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Light Regulation and Functional Characterization of Phytochrome Interacting Factor 1 (PIF1) in *Arabidopsis*

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Dedication

I dedicate this dissertation to my wonderful family. Particularly to my precious daughter Shuiyi E. Shang, who is the joy of my life, and my husband, who has given me his fullest support. I must also thank my loving mother and my mother in-laws who have helped so much with baby-sitting. Finally, I dedicate this work to my loving father, who is the first teacher in my life and taught me to be honest, brave, optimistic and perseverant.

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Light Regulation and Functional Characterization of Phytochrome Interacting Factor 1 (PIF1) in *Arabidopsis*

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Plants sense light intensity, quality and direction through a group of photoreceptors to modulate their growth and development. One family of photoreceptor is called phytochromes (phys) that perceives red and far red light. Phys transduce light signals via a sub-family of the basic Helix-Loop-Helix (bHLH) transcription factors called Phytochrome Interacting Factors (PIFs). PIFs function as negative regulators in the phy-mediated light signaling pathways. In darkness, PIFs regulate downstream gene expressions to inhibit photomorphogenesis. Upon light exposure, PIFs are phosphorylated and poly-ubiquitylated prior to their rapid degradation through the 26S proteasome pathway. One of the PIFs, PIF1, has the highest affinity for both phyA and phyB and also displayed the fastest degradation kinetics under both red and far red light. Here we showed that PIF1 directly and indirectly regulates key genes involved in chlorophyll biosynthesis to optimize the greening process in *Arabidopsis*. PIF1 binds to a G-box (CACGTG) DNA sequence element present in its direct target genes (e.g., *protochlorophyllide oxidoreductase C, PORC)* in darkness and regulates their expression. Structure-function studies revealed two separate regions called APB and APA necessary for binding to phyB and phyA, respectively, located at the amino-terminus and a novel phosphorylation site at the carboxy-terminus of PIF1. Both amino- and carboxy-terminal regions are necessary for the light-induced degradation of PIF1. However, the DNA binding is not necessary for the light-induced degradation of PIF1. Using a targeted systems biology approach, we identified new factors, HECATE proteins that promote photomorphogenesis by negatively regulating the function of PIF1. Moreover, we employed an unbiased genetic screening using luciferase imaging system to identify new mutants defective in the light-induced degradation of PIF1. The cloning and

characterization of these mutants will help identify the factors, such as the kinase and E3 ligase, responsible for the light-induced degradation of PIF1. Taken together, these data revealed detail mechanisms of how PIF1 negatively regulates photomorphogenesis and how light induces rapid degradation of PIF1 to promote photomorphogenesis.

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CHAPTER I: Introduction

Light and photoreceptors

As sessile and photoautotrophic organisms, plants constantly monitor and respond to the changes of external environment, especially light conditions. Light is the main energy source for photosynthesis in plants. At the same time, light signals also optimize plant growth and development throughout the life cycle of plants. Plants perceive the presence/absence, duration, intensity, quality and direction of light signals, and respond accordingly. These light conditions help plants monitor the presence of neighbors, day/night cycles and seasonal changes in the environment.

In some angiosperm species, such as *Arabidopsis*, a brief light exposure is sufficient to promote seed germination. The germinated seedlings follow the skotomorphogenic pattern in the dark, which show elongated hypocotyls, hypocotyl hooks and closed cotyledons with yellowish color. After exposure to light, the seedlings shift from skotomorphogenesis to photomorphogenesis. In *Arabidopsis*, photomorphogenesis is defined as shortened hypocotyl, erect and open cotyledons, and the biosynthesis of chlorophylls and photosynthetic structures. After the seedling stage, light influences the vegetative growth, such as growth direction, specified as phototropism and shade avoidance response. During the juvenile vegetative stage, light also plays a key role as a time keeper for the photoperiodic response and circadian rhythms. During the adult stage, plants monitor the light and temperature signals to determine the flowering time.

Perception, interpretation, and transduction of light signals are accomplished by four groups of regulatory photoreceptors: cryptochromes, phototropins and ZTL/LKP2/FKF1 (Zeitlupe/ LOV Kelch repeat Protein 2/ Flavin-binding, Kelch repeat, F-box) responsible for UV-A/blue region of spectrum, and phytochromes perceiving red and far-red light (Lin and Shalitin, 2003; Chen et al., 2004). Cryptochromes, phototropins and ZTL/LKP2/FKF1 group use a flavin as chromophore (Lin and Shalitin, 2003), while phytochromes use a linear tetrapyrrole chromophore, phytochromobilin, autocatalytically

attached to the apoprotein (Neff et al., 2000). Untill 2005, the photoreceptor for UV-B light has not been identified. The molecular properties of these photoreceptors enable them to perceive, integrate and transduce the light signals to downstream components to regulate the expression of genes responsible for photomorphogenesis (Chory and Wu, 2001; Quail, 2002a).

Phytochromes structure and localization

Arabidopis contains five phytochrome genes, *PHYA* to *PHYE,* each encoding a large 125 kDa protein (Mathews and Sharrock, 1997). Based on their stability under light, phytochromes have been classified into two groups, type I and type II. phyA represents the type I phytochrome, which is stable in the dark grown seedlings and degraded rapidly upon light exposure. The remaining four phytochromes, phyB to phyE, are relatively stable under light, and belong to the type II category.

There are several different classes of phytochrome responses based on the radiation energy of light required for triggering the responses. The three standard phytochrome mediated responses include the low fluence responses (LFRs), the very low fluence responses (VLFRs), and the high irradiance responses (HIRs). The LFRs are the classical red/far-red photo-reversible responses, which can be induced by a red-light pulse and reverted by a subsequent far-red light pulse (Furuya and Schäfer, 1996). The VLFRs are not photo-reversible, saturated at very low levels of active phytochromes and responsive to a broad region of the light spectrum. The HIRs require radiation with relatively high energy for a relatively long period of time. The HIRs have two subtypes, named red HIR (R-HIR) and far-red HIR (FR-HIR) (Nagy and Schäfer, 2002). The type I phytochrome, phyA, is responsible for all three types, LFR, VLFR and FR-HIR. The FR-HIR requires a short-lived intermediate (cycled Pr) during photoconversion from Pfr to Pr and is FR/R reversible instead of R/FR observed in the LFR (Shinomura et al., 2000). The type II phytochromes, primarily phyB, are red light sensors and can be reversed by far-red light with various degrees (Shinomura et al., 2000).

In plants, phyA exclusively forms homodimers, whereas the other phytochromes can selectively form heterodimeric complexes (Sharrock and Clack, 2004), although phytochromes are universally homodimers in solution (Kendrick and Kronenberg, 1994). Homodimerization and heterodimerization increase the complexity of the five phytochromes' distinct and overlapping properties. Each monomer comprises two domains: the amino-terminal photosensory domain attached to phytochromobilin chromophore and the carboxy-terminal regulatory domain. The function of the N-terminal domain is sensing and transducing light signals, whereas the C-terminal domain is mainly involved in dimerization, signal regulation and nuclear localization (Matsushita et al., 2003; Chen et al., 2005). The amino-terminal domain contains four subdomains, P1-P4. The conserved cysteine residue in the P3 sub-domain is important for the attachment of phytochromobilin to the apoproteins (Wu and Lagarias, 2000). The C-terminal regulatory domain has two sub-domains: a PAS-related domain (PRD), which is consensus in many prokaryotic and eukaryotic regulatory proteins, and a histidine kinase related domain (HKRD) which is related to the ATP-binding and histidine phosphotransferase activity (Montgomery and Lagarias, 2002).

Phytochromes exist in two stable photoconvertible forms: a red light absorbing Pr (biologically inactive) form and a far-red light absorbing Pfr (biologically active) form. The Pr form is converted into Pfr form under red light, and the Pfr form is converted back into Pr form with the far-red light exposure. The conformational change of phytochromes upon the quality of light is critical for their function as photoreceptors (Smith, 2000).

The Pr form of phytochromes is localized in the cytosol, whereas the Pfr form of phytochromes is primarily found in the nucleus. With red light exposure, the cytosolic Pr form is autoconverted into Pfr form and induced to translocate into nucleus (Kircher et al., 2002). It is worth pointing out that different phytochromes have various speed of nuclear localization. The light-induced Pr to Pfr transformation and translocalization of phyA are very rapid, whereas it takes a few hours for phyB to be accumulated in the nucleus (Neff et al., 2000). Different phytochromes have distinct ways to either self shuffle into nucleus or move into nucleus with helper proteins. For example, phyB contains nuclear-

localization signal in the PRD, which is attached to and blocked by the N-terminus of phyB in darkness. The conformational changes are induced by light. In consequence, the nuclear-localization signal of phyB is unmasked resulting in light-induced translocation of phyB into the nucleus (Chen et al., 2005). In contrast to phyB, phyA is translocated into the nucleus by association with other proteins containing nuclear-localization signals (Desnos et al., 2001; Zhou et al., 2005). The light induced accumulation of phytochromes in the nucleus results in the formation of speckles, named nuclear bodies (NBs) (Kircher et al., 2002). The strength of phyB response is correlated with the number of large NBs, and the phyB accumulation in NBs is primarily determined by the percentage of Pfr form of phyB (Chen et al., 2003). These NBs might represent the 'transcriptosome' complexes in places where the photoresponsive genes are activated (Nagy and Schäfer, 2000, 2002; Kircher et al., 2002). In conclusion, both photoactivation and nuclear translocation of phytochromes are crucial for the proper biological function of phytochromes in phytochrome mediated light signaling pathway.

The idea that Pr is the cytosolic and biologically inactive form, whereas Pfr is the nuclear localized and biologically active form, could not explain the phyA-mediated HIR. The photocycled Pr form of phyA is responsible for the HIR (Shinomura et al., 2000). In addition, phyA is translocated into the nucleus even under far-red light (Kircher et al., 1999), which suggests that the photocycled phyA could be active and accumulated in the nucleus with short half-life. The model that both Pfr and the photocycled Pr configuration of phyA migrate into the nucleus, could explain the accumulation of phyA in the nucleus with either red or far-red light treatment (Neff et al., 2000).

Phytochromes also have transcriptional and post-translational regulations in addition to photoconversion and regulation of subcellular localization by light as discussed above. phyA is highly abundant in etiolated seedlings and the amount of phyA drops rapidly upon light exposure caused both by reduced transcription and protein degradation (Quail, 1991). The rest of the phytochromes accumulate at much lower levels in etiolated and light grown seedlings. The differences in phytochrome protein abundance also contribute to their function.

The physiological functions of phytochromes

Analyses of phytochrome-deficient mutants elucidate the roles of individual phytochromes. Each of phytochromes has unique but redundant roles in the phytochrome mediated light signaling. phyA is the exclusive photoreceptor activated by far-red light, whereas phyA, phyB and phyC transduce red light signaling (Quail, 2002a; Monte et al., 2003; Franklin et al., 2003; Tepperman et al., 2004). phyA plays a major role in mediating the far-red light dependent seed germination, hypocotyl growth inhibition under far-red light and the floral promotion under modified short day condition (Whitelam et al., 1993; Parks and Quail, 1993; Nagatani et al., 1993; Johnson et al., 1994). By contrast, phyB is the dominant photoreceptor under red light (Tepperman et al., 2004). phyB functions in the inhibition of hypocotyl elongation under red light, a response redundantly modulated by phyA and phyC (Reed et al., 1994; Franklin et al., 2003). *phyB* mutant shows elongated growth of multiple tissues, including hypocotyls, stems, petioles and root hairs. In the adult stage, the deficiency of phyB results in less chlorophyll accumulation, early flowering and shade avoidance syndrome (Reed et al., 1993; Smith and Whitelam, 1997). phyC works as a co-activator with other phytochromes and blue light receptor crytochromes (Monte et al., 2003; Franklin et al., 2003). phyC plays roles in the perception of day-length and is able to promote flowering in the absence of phyA (Monte et al., 2003). In *Arabidopsis*, phyD has the closest phylogenetic relationship with phyB, and shares approximately 80% amino acid similarity. phyD functions in shade-avoidance responses by regulating flowering time and leaf area (Devlin et al., 1999). And phyE participates in light-regulated seed germination, maintenance of rosette leaves and petioles, regulation of flowering time and shade avoidance (Devlin et al., 1998; Hennig et al., 2002).

Phytochrome mediated light signal transduction pathways

Extensive research has focused on understanding the mechanisms of phytochrome mediated signal transduction (Quail, 2002a, 2002b; Chen et al., 2004). phyA is degraded rapidly after exposure to light. The degradation of phyA requires phosphorylation and ubiquitylation of Pfr form of phyA (Clough et al., 1999). As we mentioned above, the C-

terminal regulatory domain of phytochromes contains a HKRD, which is homologous to the histidine kinase domain of the bacterial two-component system (Montgomery and Lagarias, 2002). Using synthesized peptides, McMichael and Lagarias (1990) identified two candidate phosphorylation sites, Ser17 and Ser589, which were phosphorylated preferentially in the Pr and Pfr form respectively in the *Avena sativa* phytochrome (McMichael and Lagarias, 1990). Utilizing purified oat phyA extracts, the same group showed that phyA possesses serine/threonine protein kinase activity (Yeh and Lagarias, 1998). Shortly after that, a phytochrome kinase substrate 1 (PKS1) was identified by yeast two hybrid screening, which can be phosphorylated by phyA *in vitro* (Fankhauser et al., 1999). A type 5 protein phosphatase (PAPP5) was identified in the same manner as PKS1 (Ryu et al., 2005). Both the phosphorylation of phytochromes at the HKRD domain and the PAPP5-mediated dephosphorylation of phytochromes control their stability and affinity for the putative downstream signaling intermediate, (e.g., nucleoside diphosphate kinase 2, NDPK2) (Kim et al., 2004; Ryu et al., 2005). These data suggest that phytochrome signaling may involve serine/threonine phosphorylation/dephosphorylation.

Despite the extensive research emphasizing the nuclear localization and speckle formation of phytochromes, the cytosolic Pr forms of phytochromes play important roles. It is evident that most of phyA and phyB remains in cytosol even under light conditions favoring nuclear import (Nagy and Schäfer, 2002). PKS1 and NDPK2 mentioned above are present either fully or partially in the cytoplasm (Fankhauser et al., 1999; Choi et al., 1999; Chory and Wu, 2001). The pharmacological and gain of function studies showed that a heterotrimeric G protein is positioned downstream of the phytochrome mediated signal transduction and upstream of a cGMP-mediated step, which is analogous to light perception in animal system (Bowler et al., 1994; Okamoto et al., 2001). However, using both loss of function and gain of function strategies, the direct role for the heterotrimeric G protein complex in light signal transduction was precluded (Jones et al., 2003).

There are two hypotheses about the mechanisms of phytochrome-mediated regulation of gene expression (Smith, 2000). One possibility is that phytochromes might

function as kinase and act on multiple substrates to induce differential expression of nuclear genes in response to light. The phyA kinase activity and the studies on PKS1 and NDPK2 support the phytochrome kinase hypothesis (Yeh and Lagarias, 1998; Choi et al., 1999; Fankhauser et al., 1999). However, the possibility that plant phytochromes are kinases has long been controversial. The strongest argument against the hypothesis is that the kinase domain of phyB has been shown to be dispensable for phyB mediated light signaling *in vivo* (Krall and Reed, 2000; Matsushita et al., 2003). Another hypothesis is that phytochromes have one or multiple partners to control cascades of gene expression under specific conditions. The light-induced cytoplasmic/nuclear partitioning of phytochromes makes this type of regulation possible. Supporting the second hypothesis, different groups have identified a large number of signaling components that have been classified into two groups: the early intermediates and the late intermediates, downstream of phytochromes in the phytochrome mediated light signaling pathways (Fig. 1.1).

The early signaling intermediates

Genetic screens and molecular approaches from multiple laboratories have identified many signaling intermediates involved in transducing perceived light signals from phytochromes to photoresponsive genes. Using yeast two hybrid approach, researchers first identified three direct interacting components with phytochromes, PKS1 and NDPK2 as mentioned above, and Phytochrome Interacting Factor 3 (PIF3). All three belong to the early signaling intermediates which are expected to have a phenotype only under the specific light conditions (Chory and Wu, 2001; Quail, 2002a). Despite these factors identified early, additional proteins belonging to this category have been characterized later on. Some of them can interact with phytochromes and others could not or the interactions have not been examined yet.

The genetic evidence indicates that there are separate early signaling pathways used by either phyA perceiving far-red light or phyB perceiving red light only, and one shared early signaling pathway used by both phyA and phyB sensing red and far red light signals. In each pathway, there are positively and negatively acting factors (Deng and Quail, 1999; Neff et al., 2000; Fankhauser, 2001; Quail, 2002a, 2002b). Most of the

genetically identified loci have been cloned. In the phyA mediated far-red light signaling, far-red elongated hypocotyls 1 (FHY1), FHY1-like (FHL), far-red insensitive 219 (FIN219), far-red impaired response 1 (FAR1), long hypocotyls in far-red light 1 (HFR1), long after far-red light (LAF1) and phyA signal transduction 1 (PAT1) represent the positively acting early signaling intermediates (Desnos et al., 2001; Hsieh et al., 2000; Hudson et al., 1999; Fairchild et al., 2000; Ballesteros et al., 2001; Bolle et al., 2000). FHY1 and its close homolog FHL interact with phyA and translocate phyA into the nucleus in a light-dependent fashion (Hiltbrunner et al., 2005; Zhou et al., 2005). HFR1 itself binds to neither phyA nor phyB, but the direct interaction between HFR1 and PIF3 suggested that HFR1 may modulate phyA signaling though heterodimerization with PIF3. Three negative regulators have been identified, which are suppressors of phyA (SPA1), empfindlicher im dunkelroten light 1 (EID1) and short under blue light 1 (SUB1) (Hoecker, 1999; Dieterle et al., 2001; Guo et al., 2001). SPA1 is a WD40 repeat protein and localized in the nucleus. EID1 is a nuclear localized F-box protein containing leucine rich repeat (LRR) domains. Both of them are probably involved in ubiquitin-proteasome pathway. In the phyB mediated red light signaling pathway, gigantea (GI), early flowering 3 (ELF3) and *Arabidopsis* response regulator 4 (ARR4) belong to the positively acting factor category, whereas the Phytochrome Interacting Factor 4 (PIF4) functions as a negative regulator (Huq et al., 2000; Liu et al., 2001; Sweere et al., 2001; Huq and Quail, 2002). In the shared signaling pathway, the factors are able to respond to both phyA and phyB. The phytochrome direct interacting proteins fall into this category mostly functioning as negative regulators except NDPK2. However the binding affinities of these factors to phyA and phyB are different (Zhu et al., 2000).

The late signaling intermediates

The fact that distinct photoreceptor-triggered signaling events can trigger the same developmental consequences suggests the existence of common late signaling intermediates (Deng and Quail, 1999). These late signaling intermediates have generally been postulated to function downstream of the convergence of all phytochrome pathways and affect many aspects of plant growth and development.

Researchers have identified two groups of late signaling components that function antagonistically to fine tune photomorphogenesis. The first group of late signaling components includes the positive regulators of photomorphogenesis. HY5 is the first one identified as a positive regulator downstream of phytochrome mediated light signaling pathways (Ang and Deng, 1994). *HY5* encodes a basic leucine zipper type of transcription factor (Oyama et al., 1997). HY5 protein localizes to the nucleus and directly binds to the G-box motif present in the promoters of downstream target genes, such as the ribulose bisphosphate carboxylase small subunit gene (*RBCS*) and the chlorophyll a/b-binding protein gene (*CAB*) (Oyama et al., 1997; Chattopadhyay et al., 1998; Quail, 2002a). Three other positive regulators functioning in the phytochrome mediated late signaling pathways have been identified. Two of them are myb-like proteins, one G-box binding protein Circadian Clock Associated 1 (CCA1) and a closely related homolog Late Elongated Hypocotyl 1 (LHY1), which are involved in the circadian clock (Wang and Tobin, 1998; Schaffer et al., 1998). Another nuclear localized transcription factor COP1 Interacting Protein 7 (CIP7) regulates downstream target gene expression (Yamamoto et al., 1998; Deng and Quail, 1999).

The second class of late signaling intermediates characterized best are the pleiotropic COP/DET/FUS proteins acting as negative regulators. The *cop/det/fus* mutant seedlings de-etiolate and exhibit constitutive photomorphogenesis in darkness including short hypocotyls and open cotyledons without apical hooks as if they have perceived light signals except the intermediate form of the plastid development (Deng et al., 1992; Wei and Deng, 1996). Characterization of the constitutive photomorphogenic mutants revealed that the switch between skotomorphogenesis and photomorphogenesis also is regulated by a complex system repressing photomorphogenesis in darkness in contrast to photoreceptor function. Eleven recessive mutants defective in *COP/DET/FUS* genes have been identified (Wei and Deng, 1996; Deng and Quail, 1999; Quail, 2002a). The main repressor identified is constitutively photomorphogenic 1 (COP1), functions as an E3 ubiquitin-protein ligase. It contains three recognizable domains: an amino-terminal ring-finger zinc-binding domain, a coiled-coiled helix structure, and several WD-40 repeats at

the carboxyl-terminus. All three domains are involved in protein-protein interaction and the zinc-binding domain has the potential of binding to nucleic acids (Deng et al., 1992). COP1 is localized in the nucleus to suppress photomorphogenesis through direct interaction with the nuclear localized positive regulator, HY5, and destabilizing HY5 protein (Hardtke et al., 2000; Osterlund et al., 2000), and/or through repressing CIP7 gene expression in darkness (Yamamoto et al., 1998). In response to light, COP1 is inactivated in part by light-induced nuclear exclusion (Arnim and Deng, 1994). However, the depletion of COP1 in the nucleus occurs very slowly, which implies that the light-induced nuclear exclusion of COP1 may serve to inactivate COP1 in a long term manner rather than in a rapid COP1-mediated molecular event (Deng and Quail, 1999).

The multiple interactive domains of COP1 provide the basis for interaction of COP1 with multiple either upstream or downstream components mediating gene expression network in light signaling pathways (Wei and Deng, 1996). COP1 has been proven to be involved in the degradation of multiple positive regulators both in early and late signaling pathway, such as LAF1, HY5 and HFR1, in darkness (Osterlund et al., 2000; Duek et al., 2004; Seo et al., 2003; Yang et al., 2005). At least for HFR1, it gets phosphorylated prior to degradation (Duek et al., 2004). In the COP1-mediated turnover of HY5 and LAF1, SPA1 stimulates the targeted ubiquitylation and proteolysis (Saijo et al., 2003; Seo et al., 2003). However, additional data from COP1 over-expression studies also suggest that COP1 might be involved in degradation of negative regulators for photomorphogenesis besides its role in degrading positive regulators (Boccalandro et al., 2004).

Eight of eleven *COP/DET/FUS* genes encode the subunits of the COP9 signalosome complex (CSN), which is localized in the nucleus (Wei et al., 1998). The COP9 signalosome contains a high similarity to the lid subcomplex of the 26S proteasome, suggesting their functional involvement in ubiquitin-mediated nuclear protein degradation (Schwechheimer and Deng, 2001). The COP9 complex has been shown to play a central role in nuclear import or nuclear retention of GUS-COP1 (Chamovitz et al., 1996). It is possible that the COP9 complex may protect COP1 from

degradation in darkness and abolish the protection for COP1 response to light, resulting in degradation of COP1 in the nucleus under light (Deng and Quail, 1999).

Phytochrome Interacting Factors (PIFs)

The phytochrome A and/or phytochrome B interaction with PIFs points to a shortcut mechanism in which light signals are transduced from phytochromes to the particular type of transcription factors (PIFs) bound to the promoter element of some light-regulated genes (Martínez-García et al., 2000). Fig. 1.2 shows a simplified summary of PIFs function in phytochrome-mediated light signaling pathways. The first characterized phytochrome interacting factor is PIF3 (Ni et al., 1998). Physiologically, PIF3 acts predominantly as a negative regulator in phytochrome mediated light signaling pathways. *pif3* displayed shorter hypocotyls under red light, and less opened smaller cotyledons under both red light and far-red light (Kim et al., 2003). Unlike the other two interacting factors, PKS1 and NDPK2, which interact with phytochromes both in the cytoplasm and nucleus, PIF3 is a constitutively nuclear localized protein and interacts with phyB upon light-induced conversion of Pfr form of phyB (Ni et al., 1999). PIF3 is a member of basic helix-loop-helix (bHLH) superfamily of transcription factors containing a PAS domain (Ni et al., 1998; Halliday et al., 1999). The basic domain functions in DNA binding, whereas the HLH domain is involved in protein-protein interaction, such as homodimerization of PIF3 and heterodimerization with other bHLH proteins. PIF3 has been shown to form homodimers, which bind to the G-box element, a type of light response elements (LREs) found in various promoters (Ni et al., 1999; Martínez-García et al., 2000). A novel molecular recognition motif called APB (the active phyB binding) is necessary for targeting photoactivated phytochrome signaling to specific bHLH transcription factors (Khanna et al., 2004). The protein level of PIF3 has also been shown to be down regulated via polyubiquitylation and subsequent 26S proteasome-mediated degradation upon either red or far-red light exposure (Park et al., 2004; Bauer et al., 2004; Monte et al., 2004). The nuclear localized transcription factor feature of PIF3 and its regulation through posttranslational protein degradation imply its key role in

integrating photosignals perceived by photoreceptors and then regulating transcription of downstream genes that result in either inhibition or promotion of photomorphogenesis.

