ISOLATING AND CHARACTERIZING COMPONENTS IN PHYTOCHROME SIGNALING NETWORKS

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Abstract

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By

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Light is life for plants. At least three classes of photoreceptors, red/far-red light sensing phytochromes, blue/UV-A light absorbing cryptochromes and phototropins, and at least one UV-B light receptor, have been found to regulate plant growth and development.

By screening for the most severe phenotypes that severely affect light signaling, various alleles of different photoreceptor mutations and their downstream components have been isolated. There mutations completely block one or more branches of light signaling responses. Using a novel screen of an EMS mutagenized pool for less severe phenotypes under white light, three mutants, designated *de*ficient *p*hotomorphogenesis - *dep1*, *dep2* and *dep3* have been isolated.

Upon characterizing the mutants, it was demonstrated that *dep1* and *dep2* display intermediate phenotypes of several light-regulated responses involving red, far-red, and even blue light, whereas *dep3* displayed much stronger deficiencies than either *dep1* or *dep2* for all phenotypes investigated, especially in

FR-regulated responses, and approaching those of *phyA* null mutants. All *dep* mutants express wild type levels of PHYA apoprotein, and normal phyA degradation kinetics once photoconverted to the active form, indicating that *dep* mutants do not affect chromophore synthesis, its attachment to phytochromes, or the photoconvertability of phyA. DEP1 and DEP2 may represent two components downstream of shared light signaling pathways, and DEP3 is strongly affected in phyA signaling but also participates in other parts of the phytochrome transduction network.

Using SSLP, CAPs, AFLP, and SNP mapping techniques, *DEP1* and *DEP3* have been mapped to two different sites on the top of chromosome 1, and *DEP2* maps to the bottom of chromosome 4.

dep3 has been cloned and found to encode a novel mutant allele of *phyA*, designated *phyA-401*, with two missense changes in conserved amino acids within the chromophore binding pocket region. Spectrophotometry and Western Blotting data suggest that *phyA-401* produces defective phyA that is not photoreversible because it is impaired in light-induced conversion of Pfr (light unstable form) to Pr (stable), and leading to the rapid degradation of phyA upon its conversion to Pfr.

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D. Phytochrome Signaling Networks

Plants are literally rooted in place and do not enjoy the luxury of being able to change their environment by changing their location as animal do. Plants cannot move about, seeking shelter from adverse environmental conditions as animals do. Yet, plants must avoid adverse conditions if the species is to survive.

Throughout their life cycles, plants continuously respond to environmental stimuli by altering their morphology, physiology and development. Among these stimuli, light is one of most significant features in the natural environment. Light, acting through a series of specific morphogenic photoreceptors, regulates nearly every stage of whole plant growth and development. Plants grown in darkness are pale in color, have elongated hypocotyls, unopened apical hooks, and folded and unexpanded cotyledons, and whole seedling grows with a scotomorphogenic phenotype. But even a single flash of light can initiate the developmental transition from the etiolated dark-grown state to a de-etiolated light-grown state, a photomorphogenic phenotype. The intensity, wavelengths, duration, and direction of the light will be reflected in the degree of de-etiolation, and ultimately in the morphology and physiology of the plant development (Kendrick and Kronenberg, 1994).

A. Photoreceptors in Light Signaling

To monitor the light environment, plants have evolved a series of photoreceptors, including Red/Far-red light (600-750nm)-perceiving phytochromes (phy), Blue/UV-A (320-500nm) photoreceptors including cryptochromes (cry) and phototropins (phot), and at least one uncharacterized UV-B (282-320) photoreceptor (Kendrick and Kronenberg, 1994; Briggs and Huala, 1999; Briggs and Christie, 2002; Quail, 2002a).

Roles of individual photoreceptors in light perception and in regulation of various physiological responses have been addressed by studying null mutations of one or more individual receptors. Mutations in numerous presumed downstream signal transduction components have then been isolated and characterized based on the plants' physiological responsiveness to light. These genetic approaches to elucidating photomorphogenic pathways have been extremely successful, particularly for isolating phytochrome-regulated elements (Quail, 1991; Short and Briggs, 1994; Smith, 1995; Fankhauser and Chory, 1997; Quail, 2002a). By analyzing phytochrome-deficient mutants, physiological responses from seed germination, seedling de-etiolation, vegetative growth and flowering processes have been examined and assigned to specific family members and their downstream constituents (Fankhauser and Chory, 1997).

A great deal of effort has been applied to investigating the properties of phytochromes and the physiological responses controlled by them. In *Arabidopsis,* phytochrome proteins are encoded by a small gene family designated phyA, phyB, phyC, phyD and phyE that share between 50 and 80% identity (Sharrock and Quail, 1989; Quail

et al., 1995). They are divided into two classes based on their light lability: Type I phytochromes (phyA in *Arabidopsis*) are abundant in etiolated seedlings, but upon exposure to continuous red (Rc) or white light (Wc), their level drops 50- to 100-fold because of the rapid degradation of the holoprotein and repression of phy gene transcription. Type II phytochromes (phyB to phyE in *Arabidopsis*) are the most abundant phytochromes in green plants because of their light stability (Quail et al., 1995).

Phytochromes are soluble homodimers of two 120-kD polypeptides, each with a linear tetrapyrrole chromophore covalently attached to the N-terminal half of the protein. They exist in two photo-interconvertable forms: the Pr (red-light absorbing form), and the Pfr (far-red-light-absorbing form). Phytochrome is originally synthesized in its Pr form, which has a major absorption peak at 660nm. Upon illumination with red (R) light, the Pr form is converted to its Pfr form with a major absorption peak at 730nm. Irradiation of the Pfr form with far-red (FR) converts the phytochrome back to the Pr form. The conversion between Pr and Pfr forms is also associated with conformational changes of the chromophore and the protein as well as the peak absorbance change. But both Pr and Pfr forms also absorb to a greater or lesser degree over the entire visible spectrum (Kendrick and Kronenberg, 1994; Andel et al., 1996; Quail, 1997). The Pfr form is generally considered to be the biologically active form. But there is some evidence that suggests that Pr is not always biological inactive, and that the switch from Pfr to Pr (rather than Pr or Pfr themselves) can act as a short-lived signal induced during photoconversion from Pfr to Pr (Reed, 1999; Shinomura et al., 2000), or that Pr that has been cycled through its Pfr form behaves differently from the naïve Pr (Shinomura et al., 2000; Schafer and Bowle, 2002).

Exhaustive studies from numerous laboratories have shown that both the very low fluence responses (VLFR) and the far-red-light-mediated high irradiance responses (FR-HIR) act mostly through phyA excitation, whereas most of the R /FR photoreversible or low fluence responses (LFR) and the red-light-mediated high irradiance responses (R-HIR) act largely through phyB signaling, and to a lesser degree through phyC, phyD and phyE signaling (Whitelam and Harberd, 1994; Whitelam and Devlin, 1997; Franklin et al., 2003).

Another group of photoreceptors that plays an important role in plant photomorphogenesis is the cryptochromes (cry). They are flavoproteins that share amino acid similarity with DNA photolyases that catalyze blue/UV-A light-dependent DNA repair (Cashmore et al., 1999). Cryptochromes have no DNA photolyase activity; they show characteristics of blue/UV-A light receptors in plant (Sancar et al., 2000). *Arabidopsis* has at least two cryptochrome genes, *CRY1* and *CRY2*, and they show strong similarity to each other, but most of the sequence similarity is concentrated in the N-terminal photolyase-like domain (Lin et al., 1998). Interestingly, CRY2 protein is rapidly degraded in etiolated seedlings exposed to blue light (Lin et al., 1998; Guo et al., 1999), which is reminiscent of the R-light-induced degradation of phyA (Clough et al., 1999). It is not clear what functional roles the light-induced proteolysis of phyA and cry2 may play, but no diurnal change in the protein expression levels has been reported for cry2.

Genetic studies of *Arabidopsis* cryptochrome mutants, *hy4,* later referred to as *cry1*, show that they have elongated hypocotyls, and impaired blue-light-responsive anthocyanin accumulation (Ahmad and Cashmore, 1993; Ahmad et al., 1995); *cry2* mutants showed a more apparent abnormality in flowering time than in hypocotyl

elongation (Guo et al., 1998). Although cryptochromes play roles in several light-regulated responses, CRY1-mediated inhibition of hypocotyl elongation and accumulation of anthocyanin require active phytochrome for full expression (Ahmad and Cashmore, 1997).

Phototropins (phot) are another group of blue light receptors. In *Arabidopsis*, there are at least two of them, designated phot1 and phot2. Both *PHOT1* and *PHOT2* genes encode plasma membrane-associated proteins with two N-terminal LOV domains, each of which binds a flavin mononucleotide (FMN) chromophore. Like phytochromes, PHOT1 and PHOT2 also show light activated conformation changes and subsequent dark recovery (Salomon et al., 2000; Kasahara et al., 2002). *Arabidopsis PHOT2* encodes a slightly smaller protein than *PHOT1*, with 58% sequence identity and 67% sequence similarity to *PHOT1* (Jarillo, 1998). Both phot1 and phot2 have serine/threonine kinase activity in the C-terminal half with light-activated autophosphorylation, and light activated formation of a flavin C (4a)-cysterinyl adduct and its subsequent dark decay (Miller et al., 1990; Briggs et al., 2001; Crosson and Moffat, 2001; Salomon et al., 2001; Swartz et al., 2001; Christie et al., 2002). phot1 mediates phototropism in response to light pulses or low levels of continuous light, whereas phot2 responds only at high fluence rates of continuous light. phot1 also mediates light-activated chloroplast movement and accumulation at the cell surface over a wide fluence range, whereas phot2 can mediate accumulation at a low fluence rates and avoidance at higher fluence rates (Kanegae et al., 2000; Jarillo et al., 2001; Kagawa et al., 2001; Sakai et al., 2001; Kasahara et al., 2002).

B. Physiological Responses Controlled by Photoreceptors

Light affects every stage of plant development, beginning from seed germination, then directing the seedling de-etiolation process. Once a young seedling is established, light continuously regulates its vegetative architectural adaptations and influences the transition from vegetative to reproductive development, which in turn allows the seeds to start the next generation (Fankhauser and Chory, 1997). The involvement of photoreceptors on many developmental and physiological processes is summarized in the review paper (Sullivan and Deng, 2003).

Much of our understanding of the role of photoreceptors in plant development has come from research on the model plant species *Arabidopsis thaliana*. Analysis of mutants with altered functionality of one or more of the photoreceptors allow researchers to identify which photoreceptors control a particular aspect of light-regulated development. Of course, for the light-regulated responses described below, most of them are not solely controlled by light. They are also affected by other internal and environmental stimuli, such as phytohormones, temperature, stress etc. Below is a brief summary of the involvement of photoreceptors in a number of individual developmental processes.

I. Seed Germination

Light-dependent seed germination of *Arabidopsis* is mediated entirely by phytochrome (Casal, 1998). Of the five *Arabidopsis* phytochromes, phyA and phyB play a well-established role in photo-regulated seed germination (Shinomura et al., 1996).

phyB is present in dry seeds, whereas phyA is synthesized beginning several hours after imbibition (Sharrock and Clack, 2002). Once activated by R, phyB mediates largely the classical, photo-reversible LFR to induce seed germination. On the other hand, induction of germination by FR light is mediated by phyA via the VLFR or HIR systems (Shinomura et al., 1994; Casal, 1998). But the observation that *phyA/phyB* double mutants still show some R/FR reversible germination suggests the involvement of other phytochrome(s) (Poppe and Schafer, 1997). Recently, by using mutants in *phyC*, *phyD* and *phyE*, it has been demonstrated that phyE plays a role in a FR reversible manner to induce seed germination (Shinomura et al., 1994; Hennig et al., 2002).

II. Young Seedling Development

After seed germination, *Arabidopsis* seedlings follow one of two developmental patterns: in the dark, seedlings follow skotomorphogenic (or etiolated) development, with long hypocotyls, closed apical hooks, folded and unexpanded cotyledons, and development of proplastids into etioplasts; by contrast, in the light, seedlings undergo photomorphogenic development characterized by short hypocotyls, opened apical hooks, expanded cotyledons, and development of proplastids into mature green chloroplasts (Kendrick and Kronenberg, 1994; Wang and Deng, 2003). The de-etiolation process involves sophisticated signaling cascades that are regulated by both phytochromes and cryptochromes (Franklin KA, 2003; Sullivan and Deng, 2003; Franklin et al., 2003a).

Although mutations in *phyA* do not cause obvious phenotypes under white light, phyA plays a dominant role in mediating hypocotyl growth inhibition and seedling

morphogenesis under continuous FR (FRc) and FR-enriched environments (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). By contrast, phyB functions in inhibition of hypocotyl elongation under continuous R (Rc), a response enhanced by synergistic coactions with phyA (Koornneef et al., 1980; Reed and Chory, 1994). In addition, phyA and phyC appear to act redundantly with phyB in regulating hypocotyl elongation in Rc, and to function together to regulate rosette leaf morphology (Franklin et al., 2003a). Mutations in *PHYB*, *PHYD*, and *PHYE* genes and combinations of these genes have shown that there is some overlap and even partial redundancy in their activities to control leaf morphology and flowering time in response the R/FR, and shade-avoidance, particularly between phyB and phyD because of their high sequence homology (Aukerman et al., 1997; Smith, 2000; Sharrock et al., 2003, 2003). Both cry1 and cry2 function in perception of blue light to regulate the de-etiolated process (Ahmad and Cashmore, 1993; Lin et al., 1998; Quail, 2002b). Many downstream components of photoreceptors are involved in the development of seedling de-etiolation. Most of these downstream components identified to date are indicated in a simplified model of the genetic interactions in Diagram 1 (Sullivan and Deng, 2003).

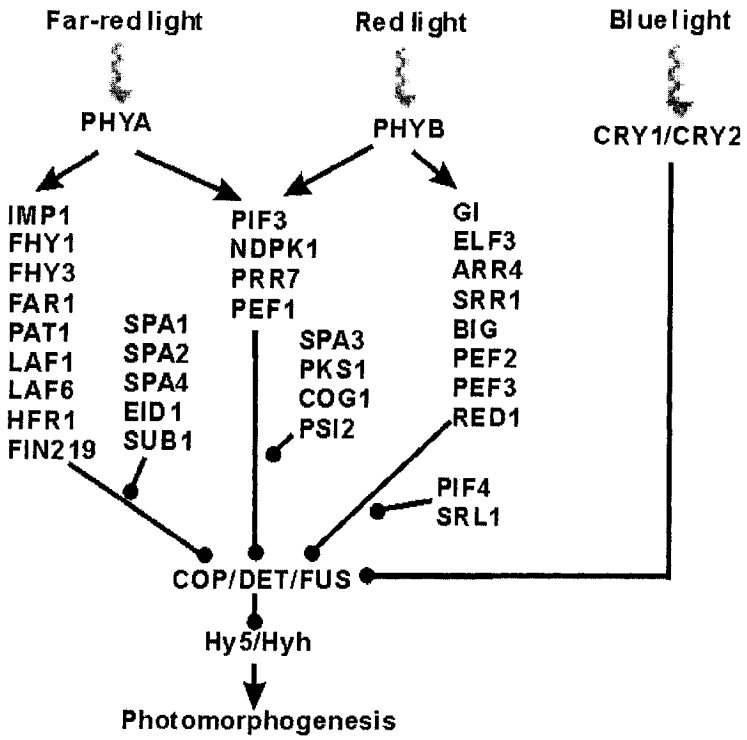


Diagram 1 Simplified model of genetic interactions regulating de-etiolation in *Arabidopsis* seedlings. phyA and phyB have both separate and shared signaling intermediates, with the COP/DET/FUS loci acting as an integration point between the phytochrome and cryptochrome signaling pathways. Arrowheads indicate positive interactions; closed circles indicate a repressive effect.

Modified from Sullivan and Deng 2003 Dev. Biol. 260(2): 289-97

III. Vegetative Growth

In the natural environment, plants constantly compete with their neighbors for various resources. If plants are grown in close proximity to each other, they will compete for light. They either adapt to the shade (shade tolerance) or attempt to grow toward light (shade avoidance). Many plant species display shade avoidance responses, the most

dramatic consequences of which are the stimulation of elongation growth of cotyledon and petiole length associated with a reduction of cotyledon and leaf expansion, increased apical dominance, and a reduction of branching. The much severe effects of the shade avoidance syndrome is the inhibition of seed germination, the acceleration of flowering, truncated fruit development, and reduced seed set (Smith and Whitelam, 1997; Morelli and Ruberti, 2000).

A fundamental function of phytochromes is the perception of changes in the light quality occurring within a plant canopy. As a plant canopy grows and fills up space, a reduction in the ratio of R:FR light occurs because FR light passes through or is reflected by vegetation, while R is absorbed by the canopy leaves. Plants have sophisticated sensing mechanisms operating through the phytochromes that perceive the R:FR ratio as an accurate indicator of neighbor proximity and leaf shading, which trigger morphological changes to avoid shade. Despite initial suggestions that the shade avoidance response is triggered by a single member of the phytochrome family, the study of *Arabidopsis* mutants deficient in one or more phytochromes has revealed that multiple phytochromes are involved (Smith, 1995).

Sensing very small changes in R:FR can also act as an early warning system for a potential shade threat for seedlings. Under laboratory conditions, *Arabidopsis* seedlings exposed to a single pulse of FR light before entering the night phase of growth will show elongated growth, a response termed the end-of-day far-red response (EOD-FR) and this can be monitored as a shade avoidance response (Bagnall et al., 1995; Casal, 1996). *Arabidopsis* mutants that lack functional phyB show constitutive shade avoidance responses with the elongated phenotype, early flowering, and increased apical dominance

under high R:FR ratio (Smith and Whitelam, 1997). However, the mutants still display some reduced response to EOD-FR. Moreover, the *phyB* mutant plants grown in the simulated vegetational shade environment flower earlier than *phyB* plants grown in normal light conditions. These observations indicate that phyB is not the sole photoreceptor involved in the regulation of the shade avoidance response. *phyD* and *phyE* monogenic mutants are essentially indistinguishable from wild-type seedlings. However, *phyB phyE* and, to a lesser extent, *phyB phyD* double mutants flower earlier and grow longer petioles than do *phyB* mutants. These results led to the proposal that, in conjunction with PHYB, both PHYD and PHYE function in the regulation of shade avoidance responses as well (Aukerman et al., 1997; Devlin et al., 1998; Devlin et al., 1999).

For a long time, it was generally accepted that phyA had little, if any, role in the shade avoidance response. But it was recently reported that *phyABDE* quadruple mutant seedlings show elongated internodes, and increased leaf length/width ratio that were clearly visible when the plants were grown under short days, but wild-type seedlings and *phyBDE* mutants lack such responses. These data indicated that phyA has an important role in inhibiting internode elongation and leaf shape in mature *Arabidopsis* (Sullivan and Deng, 2003; Franklin et al., 2003b).

IV. Flowering

Many environmental factors can initiate the transition of the apical meristem from a vegetative fate to a floral fate, such as light, phytohormones, and stress. One of the most

important factors among them is the daily duration of light - the critical photoperiod that was first discovered by Garner and Allard in the 1920s. The photoperiodic control of flowering is brought about by the interactions of genes involved in the developmental control of floral initiation, the regulation of the circadian clock, and the signal transduction of photoreceptors (Thomas B, 1997). In addition, genes involved in light perception, phytohormone metabolism, signal transduction and floral meristem specification also play roles in the regulation of flowering time.

One of the major signal transduction pathways regulating flowering time is known as the LD (flowering is accelerated in long days) promotion pathway, which relays light and photoperiodic timing signals to the floral initiation process. Mutations in genes such as *co* [*CONSTANS*; (Putterill et al., 1995)], *phyA* [*PHYTOCHROME A*; (Johnson et al., 1994)], *cry2* [*CRYPTOCHROME 2*; (Guo et al., 1998)], and *gi* [*GIGANTEA*;(Fowler S, 1999; Park DH, 1999)], are of this type. The elevated expression of the CCA1 [circadian clock associated; (Wang and Tobin, 1998)] and LHY [late elongated hypocotyl; (Schaffer et al., 1998)] genes also result in photoperiod-hyposensitive late flowering (Lin, 2002).

On the other hand, a mutant that flowers earlier than the wild type in both LD and SD (flowering is accelerated in short day) may also have reduced sensitivity to photoperiod. Early-flowering mutants in genes such as *phyB* [phytochrome B; (Goto et al., 1991)], *phyD* [phytochrome D, (Devlin et al., 1996; Aukerman et al., 1997)], *phyE* [phytochrome E,(Devlin et al., 1998)], *elf3* [early flowering, (Hicks KA, 1996; Zagotta MT, 1996)], and *pef* [phytochrome early flowering, (Ahmad and Cashmore, 1996)] belong to this group. Many genes isolated to date are associated with photoperiodic

pathways and encode either photoreceptors or proteins associated with circadian rhythms (Lin, 2002).

How an individual photoreceptor itself or in conjunction with other(s) regulates the flowering time is summarized below (Mockler et al., 2003). The inhibition of floral initiation by phyB suggests that phyB works as a repressor of flowering. That the effect of phyB on flowering is much more pronounced under SD than under LD conditions indicates a much more complicated process. The inhibitory action of phyB on flowering is also dependent on R (Guo et al., 1998). Although monogenic *phyD* has no obvious phenotypic abnormality, *phyB/phyD* plants flower earlier than the monogenic *phyB* in both LD and SD conditions, indicating that phyD inhibits flowering (Aukerman et al., 1997; Devlin et al., 1999). *phyB/phyE* double mutants flower so much earlier than the phyB monogenic mutant that an EOD-FR treatment no longer causes further acceleration of flowering in SD conditions (Devlin et al., 1998). In contrast to phyB, phyD and phyE, phyA promotes the transition from vegetative growth to flowering because *phyA* mutants flower late in LD (Johnson et al., 1994), or SD with night breaks (Reed et al., 1994), or with day extensions (Johnson et al., 1994). *phyA* mutants grown in FRc failed to flower. Moreover, *phyA* mutants flowered significantly later than the wild type in continuous R+FR light (Mockler et al., 2003). These results indicate that phyA mediates the FR promotion of flowering. But whether phyA directly promotes flowering or acts by preventing the accumulation of a floral suppressor so as to promote flowering indirectly is still unknown (Weller et al., 1997; Mockler et al., 2003). The flowering promotion function of cry2 depends on both B and R (Guo et al., 1998; Mockler et al., 1999). Therefore, cry2 may promote flowering through its suppression of the phyB-mediated R

light inhibition of floral initiation (Lin, 2002). The function of cryl in flowering seems complicated, although it may have a small promotive effect (Bagnall et al., 1996; Zagotta et al., 1996; Mockler et al., 1999).

The transition from vegetative growth to flowering is a complicate process. Based on existing data, all five R/FR light receptors and two B/UV-A light receptors are involved, and they interact redundantly or antagonistically to initiate flowering. The report that *phyB/phyD*, *phyB/phyE* and *phyB/phyD/phyE* multiple mutants show an acceleration of flowering compared with *phyB* alone indicates that phyD and phyE work redundantly with phyB in this pathway (Devlin et al., 1998; Devlin et al., 1999; Franklin KA, 2003). The monogenic *cryl*, *cry2*, or *phyA* mutants flowered at about the same time as the wild type; however, a double mutant impaired in any two of the three photoreceptors or the *cry/1cry2/phyA* triple mutant flowered significantly later than the wild type when grown in Bc light. This finding suggests that cryl, cry2, and phyA act together in mediating the direct B light promotion of flowering (Mockler et al., 2003).

