The Function and Regulation of Photobodies in Phytochrome Signaling

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Light is a critical environmental signal that regulates every phase of the plant life cycle, from germination to floral initiation. Of the many light receptors in the model plant *Arabidopsis thaliana*, the red- and far-red light-sensing phytochromes (phys) are arguably the best studied, but the earliest events in the phy signaling pathway remain poorly understood. One of the earliest phy signaling events is the translocation of photoactivated phys from the cytoplasm to the nucleus, where they localize to subnuclear foci termed photobodies; in continuous light, photobody localization correlates closely with the light-dependent inhibition of embryonic stem growth. Despite a growing body of evidence supporting the biological significance of photobodies in light signaling, photobodies have also been shown to be dispensable for seedling growth inhibition in continuous light, so their physiological importance remains controversial; additionally, the molecular components that are required for phy localization to photobodies are largely unknown. The overall goal of my dissertation research was to gain insight into the early steps of phy signaling by further defining the role of photobodies in this process and identifying additional intragenic and extragenic requirements for phy localization to photobodies.

Even though the domain structure of phys has been extensively studied, not all of the intramolecular requirements for phy localization to photobodies are known. Previous studies have shown that the entire C-terminus of phy is both necessary and sufficient for their localization to photobodies. However, the importance of the individual subdomains of the C-terminus is still unclear. For example a truncation lacking part of the most C-terminal domain, the histidine kinase-related domain (HKRD), can still localize to small photobodies in the light and behaves like a weak allele. However, a point mutation within the HKRD renders the entire molecule completely inactive. To resolve this discrepancy, I explored the hypothesis that this point
mutation might impair the dimerization of the HKRD; dimerization has been shown to occur via the C-terminus of phy and is required for more efficient signaling. I show that this point mutation impairs nuclear localization of phy as well as its subnuclear localization to photobodies. Additionally, yeast-two-hybrid analysis shows that the wild-type HKRD can homodimerize but that the HKRD containing the point mutation fails to dimerize with both itself and with wild-type HKRD. These results demonstrate that dimerization of the HKRD is required for both nuclear and photobody localization of phy.

Studies of seedlings grown in diurnal conditions show that photoactivated phy can persist into darkness to repress seedling growth; a seedling’s growth rate is therefore fastest at the end of the night. To test the idea that photobodies could be involved in regulating seedling growth in the dark, I compared the growth of two transgenic Arabidopsis lines, one in which phy can localize to photobodies (PBG), and one in which it cannot (NGB). Despite these differences in photobody morphology, both lines are capable of transducing light signals and inhibiting seedling growth in continuous light. After the transition from red light to darkness, the PBG line was able to repress seedling growth, as well as the accumulation of the growth-promoting, light-labile transcription factor PHYTOCHROME INTERACTING FACTOR 3 (PIF3), for eighteen hours, and this correlated perfectly with the presence of photobodies. Reducing the amount of active phy by either reducing the light intensity or adding a phy-inactivating far-red pulse prior to darkness led to faster accumulation of PIF3 and earlier seedling growth. In contrast, the NGB line accumulated PIF3 even in the light, and seedling growth was only repressed for six hours; this behavior was similar in NGB regardless of the light treatment. These results suggest that photobodies are required for the degradation of PIF3 and for the prolonged stabilization of active phy in darkness.
They also support the hypothesis that photobody localization of phy could serve as an instructive cue during the light-to-dark transition, thereby fine-tuning light-dependent responses in darkness.

In addition to determining an intragenic requirement for photobody localization and further exploring the significance of photobodies in phy signaling, I wanted to identify extragenic regulators of photobody localization. A recent study identified one such factor, HEMERA (HMR); *hmr* mutants do not form large photobodies, and they are tall and albino in the light. To identify other components in the HMR-mediated branch of the phy signaling pathway, I performed a forward genetic screen for suppressors of a weak *hmr* allele. Surprisingly, the first three mutants isolated from the screen were alleles of the same novel gene, *SON OF HEMERA* (*SOH*). The *soh* mutations rescue all of the phenotypes associated with the weak *hmr* allele, and they do so in an allele-specific manner, suggesting a direct interaction between SOH and HMR. Null *soh* alleles, which were isolated in an independent, tall, albino screen, are defective in photobody localization, demonstrating that SOH is an extragenic regulator of phy localization to photobodies that works in the same genetic pathway as HMR.

In this work, I show that dimerization of the HKRD is required for both the nuclear and photobody localization of phy. I also demonstrate a tight correlation between photobody localization and PIF3 degradation, further establishing the significance of photobodies in phy signaling. Finally, I identify a novel gene, *SON OF HEMERA*, whose product is necessary for phy localization to photobodies in the light, thereby isolating a new extragenic determinant of photobody localization. These results are among the first to focus exclusively on one of the earliest cellular responses to light – photobody localization of phy – and they promise to open up new avenues into the study of a poorly understood facet of the phy signaling pathway.
Dedication

To the memory of my grandmother, Madeline Ragazzo.
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1. An Overview of Phytochrome Signaling

1.1 Light Triggers Photomorphogenesis During Seedling Development

One of the most significant differences between most plant and animal species is that plants are sessile. Consequently, plants have evolved to cope with a changing environment by altering their growth and development to maximize their chances of survival. Light is a critical environmental signal that plants use to determine their developmental pattern; light regulates all aspects of plant development, from seed germination to flowering and senescence (Kami et al., 2010). One of the most dramatic examples of this phenotypic plasticity in plants is the different developmental programs exhibited by germinating seedlings such as in the reference plant *Arabidopsis thaliana* (Chen and Chory, 2011). Most seed germination occurs below the surface of the soil, in darkness. To facilitate reaching the light, the newly germinated seedling exhibits elongated embryonic stems (hypocotyls), closed embryonic leaves (cotyledons), and an apical hook that protects the meristematic tissue as the seedling grows up through the soil; this developmental program is called skotomorphogenesis (Figure 1). When the seedling emerges from the ground and into the light, a vastly different developmental program is initiated in which hypocotyl growth is inhibited, the cotyledons expand, the apical hook unfolds, and chloroplasts differentiate to allow photosynthesis to begin (Chen and Chory, 2011). This developmental program is termed photomorphogenesis, and the transition between skotomorphogenesis and photomorphogenesis is called de-etiolation (Figure 1) (Chen and Chory, 2011).
Figure 1: Two different developmental programs in Arabidopsis seedlings. The dark-grown program, skotomorphogenesis, is shown on the left, and the light-grown program, photomorphogenesis, is shown on the right.

This large phenotypic change is driven by an equally large transcriptomic change; up to one third of the Arabidopsis nuclear genome is differentially regulated by light (Ma et al., 2001; Hu et al., 2009; Leivar et al., 2009). These transcriptional changes in the nucleus need to be coordinated with the plastid genome, which encodes approximately 130 genes (Lopez-Juez and Pyke, 2005). Plastid genes are transcribed by two types of polymerases: a eubacterial-like plastid-encoded RNA polymerase (PEP), and two phage-type nuclear-encoded RNA polymerases (NEP) (Liere et al., 2011). Although both types of polymerases can transcribe most genes, the PEP preferentially transcribes photosynthesis-associated genes and the NEP preferentially transcribes housekeeping genes (Hajdukiewicz et al., 1997). One way that the nucleus and the chloroplasts are coordinated is through the light-regulated expression of sigma factors, which are nuclear-encoded PEP accessory proteins that are required for PEP function and promoter recognition (Isono et al., 1997; Tanaka et al., 1997; Fujiwara et al., 2000; Monte et al., 2004; Schweer et al., 2010). Light can also regulate chloroplast transcription via the light-dependent association of the
PEP with plastid DNA (Finster et al., 2013). Together, these huge transcriptional changes regulate light responses both in the nucleus and the chloroplasts to promote photomorphogenesis.

1.2 Photomorphogenesis is Promoted by the Red/Far-Red Light-sensing Phytochromes via Transcriptional Regulation

De-etiolation is triggered by a suite of photoreceptors that perceive varying wavelengths of light ranging from the UV-B to the red/far-red portions of the spectrum (Chen et al., 2004). Among these receptors, the phytochrome (phy) family perceives and responds to red (R) and far-red (FR) light; light in this portion of the spectrum is important because it conveys information about the availability of light for photosynthesis and the presence of neighboring plants (Chen et al., 2004; Chen and Chory, 2011).

Phys function as dimers that can convert between two relatively stable forms: a R light-absorbing Pr form, which is the form in which it is first synthesized, and a FR light-absorbing Pfr form (Franklin and Quail, 2010). When phy in the Pr form absorbs a photon of R light, it undergoes a conformational change to the Pfr form, which, in turn, is capable of absorbing a photon of FR light and converting back to the Pr form (Rockwell et al., 2006; Nagatani, 2010; Ulijasz and Vierstra, 2011). The Pfr form of phy is thermodynamically unstable; without a continual supply of R photons to maintain it in the Pfr form, phy spontaneously converts back to the Pr form in a process termed dark reversion (Rockwell et al., 2006). Because most phy-dependent physiological responses are promoted by R light and inhibited by FR light, the Pfr form of phy is generally regarded as the active form (Franklin and Quail, 2010). Importantly, conversion of phy from the Pr to the Pfr form leads to its nuclear import (Sakamoto and Nagatani, 1996; Kircher et al., 1999; Yamaguchi et al., 1999; Kim et al., 2000; Kircher et al., 2002;
Matsushita et al., 2003), which has been shown to be required for its light signaling function (Huq et al., 2003; Matsushita et al., 2003).

The phy molecule consists of a polypeptide covalently bound to a linear tetrapyrrole chromophore, phytochromobilin, which is responsible for the photoreversibility of the Pr and Pfr conformers and is required for light signaling (Wagner et al., 1996; Rockwell et al., 2006). The domain structure of phy is well studied and can be broadly divided into an N-terminal light sensing and signaling domain and a C-terminal dimerization and localization domain, with a hinge region in between (Nagatani, 2010). The N-terminus can be further subdivided into four subdomains: the N-terminal extension (NTE), which is thought to stabilize the Pfr form of phy (Cherry et al., 1992); a PAS domain; a GAF domain; and a PHY domain (Rockwell et al., 2006). Within the GAF domain is a conserved cysteine residue that serves as the chromophore attachment site (Rockwell et al., 2006). When the crystal structure of the phy chromophore binding domain from the bacterium Deinococcus radiodurans was solved, it revealed the presence of a so-called light-sensing knot formed by the NTE, PAS, and GAF domains; many mutations – both loss- and gain-of-function – have been identified in and around this knot, demonstrating its importance in phy function (Wagner et al., 2005; Nagatani, 2010). The C-terminus of phys contains two subdomains: a PAS-related domain (PRD) containing two PAS domains (PAS-A and PAS-B) and a histidine kinase-related domain (HKRD) (Rockwell et al., 2006).

In Arabidopsis, the phy family consists of five members, phyA-phyE (Sharrock and Quail, 1989; Clack et al., 1994). Among the five phys, phyA is unique in two respects; it forms only homodimers (Lagarias and Mercurio, 1985; Clack et al., 2009), and it accumulates to very high levels in darkness but is rapidly degraded in R light (Sharrock and Clack, 2002). This light-
labile property of phyA may explain why it plays a key role in the perception of FR light, as well as during the dark-to-light transition (Tepperman et al., 2006; Franklin and Quail, 2010; Kami et al., 2010; Chen and Chory, 2011). The four other phy's are comparatively stable in the light and function predominantly in R light signaling, with phyB playing the most prominent role (Franklin and Quail, 2010; Kami et al., 2010; Chen and Chory, 2011).

### 1.3 The Current Model of Phytochrome Signaling

Components of the phy signaling pathway have been identified through the extensive use of forward genetic screens, frequently using the hypocotyl length of Arabidopsis as a readout (Chory, 2010; Chen and Chory, 2011). These screens have yielded mutants that generally fall into one of two classes (Figure 2). The first class consists of mutants that are constitutively photomorphogenetic; when grown in the dark, they display short hypocotyls, expanded cotyledons, partially differentiated chloroplasts, and aberrant expression of light-regulated genes (Chen and Chory, 2011) (Figure 2). These mutants, which are almost all recessive and represent negative regulators of phy signaling, are often referred to as cop/det/fus mutants, after the founding members of this group: constitutively photomorphogenic1, de-etiolated1, and fusca (Chory et al., 1989; Deng et al., 1991; Castle and Meinke, 1994). This class also includes the quadruple mutants for suppressors of phyA-105 1-4 (spa1-4) (Laubinger et al., 2004) and phytochrome interacting factors1,3,4,5 (pifq) (Leivar et al., 2008; Leivar et al., 2009). These recessive mutants share almost all of their phenotypes with a dominant, constitutively active phyB allele, YHB (Su and Lagarias, 2007; Hu et al., 2009), suggesting that phy's initiate photomorphogenesis by repressing downstream, negatively acting signaling components (Chen and Chory, 2011).
The second class of mutants is made up of positively acting signaling components and is characterized by seedlings with a skotomorphogenetic phenotype in the light; they have long hypocotyls and underdeveloped cotyledons and can be involved in phyA signaling, phyB signaling, or both (Chen and Chory, 2011) (Figure 2). This class includes the phytochrome mutants themselves (Somers et al., 1991; Parks and Quail, 1993; Whitelam et al., 1993). phyA signaling-specific mutants include *long hypocotyl in far-red1 (hfr1)* (Fairchild et al., 2000), *long after far-red1 (laf1)* (Ballesteros et al., 2001), and *far-red elongated hypocotyl1 (fhy1)* (Whitelam et al., 1993). On the other hand, the *red elongated1 (red1)* mutant is specific for phyB signaling (Wagner et al., 1997; Hoecker et al., 2004), and the *hy5* mutant is involved in both phyA and phyB signaling (Koornneef et al., 1980; Ang et al., 1998). Another mutant that participates in both phyA and phyB signaling is *hemera (hmr, discussed below)* (Chen et al., 2010). *hmr* is the founding member of a novel subclass of photomorphogenetic mutants that is both tall and albino in the light (Chen et al., 2010; Chen and Chory, 2011). The albinism associated with the *hmr* mutation is likely due to defects in chloroplast differentiation as well as phy signaling, as the *phyABCDE* quintuple mutant can still produce chlorophyll (Hu et al., 2013). The tall, albino phenotype of *hmr* suggests roles for HMR in both the nucleus and the chloroplasts. Consistent with this idea, HMR has been shown to be dual localized to both of these compartments (Chen et al., 2010).
Given that de-etiolation involves large-scale transcriptional reprogramming, it is perhaps not surprising that many phy signaling components are involved in transcriptional regulation; these components include the positively acting bZIP transcription factor HY5 (Oyama et al., 1997), the atypical basic helix-loop-helix (bHLH) transcription factor HFR1 (Fairchild et al., 2000), and the negatively acting bHLH PIF transcription factors (Leivar et al., 2008). A major mechanism by which phy promote de-etiolation is through the regulation of the abundance of these transcriptional regulators. For instance, phy have been shown to promote the accumulation of HY5 in the light by antagonizing the nucleocytoplasmic partitioning of the E3 ubiquitin ligase COP1, which targets HY5 for proteasome-dependent destruction in the dark (von Arnim and Deng, 1994; Ang et al., 1998; Osterlund et al., 2000; Seo et al., 2003). The light-dependent accumulation of HFR1 is promoted by a similar mechanism (Duck et al., 2004; Yang et al., 2005). Additionally, photoactivated phy directly interact with the negatively acting PIFs to promote their degradation in the light (Al-Sady et al., 2006; Lorrain et al., 2008; Shen et al., 2008). As an added layer of transcriptional regulation, phy have been shown to indirectly regulate the transcriptional activity of PIFs via HFR1, which forms non-DNA binding
heterodimers with PIFs (Hornitschek et al., 2009). Phys also directly regulate PIF transcriptional activity by removing PIFs from the promoters of their target genes (Park et al., 2012).