The second phytochrome interacting factor, which interacts with both phyA and phyB is PIF4. PIF4 binds to the Pfr form of phyB, but has little affinity for phyA (Huq and Quail, 2002). Consistent with PIF3 results, PIF4 is localized in the nucleus and can bind to G-box element. The *pif4* mutant showed hypersensitive morphological phenotypes. Together with the fact that PIF4 mainly interacts with phyB, it suggests that PIF4 negatively regulates photomorphogenesis selectively in phyB signaling. In 2004, PIF1/PIL5 (PIF3-like 5) was identified (Oh et al., 2004; Huq et al., 2004). PIF1/PIL5 functions as a negative regulator in phytochrome mediated promotion of seed germination, inhibition of hypocotyl elongation and chlorophyll biosynthesis. The *pif1/pil5* mutant seeds germinated 100% under dark and far-red light conditions. These data strongly suggest that the dominant role of PIF1/PIL5 in far-red light signaling. Inhibition of seed germination by PIF1 in the dark also suggests that PIF1 might have activity in the dark before phytochrome activation. Similar to PIF3, PIF1 is degraded under red and far-red light through the 26S-proteasome pathway, but with much faster kinetics (Shen et al., 2005). PIF1/PIL5 predominantly functions in darkness to negatively regulate photomorphogenesis, but might also play roles in diurnal light/dark cycles to optimize seedling de-etiolation. Lastly, PIF5 and PIF6 were isolated and characterized in relation to phy interaction (Khanna et al., 2004). Both PIF5/6 interacted with the Pfr form of phyB. However, their biological functions were not characterized untill 2005.

Summary and the unraveled mystery in phytochrome mediated signaling pathways

Phytochrome mediated light signaling pathways constitute intricate signaling networks that affect plant growth and development throughout the life cycle. Since 1959, when a pigment named phytochrome was partially purified (Butler et al., 1959), researchers have made tremendous progress in understanding the mechanisms of how photoreceptors perceive light, how plants integrate the light signals, how light signals are transduced to trigger gene regulations, and how these regulations finally affect growth and development of plants.

The identification of PIFs points out one possible mechanism of the phytochrome mediated light signal transduction in a simple way. The PIFs mainly act as negative regulators rather than positive regulators to suppress photomorphogenesis (Duek and Fankhauser, 2005). Belonging to a family of transcription factors constitutively localized to the nucleus, PIFs bind to G-box elements present in the promoter regions of many genes and may either activate or inhibit their transcription. The direct interaction between PIFs and the biologically active Pfr form phyA and/or phyB in nucleus triggers degradation of PIFs via 26S-proteasome pathway. The turnover of PIFs releases their activation or inhibition effects from the downstream target genes resulting in promotion of photomorphogenesis. However, a few key components are still missing in this model. Although FHY and FHL have been shown to facilitate and modulate phyA translocation into the nucleus, the hypothesis about phyB nuclear translocation is still controversial, and no factor has yet been found to help import phyB into the nucleus (Hiltbrunner et al., 2005; Zhou et al., 2005). Phytochromes can form either homodimers or heterodimers (Sharrock and Clack, 2004). The biological significance of the different dimerizations is not known yet. The possibility that the homodimers and heterodimers will be translocated into nucleus with differential kinetics needs to be investigated. Whether PIFs are involved in phytochrome nuclear localization needs to be tested. For the interaction between PIFs and phyA/phyB, only APB domain has been identified as the motif critical for PIF1 and PIF3 interaction with phyB. The phyA binding domain has not been identified in any of the PIFs. The importance of the APB motif in PIF4, PIF5 or PIF6, and the presence of potential phyA binding motif need to be investigated further. Most importantly, none of the downstream target genes of PIFs has been identified. The complete signal transduction model, perception-integration-genes transcription cascade resulting in regulation of growth and morphology is missing a central part. The identification of PIF targets will provide a link between transcription factors and the changes in plant growth and development at a molecular level.

Although the mechanisms of dark-induced degradation of positive factors are well understood, little is known about the light-induced degradation of PIFs. Other than the

report that COP1 is involved in the stabilization of PIF3 (Bauer et al., 2004), the E3 ligase(s) responsible for PIFs ubiquitylation remain to be characterized. It is also possible that PIFs might be phosphorylated prior to their degradation. The identification of E3 ligase(s) degrading PIFs in a light-dependent manner will provide fundamental information on phytochrome mediated light signaling. Both RING zinc finger and F-box proteins are more abundant in *Arabidopsis* compared to *Yeast* and *Drosophila* (The Arabidopsis Genome Iniative., 2000; Gagne et al., 2002). Each individual PIF may be recognized by different specificity factor, such as F-box proteins, and negatively regulated at protein levels through the ubiquitylation and proteasome-mediated degradation by selective E3 ligases.

At last, *Arabidopsis* genome encodes 162 bHLH transcription factors (Toledo-ortiz et al., 2003). The various degrees of homodimerization and heterodimerization of bHLH proteins imply that this class of proteins may regulate extensive sets of transcriptional regulation. The different combinations of various PIFs heterodimerization need to be tested. And it is possible that the interaction between PIFs and other bHLH factors may either promote or repress the binding of PIFs to target genes resulting in activation or repression of target gene expression. Further studies are necessary to unravel the mysteries of phytochrome mediated light signaling pathways.

Therefore, in my thesis, I extended the studies of phytochrome mediated light signaling pathway by focusing on the identification of PIF1 direct target genes and the factors responsible for the light-induced degradation of PIF1. The specific objectives are:

1. Identification of PIF1 direct target genes.

2. Determine the structure-function relationship of PIF1.

3. Identification of HLH proteins which heterodimerize with PIF1 and regulate PIF1 function.

4. Identification and characterization of factors responsible for the light-induced phosphorylation and degradation of PIF1.

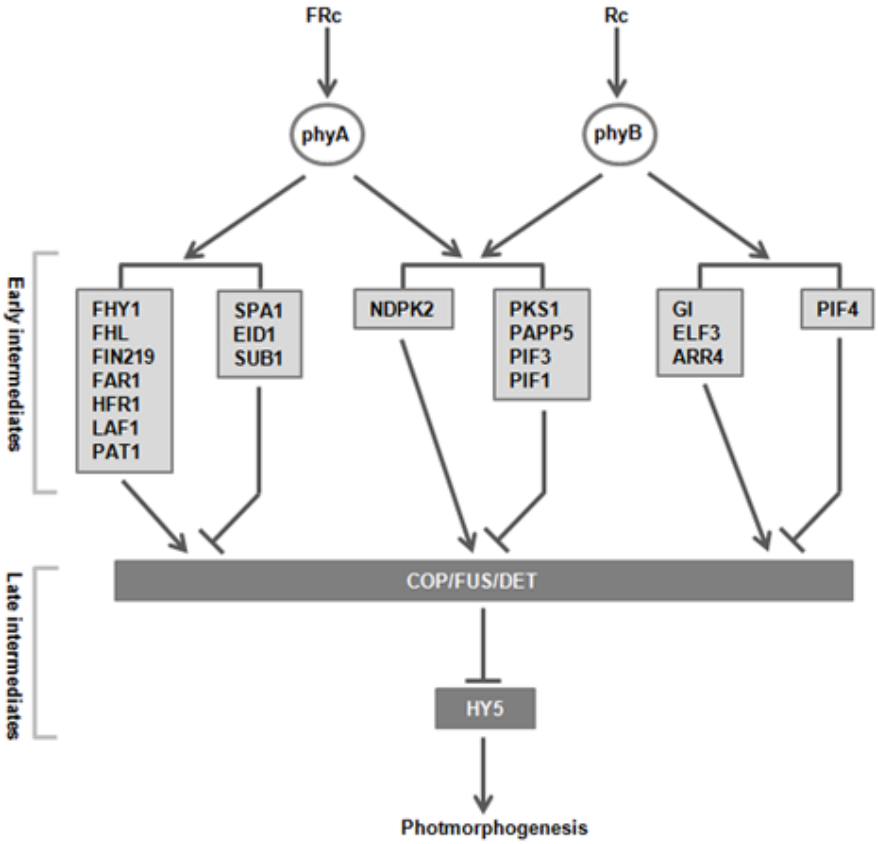

Figure 1.1: Simplified schematic diagram showing the phytochrome mediated light signaling pathways based on the molecular and genetic studies.

phyA solely perceives continuous far red light and phyB is the photoreceptor for red light sensing. One separate and one shared early signaling pathways transduce the signal to the late signaling intermediates, which is the COP/FUS/DET group. COP/FUS/DET proteins target and down regulate downstream positive components, such as HY5, in the phytochrome mediated light signaling pathways.

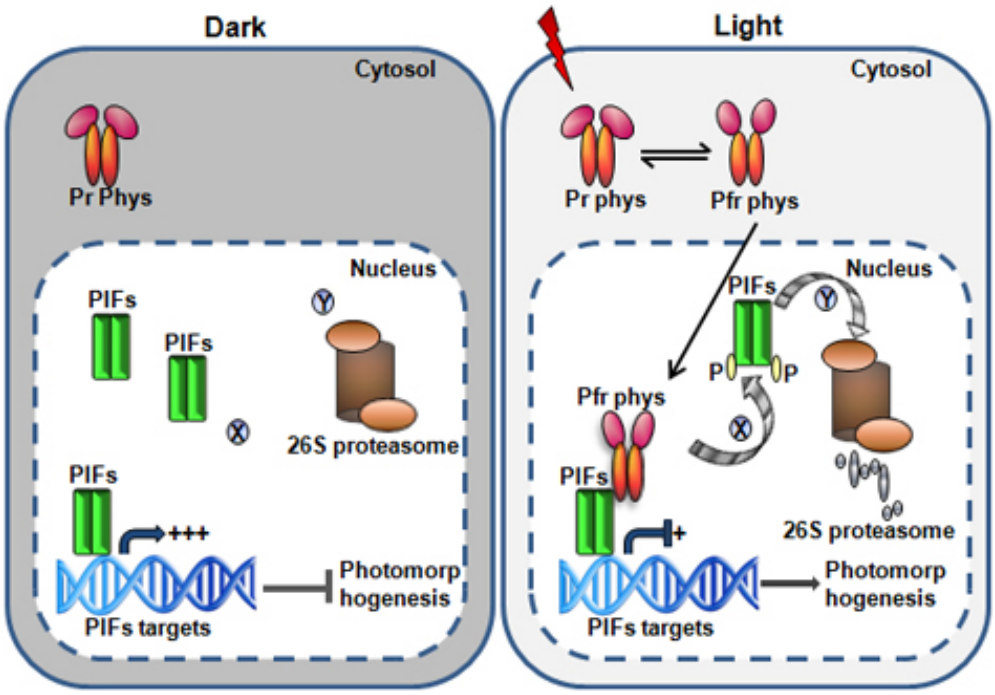

Figure 1.2: Schematic diagram of PIF-regulated phytochrome mediated light signaling pathway.

In darkness, phytochromes stay in cytosol and are biologically inactive Pr form. PIFs function as negative transcription factors in nucleus and activate downstream target gene expression to repress photomorphogenesis. Upon light perception, the Pr form of phytochromes turn into Pfr form and is translocated into nucleus. The nuclear localized Pfr form binds to PIFs. The unknown kinase(s), X(s), recognize and phosphorylate PIFs that are bound to phytochromes. The phosphorylated PIFs can be polyubiquitylated and driven into 26S proteasome for degradation. The degradation of PIFs releases the negative regulations by PIFs to promote photomorphogenesis.

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Chapter II: PIF1 directly and indirectly regulates chlorophyll biosynthesis to optimize the greening process in *Arabidopsis*

Abstract

Plants depend on light signals to modulate many aspects of their development and to optimize their photosynthetic capacity. Phytochromes (phys), a family of photoreceptors, initiate a signal transduction pathway that alters expression of a large number of genes to induce these responses. Recently, phyA and phyB were shown to bind members of a bHLH family of transcription factors called Phytochrome Interacting Factors (PIFs). PIF1 negatively regulates chlorophyll biosynthesis and seed germination in the dark, and light-induced degradation of PIF1 relieves this negative regulation to promote photomorphogenesis. Here we report that PIF1 regulates expression of a discrete set of genes in the dark, including *protochlorophyllide oxidoreductase* (*POR*), *ferrochelatase* (*FeChII*) and *heme oxygenase* (*HO3*), which are involved in controlling the chlorophyll biosynthetic pathway. Using the chromatin immunoprecipitation (ChIP) and DNA gel shift assays, we demonstrate that PIF1 directly binds to a G-box (CACGTG) DNA sequence element present in the *PORC* promoter. Moreover, in transient assays, PIF1 activates transcription of *PORC* in a G-box dependent manner. These data strongly suggest that PIF1 directly and indirectly regulates key genes involved in chlorophyll biosynthesis to optimize the greening process in *Arabidopsis*.

Keywords: bHLH transcription factors, chlorophyll biosynthesis, phytochrome signaling, photomorphogenesis, transcriptional regulation.

Introduction

Light has a profound effect on plant growth and development. Plants not only rely on light signals to regulate developmental phases, but also to provide spatial and temporal information about their environment. Within plant cells, an array of photoreceptors detects several light characteristics such as wavelength, direction, duration, and intensity. Photoreceptors such as cryptochromes, phototropins and an unidentified UV-B receptor perceive and respond to blue light, whilst phytochromes (phys) respond to the red (R) and far-red (FR) region of the spectrum (Chen et al., 2004; Whitelam and Halliday, 2007).

phys exist in two spectral forms: a R light absorbing Pr form and a FR light absorbing Pfr form. R light induces conformation of phys to the Pfr, or "active" form; FR light coverts phys to the Pr, or "inactive" form. In *Arabidopsis*, phys are encoded by a small multi-gene family (*PHYA-PHYE*). All phys are active in red light; however, phyA is light labile and activated by both R and FR light. Both phyA and phyB are predominantly in the cytosol in the Pr form. The Pfr form is induced to translocate into nucleus upon light activation either by unmasking of NLS present in the C-terminal domain (for phyB) (Chen et al., 2005), or through associated proteins (for phyA) (Hiltbrunner et al., 2006). Activation of phys by light initiates a signaling cascade, which results in changes in gene expression that drive photomorphogenesis (Whitelam and Halliday, 2007; Rockwell et al., 2006; Jiao et al., 2007).

phyA and phyB interact in a conformer-specific manner with basic helix-loop-helix (bHLH) transcription factors called Phytochrome Interacting Factors (PIFs) (Whitelam and Halliday, 2007; Castillon et al., 2007). PIFs preferentially bind a G-box (CACGTG) DNA sequence element, which is a subclass of an E-box element (CANNTG) present in many light regulated promoters (Huq and Quail, 2002; Martínez-García et al., 2000). Interactions between the Pfr form of phyB with PIF3 bound to a G-box promoter motif are hypothesized to directly regulate transcription of light responsive genes involved in photomorphogenesis (Martínez-García et al., 2000; Quail, 2002). However, recent results show that PIFs are stable in the dark and are degraded in response to R and FR light in a phy-dependent manner (Castillon et al., 2007; Bauer et

al., 2004; Shen et al., 2005; Oh et al., 2006; Shen et al., 2007; Nozue et al., 2007), suggesting that activated phys induce degradation of PIFs to promote photomorphogenesis.

Genetic analysis of PIF1 and PIF3-PIF5 suggests that these proteins function as negative regulators of distinct phy-signaling pathways (Shen et al., 2007; Castillon et al., 2007). For example, PIF3-PIF5 predominantly control hypocotyl length under R light (Huq and Quail, 2002; Khanna et al., 2004; Kim et al., 2003; Fujimori et al., 2004), while PIF1 functions as a negative regulator of chlorophyll biosynthesis in the dark and seed germination in FR light (Shen et al., 2005; Oh et al., 2006; Huq et al., 2004). PIF1 directly and indirectly regulates gibberellic acid biosynthesis and sensitivity to control seed germination (Oh et al., 2007). Compared to wild type (wt) seedlings in the dark, *pif1* seedlings accumulate higher amounts of free protochlorophyllide (pchlide), a phototoxic intermediate in the chlorophyll biosynthetic pathway. Subsequent light exposure causes photo-oxidative damage and bleaching of *pif1* seedling (Shen et al., 2005; Huq et al., 2004). PIF1 shows transcriptional activation activity in the dark, which is reduced by light-induced degradation of PIF1 to promote chlorophyll biosynthesis and seed germination in light (Shen et al., 2005; Oh et al., 2006). However, the direct target genes by which PIF1 controls chlorophyll biosynthesis have not been identified. Here, we present evidence that PIF1 directly and indirectly regulates key genes in the chlorophyll biosynthetic pathway in the dark to optimize the greening process in *Arabidopsis*.

Results

PIF1 regulates expression of tetrapyrrole pathway genes in the dark

Previously, we have shown that *pif1* seedlings have higher levels of pchlide than wt in the dark (Huq et al., 2004). Because PIF1 shows strong transcription activation activity in the dark (Shen et al., 2005; Huq et al., 2004), we reasoned that identifying the genes differentially expressed in dark-grown *pif1* and wt seedlings may provide further insight into the *pif1* phenotype. To this end, we performed whole genome expression profiling using Affymatrix Microarray chips on RNA isolated from 4 day-old dark-grown

wt and *pif1* null mutant seedlings. Using a P value of ≤0.05, the Bioconductor microarray analysis software identified only three genes (2.81X, PIF1; 1.96X, At4g17600; 1.91X, At5g44580) differentially expressed between wt and *pif1* mutants. One of the three genes is *PIF1*, which shows a 2.8-fold reduction in expression between wt and the mutant, confirming the validity of our analysis method.

Because the Bioconductor software might be too stringent to detect small expression changes in *pif1* seedlings, we used an alternative approach for data analyses as described (Hudson and Quail, 2003). Using this approach, we identified additional differentially expressed genes (data not shown). Because of PIF1's role in chlorophyll biosynthesis, we focused our analyses on genes involved in the tetrapyrrole pathway (Table 2.1) (Matsumoto et al., 2004). Interestingly, a few key genes encoding enzymes involved in tetrapyrrole pathway showed expression changes of at least 1.5-fold between the dark grown wt and *pif1* samples (Fig. 2.1A and B; Table 2.1). To independently verify our microarray results, a semi-quantitative RT-PCR assay was performed. The RT-PCR results largely support the microarray data (Fig. 2.1A; Table 2.1). Microarray analysis for *ferrochelataseI (FeChI)* (At5g26030) and *ferrochelataseII* (*FeChII*) (At2g30390), both of which are involved in the conversion of Protoporphyrin IX (PPIX) to heme (Singh et al., 2002; Tanaka and Tanaka, 2007), did not show a significant difference between the wt and *pif1* samples (Table 2.1). However, semi-quantitative and quantitative RT-PCR (qRT-PCR) analyses showed that *FeChII* is down-regulated in *pif1* seedlings compared to wt (Fig. 2.1A; Table 2.1). Taken together, these results suggest that PIF1 is a subtle regulator that controls a small set of key genes involved in chlorophyll biosynthesis.

PIF1 directly regulates *PORC* in the chlorophyll biosynthesis pathway

Because PIFs bind the E/G-box DNA sequence element (CANNTG) (Martínez-García et al., 2000; Huq et al., 2004), we analyzed the upstream promoter region of the differentially expressed genes for the presence of these elements using the PLACE web site (http://www.dna.affrc.go.jp/PLACE/signalscan.html). Results show that most of the differentially expressed genes have promoters with two or more E/G boxes (Table 2.2).

To determine if these genes are directly regulated by PIF1, we transformed *pif1* plants with a construct expressing PIF1 fused to a TAP tag, *35S:TAP-PIF1* (Fig. 2.2A) (Rubio et al., 2005). As a control, we expressed a *35S:TAP-GFP* construct in the wt background. After confirming that the *35S:TAP-PIF1* transgene complemented *pif1* phenotypes (Fig. 2.2B-G), we used both transgenic lines in a chromatin immunoprecipitation assay (ChIP). After immunoprecipitation of protein-DNA complexes using antibody to the MYC tag, enriched DNA sequences were amplified using primers to the promoter regions of the candidate genes. ChIP assay results show that the PORC promoter region was amplified from the IP fraction of *35S:TAP-PIF1* seedlings, but not in the *35S:TAP-GFP* or without antibody samples (Fig. 2.3). Under these conditions, we observed no amplification of the promoter regions of the *PORA*, *DVR, HO3* and *FeChII* genes. To determine if these genes were targeted by PIF1 in slightly younger or older seedlings, the ChIP assay was performed on tissue from a range of developmental stages, however, no amplification of these promoters was observed (data not shown). These data suggest that *PORC* is a direct target of PIF1, while *PORA*, *PORB, HO3* and *FeChII* genes are indirect targets of PIF1.

PIF1 binds G-box motifs within the *PORC* and *FeChII* promoters

Previously, we have shown that PIF1 binds a synthetic G-box motif using a gel-shift assay (Martínez-García et al., 2000; Huq et al., 2004). To determine if PIF1 directly binds the G/E boxes within PORC, a gel-shift assay was performed as described (Huq and Quail, 2002; Huq et al., 2004). Results show that PIF1 binds the labeled *PORC* G-box fragment (Fig. 2.4A). The *PORC* promoter fragment containing a mutated G-box element did not compete with the wt G-box fragment for PIF1 binding. Because the *FeChII* promoter has an identical G-box as in the *PORC* promoter and *FeChII* expression is regulated by PIF1, we also examined whether PIF1 directly binds to the G-box present in the *FeChII* promoter. Cold *FeChII* promoter probe successfully competed with labeled *PORC* fragments for PIF1 binding (Fig. 2.4B). Further, mutated G-box *FeChII* probe did not compete for PIF1 binding with *PORC*. Control proteins, *in vitro* expressed LUC and PIF3, did not bind the *PORC* G-box sequence in this assay (Fig. 2.4B). However, a

similar PIF3 preparation bound a synthetic G-box originally identified as the PIF3 binding site (data not shown) (Mart ńez-Garc á et al., 2000). PIF1 did not bind to *PORA* and *PORB* E-box sequences under these experimental conditions (data not shown). These results suggest that PIF1 directly binds to the G-box present in both *PORC* and *FeChII* promoters *in vitro* in a sequence-specific manner.

PIF1 regulates *PORC* and *FeChII* expression *in vivo*

Given that PIF1 is a transcription factor, we wanted to determine whether PIF1 can activate transcription from a native promoter *in vivo*. As a control, we transiently expressed a non-PIF1 target promoter driving GUS (*pACT2:GUS*) in wt, *pif1* and PIF1 over-expression (*35S:LUC-PIF1*) seedlings using the transient assay that we developed (Huq et al., 2004). GUS assay results show that all three genotypes express the same level of *pACT2:GUS*, suggesting that PIF1 does not control expression from this promoter (Fig. 2.5A-C). To determine whether PIF1 can activate transcription from a native promoter, we transiently expressed the native *PORC* or *FeChII* promoters driving GUS expression in wt, *pif1* and *35S:LUC-PIF1* seedlings (Fig. 2.5A). Results show that pPORC:GUS activity is significantly higher in *35S:LUC-PIF1* seedlings than in the wt or *pif1* seedlings (Fig. 2.5B). To confirm our results, we measured endogenous *PORC* expression in these lines using qRT-PCR assays and found a similar expression pattern as observed for the reporter GUS assays (Fig. 2.5B, inset). Strikingly, the increased GUS activity in *35S:LUC-PIF1* seedlings expressing *pPORC:GUS* is eliminated when the G-box within the *PORC* promoter is mutated (Fig. 2.5B). These results strongly suggest that PIF1 directly regulates *PORC* expression in a G-box dependent manner.

GUS activity in *pif1* lines expressing *pFeChII:GUS* was significantly reduced compared to GUS activity in *pFeChII:GUS* expressing wt seedlings (Fig. 2.5C). Moreover, *35S:LUC-PIF1* lines in the *pif1* background showed wt levels of *FeChII* expression, demonstrating rescue of the *pif1* phenotypes in the dark (Shen et al., 2005). However, the *35S:LUC-PIF1* seedlings did not show overexpression of *FeChII*. Using qRT-PCR, we found that endogenous *FeChII* expression levels in wt, *pif1* and *35S:LUC-PIF1* seedlings reflect the expression patterns found in the *pFeChII:GUS* assays (Fig.

2.5C, inset). In contrast to what was observed in the *pPORCGm:GUS* assays, the *pFeChGm:GUS* lines showed no significant change in GUS activity in the wt, *pif1* and *35S:LUC-PIF1* backgrounds (Fig. 2.5C). These results suggest that PIF1 is necessary for activation of *FeChII* expression in a G-box-independent manner.

Because both *PORC* and *FeChII* are modestly induced in light (Matsumoto et al., 2004), we investigated whether PIF1 plays a role in light-regulation of these genes using the qRT-PCR assays. Results show that the expression of *PORC* is modestly but significantly reduced in *pif1* seedlings compared to wt seedlings (Fig. 2.6). However, *pif1* seedlings display a wt *FeChII* expression level under these light conditions. Since PIF1 is rapidly degraded under light (Shen et al., 2005), and *PORC* and *FeChII* levels are reduced in the dark in the *pif1* seedlings compared to wt seedlings (Figs. 2.1A, 2.5B, 2.5C and 2.6), these results suggest that PIF1 does not play a significant role in the light-induced expression of these genes.

***pif1* seedlings have reduced POR enzyme activity**

Microarray and RT-PCR data show that *POR* genes are down-regulated in *pif1* seedlings compared to wt seedlings in the dark (Fig. 2.1A). To determine whether the transition from pchlide to chlorophyllide (chlide) was aberrant in *pif1* seedlings, we performed spectrofluorometric analyses on acetone extracts of 4 day-old dark-grown *pif1* and wt seedlings with or without a five minute white light treatment. The results show that although dark-grown *pif1* seedlings have a higher relative fluorescence peak at 632 nm, indicative of pchlide, the relative fluorescence peak at 670 nm, indicative of chlide, is lower in *pif1* seedlings than in wt seedlings after the light treatment (Fig. 2.7A and B). These *in vivo* enzyme assay results suggest that *pif1* seedlings have reduced levels of POR enzyme activity and, consistent with our microarray data, supports our hypothesis that PIF1 regulates expression of the *POR* genes in the dark (Fig. 2.1).