C. Phytochrome Interacting Proteins

How do photoreceptors propagate the light signal to regulate various physiological responses? It has been proposed that phytochromes may transduce their signals through cytosolic messengers such as calcium, CaM, cGMP, G-protein and others (Chory, 1994; Barnes et al., 1995; Quail et al., 1995). A set of papers published recently has changed our view of phytochrome-mediated phototransduction, and more evidence supports the proposition that phytochromes function as protein kinases, which was proposed more than a decade ago based on sequence analysis of that the C-terminal end of phytochromes show sequence homology to histidine kinase (Wong et al., 1986; Elich and Chory, 1997; Cashmore, 1998; Yeh and Lagarias, 1998; Fankhauser and Chory, 1999), although current evidence suggests that phytochrome kinase activity may not correspond to this domain. Oat phyA has long been suspected of having autophosphorylation capacity (Wong et al., 1986). The *Synecocystis* genome has an open reading frame that is transcribed and translated into a photoactive prokaryotic phytochrome; this phytochrome shows homology to the classical prokaryotic histidine sensor kinase, and is capable of transducing signals via a phospho-relay (Yeh et al., 1997; Yeh and Lagarias, 1998). But the function of phytochromes has dramatically changed by acting as serine/threonine kinases, instead of as histidine kinases, during the evolution from prokaryotic to multicellular eukaryotic systems. The phytochrome kinase hypothesis has gradually gained general acceptance. It appears that the early steps of phytochrome signal transduction involves at least two types of reactions: phytochromes

phosphorylating downstream substrates (including autophosphorylation); and phytochromes interacting with signal-transducing proteins in a light-dependent manner.

I. Phytochromes as kinases and a Phytochrome-regulated kinase

To confirm that phytochromes function as kinases, one pivotal question is the identification of the substrates of phytochrome kinases. Recently, a gene encoding PKS1 [phytochrome kinase substrate 1 (Fankhauser et al., 1999)] was identified by using a yeast two-hybrid screen with the C-terminal domain of *Arabidopsis* PHYA as the "bait". PKS1 is a cytoplasmic protein with no recognizable sequence motifs or signals for subcellular targeting. It interacts with both phyA and phyB, and it appears to bind both Pfr and Pr forms of phy. *In vitro*, PKS1 can be phosphorylated on serine and threonine residues by recombinant oat phyA with Pfr as a more active form. In keeping with PKS1 being a substrate of phytochromes, PKS1 was phosphorylated *in vivo* in a red-light-dependent manner, and the observation that PKS1 was hyperphosphorylated in transgenic plants overexpressing phyB suggested an involvement of phyB in the phosphorylation of PKS1 *in vivo*. Growth under different monochromatic light conditions indicated that PKS1 over-expressing lines were less sensitive to R light and retained normal sensitivity to B and FR light. This result indicated that phytochromes may signal by serine-threonine phosphorylation, and PKS1 as a kinase substrate acts as an inhibitor in phyB signaling (Fankhauser et al., 1999).

Another contender for phytochrome kinase substrates turns out to be cryptochrome. It has been reported that cryptochromes are phosphoproteins in *Arabidopsis* and that their mammalian homologues are phosphorylated *in vivo*; cryptochromes can interact with phyA *in vitro*, and in the yeast two-hybrid assay; recombinant CRY1 could be phosphorylated *in vitro* by recombinant oat phyA protein (Eide et al., 2002; Lin and Shalitin, 2003). Also, the phosphorylation of cry1 by phyA *in vitro* was more efficient in R light or B light than in FR or darkness (Ahmad et al., 1998). The phosphorylation of cry1 was also found to occur *in vivo* in a R-light-dependent and FR-light-reversible manner, which again suggested the involvement of a phytochrome (Ahmad et al., 1998). *Arabidopsis* CRY1 and CRY2 are substrates for phosphorylation by a purified recombinant phyA associated with kinase activity. Several mutations within the CRY1 C-terminus lead to the reduction of their phosphorylation by phytochrome preparations *in vitro* (Ahmad et al., 1998). But a recent report that the cry1 phosphorylation is B-light-specific, and little cry1 phosphorylation is detected in the seedlings at the same fluences of R and FR light suggested that the phosphorylation is independent of phytochromes (Shalitin et al., 2003). Whether CRY1 phosphorylation is phyA-dependent, and whether the phytochrome-dependent phosphorylation of cry1 is physiologically relevant, remain unclear.

Phytochrome may also regulate the activities of other protein kinases. One of the candidates identified by the yeast two-hybrid assay, nucleotide diphosphate kinase 2 (NDPK2), appears to be such a kinase (Choi et al., 1999). In an *in vitro* binding assay, the Pfr form of phyA could bind to NDPK2 with about three times higher affinity than could the Pr form. The binding of Pfr (but not Pr) to NDPK2 increased the substrate affinity of

this kinase in an NDPK2 enzymatic assay in vitro. NDPK2, localized in both the cytosol and nucleus, may play a positive role in phytochrome signal transduction. An *Arabidopsis* mutant with the *NDPK2* gene interrupted by a T-DNA insertion showed decreased sensitivity to both R and FR light in cotyledon opening and greening (Choi et al., 1999).

Although the claim that phytochromes are serine/threonine kinases has gained support from the studies summarized above, it remains to be demonstrated conclusively whether phytochrome phosphorylation occurs or is physiologically relevant *in vivo*. On the other hand, reversible protein phosphorylation is essential for controlling the flow of many signaling processes. Thus it is presumed that the de-phosphorylation process might also be involved in the regulation of phytochrome signaling. Recently, a phosphatase named FYPP for flower-specific phytochrome-associated protein phosphatase has been reported (Kim et al., 2002). The FYPP dephosphorylates phyA in a light-dependent manner, and modulates phyA-mediated light signaling in the control of flowering time (Kim et al., 2002).

II. Phytochrome interacting proteins

Increasingly, efforts to dissect phytochrome signaling pathways have been focused on isolating and characterizing phytochrome-interacting proteins (Schafer and Bowle, 2002). Many positive phytochrome-interacting proteins have been isolated, and are summarized below.

Phytochromes are soluble proteins. Where do phytochromes function inside the cell once they are photoactivated to Pfr form? For many years, it was generally believed that the cytoplasmically localized molecules might signal to the nuclear genes through some unknown second messenger pathway. Although there are some tantalizing reports, still there is no robust direct evidence for such a pathway (Quail, 1991; Millar et al., 1994; Okamoto et al., 2001). Recently, there have been several reports that phytochromes themselves translocate to the nucleus once photoactivated to the Pfr form. The intracellular localization of phytochromes has been studied using fusion protein assays. Transgenes encoding a fusion protein of a phytochrome and a marker enzyme, such as β-glucuronidase or green fluorescent protein, are expressed in plants, and the intracellular localization of the phytochrome is identified by monitoring the location of the marker or its enzymatic activity. These studies demonstrate that phyA and phyB stay mostly in the cytosol in darkness, but a large proportion of each phytochrome pool is translocated to the nucleus once photoactivated (Sakamoto and Nagatani, 1996; Kircher et al., 1999; Yamaguchi et al., 1999; Hisada et al., 2000). It has been demonstrated that a significant proportion of all five *Arabidopsis* phytochromes when fused to GFP is imported into the nuclei. Nuclear translocation of these photoreceptors into the nuclei was regulated reversibly by light, each with distinct kinetics.

To determine whether nuclear translocation of the fusion proteins reflects normal cellular activities, the full-length phyA-GFP and phyB-GFP fusion proteins were used to rescue *Arabidopsis phyA* and *phyB*-null mutants respectively. It was shown that these fusion proteins are biologically functional (Yamaguchi et al., 1999; Kim et al., 2000). It is interesting that the phy-GFP fusion proteins form speckles in the nucleus following

light-induced translocation (Kircher et al., 1999; Mas et al., 2000; Nagy et al., 2000; Nagy and Schafer, 2000). It has been proposed that these nuclear speckles might represent multimolecular 'transcriptosome' complexes that could be involved in phy-regulated gene expression at specific locations in the genome that contain photoregulated genes (Mas et al., 2000; Tepperman et al., 2001). But a recent report put into question the physiological relevance of these speckles. Dimers of the N-terminal domains of phyB fused with GFP and with a NLS (so that the whole chimeric protein will move into the nucleus) result in even distribution of the fusion protein throughout the nucleus without forming speckles. Moreover, this construct can trigger a full range of phyB responses by inhibiting hypocotyl elongation in the absence of speckle formation or the C-terminal domain (Matsushita et al., 2003). Whether speckles are necessary for phytochrome to function awaits more definitive evidence.

The exact mechanisms of phytochromes' light-dependent nuclear localization are still undetermined, although they exhibit strikingly different features as far as light quality, quantity, and the kinetics of translocation are concerned (Kircher et al., 1999). Much recent work on phytochrome signaling has focused on nuclear proteins that interact with phytochromes.

By the yeast two-hybrid assay, several nuclear-localized proteins that directly interact with phytochromes have been isolated and characterized. Phytochrome-interacting factor 3 (PIF3) was first pulled out by using the C-terminus of phyB as 'bait' to screen an *Arabidopsis* cDNA library. PIF3 is a bHLH transcriptional factor that functions in both phyA and phyB pathways (Ni et al., 1998). Once photoactivated to Pfr, full length phyB binds PIF3, and dissociates with PIF3 upon conversion to its inactive

form (Ni et al., 1999). Although phyA also binds selectively and photoreversibly to PIF3, the apparent affinity of PIF3 for phyA is ten-fold lower than for phyB (Zhu et al., 2000). Recently, a *pif3* mutant generated by T-DNA insertion and transgenic plants overexpressing the full length *PIF3* have been physiologically characterized, and they indicate that PIF3 functions in regulating several light-dependent responses including seedling hypocotyl elongation, cotyledon opening and expansion, the accumulation of anthocyanins and chlorophyll contents (Halliday et al., 1999; Kim et al., 2003). Further experiments demonstrated that the phyB-PIF3 complex binds reversibly to the "G-Box" (a DNA sequence, CACGTG, that is present in various light-regulated promoters) specifically upon light-triggered conversion of the photoreceptor to its biologically active conformer (Martinez-Garcia et al., 2000).

PIF4 is a negative regulator that interacts specifically with phyB, and selectively binds to the Pfr form of phyB, but has little affinity for phyA. It also binds to the same G-box DNA sequence motif found in various light-regulated promoters of circadian genes such as *CAA1* and *LHY* to which PIF3 binds. In contrast to PIF3, the G-box-bound PIF4 does not interact with the Pfr form of phyB, even if the PfrB concentration is 4-fold higher amount than that used for PIF3 binding (Martinez-Garcia et al., 2000; Huq and Quail, 2002). It has been demonstrated that PIF4 is not involved in regulating previously characterized G-Box-containing genes, such as *CCA1* and *LHY*, which are targets of PIF3 and involved in circadian cycle maintenance. This suggests that nucleotides outside the core G-box hexanucleotide sequence might provide *cis*-element specificity enabling the bHLH family to discriminate between target promoters in the living plant cell, as is known for other systems (Littlewood, 1998; Martinez-Garcia et al., 2000).

Early Flowering 3 (ELF3) also influences the activity of circadian clock–regulated processes and flowering time. The *ELF3* gene encodes a novel protein with 695-amino acid that may function as a transcriptional regulator (Covington et al., 2001; Hicks et al., 2001). The ELF3 protein abundance follows the pattern of circadian accumulation of *ELF3* transcript. Furthermore, the ELF3 protein interacts directly with PHYB in the yeast two-hybrid assay and in vitro. It has also been suggested that phyB and ELF3 can form a signaling complex that controls early events in plant development. Genetic analyses show that ELF3 requires functional PHYB in early morphogenesis, but not for the regulation of flowering time. Therefore, the process by which ELF3 and PHYB control flowering is more likely via independent signal transduction pathways (Covington et al., 2001; Hicks et al., 2001; Liu et al., 2001).

ZTl/ADO1 is a gene encoding a protein containing a PAS domain and localized in both the nucleus and cytosol. Under Wc or Bc light, the *ado1* mutant exhibits longer hypocotyls than those of wild-type *Arabidopsis* seedlings. Under R light, cotyledon movement and stem elongation are arrhythmic. Both yeast two-hybrid and *in vitro* binding studies show that there is a physical interaction between ADO1 and the photoreceptors cryl and phyB. These studies indicates that ADO1 is a positive regulator of phyB and cryl response pathways (Jarillo et al., 2001).

Another discovery that may elucidate photoreceptor function is the direct interaction between phyB and cry2. This relationship has been demonstrated by co-immunoprecipitation from transgenic *Arabidopsis* plants overexpressing cry2, as well as by physiological experiments (Mas et al., 2000). Seedlings of *cry2* mutants displayed a deficiency in the perception of W light, showed a longer period expression of

CAB::luciferase than those of the wild type under intermediate fluence W light, but not in R light, and a slightly increased expression under high fluence B light compared with that of wild type. This result implies that the full function of cry2 needs multiple wavelengths of light, and that some phytochrome effects might be involved. *cry2* mutants also exhibit a late flowering in Wc, but early flowering under a low R:FR ratio (W plus FR) which converts more Pfr to its Pr form (Mas et al., 2000). The late flowering of *cry2* mutants in Wc can be phenocopied by B-plus-R light, but not by either alone. This indicates that flowering promotion of cry2 is dependent on both B and R light, and suggests that phyB is involved in the process (Guo et al., 1998; Mockler et al., 1999; Lin, 2002). How phyB and cry2 interact in this process is still unknown. It has been proposed that cry2 might function either by suppressing phyB signaling directly or by removing the ability of PfrB to inhibit early flowering via another pathway, so that cry2 accelerates flowering. Although *cry2* seedlings display a wild type hypocotyl length in W light, with W plus FR they show a long hypocotyl phenotype. All these results imply that genetic interaction occurs between the phyB and cry2 pathways *in vivo*. Using fluorescent resonance energy transfer microscopy, it has been demonstrated that phyB and cry2 interact directly in the nuclear speckles that are formed in a light-dependent fashion (Mas et al., 2000).

A direct interaction between cry1 and phyA has also been confirmed with yeast two-hybrid assays using expressed C-terminal fragments of CRY1 and phyA from *Arabidopsis in vitro*. *Arabidopsis* CRY1 and CRY2 are phosphorylated by a purified recombinant phyA. *In vivo* labeling studies indicating that specific mutant alleles of CRY1 interfere with the function of phytochrome suggest the possible physiological relevance of these findings (Ahmad et al., 1998).

Auxin/indole-3-acetic acid (*Aux/IAA*) genes encode short-lived transcription factors that are induced as a primary response to the plant growth hormone IAA or auxin. Gain-of-function mutations in *Arabidopsis* genes, *SHY2/IAA3*, *AXR3/IAA17*, and *AXR2/IAA7* cause pleiotropic phenotypes consistent with enhanced auxin responses, possibly by increasing Aux/IAA protein stability. The direct interaction between Aux/IAA and phyA has been established by recombinant Aux/IAA proteins from *Arabidopsis* and pea (*Pisum sativum*) interacting with recombinant phyA from oat (*Avena sativa*) *in vitro*. Phytochrome-dependent phosphorylation of Aux/IAA proteins has been proposed to provide one molecular mechanism for integrating auxin and light signaling in plant development (Colon-Carmona et al., 2000).

The *Arabidopsis thaliana* response regulator 4 (ARR4), has similarity with the *Escherichia coli* response regulator CheY (Imamura et al., 1999; Shen et al., 2003). A direct interaction between ARR4 and phyB has been demonstrated as ARR4 co-immunoprecipitates with phyB, and ARR4 copurifies with phyB, but not with phyA. Although the Pr form and Pfr form of phytochromes exhibit different protein conformations, ARR4 binds to both. By the yeast two-hybrid assay, ARR4 interacts with full-length phyB and with the N-terminus of phyB, but not with any other phyB domains, or with the N-terminal domain of phyA. In addition, the N-terminus of phyB did not interact with ARR2, ARR5, other types of the *Arabidopsis thaliana* response regulators (Sweere et al., 2001). ARR4 is expressed in stems, leaves, and flowers but not in roots, in light-grown seedlings and dark-grown seedlings. Thus, the expression pattern of ARR4 overlaps in great part with that of phyB. Accumulation of ARR4 was induced by Wc, Rc and by a 5 minute-R pulse in dark-grown seedlings, whereas FR light was ineffective.

The inductive effect of R light could be reversed by a subsequent FR light pulse (Sweere et al., 2001). These data suggest that phyB dominates the photoregulation of ARR4 expression, although participation of other photoreceptors, especially in W light, cannot be excluded. It has been shown that ARR4 is located both in the cytosol and the nucleus. ARR4 functions by stabilizing the active Pfr form of phyB in yeast and in young *Arabidopsis* seedlings. Thus, ARR4 elevates the level of the active photoreceptor *in vivo*. Transgenic *Arabidopsis* plants overexpressing ARR4 display hypersensitivity to R light, but not to light of other wavelengths. It specifically affects phyB-dependent photomorphogenic responses (Sweere et al., 2001).

D. **Phytochrome Signaling Networks**

The discovery that each phytochrome may control subsets of unique, overlapping, or in some cases antagonistic light-mediated responses suggests complex signaling mechanisms (Quail, 2002b). Much of the current research effort on light-regulated plant responses is focused on the downstream events of signaling for different photoreceptors and their inter-connections. Microarray studies have shown that the developmental changes seen during the de-etiolation process result in a change in expression of approximately 30% of genes in the *Arabidopsis* genome (Ma et al., 2001; Tepperman et al., 2001). Such studies indicate that the expression of a fraction of genes is controlled by all or some of the photoreceptors in common, and that light signaling forms an intracellular signaling network, although each photoreceptor perceives distinct light cues (Ma et al., 2001).

Many putative light signal transduction intermediates have been identified from mutant screens aimed principally at isolating mutants displaying constitutive photomorphogenesis in darkness, or mutants that are insensitive, hyposensitive, or hypersensitive to specific or a combination of light conditions (Lin, 2002; Møller, 2002; Quail, 2002b; Wang and Deng, 2003). Here I briefly summarize some of the downstream components found so far.

One large group of *Arabidopsis* mutants, designated *cop/det/fus*, were isolated in early screens for seedlings showing constitutive de-etiolated phenotypes, as if they had perceived a light signal although they were grown in complete darkness. At least 11 loci have been isolated so far. Because these mutants are recessive, and play pleiotropic roles

involving both phytochrome signaling and cryptochrome signaling, the wild-type loci are generally considered to act negatively in darkness to suppress photomorphogenesis, and to act late in the signaling process downstream of phytochrome and cryptochrome signaling (Diagram 1). Among these loci, six (CSN1-CSN4, CSN7 and CSN8) encode six of the eight subunits of the Constitutive Photomorphogenic 9 (COP9) signalosome protein complex (Wei and Deng, 1996; Fankhauser and Chory, 1997; Wei and Deng, 1999; Hardtke, 2000; Hudson, 2000; Schwechheimer and Deng, 2000).

Constitutive Photomorphogenic 1 (COP1) is a protein with both a zinc-binding "ring figure" motif and the WD-40 repeat motif of G beta proteins. It is located in the nucleus in darkness, but undergoes a light-mediated translocation to the cytosol once the cells have been exposed to light. COP1 functions as an ubiquitin E3 ligase, targeting specific proteins for degradation by assisting in their ubiquitination (Osterlund et al., 1999). From studies on COP1 and on the COP9 complex, it has been hypothesized that they play roles in proteolysis and in chromatin remodeling (Osterlund et al., 1999; Wei and Deng, 1999; Hardtke, 2000; Osterlund et al., 2000; Schwechheimer and Deng, 2000, 2001; Schwechheimer et al., 2001; Schwechheimer et al., 2002).

Arabidopsis de-etiolated 1 (*det1*) mutants also develop like light-grown seedlings even when grow in the dark. *DET1* gene encodes a nuclear-localized protein that appears to act downstream of multiple photoreceptors to regulate morphogenesis and gene expression in response to light (Chory et al., 1989; Pepper et al., 1994). DET1 binds to nonacetylated amino-terminal tails of the core histone H2B in the context of the nucleosome, and are proposed to affect chromatin remodeling so as to regulate gene expression during photomorphogenesis. DET1 is also a component of a complex that

contains UV-DDB1, which in animal cells is part of histone acetyltransferase complexes, and DET1 may bind to the nucleosomes of light-induced genes, and may be subsequently displaced by light-dependent acetylation of histone tails, thus permitting gene expression (Benvenuto et al., 2002; Schroeder et al., 2002).

det2 mutants can de-etiolate partially in the dark (Chory et al., 1991). The deficiencies of *det2* can be restored by application of brassinosteroid. Cloning of the gene indicated that *DET2* encodes an enzyme in the brassinosteroid synthesis pathway. The gene product of *DET2* shares significant sequence identity with mammalian steroid 5 alpha-reductases, which implies that DET2 functions as a reductase in brassinolide biosynthesis (Li et al., 1996).

Under Wc conditions, besides the phytochromes and chromophore synthesis pathway mutants, the most severe light insensitive mutation is defective in a gene encoding the bZIP transcriptional factor known as HY5 (Oyama et al., 1997). The *hy5* mutation affects both phyA and phyB signaling, and cry1 signaling as well, and multiple responses including cell elongation, cell proliferation, and chloroplast development. This pattern indicates that hy5 plays a key role in the control of photomorphogenesis, and supports models postulating complicate networks other than independent linear signal transduction pathways (Oyama et al., 1997). The cellular level of HY5 protein remains low in darkness, but increases markedly in the light. COP1 interacts directly with HY5 in the nucleus to facilitate the ubiquitination of HY5, hence specifically targeting it for proteasome-mediated degradation in darkness (Osterlund et al., 2000; Schwechheimer and Deng, 2000).

Among the many known signaling pathway constituents, a subset acts specifically downstream of one photoreceptor. For example, the *FHY1*, *FHY3*, and *FAR1* gene products are apparently nuclear-localized positive regulators specifically of phyA-mediated pathways, and mutations in these genes produce pleiotropic defects on two or more FR light responses such as hypocotyl elongation, apical hook opening, cotyledon expansion, FR-preconditioned block of greening, anthocyanin accumulation, and gravitropic sensitivity (Whitelam et al., 1993; Hudson et al., 1999; Bolle et al., 2000; Desnos et al., 2001; Wang and Deng, 2002). Although *FHY3* and *FAR1* show overlapping but distinct sets of FR regulated responses, they share sequence similarity, and can form homo- or hetero-complexes as indicated by yeast-two-hybrid assays, and by co-immunoprecipitation from tissues *in vivo*. In addition, they can functionally suppress each other's mutant phenotypes. This overlap indicates that FHY3 and FAR1 may constitute on branch of a small gene family (Hudson et al., 1999; Wang and Deng, 2002). In spite of the lack of direct interaction between *FHY1* and *FHY3* or *FAR1*, and no suppression of *fhy1* mutant deficiencies by FHY3 or FAR1, the transcription levels of *FHY1* are regulated by the product of *FHY3* (Desnos et al., 2001; Wang and Deng, 2002). Furthermore, double mutants of FHY3/FAR1 are much more severely affected than is either single mutant or than would be expected from additive roles.

Some mutants only show defects in a single light-regulated response. HFR1/REP1/RSF1 and LAF1 are also localized in the nucleus, but are specific to FR inhibition of elongation; *laf1* and *hfr1* mutants have normal gravitropism, greening, and hook opening responses (Fairchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000; Ballesteros et al., 2001), and the gene-expression profiles of *laf1* and *hfr1* indicate

that these components regulate a smaller subset of phyA-regulated genes (Wang et al., 2002). Both of them are transcription factors, with LAF1 acting as a MYB-like transcription activator and HFR1 containing a bHLH domain (Fairchild et al., 2000; Ballesteros et al., 2001; Duek and Fankhauser, 2003). The *HFR1* gene is expressed abundantly under FR light. In contrast to PIF3, which can bind both phyA and phyB, HFR1 does not bind either phyA or phyB directly. But HFR1 can form homodimers or form heterodimers with PIF3, suggesting that it may function to modulate phyA signaling via heterodimerization with PIF3 (Fairchild et al., 2000). Additional experiments suggest that HFR1 mediates both phyA and cryptochrome signaling, and works in a separate pathway from HY5, even though both are involved in common response pathways (Kim et al., 2002; Duek and Fankhauser, 2003).