Collectively, these results have led to a model of phy signaling in which the later steps are fairly well defined but the early steps are still poorly understood (Figure 3). One potential reason for this relative dearth of knowledge about the early events of phy signaling is the use of hypocotyl length as a screening phenotype; light-dependent hypocotyl growth is a process that takes several days to fully manifest, leaving open the possibility that the earliest events in the phy signaling pathway will be missed (Schäfer and Nagy, 2006). Resolution of this problem would require the use of an earlier light signaling response as a screening criterion.

Figure 3: The current model of phy signaling. Positively acting signaling components are shown in red, and negatively acting components are shown in blue. Figure adapted from (Chen and Chory, 2011).
1.4 Phytochrome Localization to Photobodies is One of the Earliest Light Responses at the Cellular Level

Although it was initially thought that phy signaling occurred in the cytoplasm, experiments performed over fifteen years ago convincingly showed that upon light activation, phyB translocates from the cytoplasm to the nucleus (Sakamoto and Nagatani, 1996; Yamaguchi et al., 1999), where it is further compartmentalized into subnuclear foci termed phy nuclear bodies or photobodies (Yamaguchi et al., 1999; Chen and Chory, 2011; Van Buskirk et al., 2012). Additional studies demonstrated that all five Arabidopsis phys localize to photobodies in the light (Kim et al., 2000; Kircher et al., 2002). Although most of these observations were made based on the overexpression of fluorophore-tagged phys, photobodies have also been observed using immunolocalization against endogenous phy in both pea and Arabidopsis, suggesting that photobodies are not an artifact of phy overexpression (Hisada et al., 2000; Kircher et al., 2002).

Consistent with their differing responsiveness to varying wavelengths of light, phyA photobody localization is promoted by R, FR, and blue (B) light, while phyB photobodies form only in R light (Kim et al., 2000; Bauer et al., 2004). Phy localization to photobodies in response to R light is rapid; both phyA and phyB photobodies can be observed within a few minutes after initial R light exposure (Bauer et al., 2004), making photobody localization one of the earliest steps in phy signaling. These “early” photobodies also contain PIF3, which colocalizes with phys prior to its degradation (Bauer et al., 2004). Early photobodies disappear within an hour of light exposure, but so-called “late” photobodies form after an additional hour of R light treatment and persist into the light (Bauer et al., 2004). Because phyA is light-labile, late photobodies primarily contain phyB (Kim et al., 2000; Kircher et al., 2002).
At steady-state, the number and size of phyB photobodies is directly regulated by light via the proportion of phyB in the Pfr form (Chen et al., 2003). In bright R light, when the Pfr form is favored, phyB localizes to a few large photobodies (Chen et al., 2003). In contrast, conditions that favor the Pfr form of phyB, such as dim R light or light with a low R:FR ratio, lead to the formation of many small photobodies (Chen et al., 2003). This steady-state photobody pattern is tightly correlated with the light-dependent inhibition of hypocotyl growth; seedlings grown in dimmer light not only have many small photobodies but also have longer hypocotyls, while seedlings grown in bright light have a few large photobodies and are correspondingly short (Figure 4) (Chen et al., 2003; Van Buskirk et al., 2012). Morphologically, photobodies appear to be relatively stable subnuclear structures. However, at the molecular level, they are very dynamic; the nucleoplasmic and photobody-associated pools of phyB are rapidly exchanged as demonstrated by fluorescence recovery after photobleaching (FRAP) experiments (Rausenberger et al., 2010).

**Figure 4: The steady-state correlation between photobody morphology and hypocotyl length in the light.** Top, confocal images of 4-d old Arabidopsis nuclei expressing phyB fused to GFP (PBG) grown, from left to right, in 0.5, 1, 2, and 8 μmol m$^{-2}$ s$^{-1}$ R light. The light intensity correlates closely with the degree of hypocotyl growth inhibition (seedling images, bottom).

The ability to precisely manipulate photobody morphology using light gives photobodies great potential as a phenotype for genetic screens (Chen, 2008). Such screens have already
identified several new photobody deficient mutants, including hmr (Chen et al., 2003; Chen et al., 2010) (discussed in more detail below).

The intramolecular requirements for photobody formation have been most extensively studied using Arabidopsis phyB as a model (Van Buskirk et al., 2012). Broadly, the domains required for photobody localization have been studied using truncations, and point mutations have been used to determine specific residues that are important for photobody formation and light signal transduction (Van Buskirk et al., 2012). Truncation studies have shown that the PRD is both necessary and sufficient for phyB nuclear import, but that the entire C-terminus, including the PRD and HKRD, is required for photobody localization (Chen et al., 2005). Interestingly, a phyB truncation lacking most of the HKRD can still transduce light signals but can only localize to small photobodies (Krall and Reed, 2000; Chen et al., 2003). However, a point mutation located in the HKRD completely abolishes the signaling activity of the molecule (Krall and Reed, 2000). Consequently, the importance of the HKRD in photobody localization and phy signaling is still unclear (Van Buskirk et al., 2012). The C-terminus alone constitutively localizes to photobodies but is totally nonfunctional (Wagner et al., 1996; Matsushita et al., 2003). In contrast, the N-terminus is sufficient for signaling but does not localize to photobodies (Wagner et al., 1996; Matsushita et al., 2003).

Because light regulates both the nuclear import and photobody localization of phyB, there must be a mechanism by which the light-sensing N-terminus regulates the nuclear localization activity of the C-terminus. The current model is that this regulation occurs through a direct interaction between the two domains: when phyB is in the inactive, Pr form, the N- and C-termini tightly interact and the N-terminus masks the nuclear localization signals contained within the PRD, keeping the molecule in the cytoplasm (Chen et al., 2005). When light converts phyB to
the Pfr form, it leads to a conformational change that weakens the interaction, leading to the unmasking of the NLS and, consequently, nuclear translocation and signaling (Chen et al., 2005).

Point mutations within phyB have also shed light on the intramolecular requirements for photobody localization. Loss-of-function mutations that impair both photobody localization and phy signaling are scattered throughout the molecule; some are deficient in chromophore attachment, while others have altered spectral properties and/or accelerated dark reversion (Kircher et al., 2002; Chen et al., 2003; Oka et al., 2008; Medzihradszky et al., 2013; Nito et al., 2013; Zhang et al., 2013). An additional class of loss-of-function mutants comprises phyB alleles with apparently normal photobody localization and absorption spectra but that are unable to transduce light signals because they cannot interact with PIF3 (Oka et al., 2008; Kikis et al., 2009). These mutants demonstrate that photobody localization of phyB can be separated from its signaling function and show that photobody formation is not a consequence of phy signaling. Gain-of-function mutants have also been identified within the N-terminus of phyB; these alleles localize to large photobodies in dim light and almost universally display reduced dark reversion rates (Adam et al., 2011; Kircher et al., 2012; Medzihradszky et al., 2013; Nito et al., 2013; Zhang et al., 2013). Even more extreme is the constitutively active YHB mutant, which is locked in the active state and localizes to photobodies even in darkness (Su and Lagarias, 2007). Known missense phyB alleles with altered photobody localization are summarized in Figure 5.
Figure 5: Structural basis of phyB photobody localization and signaling. Domains are as described in Sections 1.2 and 1.3, and the chromophore is represented by four consecutive squares. Loss-of-function mutations that impair both photobody localization and phy signaling are shown in blue (Kircher et al., 2002; Chen et al., 2003; Oka et al., 2008; Medzihradszky et al., 2013; Nito et al., 2013; Zhang et al., 2013). Allele G118R does not incorporate the chromophore. Mutant D307A is impaired in the Pr-to-Pfr conversion. Alleles R322A, C327Y, and A587 have slightly faster dark reversion rates than wild-type. Mutant A372T has highly accelerated dark reversion as well as a slightly red-shifted spectrum. Mutant S86D has accelerated dark reversion and no detectable interaction with PIF3. The alleles shown in red are those with normal photobody localization but abolished signaling, possibly due to reduced interaction with PIFs (Oka et al., 2008; Kikis et al., 2009). The G767R mutation, in brown, prevents nuclear import (Matsushita et al., 2003). Gain-of-function mutations are shown in light green, and nearly all of them have been shown to have reduced dark reversion rates (Adam et al., 2011; Kircher et al., 2012; Medzihradszky et al., 2013; Nito et al., 2013; Zhang et al., 2013). The constitutively active Y276H (YHB) mutation is shown in dark green (Su and Lagarias, 2007). Figure adapted from (Van Buskirk et al., 2012).

Several models for photobody function have been proposed. One possibility, the so-called “storage depot” model, posits that phy signaling occurs in the nucleoplasm, that photobodies are storage sites for active phy but are not themselves the sites of phy signaling. In this model, photobodies serve as “slow release valves,” prolonging the half life of the Pfr form and gradually releasing it into the nucleoplasm, thereby extending the time that phy signaling can occur in the absence of light (Rausenberger et al., 2010; Van Buskirk et al., 2012). This model is
supported by the observation that phyB truncations consisting only of the N-terminus are capable of transducing light signals but do not localize to photobodies (Matsushita et al., 2003; Palagyi et al., 2010). A second model, which is not necessarily mutually exclusive, posits that photobodies are sites of protein degradation. Consistent with this model, during the dark-to-light transition, the light-labile PIF3 colocalizes with phys prior to its degradation (Bauer et al., 2004), and the photobody-deficient hmr mutant fails to degrade PIF1 and PIF3 in the light (Chen et al., 2010). Additionally, in a transgenic line overexpressing the N-terminus of phyB fused to GUS, GFP, and a nuclear localization signal (NGB), the NGB transgene product fails to localize to photobodies and Myc-tagged PIF3 accumulates in the light (Park et al., 2012). Despite these observations, or perhaps because of them, the significance of photobodies in light signaling is still debated. Further testing of these models may provide a more unified view of the physiological and molecular significance of photobodies in light signaling.

1.5 hemera Defines a Novel Class of Phytochrome Signaling Mutants

One way to better understand photobody function is to identify and characterize molecular components that regulate photobody localization. To that end, a confocal-based screen was performed to identify extragenic regulators of phyB localization to photobodies. This screen isolated a new mutant, hemera (hmr), that is defective in photobody localization in the light (Figure 6). Importantly, the extragenic hmr mutant definitively shows that there are molecules other than phyB itself that regulate phyB photobody localization.
As mentioned above, the \textit{hmr} mutant is both tall and albino in the light, making it the founding member of a new subclass of phy signaling mutants. Additional physiological characterization of \textit{hmr} showed that it is defective in both the R- and FR-mediated inhibition of hypocotyl growth while showing no equivalent phenotype in B and white (W) light (Chen et al., 2010). Therefore, \textit{hmr} is specifically involved in phy signaling. Additionally, double mutant analysis showed that \textit{HMR} acts between \textit{phys} and the master repressor of photomorphogenesis, \textit{DET1}, demonstrating that \textit{HMR} acts very early in the phy signaling pathway (Chen et al., 2010).

\textit{HMR}’s secondary structure is similar to that of the yeast protein Rad23, which shuttles polyubiquitylated proteins to the proteasome for degradation (Chen and Madura, 2002; Chen et al., 2010). In line with this similarity in structure, \textit{HMR} was able to partially rescue the function of Rad23 in mutant yeast, suggesting that \textit{HMR} plays an important role in protein degradation (Chen et al., 2010). Consistent with this possibility, several light-labile proteins, including phyA, PIF1, and PIF3, accumulate in the \textit{hmr} mutant in the light (Figure 7); \textit{hmr} is the first reported mutant with this phenotype (Chen et al., 2010).
The tall, albino phenotype of hmr suggests that HMR is involved not only in nuclear light signaling responses such as light-dependent hypocotyl growth inhibition, but also in plastid light responses such as chloroplast differentiation. Supporting this idea, in an earlier study, HMR had been isolated as a component of the plastid transcriptionally active chromosome (pTAC) and was shown to be required for normal plastid gene expression; the authors of this study named the protein pTAC12 (Pfalz et al., 2006). An analysis of HMR’s localization showed that the protein is localized to both the nucleus and the chloroplasts, providing a possible mechanism for how HMR mediates light responses in both compartments (Chen et al., 2010).

Because of HMR’s crucial role in phy signaling, and especially in the early step of photobody localization, additional studies were performed to determine how HMR itself is regulated. In line with HMR’s genetic position early in phy signaling, HMR was found to directly interact with photoactivated phys both in vivo and in vitro, and that this interaction is required for HMR’s light-dependent accumulation (Galvão et al., 2012).

1.6 Conclusions and Outstanding Questions

Over the course of several decades, hypocotyl-based forward genetic screens have led to the identification and characterization of many phy signaling components. However, because
changes in hypocotyl length take several days to manifest, the current model of phy signaling is one in which the later events are well defined but the earlier steps are comparatively poorly understood; the nature and regulation of these early steps remain among the great unanswered questions in phy signaling.

One early event that could serve as the basis for a better understanding of early phy signaling events is the light-dependent localization of phyB to photobodies, which is one of the earliest detectable events in the phy signaling pathway. Although some of the intramolecular requirements for photobody localization have been determined, there are still unresolved questions, including the relevance of the HKRD to both photobody localization and phy signaling. Because the entire phyB molecule is involved in regulating and executing light signaling responses (Wagner et al., 1996), an understanding of the role of each domain in phy localization and signaling will clarify what some of the earliest phy signaling events entail, as well as how they are regulated by phyB itself. In the second chapter of this dissertation, I will discuss my effort to understand the role of the HKRD in photobody localization by studying a point mutation in this domain that abolishes signaling.

Although photobodies are a promising system to learn more about early phy signaling events, their physiological relevance remains debated. On one hand, the tight correlation between light intensity, photobody morphology, and hypocotyl length (Chen et al., 2003) suggests that there is an intimate relationship between photobodies and physiological responses. On the other hand, a phyB truncation consisting of the N-terminus of phyB, GUS, GFP, and an NLS (NGB) does not localize to photobodies and, in continuous light, is not only capable of signaling but is even hypersensitive to lower intensities of R light, calling into question the need for photobodies in mediating physiological responses to light. Given this, a better understanding of photobodies’
roles in seedling growth is needed. The third chapter of this dissertation describes my work to ascribe a physiological function to photobodies and resolve the discrepancy between the localization and activity of the PBG and NGB molecules in phy signaling. Many of these findings have recently been published in *Plant Physiology*.

The identification of *hmr* demonstrates that photobody localization can be used as the basis for genetic screens, and *hmr*’s novel tall, albino phenotypic combination suggests that there are additional, early-acting phy signaling components that have yet to be discovered. In the fourth chapter of this dissertation, I report the isolation and characterization of a weak *hmr* allele, which is used as the background for a second genetic screen to identify additional phy signaling components in the same genetic pathway as *hmr*. This screen identified three independent alleles of a gene that we named *SON OF HEMERA (SOH)*. Surprisingly, in an independent, parallel screen looking for tall, albino mutants, we identified additional alleles of *SOH*. These loss-of-function *soh* mutants are also defective in photobody localization. These results provide clear evidence that SOH is a component of the HMR-dependent phy signaling and that SOH, like HMR, is required for photobody localization.

Collectively, the studies described in this dissertation add to our understanding of the early events of phy signaling by identifying both an intragenic and an extragenic requirement for phyB photobody localization, as well as ascribing a physiological function to photobodies.
2. Identification of an Intramolecular Requirement For the Subcellular Localization of Phytochrome B

2.1 Introduction

Previous studies have shown that the C-terminus of phyB, which contains the PRD and the HKRD, is sufficient for both nuclear import and photobody localization of phyB (Matsushita et al., 2003; Chen et al., 2005). Between these two domains, the PRD appears to be sufficient for phyB nuclear localization, but the structural requirements for photobody localization, and specifically the relevance of the HKRD, are less clear. Both the PRD and HKRD seem to be necessary for localization to large photobodies, but a phyB truncation lacking most of the HKRD, phyB-28, can still localize to small photobodies (Chen et al., 2003). Thus, the significance of the HKRD in photobody localization is still uncertain.