PIF1 regulates genes involved in heme biosynthesis

One of the major points of regulation in the chlorophyll pathway is the conversion of protoporphyrin IX (PPIX) to either Mg-protoporphyrin, which leads to chlorophyll

production, or to heme, which leads to phytochromobilin production (Fig. 2.1B) (Tanaka and Tanaka, 2007). Heme negatively regulates the chlorophyll pathway by down regulating δ-aminolevulinic acid (ALA) production (Fig. 2.1B) (Tanaka and Tanaka, 2007; Terry and Kendrick, 1999). Because *pif1* seedlings show a reduced level of *FeChII* and an increased level of *HO3* expression in the dark (Table 2.1; Figs. 2.1A and 2.5C), it is possible that *pif1* seedlings have reduced levels of heme compared to wt seedlings. Lower heme levels would result in less feedback inhibition of ALA production and a higher level of pchlide production (Tanaka and Tanaka, 2007). Because direct measurement of heme in etiolated *Arabidopsis* seedlings poses significant technical challenges, we took an indirect approach as described (Terry and Kendrick, 1999). Exogenous application of the iron chelator 2'-2'-bipyridyl (BP) prevents conversion of PPIX to heme and allows accumulation of Mg-protoporphyrin (Mg-PP) to detectable levels in seedlings. We measured Mg-PP levels in dark-grown wt and *pif1* seedlings incubated with or without BP. Our results show that after BP treatment *pif1* seedlings accumulate significantly higher amounts of Mg-PP than wt seedlings (Fig. 2.8A and B). These data suggest that *pif1* seedlings have a reduced amount of heme, possibly resulting from reduced expression of *FeChII* and an increased expression of *HO3* (Figs. 2.1A and 2.5C). Alternatively, the higher levels of Mg-PP observed in the *pif1* background may be a result of defects in the conversion of ALA to PPIX (Fig. 2.1B).

To address this, we measured PPIX levels in dark-grown seedlings treated with or without 10 mM ALA. Because pchlide and PPIX fluorescence emission spectra overlap and given that pchlide levels are higher in the *pif1* background (Fig. 2.7) (Huq et al., 2004), absorbance at 503nm was measured. The results show that *pif1* seedlings contain a wt level of PPIX (Fig. 2.9A and B), suggesting that the elevated levels in Mg-PP found in the *pif1* seedlings are a consequence of reduced levels of heme compared to wt seedlings.

Since heme is a negative feedback regulator of the early rate-limiting step in the pathway, reduced levels of heme are expected to increase the rate of ALA biosynthesis (Fig. 2.1B) (Tanaka and Tanaka, 2007). We measured the rate of ALA biosynthesis using a protocol described by (Goslings et al., 2004). The rate of ALA synthesis in *pif1*

seedlings is approximately two-fold higher than that in wt seedlings (Fig. 2.10). The modest increase in the rate of ALA synthesis is consistent with the modest increase in pchlide levels in *pif1* seedlings compared to wt seedlings (Fig. 2.7A). Taken together, these data suggest that PIF1 subtly regulates the level of heme in the dark to fine-tune the tetrapyrrole pathway in *Arabidopsis*.

Discussion

Exquisite regulation of the tetrapyyrole pathway in the dark is required to avoid photo-oxidative damage of seedlings upon illumination. This study provides genetic, molecular and biochemical evidence that PIF1 directly and indirectly regulates key genes to fine-tune the tetrapyrrole pathway. Several lines of evidence suggest that *PORC* is a direct target of PIF1. First, microarray and RT-PCR/qRT-PCR assays established that *PORC* expression is reduced in dark-grown *pif1* seedlings compared to wt seedlings (Fig. 2.1A, Table 2.1). Second, the ChIP assay shows that PIF1 binds to the promoter of *PORC in vivo* (Fig. 2.3). Third, PIF1 directly binds to the G-box element in the *PORC* promoter (Fig. 2.4A). Fourth, in transient expression assays PIF1 activates transcription of *PORC* in a G-box dependent manner (Fig. 2.5A and B). Fifth, regulation of *PORC* is consistent with our physiological data showing that after initial light exposure, chlide levels in *pif1* seedlings are reduced compared to chlide levels in wt seedlings (Fig. 2.7). Taken together, these results strongly suggest that PIF1 is a direct regulator of *PORC* expression.

Expression analyses data suggest that PIF1 regulates all three *POR* genes, with *PORA* and *PORB* displaying the most significant changes in expression (Table 2.1). However, direct interaction studies show that *PORC* is the only direct target of PIF1. One distinction between PORA, PORB and PORC is the cis-elements present in their respective promoters. *PORA* and *PORB* promoters have E-boxes, while the *PORC* promoter contains a G-box motif (Table 2.2). The PIF1 homodimer binds only G-boxes and not E-boxes in *in vitro* gel-shift assays (Fig. 2.4; data not shown). It is probable that PIF1 regulates *PORA* and *PORB* expression indirectly and *PORC* expression directly. Further, *POR* gene expression is developmentally regulated. *PORA* and *PORB* function

in young seedlings during the transition from dark to light, and *PORC* functions in light-grown plants (Tanaka and Tanaka, 2007). Therefore, PIF1 might control chlorophyll biosynthesis not only during the initial dark to light transition, but also during daily light-dark cycles.

The tetrapyrrole pathway is primarily regulated by metabolic intermediates and transcriptional regulation of metabolic enzymes (Tanaka and Tanaka, 2007). Higher pchlide content in dark-grown *pif1* seedlings suggests that PIF1 either represses genes involved in pchlide production or activates a repressor that down-regulates pchlide production. Two well-established repressors of the chlorophyll pathway are FLU and heme (Tanaka and Tanaka, 2007). Both FLU and heme are negative feedback regulators targeting early steps in the chlorophyll pathway to repress production of downstream intermediates (Tanaka and Tanaka, 2007; Goslings et al., 2004) (Fig. 2.1B). Expression analyses confirm that PIF1 does not regulate *FLU* expression or the expression of other genes involved in conversion of ALA to pchlide (Figs. 2.1A and 2.5; Table 2.1; data not shown). Conversely, PIF1 indirectly activates the expression of *FeChII* and indirectly represses the expression of *HO3* in the dark. *FeChII* encodes a ferrochelatase enzyme that converts PPIX to heme, and *HO3* encodes a heme oxygenase enzyme that converts heme to biliverdin IX (Table 2.1; Figs. 2.1 and 2.5C). Although, PIF1 regulation of *FeChII* is subtle (Fig. 2.1A), the net effect of *FeChII* and *HO3* expression may lead to lower heme content in *pif1* seedlings compared to wt seedlings. Reduced heme content relieves the feed-back inhibition of ALA synthesis and results in a higher level of pchlide in *pif1* seedlings compared to wt seedlings (Fig. 2.7A) (Huq et al., 2004). Increased levels of Mg-PP in *pif1* seedlings compared to wt seedlings after BP treatment (Fig. 2.8A), and the comparable level of PPIX after ALA treatment (Fig. 2.9A) suggest that *pif1* seedlings have less endogenous heme than wt seedlings. Moreover, *pif1* seedlings have a modest increase (~2-fold) in the rate of ALA synthesis compared to wt seedlings (Fig. 2.10). Interestingly, a reduction in plastidic *FeCh* in tobacco resulted in an increased rate of ALA synthesis and higher chlorophyll production (Tanaka and Tanaka, 2007;

Papenbrock et al., 2001), similar to our results. Combined, our data strongly suggest that PIF1 controls heme levels to optimize pchlide production in the dark.

Previous work shows that PIF1 functions as a negative regulator of chlorophyll biosynthesis under prolonged light conditions (Shen et al., 2005; Huq et al., 2004). Initially, this finding appears to contradict our conclusion that *pif1* seedlings have reduced *POR* enzyme activity. However, because *POR* expression is reduced but not eliminated in the *pif1* background (Fig. 2.1A), it is possible that the amount of pchlide, not the POR enzyme levels, is a limiting factor for chlorophyll biosynthesis under prolonged light conditions. *pif1* seedlings have an increased rate of ALA synthesis due to reduced heme content compared to wt seedlings (Figs. 2.6 and 2.10), resulting in increased pchlide synthesis in *pif1* seedlings (Fig. 2.7). Therefore, the higher pchlide level will result in higher chlorophyll synthesis in *pif1* seedlings compared to wt seedlings upon prolonged light exposure. Further experiments are necessary to determine whether the POR enzymes or their substrate (pchlide) is the rate-limiting factor under prolonged light conditions.

PIF1, PIF3 and PIF4 bind a G-box DNA sequence element present in light-regulated promoters, raising questions about how PIFs specify gene targets (Figs. 2.4 and 2.5) (Huq and Quail, 2002; Martínez-García et al., 2000; Huq et al., 2004; Shin et al., 2007). Our results show that PIF3 does not bind to the G-box present in the *PORC* and *FeChII* promoters (Fig. 2.4B). Both *PORC* and *FeChII* promoters contain the G-box sequence, **A**[CACGTG]**T**, flanked with an adenine (A) at the 5'-end and a thymine (T) at the 3'-end. Indeed, random DNA binding site selection studies for PIF3 did not isolate any G-box sequence flanked by a 3' T (Martínez-García et al., 2000). These results suggest that PIF binding is specified by the sequence flanking the G-box motif in gene promoters, as has been shown for animal bHLH DNA binding (Toledo-ortiz et al., 2003).

PIFs interact with differential affinities to phys and PIFs function in distinct phy signaling pathways (Castillon et al., 2007). However, how these interactions result in light-regulation of gene expression is still unclear. Our data show that PIF1 constitutively activates gene expression in the dark, and does not play a major role in light-regulation of

these genes (Figs. 2.1 and 2.5), which is consistent with the light-induced degradation of PIF1. These results are also consistent with recent reports that both PIF1 and PIF3 constitutively activate gene expression in the dark (Oh et al., 2007; Al-Sady et al., 2008). Therefore, how phys regulate gene expression in response to light remains to be determined.

Although PIF1 regulates key genes in the tetrapyrrole pathway, the effects are subtle. Other bHLH proteins in addition to PIF1 may also regulate the expression of PIF1 target genes. The promoters of most of these genes have multiple E/G-boxes within the 500 bp upstream of ATG (Table 2.2). It is possible that PIF1 binds E-box motifs as heterodimer(s) with other bHLH proteins. The *Arabidopsis* genome encodes ~162 bHLH proteins (Toledo-ortiz et al., 2003), and many of these factors regulate photomorphogenesis (Castillon et al., 2007). It is likely that combinatorial control by multiple factors is necessary to optimize the greening process.

In conclusion, our data show that PIF1 directly and indirectly regulates key genes in the tetrapyrrole pathway in the dark to prepare young etiolated seedlings to respond to light. PIF1 appears to act both positively and negatively to fine-tune the chlorophyll biosynthetic pathway (Fig. 2.1). Because PIF1 is degraded in light and re-accumulates in the dark (Shen et al., 2005), PIF1 might provide plants an adaptive advantage under natural light-dark cycles by reducing the daily photo-oxidative damage at dawn, and thereby ensures robustness and fitness of plants under an ambient light environment.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) and the *pif1-2* null allele was used for these experiments (Shen et al., 2005; Huq et al., 2004). All seeds were freshly harvested (2-3 months old). Plants were grown on MS media and seeds sterilized as in (Huq and Quail, 2002).

Microarray analyses

Total RNA was isolated from 4 day-old wt and *pif1* dark-grown seedlings. Microarray hybridizations and probe synthesis were performed as in (Hudson and Quail, 2003) on RNA from three independent biological samples. To identify genes that are regulated by PIF1, the data files were also analyzed using Microsoft Excel as described (Hudson and Quail, 2003).

RNA isolation, RT-PCR and qRT-PCR

Total RNA was isolated from 4 day-old dark-grown wt, *pif1*, and *35S:LUC-PIF1* transgenic seedlings using the RNase Plant Mini kit (Qiagen) and reverse transcribed using SuperScript™ II (Invitrogen) as per manufacturer's protocol. The qRT-PCR assays used the Power SYBR Green RT-PCR Reagents Kit (Applied Biosystems). Primer sequences used for RT-PCR and qRT-PCR (Table 2.3), and additional details are available in online supplemental materials.

ChIP assay

ChIP assays were performed as in (Gendrel et al., 2002), except 3 day-old dark-grown *35S:TAP-PIF1* and *35S:TAP-GFP* seedlings were vacuum infiltrated with 1% formaldehyde for 1h at 4C, and cross-linking was quenched by vacuum infiltration with 0.125M glycine for 3 min. Monoclonal antibody against MYC tag (Calbiochem) was used for immunoprecipitation.

DNA gel shift assay

DNA gel shift assays were performed as in (Huq and Quail, 2002; Martínez-García et al., 2000). PIF1, PIF3 and LUC were synthesized using the Rabbit Reticulocyte TNT system (Promega) as described (Huq and Quail, 2002). A 70bp *PORC* promoter fragment containing a G-box motif was labeled with ^{32}P-dCTP. Cold competitor probe was generated from dimerized oligos of the *PORC* or *FeChII* promoter region containing the G-box promoter motif. Probe sequences are shown in Table 2.3.

Transient transfection of promoter-GUS fusions

To construct *pPORC:GUS*, a 1.6 kb promoter region of the *PORC* gene was cloned into the *pENTR* vector (Invitrogen), sequenced and recombined into *pBGWFS7* destination vector (Karimi et al., 2005). The G-box element in the *PORC* promoter was mutated using a site-directed mutagenesis kit (Stratagene) to produce *pPORCGm:GUS*. A 1.0 kb promoter region of the *FeChII* gene was used to construct *pFeChII:GUS* and *pFeChIIGm:GUS* as described above. A 1.4 kb promoter region of the *ACT2* gene (At3g18780) was used to construct *pACT2:GUS* as described above. The DNA coated beads were bombarded into 3.5 day-old wt, *pif1*, or *35S:LUC-PIF1* transgenic seedlings under dim light as described (Huq et al., 2004). Seedlings were grown vertically in individually wrapped plates in darkness and opened just prior to bombardment. Immediately after bombardment, the seedlings were exposed to 15 min of FR light (34 $molm^{-2}s^{-1}$) before growing in the dark for 16 h. Total protein was extracted in the darkroom under safe green light and the protein concentration, Renilla Luciferase and GUS activity were determined as described (Shen et al., 2005; Huq et al., 2004).

Analysis of chlorophyll pathway intermediates

Protochlorophyllide and chlorophyllide were extracted as in (Terry and Kendrick, 1999) except 4 day-old dark-grown wt and *pif1* seedlings were used. Spectrofluorometery (TimeMaster Pro, Photon Technologies International) was performed at an excitation wavelength of 440 nm and an emission wavelength of 600-700 nm, and data curve-fitted using PeakFit v4.11 (Systat Software). The ALA feeding experiment was carried out as in (Terry and Kendrick, 1999), except ALA or buffer control was vacuum infiltrated for 5 min. at 25Hg into 4 day-old wt and *pif1* seedlings. Measurement of ALA synthesis rate was carried out as in (Goslings et al., 2004) on 3 day-old seedlings grown in 8h light/16h dark cycles, and samples were harvested at the end of the dark period.

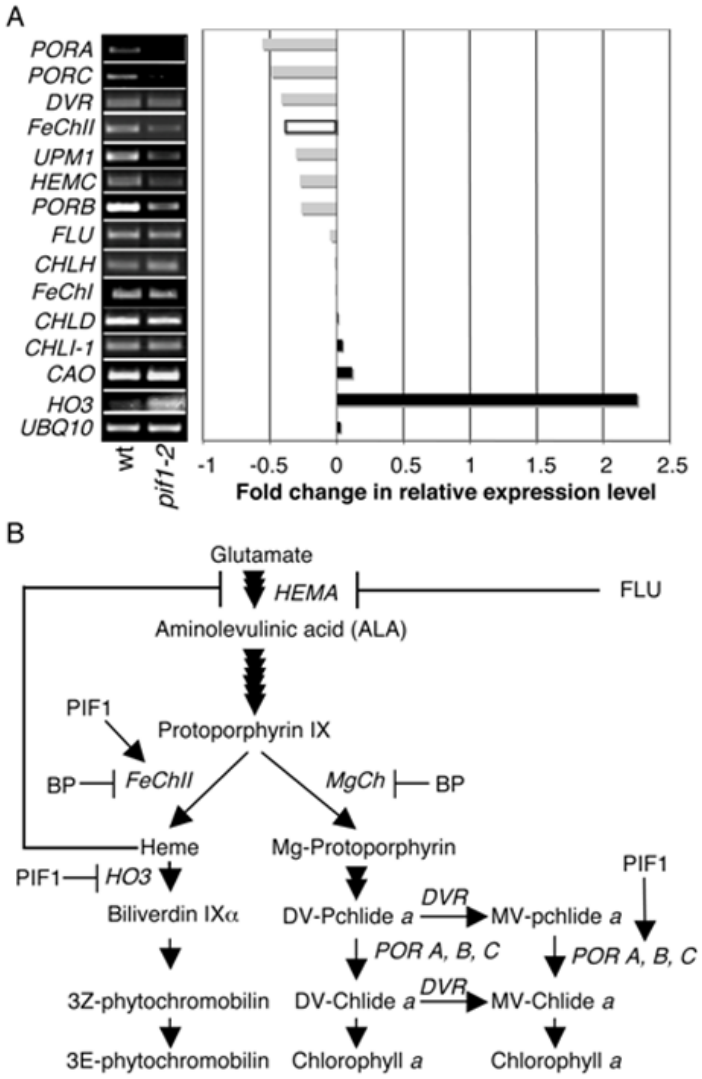

Figure 2.1: PIF1 regulates key genes involved in the regulation of the tetrapyrrole pathway.

A) Bar graph shows fold changes of selected genes in *pif1* seedlings compared to wt seedlings based on microarray (filled bars) and qRT-PCR (open bars) data. Left inset) Independent verification of microarray results using semi-quantitative RT-PCR assays of genes involved in tetrapyrrole pathway. RNA was isolated from 4 day-old etiolated seedlings. **B)** Tetrapyrrole pathway showing genes directly or indirectly regulated by PIF1. DV-Pchlide, divinylprotochlorophyllide; MV-Pchlide, monovinylprotochlorophyllide; DV-Chlide, divinylchlorophyllide; MV-Chlide, monovinylprotochlorophyllide.

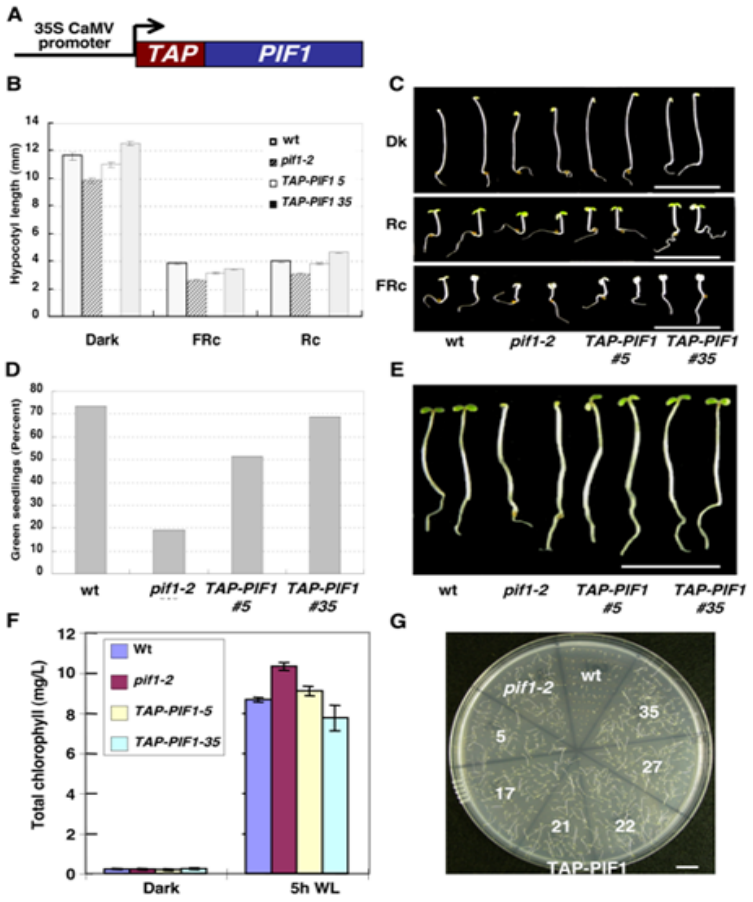

Figure 2.2: Rescue of the *pif1-2* bleaching and chlorophyll content phenotypes in *35S:TAP-PIF1* transgenic seedlings.

A) Illustration of the construct expressing TAP-PIF1 fusion protein in *pif1-2* background. **B)** Hypocotyl lengths of wild type, *pif1-2* and transgenic *35S:TAP-PIF1* seedlings grown in the dark or under red (8 $\mu molm^{-2}s^{-1}$) or far-red (0.6 $\mu molm^{-2}s^{-1}$) light for four days. **C)** Photographs of seedlings grown under the conditions described in (B). **D)** Wild type, *pif1-2* and transgenic *35S:TAP-PIF1* seedlings were grown in the dark for six days followed by two days light grown. Graph shows percentage of green seedlings in each genotype. N=30 seedlings. **E)** Representative phenotypes of wt, *pif1-2 and 35S:TAP-PIF1* transgenic seedlings from experiment described in (D). **F)** Wild type, *pif1-2* and transgenic *35S:TAP-PIF1* seedlings were grown in the dark for 3 days, then transferred to white light for 5 hours or kept in the dark. Total chlorophyll was measured as described (Huq et al., 2004). **G)** Wild type, *pif1-2* and transgenic *35S:TAP-PIF1* seedlings were surface sterilized and exposed to 15 min 3.2 $\mu molm^{-2}s^{-1}$ FR treatment or dark (control). Seedlings were grown in dark for five days at 21 ℃ and scored for germination.

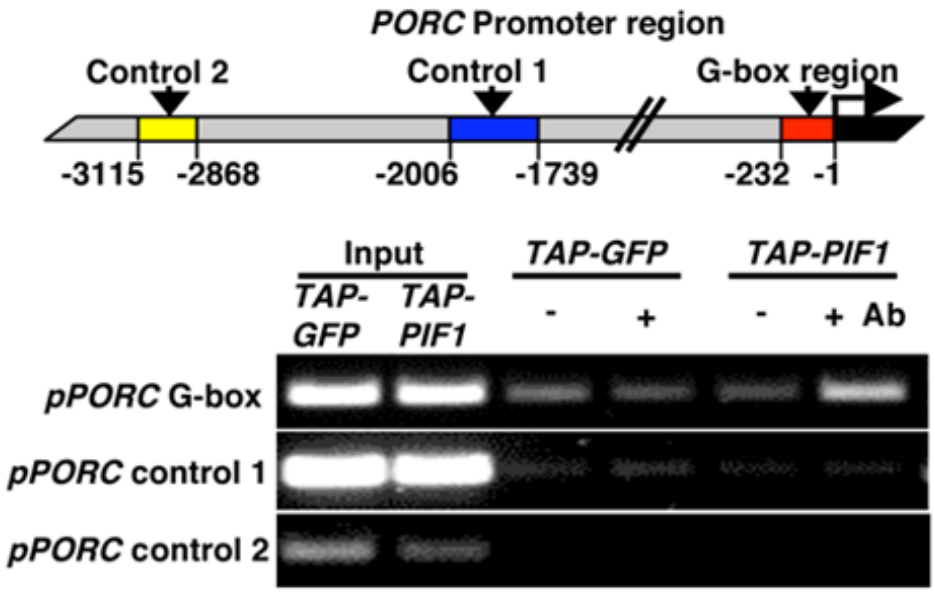

Figure 2.3: *PORC* is a direct target of PIF1.

(Top) Illustration of the *PORC* promoter region. The specific regions amplified by the ChIP assays are shown with nucleotide numbers. **(Bottom)** Gel photographs showing the amplified products from the ChIP assay. The ChIP assay was performed on 3 day-old dark-grown seedlings expressing the TAP-PIF1 or TAP-GFP fusion proteins. Antibody to the MYC tag was used to immunoprecipitate TAP-PIF1/TAP-GFP and associated DNA fragments. DNA was amplified using primers specific to the region containing the G-box element or control regions in *PORC* promoter as indicated. +/-, indicates with or without antibody; input, sample before immunoprecipitation.

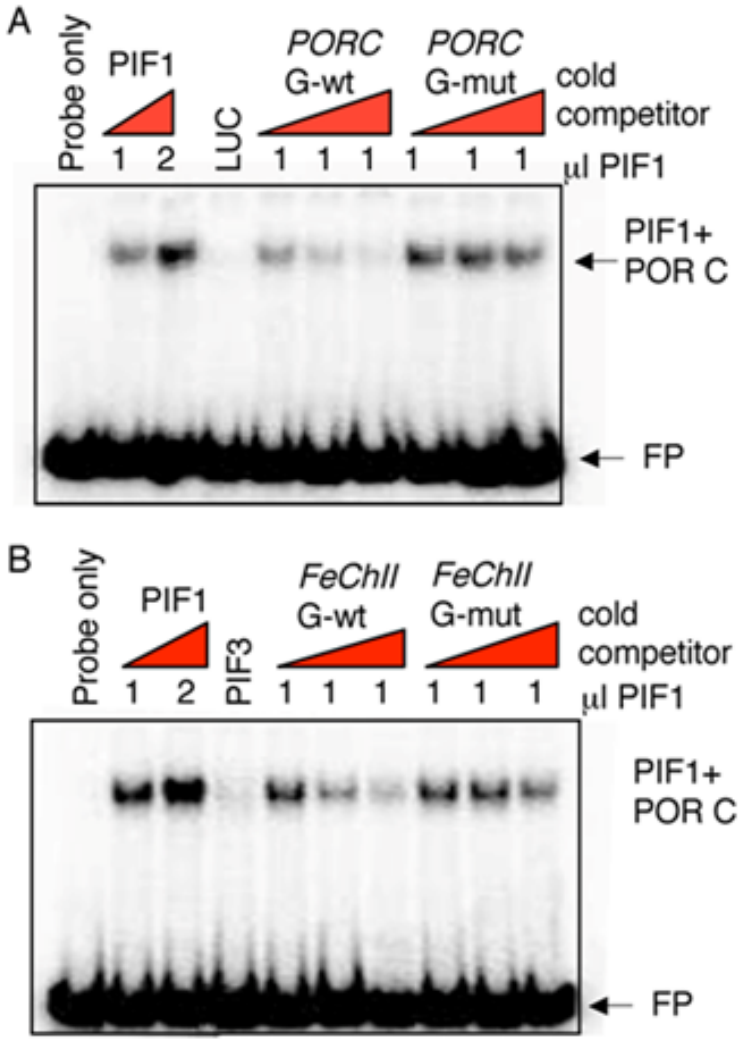

Figure 2.4: PIF1 binds the G-box motif present in *PORC* and *FeChII* native promoters *in vitro.*

A) Fifteen thousand cpm of ^{32}P-dCTP labeled *PORC* promoter fragment containing the G-box was incubated with *in vitro* TNT expressed PIF1 as indicated. Competition for PIF1 binding was performed with 5x, 25x or 125x cold *PORC* probe or mutated G-box (Gm) cold PORC probe. **B)** PIF1 binding to *PORC* labeled probe was competed by either wild type or G-box mutated *FeChII* cold probe. FP: free probe. LUC (A) and PIF3 (B) indicate *in vitro* expressed proteins used as controls.