IMP is also a nuclear-localized protein with a specific role in controlling germination induced by phyA-mediated VLFR. *imp1* mutants develop normally, and their general morphology is indistinguishable from that of wild type (Duque and Chua, 2003). IMP1 defines another group of transcription factors containing a bromodomain (BET) acting as chromatin-targeting module (Winston, 1999). IMB1 is expressed at a very low level in dry seeds, is markedly induced during seed imbibition, and its transcriptional level is again down-regulated after seed germination begins. *imp1* mutants show impaired cotyledon greening after germination in ABA and express higher levels of ABI5 [a downstream component in ABA transduction pathway that causes growth arrest after emergence of the radical from the seed coat (Lopez-Molina et al., 2002)]. IMP1 regulates a small gene family and links ABA and phyA signaling in the seed germination process (Duque and Chua, 2003).

Several phyA-specific signaling components are cytosolic [e.g. PAT1 (Bolle et al., 2000) and FIN219 (Hsieh et al., 2000)] or even plastidic [LAF6(Møller et al., 2001)]. They also play pleiotropic roles in early phyA signaling. All of them are positive regulators, but distinct from each other. The *FIN219* gene encodes a product with homology to the GH3 family of proteins whose expression is rapidly induced by auxin, and is demonstrated to be involved in light inactivation of COP1. It promotes the ubiquitin-mediated proteolysis of a subset of positive regulators (Deng et al., 1992; Seo et al., 2003). *PAT1* encodes a protein with homology to the plant-specific GRAS regulatory protein family involved in gibberellin signaling (Bolle et al., 2000). *LAF6* encodes a ATP-binding-cassette (atABC1) protein involved in the transport and correct distribution of protoporphyrin IX, which may act as a light-specific signaling factor involved in coordinating intercompartmental communication between plastids and the nucleus (Bolle et al., 2000; Hsieh et al., 2000; Møller et al., 2001).

In contrast to the mutants listed above, which result in a reduced sensitivity to FR light, *spa1* and *eid1* show enhanced phyA-specific responses (Hoecker et al., 1998; Hoecker et al., 1999; Hoecker and Quail, 2001; Baumgardt et al., 2002; Laubinger and Hoecker, 2003). SPA1 has a strong influence on the regulation of VLFR and a weaker influence on FR-HIR. In contrast, *eid1* severely alters HIR and causes almost no change on VLFR. Double mutants of *eid1/spa1* exhibit a strong interference at all fluences. These results indicate that EID1 and SPA1 are involved in different but interacting phyA-dependent signaling chains (Zhou et al., 2002). As a nuclear-localized negative regulator of phyA signaling, SPA1 directly interacts with a more broad-spectrum light-dependent proteasome-targeting component COP1 (Hoecker and Quail, 2001), and EID1 is an F-

box-containing protein that contains a leucine-zipper whose integrity is necessary for its biological function. EID1 may be involved in ubiquitin-dependent protein degradation (Buche et al., 2000; Dieterle et al., 2001). SPA1 shares highest sequence similarity with COP1. But *spa1* is hypersensitive to FR but has a normal a dark growth phenotype, whereas *cop1* exhibits photomorphogenesis in darkness. Furthermore, *spa1* is specific for phyA-signaling, while *cop1* affects multiple phy and other photoreceptor-mediated responses. This contrast indicates that these two suppressors may function at different points in the light signaling network. This idea is supported by the finding that SPA1 can stimulate LAF1 degradation mediated by COP1 (Seo et al., 2003). The *SPA1* gene also shares sequence similarity to the yeast transcription repressor TUP1, hinting that SPA1 may function as transcription repressor (Williams and Trumbly, 1990).

SPA2, identified from the *Arabidopsis* genome database by its high sequence similarity to *SPA1*, arose from a duplication of a chromosomal region that includes *SPA1* (Simillion et al., 2002; Laubinger and Hoecker, 2003). *SPA3* and *SPA4* are also homologous to *SPA1* genes within the kinase-like region, putative coiled-coil domains, and six WD-40 repeats as *SPA1* does. Like SPA1, SPA3 and SPA4 function as negative regulators in phyA-mediated FR responses. But *SPA3*, functioning as a broader suppressor of photomorphogenesis, depends not only on phyA, but on other phytochromes as well (Laubinger and Hoecker, 2003). Deletion analysis suggests that SPA3 and SPA4, like SPA1, bind to the coiled-coil domain of COP1 as a COP1-containing complex to repress photomorphogenesis. It is still too early to illustrate how SPAs and COP1 interact and regulate light signaling because neither SPA3 nor SPA4 has an apparent nuclear localization sequence, whereas COP1-mediated HY5 degradation

occurs within the nucleus (Hardtke et al., 2000; Osterlund et al., 2000; Osterlund et al., 2000; Laubinger and Hoecker, 2003). However, in the light, COP1 moves out of the nucleus and may interact with SPA3 or SPA4 in the cytosol.

Another negative regulator of phytochrome signaling networks is SUB1, which acts by suppressing light-dependent accumulation of HY5 transcript. HY5 protein undergoes COP1-dependent degradation which is relieved when COP1 is shuttled out of the nucleus in response to the light signal (Osterlund et al., 2000; Guo et al., 2001).

There are numerous other phytochrome signaling molecules specific to phyB pathways. *srr1* shows weak deficiencies in controlling hypocotyl elongation, petiole length, and flowering compared with *phyB* mutants under Wc and Rc light, but not FRc light. This mutant also shows reduced chlorophyll accumulation and EOD-FR responses (Staiger et al., 2003), and altered production of several circadian-regulated genes including *CAB*, *AtGRP7/CCR2, CCA1, CAT3, TOC1*, *GI*, and *FKF1*. This finding suggests that SRR1 is not only involved in phyB signaling, but is an important component in normal oscillator function. That the *SRR1* gene encodes a protein conserved in both plants and animals with both nuclear and cytosol localization implies an important conserved role of SRR1 (Staiger et al., 2003).

GI is a constitutively nuclear-localized protein with no sequence similarity to any other proteins in the database. The reduced de-etiolation of *gi* mutants under R but not FR light suggests that GI is an early downstream component in phyB signaling (Huq et al., 2000). Besides the role in phyB signaling, mutations in the *Arabidopsis GI* gene cause photoperiod-insensitive flowering and alteration of circadian rhythms by altering the expression patterns of *ELF3*, *LHY* and *CCA,* as well as *GI* itself. GI is required for

maintaining circadian amplitude and appropriate period length of these genes (Fowler S, 1999; Park DH, 1999).

asa1, *umb1 and tir3-1* represent 3 mutant alleles of *BIG*, a calossin-like protein, which is required for normal auxin efflux and polar transport of auxins (Gil et al., 2001; Kanyuka et al., 2003). These mutants have attenuated shade-avoidance with increased elongation of petioles and internodes, accelerated flowering and reduced leaf length and smaller leaves. BIG specifically affects phyB-mediated responses, and the *asa1/umb1/tir3-1* mutants also have organ-specific defects in response to cytokinins, ethylene, *N*-1-naphthylphthalamic acid (NPA) and gibberellins (GA). These data support that the existence of a complex sensory network in plants in which BIG mediates interactions between hormone and light responses (Kanyuka et al., 2003).

Mutations in several genes block both phyA- and phyB- signaling networks. By using a genetic screen for de-etiolation-defective seedlings, two allelic mutants were isolated that exhibited reduced sensitivity to both continuous R and FR light, suggesting involvement in both phyA and phyB signaling. The molecular lesions responsible for the phenotype were shown to be mutations in the *Arabidopsis* PSEUDO-RESPONSE REGULATOR7 (PRR7) gene, a member of small gene family in *Arabidopsis* previously suggested to be involved in circadian rhythms (Carre and Kim, 2002; Eriksson et al., 2003; Michael et al., 2003; Nakamichi et al., 2003). A PRR7:β-glucuronidase fusion protein localized to the nucleus, implying a possible function in the regulation of photoresponsive gene expression. Consistent with this suggestion, *prr7* seedlings were partially defective in the regulation of the rapidly light-induced genes *CCA1* and *LHY*, observable as a premature increase in expression level during the second peak of the

biphasic induction profile that is elicited upon initial exposure of dark-grown seedlings to light. A similar 3- to 6-h coordinated advance in peak free-running expression of CCA1, LHY, and TIMING-OF-CAB1, which are considered to encode the molecular components of the circadian oscillator in *Arabidopsis*, was observed in entrained fully green *prr7* seedlings compared with wild-type seedlings. Collectively, these data suggest that PRR7 functions as a signaling intermediate in the phytochrome-regulated gene expression responsible for both seedling de-etiolation and phasing of the circadian clock in response to light (Kaczorowski and Quail, 2003).

In contrast to *PRR7*, *COG1* gene functions as a negative regulator in both the phyA- and phyB-signaling pathways. The *COG1* gene encodes a nuclear protein with a plant-specific Dof (*D*NA binding with *o*ne *f*inger) domain, one of a family of Dof plant transcription factors (Yanagisawa, 2002; Kanyuka et al., 2003). Transgenic plants overexpressing COG1 exhibited hyposensitive responses to R and FR light in a manner inversely dependent on *COG1* mRNA levels. On the other hand, transgenic lines expressing antisense *COG1* were hypersensitive to R and FR light. Expression of the *COG1* gene is light inducible and requires phyA for FR light-induced expression and phyB for R light-induced expression. This pattern suggests that phytochromes positively regulate the expression of *COG1* as a mechanism for fine tuning the light-signaling pathway (Kanyuka et al., 2003).

As summarized above, many of the identified signaling components are nuclear localized. Most of these constituents themselves are transcription factors that are directly involved in regulating gene expression. Therefore, the light-induced translocation to the

nucleus may be especially important for initiating changes in gene expression that lead to photomorphogenesis.

Many more putative mutants deficient in FR responsiveness have been reported, but remain to be cloned and characterized molecularly (Møller, 2002), such as *fin2* (Soh et al., 1998), *fin4* (Fry et al., 2002), *fin5* (Cho et al., 2003), and the negative-regulator mutant *psi1* (Genoud et al., 1998) . Some genetically identified loci in phyB signaling are also not yet cloned, such as *red1*(Wagner et al., 1997), *pef2*, *pef3* (Ahmad and Cashmore, 1996), and *srl1* (Huq et al., 2000). Several mutants defining pathways shared by phyA and phyB have been described, including the putative positive-regulator mutant *pef1* (Ahmad and Cashmore, 1996). By cloning and characterizing these and subsequently identified genes, phyB signaling networks will be more completely understood (Ahmad and Cashmore, 1996; Wagner et al., 1997; Huq et al., 2000). However, until these genes are cloned no molecular functions or interactions can be assigned.

The range and number of phy signaling components are indicative of extremely complex transduction webs involving multiple light receptors, hormones, sugars, and other pathways. Based on the unique roles of phyC, D, and E, and overlapping functions of each individual phytochrome, there are probably many other specific or shared components to be identified in the signaling networks of the remaining phytochromes. Given that new phy signaling components are still being discovered, and that light signaling roles are being found for previously identified proteins in other pathways, it is likely that the screens for phytochrome signal transduction components are not yet saturated. In particular, since most previous screens emphasized isolation of mutants

exhibiting the most severe light phenotypes, and given that some mutant loci combinations yield enhanced phenotypes beyond those seen in the single mutants, screens for *Arabidopsis thaliana* seedlings with weak deficiencies in light-dependent inhibition of hypocotyl elongation were undertaken.

Here we report the isolation and initial characterization of two such *Arabidopsis* mutants, designated *de*ficient *p*hotomorphogenesis 1, 2 and 3 (*dep1, dep2* and *dep3*), which were isolated in a screen for seedlings growing slightly taller than wild type under white light.

Chapter II. The *Arabidopsis dep* Mutations Cause Deficiencies in Phytochrome-Regulated Growth Responses

A. Materials and Methods

B. Results

I. Isolation of *dep* mutants

II. The dep mutations affect both phyA-mediated and phyB-mediated growth responses in young seedlings

III. Mature *dep1*, *dep2* and *dep3* mutants show small deficiencies in phytochrome-controlled responses

IV. PhyA levels and photoconversion are apparently unaffected in *dep1*, *dep2* and *dep3* mutants

V. Initial genetic characterization and mapping of Deps

C. Discussion

Light, acting both through photosynthesis and through a series of specific morphogenic photoreceptors, regulates nearly every stage of whole plant growth and development. Several major classes of photoreceptors have been isolated and well characterized, including phy, cry and phot (Quail, 2002a). By characterizing mutants from these photoreceptors, the functions of individual photoreceptor in light perception and in regulation of various physiological responses have been assigned to specific photoreceptor, or group of photoreceptors (Quail, 1991; Short and Briggs, 1994; Smith, 1995; Fankhauser and Chory, 1997; Quail, 2002a).

The discovery that each phytochrome may control subsets of unique, overlapping, or in some cases antagonistic light responses suggest complex signaling mechanisms (Quail, 2002b). This intricacy is further supported by the large number of putative signal transduction components that have emerged from genetic screens, phytochrome-interacting proteins isolated by yeast two-hybrid or biochemical pulldown assays, and cellular localization or biochemical studies {Møller, 2002 #4066}. With the *Arabidopsis* genome sequencing finished and combining with other techniques, more and more downstream components have been cloned and genetically characterized recently. Among these signaling pathway constituents, some act specifically downstream of one photoreceptor, some components are involved in more than one signaling pathway (Quail, 2002a, 2002b).

Mutations in numerous presumed downstream signal transduction components have then been isolated and characterized based on the plants' physiological responsiveness to light. These genetic approaches to elucidating photomorphogenic

pathways have been extremely successful, particularly for isolating phytochrome-regulated elements. Continuous characterizing known components and figuring out their interactions with photoreceptors and other signal molecules, and isolating new signaling components analyzing their functions and involvement of signaling networks are still absolutely necessary to dissect entire light signaling.

A. Materials and Methods

Plant Materials and Growth Conditions

Seeds were surface sterilized for 20 min in a solution of 20% commercial bleach (final sodium hypochlorite concentration 0.05%[w/v]) and 0.01% SDS, then rinsed five times in sterile deionized distilled water. Seeds were sown on 100-mm Petri plates with sterile medium containing 0.8% (w/v) agar and MS salts (Murashige and Skoog, 1962) with vitamin supplement (Invitrogen, CA). Plates were kept in darkness at 4°C for 3d and then induced to germinate by exposure to cool white fluorescent light (approximately 75 μmol m^{-2} s^{-1}) for 3h, then placed in the dark at 24°C for 21h. Plates were then transferred to appropriate light conditions as indicated for each experiment.

FRc light was obtained from a high-output, 735-nm LED source (Q-beam 2001, Quantum Devices, Barneveld, WI) 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 9.7 μmol m^{-2} s^{-1} was used except as indicated. Rc was from 667-nm Q-beam LED sources (Quantum Devices, Barneveld, WI) at a fluence rate of 15 μmol m^{-2} s^{-1}. Bc was from cool-white fluorescent bulbs filtered through blue plexiglass #824 (8 μmol m^{-2} s^{-1}). Light intensity and spectral output were measured with a LI-1800 spectroradiometer (Li-Cor, Lincoln, NE).

Col-O, No-O, and Ler wild type seeds were obtained from the *Arabidopsis* Stock Center in Ohio. *phyA, phyB, cry1* and *hy1* are *phyA101, phyB101, hy4-1and hy1-1* respectively.

Mutant Isolation and Genetic Characterization

Arabidopsis thaliana seeds of the Nossen-O ecotype (No-O), were mutagenized with ethylmethanesulfonate (EMS) (Koncz et al., 1992). Approximately 10,000 M2 seedlings from families of mutagenized seeds were grown under 16h W light/8h dark cycles for 7d. Putative mutants were selected as those seedlings that grew taller than wild type but not taller than *hy5* mutants. The M2 plants were allowed to self-fertilize, and the M3 seeds re-evaluated for inheritance of the slightly elongated phenotype under W light. The original screen was done by Dr. Daniel Chamovitz in the lab of Dr. Xing-Wang Deng.

Tissue Measurements

Plants were grown as described previously (Short, 1999) for each light condition and time indicated. Seedlings were placed horizontally on 1% agar plates, photographed with Ektachrome 200 slide film (Kodak, Rochester, NY) with a reference ruler and the slides were projected onto a Kurta 1212 digitizing tablet for accurate measurements of the hypocotyls. For mature plant petiole measurements, the two oldest true leaves were excised, placed on an agar plate, photographed, and measured as above. Hypocotyl length

and petiole length were traced and the lengths and basic statistics computed with SigmaScan Pro software (SSPS, Jandel Scientific, San Rafael, CA). Measurement with seedling hypocotyl length was done with at least 50 seedlings per treatment per experiment, and with petiole length was done with at least 10 plants and total 20 leaves. Each experiment was replicated at least three times.

Electron Microscopy

Seedlings of *dep1* and No-O at 3d, 4d, and 6d after germination were fixed in fixative solution [25 mM phosphate buffer (PB; pH 7.0), 3% glutaraldehyde, 1% osmium tetroxide] at 4°C for 12-24h. Fixative was discarded and tissues treated with 1% osmium tetroxide dissolved in 25mM PB solution. Samples were incubated at 4°C, either overnight or for several days, until the solution was visibly black. The osmium solution was removed and samples were rinsed 3 times with PB. Seedlings were treated through an alcohol series for dehydration by sequentially incubating for 30 min in 30%, 50%, 75%, 89%, 95%, 100%, 100% and 100% ethanol, then kept in 100% ethanol overnight, soaked two more times in 100% ethanol, and then dried. Dried seedlings were mounted onto specimen preparation stages, and sample paste of desired thickness was spread onto the mount. Samples were placed on SEM sample holders and the paste allowed to dry overnight before coating. The SEM experiments were done by Dr. Daniel Chamovitz in Dr. Xing-Wang Deng's lab.

Immunoblot Analysis

Approximately 120 mg of seeds from each of the wild type, *dep1*, *dep2*, and *dep3* lines was plated on two identical sets of plates and treated as describes above. After inducing germination with 3 h Wc, plates were kept in darkness for 4 d. Etiolated seedlings from each set of plates corresponding to each line were harvested under dim-green light and frozen in liquid nitrogen, after one set was exposed to Rc for 4 h immediately before harvesting. Total cellular proteins were extracted under green safelight as described (Xu et al., 1995). SDS-PAGE and immunoblotting on nitrocellulose were carried out according to methods described previously (Xu et al., 1995; Short, 1999). The nitrocellulose membranes were stained with 1mg/ml Ponceau A in 1% acetic acid to confirm equivalent protein loading. Visualization of blots were performed with the PHYA apoprotein-specific monoclonal antibody 073D (developed by Dr. J. Shanklin, provided courtesy of Dr. Peter Quail) and a VectaStain ABC-AP antibody-detection system (Vector Laboratories, Burlingame, CA) using a colorimetric substrate as previously described (Short et al., 1992). The blot shown is representative of three independent replicates.

B. Results

I. Isolation of *dep* mutants

In total, approximately 10,000 M2 seedlings growing under 16 h light /8 dark cycles for 7d were screened with *hy5* used as a control. Nothing longer than *hy5* was picked, yielding 24 putative mutants to be rescreened in the M3 generation: 8 lines grew taller in FRc, 8 grew taller in Rc, and remaining 8 were tall under both conditions. Genetic crosses of the isolated individuals allowed grouping into distinct complementation groups, three of which were designated *dep1, dep2* and *dep3*. Plants were grown up to M3 and M4 populations and backcrossed twice into the parental ecotype for subsequent analyses.

To determine which light signal transduction systems are affected to yield the *dep1*, *dep2* and *dep3* phenotypes, growth deficiencies of plants from *dep1*, *dep2* and *dep3* lines were examined after growing under various light conditions for altered growth compared with those wild type No-O plants.

II. *dep* mutations affect both phyA-mediated and phyB-mediated growth responses in young seedlings

The light-dependent inhibition of hypocotyl elongation is controlled by several light signaling pathways covering various regions of the light spectrum: phyA under FRc, phyB and other phytochromes under Rc, and primarily cry1 under Bc (Fankhauser and

Chory, 1997; Lin, 2000). Because *dep* mutants were originally characterized in continuous full-spectrum white light, lack of the inhibition of hypocotyl elongation in the mutants could be on account of deficiencies in Rc, FRc, or Bc sensory pathways. The phenotypes of the mutants and wild type grown under Wc, Rc, and FRc are shown in Figure 1. Both *dep1* and *dep2* mutants grow slightly longer hypocotyls than those of wild type under Wc, Rc, and FRc light conditions, but *dep3* exhibits a more severely deficient phenotype than that of the *dep1* and *dep2* mutants, or of wild type No-O under all three light conditions, with an especially large difference in FRc. In addition to having much longer hypocotyls than *dep1, dep2*, or wild type, the FRc-grown *dep3* maintains partially appressed and unexpanded cotyledons.

The observed results were confirmed by quantitation of hypocotyl length. Seven-day-old *dep1, dep2, dep3* and wild type seedlings were grown in Wc, Rc, and FRc, and hypocotyl elongation was measured. Growth was compared with that of *hy1* [deficient in chromophore production for all phytochromes (Parks and Quail, 1991)], *phyA*, and *phyB* mutants (Figure2A). The *dep1* and *dep2* seedlings yielded what appeared to be consistently longer hypocotyls than those of the wild type under FRc, Rc and Wc, but the effect was not statistically significant in any of these cases. On the other hand, the *dep3* mutant seedlings displayed much longer hypocotyls than those of wild type, or of either *dep1* or *dep2* mutants under Rc and FRc. The difference was greatest under FRc, in which the *dep3* mutants were nearly 3-fold taller than either *dep1* or *dep2* mutants. The growth patterns of *dep1*, *dep2* and *dep3* to Wc, Rc and FRc are different from those of *phyA*, *phyB* and *hy1* mutants. Although seedlings of *dep3* grew very tall under FRc, their hypocotyls were not as long as those of *phyA* null mutants in FRc. Also unlike phyA

mutants, *dep3* seedlings unfold their apical hooks in FRc (Figure1), and grew more elongated hypocotyls under Rc than do *phyA* seedlings under the same conditions. Seedlings of *dep*1, *dep2* and *dep3* grown in darkness for 5 d were not taller than the No-O wild type seedlings (Figure 2B).

To determine whether Bc inhibition of the hypocotyl elongation is affected in *dep* seedlings, we measured the hypocotyl length of *dep1*, *dep2* and *dep3* seedlings grown under Bc for 5d. Figure 3 shows that there is not a significant difference in the hypocotyl length between the wild-type seedlings and *dep1*, *dep2* or *dep3* seedlings.

To follow the hypocotyl elongation progression and cotyledon unfolding and expansion processes, scanning electron microscopy of *dep1* seedling and wild type after 3d, 4d, and 6d in Wc growth was completed (Figure 4 a-f). After inducing germination, 3-day-old *dep1* seedlings showed elongated hypocotyls with folded cotyledons, but No-O seedlings showed a relatively short hypocotyl, and fully unfolded and expanded cotyledons (Figure 4 a, b). These differences extended to 4-day-old seedlings (Figure4 c, d). At 6d, the opening of *dep1* cotyledons still was only about 70°, whereas the wild type cotyledons were fully separated even at 3d (Figure 4 a, b, e, f). The higher magnification EM pictures indicate that *dep1* seedlings have elongated hypocotyl cells compared with those of the wild type seedlings (Figure 4 g, h). In contrast, the cotyledon cells of *dep1* seedlings are not as expanded the cotyledon cells from No-O seedlings grown under identified conditions (Figure 4 i, j).

FRc, low fluence Rc, and high fluence Bc are known to stimulate hook opening and cotyledon separation (Nagatani et al., 1993; Whitelam et al., 1993). In Wc and Rc, phyB plays the major role in mediating this response (Neff and Van Volkenburgh, 1994).

Seedlings from *dep1*, *dep2*, *dep3*, and wild type were grown in Wc, Rc, FRc and Bc for 3d and the hook opening and cotyledon separation were scored (Figure 5). While all of the seedlings had opened apical hooks under each light condition tested (data not shown), the cotyledon separation response was strongly affected in the *dep* mutants. Nearly all wild-type seedlings displayed separated cotyledons under all light conditions. However, *dep1* and *dep2* each showed a consistently higher proportion of seedlings with cotyledons less than 90 separated from one another in Wc, Rc, FRc and Bc. Although the cotyledon separation of *dep3* mutant seedlings was not significantly different from that of the *dep1* or *dep2* mutants under Wc, Rc, or Bc, nearly 100% of the *dep3* mutant seedlings maintained appressed and unexpanded cotyledons under FRc.