Functionally, the necessity of the HKRD in phy signaling is also unclear. The phyB-28 mutant can still transduce light signals, but an HKRD point mutant, phyB-18, that converts aspartic acid 1040 to a valine (Figure 8), is completely nonfunctional (Krall and Reed, 2000).

![Figure 8: Schematic of the phyB HKRD with phyB-28 and phyB-18 mutations shown.](image_url)

Because both nuclear localization and dimerization of phyB are required for its full signaling ability, and because the C-terminus of phyB is required for both of these functions, in collaboration with Meng Chen and Amit Reddy, an undergraduate researcher, I analyzed the localization and dimerization capabilities of the phyB-18 molecule. My data support the idea that
the defect in phyB-18 is due to its inability to localize to the nucleus, and that this faulty localization may stem from the inability of its HKRD to dimerize.

2.2 Results and Discussion

2.2.1 phyB-18 is Defective in Nuclear Import and Photobody Localization

In order for phyB to signal, it must be localized to the nucleus (Huq et al., 2003). Therefore, as an initial test to determine the defect in phyB-18, the full-length phyB-18 coding sequence was fused with YFP (Figure 9A) and its localization in Arabidopsis epidermal cells was analyzed via confocal microscopy. Surprisingly, instead of localizing to the nucleus, phyB-18:YFP was cytoplasmic in the light (Figure 9B).

phyB-18:YFP’s failure to localize to the nucleus does not eliminate the possibility that the molecule might still localize to photobodies if it could be imported into the nucleus. To test this possibility, a SV40 nuclear localization signal was fused to the C-terminus of phyB-18:YFP (Figure 9A) and the localization of the fusion protein in the Arabidopsis epidermis was also determined. phyB-18:YFP:NLS failed to localize to photobodies in the light (Figure 9C), demonstrating that D1040 is required for both the nuclear import and photobody localization of phyB.
Figure 9: phyB-18 is impaired in nuclear and photobody localization. (A) Schematics of phyB-18:YFP (top) and phyB-18:YFP:NLS (bottom). The phyB-18 mutation is indicated by the star. Labels above the phyB schematic indicate domains. The chromophore is represented by four consecutive squares. (B) Localization of phyB-18:YFP and (C) phyB-18:YFP:NLS in the epidermal cells of transgenic Arabidopsis seedlings. Data obtained in collaboration with Meng Chen.

2.2.2 The HKRD of phyB-18 Fails to Dimerize in vitro

Based on the above results, it seemed clear that the proximal cause of phyB-18’s null-like phenotype is its inability to localize to nucleus. One possible explanation for this lack of nuclear localization might be an inability of phyB-18 to dimerize; phyB dimerization is required for its full signaling capability (Wagner et al., 1996), and the C-terminus of phyB has been shown to be involved in both nuclear localization and dimerization (Wagner et al., 1996; Matsushita et al., 2003). Therefore, I tested the dimerization capability of both wild-type and phyB-18 mutated HKRD (phyB fragments encoding amino acids 863-1172) in a yeast two-hybrid assay (Figure 10).
Figure 10: Schematic of yeast two-hybrid plasmid inserts. Representation is as in Figure 8. The phyB-18 mutation is indicated with an asterisk.

The diploids were grown on selective media either containing or lacking the antibiotic Aureobasidin A for 2 days and then scored for growth. As shown in Figure 11, in this assay, the wild-type HKRD could interact with itself, but the mutated form could not. Additionally, the mutated HKRD could not interact with the wild-type, and vice-versa. This lack of interaction was not due to lack of expression of the proteins, as indicated by western blot (Figure 12).

Figure 11: The phyB-18 mutation inhibits HKRD dimerization in a yeast two-hybrid assay. Diploids were grown for 2 d on –Trp-Leu media with or without 125 ng/ml Aureobasidin A and growth was scored. p53/T is the positive control and Lam/T is the negative control provided with the Clontech Matchmaker Gold yeast two-hybrid system. Data obtained in collaboration with Amit Reddy.
Collectively, these results suggest that the phyB-18 mutant behaves as a null because phyB-18 cannot localize to the nucleus (Figure 9B). This inability to localize to the nucleus may be due to the D1040V mutation’s inhibitory effect on HKRD dimerization (Figure 11). This suggests a model in which dimerization of the HKRD is required for phyB’s nuclear localization and, consequently, its function in light signaling.

2.3 Materials and Methods

2.3.1 Strains and Plasmids

The phyB-18:YFP and phyB-18:YFP:NLS constructs were generated by PCR-amplifying the phyB-18 coding sequence and inserting the PCR product into the KpnI site of pMC19 either with or without the SV40 large T antigen NLS fused to the C-terminus of YFP (Chen et al., 2005).

To generate the constructs used in the yeast two-hybrid assay, a fragment encoding amino acids 863-1172 of phyB was PCR amplified from a phyB cDNA template either containing or lacking the phyB-18 mutation using primers:
F: 5'-CACGGCCATATGGCCAAGATACGGATAAGTTC-3'
R: 5'-GGCGGATCCATATGGCATCATCAGCATC-3'

The fragments were inserted into the NdeI and BamHI sites of pGBK7 and pGAD7 (Clontech, Mountainview, CA).

Y2HGold and Y187 yeast strains (Clontech) were transformed with bait and prey vectors, respectively, using the LiCl-mediated method, and the resulting haploids were mated according to the protocols described in the Clontech Yeast Protocols Handbook.

2.3.2 Confocal Microscopy

Four-day old transgenic Arabidopsis expressing either phyB-18:YFP or phyB-18:YFP:NLS were mounted on Superfrost slides in ddH2O and analyzed on a Leica SP2 inverted confocal microscope using the excitation and detection settings specified in (Chen et al., 2010). Images were processed using Adobe Photoshop.

2.3.3 Yeast Two-hybrid Assay

Yeast two-hybrid analysis was performed using the strains provided in the Matchmaker Gold Yeast Two-Hybrid System (Clontech) according to the manufacturer’s instructions. Growth on –Trp-Leu dropout media either containing or lacking 125 ng/ml Aureobasidin A was scored on the second day.

2.3.4 Western Blot

Total protein was extracted from yeast according to the protocol described in the Clontech Yeast Protocols Handbook. Proteins were run on 8% Bis-Tris SDS acrylamide gels and transferred to nitrocellulose membranes. Membranes were probed with anti-cMyc antibodies (diluted 1:1000), anti-HA antibodies (diluted 1:500), or anti-tubulin antibodies (diluted 1:8000). Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit
diluted 1:5000 (Bio-Rad, Hercules, CA). Signals were detected using SuperSignal West chemiluminescent substrate (Thermo-Fisher Scientific, Waltham, MA).
3. Photobody Localization of Phytochrome B is Tightly Correlated With Prolonged, Light-dependent Hypocotyl Growth Inhibition in Darkness

3.1 Introduction

Despite the close steady-state correlation between light intensity, photobody morphology, and hypocotyl length, the physiological relevance of photobodies in regulating seedling growth remains debated; full-length phyB fused with GFP (PBG) is capable of localizing to photobodies and transducing light signals, but a chimeric protein consisting of the N-terminus of phyB fused with GFP, GUS, and an SV40 NLS can also transduce light signals without localizing to photobodies (Chen et al., 2003; Matsushita et al., 2003). In fact, NGB plants are hypersensitive to low-intensity light, suggesting that photobodies are not required for or might even inhibit phy signaling (Matsushita et al., 2003).

To reconcile these seemingly conflicting observations, we analyzed the behavior of the PBG and NGB lines after the R light-to-dark (R-to-D) transition. In diurnal short day (8 h light, 16 h dark) conditions, although plants perceive light during the day, most hypocotyl growth occurs at the end of the night through a mechanism involving at least PIFs 1, 3, 4, and 5 (Nozue et al., 2007; Soy et al., 2012; Soy et al., 2014). The PIFs, in turn, are regulated by the circadian clock and light. In the case of PIF4 and PIF5, transcription is controlled by the circadian clock (Yamashino et al., 2003; Nozue et al., 2007); a circadian-regulated complex consisting of the EARLY FLOWERING3 (ELF3), ELF4, and LUX ARRYTHMO (LUX) proteins binds directly to the PIF4 and PIF5 promoters in the early evening and represses their expression (Nusinow et al., 2011). The abundance of the PIF4 and PIF5 proteins is controlled by activated phys (Nozue et al., 2007; Lorrain et al., 2008). In contrast, the transcription of PIF1 and PIF3 is not circadian regulated; instead, PIF1 and PIF3 are regulated solely at the post-translational level by...
photoactivated phyS (Al-Sady et al., 2006; Shen et al., 2008; Soy et al., 2012; Soy et al., 2014). Additionally, the slow dark reversion rate of phyB (Rausenberger et al., 2010) allows phyB in the Pfr form to persist into darkness, repressing the accumulation of PIFs even after the lights have turned off (Nozue et al., 2007; Soy et al., 2012; Soy et al., 2014). Especially given that photobodies have been proposed to function as stabilizers of the Pfr form of phyB (Rausenberger et al., 2010), these observations lend themselves to the possibility that the PBG and NGB lines might grow differently after the R-to-D transition due to the presence and absence of photobodies, respectively. The following sections describe how this hypothesis was tested.

3.2 Results

3.2.1 PBG Represses Hypocotyl Growth in the Dark Substantially Longer Than NGB

Previous studies have shown that during the first two days post-germination, seedlings grown in darkness or in the light are virtually indistinguishable (Wei et al., 1994). Regardless of light conditions, seedlings experience a burst of growth between the third and fifth days post-germination, but this growth is significantly more pronounced in D-grown seedlings (Wei et al., 1994; Gendreau et al., 1997). As a result, seedlings grown for 4 d in D have longer hypocotyls than seedlings grown either in continuous light or in diurnal conditions; much of the growth repression in R light-grown seedlings is due to the action of phyB (Reed et al., 1994). To assess the effect of photobodies on this growth burst, I designed a R light-to-D transition assay to measure seedling growth in PBG and NGB. In this assay, seedlings were grown for 48 h in 10 μmol m⁻² s⁻¹ R light and then transferred to darkness for 144 h (6 d), and seedling growth was monitored by measuring hypocotyl length at various time points (Figure 13A).

Consistent with previously published results (Gendreau et al., 1997), both the PBG and NGB lines grew substantially during the first 48 h after the R-to-D transition (equivalent to days
2-4 after stratification), after which both lines’ growth rates slowed (Figure 13B,C). However, the growth rate of \( PBG \) was consistently lower than that of \( NGB \), suggesting that \( PBG \) was able to repress seedling growth in darkness more effectively than \( NGB \). Given that both lines are capable of signaling in continuous light, it is possible that this difference in growth kinetics is due to the presence of photobodies in \( PBG \).

**Figure 13**: In a R-to-D transition assay, most hypocotyl growth occurs within the first 2 d after the R-to-D transition, and \( PBG \)’s growth rate is consistently slower than \( NGB \)’s. (A) Schematic of growth conditions and sampling time points for the 10 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) R-to-D transition assay. (B) Absolute hypocotyl lengths of \( PBG \) and \( NGB \) seedlings grown in the conditions shown in (A). (C) Data shown in (B) normalized to the hypocotyl length at time 0.

Because both lines stopped growing approximately 48 h after the R-to-D transition, I performed a more detailed growth kinetics experiment focusing exclusively on this time period (Figure 14A). We arbitrarily defined “growth” as the average seedling height being at least 30% taller than that at time 0. Based on this threshold, the \( PBG \) line could repress growth in darkness for 18 h, while the \( NGB \) line could only repress growth for 6 h, or one third as long as \( PBG \) (Figure 14B). Because the levels of the PBG and NGB transgene products remained relatively constant during the course of the assay (Figure 15), it is likely that the observed growth kinetics are not due to changes in phyB levels. Instead, \( PBG \)’s ability to repress growth in darkness may lie in its ability to localize phyB to photobodies.
Because both \textit{PBG} and \textit{NGB} are transgenic lines that overexpress phyB derivatives, I tested whether similar behavior could be observed in wild-type seedlings and whether, as predicted, this growth was promoted by PIFs and repressed by phys. As shown in Figure 14C, Col-0 was able to repress hypocotyl elongation for 6 h. In contrast, \textit{phyB-9} seedlings began to grow immediately after the R-to-D transition, demonstrating that hypocotyl growth repression in this condition is phyB-dependent. Additionally, the \textit{pifq} mutant displayed negligible growth, showing that the hypocotyl elongation after the R-to-D transition is PIF-dependent.

![Figure 14: PBG can repress growth in darkness 3 times longer than NGB.](image)

(A) Schematic of growth conditions and sampling time points for the shortened 10 µmol m$^{-2}$ s$^{-1}$ R-to-D transition assay. (B) Normalized growth kinetics of \textit{PBG} and \textit{NGB} when grown in the conditions shown in (A). Error bars indicate mean ± SEM of 3 independent experiments. (C) Normalized growth kinetics of Col-0, \textit{phyB-9}, and \textit{pifq} seedlings grown in the conditions shown in (A). Error bars represent the mean ± SD of at least 15 seedlings per genotype.

![Figure 15: PBG and NGB transgene product levels do not change substantially during the 10 µmol m$^{-2}$ s$^{-1}$ R-to-D transition.](image)

Transgene product levels are indicated with an anti-GFP blot; RPN6 was used as a loading control.
3.2.2 *PBG, but not NGB, Can Repress PIF3 Accumulation in Both the Light and the Dark*

Hypocotyl growth is promoted by the PIF family of bHLH transcription factors (Leivar and Quail, 2011). Among the PIFs, PIF1, PIF3, PIF4, and PIF5 have been shown to be important in promoting hypocotyl growth in diurnal conditions. Of these, *PIF4* and *PIF5* transcription is regulated by the circadian clock (Yamashino et al., 2003; Nozue et al., 2007), while *PIF1* and *PIF3* transcription is not (Soy et al., 2012; Soy et al., 2014). Because *PIF3* transcription is not circadian-regulated, and because the half-life of PIF3 in the light is longer than that of PIF1 (Al-Sady et al., 2006; Shen et al., 2008), I used PIF3 as a model to test whether *PBG* and *NGB* differentially regulate PIF abundance.

Using a shortened version of the 10 µmol m⁻² s⁻¹ R-to-D transition assay (Figure 16A), I analyzed PIF3 levels in *PBG* and *NGB* during the R-to-D transition. In *PBG*, PIF3 did not accumulate to detectable levels until 12-18 h after the R-to-D transition (Figure 16B), which perfectly correlated with the onset of growth in this line. Further supporting the idea that PIF3 promotes hypocotyl elongation, the accumulation of PIF3 in Col-0 also correlated with the initiation of growth in wild-type plants – approximately 6 h after the R-to-D transition (Figure 16B). The earlier onset of hypocotyl elongation and PIF3 accumulation in Col-0 compared with *PBG* also suggests that the amount of active phyB is important in determining the length of time that seedling growth can be repressed in darkness.

In striking contrast to *PBG* and Col-0, in both *NGB* and *phyB*-9, PIF3 accumulated in the light and remained detectable for the duration of the experiment (Figure 16B); these results are consistent with those of a previously published report showing that *NGB* accumulates PIFs in the light (Park et al., 2012). Because the transcript levels of *PIF3* did not differ between *PBG* and *NGB* (Figure 17A), as well as between Col-0 and *phyB*-9 (Figure 17B), this discrepancy in PIF3 levels likely occurs at the post-transcriptional level, and possibly due to the effect of photobodies.
Because PIFs are transcription factors, I next wanted to assess the transcriptional activity of PIFs in the assay condition by analyzing the transcript levels of four well-characterized PIF target genes: *PIL1*, *XTR7*, *IAA29*, and *ATHB-2* (Leivar et al., 2009; Hornitschek et al., 2012; Leivar et al., 2012). Consistent with the critical role that PIFs play in activating these genes, none of them were induced in *pifq* at any time during the experiment (Figure 16C). In both Col-0 and *phyB-9*, the expression of these genes was correlated with the abundance of PIF3; in Col-0, their induction began approximately 6 h after the R-to-D transition, while in *phyB-9*, they were already induced in the light relative to Col-0, and their expression increased even more after the transition to darkness (Figure 16C).