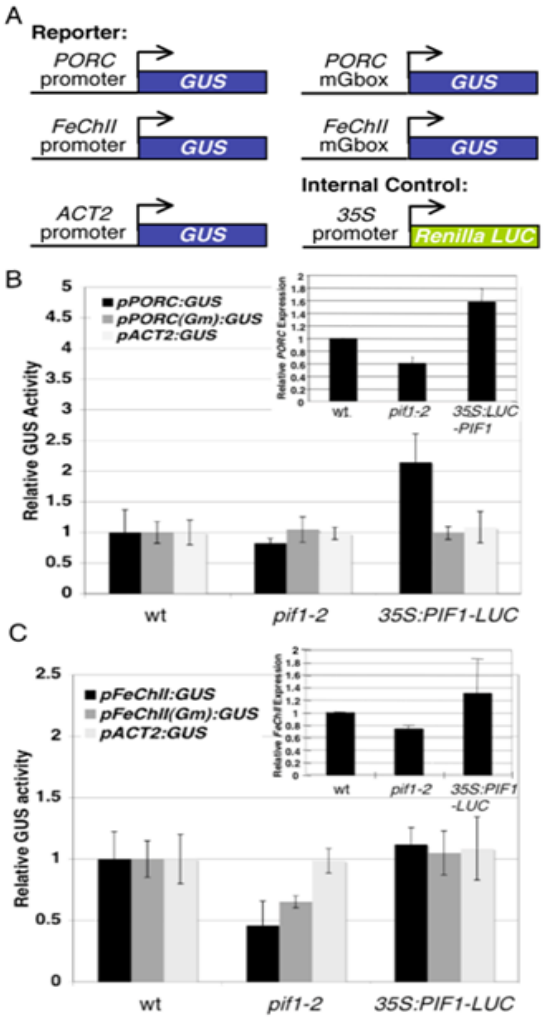

Figure 2.5: PIF1 activates transcription from *PORC* and *FeChII* promoters *in vivo.*

A) Illustration of reporter and internal control constructs used in transient promoter activation assay. **B)** 3.5 day-old dark-grown wt, *pif1* or *35S:LUC-PIF1* seedlings were transiently transformed with *pACT2:GUS* or *pPORC:GUS* or plasmid containing a mutated promoter G-box motif (*pPORCGm:GUS*). Relative expression of GUS was measured. Wt GUS expression levels are set to 1. (N=3 biological replicates, ±SE). Inset: qRT-PCR data showing relative expression of *PORC* in wt, *pif1* and *35S:LUC-PIF1* seedlings. Wt *PORC* expression levels are set to 1. (N=5 trials, each with 3 technical replicates, ±SE). **C)** As in B) except seedlings were transformed with *pFeChII:GUS* or *pFeChIIGm:GUS*. (N=3 biological replicates, ±SE). Inset: qRT-PCR data showing relative expression of *FeChII* in wt, *pif1* and *35S:LUC-PIF1* seedlings. Wt *FeChII* expression levels have been set to 1. (N=3 trials, each with 3 technical replicates, ±SE).

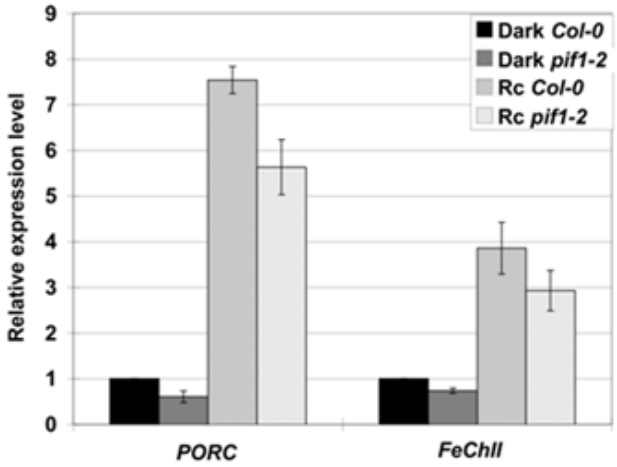

Figure 2.6: Light-regulation of *PORC and FECHII* expression.

The relative expression level was determined by qRT-PCR assays from RNA samples isolated from wild type and *pif1-2* seedlings grown in the dark for four days or four day-old darkgrown seedlings exposed to Rc (10 $\mu molm^{-2}s^{-1}$) for 6 hrs. The level of *UBQ10* was used to normalize the data, and calculate the relative expression levels.

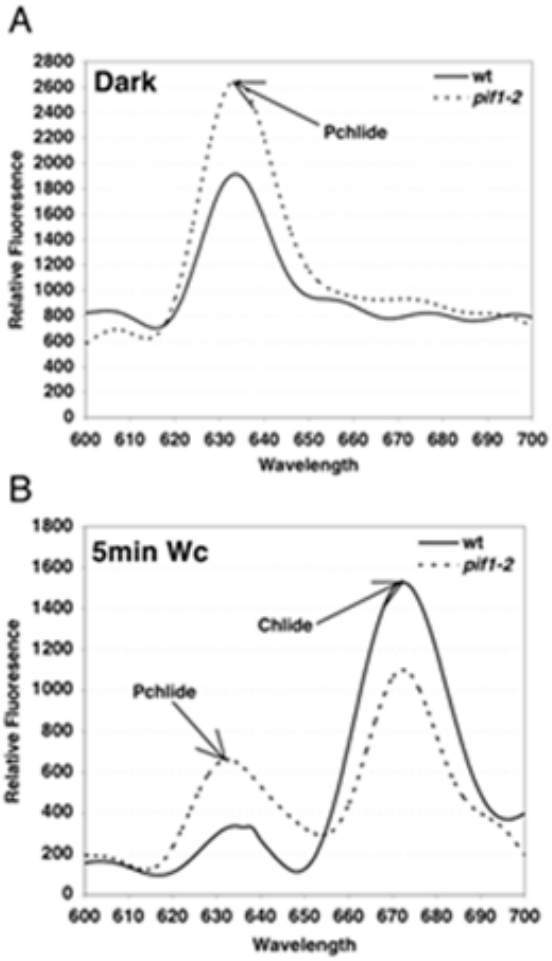

Figure 2.7: *pif1* seedlings have altered pchlide and chlide levels compared to wt seedlings.

A) Relative fluorescence of protochlorophyllide (632 nm) in 4 day-old dark grown wt or *pif1* seedlings. **B)** Relative fluorescence of protochlorophyllide and chlorophyllide (670 nm) in 4 day-old dark-grown seedlings exposed to 5 min 80 $mmolm^{-2}s^{-1}$ white light.

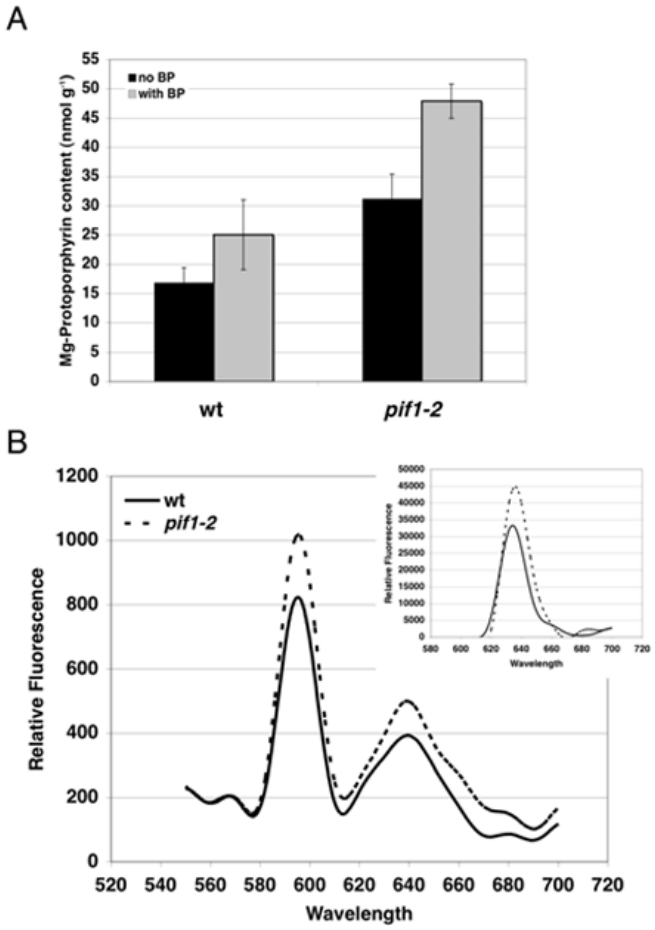

Figure 2.8: Spectrofluorometry of BP treated seedling extracts shows that *pif1-2* seedlings might have reduced level of heme than wt.

A) Mg-protoporphyrin content in wt and *pif1-2* 4 day-old seedlings with or without 20 hr 2'2'-bipyridyl treatment. (N=3 biological replicates, ± SE). **B)** Relative fluorescence of Mg-protoporphyrin (peak at 598 nm) in 4 day-old wt or *pif1-2* seedlings treated with 2'2'-bipyridyl for 20 hr. Excitation at 410 nm. Inset: relative fluorescence of the same samples after excitation at 440 nm, showing protochlorophyllide peak at 640 nm.

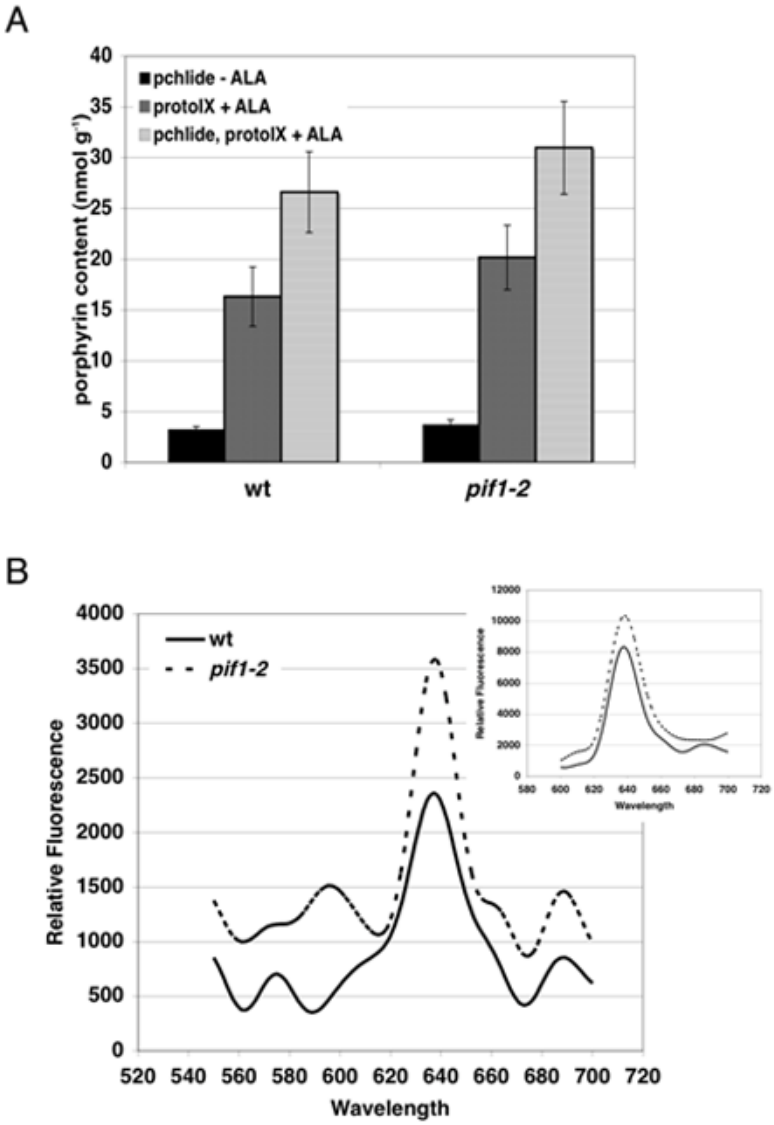

Figure 2.9: Spectrofluorometry of ALA treated seedling extracts shows defects in chlorophyll biosynthesis in *pif1-2* seedlings is upstream of ALA biosynthesis.

A) Porphyrin content in wt and *pif1-2* 4 day-old dark-grown seedlings. (N=3 biological replicates, ±SE). **B)** Relative fluorescence of porphyrin in 4 day-old wt or *pif1-2* seedlings fed 10 mM ALA for 20 hr. Excitation at 410 nm. Inset: relative fluorescence of the same samples after excitation at 440 nm, showing protochlorophyllide content.

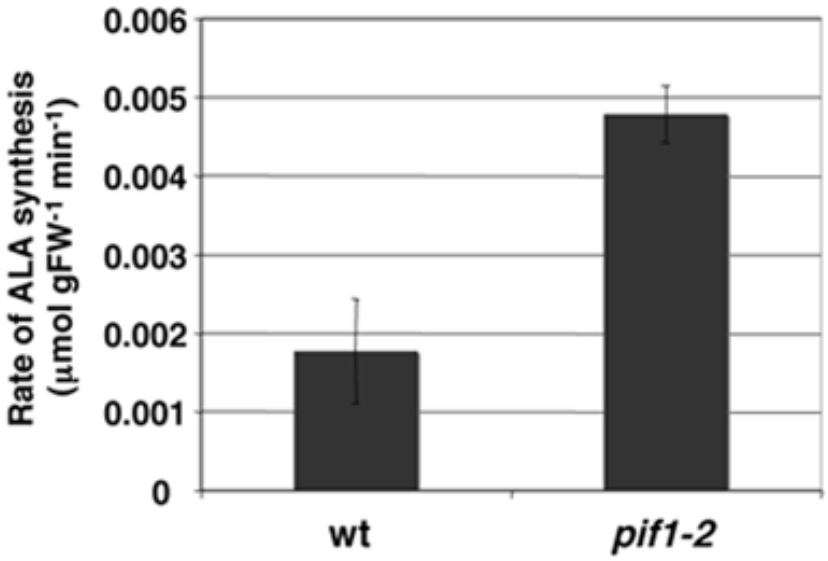

Figure 2.10: Increased rate of ALA synthesis in *pif1* seedlings compared to wt seedlings.

Rate of ALA synthesis measured by absorbance at 553 nm in 3 day-old wt and *pif1* seedlings grown in 8 hr L/16 hr D cycles. (N=6 biological replicates, ±SE). Samples were harvested at the end of the dark period before the onset of light.

Table 2.1: Expression of tetrapyrrole biosynthesis/regulatory genes in wild type vs *pif1* mutant seedlings.

		Microarray comparison (Wt/*pif1*)				RT-PCR	qRT-PCR	G-box
Gene name	**Locus Identifier**	Wt Av	*pif1* Av	F. C. (Wt/*pif1*)	P-value	F. C. (Wt/*pif1*)	F. C. (Wt/*pif1*)	Y/N
PORA	AT5G54190	7625.70	3406.57	2.24	0.01	4.59		
PORC	AT1G03630	355.20	181.03	1.96	0.07	2.08	2.25	Y
DVR	AT5G18660	155.37	91.10	1.71	0.01	1.22		
UPM1	AT5G40850	559.10	387.57	1.44	0.06	2.43		
PBGD/HEMC	AT5G08280	1528.90	1112.70	1.37	0.02	2.32		
HO2	AT2G26550	189.63	138.47	1.37	0.14			
PORB	AT4G27440	9147.50	6715.30	1.36	0.17	2.34		
GUN4	AT3G59400	782.37	582.73	1.34	0.16			
ALAD2/HEMB2	AT1G44318	103.13	81.00	1.27	0.04			Y
GSA2	AT3G48730	823.87	678.27	1.21	0.07			
URO1/HEME2	AT2G40490	1142.53	942.30	1.21	0.06			
URO2/HEME1	AT3G14930	512.23	423.70	1.21	0.33			
CHLI2	AT5G45930	275.23	235.50	1.17	0.29			
GSA1	AT5G63570	841.90	721.10	1.17	0.11			
CRD1	AT3G56940	3747.63	3229.00	1.16	0.39			Y
CHLG	AT3G51820	492.87	439.27	1.12	0.40			Y
HO1/HY1	AT2G26670	4221.43	3779.07	1.12	0.34			
FLU	AT3G14110	874.77	833.27	1.05	0.64	1.03		
CPO1/LIN2/HEMF1	AT1G03475	533.07	508.30	1.05	0.11			
HEMA2	AT1G09940	459.30	443.83	1.03	0.38			
ALAD1/HEMB1	AT1G69740	1229.70	1190.63	1.03	0.60			
CHLH/GUN5	AT5G13630	1203.40	1185.60	1.02	0.90	0.94		Y
FC1	AT5G26030	781.87	774.27	1.01	0.93	1.13		

HY2	AT3G09150	99.83	100.37	0.99	0.96			Y
CHLD	AT1G08520	833.23	842.57	0.99	0.90	1.19		
PPO1/HEMG2	AT5G14220	629.23	644.50	0.98	0.83			
CHLI1	AT4G18490	24.20	25.30	0.96	0.96	1.00		
CPO2/HEMF2	AT4G03205	110.17	120.00	0.92	0.80			
CHLM	AT4G25080	2994.40	3275.60	0.91	0.25			
PPO2/HEMG1	AT4G01690	757.70	837.10	0.91	0.33			
CAO	AT1G44446	407.43	456.07	0.89	0.36	0.94		Y
HEMA1	AT1G58290	761.63	858.93	0.89	0.34			
UROS/HEMD	AT2G26540	291.67	335.83	0.87	0.47			Y
CPO3/HEMF3	AT5G63290	73.77	88.93	0.83	0.33			
FC2	AT2G30390	292.63	377.77	0.77	0.33	1.98	1.61	Y
HO4	AT1G58300	6.37	8.63	0.74	0.30			
HEMA3	AT2G31250	18.70	52.83	0.35	0.23	1.38		
HO3	AT1G69720	14.07	45.80	0.31	0.02	0.48	0.44	
UBQ10	AT4G05320	15522.10	15984.93	0.97	0.69	0.97	1.00	

Table 2.2: E/G boxes present in the promoters of genes for the tetrapyrrole pathway in *Arabidopsis.*

Gene name	Atg Number	5' Region from ATG	Position	Box name	E-Box sequence
PORA	AT5G54190	2004 bp	-1977	E	TTATACAACACACATGGTACATAAGC
			-1949	E	AGAACTTACTCATCTGAAACTTGAGT
			-1657	E	GTTCTCGTCACATCTGCAGATTCACG
			-1487	E	ATCTAAGATACACATGAGTCCCACAT
			-1080	E	AACCCTCCGACACCTGTCAACGATCC
			-111	E	CTAGGAAACACATATGGGAGGGGACC
			-49	E	CAAGCACAGACATTTGCATAACATTC
PORC	AT1G03630	2000 bp	-1853	E	ATAAACTAATCACATGCATATTTCTA
			-1773	E	AATATTTAGCCAAATGAGCTATTACG
			-1323	E	GAGATGGACACAAATGTTCAAAGAAT
			-563	E	CTGTTATAGGCATATGAACAAGACAA
			-270	E	GTTCTTGACACATTTGGACGGTCCAG
			-219	E	GTGATCAGATCAGTTGAGAGTTAACA
			-103	G	GCCTGGAAGACACGTGTCACTAACCC
DVR	AT5G18660	2076 bp	-2021	E	AGTTAAACGACACTTGTGACACTGTG
			-1979	E	TAGAATTAGTCAATTGGACATAGTGG
			-1608	E	TTGAATAGAGCATATGTCTATCAAAT
			-1140	E	ATTCTCTTTACACATGTATGTTTTGG
			-472	E	TTGTGGTTGTCATTTGTCTCTCCATC
			-425	E	GAACGCCGTACATCTGTTCGTTACGT
UPM1	AT5G40850	2035 bp	-2001	E	TGCCTCTATACATATGATAAAGTTTC
			-1734	E	CCCGCAACCGCAAATGCTTGCGGGAA

			-1290	E	GTGTAGATGTCAAGTGTTTTGCTAAA
			-1044	E	TGGGAAGATCCAGCTGCATCTCCATC
			-702	E	GTGTCTCCAACATGTGCATATTCACA
			-589	E	TCCTAGCCGCCATTTGTTCTCCACAT
			-257	E	GGTTTTGTGCCAATTGAAGAAATCAG
			-164	E	TTGTAGTAATCATATGATTTACTAAC
PBGD/HEMC	AT5G08280	2006 bp	-1999	E	TAAGCCTTGCCACTTGTCTAGACATG
			-1869	E	TTTGAAGAATCAGCTGAGATCAAAAG
			-1608	E	ACTTGGTAGACAAATGGTGTCACATA
			-1404	E	TCGGGATATTCAGCTGACCAGAATCA
			-1224	E	CTTGAGAGCCCAGTTGATCTTGTTGT
			-977	E	ATACTAGAATCAATTGTAAGTGAAGC
			-472	E	AACCGGAGTGCAGATGTGGAAGAAGA
			-106	E	CCATCAACTCCATTTGAAGCGAAATT
			-13	E	TTGGCTCCTCCACCTGAATCCATGGA
HO2	AT2G26550	1999 bp	-1553	E	ATCTCAACTTCAGCTGCCCCTACATA
			-1193	E	GGGGAAGTAACAAATGAGATCTCAGG
			-1148	E	CCAATAAGACCAAATGATTACGGAAG
			-387	E	ACATTGTGGTCACTTGTACGTTTGAA
			-155	E	TTATTTTTTACATCTGCCCTATTTAG
PORB	AT4G27440	2056 bp	-631	E	AGTGATGGTACACATGAGATTTGTAG
			-540	E	GATTTTGTGACATATGACATAATGCT
			-443	E	GACAGAGTAACAATTGTTTATTACTG
			-295	E	CCCTTTATTGCAACTGTTCCATTTGT
			-286	E	GCAACTGTTCCATTTGTTAACCCAAA
			-101	E	CCGAATATCTCATTTGCTAGTACATA
GUN4	AT3G59400	2048 bp	-2017	E	CATAAACAGTCATTTGGGTGAATATA

			-1764	E	TTCTCAGCATCAGCTGTTGTCAACCC
			-1720	E	AAGCTTGGGGCACTTGTAAGGATATC
			-1217	E	TTCCCTGTAACATCTGAACGATCAAA
			-961	E	CATGGTCTTTCAGCTGACCCCTTCTT
			-506	E	GTATTTGTATCAAATGATAATGGGCC
ALAD2/HEMB2	AT1G44318	2058 bp	-1911	E	ATGCATAAGACATGTGTACACTCAAC
			-1896	E	GTACACTCAACATTTGTATAATCTAG
			-1677	E	GTATGTGTGTCACATGGAGTCATCAA
			-1303	E	TTGCCTTGGTCAGGTGGTTTAGTCTT
			-1275	E	TTGACTATCCCAAATGACCCTAGATT
			-660	E	GGAATCGAACCATCTGACCATCTGAC
			-652	E	ACCATCTGACCATCTGACCAAGACAA
			-42	G	CGGTCTCATTCACGTGTTTATTCTCC
GSA2	AT3G48730	1984 bp	-1773	E	CGACATGACTCAAATGGCAAGTCAAC
			-1561	E	AATATAGTAACAAATGATCAGACGTT
			-1267	E	TTTTTTTTTTCAGGTGACAAATTTCA
			-1253	E	TGACAAATTTCAAATGTGGGGGTTTT
			-492	E	TTGTTCTTACCAAGTGGTGATGATAT
			-42	E	CTTACGAGCGCAAGTGAGAGAGTAAC
URO1/HEME2	AT2G40490	2041 bp	-1204	E	GCAGCGGAAGCACTTGCGATTGCTGA
			-943	E	AAGCAACTTGCAGCTGAAAATGCTTT
			-851	E	CTTTTTCAGCCATTTGAACAAGCATC
			-829	E	CATCACGAGCCACTTGACATTCTCCC
			-680	E	GAATTTGAGCCAAATGTTGAAGGAAC
URO2/HEME1	AT3G14930	1984 bp	-1321	E	TTTAGTGTTACAAATGCTTTGAATGT
			-1028	E	GATCATAGATCAATTGATCCAAGCTG
			-737	E	AAGCACACAACAAGTGTATAACTATG

			-42	E	AATCAGAAAGCATTTGATCTTCAAAC
CHLI2	AT5G45930	2024 bp	-1797	E	CAATAAAACACATTTGAAGCAAATCA
			-1739	E	CGGTTTAGTCCATGTGGTAATCTAGT
			-1615	E	ACCATTTATTCACTTGTGTATGGAAA
			-1454	E	AGCAGGCGTCCAGCTGACCATATATG
			-1411	E	GGTCACCAATCATTTGAATCAAGTTG
			-1338	E	TGCAAATAATCATCTGCAAAATTGAA
			-1293	E	AACACAAAAGCAAGTGTTTGGGGAAT
			-754	E	TAAGATCTAGCAAGTGAAGAACTGGA
			-706	E	GGTTTTGGAGCAATTGAATGAACTTT
			-312	E	ACTAAAGATTCACATGAATGTAACTG
GSA1	AT5G63570	2085 bp	-1559	E	TGACAAATTTCAAGTGTGGAGGGTTC
			-1152	E	CTCTTTGCTGCAGATGGAAGGTCGAG
			-1068	E	GTTACAACGGCAGGTGAAGTGACTTT
			-813	E	CTGTTTCTGCCATGTGGTAGTGACAC
			-329	E	AGAAAAAAATCATTTGTAAGATTATT
CRD1	AT3G56940	2075 bp	-1875	E	AATTCTGTGACACTTGTTTGCTTTAC
			-1833	E	CTCATTGTTCCATATGCAACAACTGT
			-1824	E	CCATATGCAACAACTGTGTCCAGAGA
			-1601	E	ATTTCAAAGACATATGGATGAAAAGA
			-1581	E	AAAAGATTAGCATCTGGAAAGCTATA
			-1476	E	ATTCCTATCTCATCTGTACTAACCAG
			-1296	E	TTTTGGGGAACATATGGGAAATATTT
			-1243	E	AAGTTTAGATCATTTGTCAAGGAAGA
			-892	E	GGAGGATCTTCAAGTGATGGTGAAAA
			-853	E	GTTCGGGTCTCAGATGAAGATGAAGA
			-143	G	TAATTATCGCCACGTGTCTGGCTCCC