The negative gravitropism of seedlings under Rc has been suggested to be dependent largely on the level of phyB in its active (PfrB) form (Liscum and Hangarter, 1993; Liscum, 1993; Poppe et al., 1996; Robson and Smith, 1996). To determine whether mutations in the *DEP* genes lead to reductions in the negative gravitropic response, *dep1*, *dep2*, *dep3*, and wild-type seedlings were grown under Rc for 5d. Figure 6 shows that *dep1*, *dep2*, and *dep3* all display decreased directional gravitropism compared with wild type.

To quantitatively test the Rc-regulated negative gravitropic response, we plated seeds from mutants and wild-type lines onto square plates and allowed the plants to grow on the vertical surface in Rc for 5d after stratifying and inducing seed germination. Then the exact angle of each seedling relative to vertical was measured. The data are shown in Figure 7. *dep1* seedlings showed a much more randomized growth pattern compared with wild type seedlings, whereas the growth directions of *dep2* seedlings were not

significantly different from those of the wild type. On the other hand, *dep3* seedlings showed much more randomized growth than *dep1*, *dep2* or wild type seedlings.

In young seedlings, characterization of *dep1*, *dep2*, and *dep3* mutants under different light conditions indicated that they played pleiotropic roles in several light-regulated processes, and these mutants show stronger deficiencies under FRc, especially the *dep3* mutants which display some phenotypes comparable to those of *phyA* null mutants. These data indicate that *dep1*, *dep2* and *dep3* may play roles in phytochrome signaling networks, with *dep3* strongly blocked in phyA signaling. However, if other light signaling pathways are affected, it is possible that mature plant growth and development may be affected as well.

III. Mature *dep1*, *dep2* and *dep3* mutants show small deficiencies in phytochrome-controlled responses

The findings that *dep1*, *dep2* and *dep3* all show weak deficiencies in seedling responses to Rc, including inhibition of hypocotyl elongation, cotyledon opening, and gravitropism, suggest that DEP*s* may be involved in phyB signaling pathways as well as in phyA-controlled responses. To determine whether the reduced seedling responsiveness to light continues into maturity, several phytochrome-mediated responses were measured in older plants. Petiole length of the two oldest true leaves was measured after 25d growth in Wc (Figure 8). Although neither *dep1* nor *dep2* mutation was sufficient to yield statistically significant elongation of petioles beyond that of wild-type

plants, the *dep3* mutant displayed a small but significant ($p<0.02$) increase in petiole length consistent with the effects of phyB (Reed et al., 1993).

In addition to regulating plant vegetative growth, another major process regulated by phytochrome in light-grown plants is the perception of day-length to control flowering. *phyB, D,* and *E* mutants all contribute to accelerated flowering in short days (SD) (Smith and Whitelam, 1997; Whitelam and Devlin, 1997), but *phyA* mutants flower later in long days (LD) (Johnson et al., 1994; Lin, 2000). To investigate whether *dep* mutants show an altered flowering response, plants were grown in Wc for the LD treatment and 8/16 light-dark cycles as SD treatment until the inflorescence bolt was first detectable. At that time, the number of days since initiation of germination and the number of rosette leaves were recorded (Table1). The *dep1*, *dep2*, and *dep3* mutants all showed a small delay in flowering time compared with the wild type No-O both under LD and SD treatments. However, the number of rosette leaves present at the flowering time was not statistically different among the mutants and wild type either under LD or SD. In all genotypes, flowering occurred when the plants produced 7-9 rosette leaves under LD and 20-28 rosette leaves as under SD. These data suggest that the *dep* mutants show a delay in time to reach the level of development at which flowering occurs, not the developmental stage at which flowering is initiated.

IV. PhyA levels and photoconversion are apparently unaffected in *dep1*, *dep2* and *dep3* mutants

The absence of the PHYA apoprotein strongly affects FRc responses, while deficiencies in phytochromobilin production decrease responsiveness to both FRc and Rc by depriving all phytochromes of chromophore (Parks et al., 1989; Dehesh et al., 1993; Parks and Quail, 1993). The formal possibility that *dep1* and *dep2* lead to decreased production of phyA holoprotein was tested by immunoblotting seedling extracts and probing with phyA-specific monoclonal antibodies (Xu et al., 1995; Short, 1999). Western blots with extracts of No-O, *dep1*, *dep2*, and *dep3* mutants grown for 4 d in darkness and either left in darkness or exposed to 4 h Rc were tested for the dark accumulation of PHYA protein and for the light-induced degradation of that protein (Figure 9). None of the *dep1*, *dep2* and *dep3* seedlings maintained decreased PHYA levels compared with that of the wild type. Furthermore, exposure to Rc for four hours led to the light-dependent loss of immunochemically detectable PHYA protein, comparable to that of wild-type seedlings also.

V. Initial genetic characterization and mapping of Deps

To examine the genetic properties of *dep1, dep2* and *dep3*, mutants were backcrossed to the No-O parental background, and phenotypes of the F1 seedlings and of the segregating F2 seedlings were scored (Figure 10). Because of the weak hypocotyl elongation phenotype of *dep1* and *dep2*, the dominance of the mutations was

inconclusive in the F1. The size distributions of the *dep1* and *dep2* compared with those of wild type (Figure 10 A-C) overlap to such a great extent that most of the progeny fall within the range of sizes that could be attributed to either parental type. However, the segregating F2 populations of the backcrosses suggest that the size distribution of the *dep2* segregating population is not fully recessive (Figure 10 E-F). However, the phenotype of F1 seedlings from *dep3* backcross to No-O growing under Wc is comparable to wild type No-O seedlings. The size distribution of F2 populations of the *dep3* included about one quarter (192/780) of seedlings growing like *dep3* with very long hypocotyls, and three out of four (588/780) seedlings growing like wild type (Figure 10G), indicating that *dep3* is a classic recessive mutant allele (Table 2). Based on the distribution of seedlings from Figure 10A and Figure 10D, and supposing that *dep3* was completely recessive, the pattern of the predicted distribution of *dep3* F2 seedlings (Figure 10H) is comparable to the actual distributions of F2 seedlings (Figure 10G).

Because *dep1* and *dep2* display only marginally different phenotypes from those of wild type for all responses examined, selecting homozygous individuals from among the F2 populations for genetic mapping proved unreliable, even after rescreening the F3 families for segregation.

On the other hand, the progeny of No-O X *dep3* exhibit the typical recessive Mendelian segregation in the phenotypes of F1 seedlings, and in the distribution of F2 populations (Table 2, Figure 10 G). The phenotypes of *dep3* mutants are strong, distinct, and easily scored phenotypes under FRc. Therefore, additional mapping populations were developed by crossing the *dep3* mutants to Col-O and Ler ecotypes, and the F2 seedlings with very long hypocotyls after 3 d FRc and the ability to accumulate

chlorophyll after later transferring to 1 d Wc were selected and grown to maturity. To make sure the F2 seedlings are homozygous, each line required retesting in the F3 generation to eliminate individuals that were potentially heterozygous. Upon confirmation of homozygosity in each F2 by the lack of F3 segregation, the DNA that had been extracted from F2 seedlings was used for mapping *dep3*. The No-O allele of SSLP marker nga63 cosegregated with the *dep3* allele in 3 out of 544 chromosomes, indicating its location to within 0.05 map units of the marker on the short arm of chromosome I. The *DEP1* locus maps to the short arm of chromosome I between nga63 (73 out of 270) and Athso392 (64 out of 202). The *dep2* mutant allele maps to the long arm of chromosome IV between CAPS marker PG11 (35 out of 208 chromosomes) and SSLP marker nga1107 (22 out of 100 chromosomes).

C. Discussion

Screens for severely altered photomorphogenic phenotypes under controlled light conditions have proven effective for isolating mutants in light signaling pathways (M鈥 ler et al., 2002; Quail, 2002b). However, mounting evidence for parallel pathways, redundant components, and integrative signaling networks suggests that the loss of function of some signaling constituents may result in less dramatic phenotypes. By screening for mutants growing only slightly taller than wild type under Wc, we have isolated a class of mutants that would not have been recognized using most previous approaches.

The deficiencies observed in the *dep* mutants are light-dependent. When the mutants are grown in darkness, *dep1*, *dep2* and *dep3* are indistinguishable from the wild type. This result suggests that Dep1, Dep2 and Dep3 are required for normal light signaling, rather than components of general growth maintenance. To determine which wavelengths are most effective at differentiating *dep*s from wild-type seedling growth and development—and thereby which photoreceptor systems are likely involved—plants were grown under various light regimes and known growth responses were measured to clarity in detail the involvements of Deps in light signaling.

Based on the data we have presented, *dep* mutants grow with elongated hypocotyls, and, in some cases, cotyledons remain closed and poorly expanded under all light conditions tested. The EM pictures of *dep1* young seedling suggest that the long hypocotyl can be explained by increased elongation of hypocotyl cells, and does not

require a change in proliferation of the hypocotyl cells. Conversely, the mutant cotyledon cells are not as well expanded as those of wild type that leads to the smaller size of the cotyledons than that of the wild type No-O (Figure e,f). Therefore, at least in the case *dep1,* deficiencies in the inhibition of hypocotyl cell elongation and inducement of cotyledon cell expansion during young seedling de-etiolation mimic processes observed in *phyA* and *phyB* mutants (Somers et al., 1991; Dehesh et al., 1993; Parks and Quail, 1993).

Deps are components of the phyA signal transduction network

The most severe developmental deficiencies of *dep* mutant seedlings, especially for *dep3,* were observed under FRc. Among the phenotypes observed in the individual mutants were longer hypocotyls and a higher proportion of *dep1* and *dep2* seedlings - and almost a hundred percent of *dep3* seedlings - exhibiting closed cotyledons but opened apical hooks. The phyA photoreceptor is known to be the primary mediator of the FR-HIRs such as inhibition of elongation and cotyledon separation (Quail et al., 1995), indicating that phyA response pathways are affected by *dep1, dep2* and *dep3.* What was most unexpected about the *dep* responses was their relative severity in the *dep3* mutants when compared with the *dep1* and *dep2* mutants although they all grew with relatively weak deficiencies under Wc. The FR-HIR growth responses of *dep3* seedlings were more comparable to those of *phyA* null mutants. Still, *dep3* seedlings have unfolded apical hooks that are different from *phyA* seedlings, and other responses are quantitatively distinct from previously reported *phyA* mutant responses to FRc (Dehesh et al., 1993;

Parks and Quail, 1993; Whitelam et al., 1993). These data are consistent with Deps functioning as components in phyA signaling. The pleiotropic nature of the phenotypes of *dep* mutants indicates that most, if not all, FR-HIR growth responses are modulated through the network containing the *DEP* gene products.

In addition to the seedling growth phenotypes affected by mutations in the *Dep* genes, adult plants may also be defective in phyA-mediated developmental responses. While phyA has comparatively minor effects on the growth of mature plants, the *phyA* null mutation has been shown to increase the time to initiation of flowering (Johnson et al., 1994). Mature *dep* plants consistently show a brief delay in flowering time in both SD and LD conditions compared with that of the wild type, regardless of whether the plants carry the *dep1*, *dep2*, or *dep3* mutation. However, the numbers of rosette leaves produced when bolting begins are not statistically different under the SD and LD conditions tested. Thus, the observed flowering delay may be explained by a general slowing of development in the mutants rather than by a direct phyA-mediated late flowering response.

Deps function in phyB signaling pathways as well as in phyA signaling

The finding that *dep1, dep2* and *dep3* mutants also had longer hypocotyls than those of the wild type under Rc and Wc is inconsistent with DEPs functioning solely in phyA signaling. Similarly, *dep1*, and *dep3* seedlings have a reduced regulation of gravitropic responsiveness in seedlings grown under Rc, as do *phyB* but not *phyA* mutants (Poppe et al., 1996). This apparent involvement of DEP1 and DEP3 in phyB-mediated

responses in young seedlings can also be observed in mature plants; the *dep3* mutants produce significantly longer petioles than the wild type, although the difference is small.

That the data show effects of *dep1, dep2* and *dep3* on phyA- and phyB-regulated responses could be interpreted several ways. One formal possibility to explain the loss of light responsiveness is that the production of available functional phytochromobilin is reduced as a result of the *dep* mutations, just as *hy1* and *hy2* do (Parks and Quail, 1991). However, immunoblots with PHYA apoprotein-specific antibodies indicate that *dep1, dep2* and *dep3* are not inhibiting accumulation of the receptor apoprotein, and the light-dependent rapid degradation of phyA in *dep* mutants, as in wild type, demonstrates that neither the chromophore biosynthesis or transport pathways, autocatalytic chromophore attachment, nor the phyA holoprotein's photoconvertibility to the labile Pfr form is affected substantively (Xu et al., 1995). Therefore, the *dep1*, *dep2* and *dep3* mutations are apparently affecting steps downstream of the receptors.

The most likely remaining explanations are that a signaling intermediate interacting with both phyA and phyB pathways is functionally sequestered, or that the Dep proteins themselves are downstream components of both phyA and phyB transduction chains. As detailed in the introduction, several examples of these kinds of components have already been found. In some cases, proteins interact directly with both phyA and phyB photoreceptors, while in other cases mutations in individual genes affect both phyA- and phyB-mediated responses.

There is considerable evidence that, at least in *Arabidopsis*, the functions of cryptochromes in plant photomorphogenesis overlap broadly with the functions of phytochromes (Lin, 2002). The direct interaction of CRY2 and PHYB has been shown by

yeast two hybrid and co-immunoprecipitation methods (Mas et al., 2000). CRY1 and COP1 direct interaction has been found in yeast two-hybrid assays (Wang et al., 2001). SUB1, as a component in blue light signaling pathway, functions as a modulator in phytochrome signaling pathway (Guo et al., 2001). While the hypocotyl lengths of *dep* mutants under B light were not significantly different from those of wild type (Figure 3), proportionally more *dep3* seedlings did have unexpanded cotyledons in Bc (Figure 5). Therefore, the *dep* mutants may also have a limited role in B light signal transduction, or they may alter the sensitivity to B under low fluence rate light conditions. This observation is being investigated further.

Deps play pleiotropic roles in the complex phytochrome signaling networks

DEP1, DEP2 and DEP3 are involved downstream of both phyA and phyB signaling networks. This is not surprising; there is ample evidence from *phyA* and *phyB* mutants (reviewed in (M鮄ler et al., 2002; Wang and Deng, 2003) and overexpressors (Boylan and Quail, 1991; Wagner et al., 1991; Short, 1999) that phyA and phyB signaling networks modulate overlapping, as well as unique, sets of responses. Subsequently, numerous individual mutants have been found that affect both phyA- and phyB-mediated pathways [e.g. *hy5* (Koornneef et al., 1980); *pef1* (Ahmad and Cashmore, 1996); *psi1* (Genoud et al., 1998); *dfl1* (Nakazawa et al., 2001); and *shl* (Pepper et al., 2001)] or that bind to both phyA and phyB [PIF3 (Ni et al., 1998); PKS1 (Fankhauser et al., 1999); NDPK2 (Choi et al., 1999)].

Based on the strong deficiencies of *dep3* in FRc-controlled responses, DEP3 is necessary for light-mediated signaling, especially in FRc. The effect of *dep3* on FRc-regulated responses is in many respects comparable to that in *phyA* null mutants (Figure 3, 5), but unlike *phyA*, *dep3* seedlings also showed unfolded apical hooks. In addition, *dep3* seedlings also exhibit weak deficiencies in some Rc-regulated responses such as inhibition of hypocotyl elongation in Rc (Figure 3), closed cotyledons under Rc (Figure5), Rc-mediated gravitropic response (Figure 6, 7), and Rc-regulated petiole elongation (Figure 8). These data are inconsistent with *dep3* behaving identically with *phyA* null mutants. *dep3* also accumulates normal levels of PHYA apoprotein and the phyA can be photoconverted to Pfr for subsequent ubiquitination-dependent degradation. All these data suggest that *dep3* is different from previously reported *phyA* mutations.

Among the phyA downstream components found so far, *hy5* exhibits strong deficiencies as *dep3* did in FRc-regulated hypocotyl elongation and unfolded cotyledon expansion, but *hy5* also showed strong deficiencies under red and blue light that are different from *dep3* mutant (Oyama et al., 1997). Among the described phyA-signaling components, only *dep3* shows strong deficiencies in FRc and weak deficiencies in Rc-regulated responses, making this mutant phenotypically distinct from known phyA downstream positive regulators such as FAR1, FHY1, FHY3, FIN5, PAT1 and FIN219 (Hudson et al., 1999; Bolle, 2000; Hsieh et al., 2000; Wang and Deng, 2002; Cho et al., 2003).

As more members in the phytochrome signaling web have been characterized at the molecular level, some components are involved in both phyA and phyB pathway, such as NDPK2 (Choi et al., 1999), PIF3 (Ni et al., 1998), and PKS1(Fankhauser et al.,

1999). But *pif3* shows short hypocotyl length under Rc and FRc (Kim, 2003), which is different from the defects caused by *dep1* and *dep2* mutants. *ndpk2* mutant displays a partial defect in responses to both R and FR including cotyledon opening and greening (Choi et al., 1999). PKS1 over-expressing line shows less sensitive to B and FR light (Fankhauser et al., 1999). Like *dep1* and *dep2,* these three mutants only affect some of the light-regulated responses, and showed weak deficiencies in most responses tested. This provides evidences to understand the position that DEP1 and DEP2 proteins hold in phy signaling as they might function as common factors in both phyA and phyB signaling. But *dep1* and *dep2* are different from NPDK2, PIF3 and PKS1 by their mapping positions. On the other hand, based on the weak deficiencies of *dep1* and *dep2,* it is also possible that they function at a branched pathway in the complicated light signaling networks, and block of only one branch only leads to leaky phenotypes like dep1 and dep2 mutants do.

To determine what roles DEPs play in phytochrome signaling, we need to further characterize and clone these genes. By examining the protein sequences and components that interact with each of the DEPs, it may be possible to link them into an emerging model of light signaling in plants.

Chapter III. Missense Mutations in the Chromophore Binding Domain of Phytochrome A May Impair the Photoconversion of P_{fr} to P_r

As described above, isolating and characterizing new signaling components is still necessary to figure out the entire phytochrome signaling network. By screening for seedlings with intermediate length hypocotyls under Wc, we isolated a group of mutants and characterized three of them, designated *dep1*, *dep2* and *dep3*.

Under Wc, Rc, and FRc, *dep1* and *dep2* showed slightly deficiencies in several Rc and FRc regulated responses during both young seedling development and mature plants photomorphogenesis while comparing those with wild type. They also showed slight deficiencies in some of B regulated responses. These results indicated that DEP1 and DEP2 play pleiotropic roles involving in more than one photoreceptor signaling system. Because of their weak phenotypes, it was impractical to map or clone *DEP1* and *DEP2* genes in order to assign them with specific functions, or to locate their exact positions in light signaling networks

On the other hand, *dep3* shows comparable weak deficiencies to *dep1* and *dep2* under Wc and Rc, but it exhibits much stronger defects in FR light-regulated responses. The effects in FRc are nearly as strong in *dep3* as they are in *phyA* null mutants. This finding suggests that DEP3 plays an important role in phyA signaling. The fact that *dep3* displayed deficiencies under Wc, Rc, and FRc also provides further evidence that different signaling pathways from different photoreceptor overlap with each other, and form complicated signaling networks. The photoreceptors may control some common signaling pathway components to regulate the same or similar photoresponses in redundant, synergistic or antagonistic ways.

To continue characterizing *dep3*, phyA-specific biochemical and physiological responses were investigated to further define the roles of DEP3 in phyA signaling. Because of the extremely long hypocotyls of *dep3* mutants under FRc and its completely recessive genotype, it was possible to select F2 homozygous seedlings reliably for fine mapping the *DEP3* gene within one BAC and eventually to clone the *DEP3* gene.

A. Materials and Methods

Plant Materials and Growth Conditions are described in the materials and methods in the previous chapter.

Analysis of Anthocyanin Content in Seedlings

After surface sterilization, seeds from each line were sown on two sets of plates with MS + vitamin medium supplemented with 2% sucrose. Seeds were stratified at 4°C for 3d. After inducing seed germination by exposing plates to Wc for 3h, and returning the plates to darkness for 21h, one set was transferred to FRc (9.7 μmol m^{-2} sec^{-1}) at 24°C and the other set to darkness at 24°C for 2d. Subsequently, anthocyanins were extracted from seedlings by the acidic methanol method of Schmidt and Mohr (1981), and the total amount of anthocyanins was quantified spectrophotometrically. Anthocyanin content per seedling was calculated according to the following formula:

$(A_{530}-0.25*A_{670})$/number of seedlings

At least fifty seedlings were used per extraction and each data point represents the mean of at least three independent experiments.

Total RNA extraction and RT-PCR

After inducing germination, seedlings of *dep* mutants and No-O grown for 2d in FRc were collected and weighed, then frozen in liquid nitrogen. The total RNA was isolated from the frozen seedlings by using a TOTALLY RNA^{TM} Kit for Isolation of Total Cellular RNA (Ambion, Austin, TX) using the manufacture's instructions. The total RNA concentration was measured spectrophotometrically (A_{260}), and the quality of the RNA was checked by gel electrophoresis. Reverse transcription (RT) for RT-PCR was done with the two-step RT-PCR using the RETROscript kit (Reverse transcription for RT-PCR; Ambion, Austin, TX) according to the manufacture's instructions. The RT step employed oligo(dT) as primer, and the second step used nested primers specific for each target gene. All the primers were designed to span at least one intron, so as to exclude amplification of contaminating genomic DNA and confirmed by sizing the RT-PCR products using agarose gel electrophoresis. The actin control forward primer (GGTTGGATCCTCCATTTCTCTATCTTTC) and reverse primer (GGTTAAGCTTCATGAGTGAGTCTGTGAG) are expected to amplify a RT-PCR product of 654bp and genomic DNA product of 1374bp; the chalcone synthase (CHS) forward primer (GGTTGGATCCCTGAGAACCATGTGCTTCAG), and reverse primer (GGTTGGATCCGGAGGTAGTGCAGAAGACG) yield a RT-PCR product of 324bp and genomic DNA product of 410bp; the chalcone isomerase (CHI) forward primer (GGTTGGATCCTCAACAGAGTGATCACTAAC), and reverse primer (GGTTGAATTCCAACCGACTCAACAATG) yield a RT-PCR product of 738bp and genomic DNA product of 1165bp; and the dihydroflavanol reductase (DFR) forward

primer (GGTTGGATCCTCGAGACTATACTTGAAG), and reverse primer (GGTTAAGCTTTGTTCGTGCCACCGTTCG) yield a RT-PCR product of 840bp and genomic DNA product of 1260bp.

Measurement of Total Chlorophyll

At least fifty seeds per genotype were plated in triplicate on MS + vitamin medium without sucrose. Cold treatment for stratifying seed to synchronize germination was done as described above. One set of plates was put under FRc (9.7 μmol m^{-2} sec^{-1}) at 24°C for 1d, another set for 2d, and the third set for 3d respectively, then transferred to Wc (75 μmol m^{-2} sec^{-1}) for 3d. Seedlings were collected and homogenized directly or treated by overnight shaking in darkness in 80% acetone for total chlorophyll extraction. After centrifugation to remove the debris, the total chlorophyll concentration in supernatant was determined spectrophotometrically, as described (Chory et al., 1991). Total chlorophyll concentration is calculated by chlorophyll a + chlorophyll b using the formula:

Chl a=$(12.7*A_{663})-(2.69*A_{645})$; Chl b=$(22.9*A_{645})-(4.48*A_{663})$

Each data point represents the mean of at least three independent experiments. Chlorophyll content was normalized to seedling number or to fresh weight as indicated below.