Similarly, in *PBG*, the four targets were induced between 12 and 18 h after the light-to-dark transition, again consistent with the onset of hypocotyl growth and PIF3 accumulation (Figure 16B,D, and Figure 14B). In *NGB*, these targets were induced approximately 6 h after the R-to-D transition, which was consistent with the initiation of growth in this line but inconsistent with PIF3 levels (Figure 16B,D and Figure 14B). Together, these results suggest that there might be two mechanisms by which phyB regulates seedling growth: through regulating PIF abundance and/or PIF transcriptional activity. This series of experiments cannot distinguish between these two possibilities for *PBG* and Col-0, which may employ both mechanisms simultaneously. However, the high levels of PIF3 in *NGB* suggest that this line may regulate hypocotyl growth only via the latter mechanism.
Figure 16: *PBG*, but not *NGB* can repress PIF3 in both light and dark. (A) Schematic of the growth conditions and collection time points for the assay. (B) PIF3 abundance in *PBG, NGB, Col-0*, and *phyB-9*. PIF3 levels relative to the mean overall PIF3 level within each line are shown below each blot. RPN6 was used as a loading control. “D,” dark-grown control. (C) Expression of PIF target genes in Col-0, *phyB-9*, and *pifq*. (D) Expression of PIF target genes in *PBG* and *NGB*. Expression levels in (C) and (D) are normalized to those of *PP2A*, and error bars represent the mean ± SD of 3 technical replicates.
Figure 17: *PIF3* transcript levels during the 10 μmol m⁻² s⁻¹ R-to-D transition. Seedlings were grown and sampled as in Figure 16A. (A) *PIF3* expression in *PBG* and *NGB*. (B) *PIF3* expression in Col-0 and *phyB-9*. Expression levels were normalized to those of *PP2A*, and error bars represent the mean ± SD of 3 technical replicates.

### 3.2.3 Phobody Disassembly in *PBG* Correlates With PIF3 Accumulation and Hypocotyl Growth

The differences between the *PBG* and *NGB* lines in hypocotyl growth, PIF3 accumulation, and PIF target gene transcription provided an opportunity to determine whether phohbodies might be involved in one or more of these processes during the R-to-D transition. Previous studies of photobodies have primarily utilized 2D maximum projections to characterize photobody number and size (Yamaguchi et al., 1999; Kircher et al., 2002; Chen et al., 2003; Chen et al., 2010). While this approach is useful when there are only a few large photobodies in the nucleus, in conditions in which phyB is localized to numerous small photobodies, it is possible
that a maximum projection would cause multiple photobodies in different planes to be merged into a single photobody. Consequently, in such conditions, a maximum projection-based approach could lead to the loss of information about photobody number and size.

To circumvent this problem, we took advantage of Huygens 3D imaging software, which allowed for volumetric analysis of photobodies from multiple optical sections. In this analysis, we excluded any object with a volume less than $5 \times 10^{-3} \, \mu m^3$; assuming photobodies are spherical, this volume corresponds to a photobody with a diameter of 0.1 µm. Any object with a volume greater than $5 \times 10^{-3} \, \mu m^3$ and less than $0.2 \, \mu m^3$ (approximately 0.72 µm in diameter) was considered a small photobody, and objects with a volume equal to or greater than $0.2 \, \mu m^3$ were considered large photobodies.

Because the major differences between PBG and NGB in hypocotyl growth, PIF3 accumulation, and PIF target gene transcription occurred within the first 24 h after the light-to-dark transition (Figures 15B, 17B, and 17D), I focused on analyzing the photobody phenotypes of these lines during this time period. As expected, in continuous light, the phyB-GFP in the PBG line localized almost exclusively to large photobodies in all hypocotyl nuclei; although there were some nuclei that contained small photobodies, these were rare (Figure 18). After the R-to-D transition, the photobody phenotype of PBG underwent two major changes. First, during the initial 12 h in darkness, although almost all hypocotyl nuclei still had photobodies, the large photobodies began to disassemble, and many small photobodies began to appear instead (Figure 18). Second, between 12 and 18 h in darkness, photobodies were entirely lost from the majority of hypocotyl nuclei (Figure 18, broken red line). The timing of this second transition correlated tightly with the onset of hypocotyl growth, PIF3 accumulation, and PIF target gene transcription in PBG. In the NGB line, inconsistent with previous reports (Matsushita et al., 2003), we observed a small proportion of hypocotyl nuclei (approximately 15%) with photobodies (Figure
However, the fraction was small enough to still be consistent with the idea that photobodies are required for PIF degradation in the light and for prolonged inhibition of PIF3 accumulation in darkness. These observations regarding NGB suggest that this line can repress PIF3 activity independently of photobodies.

![Figure 18: Photobody morphology in PBG and NGB after the 10 µmol m\(^{-2}\) s\(^{-1}\) R-to-D transition.](image)

Seedlings were grown according to the schematic at the top of the figure. For each line at each time point, a confocal image representative of the majority of nuclei is given. The percentages within the images represent the mean proportion of nuclei displaying the phenotype shown, ± the SEM of at least 3 independent experiments; \(n\) indicates the total number of nuclei analyzed to generate the percentage. Scale bars in the confocal images represent 5 µm. For the PBG line, the time points at which at least half of all nuclei have photobodies also include quantification of the number and size of those photobodies. Blue bars represent small photobodies and yellow bars, plotted on the secondary y-axis, represent large photobodies. The photobodies were binned into the following volume ranges: a, 0.0005-0.0019; b, 0.002-0.0049; c, 0.005-0.099; d, 0.01-0.019; e, 0.02-0.039; f, 0.04-0.079; g, 0.08-0.119; h, 0.12-0.159; i, 0.16-0.199; L, ≥0.20 µm\(^3\). Error bars represent standard deviation, and \(n\) indicates the number of nuclei analyzed to generate the distributions. The vertical broken red line indicates the transition point in PBG after which photobodies disappear, PIF3 accumulates, PIF target genes are transcribed, and hypocotyl growth begins.

3.2.4. Lower Light Intensity Leads to Faster Photobody Disassembly and a Reduced Capacity for Hypocotyl Growth Inhibition in PBG in the Dark

At steady state, photobody number and size can be precisely manipulated by changing light conditions; light treatments that shift the equilibrium in favor of the Pfr form lead to fewer,
larger photobodies, while light treatments that favor the Pr form lead to more, smaller photobodies (Chen et al., 2003; Van Buskirk et al., 2012). Because of this, we reasoned that if photobodies are involved in repressing hypocotyl growth and PIF3 accumulation in the dark, manipulating photobody morphology by changing the light conditions in my assay should also lead to changes in these responses. To test this hypothesis, I first grew PBG and NGB seedlings in a lower light intensity of 1 μmol m$^{-2}$ s$^{-1}$ R light, transferred them to darkness, and monitored the photobody morphology of the lines. Consistent with previous reports (Chen et al., 2003; Van Buskirk et al., 2012; Medzihradszky et al., 2013), at this lower fluence rate, phyB-GFP localized to a mixture of small and large photobodies (compare broken red lines in Figure 19 and Figure 18). These photobodies were lost more rapidly from PBG after the R-to-D transition, disappearing from 80% of all hypocotyl nuclei within 12 h, or 6 h faster than in the high intensity assay (Figure 19). Additionally, large photobodies disassembled and smaller photobodies appeared more quickly in PBG in this condition compared with the higher intensity condition (Figure 19, graphs). In NGB, a small fraction of nuclei had photobodies in the light, but this proportion was smaller than that in the high intensity light condition, suggesting that the photobodies in NGB are also light dependent (Figure 19). However, similar to its behavior in the 10 μmol m$^{-2}$ s$^{-1}$ R light assay, these photobodies disappeared rapidly after the R-to-D transition and were completely lost from all hypocotyl nuclei within the first 6 h in darkness (Figure 19).
Having shown that photobodies disassemble more rapidly in PBG after the 1 µmol m$^{-2}$ s$^{-1}$ R-to-D transition than after the 10 µmol m$^{-2}$ s$^{-1}$ R-to-D transition, I next wanted to determine whether this more rapid photobody disassembly correlated with a more rapid induction of hypocotyl growth, PIF3 accumulation, and PIF target gene transcription (Figure 20A). In the PBG line, the correlation between these responses remained very tight. Consistent with photobodies being lost from the line approximately 12 h after the R-to-D transition, hypocotyl growth also began in PBG 12 h after the R-to-D transition (Figure 20B). The onset of growth coincided with the accumulation of PIF3, as well as the induction of PIF target gene transcription (Figure 20C,D). In contrast, NGB behaved similarly between the 1 µmol m$^{-2}$ s$^{-1}$ and 10 µmol m$^{-2}$ s$^{-1}$ R-to-D transition experiments; hypocotyl growth and PIF target gene transcription began approximately 6 h after the transition to darkness (compare Figure 14A and Figure 20B, Figure 16D and Figure 20D), and PIF3 accumulated in the light and persisted into darkness (compare

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**Figure 19:** Photobodies disassemble more rapidly in PBG after the 1 µmol m$^{-2}$ s$^{-1}$ R-to-D transition. Seedlings were grown according to the schematic at the top of the figure and were sampled and imaged at the indicated time points. Percentages, $n$s, scale bars, and bin volume ranges are as in Figure 18. The vertical broken red line indicates the point at which PBG transitions to having a majority of nuclei with photobodies to having a majority of nuclei without photobodies.
Figure 16B and Figure 20C). Together, these results suggest that photobody localization patterns are responsible for retaining light quantity information during the day and using that information to fine-tune hypocotyl growth responses in darkness.

3.2.5 Prolonged Hypocotyl Growth Inhibition in PBG is Due to Stabilization of the Pfr Form of Phytochrome B

Despite the reported hypersensitivity of NGB to continuous light, the experiments described above clearly show that NGB is not nearly as effective as PBG in repressing hypocotyl growth in the dark. One possible explanation for this difference could be differences in the stability of the Pfr form of phyB in the dark; in other words, PBG and NGB might have different dark reversion rates. Although PBG and NGB have similar dark reversion rates \textit{in vitro} (Oka et al., 2004), \textit{in vivo} dark reversion rates are generally much slower, and photobodies may
contribute to this by stabilizing the Pfr form of phy (Rausenberger et al., 2010; Zhang et al., 2013). To test this possibility, I treated PBG and NGB seedlings with a terminal FR pulse prior to transferring them to darkness, thereby converting all of the active phyB to the inactive Pr form (Figure 21A). Because photobody localization is dependent on phyB being in the Pfr form (Chen et al., 2003), we expected that this FR treatment would lead to the rapid dissociation of PBG photobodies. Consistent with this hypothesis, as well as with previous reports (Rausenberger et al., 2010; Adam et al., 2011), photobodies were completely lost from PBG within 1 h of the FR pulse (Figure 21A). Additionally, the small photobodies seen in a fraction of NGB nuclei were also lost (Figure 21A), suggesting that the formation of these photobodies is also dependent on the Pfr form of phyB.

Next, we analyzed the growth kinetics of PBG and NGB after FR treatment. The FR pulse had a profound impact on the growth of PBG, reducing its growth repression in the dark by half, from 18 h to 9 h (compare Figure 14B and Figure 21B). In contrast, NGB behaved similarly in both conditions, with hypocotyl elongation beginning after 6 h in each case (compare Figure 14B and Figure 21B). This result suggests that the prolonged growth inhibition in PBG after the R-to-D transition is due to photobody stabilization of the Pfr form of phyB. However, even after FR treatment, hypocotyl elongation was still repressed 3 h longer in PBG than in NGB (Figure 21B), suggesting that mechanisms besides Pfr stabilization might be involved in growth repression in PBG.

Consistent with previous reports on PIF3 dynamics (Monte et al., 2004; Leivar et al., 2008), FR treatment led to a rapid (within 1 h) increase in PIF3 in PBG, which correlated perfectly with the dissociation of photobodies in this line (Figure 21A,C). Because under normal circumstances, PIF3 degradation is triggered by the Pfr form of phyB, the presence of PIF3 in PBG can be used as a proxy for the presence of the Pfr form of phyB in this line. Therefore, in
*PBG*, the Pfr form lasts for 18 h after the 10 μmol m\(^{-2}\) s\(^{-1}\) R-to-D transition (Figure 16B), and for 12 h after the 1 μmol m\(^{-2}\) s\(^{-1}\) R-to-D transition (Figure 20C). On the other hand, *NGB* accumulated PIF3 in the light, and the PIF3 level remained steady for the duration of the experiment, even after FR treatment (Figure 21C).

Despite the rapid accumulation of PIF3 in *PBG* after FR treatment, the expression of PIF target genes remained repressed for 9 h (Figure 21D), correlating perfectly with the repression of hypocotyl growth (Figure 21B) and suggesting that hypocotyl growth repression in this condition in *PBG* might be mediated through an unknown mechanism that works independently of photobodies and of the Pfr form of phyB. In contrast, the expression of all four genes was induced immediately in *NGB* after FR treatment (Figure 21D), suggesting that the repression of PIF activity in the dark in this line is dependent on the Pfr form. This suggests that the transcription of PIF target genes in *NGB* can be used as a readout for the presence of the Pfr form; the Pfr form of NGB therefore lasts for 6 h in darkness, regardless of the light intensity (Figure 16D, Figure 20D, and Figure 21D). Together, these results show that the Pfr form of PBG can last up to 12 h longer than the corresponding Pfr form of NGB.
Figure 21: Photobody localization of PBG correlates with the inhibition of PIF3 accumulation, but not with the repression of hypocotyl growth or PIF target gene transcription after the R-FR-D transition. (A) (Top) Schematic of growth conditions and sampling time points, and (bottom) representative confocal images of PBG and NGB localization. One hour after the FR treatment, all photobodies are gone from PBG (broken vertical red line). The percent values indicate the percent of all analyzed nuclei with the phenotype shown in the image (with or without photobodies, mean ± SD of at least 2 independent experiments); n indicates the number of nuclei analyzed to generate the percentage; and the scale bars represent 5 µm. B, Growth kinetics of PBG (black line) and NGB (gray line). The horizontal gray line is the threshold value of 1.3, and black and gray arrows point to where PBG and NGB cross this threshold after FR treatment (9 and 6 h, respectively). Error bars indicate the mean ± SEM of 3 independent experiments. C, Western blot showing PIF3 abundance in PBG and NGB after FR treatment. PIF3 levels relative to the mean overall PIF3 level within each line are given below the blots. RPN6 was used as a loading control. pifq, pif1pif3pif4pif5 quadruple mutant, “D,” dark-grown control. D, Transcript levels of 4 well defined PIF target genes in PBG (black lines) and NGB (gray lines) after FR treatment. Data were normalized to PP2A. Error bars represent the mean ± SD of 3 technical replicates.

3.2.6 A Photobody-deficient Mutant is Impaired in Light-dependent Hypocotyl Growth Inhibition

To add genetic evidence to the idea that photobodies and hypocotyl elongation in the dark are tightly linked, I turned to a mutant that is known to be defective in photobody localization:
hmr-1/PBG (Chen et al., 2010). I first confirmed that hmr-1/PBG is defective in photobody localization in the assay condition. Inconsistent with the initial report by (Chen et al., 2010), after 48 h in continuous R, the hmr-1/PBG mutant was capable of forming large photobodies. However, the overall photobody localization pattern of the mutant more closely resembled that of PBG grown for 48 h in 1 µmol m$^{-2}$ s$^{-1}$, rather than 10 µmol m$^{-2}$ s$^{-1}$, R light (Compare Figure 19 and Figure 22A). This result demonstrates that hmr-1/PBG mutants are defective in photobody localization as early as 2 d post-germination. The difference in localization pattern may have to do with the age of the seedlings; it is possible that large photobodies form more readily in younger seedlings than in older seedlings.