CHLG	AT3G51820	1992 bp	-1668	E	AAACATTTTCCACCTGCTAAGAGCCT
			-1135	G	TGACTGTTGCCACGTGTCTCTCAACC
			-956	E	TTGATTGTTCCACATGTAGTAAAAAT
			-168	E	AGGATCGTGCCACATGGCAGTTATTG
HO1/HY1	AT2G26670	1996 bp	-1985	E	GTTCTTTTCTCATATGTTCTAATCAT
			-1929	E	AGAACAAGCTCACATGTTGGACAATT
			-1918	E	ACATGTTGGACAATTGATAGTTTTTA
			-1704	E	GAAATGTGCCCACATGAAGGAATATG
			-1659	E	CCACTCATCGCAGATGCTTTCTTTAT
			-1467	E	CCTTTCTTCACAACTGACCTACTCAA
			-1449	E	CTACTCAACACATTTGTGAAAGAGTG
			-1179	E	CCGTTACCGGCAAGTGGATTAGAAGA
			-1036	E	TATACGCTTACAGCTGTAAAAGATGA
			-843	E	TGCATTTTAGCATCTGTGGGTCAATC
			-252	E	GACATTCAGACATGTGCAACACTCAA
			-159	E	TCGTTATCTTCATTTGGAAACAACTA
FLU	AT3G14110	1994 bp	-1991	E	CTTTCCAAGTCAAGTGATTTTGAAGC
			-1962	E	TTCTTCAAGCCACATGACTATCCGAA
			-1900	E	GACAAGCCTCCAAATGAGATGTTGAT
			-1820	E	AAGAATGATTCATTTGAAAAACGACT
			-1155	E	GAGAACCACACACATGCATGCAGAAA
			-761	E	TTCTCGCTGACATTTGAACTCAACAT
			-740	E	AACATTTCCTCATGTGGCGATGTAGA
CPO1/	AT1G03475	2027 bp	-1988	E	TTTAAGTACACAATTGAAATTCATAG
LIN2/HEMF1			-1812	E	ATACACTAACCATATGTCTATAGTAA
			-1223	E	TGTTCTCCGCCACCTGAGTCTGATGA
			-1015	E	CACCCTTTTGCATGTGTCCGTCCTTG

			-972	E	GTTGCTTCTCCAAGTGCCGCCTTCGC
HEMA2	AT1G09940	2039 bp	-1507	E	CGGGCTCAACCACTTGATCCTGAGAT
			-1012	E	TAGAGATTGCCAGATGAGGGAGTAAA
			-661	E	ATTATGCAAACAAATGTCAACTGGTT
			-654	E	AAACAAATGTCAACTGGTTATATCAT
			-214	E	GACCCGGATCCACCTGCTTCTTTCAA
ALAD1/HEMB1	AT1G69740	2062 bp	-2050	E	CAAATTGACACATTTGTTGGTCCCAC
			-1961	E	CTCTTCTTCTCAAATGAACGATTTTT
			-1944	E	ACGATTTTTTCATATGCTTACTTTGA
			-1310	E	TGATATGCGACATTTGTCAGCGATGC
CHLH/GUN5	AT5G13630	2018 bp	-1964	E	GAAGAAGCAACAAGTGCGTGATCTCA
			-1503	E	ACCACCTCTTCAGATGGCGGCGTCGA
			-989	E	AACAATATTACACTTGGGAAATGACA
			-663	E	TTAACTTTTACATTTGTTGTTACAAT
			-651	E	TTTGTTGTTACAATTGTTATGAGTCT
			-206	G	ACTATTCGTCCACGTGTCCTTCCCTC
			-109	E	CATAAACTCCCACTTGGAGCTCAAAA
FC1	AT5G26030	2080 bp	-1882	E	AAAAAAAACTCAAATGATTCCATTTA
			-1782	E	AAATCTACTCCAAATGATCAATACAA
			-1528	E	TTTGACTAGTCAGGTGGCCGCTGTTC
			-1374	E	ATCTTTTATACAAGTGTGTCAATTTG
			-1202	E	AGGTTTTTACCAATTGAACTAACGAC
			-1187	E	GAACTAACGACACTTGGTAGATGATG
HO3	AT1G69720	2043 bp	-2019	E	GAAAACGCCACAACTGTTACAACTGT
			-2010	E	ACAACTGTTACAACTGTGGTCTGGTA
			-1948	E	GCTGCTCTGGCACCTGACATGAACAA
			-1822	E	CGGAAATCGACAGATGTTTCAGAAAG

			-1501	E	TATGAAGTAGCAGCTGCAGAATCTAA
			-1259	E	GCAATGGCAGCAGATGAAGCAGAGGA
			-1175	E	GACTCTACTACACATGTTTGTCTCTT
			-1125	E	ATGGTTTTCGCAGTTGAAGGAAATGG
			-124	E	TTAAATTCGTCAATTGTTATTTTTTT
HY2	AT3G09150	2072 bp	-1917	E	CAGACAGATTCAAGTGGCAAAGCTAG
			-1830	E	GGTTTTCTAACAAATGCTTTAAAAGT
			-1742	E	GAGATCTGATCATTTGCAATGAGCTT
			-1461	E	ACTTTCAGATCATCTGTTATAATGAA
			-1432	E	AGAAGCTTTACAGCTGAAAAAAACTC
			-1073	E	ATCCTATACCCACTTGAGGCGATTTT
			-218	G	CTGTGCATTCCACGTGGCGGATGTGG
			-150	E	TTGTCGTTGCCAATTGCGTTTGTCTC
CHLD	AT1G08520	2043 bp	-1994	E	AATTTTTATTCATATGGACTACAAAT
			-1772	E	CAACAAGGTACATGTGCATCAATCTC
			-1496	E	ATAGCCAATCCAAATGTTTAGGAGAG
			-1242	E	AAATGTGAGACACATGCATGCATGAG
			-1076	E	CTTTGTAGAGCATGTGATCTACTAGT
			-1047	E	TCCGAAGAGCCATATGGAGAAGGAGA
			-823	E	TTAAATCTCTCATTTGGACGCTTTAA
			-661	E	CCTTTAGTTGCATTTGCATATTTATT
			-626	E	CAAAGAAGTACAGATGATTATGCCTT
			-414	E	TATTAGTTACCACTTGTTTACTACAT
PPO1/HEMG2	AT5G14220	2032 bp	-943	E	TCTTATTGTACAAATGGGCCACGTAA
			-517	E	AATAGGTCATCAAGTGTAGTTTGTGT
			-273	E	GTTTAGTTTTCACATGAGTTCAAACA
CHLI1	AT4G18490	2000 bp	-1565	E	ACTACATATACAACTGCAAAGCGATT

			-939	E	TTTTTCTTTGCAGTTGGTTTTCAAGA
			-894	E	GCCATGTCTGCACCTGCTAAAAGAAG
			-835	E	CGGTATGGTTCAAATGTTCATGTGAT
			-827	E	TTCAAATGTTCATGTGATATGATTCA
			-484	E	AAGTCAATGACAGATGATGATCCAAT
			-97	E	AAAAAGAATCCATTTGCCTTCTCTTA
CPO2/HEMF2	AT4G03205	2016 bp	-1983	E	GACATGATTGCACCTGAAGGTGGAAT
			-1962	E	GGAATATTCTCAGCTGAAGATGCCGA
			-1818	E	TGCATACTGACATTTGTTTTGCGTGT
			-438	E	TTTTACTGAGCAGGTGATTCACATAG
			-307	E	GTCAACACTTCACTTGCAGTCCTCCT
CHLM	AT4G25080	2027 bp	-1974	E	GCCAAGGGTTCAAATGGTTTTGAAGA
			-1917	E	ATGATTAGAGCATATGCATGTTTCCC
			-1828	E	GCTAGCTGATCATTTGCCCTTATTTG
			-1575	E	TCACATCTCGCAGGTGGTAGCGATCG
			-1559	E	GTAGCGATCGCACTTGGAGCAAAGTC
			-1318	E	TATAGACAGACAAATGTGGCTCGACA
			-1170	E	TTCTCCGAAACATTTGGTGAGTCACA
			-764	E	CATTCATCTTCACATGATGCAATTGA
			-755	E	TCACATGATGCAATTGATGGTATATT
			-632	E	AATAGAACAACAATTGTGGGAAACAT
			-404	E	ATTCATACTACATCTGTTTTGCTTTC
			-377	E	GACTGTGTTCCAATTGTTGATATATG
PPO2/HEMG1	AT4G01690	2031 bp	-1829	E	TATTTGCTGGCAAGTGGCAGTGAAGT
			-1545	E	GAAAGTCTGTCAGTTGGGTTTTGGCC
			-1529	E	GGTTTTGGCCCAATTGATTTACCTGG
			-1112	E	TGATGGGATACAAGTGAACACGACAC

			-931	E	TAGGGGATGACAATTGACAACCAATT
			-665	E	ACAGCATAAACACATGCACTAGTCCT
			-598	E	TTTGATTAACCAAGTGGTCATAATTA
			-104	E	TGTGTGATTGCAGGTGAATATTTCTC
CAO	AT1G44446	2011 bp	-1892	E	ATAGCACAGACAGGTGATCCAACCGG
			-1771	E	TGTATATATACATGTGTTGTTTGGAG
			-1731	E	TGTTCTTACGCAGATGGACGGTACTT
			-510	E	CAAATTATCTCAAATGCCAATCCCAA
			-193	E	TCTTCATCATCATGTGGATATTAATA
			-173	G	TTAATACCGCCACGTGTTCAATTCTC
			-153	G	ATTCTCTCTACACGTGTCATCTTCCC
HEMA1	AT1G58290	2087 bp	-2061	E	GATGAATGGGCAGTTGTGGACGAGAG
			-1919	E	GGAAGGCCATCAAGTGTACGTATGAA
			-1868	E	AGTCTCCAAGCACATGTCTTTACTCT
			-1782	E	CTTAGTTGTTCACTTGTTTCTTTTCC
			-1699	E	ACAATTTGGTCAATTGGTTAGCATCC
			-925	E	CCGGACTCAACATATGTAAGAAACAA
			-515	E	TCATATTTTTCATATGGTTCTCCTAA
			-324	E	CGTGTAAGAACAAATGCCACCAAATA
UROS/HEMD	AT2G26540	2001 bp	-1676	E	GCGAGGTGTTCAAATGTAACTGATCA
			-1662	E	TGTAACTGATCAGTTGATTCATTAAG
			-1500	E	GAAGCTTAACCAGTTGCTAAAATCTG
			-1431	E	ACGAGAAGAACAAATGCTTGAAGGAA
			-1275	E	CTTTCATTGTCAACTGTCTCTTCACT
			-887	E	CATAATAGTTCATTTGGCTTTTTACC
			-203	E	AAATTGGGCTCAAATGAAAAAACAAA
			-161	G	CATTAAAACGCACGTGGAGATAGAAT

CPO3/HEMF3	AT5G63290	2083 bp	-2027	E	GTGTTTATCTCAATTGCCAGGAGCAT
			-1950	E	ACTTCAGGTCCAGTTGTGTGTATGGT
			-1868	E	GAGTTAATTGCATGTGATGTTTTAAA
			-1335	E	TTTTAGTTTTCATTTGCTGACATTTT
			-1269	E	GCGAGCTATGCAAGTGGGATTCGGCT
			-1084	E	TATCTGTGATCACTTGTCATGTAATC
			-1051	E	GTTACAGATTCATCTGCATTTGGTTC
			-1045	E	GATTCATCTGCATTTGGTTCTACGTT
			-928	E	TTACAAAACGCAGATGCTGCGTGATT
			-514	E	AATCGTTAACCAGCTGCTCCTCCGAT
			-361	E	TCGTCATCGACAAATGCTGTGACGTT
			-174	E	TAATATTTTTCAATTGTAATTAGTGT
FC2	AT2G30390	2041 bp	-1912	E	AAAAGTTTAACATATGTGAATCTATC
			-1739	E	TACAGATACACAAATGATGAAATTAA
			-1564	E	CTTTCCAGATCAAATGTTTTGGCAAC
			-1464	E	TAAATAAGCACAAGTGATTATTCATA
			-1301	E	AGCCACCAAACATTTGCAATAAGAAA
			-631	E	GCAAGAGATACATTTGAAGACAATGC
			-575	E	CTATCTGATTCATCTGATTGTTGAAG
			-436	E	GACTTGTTTTCAGGTGAATGAACAGA
			-191	E	AAAATTATCACAACTGTGTGGACACG
			-179	G	ACTGTGTGGACACGTGTGGTTCTCCG
			-131	E	CATTTTATCCCAACTGTGTCTGGTCT
HO4	AT1G58300	2042 bp	-832	E	CTTCCCATACCAAATGAAGACTATCT
			-271	E	ATTATTTTGTCAAGTGGCGCGGCGGT
CHLD	AT1G08520	2043 bp	-1994	E	AATTTTTATTCATATGGACTACAAAT
			-1772	E	CAACAAGGTACATGTGCATCAATCTC

			-1496	E	ATAGCCAATCCAAATGTTTAGGAGAG
			-1242	E	AAATGTGAGACACATGCATGCATGAG
			-1076	E	CTTTGTAGAGCATGTGATCTACTAGT
			-1047	E	TCCGAAGAGCCATATGGAGAAGGAGA
			-823	E	TTAAATCTCTCATTTGGACGCTTTAA
			-661	E	CCTTTAGTTGCATTTGCATATTTATT
			-626	E	CAAAGAAGTACAGATGATTATGCCTT
			-414	E	TATTAGTTACCACTTGTTTACTACAT
HEMA3	AT2G31250	2000 bp	-1931	E	AAGGATTTCACAGTTGATATTGAAGG
			-1284	E	ATAGAGAGAGCAAATGAAATCTTAAC
			-1011	E	TTCATCGAGCCACCTGATTTTATAAA
			-466	E	ATCCTCACCTCACCTGATACAGAGCA
HO3	AT1G69720	2043 bp	-2019	E	GAAAACGCCACAACTGTTACAACTGT
			-2010	E	ACAACTGTTACAACTGTGGTCTGGTA
			-1948	E	GCTGCTCTGGCACCTGACATGAACAA
			-1822	E	CGGAAATCGACAGATGTTTCAGAAAG
			-1501	E	TATGAAGTAGCAGCTGCAGAATCTAA
			-1259	E	GCAATGGCAGCAGATGAAGCAGAGGA
			-1175	E	GACTCTACTACACATGTTTGTCTCTT
			-1125	E	ATGGTTTTCGCAGTTGAAGGAAATGG
			-124	E	TTAAATTCGTCAATTGTTATTTTTTT

Table 2.3: Oligo sequences used in experiments described in text.

Gene	Atg Number	Forward	Reverse
RT-PCR			
HEMA3	At2g31250	GCGTGAGAGGCTTGCTATTC	GTCAAAACAGAGCAAAAACTCAAA
HEMC	At5g08280	AGGATGTTCGAGATGCGTTTAT	TGTGTACTAGTTTCATCGTGGGA
UPM1	At5g40850	TCCAAATTCCGACATAACTATCC	GCCGACATAAAGAAGTCTAGCAT
CHLD	At1g08520	GTTTTGGAAGCTGTCCGAGGT	GCGCAATTTCTGGTATGGTG
CHLH	At5g13630	TCGTTTGCGTTTGATAGTGATG	TCTTGTCTTCCACCTGCGAGTA
CHLI-1	At4g18480	TTAGAGATGCTGATTTACGGGTC	TGGTTCTTCTCACCGTTTACAG
CAO	At1g44446	TTGATCTTGGCACAGTGAACGA	ACCGAACTCCGAGCTTGTCATA
PORA	At5g54190	CACATTACACTCTTTAAGTC	GAAGCTCCCGTGACAACCACG
PORB	At4g27440	CTTCAGCAATCACACTCTCTC	GGCTAGACCTAACCCAGACG
PORC	At1g03630	GGCAAACCTTGGAGACCTAAGA	CAAACCAACAAGCTTCTCGCTA
FLU	At3g14110	CTCAGAGAGCTTTCTTTAGTG	TCAGTCAGTCTCTAACCGAGC
FeChI	At5g26030	TCCTTCATCACCGACTT	AACTACTTACCCACATCAGC
FeChII	At2g30390	TCACTGAGGAAGCCATTGAACA	TTGAGACAGCCATAGCACCAAC
DVR	At5g18660	CGTTTATAGCGGATTGCGTGT	GCAAAGAAATCCTCAAGCGTG
HO3	At1g69720	AGAGAAGAAAAGAGTCATTGC	GTATTGGTTCCACGAAACTA
		GATCTTTGCCGGAAAACAATTG	CGACTTGTCATTAGAAAGAAAGAG
UBQ10	At4g05320	GAGGATGGT	ATAACAGG
qRT-PCR			
PORC	At1g03630	GGGCAAAACAGTTCAATGA	GGAAAAAGAAGCCGAAACAG
FeChII	At2g30390	ACGGAAGAGCAGCAATGTTAG	TTGGATTCTTGGATTGGGTT
HO3	At1g69720	AGAGAAGAAAAGAGTCATTGC	GTATTGGTTCCACGAAACTA
UBQ10	At4g05320	TTCGTGGTGGTTTCTAAATC	GAAAGAGATAACAGGAACGG

ChIP assay			
PORA	At5g54190	CATACACAAAGAGATGGAGTCCCATG	TGTTTCGTTTAAGACTTAAAGAGTG
PORC	At1g03630	CAACGGTGATCAGATCAGTTGAGAG	TGTTGTACGGAACTGAAGGTGCTAG
PORC control-1	At1g03630	CTGCCTTGTCTTGACGTCTATT	CAAGTAGTTCCGCTCACGTAAT
PORC control-2	At1g03630	ACAGTATAGTACATCGAATGCC	CACATACGATTTCGATTCTAA
FeChII	At2g30390	TCTGTATCTGTCAGTACTTGTGG	TTAATCAGACCAGACACAGTTGG
DVR	At5g18660	GTGGTGGTGACGATGAA	AAGACAAAAACCTGTACCGTA
HO3	At1g69720	ATTCCAAGGTATGTGGTTGT	AGGAAGTGTAAGCATCAAGC
Gel Shift			
PORC	At1g03630	CTAGAACTAGTGGATCCCCCGGGCTGCAGG-AATTCAAGCTGTAGCCTGGAAGA**CACGTG**TCACTAACCCAAAACTAGTCGAC-TCGACCTCGAGG	
PORCm	At1g03630	CTAGAACTAGTGGATCCCCCGGGCTGCAGG-AATTCAAGCTGTAGCCTGGAAGA**CCCGTA**TCACTAACCCAAAACTAGTCGAC-TCGACCTCGAGG	
FeChII	At2g30390	AGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATT CGCCCTT-CTTGAGGAGTGAGGACGGAGAACCA**CACGTG**TCCACACAGTT-AAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGC	
FeChIIm	At2g30390	AGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATT CGCCCTT- GGACGGAGAACCA**CTTTTG**TCCACACAGTT-AAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGC	

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Chapter III: The structure-function relationship of PIF1

Abstract

The phytochrome (phy) family of photoreceptors regulates changes in gene expression in response to red/far-red light signals in part by physically interacting with constitutively nuclear-localized PIFs, phy-interacting bHLH transcription factors. PIF1, the member with the highest affinity for phys, is rapidly phosphorylated and ubiquitinated under red and far-red light prior to its degradation. Here we show that PIF1 interacts with phyA through a novel active phyA binding (APA) motif whereas it interacts with phyB through a conserved active phyB binding (APB) motif. phy interaction is necessary, but not sufficient for the light-induced phosphorylation and degradation of PIF1. DNA binding is not necessary for the light-induced degradation of PIF1. Domain mapping studies revealed that the phy-interaction and light-induced degradation domains are located at the N-terminal 150 amino acid region of PIF1. Moreover, both N-terminus and C-terminus are necessary for light-induced degradation of PIF1. The mutant PIF1 with the Ser to Ala (S464AS465AS466A) mutations at the C-terminus had significantly reduced degradation rate compared to wild type PIF1 in transgenic plants. In addition, hypocotyl lengths of the mutant PIF1 transgenic plants were much longer than the wild type PIF1 transgenic plants under light, suggesting that the mutant PIF1 is suppressing photomorphogenesis. Taken together, these structure-function studies suggest that PIFs have conserved phyA and phyB binding domains, and multiple domains of PIF proteins are necessary for the light-induced degradation of PIFs.

Keywords: bHLH factor, domain mapping, phosphorylation, photomorphogenesis, proteasomal degradation.

Introduction

Growth and development are highly regulated by environmental light signals at all phases of a plant's life cycle. Plants have evolved several light receptors: the phytochrome (phy) family of photoreceptors to monitor the red (R)/far-red (FR) region; the cryptochromes (crys), phototropins (phots) and ZTL/FKF1 family of F-box proteins to monitor the UV-A/blue region; and an unidentified receptor to monitor the UV-B region of the spectrum (Lin and Shalitin, 2003; Chen et al., 2004). The phy family in *Arabidopsis thaliana* (*PHYA-PHYE*) encodes ~125 kDa soluble proteins that can form selective homo- or hetero-dimers between the family members (Mathews and Sharrock, 1997; Sharrock and Clack, 2004). Their photosensitivity relies on the acquisition of a covalently attached bilin chromophore that enables the existence of two inter-convertible forms of phys: the Pr form (biologically inactive) with maximal absorbance in the R region of the spectrum and the Pfr form (biologically active) with maximal absorbance in the FR region of the spectrum. The Pr form is converted to the biologically active Pfr form under R light, and the Pfr form is converted back to the inactive Pr form under FR light(Rockwell et al., 2006). The array of photoreceptors allows plants to monitor and respond to a number of parameters of ambient light signals for optimum photomorphogenic development (Whitelam and Halliday, 2007).

phys in the Pr form are predominantly in the cytosol, but they are induced to translocate into the nucleus upon light activation (Kircher et al., 2002). Light induces nuclear import of phys via either a conformation change (in phyB) resulting in the unmasking of a nuclear localization signal (NLS) present in its C-terminal domain (Chen et al., 2005), or an association (of phyA) with other proteins containing an NLS (Zhou et al., 2005; Hiltbrunner et al., 2006; Rösler et al., 2007). Light-induced nuclear translocation is necessary for the majority of the biological functions of phyA and phyB (Huq et al., 2003; Matsushita et al., 2003; Hiltbrunner et al., 2006; Rösler et al., 2007). However, cytosolic phyA regulates negative gravitropism in blue light as well as red light-enhanced phototropism (Rösler et al., 2007). In the nucleus, phys interact with a group of unrelated proteins (Whitelam and Halliday, 2007), and initiate signaling

cascades that result in changes in expression of ~10% of the genome (Rockwell et al., 2006; Jiao et al., 2007; Whitelam and Halliday, 2007). However, the primary biochemical mechanism of signal transfer from photoactivated phys to signaling partners is still unknown.

Among the phy-interacting proteins, the PHYTOCHROME INTERACTING FACTOR (PIF) family of bHLH transcription factors constitutes the best model for understanding phy-regulated gene expression (Duek and Fankhauser, 2005; Castillon et al., 2007; Whitelam and Halliday, 2007). Six closely related genes of the *Arabidopsis* bHLH superfamily encode PIF1 and PIF3-PIF7 (Toledo-ortiz et al., 2003; Castillon et al., 2007; Leivar, Monte, Al-Sady, Carle, Storer, Alonso, Ecker, and Quail, 2008a). PIFs interact selectively with the Pfr forms of phys with differential affinities *in vitro*. For example, PIF1 and PIF3 interact with the Pfr forms of both phyA and phyB, while all other PIFs interact with the Pfr form of phyB only (Ni et al., 1999; Huq and Quail, 2002; Huq et al., 2004; Khanna, Huq, Kikis, Al-sady, Lanzatella, and Quail, 2004a; Leivar, Monte, Al-Sady, Carle, Storer, Alonso, Ecker, and Quail, 2008a). Interaction of PIFs with other phys has not been detected. An N-terminal conserved region of PIFs, called the APB (active phyB binding) motif is necessary for the physical interactions between PIFs and the photoactivated phyB (Khanna, Huq, Kikis, Al-sady, Lanzatella, and Quail, 2004a). Similarly, an APA (active phyA binding) motif within the N-terminal region of PIF3, distinct from the ABP motif, is necessary for the interaction of PIF3 and phyA (Al-Sady, Ni, Kircher, Schäfer, and Quail, 2006a).

In darkness, PIFs directly bind to downstream targets in nucleus to regulate the cascade of gene expression to repress photomorphogenesis. Belonging to the bHLH transcription factor subfamily, PIFs bind to G-box DNA sequence motifs (CACGTG) through their basic domains and regulate gene expression (Martínez-García et al., 2000; Oh et al., 2009; Moon et al., 2008; Oh et al., 2007; Hornitschek et al., 2009; Toledo-Ortiz et al., 2010). PIF3 positively regulate anthocyanin biosynthesis by activating the transcription of anthocyanin biosynthetic genes (Shin et al., 2007). PIF1 regulates gibberellic acid metabolic and signaling genes to suppress seed germination (Oh et al.,

2006, 2007). PIF1 also directly and indirectly regulates chlorophyll biosynthetic genes to optimize the greening process in *Arabidopsis* (Moon et al., 2008). PIF4 and PIF5 promote the shade avoidance syndrome by directly binding to G-boxes present in the promoter of shade marker genes (Hornitschek et al., 2009).