Biliverdin IX Rescue

Biliverdin IX hydrochloride (BV) was purchased from Frontier Scientific (P.O. Box 31 Logan, Utah), and an approximately 10mM stock solution was made in fresh DMSO. A portion was diluted into 5% HCl in MeOH to an approximate final concentration of 10μM, and the absorption peaks of the 10uM solution was measured. The expected peaks at 377 and 696 were used to determine BV concentration based on molar extinction coefficients of 66,200 M^{-1} cm^{-1} at 377nm, and 30,800 M^{-1} cm^{-1} at 696nm. The stock solution concentration was adjusted, and then diluted in MS medium at a concentration at 0.1M for chromophore supplementation experiments.

Mapping

Plants of *dep3* were outcrossed to Columbia-O (Col-O) and Landsberg *erecta* (Ler) ecotypes. F2 progeny were screened for long hypocotyls under 3d FRc and for the ability to produce chlorophyll after transfer to 3d Wc. Over a thousand seedlings meeting these criteria were transferred to soil and grown for two weeks before a leaf was removed for DNA extraction. F3 plants were rescreened to confirm the homozygosity of their F2 parents. A slightly modified version of the method by Dellaporta (Dellaporta, 1983) was used to extract the total genomic DNA. The DNA isolated from the leaves of plants lacking segregation in the F3 generation were used for mapping by PCR-based simple sequence length polymorphism [SSLP; (Bell, 1994)], cleaved amplified polymorphic

sequences [CAPS (Konieczny and Ausubel, 1993)], and single nucleotide polymorphism [SNP (Ye, 2001)] methods.

Amplification of Full Length Gene Fragments

Eight sets of the forward and reverse primers were designed for each candidate gene including at least one thousand base pairs of upstream and one thousand base pairs downstream of the gene to maximize inclusion of regulatory elements. Primers were designed using a web-based program at:

http://alces.med.umn.edu/websub.html

An ApaI or a SmaI site was engineered onto the 5' end of forward and reverse primers, respectively, along with 3 to 5 additional nucleotides to improve subsequent restriction digestion and cloning of the amplified genes. Each set of primers was used to run PCR reactions with BAC-F14J9 as template to amplify the target fragments with the Takara *LA Taq*™ proofreading DNA polymerase mix (PanVera, Madison, WI).

The amplified fragments form PCR reactions were ligated into T-easy vector (a vector designed to clone the PCR fragment [pGEM-T and T-easy vector system (Promega, Madison, WI)]), and the ligation products were used to transform JM109 competent cells by heat shock (Sambrook et al., 1989).

JM109 and DH5α *E. coli* strains were grown in sterilized LB (10g tryptone, 5g yeast extract, 5g NaCl per liter) medium containing selective antibiotic ampicilin (50ug/ml) for JM109 or kanamycin (50ug/ml) for DH5α. The LB plates supplemented with 1% agar were cultured at 37°C overnight to screen for positive colonies.

Positive clones were selected on plates with X-gal (50μg/ml) and IPTG (20μg/ml) for loss of β-galactosidase activity for further screening. Only those white colonies that were shown to contain the target insertions by PCR screening were picked for growth in liquid culture, and transferred to LB liquid medium with antibiotic grown at 37°C while shaking at 250 rpm overnight prior to plasmid extraction. Plasmids were extracted by *Wizard plus SV miniprep DNA purification system* (Promega, Madison, WI). The inserted fragments were excised with ApaI and SmaI for later insertion into the pGreen binary vector (Hellens et al., 2000). For each gene, at least three individual clones were selected to minimize the chance of propagating a PCR amplification error. The fragments were then religated to the pGreen (www.pGreen.ac.uk) binary vector, and transformed by heat shock to DH5α competent cells generated by the $CaCl_2$ method (Sambrook et al., 1989). Positive colonies were screened and plasmids were extracted as above. Those pGreen vectors with the genomic fragment insertions, along with pSoup plasmid required for pGreen maintenance and virulence of the host *Agrobacterium*, were used to co-transform *Agrobacterium tumefaciens* strain GV3101 (Koncz, 1986) by electroporation with Bio-Rad and Gene Pulser II with the Capacitance Extender Plus module (Hercules, CA). Positive clones identified on kanamycin (50μg/ml) and tetracycline (5μg/ml) were used to transform *dep3* mutant *Arabidopsis* plants as described below.

Plant Transformation

Agrobacterium was cultured in sterilized LC (10g tryptone, 5g yeast extract, 8g NaCl per liter) medium containing selective antibiotics kanamycin (50ug/ml) and

tetracycline (5ug/ml). Plates containing LC plus 1% agar were grown for 3d at 28°C to screen for positive colonies.

Liquid culture of *Agrobacterium* was typically started by picking a positive clone from each construct, growing in 250ml liquid LC medium with 50ug/ml kanamycin and tetracycline (5ug/ml) overnight (roughly 18 hours). Cells were harvested by centrifuge at 5000g for 20 min at room temperature, then resuspended in a solution containing of 5.0% sucrose and 0.02% Silwet L-77 (Lehle Seeds, Round Rock, TX) to a final cell density of approximately 0.80 OD_{650} prior to use.

The plants selected for *Agrobacterium* transformation had their initial bolt removed to encourage multiple bolt stems. After about 3d, new bolts were dipped in the *Agrobacterium* inoculum in a 50ml falcon tube. Plants were inverted into the suspension such that all above ground tissue were submerged, and plants were removed after 3-5 sec with gentle agitation. Plants were covered with domes, and allowed to recover overnight in low light intensity. Domes were removed approximately 24 hours after the treatment. After 7d growth, the same plants were dipped again using an identical inoculum, but with 0.04% Silwet L-77. Plants were grown for about four weeks until siliques were brown and dry, and seeds were harvested.

Selection of the Putative Transformants

Seeds harvested from the potentially transformed plants were plated on a 0.8% agar medium containing MS + vitamins supplemented with 50ug/ml kanamycin. Seeds were stratified and induced to germinate as described previously, then exposed to FRc

(9.7 umol m^{-1} sec^{-1}) at 24°C for 3d. Transformed plants scored by the kanamycin resistant phenotypes were screened for rescue from the *dep3* phenotypes. Those that grew tall and greened after one week in Wc lacked rescue, whereas those that were fully de-etiolated but could not green in Wc were restored to the wild type phenotype.

Phytochrome Extractions and Spectrophotometric Assays

Fresh or frozen *Arabidopsis* seedlings grown in darkness for 4d were rapidly homogenized in equal volume of cold extraction buffer (100mM Tris-HCl, 140mM $(NH_4)_2SO_4$, 50% ethylene glycol, 5mM EDTA, and 0.5 mM PMSF [pH 8.3]), with 10% v/v pH7.8 polyethylenimine (PEI), then centrifuged at 25,000 g for 15 min, and the supernatants were precipitated by chilled saturated $(NH_4)_2SO_4$ to a final concentration of 36%, left on ice for 1~2 min, and centrifuged at 25,000 g for 20 min. The pellets were resuspended in Resuspension Buffer (50mM Tris, pH7.8, 5.0 mM EDTA 10mM iodoacetamide [added immediately before use]), and were used for spectrophotometric scans using a dual wavelength spectrophotometer (AVIV UV-VIS-IR Model 14DS, Beckman Coulter, Fullerton, CA). The absorbance scan measurement for Pfr was taken after the micro-cuvette with 50µl phytochrome-enriched crude extract was exposed to a saturating R pulse for 60 sec, and the measurement for Pr was taken after a saturating FR treatment for 120 sec. The R and FR were generated by LED light sources as in Plant Materials and Growth Conditions.

Sequencing of *PHYA* gene and At09575

Genomic DNA was isolated from wild type No-O and the *dep3* mutant. A set of forward and reverse primers was used to amplify entire *PHYA* genes. The PCR products were purified with the PCR Purification kit (Qiagen USA, Valencia, CA), and then inserted in pGEM-T vector as described previously. For each genotype, two independent clones were identified and their plasmids extracted to serve as template for sequencing. A series sequencing primers were designed based on the PHYA sequence from TAIR data base, and the cloned genes sequenced at the Rockefeller DNA Sequencing Center.

B. Results

I. *dep3* Has Altered Seed Germination under HIR-FR and Wc

In *Arabidopsis*, photo-reversible induction and inhibition of seed germination is mediated by phytochrome. Dormant seeds germinate poorly when they are imbibed in darkness, and the germination rate is affected by the Pr:Pfr ratio of phyB stored inside the seeds, which is determined by the light regime under which the parent plant was grown (McCullough, 1970). By contrast, nearly 100% of seeds germinate when put under Wc and Rc (Shinomura et al., 1994). The germination process is solely regulated by phytochrome, and dominated by phyA and phyB. Among them, phyB principally regulates seed germination in darkness and in continuous irradiation with W and R light, but phyA plays the role of regulating seed germination under the VLFR and FR-HIR conditions.

dep mutants were examined for deficiencies in phyA-regulated seed germination. After sterilizing and plating the seeds from different lines, but before putting in 4°C darkness for stratification, seeds were irradiated with 3h FRc to convert nearly all the Pfr form of phyB stored inside the dormant seeds to Pr. After stratifying, the plates were placed directly under FRc for 1d or 1.5 d so that the effect of phyB on seed germination was minimized. This treatment allows the effect of newly synthesized phyA to be tested by subsequent FR treatments (Figure 11a,b). After 1d FRc treatment, *dep3* seeds did not show any germination at all, while *phyA* seeds germinated with a rate of less than 10%.

Under the same conditions, 40-60% of the seeds from wild type, *dep1*, *dep2*, *hy1*, *phyB*, and *hy5* germinated. By extending the FRc to 1.5d, 30% of *dep3* seeds germinated, while more than 80% or more of the seeds from other lines germinated. This result indicates that under FRc the seeds from *dep3* showed a significant delay in seed germination and a low germination frequency. Thus, *dep3* is more strongly affected than *phyA* null mutants, although both of them displayed a slowed germination rate compared with that of the other lines under the conditions tested (Figure 11a, b).

To determine whether the effect *dep3* mutant is specific to FRc, germination rates from different lines under Wc light for 1d and 1.5d were also tested (Figure11 a, b). The *dep1* and *dep2* seeds did not show a significant difference compared with wild type, although *dep2* show a slightly delayed seed germination with the one-day treatment. As expected, *hy1* and *phyB* seeds had lower germination rates after the one-day treatment. But after one and a half day, all lines had reached more than 80% germination except the *dep3* line. The *dep3* seeds showed very poor seed germination, no matter one day Wc with less than 20% germination rate or one and a half day Wc treatments with about 50% germination rate. Even after extended growth in Wc, dep3 seeds did not germinate fully (data not shown).

II. *dep3* Has Reduced Anthocyanin Accumulation

The accumulation of anthocyanins in juvenile plants is strictly regulated by light, and is amplified by exogenous sucrose (Kunkel et al., 1996; Mita et al., 1997; Short, 1999). The process is not only an important defensive response for plants reacting to

environment signals, but also a useful process to investigate the phyA signaling networks. In young *Arabidopsis* seedlings, anthocyanin accumulation is predominantly controlled by phyA. phyB cannot induce the accumulation of juvenile anthocyanins, whereas phyA can act independently of other photoreceptors for this process. The presence of functional phyA and complete phyA signaling pathway is required for *Arabidopsis* seedlings to accumulate anthocyanins under FRc (Kunkel, 1996). Therefore, anthocyanin pigment accumulation can serve as a marker to characterize components specifically in phyA signaling. To examine whether *deps* show any deficiencies in this metabolic response, we tested the anthocyanin accumulation of each *dep* mutant and other photomorphogenic mutant lines, and compared their pigment levels with those of wild type.

After inducing seed germination, seedlings of wild type and different mutants were grown in FRc or darkness for 2d, and the quantities of anthocyanin accumulated in FR light were measured spectrophotometrically and normalized to seedling number (Figure 12). FRc strongly induced the accumulation of anthocyanin in both No-O and RLD wild type seedlings. Both *dep*1 and *dep*2 also accumulated levels of anthocyanins not significantly different from those of wild type. However, *dep3* displayed significantly lower levels of anthocyanins, and closer to those in *phyA* null mutants. All the mutants and wild types accumulated very low levels of anthocyanins in darkness. These results indicate that *dep3* blocks phyA signaling strongly, whereas neither *dep*1 nor *dep*2 could significantly affect this branch of phyA signaling. None the tested lines accumulate anthocyanins under darkness, so the deficiencies shown here are FR-dependant.

Figure 13 shows a simplified version of the anthocyanin biosynthetic pathway. There are at least four key enzymes known to control this pathway. They include

phenylalanine ammonia-lyase (PAL), the first enzyme in the general phenylpropanoid pathway; chalcone synthase (CHS), the first enzyme in the flavonoid pathway; chalcone isomerase (CHI), the second enzyme in the flavonoid pathway; and the dihydroflavonol reductase (DFR), the first enzyme leading to the production of anthocyanins (Kubasek et al., 1992; Noh and Spalding, 1998). *Arabidopsis* contains at least three genes that encode PAL1, PAL2, and PAL3. Among them, PAL1 is induced by wounding and pathogen attachment, and is expressed in a tissue-specific manner in *Arabidopsis* seedlings (Ohl et al., 1990). The three genes in *Arabidopsis* that encode PAL provide a point for carbon to enter the anthocyanin biosynthetic pathway. Seedlings grown in darkness express *PAL1* mRNA, but mRNA levels of *CHS*, *CHI* and *DFR* are very low (Kubasek et al., 1992). The late pathway enzymes are all represented by single genes in the *Arabidopsis* genome.

We tested the mRNA levels of *CHS*, *CHI* and *DFR* of different mutant seedlings growing under 2d FR, and compared them to those of the wild type by RT-PCR. The mRNA level of the constitutively expressed *ACTIN* gene was used as control and for equal loading (Figure 14). Despite showing wild type levels of anthocyanin, *dep2* displayed a distinct decrease in both CHS and CHI mRNA levels compared with those of wild type, while dep1 was only affected in CHI message accumulation. *dep1* actually expressed higher levels of CHS mRNA than did wild type. On the other hand, *dep3* seedlings expressed very low levels of CHS, CHI and DFR messages, consistent with the low anthocyanin content of the seedlings.

III. The FR-preconditioned Greening Response is Defective in *dep3*

Although wild type *Arabidopsis* seedlings de-etiolate morphologically in FRc light, the chloroplast development process cannot be completed in this condition. This effect is active resulting in the inability of cotyledons to green even upon subsequent transfer to W. The severe repression of protochlorophyllide oxido-reductase (POR) genes by FRc coupled with irreversible plastid damage led to temporal separation of phytochrome-mediated POR repression from light-dependent protochlorophyllide reduction, but both processes can occur coordinately in Wc (Barnes et al., 1996). Recent studies on the key chlorophyll synthesis gene *HEMA1* demonstrates that the FR block of greening comprises two separate responses: a white light intensity-independent response that requires three days far-red and is associated with a loss of expression of the nuclear genes *HEMA1* and *LHCB* following the transfer to white light (a transcriptionally coupled response), and a white light intensity-dependent response that is induced by 1d FR and is transcriptionally uncoupled. Both pathways lead to the loss of nuclear gene expression (McCormac and Terry, 2002). In contrast, seedlings initially grown in darkness without FRc pre-illumination can green within 1 or 2d after the onset of illumination with white light. It has been demonstrated that this far-red preconditioned block of greening is dependent on functional phyA and an intact phyA signal transduction pathway. Hence, *phyA* null mutants or mutants in components downstream of *phyA,* for example *fhy1* and *fhy3*, are capable of greening after transfer from FRc to Wc, whereas the wild type cannot green and will die.

To characterize further the range of phenotypes in the *dep* mutants, we examined whether they can prevent the FRc-preconditioned block of greening response. After inducing germination, seedlings of wild type and mutant lines were illuminated for 3d with FRc and transferred to Wc for another 3d. Total chlorophyll from seedlings of each line was extracted and quantified spectrophotometrically (Figure 15). None of the wild-type seedlings could green, while a small proportion of *dep1* and *dep2* seedlings green fully and develop some chlorophyll after transferring to white light. However, all the *dep3* seedlings were fully able to green, as the seedlings of the *phyA* mutants did under the same conditions. When the green *dep3* seedlings were transplanted to soil, they all grew to maturity and produced viable seeds. The wild type and most of the *dep1* and *dep2* seedlings died from their inability to produce chlorophyll. In contrast, the seedlings grown in 3d darkness following 3d W-light treatment were able to green fully regardless of genotype. To exclude the possibility that the *dep1*and *dep2* seedlings might exhibit a shift in the sensitivity to FR light, FRc treatments of 1d or 2d instead of 3d were tested (Figure 15). The result showed that 1d FRc treatment was not sufficient to block the seedlings greening response in *dep1*, *dep2* or wild type after illuminating with Wc. However, 2d FRc prevents most of the wild type, as well as *dep1* and *dep2* mutant seedlings from greening. On the other hand, *dep3* seedlings green fully after 3d Wc. This means that both *dep1* and *dep2* mutants did not show any significant decrease of their sensitivity to FRc, but *dep3* did.

IV. *dep3* Phenotypes Are not Rescued by Exogenous Biliverdin Chromophore

While *dep1* and *dep2* mutations resulted in weak deficiencies in the phyA-specific responses, *dep3* mutation yields very strong deficiencies in those responses, and the deficits are comparable to those observed in *phyA* null mutants. Therefore, we hypothesized that *dep3* may contain defects either in the *PHYA* gene or in downstream components in phyA signaling pathway, which block signaling or affect the expression of the *PHYA* gene. Because of the phenotypes observed in R, it was also possible that the altered light responses could also be caused by mutation(s) that affect functional chromophore production. It has been reported that exogenous biliverdin (BV), a direct precursor to phytochromobilin, is biologically functional in associating with the phytochrome apoprotein, and can rescue the deficiencies of *hy1* and *hy2* (two chromophore synthesis pathway mutants) mutations (Parks and Quail, 1991).

To address the later possibility, we tested whether exogenous BV could restore the wild type light-grown phenotypes to the *dep* mutants (Figure 16). Seedlings of No-O, *dep1, dep2, and dep3*, and *hy1* were sown on the medium with BV (+BV row) or without BV (-BV row), and grown in FRc for 4d. The *hy1* mutant was used as a positive control. As expected, *hy1* seedlings growing on the medium without supplemented BV displayed etiolated phenotypes including elongated hypocotyls, closed apical hooks, and folded and unexpanded cotyledons. The *hy1* seedlings growing with added BV displayed partially restored photomorphogenesis with shorter hypocotyls, opened apical hooks, and well expanded cotyledons. On the other hand, BV

failed to rescue the deficiencies of *dep* seedlings, with *dep1* and *dep2* seedlings still growing slightly taller than the wild type seedlings, and *dep3* seedlings growing extremely tall and with closed cotyledon. The seedlings of *dep*s growing on BV containing medium showed the same phenotypes as the seedlings growing on the medium without supplemental BV.

V. Fine Mapping of *dep3*

Based on linkage to SSLP based markers that we showed in previous chapter, the *DEP1* gene maps to the north arm of chromosome 1 between SSLP markers nga63 and Athso392, *DEP2* maps south arm of chromosome 4 between CAPS marker AG11 and SSLP maker nga1107, and *DEP3* maps the north arm of chromosome 1 near SSLP nga63. Because the weak phenotypes of *dep1* and *dep2* mutants and the natural variability of wild type, screening for F2 homozygous seedlings proved too imprecise for reliable fine mapping even after re-screening the F3 seedlings. Therefore, we concentrated on the fine mapping *DEP3*.

To continue fine mapping *DEP3* and clone the gene, SNP method was used. As this method is easy to deal with many samples by only run PCR to check the F2 polymorphism, and there is an available program for designing primers. With the *Arabidopsis* whole genome sequencing finished in 2001, tons of SNP markers are available so that we have much more choices to find the desired markers for fine mapping.

Having narrowed the location of *DEP3* to a small region of Chromosome I, 1,112 F2 DNA samples were used for the fine mapping of *DEP3*, and the results are shown in Figure 17. The first SNP marker located within BAC- F7G19 at position of 6807, and revealed 16 out of 1066 recombinants, and indicating a position within 1.5 map units from *DEP3*. A second marker was within BAC-F21M12 at position of 28013 and yielded 1 out of 2224 chromosomes recombinant (<0.1 map unit). In addition, the DNA sample showing recombinant with F21M12-28013 is different from the DNA samples that showed recombination with F7G19-6807. These data suggested that *DEP3* is located between these two markers, and possibly within F21M12 (0-28013) or the adjacent BAC-F14J9. To further narrow the location of *DEP3*, two more markers within F14J9 at 49,374bp and 86,238bp were picked, yielded 3 out 1066 and 1 out of 2224 recombinants. In addition, the 3 recombinants DNA samples of F14J9-49374 are within the 16 DNA samples shown recombinants to F7G19-6807 and the DNA sample of the recombinant to F14J9-86238 is the same DNA sample that shows recombinant to F21M12-28013. The results indicated that *DEP3* is located between 49,374bp and 86,238bp within F14J9.

Within the region *DEP3* maps, there are eight genes or putative genes (Figure 17). These include At1g09510, cinnamyl-alcohol dehydrogenase (CAD); At1g09520, a putative expressed protein with unknown function; At1g09530, phytochrome interacting factor 3 (PIF3); At1g09540, MYB family transcription factor (MYB61); At1g09550, a putative pectinacetylesterase; At1g09560, germin-like protein (GLP4 and GLP5); At1g09570, phytochrome A (PHYA); and At1g09575, an expressed protein of unknown function.

VI. Cloning *dep3*

Each of the eight putative proteins corresponding to the region bounded by the flanking SNP markers described above was subcloned and used to transform the *dep3* mutant plants. The seeds from plants potentially transformed by *Agrobacterium* were plated on MS + Vitamin medium containing kanamycin (50mg/ml), and stratified, and induced to germinate. The seedlings were grown under FRc for 3d, and then transferred to Wc. The phenotypes for transformed and rescued plants were identified as described in methods. The transformants containing At05920, At05930, At05940, or At09550 could not rescue the *dep3* mutant phenotypes. Two of remaining genes, cinnamyl-alcohol dehydrogenase (CAD) and germin-like protein (GLP4) (GLP5),are primarily metabolic genes, and therefore unlikely to be involved directly in phytochrome signaling. Therefore, we chose to concentrate on the remaining genes and sequence the DNA corresponding to the putative protein and the *PHYA* gene from the mutant and wild type plants. No mutations were found in At09575 corresponding to putative protein.

On the other hand, At09570 (encodes phyA apoprotein) carried two point mutations in *dep3,* including a T to C change in codon 331 resulting in a missense mutation with Met to Val (M331V), eight amino acids downstream of the chromophore-binding site Cys_{323}. The second mutation is a G to A base change in codon 463 resulting in a second missense mutation of Pro to Ser (P463S) (Figure 18). The M331V mutation is located in the GAF chromophore-binding domain, found in the N-terminal half of the

phytochrome protein. Both M331 and P463 are highly conserved in all *Arabidopsis* phytochromes (Figure 18).

A large number of mutations within the *PHYA* gene have been reported and entered in GenBank (Figure 19). Most of them are clustered within the two PAS domains and Quail domain, all located in the C-terminal half of the protein. The mutations in *dep3* are different from those of any phyA mutants previously described. It is a novel mutants allele of *PHYA* mutants, which we have designated *phyA-401*.

Sequence comparisons with phytochromes from a variety of species reveal that both of the residues are highly conserved. Moreover, alignments indicate that the residues are not only highly conserved within higher plants (both monocots and dicots), they are also maintained in lower plant phytochromes and cyanobacteria. The M_{331} is even conserved in phytochromes isolated from fungi and red algae (Figure 20).