In addition to impaired photobody localization in continuous R light, the large photobodies in the hmr-1/PBG mutant dissociated rapidly after the transfer to darkness, with the large photobodies dissociating into many smaller ones 6 h after the R-to-D transition, also similar to the behavior of PBG during the 1 µmol m$^{-2}$ s$^{-1}$ R-to-D transition (Compare Figure 19 and Figure 22A). Because of difficulties in identifying the hmr-1/PBG mutant seedlings after more than 6 h in darkness, the analysis of photobodies in hmr-1/PBG could not be extended beyond 6 h after the R-to-D transition. Regardless, these results confirm that hmr-1/PBG is impaired in photobody localization in our assay condition.

Having characterized the photobody mislocalization phenotype of hmr-1/PBG during the R-to-D transition, I next asked whether the mutant would be defective in light-dependent hypocotyl growth inhibition in the dark. Consistent with my hypothesis, hmr-1/PBG grew much more quickly than PBG after the R-to-D transition, with the onset of growth occurring after 6 h in darkness (Figure 22B). However, the timing of growth in hmr-1/PBG did not correlate with the complete loss of photobodies from this line. This result suggests that HMR may regulate additional downstream signaling components, and not just phy localization to photobodies.
To determine whether *hmr*’s effect on hypocotyl growth is photobody-dependent, I analyzed the behavior of *hmr-1/NGB* in the R-to-D transition. I crossed *hmr-1/PBG* to *NGB* and identified a single F2 plant that was homozygous for the NGB transgene, had lost the PBG transgene, and was heterozygous for *hmr-1*. I then grew these seedlings in 10 µmol m$^{-2}$ s$^{-1}$ R light for 48 h, then transferred them to darkness for 48 h. At the end of this time, the seedlings were separated by color (green or white, with white seedlings been homozygous for the *hmr-1* mutation) and measured. Consistent with previous reports (Chen et al., 2010; Galvão et al., 2012), in the background of the full-length, photobody-localized *PBG* line, the *hmr* mutation increased hypocotyl length (Figure 22C). However, in the nucleoplasmic NGB background, the *hmr* mutation had a comparatively marginal effect (Figure 22C), suggesting that *hmr*’s phenotype is photobody-dependent.
Figure 22: *hmr-1/PBG* is impaired in hypocotyl growth inhibition in darkness, and its phenotype is photobody-dependent. (A) The photobody phenotypes of *PBG* (left) and *hmr-1/PBG* (right) after being grown for 48 h in 10 µmol m⁻² s⁻¹ R light (Time 0) and 6 h after the R-to-D transition. Graphs, percentages, ns, and scale bars are as in Figure 18. (B) Growth kinetics of *PBG* (black line) and *hmr-1/PBG* (red line). The horizontal broken gray line represents the growth threshold of 1.3, and the red arrow points to the time at which *hmr-1/PBG* crosses this threshold: 6 h after the R-to-D transition. Error bars represent the mean ± SEM of 3 independent experiments. Growth conditions and sampling time points are as in Figure 14A. (C) Hypocotyl lengths of seedlings of the indicated genotypes after being grown for 48 h in 10 µmol m⁻² s⁻¹ R light, then being transferred to darkness for an additional 48 h. The length of the green seedlings is shown in black, and the length of the white seedlings is shown in white. The percent increase in hypocotyl length of the white seedlings over the green seedlings is plotted in gray on the secondary axis. Error bars indicate the mean ± SEM of 3 independent experiments.

### 3.3 Discussion

Although a growing number of reports support the physiological relevance of photobodies in light signaling (Van Buskirk et al., 2012), in continuous light, the nucleoplasmic NGB can also inhibit hypocotyl growth, especially at lower intensities of light (Matsushita et al., 2003; Palagyi et al., 2010). These observations of NGB suggest that photobodies are unnecessary for, and might even act negatively in, light-dependent hypocotyl growth inhibition. In this work, I
developed a R-to-D transition assay to determine the relationship between photobody morphology, hypocotyl growth in the dark, the accumulation of PIF3, and the transcription of PIF target genes. Comparison of the behavior of PBG and NGB lines demonstrates a tight correlation between photobody localization and the repression PIF3 accumulation in both the light and the dark. I also add genetic evidence to this correlation by showing that the photobody-deficient hmr-1/PBG mutant (Chen et al., 2010) is impaired in light-dependent hypocotyl growth inhibition. These results support a model in which photobodies repress the accumulation of PIF3 and inhibit hypocotyl elongation in the dark by stabilizing the active, Pfr form of phyB (Figure 23).

![Figure 23: Model for the function of photobodies in regulating hypocotyl growth in darkness.](image)

The Pfr forms of both PBG and NGB can persist into darkness to repress hypocotyl elongation (cell growth, yellow dotted rectangles). Both PBG and NGB can repress PIF activity and, consequently, cell growth, for a certain period of time. However, photobody localization of PBG also permits (1) repression of PIF accumulation and (2) slowed dark reversion of the Pfr form, thereby prolonging the effects of the Pfr form and facilitating extended hypocotyl growth inhibition in darkness. This photobody-dependent repression of hypocotyl growth allows seedlings to fine-tune the growth response to changing light environments.

### 3.3.1 Photobody Localization of Phytochrome B Closely Correlates with PIF3 Degradation

Accumulating evidence suggests that photoactivated phys regulate PIFs by two mechanisms: promoting their degradation in the light (Al-Sady et al., 2006; Lorrain et al., 2008; Shen et al., 2008) and repressing their transcriptional activity (Park et al., 2012). The data presented here provide two lines of evidence in support of the idea that photobodies are required...
for PIF3 degradation both in the light and in darkness, after the R-to-D transition. First, NGB, which does not localize to photobodies, fails to degrade PIF3 in the light and after the R-to-D transition (Figure 16B). This observation is in line with results obtained by (Park et al., 2012), who showed that NGB cannot not promote the degradation of overexpressed His- and Myc-tagged PIF3 in continuous light. Second, in every condition tested, including the 10 µmol m$^{-2}$ s$^{-1}$ and the 1 µmol m$^{-2}$ s$^{-1}$ R-to-D transition, as well as the R-FR-D transition, when photobodies were absent from at least 50% of nuclei, PIF3 accumulated in PBG (Figure 14B, Figure 20C, and Figure 21C).

Due to the technical difficulty associated with obtaining large quantities of hmr mutant seedlings, especially after only 2 d post-germination, the PIF3 levels in hmr-1/PBG during the R-to-D transition were not determined. However, in 4-d old hmr seedlings, PIF1 and PIF3 both accumulate (Chen et al., 2010), consistent with hmr’s photobody-deficient phenotype. It is therefore reasonable to hypothesize that 2-d old hmr seedlings would also accumulate PIF3 in the light. Collectively, these results support the model that photobody localization of phy is required for PIF3 degradation.

Although it cannot be ruled out that photobody localization and PIF3 degradation are parallel consequences of the photoactivation of phy, the conclusion that photobodies are required for PIF3 degradation is supported by several other observations reported in the literature. The constitutively active YHB mutant also constitutively localizes to photobodies, and PIF3 fails to accumulate in this line even in darkness (Hu et al., 2009; Galvão et al., 2012). Introducing the hmr mutation into the YHB background results in inhibited photobody localization and reversal of the PIF3 degradation phenotype of the line (Chen et al., 2010; Galvão et al., 2012). Additionally, PIF3 colocalizes with active phyB prior to its degradation (Bauer et al., 2004). Mutant forms of phyB that cannot interact with PIF3 localize to apparently normal photobodies but cannot mediate
light-dependent hypocotyl growth inhibition (Oka et al., 2008; Kikis et al., 2009), suggesting that PIF3 recruitment to photobodies is required for its degradation and the subsequent derepression of the photomorphogenic developmental program. These observations, as well as my own, support the model that photobody localization of phyB is required for PIF3 degradation in both the light and the dark.

Despite these observations, the molecular function of photobodies remains unknown. Photobodies could themselves be the sites of PIF3 degradation. However, repeated attempts at colocalizing photobodies with components of the ubiquitin-proteasome system by immunocytochemical methods failed to show specific colocalization of photobodies with polyubiquitylated proteins, as well as the proteasome itself (data not shown). Alternatively, photobodies might be sites for post-translational modification of PIFs, with PIFs subsequently degraded elsewhere (Van Buskirk et al., 2012). For example, the degradation of PIFs is preceded by their phosphorylation (Al-Sady et al., 2006; Lorrain et al., 2008; Shen et al., 2008), and photobodies could be sites where phosphorylation occurs. Consistent with this possibility, PIF3 is only partially phosphorylated in NGB (Park et al., 2012).

Based on the R-FR-D experiment, it seems that the mechanism by which PBG represses PIFs is fundamentally different from that of NGB. A previous report has shown that NGB transduces light signals by removing PIFs from the promoters of their target genes (Park et al., 2012). Consistent with this observation, a FR pulse quickly inactivated NGB, PIF activity was derepressed, and PIF targets were transcribed (Figure 21D). The same FR treatment clearly led to PBG’s inactivation as well, as PIF3 accumulated within an hour (Figure 21C). However, the transcription of PIF targets remained repressed (Figure 21D). This observation implies that there must be an additional mechanism by which PIFs are repressed in PBG but not in NGB. One possibility is that there are repressor proteins present in PBG that are absent in NGB, and the
delay in PIF target induction reflects the time needed to remove these additional repressors. Another possibility is that the chromatin states of the two lines might be different. In NGB, the chromatin might be organized in such a way that PIF target genes are primed for induction, while in PBG these loci are contained within compacted chromatin that needs to be “opened up” in order for transcription to occur. Given that phyB has previously been shown to be involved in chromatin compaction (Tessadori et al., 2009), this is an intriguing possibility.

### 3.3.2 Photobodies Inhibit Hypocotyl Growth in the Dark by Stabilizing the Active Form of Phytochrome B

The dark reversion rate of phyB plays an important role in regulating hypocotyl growth in the dark in diurnal conditions. This importance is perhaps best illustrated through end-of-day FR treatment, in which seedlings grown in light/dark cycles are treated with a pulse of FR light prior to being transferred to darkness; this treatment converts phyB from the active Pfr form to the inactive Pr form (Elich and Chory, 1997). Compared with untreated seedlings, seedlings subjected to end-of-day FR treatment are much taller, demonstrating that the persistence of active phyB is critical for hypocotyl growth inhibition in darkness (Elich and Chory, 1997). The ability of the Pfr form to persist in the absence of light is heavily influenced by its dark reversion rate (Rockwell et al., 2006). Although the dark reversion rates of PBG and NGB are comparable in vitro (Oka et al., 2004), the in vivo dark reversion rate of phyB does not necessarily correlate with that measured in vitro. In the case of full-length phyB, the in vivo dark reversion rate is much slower than the in vitro rate (Hennig and Schafer, 2001), and mathematical modeling has suggested that photobodies are one of the reasons behind this slowed dark reversion in vivo by stabilizing the Pfr form in the dark (Rausenberger et al., 2010). A recent report on the photobody localization patterns of phyB mutants also supports this idea; mutants with reduced dark reversion rates associate with photobodies more easily, and these photobodies are more stable, while
mutants with accelerated dark reversion rates require higher light intensities to localize to photobodies, and these photobodies are not as stable (Zhang et al., 2013). Interestingly, it has recently been shown that the repression of accumulation of PIF1 and PIF3 and, consequently, hypocotyl elongation in the dark, requires the persistence of photoactivated phyB (Soy et al., 2012; Soy et al., 2014).

By comparing the behavior of the photobody-localized PBG and the nucleoplasmic NGB, I provide experimental evidence in support of the model that photobodies stabilize the Pfr form of phyB in the dark. Because the presence of the Pfr form of PBG is closely correlated with the repression of PIF3 accumulation, PIF3 abundance can be used as a way to estimate the persistence of the Pfr form of PBG. In the case of NGB, the presence of the Pfr form is most closely correlated with the repression of PIF target gene transcription; we can therefore use the induction of these targets to estimate the persistence of the Pfr form of NGB. Based on these readouts, the 10 µmol m$^{-2}$ s$^{-1}$ R-to-D transition shows that the Pfr form of PBG can last for 18 h in darkness (Figure 16B), while the Pfr form of NGB can only last for 6 h (Figure 16D). Because PBG and NGB have similar dark reversion rates in vitro (Oka et al., 2004), these results suggest that in vivo, photobody localization of PBG extends the lifetime of its Pfr form three times longer than that of the nucleoplasmic NGB. Comparison of these results with those of the 10 µmol m$^{-2}$ s$^{-1}$ R-FR-D transition shows that FR-mediated dissociation of photobodies in PBG reduces the duration of hypocotyl growth inhibition by half, from 18 h to 9 h (Figure 14B, Figure 21B), suggesting that photobody localization of PBG permits extended repression of hypocotyl growth by stabilizing the Pfr form of PBG.

The persistence of the Pfr form of phyB can also be fine-tuned by altering the intensity of the light in which a seedling is grown. Growing PBG in 1 µmol m$^{-2}$ s$^{-1}$ R light led to the formation of both large and small photobodies, and the lifetime of the Pfr form of PBG in the
dark was reduced from 18 h in the 10 µmol m$^{-2}$ s$^{-1}$ R-to-D transition, to 12 h (Figure 18 and Figure 19). In contrast, the Pfr form of NGB lasted the same length of time in both conditions. These results suggest that photobody localization of phyB can be used to fine-tune hypocotyl growth in darkness in response to varying intensities of light by modulating the dark reversion rate of phyB.

Although these experiments were not performed in diurnal conditions, it is likely that a mechanism similar to that of the R-to-D transition assay is also at work during the day-to-night transition in diurnal conditions. In both cases, hypocotyl growth is promoted by the accumulation of PIFs and repressed by active phys (Soy et al., 2012; Soy et al., 2014). Additionally, because photobodies have similar disassembly dynamics after the R-to-D transition and after the day-to-night transition (Kircher et al., 2002), it is quite possible that photobody localization of phyB is involved in the repression of PIF accumulation and hypocotyl growth seen in the early evening in diurnal conditions. Collectively, these results support the model that photobody localization of active phyB is required for the fine-tuning of seedling growth in the dark in response to changing light conditions.

3.4 Materials and Methods

3.4.1 Plant Materials, Growth Conditions, and Hypocotyl Measurements

The *PBG* (Yamaguchi et al., 1999), *NGB* (Matsushita et al., 2003), *phyB-9* (Reed et al., 1993), and *pifq* (Leivar et al., 2008) lines were as previously described. Seeds were surface sterilized with a rinse in 70% EtOH, followed by 15 min in 50% bleach supplemented with 0.02% Triton-X-100. Seeds were rinsed 5 times with ddH$_2$O prior to plating on half-strength Murashige and Skoog medium supplemented with B vitamins (Caisson) and containing 0.6% phyto agar (Caisson). Seeds were stratified for 5 d in darkness prior to being treated with the indicated light
conditions. The light intensity in the R and FR light LED chambers (Percival Scientific, Perry, IA) was measured using a fiber optic probe and SpectraWiz software (StellarNet, Inc., Tampa, FL). Seedling images were obtained by laying seedlings on a transparency and scanning using an Epson Perfection V700 photo scanner. Hypocotyl lengths were measured using ImageJ software (http://rsbweb.nih.gov/ij/).

### 3.4.2 Protein Extraction and Western Blot

Total protein was extracted from seedlings using a mortar and pestle and 3 volumes of extraction buffer containing bromophenol blue as described previously (Galvão et al., 2012). Protein samples were run on 8% Bis-tris-SDS-acrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Polyclonal antibodies against PIF3 (Chen et al., 2010) were used at a 1:500 dilution, polyclonal antibodies against GFP (Life Technologies) were used at a 1:2000 dilution, and polyclonal antibodies against RPN6 (Enzo Life Sciences, Farmingdale, NY, Cat. #BML-PW8370-0025) were used at a 1:1000 dilution. Secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and were used at a 1:5000 dilution. Blots were visualized on X-ray film using SuperSignal West chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA). PIF3 and GFP levels were quantified using QuantityOne software (Bio-Rad), followed by a multi-step normalization: after background subtraction, the intensity of the PIF3 or GFP band was divided by the intensity of the corresponding RPN6 band. Then, the mean PIF3 or GFP intensity was calculated, and the normalized PIF3 or GFP intensity for each individual time point within each line was divided by this mean. This is the value that is reported in the figures.