In wild type seedlings, light signals perceived by phys promote degradation of PIFs through the ubiquitin (ubi)/26S proteasomal pathway to derepress gene expression and promote photomorphogenesis (Castillon et al., 2007; Leivar and Quail, 2011; Moon et al., 2008). PIF3 is degraded under both R and FR light conditions in a phy-dependent manner (Bauer et al., 2004). Subsequently, it was shown that PIF1 and PIF3-PIF5 are degraded under light through the ubiquitin (ubi)/26S-proteasomal pathway (Monte et al., 2004; Park et al., 2004; Shen et al., 2005; Oh et al., 2006; Nozue et al., 2007; Shen et al., 2007; Lorrain et al., 2008). PIF3-PIF5 are also phosphorylated specifically in response to R light, and the phosphorylated form is presumably degraded under light (Al-Sady, Ni, Kircher, Schäfer, and Quail, 2006b; Shen et al., 2007; Lorrain et al., 2008).

The phosphorylation, ubiquitylation and subsequent degradation of PIFs are crucial in phytochrome mediated light signaling pathway. Both APA and APB motifs are necessary for the light-induced phosphorylation and subsequent degradation of PIF3. Despite the fact that PIF1 has the strongest affinity among the PIFs for both phyA and phyB (Huq et al., 2004), the functional significance of its direct physical interaction with photoactivated phys has not been demonstrated. Moreover, the domains responsible for either the phosphorylation or the degradation of PIFs have not been identified yet. Here we show that although PIF1 has an APB motif similar to other PIFs, it has a different APA motif than PIF3. Similar to PIF3, the direct physical interaction of PIF1 with phyA or phyB was necessary for light-induced phosphorylation and degradation. Moreover, the DNA binding is not necessary for the light induced degradation of PIF1. However the C-terminus of PIF1 is required for PIF1 turnover and the mutant PIF1 S464-466A has the reduced rate of degradation compared with wild type PIF1.

Results

The APB and APA motifs present in the N-terminal 150 amino acid region are necessary for the Pfr-specific interaction of PIF1 with phyA and phyB both *in vitro* and *in vivo*.

To understand the functional significance of PIF1-phy interactions, we are mapping the phy interaction motifs in PIF1. Recent reports showed that a small motif, named the active phytochrome B binding motif (APB), present in many phy-interacting bHLH factors, is necessary for the physical interaction with the Pfr form of phyB (Fig. 3.1A; Khanna et al., 2004; Al-Sady, Ni, Kircher, Schäfer, and Quail, 2006a; Shen et al., 2007). Alanine scanning by site-directed mutagenesis of conserved amino acids in this region reduced PIF1's interaction with the Pfr form of phyB either severely (E41A, L42A or G47A) or partially (W44A) (Figs. 3.1A, 3.2A and 3.2B), suggesting that the putative APB motif in PIF1 is also necessary for the interaction with the Pfr form of phyB.

Al-Sady et al. (2006) showed that the two phenylalanine residues (F203 and F209) in PIF3 are necessary for its interaction with phyA. Interestingly, mutations in the corresponding amino acids in PIF1 (F148 and F155) did not disrupt the Pfr-specific binding of PIF1 to phyA (Figs. 3.1B, 3.3A and 3.3B). However, deletion of 11 (positions 85 to 95) or 34 (positions 84 to 117) amino acid residues markedly reduced the Pfr-specific interaction of PIF1 with phyA (Fig. 3.3A and C). Deletion of 43 amino acid residues (positions 118 to 160) severely reduced the Pfr-specific interaction of PIF1 to phyA (Fig. 3.3A and C). This region of PIF1 (from residue 84 to residue 160) was scrutinized to identify specific amino acids critical for the PIF1-phyA interaction. Site-directed mutagenesis of leucine 95 to alanine showed a similar binding capacity as that of the 11 or 34 amino acid deletion mutants (Figs. 3.2C and 3.3C). Site-directed mutagenesis of serine 123, glycine 153 and glycine 160 to alanines in the leucine 95 mutant background did not show significant differences in binding compared to the leucine 95 single mutant (data not shown). However, site-directed mutagenesis of asparagine 144 to alanine in the leucine 95 mutant background showed that these two amino acid residues were necessary for the interaction with the Pfr form of phyA *in vitro*

(Figs. 3.2C and 3.1C). These results suggested that the phyA binding sites were different between PIF1 and PIF3.

Although PIFs have been shown to interact with phys in experiments using *in vitro* transcribed and translated PIFs and phys, neither *in vivo* interactions nor interactions between plant-expressed proteins have been demonstrated. To investigate whether PIF1 interacts with phyA or phyB *in vivo* and to examine the involvement of specific amino acids in PIF1-phy interactions *in vivo*, we generated homozygous transgenic plants expressing LUC-PIF1-3M (a luciferase-PIF1 fusion protein with three mutations in PIF1: G47A, L95A and N144A) in the *pif1* mutant background. We performed co-immunoprecipitation assays using the anti-PIF1 antibody on samples prepared from dark and light-exposed plants. Results showed that LUC-PIF1 could efficiently interact with both phyA and phyB from plant extracts (Fig. 3.4). However, co-immunoprecipitations of LUC-PIF1-3M recovered much less phyA and phyB under R light compared to LUC-PIF1 co-immunoprecipitations (Fig. 3.4). These results were consistent with the *in vitro* interactions shown in Fig. 3.2B and C. Taken together, these data suggested that the three amino acids (G47, L95 and N144) in PIF1 were critical for physical interactions with the Pfr forms of phyA and phyB both *in vitro* and *in vivo*.

Direct interactions with the Pfr forms of either phyA or phyB are necessary for the light-induced phosphorylation and degradation of PIF1

To investigate whether direct physical interactions with phys are necessary for the degradation of PIF1 in light, we generated homozygous transgenic plants expressing LUC-PIF1G47A or LUC-PIF1-2M (containing two mutations in PIF1: L95A and N144A) in the *pif1* background. LUC-PIF1-3M (containing three mutations in PIF1: G47A, L95A and N144A) is described above. Luciferase assays showed that the degradation of the LUC-PIF1G47A (deficient in interaction with phyB) was slightly reduced under prolonged R light (Fig. 3.5). The triple mutant LUC-PIF1-3M (deficient in interaction with phyA and with phyB) was completely stable under FR light and only partially degraded under prolonged R light (Fig. 3.5). To investigate the early kinetics of degradation, we performed cycloheximide (CHX) chase assays for the wt and the mutant

forms of PIF1 fused to LUC after a pulse of R light followed by dark incubation (Fig. 3.6A). The degradation rate of LUC-PIF1G47A was similar to the wt LUC-PIF1 under these conditions (Fig. 3.6B), suggesting that phyB plays a minor role in early PIF1 degradation under limited R light. However, the degradation rates of both LUC-PIF1-2M and LUC-PIF1-3M were greatly reduced after a pulse of R light compared to those of LUC-PIF1 (Fig. 3.6C). Moreover, LUC-PIF1-3M was neither phosphorylated nor degraded up to 20 min after a pulse of R light, whereas wt LUC-PIF1 was both phosphorylated and degraded under these conditions (Fig. 3.6D). These results, and those depicted in Fig. 3.4, suggested that direct interactions of PIF1 with phys were necessary for the light-induced phosphorylation and degradation of PIF1.

Both N- and C-terminal domains of PIF1 are necessary for the light-induced degradation of PIF1.

Since PIF1 was degraded under R and FR light, we initiated mapping of the degradation domain of PIF1. To this end, we generated translational fusions of LUC with one of two regions of PIF1 (amino acid residues 1-150, responsible for phy interaction and including the transcriptional activation domain of PIF1, and residues 151-478, responsible for dimerization and DNA binding) as described (Shen et al., 2005), and produced transgenic plants. To examine whether dimerization was necessary for PIF1 degradation, we also produced transgenic plants expressing LUC fused to the 1-150 amino acid region of PIF1 along with the bHLH domain (Fig. 3.7A). We measured LUC activity as an indicator of fusion protein stability under dark and light conditions as described (Shen et al., 2005). All three truncated fusion proteins were stable under both R and FR light, while the full-length LUC-PIF1 fusion protein was degraded under those conditions as expected (Fig 3.7B). Western blot analyses of two of the truncated proteins (LUC-PIF1-N150 and LUC-PIF1-C327) showed that these fusion proteins were neither phosphorylated nor degraded under R light (Fig. 3.7C). These results strongly suggested that both the N- and C-terminal regions of PIF1 were necessary, but not sufficient, for the light-induced degradation of PIF1. In addition, since the phy-interaction motifs were present in the 1-150 amino acid region of PIF1, these results together with the above

point mutations (Figs. 3.2 and 3.6) suggest that phy binding was necessary, but not sufficient, for PIF1's light-induced degradation.

S464-466 are necessary for the rapid light-induced degradation of PIF1

To map the C-terminal amino acids necessary for light-induced phosphorylation and degradation, we mutated two clusters of three serine residues at the C-terminal region of PIF1 (S459-461 and S464-466) to Ala separately. The mutant PIF1s were fused to luciferase (LUC) and expressed using a constitutive (CaMV35S) promoter in the *pif1* background. Homozygous transgenic plants were selected and assayed for PIF1 stability and photomorphogenic phenotypes. Results show that the rate of degradation of the mutant LUC-PIF1 (S459-461A) was similar to that of wild type LUC-PIF1 under R light (Fig. 3.8A, top). Strikingly, the rate of degradation of the mutant LUC-PIF1(S464-466A) was strongly reduced compared to wild type LUC-PIF1, despite the presence of the light-induced phosphorylation in this mutant PIF1 (Fig. 3.8A, bottom). Consistent with these data, the hypocotyl lengths of the mutant *LUC-PIF1(S464-466A)* were much longer than the wild type *LUC-PIF1*, while the hypocotyl lengths of the mutant *LUC-PIF1 (S459-461A)* were largely similar to that of the wild type *LUC-PIF1* (Fig. 3.8B and C). We have also included *LUC-PIF1-3M* transgenic line as a control. *LUC-PIF1 (S464-466A)* transgenic plants showed similar phenotype compared to that of the *LUC-PIF1-3M* transgenic lines (Fig. 3.8B and C). These data strongly suggest that S464-466 are necessary for the rapid light-induced degradation of PIF1.

DNA binding is not necessary for the light-induced degradation of PIF1

In other systems, transcription factors are often tagged for subsequent degradation by the ubi/26S proteasomal pathway while they are assembled in the transcription initiation complex bound to their DNA target (Mayr and Montminy, 2001; Muratani and Tansey, 2003). Davis et al. (1990) showed that a single amino acid substitution (E118D) in MYOD, a bHLH protein, abolished its DNA binding activity (Davis et al., 1990). To investigate whether DNA binding was necessary for the light-induced degradation of PIF1, we introduced the above missense mutation in the corresponding amino acid of

PIF1 (PIF1E293D), and compared the DNA binding activity of the wt and mutant PIF1. The mutant PIF1 did not bind to the target DNA while the wt PIF1 showed robust binding (Fig. 3.9A and B). We made a LUC-PIF1E293D fusion construct and generated homozygous transgenic plants expressing the fusion protein in the *pif1* mutant background. LUC assays showed that this mutant PIF1 (PIF1E293D) was degraded significantly more than the wild type PIF1 under R light (Fig. 3.9C). The data suggested that DNA binding was not necessary for, and might have retarded, light-induced PIF1 degradation.

Discussion

Because PIFs physically interact with the photoactivated phy molecules, PIFs were thought to receive light signals from phys and induce photomorphogenesis (Ni et al., 1998, 1999; Quail, 2002). However, contrary to our expectation, the majority of the biological functions of the PIF family members are to negatively regulate phy signaling (Castillon et al., 2007; Monte et al., 2007). To remove this negative regulation, phys induce degradation of PIFs in order to promote photomorphogenesis. Here we present evidence that, using diverse sequences, phys interact with PIF1 to induce its phosphorylation, poly-ubiquitination and subsequent degradation under both R and FR light conditions.

PIFs have been shown to interact selectively with the Pfr form of phys *in vitro* (Ni et al., 1999; Huq et al., 2004). Sequence alignment and site-directed mutagenesis revealed that an N-terminal motif, named the active phyB binding motif (APB), is necessary for the physical interactions between PIFs 3-7 and phyB *in vitro* (Khanna, Huq, Kikis, Al-sady, Lanzatella, and Quail, 2004b; Shen et al., 2007; Leivar, Monte, Al-Sady, Carle, Storer, Alonso, Ecker, and Quail, 2008b). A second motif immediately downstream of the APB motif, named the active phyA binding motif (APA) (Fig. 3.1B), has been shown to mediate interactions between PIF3 and phyA (Al-Sady, Ni, Kircher, Schäfer, and Quail, 2006b). Here we show that while PIF1 had a functionally conserved APB motif (Fig. 3.2), it used a novel APA motif for interaction with the Pfr form of phyA (Figs. 3.1C and 3.2C). The APA and APB motifs were necessary for the robust

interaction with phyA and phyB, respectively both *in vitro* and *in vivo* (Fig. 3.2C and 3.4). Moreover, because the triple mutant still interacted with phyA/phyB *in vivo*, perhaps additional amino acid residues in PIF1 participate in physical interactions between PIF1 and phys *in vivo*. Combined, these data suggest that although phyB uses a highly conserved sequence motif for physical interactions with PIFs, phyA uses a more diverse sequence for physical interactions with PIFs. Identification and functional characterization of additional phyA interacting factors might reveal whether phyA uses any conserved sequence motif for physical interaction.

The functional significance of PIF-phy physical interactions appears antagonistic. Direct interactions with phys are necessary for the light-induced phosphorylation and degradation of PIF1, because a PIF1 triple mutant deficient in phy interaction displayed reduced levels of phosphorylation and degradation under light (Fig. 3.6). These results are consistent with the recent reports that physical interactions with phys are necessary for the light-induced phosphorylation and degradation of PIF3/PIF5 (Al-Sady, Ni, Kircher, Schäfer, and Quail, 2006a; Lorrain et al., 2008; Shen et al., 2007). However, expression of two separate regions of PIF1 (1-150 aa containing the APA and APB motifs, and 151-478 aa containing the dimerization domain) in transgenic plants showed that these isolated regions were neither phosphorylated nor degraded under either R or FR light conditions (Fig. 3.7). Because the phy-interaction motifs are present at the N-terminal 150 amino acid region of PIF1 (Fig. 3.2), these results demonstrate that although the physical interactions between PIF1 and phys are necessary, they are not sufficient for the light-induced phosphorylation and degradation of PIF1. In addition, the reduced degradation of LUC-PIF1(S464-466A) suggest that the C-terminus is also necessary for the rapid light-induced degradation of PIF1. Strikingly, LUC-PIF1(S464-466A) mutant is still robustly phoshorylated in response to light in vivo as previously observed for wild type PIF1 (Figs. 3.8A). These data suggest that light-induced phosphorylation is necessary, but not sufficient for the degradation of PIF1. Taken together, these data suggest that both N- and C-terminal domains are necessary for the light-induced degradation of PIF1.

The enhanced degradation of the PIF1 mutant that failed to bind to DNA suggests that DNA binding may protect PIF1 from degradation (Fig. 3.9). These results are consistent with previous reports that a small fraction of PIF1 (20-30%) was not degraded even under continuous light exposure (Shen et al., 2005). Taken together, these results suggest that the light-induced degradation of PIF1 might be nucleoplasmic and is uncoupled from the transcription complex.

In conclusion, our data and those of others show that light-activated photoreceptors directly interact with PIF1 and other PIFs to induce their phosphorylation, poly-ubiquitination and subsequent degradation via the ubi/26S proteasomal pathway in order to promote photomorphogenesis. Both direct physical interactions of PIF1 with phys and phosphorylation of PIF1 are necessary for the light-induced degradation of PIF1 *in vivo*. Because phy-interaction is necessary for the light-induced phosphorylation and degradation of PIFs (Henriques et al., 2009; Castillon et al., 2007), and because phyA has been shown to have Ser/Thr kinase activity(Yeh and Lagarias, 1998), it is possible that phys might directly phosphoryate PIFs in response to light. However, convincing in vivo evidence for the phyA kinase hypothesis is still lacking. Therefore, it remains to be determined whether the light-induced phosphorylation of PIFs represents the primary biochemical mechanism of phy signal transfer or whether phys simply function as scaffold proteins to bring the PIFs and another unknown kinase together for the phosphorylation event.

Materials and methods

Plant growth conditions and phenotypic analyses

Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture, Bellevue, WA) under 24 hour light at 24 ℃ ± 0.5 ℃. Monochromatic R and FR light sources are as described (Shen et al., 2005). Light fluence rates were measured using a spectroradiometer (Model EPP2000, StellarNet Inc., Tampa, FL) as described (Shen et al., 2005). Seeds were surface sterilized and plated on Murashige-Skoog (MS) growth medium (GM) containing 0.9% agar without sucrose (GM-Suc) as described (Shen et al.,

2005). After 3-4 days of moist chilling at 4℃ in the dark, seeds were exposed to 3 hours white light at room temperature in order to satisfy this requirement for the completion of germination before placing them in the dark for another 4 days. For transgenic plants, the 35S:LUC-PIF1 (LP), 35S:LUC-GFP (LG) lines were generated as described (Shen et al., 2005). For quantitation of hypocotyl lengths, digital photograph were taken and at least 30 seedlings were measured using the publicly available software ImageJ (http://rsb.info.nih.gov/ij/), and the experiments were repeated at least three times.

Protein extraction and Western blotting

Four day-old seedlings were either kept in darkness or exposed to R or FR light (amount of light is indicated on individual figures) and incubated in the dark for various times before protein extraction. For detecting LUC-PIF1 proteins in transgenic plants, boiling denaturing buffer (100 mM MOPS, pH 7.6, 5% SDS, 10% Glycerol, 4 mM EDTA, 40 mM β-mercaptoethanol) was added at a 1:3 (w/v) ratio before grinding. Protease inhibitor cocktail (1X) (F. Hoffmann-La Roche Ltd, Basel, Switzerland) and 2 mM PMSF were also added during extraction. Total protein supernatants were separated on 6.5% SDS-PAGE gels, blotted onto PVDF membrane and probed with anti-luciferase (1:750) (70C-CR2029RAP, Fitzgerald Industries International, Concord, MA). For the immunoblot analyses, the membranes were blocked with 1X TBST plus 0.5% non-fat milk buffer at 4 ℃ overnight with the primary anti-luciferase antibody. Peroxidase-labeled goat anti-rabbit (anti-mouse for tubulin) antibody (KPL Inc., Gaithersburg, MD) in a 1:50,000 dilution was used as secondary antibody. Membranes were developed with SuperSignal West Pico Chemiluminescent substrate kit (Pierce Biotechnology Inc., Rockford, IL), and visualized on an X-ray film.

Construction of plasmids and *in vitro*/*in vivo* co-immunoprecipitation assays

The DNA constructs for expressing full-length phyA, phyB, GAD and GAD-PIF1 have been described previously(Huq et al., 2004). The phyB deletion constructs are as described (Zhu et al., 2000). Various fragments of PIF1 or phyA were amplified by PCR using PfuTurbo enzyme and then cloned into the pET17b vector (EMD Biosciences Inc.,

Madison, WI) for *in vitro* expression. The specific amino acid mutations in full-length PIF1 were introduced using a site-directed mutagensis kit (Stratagene, La Jolla, CA). Restriction enzyme sites (EcoRI-SalI or EcoRI-XhoI for PIF1 and NdeI-XhoI for phyA) were introduced into the PCR primers (Supplementary Table), and all the constructs were sequenced completely. For *in vitro* co-immunoprecipitation assays, all proteins were expressed in the TnT *in vitro* transcription/translation system (Promega, Madison, WI) in the presence of ^{35}S-methionine using the T7 promoter. *In vitro* co-immunoprecipitation experiments and sample preparation were performed as described (Huq et al., 2004; Ni et al., 1999).

For *in vivo* co-immunoprecipitation assays, seedlings were pretreated with MG132 as described above. Total proteins were extracted from ~0.4 g seedlings (either kept in darkness or treated with 3000 molm^{-2} of Rp followed by dark) with 1 mL native extraction buffer (100 mM NaH_2PO_4, pH 7.8, 100 mM NaCl, 0.05% NP-40, 1X Protease inhibitor cocktail [F. Hoffmann-La Roche Ltd, Basel, Switzerland], 2 mM PMSF, 10 μM MG132, 25 mM β-GP, 10 mM NaF, 2 mM Na orthovanadate and 100 nM calyculin A) and cleared by centrifugation at 16,000 g for 15 min at 4℃. Anti-PIF1 antibody was incubated with Dynabead (Invitrogen Inc., Carlsbad, CA) (20 L/ g antibody) for 30 min at 4℃ and the beads were washed twice with the extraction buffer to remove the unbound antibody. The bound antibody-beads were added to a total of 500 g total protein extracts and rotated for another 3 h at 4℃ in the dark. The beads were collected using a magnet, washed three times with wash buffer, dissolved in 1X SDS-Loading buffer and heated at 65℃ for 5 min. The immunoprecipitated proteins were separated on an 8% SDS-PAGE gel, blotted onto PVDF membrane, and probed with anti-phyA, anti-phyB or anti-LUC antibodies as described above.

Cyclohexamide chase and luciferase assays

For cycloheximide chase assays, 4 day-old dark-grown seedlings were pretreated with 50 μM cycloheximide or solvent control DMSO in MS-Suc liquid medium for 3 hours in darkness as described (Shen et al., 2005). After pretreatment, the seedlings were exposed to 3000 μmolm^{-2} of R light (Rp) for 1 min, and then kept in darkness before

harvesting at different time points indicated in the figures. For luciferase assays, samples were collected in liquid nitrogen and total protein was extracted using 1X Luciferase Cell Culture Lysis Reagent (CCLR) (Promega, Madison, WI) with 2mM PMSF and 1X complete protease inhibitor cocktail (F. Hoffmann-La Roche Ltd, Basel, Switzerland). Luciferase activity was measured as described (Shen et al., 2005).

***In vitro* gel-shift assays**

DNA gel shift assays were performed as described (Huq and Quail, 2002). PIF1, PIF1E293D and LUC were synthesized using the Rabbit Reticulocyte TNT system (Promega, Madison, WI). A 70 bp *POR C* promoter fragment containing a G-box motif known to be a PIF1 binding site, was labeled with ^{32}P-dCTP (Su et al., 2001; Moon et al., 2008). The binding conditions and gel compositions are as described (Huq and Quail, 2002).

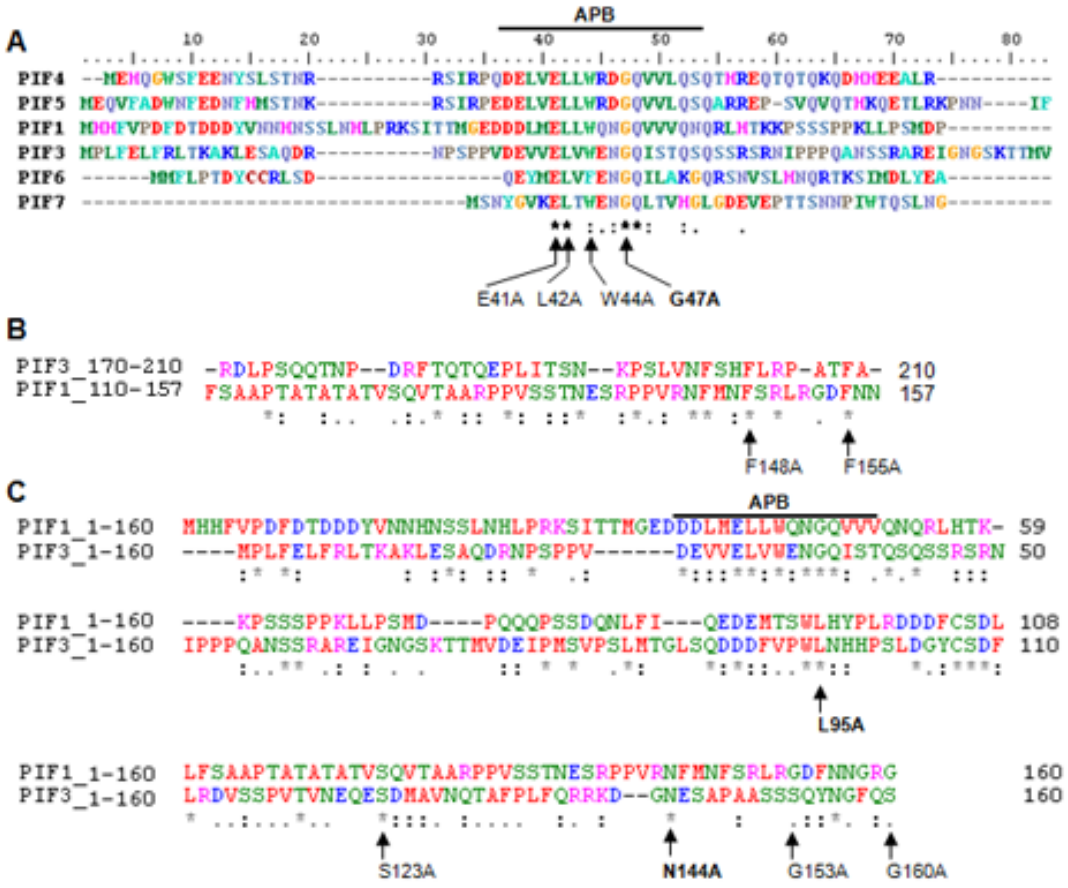

Figure 3.1: Sequence alignments of the APB and APA motifs in PIFs.

A) Alignment of the predicted amino acid sequences from the N-terminal regions of PIF1, and PIF3-PIF7. The putative APB motif is indicated by a thick line on the top. The amino acid residues mutated in PIF1 are shown at the bottom. **B)** Sequence alignment of the putative APA motif present in PIF1 and PIF3. Amino acid regions in PIF1 (110-157) and PIF3 (170-210) are aligned. The phenylalanine residues critical for interaction between PIF3 and phyA are indicated by arrows. **C)** Alignment of the predicted amino acid sequences from the N-terminal 160 amino acid regions of PIF1 and PIF3. The amino acid residues (Leucine 95 and Asparagine 144) responsible for interaction between PIF1 and phyA are shown in bold.