VII. *phyA-401* lacks photoreversible phyA

Previous western blotting data have shown that seedlings of *phyA401* do not show reduced expression of PHYA apoprotein compared with the PHYA level in wild type seedlings, and the phyA Pr in *phyA401* seedlings could be photoconverted to Pfr and undergo normal ubiquitination-mediated degradation. However, for normal function, phyA must be able to photoconvert back to Pr from the Pfr form. One of the missense mutations in *phyA401* is located at residue 331, eight amino acids downstream of the Cys at which the chromophore is attached to phytochrome. Because light-dependent degradation of phyA is only an indirect measurement of photo-

convertibility, and does not measure reversibility of the photoreaction, photo-reversibility of phyA in the *phyA-401* mutants and wild type seedlings were measured spectrophotometrically as well (Figure 21). Phytochrome-enriched extracts from No-O seedlings reliably yielded photo-convertible phyA in darkness, and showed typical light-dependent degradation kinetics within 4 h Rc. However, similar extractions from *phyA401* seedlings did not show the R/FR inter-conversion even in extracts from dark grown seedlings, as well as from seedlings from exposed to various Rc treatments.

Phytochrome-enriched extracts from *dep1* and *dep2* seedlings show apparently normal R/FR inter-conversion comparable to that seen in wild type extracts. The results indicate that both *dep1* and *dep2* seedling express light-convertible phyA and exhibit normal R light-dependent degradation (data not shown).

C. Discussion

PhyA-401 is a novel allele with mutations in the GAF domain and a phytochrome-specific domain that impairs the photoconversion of phyA from Pfr to Pr

Using a variety of screens, several *phyA* mutation have been isolated in other laboratories including *phyA-101, phyA-103-1, phyA-103 -2, phyA-103 -3, phyA-104, phyA-105, phyA-106, phyA-107, phyA-108, phyA-109, phyA-110, phyA-205, phyA-301, phyA-300D, phyA-302-1, phyA-302-2.* Among them, *phyA-101* has a single nucleotide transition that introduces a stop codon and leads to a null mutation with no detectable PHYA apoprotein. *phyA-110* contains an R to S missense mutation at position 279, just 44 amino acids upstream of the Cys323 where chromophore is attached covalently. Although the PHYA polypeptide is still present at wild type levels in *phyA-110* and it can also undergo light-induced degradation, it lacks photochemical activity. It has been proposed that the mutation disrupts reversible photoconversion of phyA from the Pfr to Pr form, but to date no experimental proof of this contention has been reported. It is still uncertain how the mutation in *phyA-109* leads to non-functional phyA, but it is clear that the phyA also lacks photochemical activity tested spectrophotometrically. All the other *phyA* mutants reported in the literature show normal expression of PHYA, their phyA is photochemically active as tested by spectrophotometry, and the aberrant phyA has normal *in vivo* protein turnover properties. Most of the lesions are clustered in the PAS domains or "Quail Box", and are thought to have selective effects in phyA regulatory

activities (Whitelam et al., 1993; Reed et al., 1994; Xu et al., 1995; Fry et al., 2002; Yanovsky et al., 2002). Here we report a novel mutant allele of *phyA*, designated *phyA-401*, in which Met_{331} (a residue highly conserved throughout plant and non-plant phytochromes) is changed to Val, and a second mutation in which a Pro_{463} (conserved among plant phytochromes) is converted to a Ser (Figure 18, Figure 19). Both of these amino acid changes are located toward the N-terminus with no change in the C-terminal half of the molecule.

Molecular and biochemical characterization of phyA indicates that the apoprotein folds into two major structural domains: a globular N-terminal domain cradling the covalently attached chromophore in a hydrophobic pocket; and a more extended C-terminal domain. A short flexible hinge in the core region connects the two domains (Quail, 1991; Quail, 1997; Park et al., 2000). Both of the amino acid changes in *phyA-401* are located in the N-terminal domain within the hydrophobic pocket (positions 1-673), which is sufficient for spectral integrity in the absence of the C-terminal putative signaling domains (Boylan and Quail, 1991; Cherry et al., 1993; Boylan et al., 1994; Cherry and Vierstra, 1994; Quail, 1997). It has been reported that the N-terminal domains of phytochromes A and B determine their photosensory specificity and differential light lability (Quail et al., 1996; Wagner et al., 1996; Park et al., 2000). The amino acid changes from Met to Val, which causes a change from a more extended side chain to a relatively small side chain, and from Pro to Ser, a change from structurally stiff amino acid with nonpolar side chain to an amino acid with a small uncharged polar side chain, might lead to a structural change of the N-terminal hydrophobic pocket. These changes could affect the three-dimensional structure of the holoprotein so that the *phyA-401* might

lock and stabilize the tetrapyrrole chromophore in its Pfr isomer without converting back to the Pr isomer even though under FR treatment, which results in *phyA-401* losing its photochemical reversibility, as the chromophore, not the PHYA protein, is what is being measured spectrophotometrically. It has been demonstrated in our experiments that the PHYA molecule in *phyA-401* is not inherently unstable, as the protein is detectable in darkness at wild type levels. Yet, the protein is degraded in a light-dependent manner, indicating that it can bind chromophore normally, and is at least initially photoconverted to the labile Pfr form (Figure 9).

Based on published biochemical, immunochemical, and physiological studies on extracted or purified phyA, the conformational changes between Pr and Pfr of phyA have been mapped in phyA to the N-terminal segment (between amino acids 37-136), to a segment around position 406 near the chromophore attachment site, to a segment in or near the linker region (between amino acid 684-731), and to a region around position 814 (Lagarias, 1985; Wong et al., 1986; Grimm et al., 1988; McMichael and Lagarias, 1990; Quail, 1991; Furuya and Song, 1994; Quail, 1997). The M331V change is very near the Cys_{323} chromophore attachment site. Therefore, it is quite possible that M331V affects the conformational change of the protein and leads to the observed deficiencies of *phyA-401*. This is the first report suggesting that Met_{331} and/or Pro_{460} play a role(s) in determining phyA photosensory function.

Like *phyA-110*, the *phyA-401* has wild type expression levels of PHYA apoprotein, and the apoprotein undergoes R-light-induced photoconversion and degradation as wild type phyA does after 4 hours R light exposure (Figure 9). But the normal FR-HIR responses require the continuous cycling of phyA between the Pr and Pfr

forms. If the Pfr of *phyA-401* does not convert back to Pr in FR, all of the *phyA-401* will quickly be converted to the Pfr form and stabilized in the Pfr form. This form is unstable and undergoes rapid degradation via the ubiquitination pathway, which causes a loss of the resource pool of phyA, and leads to the strong deficiencies of *phyA-401* reported here. The two residues, M_{331} and P_{461} are completely conserved in all the higher and lower plants for which sequences have been entered in the GenBank database, and are also conserved in the blue-green bacterium photoreceptor *Synechocystis spp.* phytochrome, and M_{331} is conserved in fungi and some bacterium phytochrome-like photoreceptors (Figure 20). This indicates M_{331} and P_{461} are probably necessary for maintaining phytochrome function.

Previous studies have shown that overexpression of only the N-terminal domain of phyA in transgenic *Arabidopsis* resulted in photochemically active but biologically defective chromoproteins, indicating the importance of the N-terminus for producing functional phyA (Boylan et al., 1994). Here, we also provide evidence that the N-terminus is necessary for maintaining the photochemical activity of phyA. The mutations in *phyA-401* do not affect its Pfr stability because the immunochemically detectable PHYA level in *phyA-401* after 2h or 4h Rc treatment do not show more rapid degradation kinetics or a reduced level of apoprotein than that from No-O wild type. This result coincides with previous reports that the N-terminal domain of phyA alone is light stable *in vivo*, and the C-terminus of phyA is required for Pfr degradation to proceed (Boylan et al., 1994; Wagner et al., 1996). The fact that *phyA-401* has a normal C-terminal domain explains why the phyA in *phyA-401* does not degrade faster than that of wild type after R irradiation. We propose that *phyA-401* has a normal regulatory core region including the

two PAS domains and "Quail box" for signaling activities, and in C-terminal-mediated degradation because the phytochrome sequence is not mutated within these regions.

Based on the physiological responses tested, the *phyA-401* mutants had partially functional phyA that can lead to opened apical hooks, less elongated hypocotyls in FRc, slightly higher anthocyanin levels (Figure 12), and marginally higher sensitivity to the FR-preconditioned block of greening response compared with those of the *phyA-101* null mutant (Figure 15). This slight phyA responsiveness might be explained as the initial functional signaling by the Pfr form of phyA inducing physiological responses before the phyA is lost to degradation. All these experiments indicate that *phyA-401* does not abolish all functional phyA as *phyA* null mutants do, but rather that they may lack a sufficient Pr and Pfr pool of phyA over time.

If mutations in *phyA-401* lead to a constitutively active form of phyA, it may be very useful for future research to investigate the functions of phyA in eliciting responses that are not detectable with normal phyA and known *phyA* mutants. For example, over experiments show some Rc deficiencies of *phyA-401* that were not found with other *phyA* mutations; previous alleles of *phyA* null mutants did not show deficiencies but wild type phenotype under Wc light (Nagatani et al., 1993; Whitelam et al., 1993). However, experiments with overexpressers of phyA or phyB have shown that phyA can antagonize phyB responses and *vice verse* (Short, 1999; Hennig et al., 2001).The two amino acids mutated in *phyA-401* are highly conserved, especially for the Met_{331}. Therefore, these mutants could be important for investigation of evolution and other photoreceptors' functions in various species.

phyA-401 yields more severe defects in seed germination compared with the _phyA-101_ null mutant.

As mentioned above, *phyA-401* mutants display fewer or less severe deficiencies in most tested phyA-regulated responses than those of *phyA* null mutant, but this determination does not apply to seed germination. *phyA-401* mutants display a delay of germination and lower germination frequencies than those of *phyA* null mutants under Wc and FRc, indicating a role for multiple phytochrome signaling.

Both phyA and phyB are involved in inducing seed germination under Wc, although phyB plays the principle role in inducing seed germination (Shinomura et al., 1994). A small pool of phyB is stored in seeds, and its form is dependent on the light conditions under which the parent plant was grown. To limit the effect of phyB stored inside of dormant seeds, we first gave the seeds 3h FRc to convert existing phyB to its Pr form before transferring them to darkness at 4°C. After 3d stratification, the seeds were put directly into FRc. The results from these measurements indicated that the seed germination of *dep1* was not apparently affected, that *dep2* showed a slight delay of seed germination that might be caused by *dep2* affecting phyA signaling, but that *phyA-401* mutants showed a significant delay and lower frequencies of germination compared with that of the wild type in both Wc and FRc (Figure 12).

In addition, the defect is even stronger in *phyA-401* than in *phyA* null mutants, in both Wc and FRc. If *phyA-401* were only affected in the photoconversion of Pfr to Pr, and the Pfr undergoes rapid degradation, *phyA-401* would be expected to exhibit a similar germination rate to the *phyA* null mutant, or even a slightly higher germination rate

because of the brief window in which phyA Pfr is abundant. This result suggests that *dep* mutants may be affected not only in phyA signaling, but also in other signaling pathway(s). The other pathway affecting this germination response is not likely to be phyB signaling because the FRc treatment just after plating the seed would be expected to convert phyB to its inactive Pr form, and it has been reported that phyB plays an antagonistic role to phyA in FR-mediated seed germination (Shinomura et al., 1994). If the phyB pathway were affected in FRc-induced seed germination, *phyA-401* should yield a higher germination frequency than that of *phyA-101* (Shinomura et al., 1994).

On the other hand, besides phyA and phyB, phyE also is involved in the process of seed germination in *Arabidopsis* seed germination, and that phyE is required to induce seed germination in FRc, as well as being directly involved in R/FR-reversible seed germination (Hennig et al., 2002). To explain the strongest defects of *phyA-401* in germination induction, one possibility is that the two pathways of phyA and phyE may share some common downstream components, and that the Pfr form of phyA can still binds these downstream components and either maintain them in non-productive complexes or targeting them for degradation such that they display dominant negative effects to delay the seed germination rate and reduce the frequencies of germination.

It has been reported that gibberellins also can induce seed germination even in phytochrome deficiency mutants (Yang et al., 1995). The dominant negative proposal may apply to this pathway as well.

Another formal possibility is that another mutation unrelated to phytochrome is responsible for an overall lower germination under all light conditions.

phyA-401 exhibits defects in Rc-regulated responses

The finding that *phyA-401* mutants also had longer hypocotyls than those of wild type and *phyA* null mutants under Rc is inconsistent with *phyA-401* affecting solely phyA signaling. Similarly, a consistently high percentage of seedlings of *phyA-401* displayed closed cotyledons in Wc and Rc (Figure 5). *phyA-401* seedlings have reduced regulation of gravitropic responsiveness under Rc (Figure 6), as do *phyB* but not *phyA* null mutants (Poppe et al., 1996). This apparent involvement of *phyA-401* in phyB-mediated responses in seedlings can also be observed in mature plants: the *phyA-401* mutants produce significantly longer petioles than the wild type, although the difference is small (Figure 8).

That *phyA-401* exhibits defects in both phyA- and phyB-regulated responses could be interpreted several ways. One formal possibility to explain the loss of light responsiveness is that the production of available functional phytochromobilin is reduced as a result of the *dep* mutations, as occurs in the *hy1* and *hy2* mutants (Parks and Quail, 1991). However, immunoblots with PHYA apoprotein-specific antibodies indicate that *phyA-401* plants are not inhibited in the accumulation of the receptor apoprotein. Moreover, the light-dependent rapid degradation of phyA in *phyA-401* mutants, comparable to that in wild type demonstrates that neither the chromophore biosynthesis or transport pathways, autocatalytic chromophore attachment, nor the phyA holoprotein's photoconvertibility to the labile Pfr form is affected substantively (Xu et al., 1995). This contention is further supported by the failure of BV to rescue the *phyA-401* phenotype (Figure 5).

Another possibility is that the Pfr of *phyA-401* could still bind to some common downstream components for both phyA and phyB, for example PIF3, PSK1, or NDKP2, and either restrict their functions or target them for degradation such that they display dominant negative effects in Rc. This hypothesis might also explain the observed elongated petioles (Ni et al., 1998; Fankhauser et al., 1999).

It has been reported that PIF3 interacts with both phyA and phyB in the Pfr form. However, PIF3 also interacts with the N-terminal domain, and binds more strongly with full length phytochrome than with the C-terminus alone, indicating that the associations of PIF3 to the N- and C-termini are synergistic (Ni et al., 1998, 1999). PKS1 can bind to phytochrome both in the Pr and the Pfr forms, but PKS1 is phosphorylated on serine and threonine residues in a R-dependent manner, and is thought to be a negative regulator for phytochrome signaling (Fankhauser et al., 1999). NDPK2 binds to the Quail box of phytochrome preferentially in the Pfr form (Choi et al., 1999). It is surprising that phospho-PKS1 is subsequently released from the photoactived Pfr of phytochrome, which can then associate with both PIF3 and NDPK2 (Smith, 1999). It is possible that one of these phytochrome-associated proteins or another phytochrome-binding protein becomes non-productively associated with the mutant *phyA-401*, preventing its productive association with phyB.

SUMMARY

The deficiencies exhibited in *dep1, dep2* and *dep3* mutants are summarized in following table:

Responses tested	*dep1*	*dep2*	*dep3*	Photoreceptor(s) controlling the response
Inhibition of hypocotyl elongation	Yes	Yes	Yes	phyA, phyB
Apical hook unfolding	No	No	No	phyA, phyB
Cotyledon opening and expansion	Yes	Yes	Yes	phyA, phyB
Rc-mediated gravitropism	Yes	Yes	Yes	phyB
Germination in Wc	No	Weak	Yes	phyA, phyB
Petiole length	No	No	Yes	phyB
Flowering time	Yes	Yes	Yes	phyA, phyB
Germination in FRc	No	Weak	Yes	phyA
FR-preconditioned loss of greening	No	Weak	Yes	phyA
Anthocyanin accumulation	No	Weak	Yes	phyA

The above table summarizes the results showing that *dep1* and *dep2* exhibit deficiencies in both phyA and phyB-mediated light responses, with *dep2* affected more broadly than *dep1*. This analysis suggests that DEP1 and DEP2 are two components that function in both phyA and phyB signaling, and that DEP2 may function upstream of DEP1, or that DEP1 and DEP2 may feed into different subsets of signaling pathways.

phyA-401 (*dep3*) shows several response phenotypes that differ from those of previously described *phyA* mutant alleles. The physiological characterization of *dep3* reveals distinct responses from *phyA-101* in two major respects. 1) Several responses are

less affected by the *phyA-401* mutations than by the null mutation in *phyA-101*. For example, the *dep3* seedling hypocotyls are slightly less elongated than are those of the null *phyA* in FRc, they display opened apical hooks, and are more susceptible to the FR-preconditioned block of greening and accumulate more anthocyanins than do *phyA-101* seedlings. 2) Other responses are more severe in *phyA-401* than in seedlings carrying the null mutant allele. These effects include Rc-response deficiencies such as elongated hypocotyls in Wc and Rc, loss of gravitropic growth orientation, and more elongated petioles that are not observed in *phyA-101*, as well as stronger deficiencies in seed germination under Wc and FRc. Although these response data suggested that *dep3* was a downstream transduction component that affects multiple photoresponse pathways, mapping and sequencing the *DEP3* gene showed that the phenotype is the result of a novel allele of *phyA*.

PhyA-401 is an important allele of *phyA* that could be used to study the structure of phytochromes so as to illustrate its physical characteristics and functions, to investigate physiological responses that are not altered in other known *phyA* mutants, and to examine direct or indirect phyA-phyB interactions or competition among phytochromes for common reaction partners. The high degree of conservation of the two amino acids found to be changed in *phyA-401* may also prove useful for addressing questions of photoreceptor evolution.

Tables

Table 1. Flowering times under Long Day (LD) and Short Day (SD) conditions.

Plants were grown under Wc, and the number of days and of rosette leaves at the time when bolting was detected were scored. Each cell represents the mean values for at least 16 plants +/- 1 standard deviation. *dep1, dep2* and *dep3* show a significant delay of flowering time measured with days under LD and SD treatment by Student t-Test ($p<0.01$), but the difference in rosette leaf number between mutants and wild type at the time of flowering was not significant under any conditions.

	No-O	*dep1*	*dep2*	*dep3*
Days to Flowering (LD)	22.6 ±1.5	25.4 ±0.9	24.7 ±1.6	25.9 ±1.5
Rosette Leaf Number at Flowering (LD)	8.0 ±0.7	8.5 ±0.7	8.9 ±0.7	8.6 ±0.9
Days to Flowering (SD)	117.2 ±14.5	132.1 ±11.5	128.7 ±11.5	147.8 ±9.9
Rosette Leaf Number at Flowering (SD)	25.4 ±2.3	24.9 ±3.6	26.5 ±2.8	24.0 ±4.3

Table 2. Genetic characterization of *dep3* mutants

Plants of *dep3* were backcrossed to No-O wild type. F1 progeny were grown under Wc, and their hypocotyl lengths were compared with those of wild type No-O. F2 progeny were grown for 5 days under FRc, the extremely tall hypocotyl seedlings from *dep3* and short hypocotyl seedlings growing like wild type were counted. The *dep3* segregation pattern is consistent with Mendelian recessiveness of the mutant *DEP3* allele.

Cross	Generation	Seedlings with Short Hypocotyls	Seedlings with Long Hypocotyls	χ^2
No-O X *dep3*	F1	40	0	P<4E-14
	F2	588	192	P<0.06

Figures and Figure Legends

Figure 1. Visual phenotypes of *dep* mutants grown under various light conditions.

Seedlings were prepared as described in the text and grown under Wc, Rc and FRc for 7d. Under all conditions, *dep1* and *dep2* mutants showed slightly longer hypocotyls than those of the wild type, but the *dep3* mutants yield the strongest phenotype, including longer hypocotyls and reduced cotyledon separation and expansion.

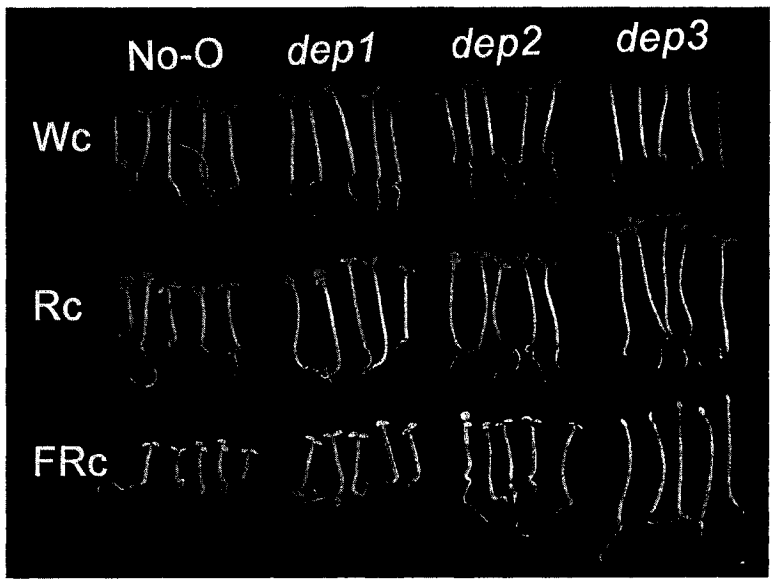
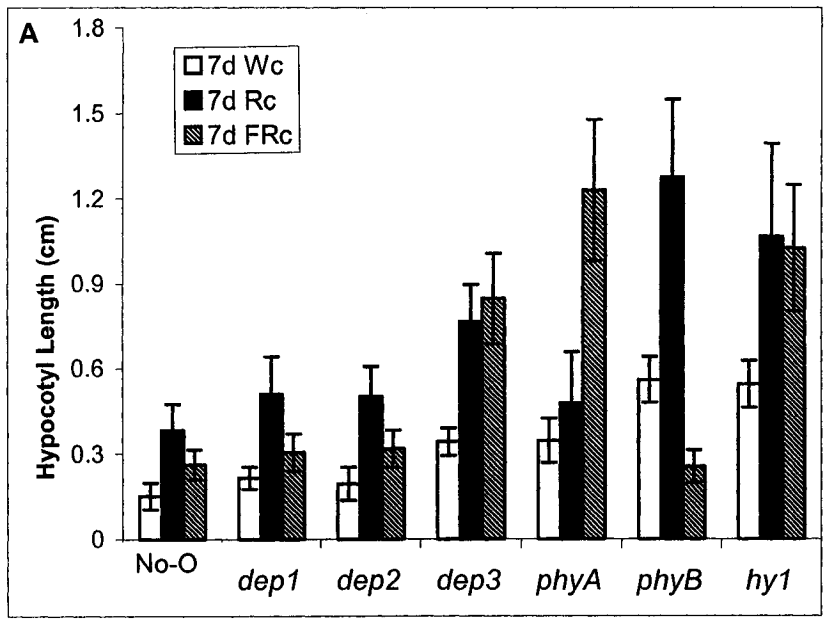


Figure 1

Figure 2. Hypocotyl growth under different light conditions.

(A) Growth of seedlings for 7 d under Wc, Rc, or FRc at 24°C. Seeds plated on MS-agar were stratified and induced to germinate in 3 h Wc, transferred to dark for 21 h, and exposed to 75 μmol m^{-2} s^{-1} Wc, 15 μmol m^{-2} s^{-1} Rc, or 9.7μmol m^{-2} s^{-1} FRc for 7 d. The *dep1* and *dep2* mutants were only slightly taller than wild type No-O, while the *dep3* mutants grew significantly taller in both Rc and FRc, comparable to *hy1* and *phyA* mutants. At least 40 seedlings were measured for each treatment in each experiment. Four replicate experiments were performed and the results combined. Error bars indicate +/- 1 standard deviation.

(B) Growth of seedlings for 5 d in darkness at 24°C. Seeds plated on MS-agar were stratified and induced to germinate in 3 h Wc, then transferred to dark 5 d. Mutants and wild type were indistinguishable under darkness. Hypocotyl lengths of at least 40 seedlings were measured in each of 3 experiments and the data were pooled. Error bars indicate +/- 1 standard deviation.