### 3.4.3 RNA Extraction and qRT-PCR

Seedlings were flash-frozen in liquid nitrogen and ground to a powder using a plastic mortar and wooden pestle. Total RNA was then extracted using the Spectrum Plant Total RNA
kit (Sigma-Aldrich, St. Louis, MO, Cat. #STRN-250) with on-column DNase treatment (Sigma Cat. #DNASE10-1SET) and yield was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Five micrograms of total RNA was then used for cDNA synthesis using SuperScript II Reverse Transcriptase (Life Technologies, Grand Island, NY; Cat. #18064-014) using oligo(dT)\textsubscript{12-18} primers (Life Technologies, Grand Island, NY; Cat. #18418-012) according to the manufacturer’s instructions. qRT-PCR was performed using FastStart Universal SYBR Green (Roche Applied Science, Indianapolis, IN; Cat. # 04913914001) and a Mastercycler ep realplex qPCR machine with realplex software (Eppendorf). qPCR primers are listed in Appendix A.

3.4.4 Confocal Live-cell Imaging and Quantification of Photobody Morphology

Seedlings were mounted on Superfrost slides (VWR, Radnor, PA; Cat. #48311-600) using ddH\textsubscript{2}O and 22 x 40 mm coverslips (no. 1.5, VWR, Radnor, PA; Cat. #48393-172). Nuclei from hypocotyl epidermal cells were imaged using a Zeiss LSM 510 inverted confocal microscope (Carl Zeiss, Jena, Germany). GFP was detected using a 100x Plan-Apochromat oil immersion objective, 488 nm excitation from an argon laser, and the manufacturer’s default “Green only” detection setting (505-550 nm bandpass detector). Images were collected using LSM 510 software version 4.2. Images were processed using Adobe Photoshop CS5 software (Adobe Systems, Inc., San Jose, CA).

To calculate the overall proportion of nuclei possessing photobodies, maximum projections of optical sections of the hypocotyl cells were generated using LSM Image Browser software version 4.2.0.121 (Carl Zeiss, Jena, Germany). Then, the number of nuclei with photobodies was manually scored. To determine the size and number of photobodies, stacks of optical sections were loaded into Huygens Essential software (Scientific Volume Imaging,
The object analyzer tool was used to threshold the image and to calculate the number and the volume of photobodies in the image. For each nucleus, the photobodies were sorted by size and then manually binned using Microsoft Excel.
4. SON OF HEMERA is a Novel, Early-acting Phytochrome Signaling Component That is Required For Phytochrome Localization to Photobodies

4.1 Introduction

The isolation and characterization of the \textit{hmr} mutant was an extremely exciting discovery; it was the first extragenic phyB photobody mutant to be thoroughly characterized, and it was also the founding member of a novel subclass of phy signaling mutants that is both tall and albino (Chen and Chory, 2011). The novel phenotypes associated with \textit{hmr} meant that this mutant opened a new avenue of research into the early events of phy signaling.

But with the promises that came with \textit{hmr}, came challenges, as well. The albinism associated with the \textit{hmr} null phenotype meant that the mutation was seedling lethal, thereby restricting the types of experiments that could be performed on \textit{hmr} null mutants. For example, in the study of photobody-dependent hypocotyl growth inhibition described in the previous chapter, no analysis of PIF3 accumulation or PIF target gene transcription could be performed because the mutant cannot be easily isolated from a segregating population of 2-d old seedlings. Additionally, because \textit{hmr} null mutants do not have a phentoype in the dark, HMR’s function in the dark and during the dark-to-light transition cannot be studied using \textit{hmr} null mutants. In order to understand HMR’s role in these conditions, an alternative strategy would need to be employed, preferably in the form of a viable, weak allele.

Through the services of the Seattle \textit{Arabidopsis} TILLING project (Till et al., 2003), one such allele was identified, \textit{hmr-22} (Figure 24A). The \textit{hmr-22} mutation converts a highly conserved aspartic acid residue, Asp516, to an asparagine (Figure 24B). \textit{hmr-22} mutants can survive into adulthood and are fully fertile, meaning that, unlike \textit{hmr} null alleles, the \textit{hmr-22} allele can be maintained in a homozygous stock. Analysis of the \textit{hmr-22} protein level in various
light conditions demonstrated that the hmr-22 mutation does not affect the protein’s stability, suggesting that the mutation instead affects HMR’s activity (Li et al., unpublished data).

**Figure 24:** The hmr-22 mutation. (A) Schematic of the HMR protein with null alleles (hmr-2 and hmr-5) and the weak hmr-22 allele highlighted in red. PIR, phy-interacting region. (B) Amino acid sequence alignment of the C-terminus of HMR. The aspartic acid residue that is mutated in hmr-22 is highlighted by a red rectangle. Data obtained by Meina Li and Rafaelo Galvão.

Use of the hmr-22 mutant to study HMR’s role during the dark-to-light transition demonstrated that HMR is required for the degradation of PIF3 at a step subsequent to its light-dependent phosphorylation (Li et al., unpublished data). Additionally, analysis of hmr-22 in the dark showed that it has normal levels of PIF1 and PIF3, indicating that HMR is not involved in the accumulation of PIFs in the dark (Li et al., unpublished data).

Microarray analysis of 4-d old hmr-5 and hmr-22 seedlings showed that over 1,300 genes are statistically significantly changed by twofold (SSTF, (Hu et al., 2009)) in hmr-5 (Figure 25A). In hmr-22 nearly 400 genes were SSTF, and nearly 90% of those were also misregulated in hmr-5 (Li et al., unpublished data) (Figure 25A), further demonstrating that hmr-22 is a true weak, loss-of-function HMR allele. In both mutants, many plastid genes were also SSTF, validating HMR’s importance in regulating both nuclear and plastidial transcription (Li et al., unpublished data).
Because PIF1 and PIF3 accumulate in hmr mutants (Chen et al., 2010; Galvão et al., 2012), the SSTF genes in hmr-5 were compared with SSTF genes in pifq (i.e. genes whose expression is dependent on PIFs), and 203 overlapping genes were identified (Figure 25B). These genes were divided into four classes based on the effect that PIFs and HMR had on their expression: Class I, repressed by PIFs and repressed in hmr; Class II, induced by PIFs and induced in hmr; Class III, repressed by PIFs and induced in hmr; and Class IV, induced by PIFs and repressed in hmr (Li et al., unpublished data).

![Figure 25: Microarray analysis of hmr-5 and hmr-22.](image)

**Figure 25: Microarray analysis of hmr-5 and hmr-22.** (A) Seedlings were grown for 4 d in 10 μmol m⁻² s⁻¹ R light. The Venn diagram shows the number of SSTF genes in hmr-5 (left), hmr-22 (right), and those genes that are misregulated in both genotypes. (B) Comparison of SSTF genes hmr-5 seedlings (left) and pifq seedlings (right).

In this chapter, I describe additional characterization of hmr-22. I then describe the effort to identify genes that act in the same signaling pathway as HMR by performing a suppressor screen using hmr-22 as the genetic background. Unexpectedly, the first three mutants isolated from this screen represented three independent alleles of the same gene, which we named SUPPRESSOR OF HEMERA (SOH). The soh alleles all suppress hmr-22 in an allele-specific manner, suggesting a direct interaction between SOH and HMR. Surprisingly, an independent, parallel screen based on hmr’s tall, albino phenotype also identified alleles of SOH, leading us to rename SUPPRESSOR OF HEMERA as SON OF HEMERA. These null soh alleles have similar phenotypes as hmr null mutants, including impaired localization of phyB to photobodies. Many of these experiments were performed in collaboration with other members of the Chen lab, and
their contributions are noted where applicable. The results described in this chapter collectively establish \textit{hmr-22} as a true weak, loss-of-function \textit{hmr} allele and identify \textit{SOH} as an early-acting component of phy signaling that works in the same genetic pathway as \textit{HMR}. They also establish \textit{SOH} as a new, extragenic factor required for phyB photobody localization.

\textbf{4.2 Results}

\textbf{4.2.1 \textit{hmr-22} Has Defective Plastids in the Light, but not in the Dark}

The \textit{hmr-22} mutant has a virescent phenotype; the cotyledons and new leaves emerge yellow, then gradually turn green. Consequently, 4-d old \textit{hmr-22} seedlings are much paler than their wild-type counterparts and contain substantially less chlorophyll (Figure 26A). Examining the ultrastructure of the plastids in 2-d old \textit{Col-0}, \textit{hmr-22}, and \textit{hmr-2} mutants showed that \textit{Col-0} chloroplasts had thylakoid membranes and small stacks of grana (Figure 26B). In contrast, both \textit{hmr-2} and \textit{hmr-22} had clearly abnormal plastids with only rudimentary membrane structures (Figure 26B). However, despite the clear chloroplast defects in \textit{hmr-22} mutants, they were still able to survive into adulthood.

Interestingly, transmission electron microscopy performed on the plastids of 2-d old, dark-grown \textit{Col-0} and \textit{hmr-22} seedlings showed no obvious difference between the plastids of either line; both had clearly defined prolamellar bodies, suggesting that \textit{HMR} is not required for normal etioplast formation in the dark (Figure 26C).
Figure 26: *hmr*-22 has defective plastids in the light, but normal plastids in the dark. (A) (Left) Images of 4-d old seedlings grown in W light. (Right) Quantification of chlorophyll levels in the seedlings shown at left. Error bars indicate the mean ± SEM of 3 independent experiments. (B) Transmission electron micrographs of plastids from 2-d old seedlings grown in 8 μmol m⁻² s⁻¹ R light. Scale bars indicate 500 nm. (C) Transmission electron micrographs of plastids from 2-d old dark-grown seedlings. Scale bars indicate 200 nm. Data obtained in collaboration with Meina Li and Meng Chen.

4.2.2 *hmr*-22 is Defective in All Analyzed Phytochrome-mediated Responses

There are several responses that are mediated by phyA: phyA mediates the very low fluence response (VLFR) and the high irradiance response to FR light (HIR-FR), while phyB mediates the low fluence response (LFR) and the high irradiance response to R light (HIR-R); *hmr* null mutants are defective in all of these responses (Chen et al., 2010). To determine whether *hmr*-22’s phenotypes are similar to those of *hmr* null mutants, we analyzed all of these responses.
in hmr-22. First, we tested the VLFR by growing hmr-22 for 4 d under hourly pulses of dim FR light (Casal et al., 2000). Similar to both phyA-211 and hmr-2, the cotyledons of hmr-22 remained closed in this condition, indicating that hmr-22 is deficient in the VLFR and has a response similar to that of hmr-2 (Figure 27A). Then, we analyzed the LFR by growing Col-0, hmr-22, hmr-2, and phyB-9 plants for 5 d in short-day conditions either with or without end-of-day FR (EODFR) treatment, which consists of a 15 min pulse of FR light at the end of each light period (Elich and Chory, 1997). The hmr-22 mutant had an intermediate response to this treatment, responding more strongly than hmr-2 but more weakly than Col-0, demonstrating that hmr-22 is impaired in the LFR, but not as severely as the hmr null mutant (Figure 27B). Similarly, hmr-22 had reduced HIR-R and HIR-FR responses (Figure 27C,D). However, like the hmr null mutant, hmr-22 had a normal cryptochrome-mediated B light response. Collectively, these results show that hmr-22 is defective in all phy modes of action and generally has a weaker phenotype than hmr-2.
Figure 27: *hmr-22* is defective in all *phy* modes of action. (A) Images of the cotyledons of seedlings grown for 4 d under hourly pulses of FR light to test the VLFR. (B) Seedling responses to EODFR treatment. Hypocotyl lengths of seedlings grown without EODFR treatment are shown in gray, and with EODFR treatment are shown in white. The percent increase in hypocotyl length after EODFR treatment is plotted in red on the secondary axis. (C) R light fluence response curve. (D) FR light fluence response curve. Error bars represent SEM. Data obtained in collaboration with Rafaelo Galvão.

4.2.3 Identification of **SUPPRESSOR OF HEMERA (SOH)** via *hmr-22* Suppressor Screen

The yellow cotyledons of *hmr-22* mutants, and the ability to maintain *hmr-22* in a homozygous stock, made it an ideal genetic background for a suppressor screen. In this screen, we used EMS mutagenesis to identify second-site mutations that restore normal greening to *hmr-22*. We grew mutagenized *hmr-22* seeds in strong white light for 4 d and looked for mutants with green, rather than yellow, cotyledons. Using this screening strategy, we were able to identify three independent mutants that we named *suppressors of hmr (soh)*. All three of these mutants were able to fully rescue the greening defect seen in *hmr-22* seedlings (Figure 28A). Consistent with the full rescue of greening, the *soh* mutants were also able to fully rescue *hmr-22*’s defects in chloroplast gene transcription (Figure 28B).
We then crossed the suppressors, which were in the Col-0 background, to the Landsberg erecta (Ler) ecotype and generated an F2 mapping population for use in map-based cloning. Surprisingly, rough mapping of all of the suppressors placed them in the same region of the genome, on the long arm of chromosome 4. Additional fine mapping of one of the mutants, soh-102, further narrowed down the region (Figure 29), and sequencing revealed the mutation to be a single C-to-T mutation in At4g28590. Subsequent sequencing of SOH in the other two mutants demonstrated that both also contained mutations in this gene.
Figure 29: Map-based cloning of soh-102. The soh-102 locus is shown in between marker pairs 923/924 and 937/938 on Chromosome 4. Three recombinants out of 1014 chromosomes indicated that the soh-102 mutation was in a gene located on BAC T5F17.

The SOH protein consists of four predicted domains: a transit peptide spanning amino acids 1-92 (ChloroP1.1, http://www.cbs.dtu.dk/services/ChloroP/) (Emanuelsson et al., 1999); a monopartite nuclear localization signal (NLS) spanning amino acids 90-100 (NLS mapper, http://nls-mapper.iab.keio.ac.jp) (Kosugi et al., 2009); a PEST domain spanning amino acids 140-172 (epestfind, http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind); and a thioredoxin (Trx)-like fold lacking the canonical CXXC catalytic site that spans amino acids 200-313 (Phyre2, http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) (Kelley and Sternberg, 2009) (Figure 30). A BLAST search performed by Chan Yul Yoo using the full-length SOH amino acid sequence identified SOH orthologs within most plant species, including dicots, monocots, and moss. SOH orthologs in the Brassica family all possess a transit peptide, a monopartite NLS, a PEST domain, and a Trx-like fold, suggesting functional conservation of these domains in the Brassicaceae.
Figure 30: Schematic of the domain structure of SOH, with mutations shown. Mutations shown in green are \textit{hmr}-22 suppressors, and mutations shown in red are null or null-like (discussed below). Data were obtained in collaboration with Tao Ma, He Wang, and Chan Yul Yoo.

Through previous genetic screens looking specifically at chloroplast phenotypes, SOH has been identified as a factor that is essential for chloroplast biogenesis and PEP-mediated chloroplast gene transcription (Qiao et al., 2011; Yu et al., 2011; Powikrowska et al., 2014; Yua et al., 2014). Perhaps not surprisingly, then, several of these reports have shown that SOH is a chloroplast-localized protein that colocalizes with the nucleoid marker PEND and interacts with components of the chloroplast transcriptional machinery (Qiao et al., 2011; Yu et al., 2011; Powikrowska et al., 2014; Yua et al., 2014). One study suggested that SOH possesses disulfide reductase activity despite the lack of the canonical catalytic site (Yua et al., 2014). Collectively, these studies demonstrate that SOH is critical for proper chloroplast development and function, consistent with the \textit{soh} mutants rescuing greening and plastid gene expression in the \textit{hmr}-22 background.

