165.

Zupan, J.R., and Zambryski, P. (1997). The Agrobacterium DNA transfer complex. Crit Rev Plant Sci **16,** 279-295.

Zupan, J.R., Citovsky, V., and Zambryski, P. (1996). *Agrobacterium* VirE2 protein mediates nuclear uptake of single-stranded DNA in plant cells. Proc Natl Acad Sci USA **93,** 2392-2397.