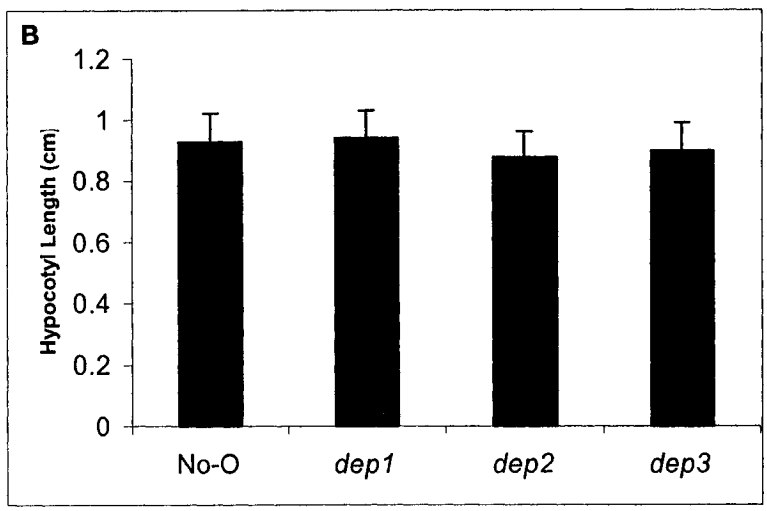


Figure 2

Figure 3. *dep* mutants grow comparably to wild-type after 5d Bc

Hypocotyl length of seedlings was measured after growth at 24°C for 5d in Bc. Seeds plated on MS-agar were stratified and induced to germinate in 3 h Wc, then put into darkness 21h, then transferred to Bc (20 μmol m^{-2} s^{-1}) for 5d. The mutants and wild type were not significantly different under these conditions. Hypocotyl lengths of at least 40 seedlings were measured in each of 3 experiments and the data were pooled. Error bars indicate +/- 1 standard deviation.

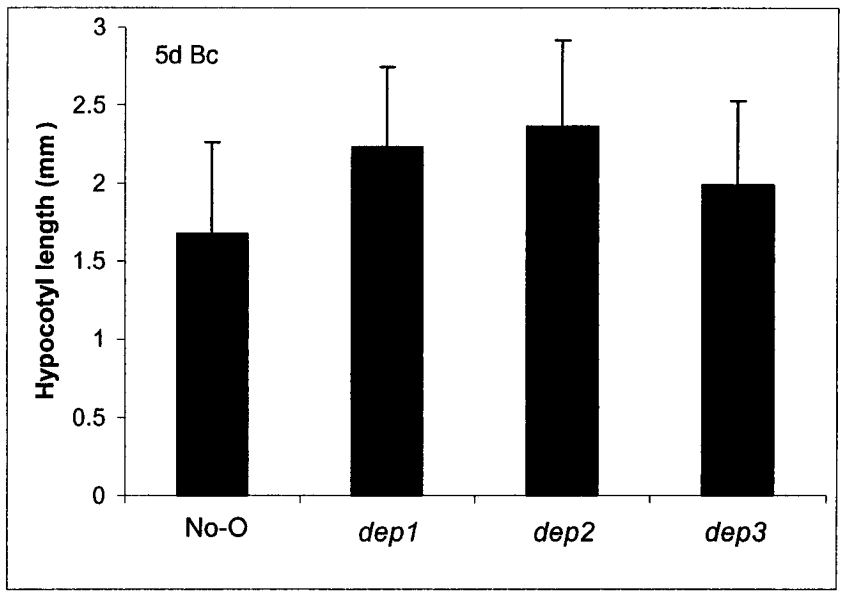


Figure 3

Figure 4. *dep1* and No-O seedlings show differential growth of hypocotyl cells and cotyledon cells in Wc .

The *dep1* mutant allele exhibits increased hypocotyl cell elongation, and slowed cotyledon expansion and separation. Wild type No-O or *dep1* plants were grown for 3d, 4d, or 6d under Wc, fixed, and subjected to scanning electron microscopy. Six-day-old seedlings were examined at higher magnification, showing that increased hypocotyl growth results from increased cell elongation, and that *dep1* has decreased cotyledon cell expansion. Scale bars represent 100 μm in the whole plant panels, and 10 μm in the close-ups of the hypocotyl and cotyledon lower surface.

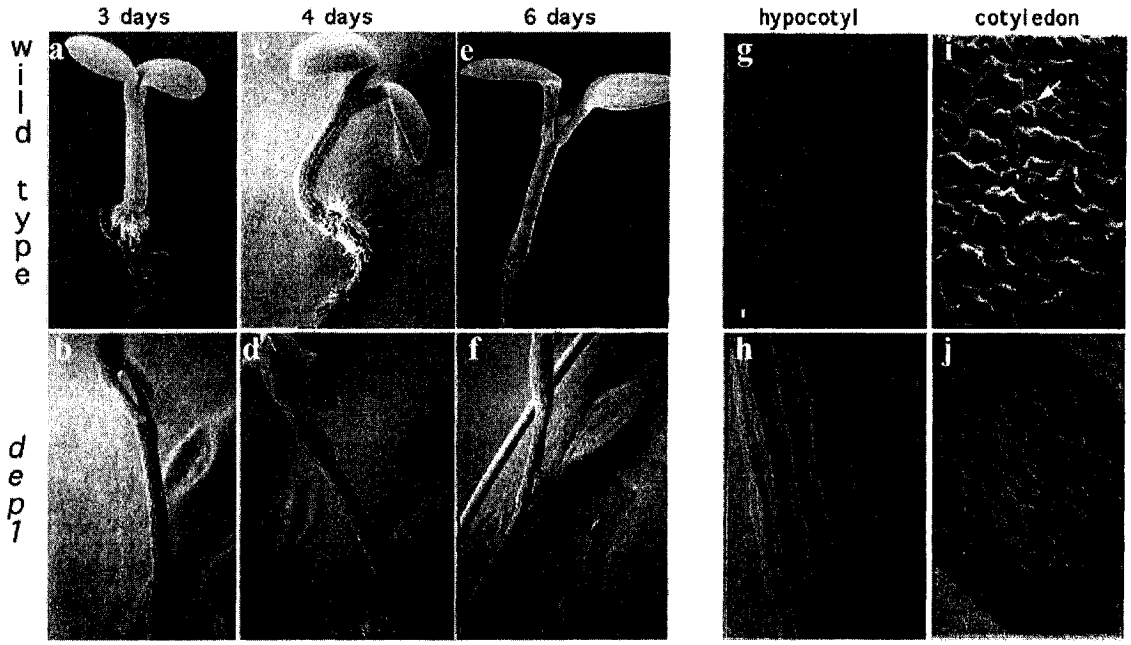


Figure 4

Figure 5. Proportion of wild-type and mutant seedlings with closed cotyledons under various light conditions.

Seeds plated on MS-agar were stratified and induced to germinate in 3 h Wc, transferred to dark for 21 h, and exposed to 75 μmol m^{-2} s^{-1} Wc, 15 μmol m^{-2} s^{-1} Rc, 9.7 μmol $m^{-2}s^{-1}$ FRc, and 20 μmol m^{-2} s^{-1} Bc for 3 d at 24°C. Seedlings with cotyledons separated by less than 45° with respect to one another were counted as closed. Wild type seedling display 100% opened cotyledons, and except *dep3* which maintained 100% of cotyledon closed in FRc, *dep* mutants showed 20~40% closed cotyledons under Wc, Rc, FRc and Bc. At least 40 seedlings were scored for each treatment in each of three experiments. Error bars indicate +/- 1 standard deviation.

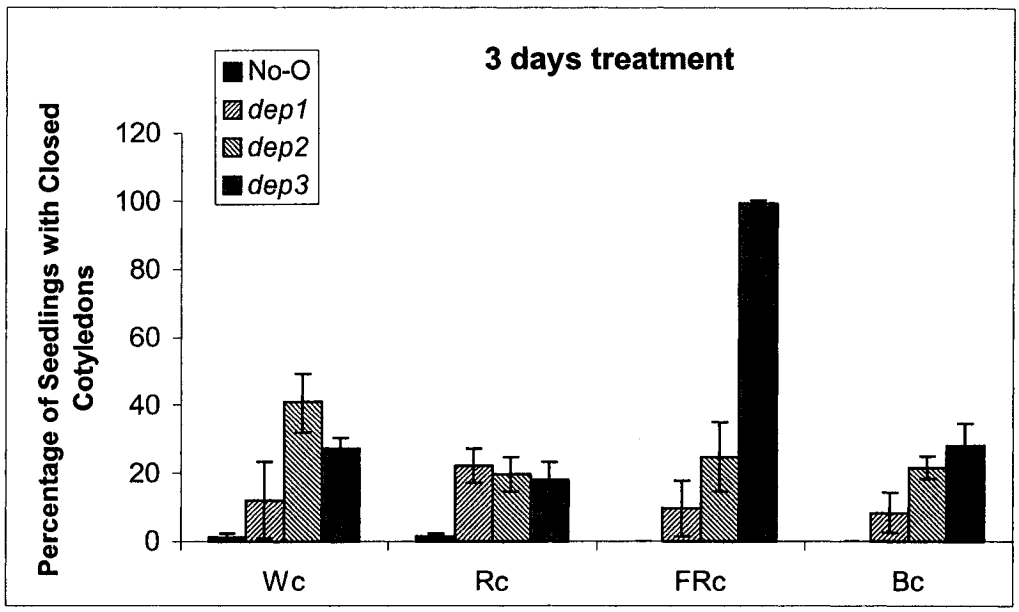


Figure 5

Figure 6. Seedlings grown under Rc lose gravitropism.

Visual comparison of wild type, *dep1, dep2,* and *dep3* mutant seedlings grown under Rc on standard horizontal plates. The seedlings were grown on MS medium in Rc (15 μmol m^{-2} s^{-1}) for three days at 24°C.

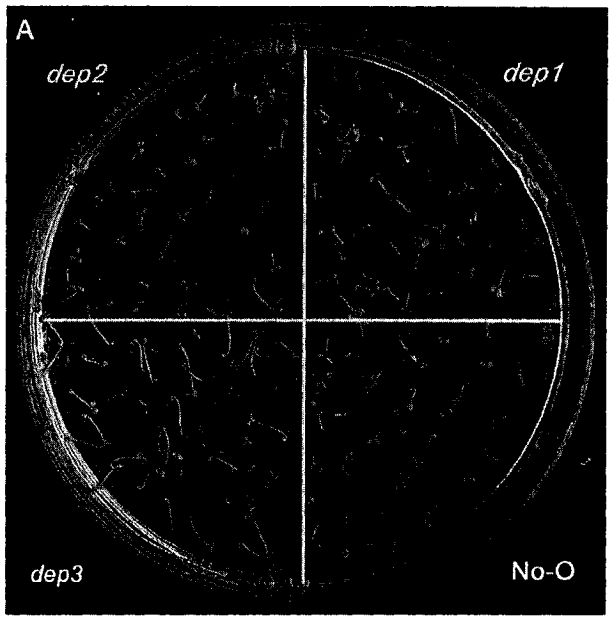


Figure 6

Figure 7. Directional seedling growth deviation from vertical.

The angles that mutants and wild-type seedlings grew were scored as individual growth angle deviation from the vertical. 0° represents seedling growth parallel to the gravity vector. *dep* mutants grew in more randomized directions, with *dep3* the least directional, *dep1* slightly more so, and *dep2* only marginally different from that of the wild type seedlings. The seedlings were grown on MS medium on square plates turned vertically in Rc (15 μmol m^{-2} s^{-1}) for five days at 24°C. The experiments were repeated three times, each time with approximately 40 seedlings per genotype.

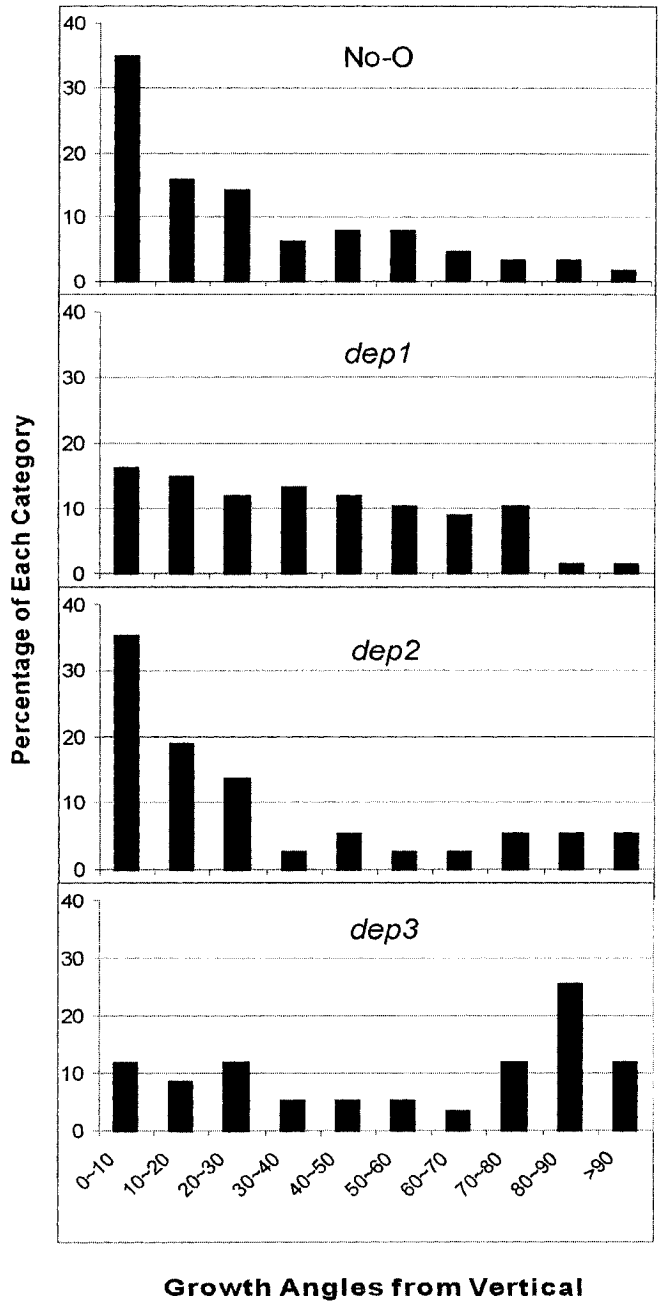


Figure 7

Figure 8. Petiole length of leaves from wild type, *dep1*, *dep2*, and *dep3* plants grown under Wc.

Petiole lengths of the two oldest true leaves were measured after growth for 25 d in Wc. In total, 20 leaves from 10 plants were measured. The *dep3* mutant petioles were marginally different from No-O and from *dep1* alone, using the Student t-Test ($p<0.02$). No other comparisons yielded statistically significant differences. Error bars indicate +/- 1 standard deviation.

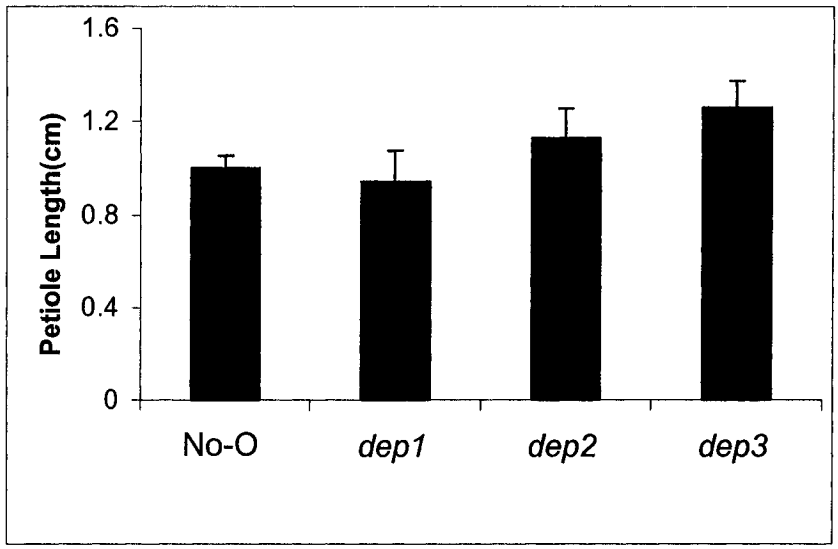


Figure 8

Figure 9. PHYA accumulation in darkness and its light-induced degradation in wild type and *dep* mutants.

Phytochrome-enriched protein extracts were separated by SDS-PAGE, blotted, and probed with phyA-specific monoclonal antibodies. The *dep* mutants accumulate PhyA apoprotein to levels at least as high as those in wild type, and the PhyA is sufficiently photoactive to exhibit apparently normal light-induced degradation after 4h R.

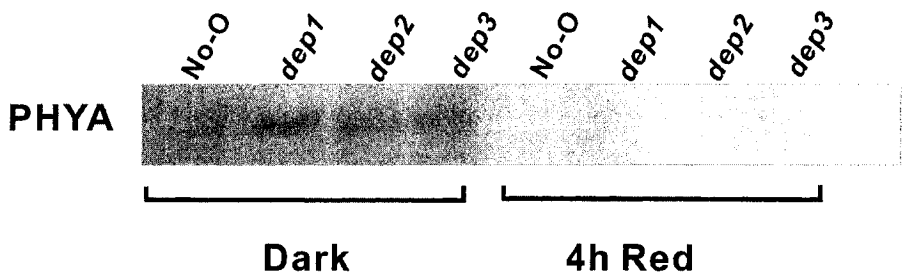


Figure 9

Figure 10. Comparisons of hypocotyl length of wild type, *dep* mutants and their F2 progeny.

Hypocotyl lengths of wild type, homozygous *dep1, dep2,* and *dep3* seedlings, and segregating populations of F2 seedlings from No-O X *dep1,* No-O X *dep2,* and No-O X *dep3* were measured after growth under FRc for seven days at 24 C. Each figure contains size distribution measurements pooled from three independent experiments, each with at least 50 seedlings per genotype. (A) No-O; (B) *dep1*; (C) *dep2*; (D) *dep3* hypocotyl length distributions; (E) segregating F2 seedlings of *dep1* backcrossed to No-O; (F) segregating F2 seedlings of *dep2* backcrossed to No-O; (G) segregating F2 seedlings of *dep3* backcrossed to No-O.

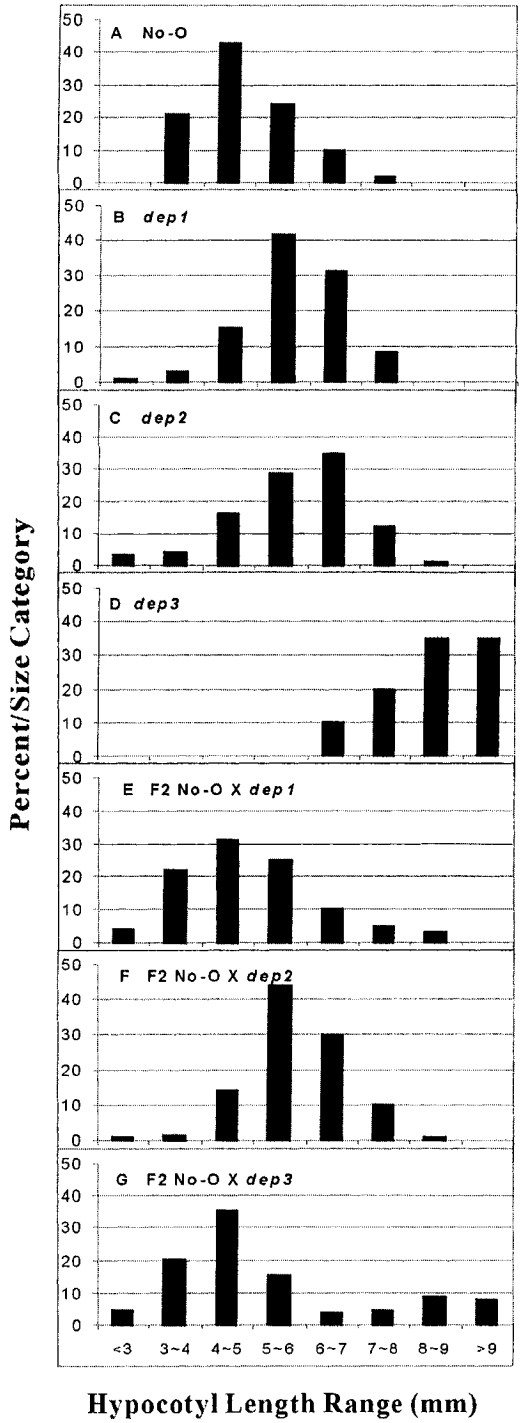
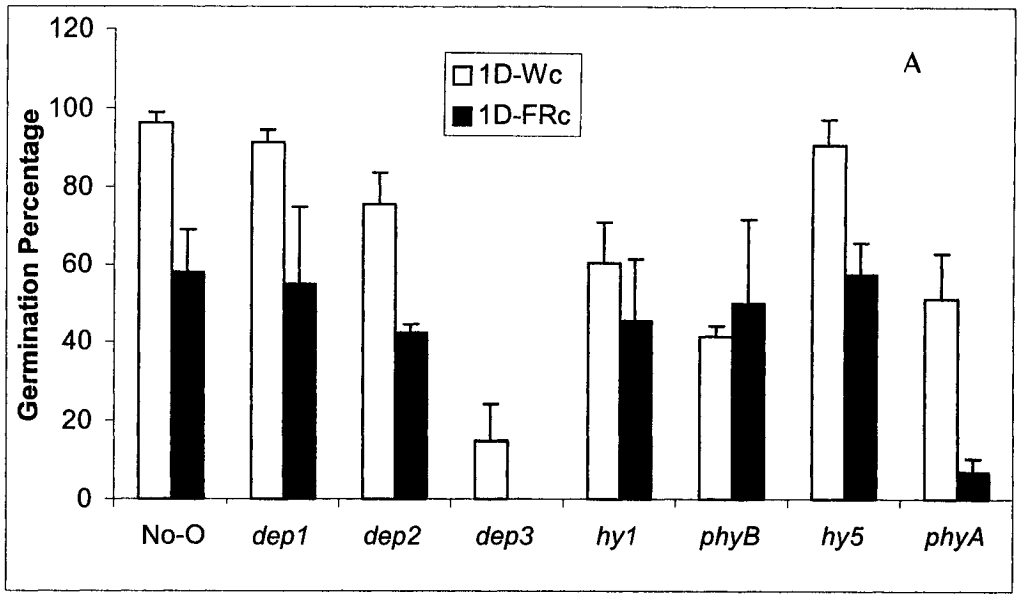


Figure 10

Figure 11. Comparison of germination rates of *dep* mutants to those of wild type and related mutant lines.

Germination was scored after 1d and 1.5d in Wc (75 μmol $m^{-2}s^{-1}$) or FRc (9.7 μmol $m^{-2}s^{-1}$). At least 50 seeds of each line were sterilized and plated on MS plates, then irradiated with 3 h FR (9.7 μmol $m^{-2}s^{-1}$). The plates were put at 4°C in darkness for 3d to stratify, then transferred to Wc or FRc for 1d (A) or 1.5d (B). *dep3* displayed a significant delay of germination under Wc and FRc. Each experiment was repeated 3 times, and error bars indicate +/- 1 standard deviation.