4.2.4 The \textit{soh} Alleles Rescue the Nuclear Defects of \textit{hmr}-22

In addition to its plastid defects, \textit{hmr}-22 is also impaired in nuclear responses to light, including the HIR-R, HIR-FR, PIF accumulation, and PIF-dependent gene transcription. To determine whether the \textit{soh} alleles could also rescue the nuclear defects of \textit{hmr}-22, I analyzed these phenotypes in the \textit{sohhmr}-22 double mutants.

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Growing the seedlings for 4 d in either R or FR light showed that all three suppressors were able to fully rescue the long hypocotyl of *hmr-22* in either condition (Figure 31A). All three suppressors could also rescue the accumulation of phyA and PIF3 in *hmr-22* in both R and FR light (Figure 31B). Consistent with the rescue of PIF3 levels, the transcription of PIF target genes from Class II (induced by PIFs, induced in *hmr*) and Class IV (induced by PIFs, repressed in *hmr*) was also rescued in all three suppressor lines (Figure 31C). Collectively, these results show that the soh alleles can rescue not only the chloroplast, but also the nuclear phenotypes of *hmr-22*.

**Figure 31:** The suppressors rescue the nuclear defects of *hmr-22*. (A) Hypocotyl lengths of seedlings grown for 4 d in either 2.2 µmol m⁻² s⁻¹ R light (left) or FR light (right). *, significantly different from *hmr-22*, p < 0.1. Error bars indicate the mean ± SEM of 3 independent experiments. (B) Protein accumulation phenotypes of Col-0, *hmr-22*, and *hmr-22soh* seedlings grown in 10 µmol m⁻² s⁻¹ R light (top) or FR light (bottom). (C) Transcript levels of Class II (PIF-induced, induced in *hmr*) and Class IV (PIF-induced, repressed in *hmr*) genes in seedlings grown for 4 d in 10 µmol m⁻² s⁻¹ R light. Data obtained in collaboration with Chan Yul Yoo and Yiting Shi.
4.2.5 The Suppressors Are Allele-specific

To determine the allele specificity of the suppressors, I crossed all three soh alleles to the hmr-5 null mutant and generated F2 plants that were homozygous for the given suppressor allele and heterozygous for hmr-5. I then grew the seedlings for 4 d in 10 μmol m⁻² s⁻¹ R light and analyzed the phenotype of the double mutants. None of the soh alleles were able to rescue the tall, albino phenotype of hmr-5 (Figure 32A,B), suggesting that all three suppressors are allele-specific for hmr-22. This result, in turn, suggests that HMR and SOH might directly interact.

**Figure 32:** The hmr-22 suppressors are allele-specific. (A) Images of seedlings of the indicated genotypes grown for 4 d in 10 μmol m⁻² s⁻¹ R light. (B) Quantification of the hypocotyl lengths of the seedlings shown in (A). *, statistically significantly different from Col-0, p < 0.001. Error bars indicate the mean ± SD.

4.2.6 Null soh Mutations Confer a hmr-like Phenotype

Because the hmr-22 suppressor mutations of SOH are missense alleles, one question that arose during their characterization was the nature of the phenotype conferred by soh null mutations. As mentioned above, chloroplast-specific screens performed by other groups had already demonstrated that SOH is critical for chloroplast differentiation and the transcription of plastid-encoded genes, but nobody had characterized nuclear responses to light in these mutants. Therefore, I characterized phy signaling responses in soh null mutants, but in an unconventional and highly unexpected manner.
At the same time as the hmr-22 suppressor screen was being performed, other members of the lab, including Tao Ma, and He Wang, were performing a hmr-like screen looking for mutants that were tall and albino in R light. Several mutants were identified, and we collaborated with Drs. Jun Cao and Detlef Weigel, to performed simultaneous mapping and deep sequencing of the mutants via SHOREmap (Schneeberger et al., 2009). Surprisingly, three of the mutants isolated from this screen were null alleles of SOH (Figure 30). All three mutants were both tall and albino in the light (Figure 33), and all three had photobody localization defects (Figure 34), just like hmr-1/PBG. We also obtained a T-DNA insertion allele of SOH (SALK_075057) (Alonso et al., 2003), soh-10 (Figure 30), and found that it and soh-1/PBG, like hmr null mutants, accumulate PIF1, PIF3, and phyA in the light. The identification of these soh alleles prompted us to rename SOH from SUPPRESSOR OF HMR to SON OF HEMERA.

Figure 33: soh null mutants are tall and albino in R light. Seedlings were grown for 4 d in 10 µmol m\(^{-2}\) s\(^{-1}\) R light. (A) Images of the parental PBG line, as well as the soh-1, soh-2, and soh-3 mutants. The hmr-1 mutant is also shown for comparison. (B) Quantification of hypocotyl length of soh mutants. Data were obtained in collaboration with He Wang and Chan Yul Yoo.
Genetic analysis of soh-10 demonstrated that SOH, like HMR, is genetically between phys and the master repressor of photomorphogenesis DET1. Additional double mutant analysis of soh-10hmr-5 showed that the double mutant phenotype was not additive, providing an additional line of evidence supporting the idea that SOH and HMR act in the same genetic pathway. Collectively, these results demonstrate that, in addition to its role in chloroplast differentiation, SOH also regulates light responses in the nucleus, including the localization of phyB to photobodies. Further confirming the results of the hmr-22 suppressor screen, SOH and HMR work in the same genetic pathway to mediate photomorphogenesis.

### 4.2.7 SOH is Dual-localized to the Nucleus and Chloroplasts

Although previous reports have clearly demonstrated that SOH is localized to the chloroplasts (Qiao et al., 2011; Yu et al., 2011; Powikrowska et al., 2014; Yua et al., 2014), our detection of an NLS within the protein, the many nuclear phenotypes of soh, and the direct interaction between SOH and HMR all pointed to the possibility that SOH, like HMR, is dual-localized to both the chloroplasts and the nucleus. To test this possibility, I transiently expressed SOH-CFP in tobacco epidermal cells and observed the fusion protein’s localization via confocal microscopy. Consistent with my prediction, I observed fluorescence in the chloroplasts, where
SOH-CFP localized to punctate structures, which was consistent with a previous report (Qiao et al., 2011), as well as diffusely in the nucleoplasm (Figure 35). In this condition, SOH-CFP appeared to localize more strongly in the chloroplasts than in nuclei (Figure 35).

### Figure 35: SOH is dual-localized to the nucleus and the plastids in tobacco epidermal cells

Transient expression analysis of either CFP (top) or SOH-CFP (bottom) in tobacco epidermal cells. N, nucleus, P, plastid.

#### 4.2.8 SOH and HMR Colocalize on Nuclear Speckles in Tobacco

The lack of a canonical catalytic site in SOH’s thioredoxin-like fold suggests that rather than, or perhaps in addition to, catalyzing the reduction of disulfide bonds, this domain might be involved in protein-protein interactions. Given the predicted disulfide bonds in HMR’s N-terminus, it is tempting to hypothesize that this interaction has something to do with the suppressors’ ability to suppress *hmr-22*’s phenotypes. At least one report has shown that a direct interaction between two proteins results in the masking of the localization signals of one of the partners, providing a mechanism by which its partitioning, and therefore its function, is regulated (Chen et al., 2012). To test whether this might be true in the case of HMR and SOH, I transiently coexpressed SOH-CFP and HMR-(PT)_4P-HA-YFP (hereafter referred to as HMR-HA-YFP) in tobacco epidermal cells. When expressed alone, SOH-CFP clearly localized to both the nucleus, where it was diffusely distributed, and the chloroplasts, where it localized to punctate structures
(Figure 35, bottom and Figure 36, top). In contrast, although HMR localizes to both the nucleus and the chloroplasts, when expressed alone in tobacco, YFP fluorescence from the HMR-HA-YFP construct was only visible in punctate structures in the chloroplasts (Figure 36, middle). Surprisingly, in cells expressing both SOH-CFP and HMR-HA-YFP, the nuclear fluorescence of both proteins became much higher, and the proteins colocalized on nuclear speckles (Figure 36, bottom). This result suggests that SOH and HMR might regulate each other’s localization, promoting the accumulation of both proteins in the nucleus.

Figure 36: SOH and HMR alter each other’s localization in tobacco epidermal cells. Top, SOH-CFP expressed alone; middle, HMR-HA-YFP expressed alone; bottom, SOH-CFP and HMR-HA-YFP expressed together.

To test whether altered HMR localization might be the reason behind \textit{hmr-22}’s phenotypes, the \textit{hmr-22} mutation was introduced into HMR-HA-YFP and transiently expressed in tobacco epidermal cells. Contrary to the hypothesis, the localization pattern of hmr-22-HA-YFP was very similar to that of HMR-HA-YFP (Figure 37A), suggesting that the \textit{hmr-22} mutation does not affect HMR’s localization in tobacco in the absence of SOH. However, it was still
possible that, when coexpressed with SOH, the localization of the two proteins would be different. Therefore, I coexpressed hmr-22-HA-YFP with wild-type SOH-CFP, but again, I found no difference in localization compared with wild-type HMR and wild-type SOH together (Figure 36 and Figure 37A). Finally, I tested the effect of soh-102 on localization when expressed alone or with HMR or hmr-22. By itself, soh-102 had a similar localization pattern as SOH: diffuse nucleoplasmic fluorescence, and punctate localization in the chloroplasts (Figure 37B). Coexpression of soh-102 with either wild-type HMR or hmr-22 resulted in a similar localization pattern as that seen with wild-type SOH: punctate localization of both proteins in the nucleus and a concurrent reduction of fluorescence in the chloroplasts (Figure 36 and Figure 37B). Together, these results demonstrate that although SOH and HMR clearly have an effect on each other’s localization in tobacco, this effect does not appear to be altered by either hmr-22 or soh-102. These studies do need to be expanded by looking at the functionality of both SOH and HMR in this condition. However, at least preliminarily, these results suggest that altered localization is neither the cause of hmr-22’s defect nor the mechanism behind the suppressors’ ability to suppress it. Regardless, the colocalization of SOH and HMR still supports the idea that SOH and HMR directly interact.
Figure 37: hmr-22 and soh-102 do not affect the localization patterns and colocalization of SOH and HMR. (A) Top, localization of wild-type HMR; middle, localization of hmr-22; bottom, colocalization of hmr-22 and wild-type SOH. (B) First row, localization of wild-type SOH; second row, localization of soh102; third row, colocalization of soh-102 with wild-type HMR; fourth row, colocalization of soh-102 with hmr-22.
4.3 Discussion

Studies of hmr null mutants clearly demonstrated that HMR is a positive regulator of photomorphogenesis that acts early in phy signaling and is required for phyB localization to photobodies (Chen et al., 2010). hmr’s unique phenotypes opened up many new possibilities in the study of phy signaling, but they also led to unique challenges associated with working with an albino mutant. In particular, because hmr null mutants die as seedlings and have no discernable dark phenotype, studies on HMR’s function in the dark and during the dark-to-light transition could not be easily performed using null mutants. Here, I describe the characterization of a viable, weak hmr allele, hmr-22, that recapitulates all analyzed hmr null phenotypes, albeit more weakly (Figure 26 and Figure 27).

The isolation of hmr-22 also provided the genetic material needed for a hmr suppressor screen, which yielded several alleles of the same gene, SON OF HEMERA (SOH) (Figure 28 and Figure 30). Although SOH had been previously shown to be involved in chloroplast biogenesis, my characterization showed that SOH also functions in the nucleus and is able to rescue the phenotypes associated with hmr-22 in both compartments (Figure 28 and Figure 31). Surprisingly, a parallel screen performed in the lab looking for tall, albino (hmr-like) mutants also yielded null alleles of SOH (Figure 30). These alleles shared similar phenotypes as hmr null mutants, including impaired phyB localization to photobodies (Figure 34). Consistent with soh having both nuclear and chloroplast phenotypes, I show that SOH is dual-localized to both compartments (Figure 35). The hmr-22 suppressor alleles of SOH work in an allele-specific manner (Figure 32), suggesting that SOH and HMR directly interact. Together, these results support a model in which SOH and HMR work together via direct interaction both in the nucleus and in chloroplasts (Figure 38). In the nucleus, this SOH-HMR complex mediates degradation of light-labile proteins and phyB localization to photobodies. In the chloroplasts, these two proteins
work together to regulate plastid gene transcription. Working in concert, SOH and HMR together promote photomorphogenesis in both the nucleus and the chloroplasts (Figure 38).

![Figure 38: Model for the function of SOH and HMR in promoting photomorphogenesis.](image)

In the nucleus, SOH and HMR work in a complex downstream of phys to promote photobody localization and inhibit hypocotyl growth. In the chloroplasts, SOH and HMR are also part of a complex, this one promoting the expression of photosynthesis-related genes encoded in the plastids to promote chloroplast differentiation. Through their functions in both compartments, SOH and HMR work in concert to promote photomorphogenesis.

The discovery of hmr suggested that there is an entire branch of the phy signaling pathway that has yet to be discovered. One way to find new components of this signaling pathway is through a hmr suppressor screen, which would identify components that specifically act in the same branch as HMR itself. While the hmr null background would have made such a screen prohibitively difficult, the hmr-22 allele made it possible. The identification of three independent alleles of SOH through this screen (Figure 28) illustrates that it is a viable strategy for isolating new phy signaling components, and that the screen is very specific.

The identification of loss-of-function soh mutants through a parallel, independent hmr-like screen further demonstrates the importance of SOH in phy signaling in general, and in the
HMR-mediated branch of phy signaling specifically. All of the phenotypes examined in soh are similar to those found in hmr. In particular, phyB localization to photobodies is impaired (Figure 34), making SOH the second known extragenic regulator of large photobody formation in the light.

Both the loss-of-function and hmr-22 suppressor alleles of SOH have nuclear and plastid phenotypes. The importance of SOH in chloroplast differentiation has been shown in previous studies (Qiao et al., 2011; Yu et al., 2011; Powikrowska et al., 2014; Yua et al., 2014), and our results confirm and extend these findings. However, presumably because of their focus on the chloroplasts, these reports have all missed SOH’s role in the nucleus, even, in one case, in the face of clear evidence of SOH’s nuclear localization (Qiao et al., 2011). Our results clearly demonstrate that SOH plays an equally important role in the nucleus, controlling the localization of phyB to large photobodies and inhibiting hypocotyl elongation (Figure 33 and Figure 34). Consistent with SOH’s role in both compartments, I show that SOH also localizes to both places (Figure 35), making in among the few known proteins in plants that localize to both the nucleus and the chloroplasts.

SOH has a paralog in the Arabidopsis genome, and this gene has also been identified in the chloroplast-based screens that isolated SOH (Qiao et al., 2011; Powikrowska et al., 2014). We also identified an allele of the paralog in our hmr-like screen and named it DAUGHTER OF HEMERA (DOH). As in both hmr and soh, phyB cannot localize to large photobodies in the doh null mutant. Interestingly, although we recovered DOH from the hmr-like screen, we have yet to isolate it from the hmr-22 suppressor screen, suggesting that SOH and DOH perform nonredundant functions. Consistent with the idea of nonoverlapping functionality, the localization of these two proteins also appears to be different (Qiao et al., 2011; Powikrowska et al., 2014).
The allele specificity of the suppressors and the colocalization of SOH and HMR on nuclear speckles hinted at the possibility of a direct interaction between SOH and HMR. In fact, \textit{in vivo} co-immunoprecipitation and \textit{in vitro} GST pulldowns performed by Chan Yul Yoo also indicate that this is the case both \textit{in vivo} and \textit{in vitro}. This interaction is not merely an artifact, as a different allele of \textit{SOH} was identified in a suppressor screen for the \textit{var2} mutant and was found to work as a bypass mutation in this case (Yu et al., 2011).