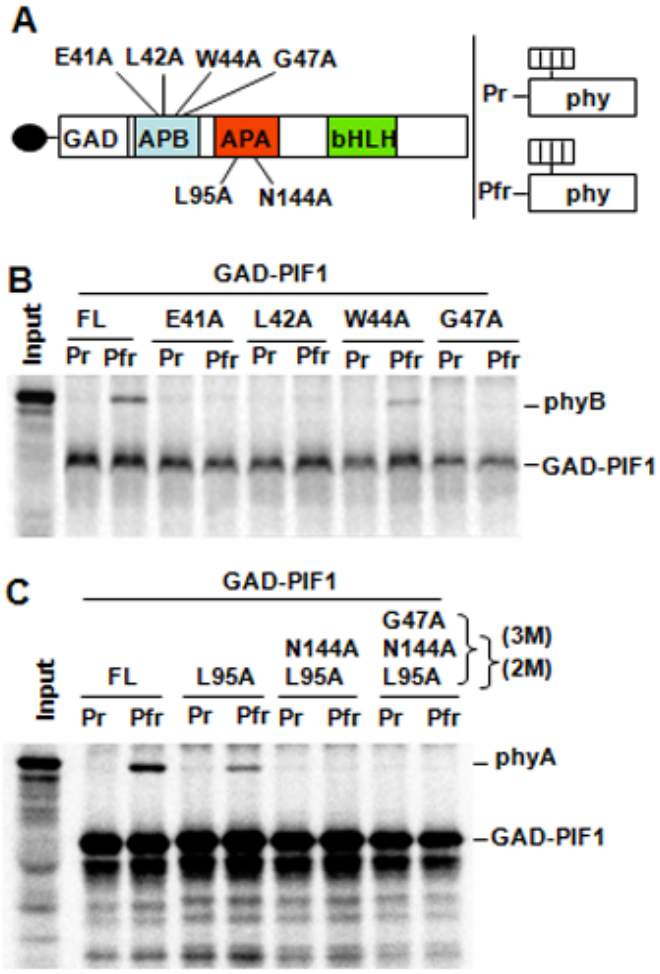

Figure 3.2: The APB and APA motif present in the N-terminal 150 amino acid region of PIF1 is necessary for its Pfr-specific interaction with phyA and phyB *in vitro*.

A) Schematic representation of the gal4 activation domain-PIF1 (GAD-PIF1) baits (left) and full-length phy (phy) preys (right) used in co-immunoprecipitation assays. Mutations made in GAD-PIF1 for testing phyB binding are shown above the schematic, and those for testing phyA binding are below. Autoradiographs show *in vitro* interactions of wild type PIF1 or each of four PIF1 mutants with the Pr or Pfr forms of phyB **(B)** or single, double (2M) or triple (3M) mutants of PIF1 with the Pr or Pfr forms of phyA **(C)**. The leftmost lane of each panel shows the input and and the others show the pellet fractions from co-immunoprecipitation assays performed with *in vitro* synthesized bait and prey proteins. The phyA and phyB holoproteins were reconstituted by adding the chromophore. The baits were immunoprecipitated using anti-GAD antibody.

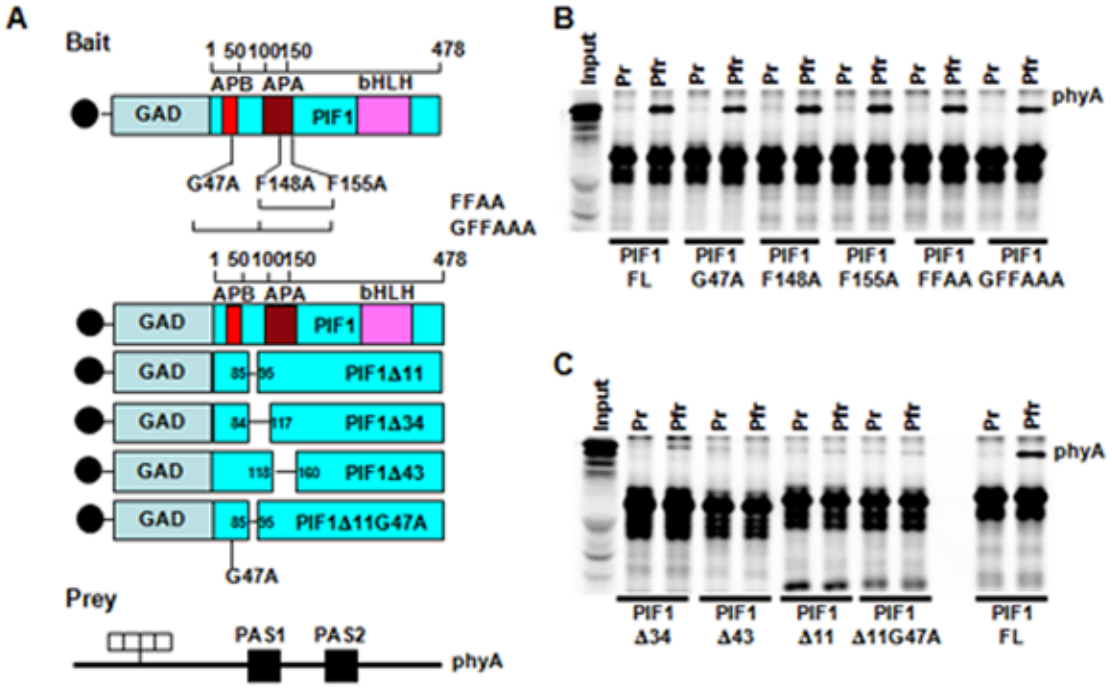

Figure 3.3: The putative APA motif present in PIF3 is not responsible for the Pfr-specific interaction of PIF1 with phyA.

A) Schematic diagram of the bait and prey used for the co-immunoprecipitation assays. The amino acid residues mutated or deleted in each construct are marked. **B)** Autoradiograph showing interactions of wild type PIF1, PIF1G47A, PIF1F148A, PIF1F155A, PIF1FFAA and PIF1GFFAAA with the Pr and Pfr forms of phyA. Left panel shows the input and the right panel shows the pellet fraction from the *in vitro* co-immunoprecipitation assays. **C)** Autoradiograph showing interactions of wild type and various deletion mutants of PIF1 with the Pr and Pfr forms of phyA. Left panel shows the input and the right panel shows the pellet fraction from the *in vitro* co-immunoprecipitation assays.

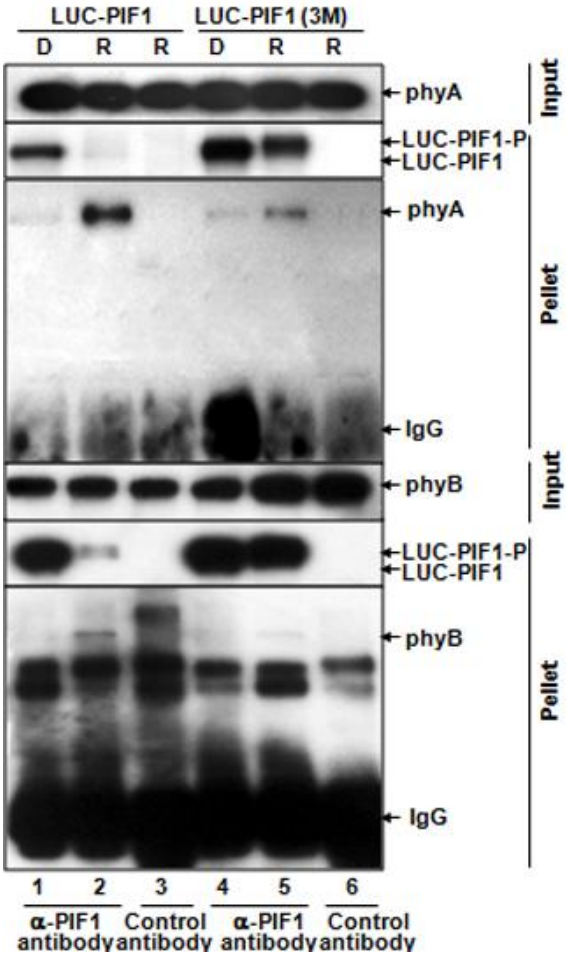

Figure 3.4: The APB and APA motif present in the N-terminal 150 amino acid region of PIF1 is necessary for its Pfr-specific interaction with phyA and phyB *in vivo*.

LUC-PIF1-3M shows much less affinity for the Pfr forms of phyA and phyB compared to LUC-PIF1 in *in vivo* co-imunoprecipitation assays. The input and pellet fractions from *in vivo* co-immunoprecipitation assays are indicated. Total protein was extracted from four day-old dark-grown seedlings either exposed to Rp light (R; 3000 $mmolm^{-2}$) or kept in the dark. Co-immunoprecipitations were carried out using the anti-PIF1 antibody (lanes 1, 2, 4 and 5) or with and unrelated IgG as a control (lanes 3 and 6). The immunoprecipitated samples were then probed with anti-phyA, anti-phyB or anti-LUC antibodies.

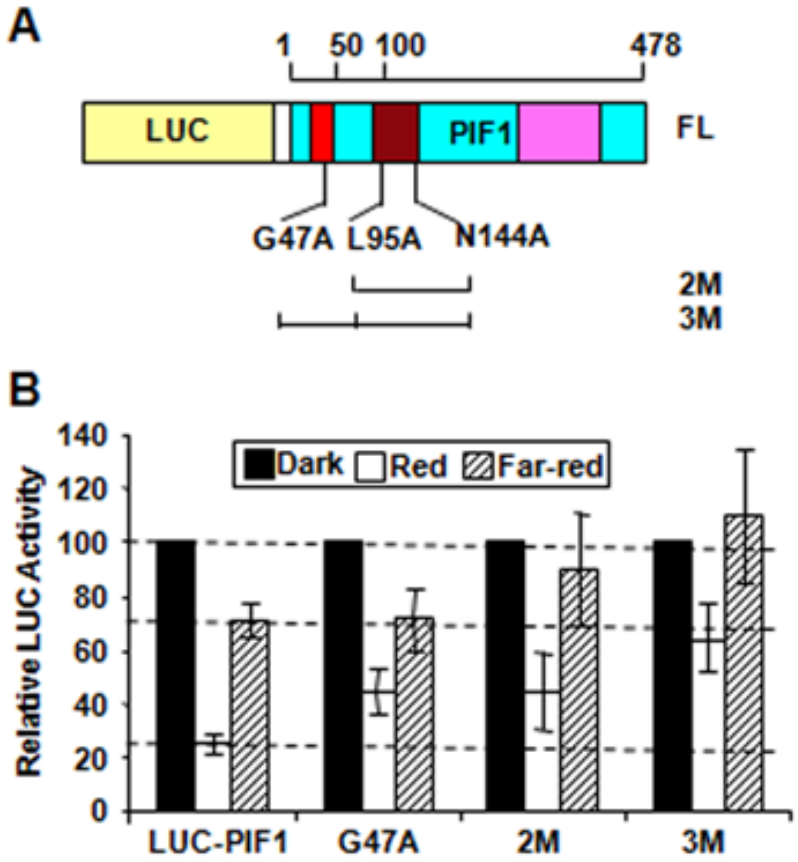

Figure 3.5: Direct interactions of PIF1 with phyA and/or phyB are necessary for the light-induced degradation of PIF1.

A) The amino acid residues mutated in each construct are shown. **B)** LUC activity was measured from 4-day-old dark-grown seedlings transferred to continuous R (10 mmolm^{-2}s^{-1}) or FR (10 mmolm^{-2}s^{-1}) light for 1 h as described (Shen et al., 2005). Error bars, SEM.

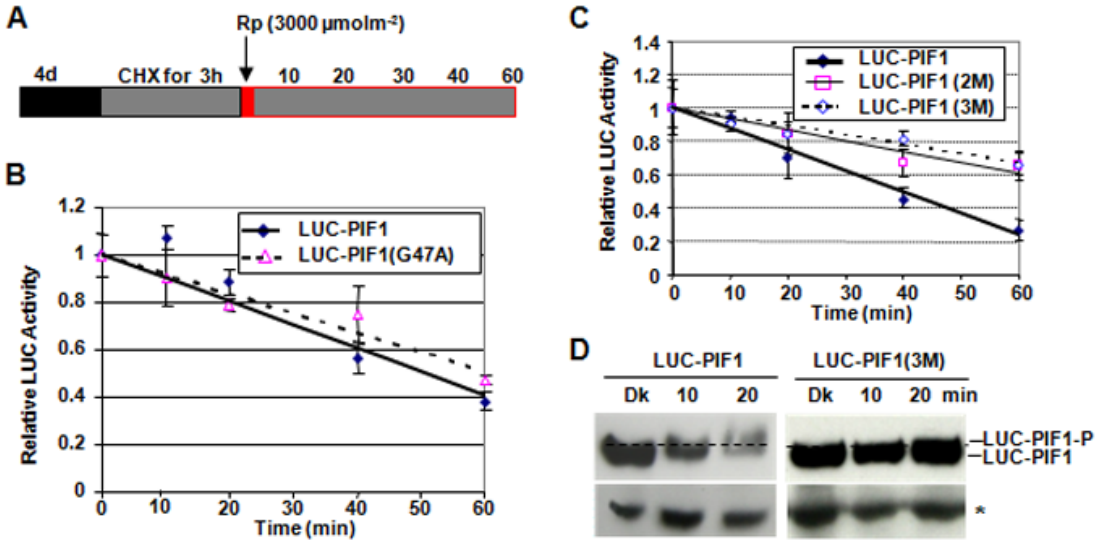

Figure 3.6: Interactions with the Pfr form of phyA and phyB are necessary for the light-induced phosphorylation and degradation of PIF1.

A) Design of the cycloheximide chase assays. Relative lucerifase activity for phy-interaction deficient mutants was measured in 4-day-old (4d) dark-grown seedlings pretreated with cycloheximide (CHX) in the dark for 3h, exposed to R (3000 $mmolm^{-2}$) light and then incubated in the dark for the indicated time (min). Assays show the kinetics of degradation of LUC-PIF1-G47A **(B)** and LUC-PIF1-2M and LUC-PIF1-3M **(C)** compared to wt LUC-PIF1. LUC-PIF1G47A is deficient in phyB interaction, LUC-PIF1-2M is deficient in phyA interaction and LUC-PIF1-3M is deficient in both phyA and phyB interaction as shown in Fig. 3.2. Means ± SE of five biological replicates are shown. **D)** The abundance and phosphorylation status of LUC-PIF1 or LUC-PIF1-3M fusion proteins prior to and after exposure to a Rp determined in Western blots using anti-LUC antibody. A dotted line separates the two forms of PIF1. The asterisk denotes a cross-reacting band.

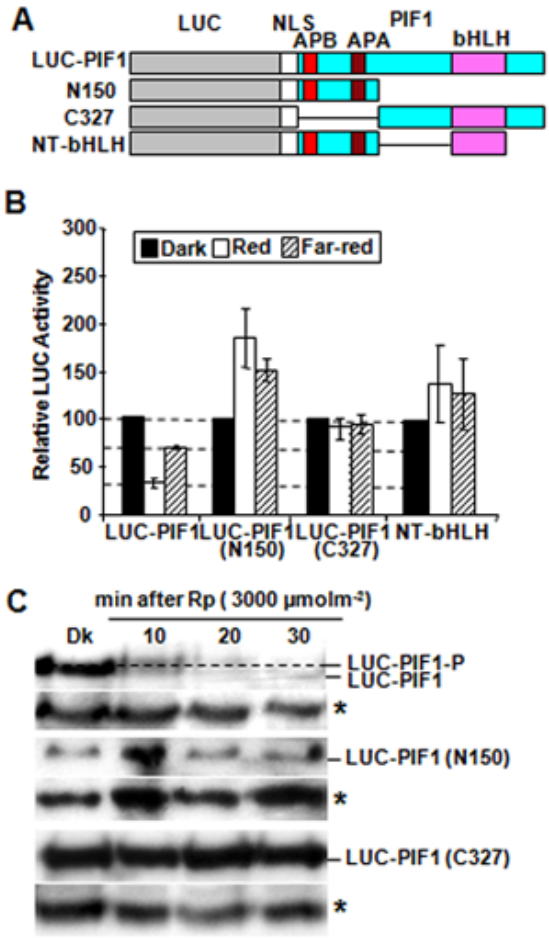

Figure 3.7: Both the N- and C-terminus of PIF1 are necessary for the light-induced degradation of PIF1.

A) Design of the PIF1 deletion constructs fused to LUC. The white box represents a nuclear localization signal (NLS). **B)** LUC activity was measured from 4-day-old dark-grown seedlings transferred to R (10 $mmolm^{-2}s^{-1}$) or FR (10 $mmolm^{-2}s^{-1}$) light for 1 h as described (Shen et al., 2005). Means ± SE of five biological replicates are shown. Some constructs showed greater stability of the fusion protein in light relative to darkness for unknown reasons. **C)** Western blots showing truncated PIF1 fusion proteins are neither phosphorylated nor degraded under light, but the wt LUC-PIF1 is both phosphorylated and degraded under light. A dotted line separates the two forms of PIF1. Asterisks denote a cross-reacting band.

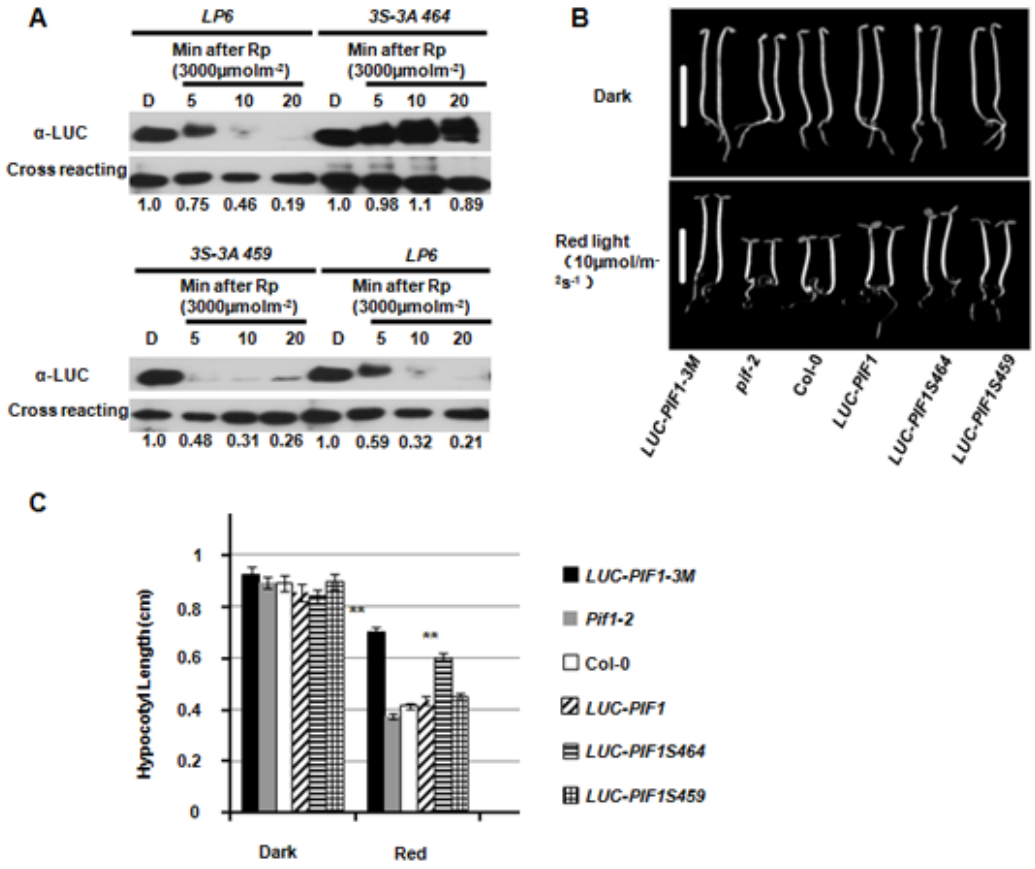

Figure 3.8: S464-466 are Necessary for the Rapid Light-Induced Degradation of PIF1.

A) Light-induced phosphorylation and degradation of a PIF1 containing either S459-461 to Ala (top) or S464-466 to Ala (bottom) compared to wild type LUC-PIF1. The rate of light-induced degradation of LUC-PIF1(S464-466A) is strongly reduced compared to wild type LUC-PIF1 (Bottom). *, indicates cross-reacting band. Numbers under the protein gel blots show relative PIF1 level in wild type LUC-PIF1, LUC-PIF1(S459-461A) and LUC-PIF1(S464-466A) transgenic lines. PIF1 level in each dark samples is set as 1. **B)** LUC-PIF1(S464-466A) promotes hypocotyl growth under red light. Photographs of seedlings of various genotypes grown in the dark or under R light (7 $mmolm^{-2}s^{-1}$) for four days. Bar = 5 mm. A second allele of LUC-PIF1(S464-466A)#33 displaying similar long hypocotyl phenotype under red light is shown in supplementary Fig. 3.5. C) Bar graph showing the mean hypocotyl lengths of various genotypes as indicated. Seedlings were grown as described in B. Error bars represent standard error of mean ($n \geq 30$). *, indicates significant difference ($p<0.05$).

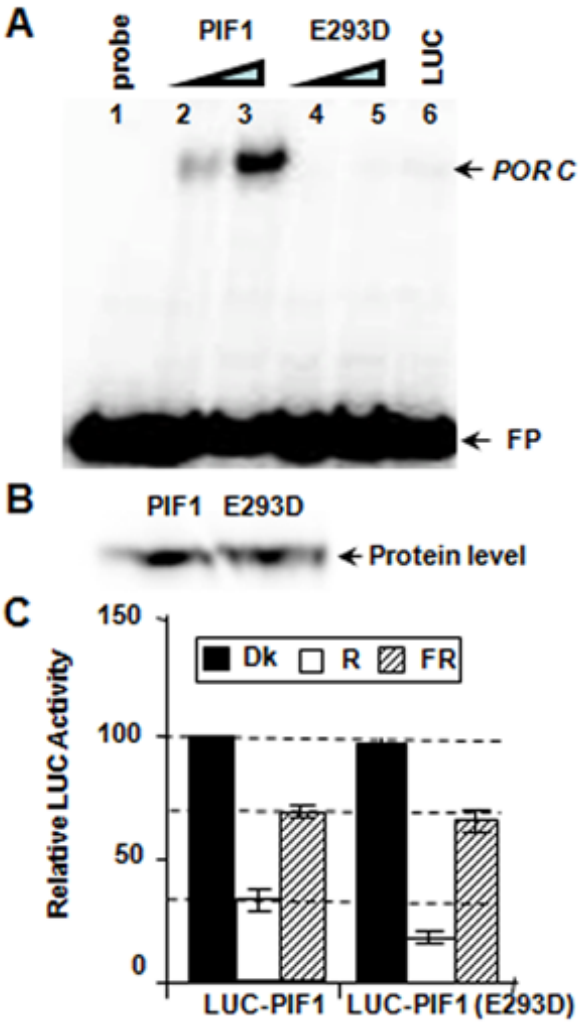

Figure 3.9: DNA binding is not necessary for the light-induced degradation of PIF1.

A) The PIF1E293D mutant does not bind to a G-box DNA sequence element (*POR C*; Su et al., 2001; Moon et al., 2008). *In vitro* translated PIF1 or PIF1E293D was incubated with a radiolabeled fragment of *POR C* in a DNA gel shift assay. Lane 1, free probe; lanes 2-3, increasing amount of wt PIF1; lanes 4-5, increasing amount of PIF1E293D mutant protein, and lane 6, unrelated luciferase protein as a negative control. FP, free probes. **B)** Comparison of the levels of wt and mutant PIF1 proteins produced by *in vitro* transcription and translation. **C)** Relative LUC assays were performed under conditions described in Figure 8. Means ± SE of five biological replicates are shown.

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Chapter IV: The HECATE proteins promote photomorphogenesis by negatively regulating the function of PIF1 in *Arabidopsis*

Abstract

The Phytochrome Interacting Factors (PIFs), a small group of bHLH transcription factors repress photomorphogenesis both in the dark and light. Light signals perceived by the phytochrome family of photoreceptors induce rapid degradation of PIFs to promote photomorphogenesis. Here we show that HECATE proteins, another small group of bHLH proteins antagonistically regulate PIF1 function to promote photomorphogenesis. Both HEC1 and HEC2 heterodimerized with PIF1 in yeast-two-hybrid assays and in both *in vitro* and *in vivo* co-immunoprecipitation assays. *PIF1* and *HEC* genes are co-expressed in the same tissues and the proteins are co-localized in the nucleus. *HEC2* RNAi lines and *hec1* mutant showed hyposensitivity to light-induced seed germination and chlorophyll and carotenoid accumulation, two hallmark processes oppositely regulated by PIF1. By contrast, constitutive overexpression of *HEC2* induced seed germination after FR light exposure in a GA-dependent manner, and increased chlorophyll and carotenoid accumulation compared to wild type. The seed germination phenotypes of *hec1* or *hec2* RNAi lines are eliminated in the *pif1* background, suggesting that *pif1* is epistatic to *hec* functions. *HEC2* overexpression also reduced the light-induced degradation of PIF1. Taken together, these data suggest the HECATE proteins promote photomorphogenesis by negatively regulating the function of PIF1 and possibly other PIFs in *Arabidopsis*.

Keywords: *Arabidopsis*/bHLH transcription factor/ HLH transcription factor/hetero-dimerization/photomorphogenesis.