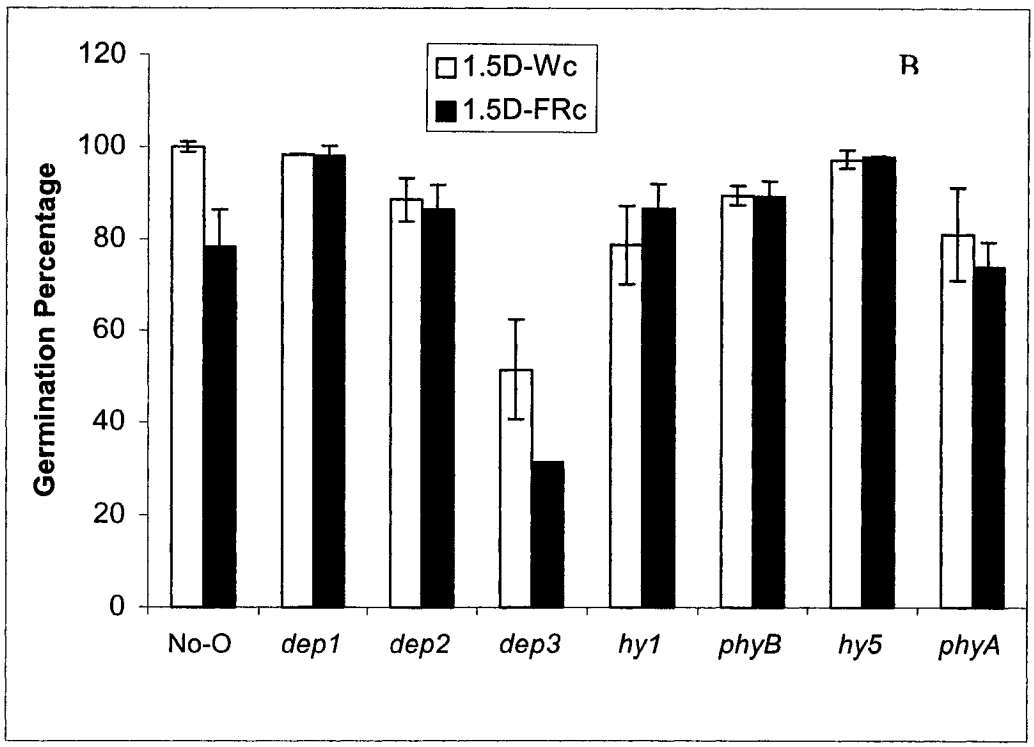


Figure 11

Figure 12. *dep3* accumulates anthocyanins at very low levels.

Seeds from each line were plated on MS-agar + 2% sucrose, and were stratified and induced to germinate in 3 h Wc, transferred to dark for 21 h, and exposed to 4 μmol m^{-2} s^{-1} FRc for 2 d. Anthocyanins were extracted in acidic methanol and measured spectrophotometrically. Both *dep1* and *dep2* accumulate anthocyanins at levels near those of wild type No-O, while the *dep3* mutant response more nearly resembles that of the *phyA* null mutant. Each experiment was repeated 3 times, and error bars indicate +/- 1 standard deviation.

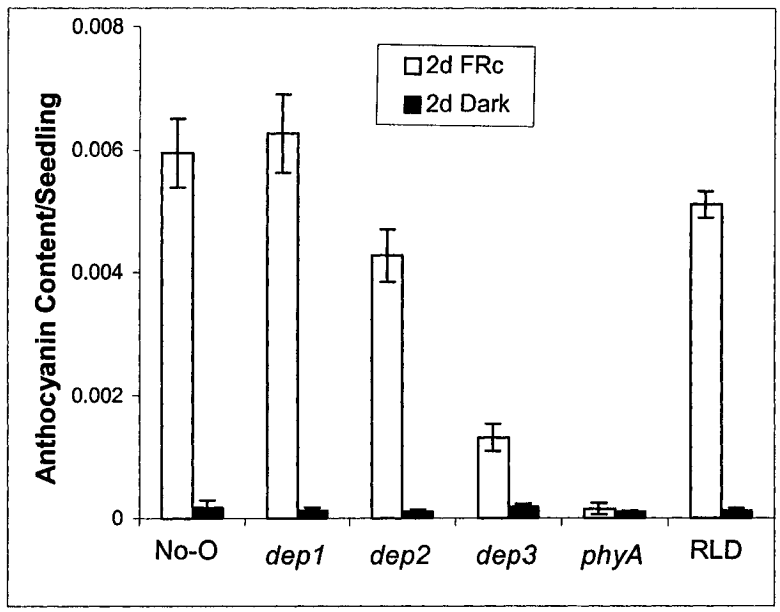


Figure 12

Figure 13. Simplified Anthocyanin synthesis pathway

A simplified pathway for anthocyanin synthesis shows four known regulatory points for the synthesis process. The three late enzymes, *CHS*, *CHI*, and *DFR*, are light inducible and control late steps feeding into anthocyanin synthesis.

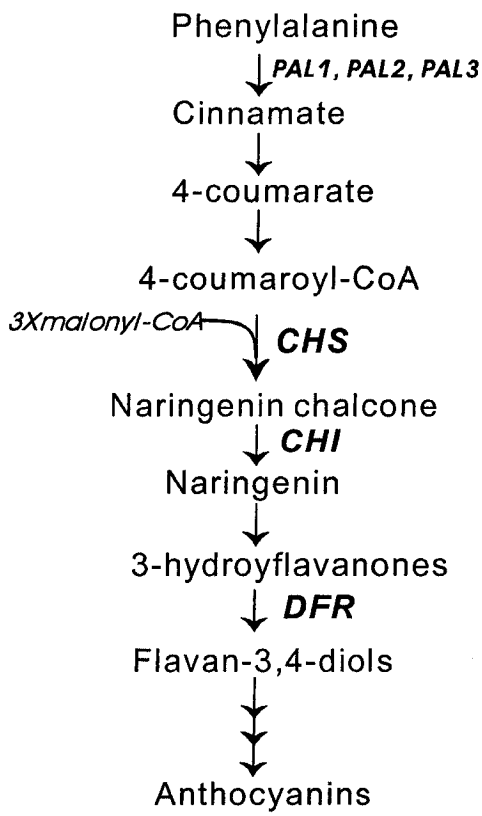


Figure 13

Figure 14. Steady state mRNA levels of the anthocyanin pathway genes *CHS*, *CHI*, and *DFR*.

Seeds from different lines were plated on MS-agar + 2% sucrose, stratified, induced to germinate in 3 h Wc, transferred to dark for 21 h, and exposed to 9.7 μmol m^{-2} s^{-1} FRc for 2d. Total RNA was extracted and subjected to two-step RT-PCR with *ACTIN* as quantitative control. The first step used a poly-T primer and the second step used primers specifically for the open-reading-frame for each enzyme. *dep1* did not show a significant change in the expression of any of the three messages, *dep2* displayed reduced expression of *CHS* and *CHI*, while *dep3* mutants exhibited significant down-regulation of all three enzyme mRNAs. N=No-O; *d1=dep1; d2=dep2; d3=dep3.* The figure is the representative of three independent experiments.

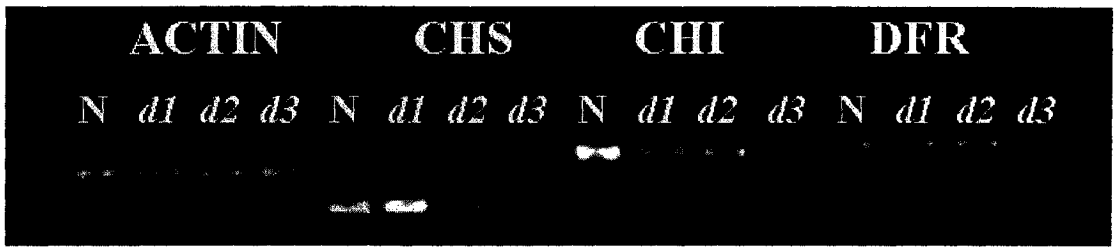


Figure 14

Figure 15. *dep3* affects the FR-induced suppression of greening.

Seeds plated on MS-agar were stratified and induced to germinate in 3 h Wc, transferred to dark for 21 h, and exposed to 9.7 μmol m^{-2} s^{-1} FRc for 1, 2, or 3 d. Plates were then transferred to Wc for 3 d. Total chlorophyll was extracted in 80% acetone and measured spectrophotometrically. Both *dep1* and *dep2* accumulate chlorophyll comparably to wild type No-O, while the *dep3* mutant response resembles that of *phyA* null mutant.

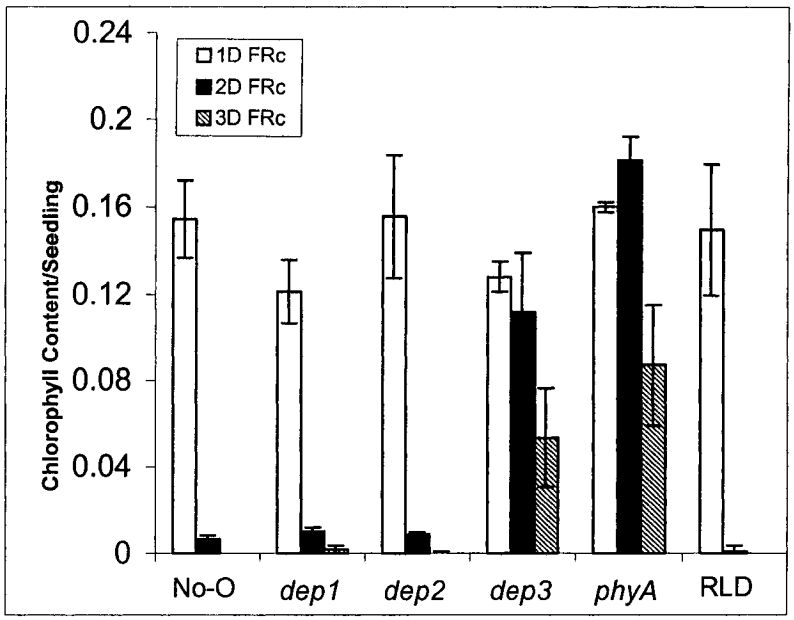


Figure 15

Figure 16. Biliverdin does not rescue the light response deficiency phenotypes of *dep* mutants

No-O, *dep1, dep2, dep3*, and *hy1* seeds were plated on duplicate plates of MS medium, one of each supplemented with 0.1 mM biliverdin (+BV), a functional chromophore precursor. Seedlings grown without BV are shown in the top (-BV) row. After stratification and induction to germinate with 3 h W, the seeds were transferred to dark for 21 h, and exposed to 9.7 μmol m^{-2} s^{-1} FRc for 4d. The *dep1*and *dep2* mutants grew slightly longer hypocotyls compared with the No-O wild type, while *dep3* and *hy1* grew very long hypocotyls with folded cotyledons, and *hy1* also maintained closed apical hooks. With exogenous BV (+BV), only *hy1* seedlings were rescued, yielding shorter hypocotyls, opened apical hooks, and well expanded cotyledons. BV did not rescue the long hypocotyl phenotype of *dep1, dep2,* or *dep3,* or the closed cotyledon phenotype of *dep3*.

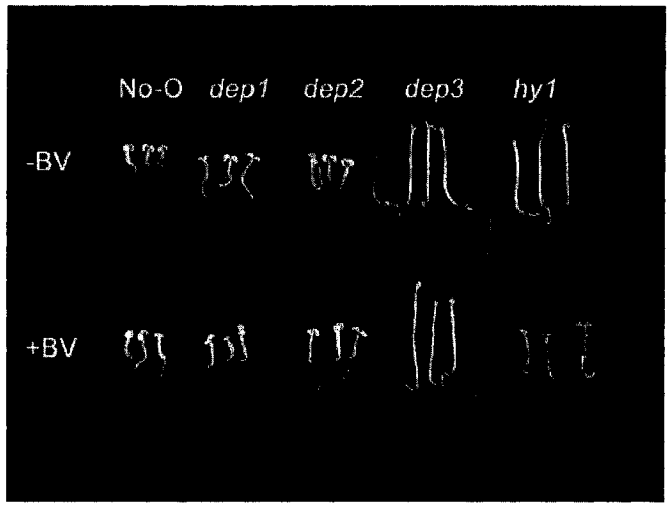


Figure 16

Figure 17. Mapping of *dep3*.

The *DEP3* gene was linked to SSLP markers near the top of chromosome 1 encompassing a region covered by 5 BAC clones. Fine mapping with SNP markers further narrowed the region containing *DEP3* to a fragment of BAC F14J9 between 49374bp and 86238bp that included eight presumptive genes. The numbers of recombinants and possible recombination events are indicated.

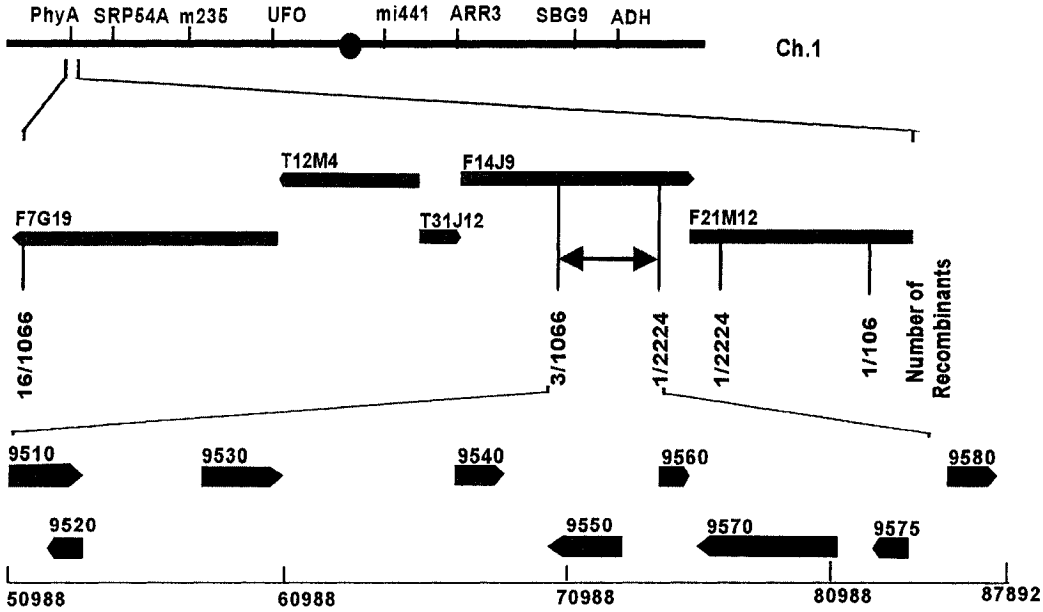


Figure 17

Figure 18. *dep3* contains two point mutations within the *PHYA* gene.

Sequencing the *PHYA* gene of *dep3* yielded two point mutations within the coding region. One T to C change at base 991 and a G to A change at base 1387 (counted from the cDNA presumptive translation initiation ATG), leads to amino acid changes of $Met_{331}$to Val (eight amino acids downstream of Cys_{323} to which the chromophore attaches) and Pro_{461} to Ser.

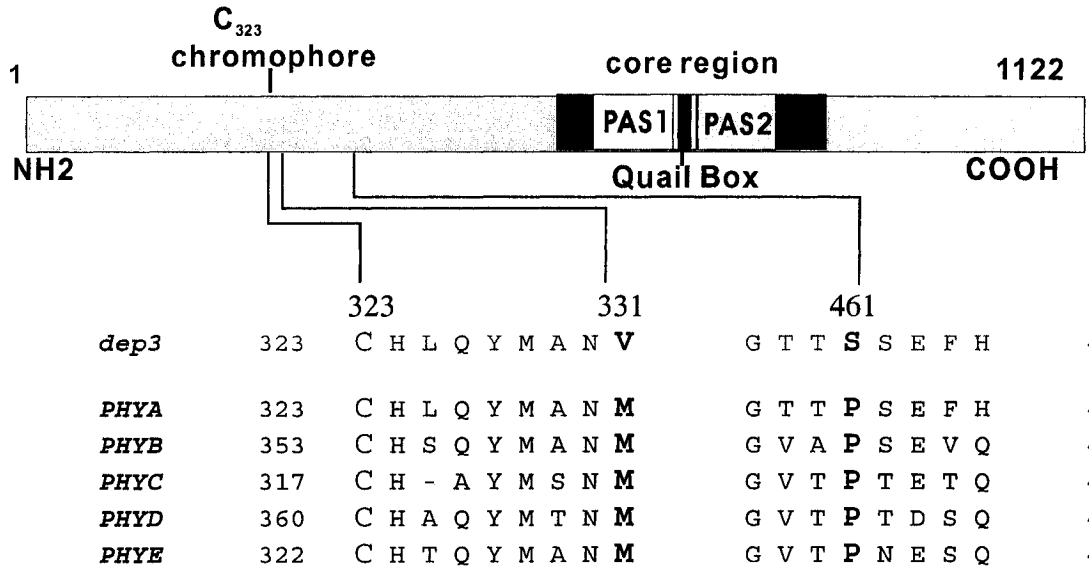


Figure 18

Figure 19. *dep3* is a novel mutant allele of phyA

The *dep3* mutation is different from previous reported phyA alleles. It contains novel mutations located in the N-terminal domain near the chromophore attachment site. Other known alleles are primarily clustered in the C-terminal domain, especially within the PAS domains or "Quail Box" (Q). HK is the Histine-Kinase-like domain.

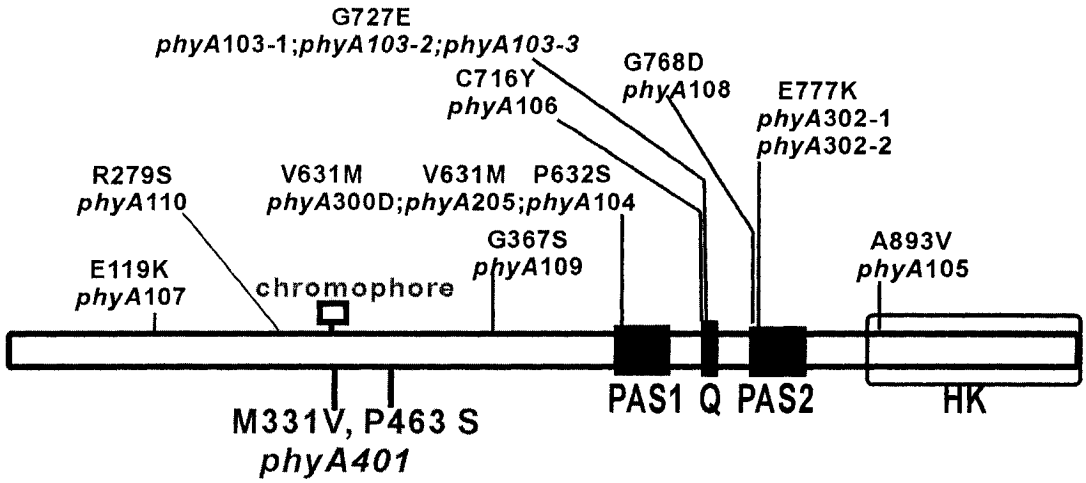


Figure 19

Figure 20. The two amino acids mutated in phyA-401 are highly conserved.

The two amino acids M_{331} and P_{461} are highly conserved among phytochromes from higher plants including both dicots and monocots, from lower plant such as ferns and moss, from green algae, and from cyanobacteria. The M_{331} is also conserved in fungal and diatom phytochromes. This conservation suggests that these two amino acids are critical for maintaining normal phytochrome function.

Dicots		
Arabidopsis - PhyA	C H L Q Y M A N **M** D	G T T **P** S E
Soybean-phyA	C H A Q Y M A N **M** D	G V T **P** S E
Pisum sativum-phyA	C H L Q Y M A N **M** D	G A T **P** T E
Nicotiana tabacum	C H L Q Y M E N **M** S	G M T **P** S D
Solanum tuberosum-phyA	C H L Q Y M E N **M** N	G M N **P** S D
Parsley- phyA	C H L Q Y M E N **M** N	G A T **P** S D
Zucchini-phyA	C H L Q Y M E N **M** N	G L T **P** N D
Monocots		
Zea mays	C H L K Y M E N **M** N	Q T A **P** T E
Oryza sativa	C H L Q Y M E N **M** N	Q N A **P** T E
Lower plants		
Adiantum capillus-PHY2	C H S Q Y M A N **M** G	G T T **P** I E
Selaginella martensii-PHY1	C H A Q Y M G N **M** G	G I T **P** S E
Green algae		
Ceratodon purpureus-PHY2	C H A Q Y M G N **M** G	G T T **P** I E
Cyanobacteria		
Synechocystis sp.	C H L T Y L K N **M** G	G E T **P** D E
Fungi		
Neurospora crassa	V H L K Y L S N **M** G	
Diatoms		
Deinococcus radiodurans	M H M Q Y L R N **M** G	
Non-photosynthetic bacteria		
Bradyrhizobium sp.	I H Q K Y L Q N **M** G	

Figure 20

Figure 21. *phyA-401* lacks photoreversible phyA.

No-O and *phyA-401* seeds were plated on four sets of MS medium plates. After stratification and germination induction with 3 h Wc, the plates were kept in darkness for 5d, and exposed to 15 μmol m^{-2} s^{-1} Rc for 0, 1h, 2h, 4h. Total proteins enriched for phytochromes were extracted, and absorption measured spectrophotometrically in the Pfr and Pr forms by exposing the samples to 1 min R or 2 min FR, respectively. The absorption difference between Pr and Pfr scans was plotted. The scans show that phyA in No-O seedlings is photochemically active and undergoes normal R-mediated degradation as indicated by peaks at 667nm and valleys at 710nm, but photoreversible phyA in *phyA401* seedlings is not detectable even in dark control plant extracts.

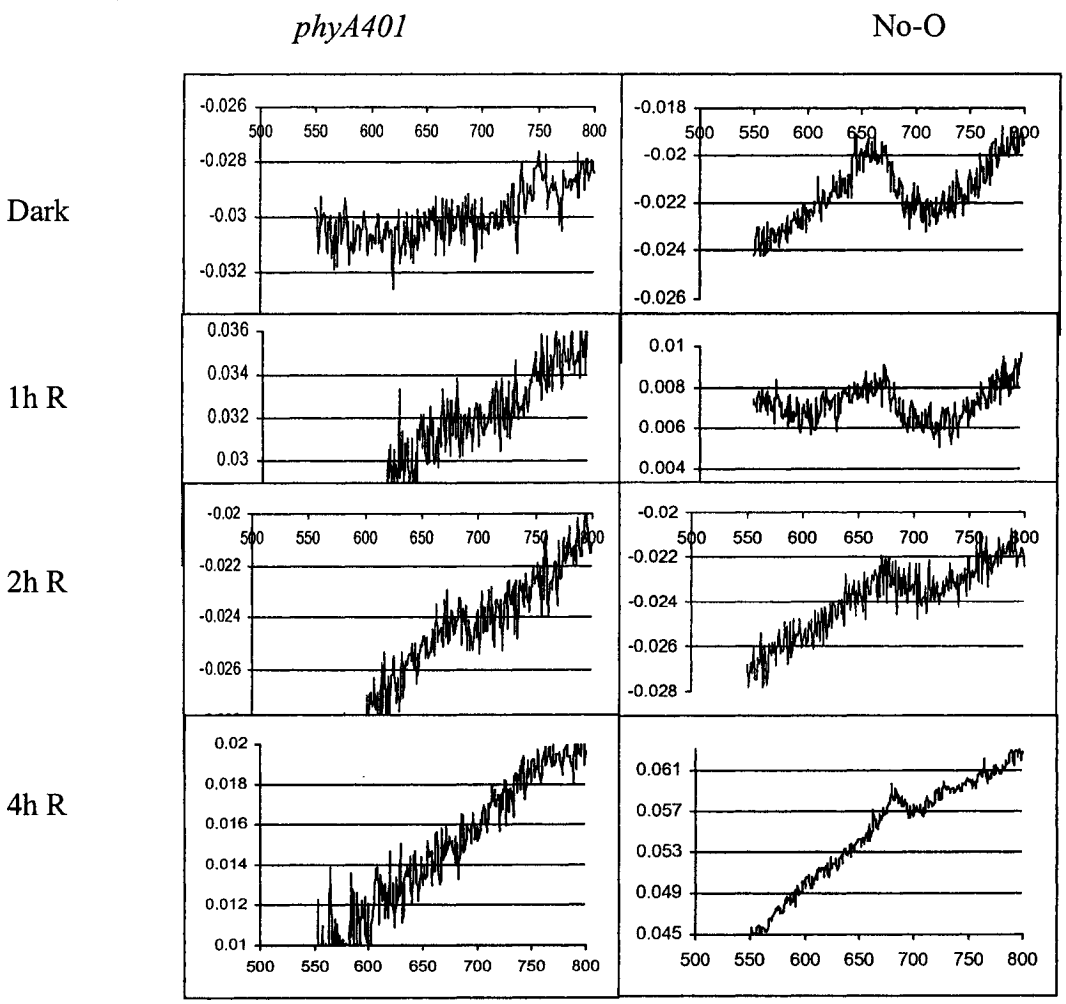


Figure 21

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