Despite the genetic, cell biological, and biochemical evidence indicating a direct interaction between HMR and SOH, the significance of this interaction is still unclear. Although it is apparent that coexpression of HMR and SOH results in substantial changes in the localization of both proteins, this localization pattern is not affected by \textit{hmr22} or \textit{soh102} (Figure 37). Therefore, although SOH and HMR do appear to regulate each other’s localization, this change in localization does not seem to be the reason behind the suppressors’ ability to suppress \textit{hmr-22}. Instead, the suppressors may work by regulating the activity of \textit{hmr-22}. This regulation of activity could occur by SOH serving as a “bridge” between HMR and an additional protein that is required for HMR’s function. Regardless, when the mechanism by which the suppressors suppress \textit{hmr-22} is determined, it will give great insight into the regulation of the early events of phy signaling.

The question still remains, then: what are the speckles to which SOH and HMR colocalize? Because HMR and SOH are the first two extragenic regulators of photobody formation to be characterized, and because HMR has previously been shown to localize to the periphery of photobodies (Chen et al., 2010) one logical possibility is that these speckles are photobodies. Testing whether the colocalization of SOH and HMR is light-dependent, and/or tagging phyB with a third fluorophore and using it in the tobacco colocalization analysis might resolve this question.
4.4 Materials and Methods

4.4.1 Plasmids

The HMR-HA-YFP vector was generated by Tao Ma by PCR amplification of the HMR coding sequence, with a sequence encoding a (PT)$_4$P linker and 3xHA tag contained within the antisense primer. The PCR product was digested with BglII and NheI and inserted into the BamHI and XbaI sites of pMC19 (Chen et al., 2005).

The hmr-22-HA-YFP vector was generated by Chan Yul Yoo by introducing the hmr-22 mutation into the HMR coding sequence via site-directed mutagenesis. Then, Gibson assembly (Gibson et al., 2009) (NEB, Ipswitch, MA, Cat.# E2611) was used to assemble three fragments: pCHF1 digested with BamHI and PstI; the hmr-22 fragment; and a fragment encoding a (PT)$_4$P linker, 3xHA, and YFP.

The SOH-CFP vector was generated by PCR-amplifying the SOH coding sequence, then ligating the product into the KpnI and SacI sites of pMC27 (Chen et al., 2005). A similar procedure was followed for generating soh102-CFP.

4.4.2 Plant Materials and Growth Conditions, and Cloning of SOH Alleles

Seed sterilization, imbibition, and stratification were performed according to the protocol described in Section 3.4.1. Growth chambers and measurement of light intensity are also as described in Section 3.4.1. The hmr-22 TILLING allele was backcrossed to Col-0 three times before use in experiments.

For the hmr-22 suppressor screen, hmr-22 seeds were treated with EMS according to (Weigel and Glazebrook, 2002). M2 families were screened under strong W light for seedlings with normal greening. Candidate mutants were crossed to Ler to generate an F2 mapping population, and simple sequence length polymorphism; cleaved amplified polymorphic sequences
(CAPS) (Konieczny and Ausubel, 1993); and derived CAPS (Neff et al., 1998) markers were used to map the positions of the mutations.

4.4.3 Measurement of Chlorophyll Content

Chlorophyll from 100 mg of 4 d old seedlings was extracted in 3 ml of 100% DMSO with incubation at 65°C for 30 min. Then the absorbance at 663 nm and 645 nm was measured by spectrophotometry. Chlorophyll was quantified as described previously (Chen et al., 2000).

4.4.4 Transmission Electron Microscopy

Seedlings were grown for 2 d in the indicated light conditions, and then fixed in 4% glutaraldehyde diluted in 0.1 N sodium cacodylate buffer. After fixation, seedlings were washed 2 × 30 min in a solution of 0.1 N sodium cacodylate and 7.5% sucrose, and then rinsed in 1% OsO₄ for 3 h. They were then washed 2 x 30 min in 0.1 N veronal acetate buffer, followed by an overnight wash in the same buffer. The following day, seedlings were dehydrated in serial dilutions of acetone and then impregnated with a Spurr resin/acetone mixture: 30/70 overnight, then 50/50 for 8 h, then 70/30 overnight. Finally, the seedlings were treated with 100% Spurr resin for 2 x 6 h, then for 3 d, then 2 x 1 d. The seedlings were then embedded in fresh Spurr resin and incubated at 60° for 48 h. Ultrathin sections of 70-90 nm were cut using an Ultracut E ultramicrotome (Reichert-Jung, Wetzlar, Germany) and collected on Maxtaform HR24 copper/rhodium grids (Electron Microscopy Sciences, Hatfield, PA). The sections were post-stained in 2% uranium acetate for 15 min and rinsed in ddH₂O. Then, they were stained in lead citrate for 7 min, rinsed, and air dried. The sections were viewed using a Phillips/FEI CM-12 transmission electron microscope (Hillsboro, OR) and an XR-100 2Vu digital camera system (Advanced Microscopy Techniques, Woburn, MA).
4.4.5 Protein Extraction and Western Blot

Protein was extracted and western blots were performed according to the protocol described in Section 3.4.2. In addition to the antibodies mentioned in that section, the anti-phyA antibody, a gift from Dr. Peter Quail, was used at a 1:1000 dilution.

4.4.6 RNA Extraction and qRT-PCR

RNA extraction and qRT-PCR was performed according to the protocol described in Section 3.4.3. qPCR primers used are listed in Appendix A and Appendix B.

4.4.7 Transient Protein Expression in Tobacco Epidermal Cells, Live-cell Imaging, and Confocal Microscopy

To analyze the photobody phenotype of the soh null alleles, seedlings were grown for 4 d in 10 μmol m⁻² s⁻¹ R light, then mounted on Superfrost microscope slides (VWR, Radnor, PA) in ddH₂O. Epidermal nuclei from the top third of the hypocotyl were imaged on a Leica SP5 inverted confocal (Leica Microsystems, Buffalo Grove, IL) using a 100x HCX PL APO oil immersion objective with excitation and emission settings for GFP as described in (Chen et al., 2010). Images were collected using LAS AF software version 2.6 and processed using Adobe Photoshop CS5 software (Adobe Systems, Inc., San Jose, CA). Photobody diameters were measured by Laura Dixon using ImageJ software (http://rsbweb.nih.gov/ij/).

Transient expression assays of SOH and HMR localization were performed using greenhouse-grown Nicotiana benthamiana according to (Roberts et al., 2011), but with the OD at infiltration set to 1.0 instead of 0.5. Seventy-two hours after infiltration, leaf punches were mounted on Superfrost slides in ddH₂O and imaged on a Zeiss LSM510 inverted confocal microscope using a 40x Plan-Apochromat dry objective (Carl Zeiss, Jena, Germany) using sequential detection of each fluorescent molecule with LSM 510 software version 4.2. DAPI was monitored using excitation from a 405 nm diode laser and a 420-480 bandpass detector;
chlorophyll was monitored using excitation from a 405 nm diode laser and a 615 nm longpass detector; CFP was monitored using 458 nm excitation from an argon laser and a 465-510 nm bandpass detector; and YFP was monitored using 514 nm excitation from an argon laser and a 505-550 nm bandpass detector. Images were processed using LSM Image Browser software version 4.2.0.121 (Carl Zeiss, Jena, Germany) and Adobe Photoshop CS5 software (Adobe Systems, Inc., San Jose, CA).
5. Summary

5.1 The Histidine Kinase-related Domain of Phytochrome B is Required for its Nuclear and Photobody Localization

Structural studies of phyB have shown that the N-terminus of the molecule is required for light sensing and signaling and that the C-terminus is involved in dimerization and is sufficient for nuclear import and photobody localization (Chen et al., 2005; Rockwell et al., 2006). However, each domain of phyB appears to have a function in photobody localization, as point mutations that affect photobody localization are scattered throughout the molecule (Figure 5). In particular, the role of the HKRD in phy signaling is unclear, as the phyB-28 truncation still localizes to small photobodies and can transduce light signals, while the phyB-18 point mutant is not functional at all. Here, I showed that the phyB-18 impairs nuclear localization (Figure 9B), providing an explanation for why the mutant is functionally null. Additionally, incorporating a nuclear localization signal into the phyB-18 transgene product failed to rescue photobody localization (Figure 9C), demonstrating that D1040 is required for both nuclear and photobody localization of phyB. Yeast two-hybrid analysis suggested that the phyB-18 mutation impairs HKRD dimerization in vitro. These results support the model that HKRD dimerization is an intramolecular determinant of both nuclear and photobody localization of phyB.

5.2 Photobody Localization of Phytochrome B is Tightly Correlated With Hypocotyl Growth Inhibition and Repression of PIF3 Accumulation in Darkness

At steady-state, photobody localization and hypocotyl growth inhibition are tightly correlated with the intensity of the light in which a seedling is grown (Figure 4). Additionally, there is a close relationship between the activity of most phyB mutants (loss- or gain-of function) and the ease with which they localize to photobodies (less and more easily, respectively) (Figure
siting that photobody localization of phyB is important for mediating normal light responses. However, at least one report describing a phyB truncation that is functional for signaling but fails to localize to photobodies (NGB) (Matsushita et al., 2003) called into question the significance of photobodies in phy signaling. My goal was to reconcile these conflicting conclusions.

Because photoactivated phy has been shown to persist into darkness to regulate hypocotyl growth via PIFs in diurnal conditions, I explored the possibility that photobodies might be involved in this process during the light-to-dark transition. I demonstrated a close correlation between the localization of phyB to photobodies and the degradation of PIF3. In a phyB-overexpressing transgenic line with normal photobody localization (PBG), the presence of photobodies correlated with the repression of PIF3 accumulation. In all examined cases, when photobodies were lost from at least half of all nuclei, PIF3 accumulated (Figure 16B, Figure 18, Figure 19, Figure 20B, and Figure 21A,C). In the NGB line, PIF3 accumulated even in the light (Figure 16B, Figure 20B, and Figure 21C), suggesting that photobodies are required for PIF3 degradation.

The dark reversion rate of phyB appears to play an important role in mediating both photobody localization and hypocotyl growth (Elich and Chory, 1997; Rausenberger et al., 2010; Zhang et al., 2013). Although PBG and NGB have been shown to have similar dark reversion rates in vitro (Oka et al., 2004), here, I provided evidence that their dark reversion rates in vivo are different. Based on their respective readouts for the persistence of the Pfr form, the Pfr form of PBG can last up to three times longer than that of NGB in darkness. Additionally, PBG can adjust its growth to varying light intensities, while NGB responds identically to both low- and high-intensity light (Figure 14B and Figure 20B). Together, these results suggest that photobody
localization of phyB stabilizes its Pfr form and allows for fine-tuning of growth in response to changing light conditions.

5.3 SON OF HEMERA is a Novel Extragenic Regulator of Photobody Localization

The identification of hmr as the founding member of the tall, albino subclass of light signaling mutants suggested that an entire branch of the phy signaling pathway had been missed in previous screens. To identify new components of this HMR-dependent signaling pathway, I performed a forward genetic screen to identify suppressors of a weak hmr allele. This screen isolated three alleles of the same gene, SON OF HEMERA (SOH). All three soh alleles were able to rescue all analyzed nuclear and plastid defects of the weak hmr allele in an allele-specific manner, suggesting that SOH and HMR directly interact. Further supporting the idea of a direct interaction, transient coexpression of SOH and HMR in tobacco epidermal cells showed that both proteins colocalize on nuclear speckles. Interestingly, a parallel tall, albino screen conducted by other members of the lab identified three null alleles of soh, all of which were defective in photobody localization in the light. Together, these results demonstrate that SOH and HMR act in the same signaling pathway to mediate nuclear and chloroplast responses to light, including the localization of phyB to photobodies.

5.4 Conclusions

Although phy signaling has been studied for decades, the earliest events in this signaling pathway are poorly understood. To gain insight into the nature of these early steps, as well as how those steps are regulated, I studied the photobody localization of phyB, which is the earliest known cellular event in phy signaling. My goals were to identify new intragenic and extragenic requirements for photobody localization, as well as to better define the role of photobodies themselves in phy signaling. By studying a point mutation in the HKRD of phyB, I determined
that HKRD dimerization is required for photobody localization, thus defining an intragenic determinant of photobody localization. By comparing the PBG and NGB transgenic lines, I showed a close correlation between photobody localization and the repression of PIF3 accumulation and provide evidence that photobodies stabilize the active form of phyB in the dark, providing a mechanism by which plants can fine-tune their growth in response to varying light conditions. Finally, I conducted a hmr suppressor screen and identified SOH, a novel gene that works in the same branch of the phy signaling pathway as HMR. Null mutants of soh, identified in a parallel screen, have hmr-like phenotypes including photobody mislocalization, making SOH the second extragenic determinant of photobody localization to be identified. I provide evidence in support of the model that SOH and HMR interact to mediate light responses in both the chloroplasts and the nucleus, including phyB localization to photobodies. Collectively, these studies enhance our understanding of the earliest events in phy signaling by identifying new intragenic and extragenic regulators of photobody localization, as well as by deepening our understanding of the role photobodies play both in early signaling, such as the degradation of PIF3, as well as longer-term signaling outputs such as hypocotyl elongation.
# Appendix A

Table 1: qPCR primers used to analyze the expression of nuclear genes.

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<th>Sense primer</th>
<th>Antisense primer</th>
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<tr>
<td>AT1G69960</td>
<td>PP2A</td>
<td>TATCGGATGACGATTCCTCGTGAG</td>
<td>GCTTGTCGACTATCGAATGAGA</td>
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<tr>
<td>AT1G09530</td>
<td>PIF3</td>
<td>CTTCAACTTCAAGTGCAGATC</td>
<td>GCAAGCCCATTGCTATAAG</td>
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<td>AT2G46970</td>
<td>PIL1</td>
<td>AAATTTGCTCTCAGCCATTCTGGA</td>
<td>TTCTAAGTTGAGGCGGCACGAG</td>
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<tr>
<td>AT4G16780</td>
<td>ATHB2</td>
<td>TCACAGTACTCTCAATTCGAAGC</td>
<td>CCGTAAGAACTCGCAGTCTAC</td>
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<tr>
<td>AT4G32280</td>
<td>IAA29</td>
<td>CACCATCATGGCCGTATCA</td>
<td>CCACAGTACCGGTGTTTGA</td>
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<tr>
<td>AT4G14130</td>
<td>XTR7</td>
<td>CACCAGTACCCTGCTACTTG</td>
<td>CATTGGTGTAGAAGAATAG</td>
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<td>AT1G03790</td>
<td>SOMNUS</td>
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<td>TCAAGTCAAGAGATCCATGACCCATCAC</td>
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<tr>
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<td>AT5G64120</td>
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<td>GTGACACATGCTCTACT</td>
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### Appendix B

Table 2: qPCR primers used to analyze the expression of plastid genes.

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<td>rbcL</td>
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<td>GGAGATGATTCTGTACTACAAT GTCCCTATTACGAGCTTTGAC</td>
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<td>ATCG00680</td>
<td>psbB</td>
<td>CATCCAAATCTGGATCAATACCAG</td>
<td>ACATTCTCTTAGCGGCTTT CGTCCCTTGACTTAACCTGTA</td>
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<td>ATCG00180</td>
<td>rpoC1</td>
<td>GTATAGCTTCCTGAGTCTCAG</td>
<td>GATGCAATTGGAGCTTATCG CGATAGGAACCTCTTCTTTGAAGC</td>
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<tr>
<td>ATCG00190</td>
<td>rpoB</td>
<td>CAATGATAGTGGTACCAAGTACCTC</td>
<td>CTagTGGACATTAGCATCTTGT CAGATTCTAAAGTAAGCATCTCTTG</td>
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Biography

Elise Kristen Van Buskirk was born on November 28, 1984 in Edison, New Jersey to Mildred Ragazzo and Carl Pasoreck. She attended Cornell University in Ithaca, New York and graduated in 2007 with an A.B. in Biological Sciences and Spanish with Distinction in All Subjects. She will receive her Ph.D. in Biology, with a certificate in Cell and Molecular Biology, from Duke University in September 2014.

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