Introduction

Phytochrome Interacting Factors (PIFs) belong to the basic helix-loop-helix (bHLH) superfamily of transcription factors (Toledo-ortiz et al., 2003; Duek and Fankhauser, 2005; Leivar and Quail, 2011). The signature feature of the bHLH factors is the presence of a bipartite signature domain, the bHLH domain, which contains an N-terminal DNA binding basic region (b) and a C-terminal dimerization region (HLH). The DNA binding region is composed of approximately 15 amino acids with a high percentage of basic residues. The HLH region consists of approximately 60 amino acids containing two a-helices joined by a variable loop and mediates homodimerizaton and/or heterodimerization with other bHLH proteins (Littlewood and Evan, 1998). These factors can bind to cis-acting regulatory elements found in the promoter regions of target genes either as homodimers and/or heterodimers. The most common of these cis-elements is the E-box (5'-CANNTG-3'). E-boxes are classified into different types depending on the central two nucleotides. For example, a G-box (5'-CACGTG-3') has CG as the central two nucleotides. It has been proposed that the nucleotide regions flanking the E/G-box as well as the central two nucleotides play important roles in specifying which bHLH factors bind to that region (Littlewood and Evan, 1998). In the case of bHLH factors that form heterodimers with multiple partners, each different combination would bind to slightly different promoter regions and thereby increase the diversity of target genes.

One subclass of bHLH factors (group D) lacks the basic DNA binding region of the bHLH domain and is designated as HLH proteins (Benezra et al., 1990). Predictably, these proteins lack the ability to bind DNA but are still able to heterodimerize with other bHLH proteins through interaction of the HLH domains. Heterodimerization between HLH and other bHLH proteins prevents the DNA binding and transcriptional activation activities of the bound bHLH protein. Consequently, HLH proteins are considered dominant negative regulators of bHLH proteins (Benezra et al., 1990) and are involved in a number of developmental processes in animal systems (Littlewood and Evan, 1998).

PIFs consist of six family members (PIF1 and PIF3-7) from group 15 of the *Arabidopsis* bHLH superfamily (Toledo-ortiz et al., 2003; Duek and Fankhauser, 2005;

Castillon et al., 2007). In addition to the bHLH domain, PIFs have either an active phyB binding (APB) domain and/or active phyA binding (APA) domain located at the N-terminus of these proteins (Khanna et al., 2004; Duek and Fankhauser, 2005; Castillon et al., 2007). PIFs interact with the biologically active form of phytochromes (phys), the red/far-red light photoreceptors, using the APA and/or APB domains (Khanna et al., 2004; Al-Sady et al., 2006; Shen et al., 2008). Although PIFs are highly homologous proteins, they have selective homo- and heterodimerization preferences. For example, PIF6 and PIF7 preferentially form hetero-dimers with other PIFs, but failed to form homo-dimers in yeast two-hybrid assays (Bu, Castillon, et al., 2011). Moreover, monogenic *pif* mutants displayed distinct morphological phenotypes (Castillon et al., 2007; Leivar and Quail, 2011). *pif3-pif7* single mutants displayed short hypocotyl phenotypes under red and/or far-red light conditions, while *pif1* mutant showed strong effect on seed germination, chlorophyll and carotenoid accumulation in response to light (Huq et al., 2004; Oh et al., 2004; Toledo-Ortiz et al., 2010). *pif1* mutants germinate after far-red light exposure due to a mis-regulation of various hormone biosynthetic and signaling genes (Oh et al., 2009). *pif1* seedlings exhibit photooxidative damage (bleaching) and fail to green when dark-grown seedlings are transferred to light primarily due to mis-regulation of chlorophyll and carotenoid biosynthetic genes in the dark (Toledo-Ortiz et al., 2010; Stephenson et al., 2009; Moon et al., 2008). A quadruple *pif1pif3pif4pif5* mutant displayed constitutively photomorphogenic phenotypes (both morphologically and at the gene expression level) in the dark (Leivar et al., 2009, 2008; Shin et al., 2009), suggesting that PIFs repress photomorphogenic growth pattern in the dark.

In contrast to PIFs, the phy family of photoreceptors (phyA-phyE in *Arabidopsis*) perceives red/far-red/blue light signals in surrounding environment and promotes photomorphogenic development of plants (Bae and Choi, 2008; Quail, 2010). phys are synthesized as the Pr form in the cytosol. Upon perceiving light signals using the bilin chromophore attached to the N-terminal domains of phys, they convert to a biologically active Pfr form and translocate into the nucleus as either a homodimer or as a

heterodimer (Clack et al., 2009; Fankhauser and Chen, 2008). Within the nucleus, phys interact with the PIFs using the APA and/or APB domains, and inhibit PIF functions to promote photomorphoegnesis. Recent data suggest that phys inhibit PIF functions by at least two mechanisms. First, phys directly interact with PIFs and induce rapid phosphorylation, poly-ubiquitylation and 26S proteasome-mediated degradation of PIFs (Castillon et al., 2007; Henriques et al., 2009). Except PIF7, the other PIFs (PIF1 and PIF3-PIF6) are rapidly degraded in response to light signals with differential kinetics (Castillon et al., 2007; Leivar and Quail, 2011). Second, phys stabilize positively acting HLH protein, the dominant negative regulator of bHLH proteins, in response to light signals (Castillon et al., 2007; Henriques et al., 2009). For example, HFR1 is a HLH protein that is degraded in the dark through the COP1-SPA complex, but is stabilized under light through the phy-mediated light signaling pathways (Henriques et al., 2009). Although PIF4 and PIF5 are degraded under light (Henriques et al., 2009), HFR1 interacts with the residual PIF4 and PIF5, and inhibits their DNA binding ability and target gene expression (Hornitschek et al., 2009). This dual level of regulation under light largely eliminates PIFs repressive function to drive photomorphogenesis.

Among the PIFs, PIF1 has the highest affinity for both phyA and phyB and is degraded in response to light (Huq et al., 2004; Shen et al., 2008; Castillon et al., 2007). PIF1 is also phosphorylated by *Arabidopsis* CK2, and this phosphorylation enhances the light-induced degradation of PIF1 (Bu, Zhu, et al., 2011). PIF1 also interacts with HFR1 in yeast two-hybrid assays (Bu, Castillon, et al., 2011). However, the biological significance of this interaction is still not known. Therefore, the HLH protein(s) that may regulate PIF1 activity have not been identified yet. In this study, we used a systems biology approach to identify a group of HLH proteins (the HECATE family) that interacts with PIF1. We show that HECATE proteins function antagonistically to PIF1 and possibly other PIFs to promote photomorphogenesis.

Results

PIF1 interacts with HECATE proteins

To identify and characterize potential regulators of PIF1, we focused on a group of small bHLH proteins that are predicted not to bind to DNA (the HLH proteins) (Toledo-ortiz et al., 2003; Bailey et al., 2003). Reasoning that light-regulated *bHLH* genes might play roles in light signaling pathways as previously observed (Fairchild et al., 2000; Huq and Quail, 2002), we focused on *HECATE* family, as *HEC1* expression was shown to be down-regulated by light in Genevestigator database (Fig. 4.1) (Zimmermann et al., 2004). The bHLH domains of HEC1 and HEC2 displayed high similarity to the bHLH domains of PIFs and HFR1, bHLH proteins previously shown to function in light signaling pathway (Fig. 4.2). To examine whether HECATE proteins interact with PIFs, we cloned *HEC1* and *HEC2* genes into yeast-two-hybrid vectors and performed yeast-two-hybrid interaction assays. Fig. 4.3 shows that both HEC1 and HEC2 interacted with PIF1 and PIF3 in a yeast-two-hybrid liquid β-galactosidase assay (Fig. 4.3A), suggesting that HECATE proteins heterodimerize with PIF1 and PIF3 and possibly other PIFs.

To independently verify the physical interaction between HEC and PIF proteins, we cloned *HEC2* into an *in vitro* expression vector (pET17b) as a fusion protein with GAL4 activation domain (GAD). We have co-expressed either GAD alone with PIF1 and PIF3 or GAD-HEC2 with PIF1 and PIF3 in TnT system as described (Toledo-ortiz et al., 2003; Huq et al., 2004; Huq and Quail, 2002), and co-immunoprecipitated using antibody against GAD. Fig. 4.3B shows that GAD-HEC2 efficiently co-immunoprecipitated both PIF1 and PIF3, which is consistent with the yeast-two-hybrid assay results shown in Fig. 4.3A.

To demonstrate that HEC and PIF proteins interact *in vivo*, we made transgenic plants expressing TAP-PIF1 with endogenous *PIF1* promoter and HEC2-GFP fusion protein expressed from a constitutively active 35S promoter. We crossed these two transgenic lines and produced homozygous lines expressing both the TAP-PIF1 and

HEC2-GFP fusion proteins. These transgenic lines were used to perform *in vivo* co-immunoprecipitation assays using α-GFP antibody. Results show that HEC2-GFP efficiently co-immunoprecipitated TAP-PIF1 from plant extracts (Fig. 4.3C). Taken together, these data suggest that HEC1 and HEC2 interact with PIF1, and might function in light signaling pathways by regulating PIF1, PIF3 and possibly other PIFs.

HEC1 and HEC2 positively regulate seed germination

HECATE proteins have been shown to regulate female reproductive tract development in *Arabidopsis* (Gremski et al., 2007). To investigate the biological functions of HECATE proteins in light signaling pathways, we employed reverse genetic approaches and produced *HEC2* RNAi and overexpression plants. Semi-quantitative RT-PCR was used to examine the mRNA levels for these plants (Fig. 4.4). *hec1* mutant was previously described (Gremski et al., 2007). We used these lines to investigate seed germination phenotypes in response to light, one of the major biological processes regulated by PIF1 (Oh et al., 2004; Shen et al., 2005). PIF1 directly and indirectly regulates multiple signaling pathways including GA signaling to repress seed germination in *Arabidopsis* (Oh et al., 2004, 2009). Light-induced degradation of PIF1 relieves this negative regulation and promotes seed germination (Oh et al., 2006; Shen et al., 2005). Fig. 4.5A shows that *hec1* and three independent RNAi lines of *hec2* displayed much reduced level of seed germination compared to wt under increasing amount of R light. These seeds eventually germinate under prolonged exposure (>3 h) to light (Fig. 4.5A), suggesting that they are not permanently dormant. To assess whether HEC1 and HEC2 promote seed germination through inhibition of PIF1 function, we crossed *hec1* and *hec2* RNAi lines into *pif1* background and examined their seed germination phenotypes. Results show that the reduced seed germination of *hec1* and *hec2* RNAi lines in response to light is eliminated in the *pif1* background. The double mutant seeds germinated similar to the *pif1* single mutant (Fig. 4.5B). These data suggest that *pif1* is epistatic to *hec* function in regulating seed germination.

In contrast to the *hec* mutants, two independent *HEC2* overexpression lines showed ~100% seed germination after FR exposure similar to *pif1* mutant (Fig. 4.5C). The opposite phenotypes of the mutant/RNAi lines compared to the overexpression lines provide strong evidence that HECATE proteins regulate seed germination in response to light. To examine whether *HEC2* overexpression lines promote seed germination through mis-regulation of GA pathway, we investigated their seed germination response in the presence and absence of paclobutrazol (PAC), a biosynthetic inhibitor of GA. The high germination rate of *HEC2* overexpression lines is completely eliminated in the presence of PAC (Fig. 4.5C). These data strongly suggest that HEC2 regulates GA biosynthesis/signaling to promote seed germination in response to light.

HEC1 and HEC2 positively regulate seedling deetiolation

Since *hec1* and *hec2* RNAi lines showed hyposensitive seed germination phenotypes (Fig. 4.5), we investigated the seedling deetiolation phenotypes of these lines in response to R and FR light conditions. Results showed that the hypocotyl lengths for the *hec1* and *hec2* RNAi lines were longer than that of wt under both R and FR light conditions (Fig. 4.6A, B and C). The cotyledon areas were largely similar to wt (data not shown). Although the hypocotyl length of *hec1* was longer than the wild type in the dark (Fig. 4.6A, B and C), the hypocotyl lengths for the *hec2* RNAi lines were largely similar to the wild type in the dark, suggesting that the long hypocotyl phenotypes are light-dependent. Overall, these data suggest that both HEC1 and HEC2 function as positive regulators of phy signaling pathways. This is in contrast to the PIF functions, where majority of the PIFs function as negative regulator of phy signaling pathways.

HEC1 and HEC2 positively regulate chlorophyll and carotenoid biosynthesis

Chlorophyll and carotenoid biosynthesis is coordinately regulated in *Arabidopsis* in response to light, and PIF1 plays a critical role in directly regulating both of these pathways (Huq et al., 2004; Toledo-Ortiz et al., 2010; Stephenson et al., 2009; Moon et al., 2008). To assess the roles of HECATE proteins in regulating these pathways, we measured chlorophyll and carotenoid levels in *hec1* and *hec2* RNAi lines as well as

HEC2 overexpression lines. Seedlings were grown for 2.5 days in the dark and then exposed to white light over time before harvesting for pigment measurement. Results show that *hec1* and *hec2* RNAi lines display reduced level of both chlorophyll and carotenoid in response to light (Fig. 4.7A and B). By contrast, two independent *HEC2OX* lines and one *HEC2-GFP* overexpression line displayed much higher level of chlorophyll and carotenoid levels compared to the wild type seedlings. The *HEC2OX* phenotypes were stronger than the *pif1* mutant, suggesting that HEC proteins might be negatively regulating not only PIF1, but also other PIFs to promote chlorophyll and carotenoid biosynthesis. These data suggest that HEC and PIF proteins are functioning antagonistically to regulate these pathways.

PIF1 regulated genes are oppositely regulated by HEC1 and HEC2

To investigate the molecular phenotypes of *hec* mutants and also to examine whether *hec* mutants function antagonistically to *pif1* at the gene expression level, we performed qRT-PCR assays for PIF1 target genes involved in seed germination. To eliminate the influence of GA on PIF1 target gene expression, we crossed the *hec* mutants and *HEC2* overexpression lines into *ga1* mutant background as previously performed (Oh et al., 2007). Seeds of various genotypes were sterilized and plated within one hour of imbibition, and then exposed to a saturated FR light to inhibit germination. Then the plates were either kept in the dark or exposed to a pulse of R light followed by incubation in the dark for an additional 12 hours. RNA was isolated from these seeds and qRT-PCR was performed for *GAI* and *RGA*, two DELLA genes that are direct targets of PIF1 as previously observed (Oh et al., 2007). Results show that the expression of *GAI* and *RGA* is reduced in the *hec1* and *hec2* RNAi lines compared to wild type (Fig. 4.8). By contrast, the expression of *GAI* and *RGA* is stimulated in the *HEC2* overexpression lines compared to wild type. The data also show that HEC1 and HEC2 regulate the expression of *GAI* and *RGA* in a manner opposite to that of PIF1. Thus, HEC1/2 and PIF1 function antagonistically to regulate gene expression.

HEC2 blocks the DNA binding ability of PIF1

Previously, we have shown that PIF1 binds to a G-box motif present in *PORC* and *PSY* promoters using a gel-shift assay (Moon et al., 2008; Toledo-Ortiz et al., 2010). To determine if HEC2 can block the DNA binding ability of PIF1, we co-expressed PIF1 and HEC2 using the TnT system and performed a gel-shift assay as described (Huq et al., 2004; Huq and Quail, 2002; Moon et al., 2008). Results show that HEC2 prevents the binding of PIF1 to the *PORC* G-box fragment (Fig. 4.9). To examine the specificity of this inhibition, we created a mutant version of HEC2 that has strongly reduced affinity for PIF1 (Fig. 4.10). We co-expressed the mutant form of HEC2 and used as controls in these binding assays. Results showed that the mutant form of HEC2 reduced the DNA binding ability of PIF1 much less efficiently compared to the wild type HEC2 (Fig. 4.9). These results suggest that HEC2 heterodimerizes with PIF1 and prevents PIF1 from binding to its target promoters.

***PIF1, HEC1* and *HEC2* genes are co-expressed**

Because PIF1 and HEC proteins heterodimerize, co-expression of *PIF1* and *HEC* genes would be a prerequisite for such heterodimers to be functionally relevant *in vivo*. We have analyzed the spatial regulation of expression of *PIF1* and *HEC* genes using eFP browser (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) (Fig. 4.11). However, because *HEC2* probe is absent on microarray chips, data for *HEC2* were not available. To compare tissue-specific or developmental expression patterns of *PIF1* and *HEC* genes, we used a promoter:reporter fusion strategy. We cloned ~2 kb promoter region upstream of the ATG start codon of *PIF1*, *HEC1* and *HEC2* genes into a pENTRY vector, and then recombined with a Gateway compatible destination vector containing the *GUS* gene as a transcriptional fusion (Karimi et al., 2005). These constructs have been transformed into wild type *Arabidopsis* and single insert homozygous transgenic plants have been selected. Histochemical GUS assays have been performed using X-Gluc as a substrate as described (Shen et al., 2007). Results showed that all three genes are co-expressed at the seedlings stage in a tissue-specific manner (Fig. 4.12A). Moreover, these genes are co-expressed in seedlings grown in the dark or light (R, FR and white light) conditions. The striking co-

expression of *PIF1*, *HEC1* and *HEC2* in the imbibed seeds (Fig. 4.11) as well as seedlings grown under different conditions (Fig. 4.12A) suggest that these genes function together in a tissue- and developmental-stage-specific manner.

HEC2 is localized in the nucleus

Because PIF1 and HEC proteins interact with each other, they are expected to be in the same subcellular location. To investigate the subcellular localization of HEC1 and HEC2 proteins, we transformed wt *Arabidopsis* with *35S:HEC1-YFP* and *35S:HEC2-GFP* constructs. Homozygous transgenic lines for HEC1-YFP were lethal. However, single insert homozygous HEC2-GFP lines were viable. We investigated the subcellular localization in stable transgenic background using fluorescence microscope. Results showed that HEC2-GFP is localized in the nucleus (Fig. 4.12B). This is also consistent with the predicted subcellular localization of HEC2 using PRORT (http://psort.ims.u-tokyo.ac.jp/form.html; version 6.4). Previously, PIF1 was shown to localize in the nucleus (Huq et al., 2004). Co-localization of these proteins within the nucleus suggests that they may regulate same biological processes as described above.

***HEC1* and *HEC2* expressions are modestly regulated by light**

To examine the kinetics of light regulation of *HEC1* and *HEC2* expression, we performed semi-quantitative RT-PCR under various light regimens for different time periods. Four-day old dark grown seedlings were exposed to either continuous R or FR lights for 1, 3, 6, 12 and 24 hr or kept in darkness. Total RNA was isolated from these samples for RT-PCR experiments. Results showed that both *HEC1* and *HEC2* mRNA levels were gradually down-regulated under R and FR light conditions. PIF1 transcript level was mostly unaffected under these conditions (Fig. 4.13A).

Post-translational regulation of oppositely acting transcription factors has been shown to be central in light signaling pathways (Huq, 2006). For example, HY5, LAF1 and HFR1 (positive regulators) are degraded in the dark to repress photomorphogenesis, while PIFs (negative regulators) are degraded in light to promote photomorphogenesis (Henriques et al., 2009; Huq, 2006). To investigate the effect of light on HEC protein

levels, we used antibody against GFP to examine the HEC2-GFP protein levels in the dark and dark-grown seedlings exposed to white light over time. Results show that HEC2-GFP is modestly stabilized in response to prolonged light conditions (Fig. 4.13B). However, unlike the previously described positively acting components in light signaling (e.g., HY5, LAF1 and HFR1), HEC2-GFP was relatively abundant in dark-grown seedlings. These data suggest that HEC2 may function both in the dark and light conditions.

HEC2 reduces the light-induced degradation of PIF1

Previously, it was shown that PIF1 and PIF3-6 are degraded in response to light (Henriques et al., 2009; Castillon et al., 2007). PIFs interact with the Pfr forms of phyA and phyB using the APA and APB domains, respectively, and this interaction is necessary for the light-induced degradation of PIFs (Henriques et al., 2009; Castillon et al., 2007). PIF3 has been shown to interact with phyB in a 1:1 stoichiometry, suggesting that PIF3 dimer interacts with phyB dimer (Zhu et al., 2000). Because HEC proteins interact with PIF1, we examined whether this heterodimerization prevents the light-induced degradation of PIF1. Results show that the light-induced degradation of PIF1 is greatly reduced in *HEC2* overexpression line in response to both R and FR light conditions (Fig. 4.14A and B). This stabilization is at the post-translational level, as the mRNA for *PIF1* is not altered in the *HEC2* overexpression line under identical conditions (Fig. 4.14C). PIF1 is rapidly degraded in the wt background under the same conditions, suggesting that HEC2 stabilizes PIF1 at the protein level. However, PIF1-HEC2 heterodimer might be non-functional as the heterodimer fails to bind to DNA (Fig. 4.9).

Discussion

PIFs have been shown to function as cellular hubs for various signaling pathways, including their central roles in phytochrome signaling (Castillon et al., 2007; Leivar and Quail, 2011). PIF1 has the highest affinity for phyA and phyB among all the PIFs and functions as a critical negative regulator of light-induced seed germination, chlorophyll and carotenoid biosynthesis, hypocotyl suppression and hypocotyl negative gravitropism

(Oh et al., 2004; Huq et al., 2004; Toledo-Ortiz et al., 2010). In this study, we identify new factors that regulate PIF1 activity and protein level using a targeted systems biology approach. A simplified model with the new factors was demonstrated in Fig. 4.15.

The genetic, photobiological and biochemical data presented here provide strong evidence that HECATE proteins function positively in phy signaling pathways. First, *hec1* and *hec2* RNAi lines showed reduced seed germination in response to light, while *HEC2* overexpression lines germinate constitutively in the dark after FR pulse. Second, *hec1* and *hec2* RNAi lines displayed longer hypocotyls compared to wt seedlings under both R and FR light conditions. By contrast, the hypocotyl lengths of *hec1* and *hec2* RNAi lines were largely similar to that of wt seedlings in darkness, suggesting the phenotypes are light dependent. Third, both *hec1* and *hec2* RNAi lines have reduced levels of chlorophyll and carotenoid compared to wt seedlings. Hyposensitive phenotypes of the above hallmark biological processes strongly suggest that HECATE proteins are positively acting components in phy signaling pathways.

Previously, positively acting components in phy signaling pathways have been described (Huq and Quail, 2005). Among those, only HFR1 is a HLH protein that functions positively in FR and blue light signaling pathways (Fairchild et al., 2000; Fankhauser and Chory, 2000; Duek and Fankhauser, 2003). *hfr1* mutants did not show any phenotype under R light conditions. In contrast, *hec* mutants are hyposensitive to both R and FR light conditions (Fig. 4.6), suggesting HECATE proteins represent new players in light signaling pathways.

HECATE proteins appear to function antagonistically to PIF1 and possibly other PIFs in light signaling pathways. First, HECATE proteins interact with PIF1 in yeast-two-hybrid assays, in vitro and in vivo co-immunoprecipitation assays. Second, all the hallmark biological processes that are regulated by PIF1 (e.g., repression of seed germination, elongation of hypocotyls, repression of chlorophyll and carotenoid biosynthesis) are oppositely regulated by HECATE proteins (Figs. 4.5, 4.6 and 4.7). Third, *PIF1* and *HECATE* genes are expressed in the same tissues at similar developmental stages. Fourth, PIF1 and HECATE proteins are localized in the same

subcellular compartment, nucleus, potentially functioning in the same pathway. Fifth, the DNA binding activity of PIF1 is inhibited by wt HEC2 in vitro, but not by a mutant HEC2 that does not interact with PIF1 (Fig. 4.9). Sixth, the expression of PIF1 target genes are oppositely regulated in *hec* mutants compared to *pif1* seedlings (Fig. 4.8). These data suggest that HECATE proteins directly bind to PIF1 and prevent PIF1 function. The mechanisms by which HECATE proteins inhibit PIF1 functions appear to be similar to Id proteins in animal system or HFR1/ILI1 in plants (Benezra et al., 1990; Perk et al., 2005; Hornitschek et al., 2009; Zhang et al., 2009). In these cases for example, HFR1 binds to PIF4 and PIF5 and prevent their DNA binding and transcriptional regulation of their target genes. Although HFR1 also interacts with PIF1 in yeast-two-hybrid assays, the biological significance of this interaction is still lacking. Therefore, HECATE proteins represent new components in light signaling pathways that function through PIF1 and potentially other PIFs in *Arabidopsis*.

Previously, HLH proteins have been shown to regulate the activity of bHLH proteins by forming a dominant negative heterodimer complex. However, this heterodimerization has not been shown to regulate the abundance of the partner bHLH proteins. Our data showing that HEC2 overexpression strongly reduces the light-induced degradation of PIF1 *in vivo* (Fig. 4.14) suggest that HLH proteins not only inhibit the DNA binding activity, but also might regulate the stability of their interacting bHLH partners. Although PIF1 is more stable in *HEC2* overexpression background, PIF1 does not show overexpression phenotypes in this background as expected as it forms non-DNA binding heterodimer complex with HEC2. In *Arabidopsis*, there are >162 bHLH proteins of which >27 are predicted to be non-DNA binding HLH proteins. Both the bHLH and HLH proteins have been expanded in plants compared to animal system, and has been shown to function in many signaling pathways in plants. It is possible that these antagonistically acting pairs of proteins have co-evolved in multiple signaling pathways in plants for fine-tuning these pathways. Furthers studies are necessary to test whether the bHLH and HLH proteins have co-evolved in plants.

In summary, our targeted systems biology approach has uncovered new players in light signaling pathways that have not been identified through other methods. As more genomics data are available for many organisms, this approach has the potential to advance our understanding of signaling pathways that involve multiple redundant and/or overlapping genes.

Materials and methods

Plant growth conditions, light treatments and phenotypic analyses

Seeds were sterilized with 20% bleach + 0.3% SDS for ten minutes, washed five times with water and then plated on Murashige-Skoog (MS) growth medium (GM) containing 0.9% agar without sucrose (GM-Suc). After 4 days of stratification at 4 ºC in the dark, seeds were exposed to 1 hour white light at room temperature to induce germination and kept in darkness for 23 hours. After this time period, the plates were transferred to growth chambers under red, far-red, or blue light conditions for an additional 3 days. Light fluence rates were measured using a spectroradiometer (Model EPP2000, StellarNet Inc., Tampa, FL) as described (Shen et al., 2008). Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture, Bellevue, WA) under continuous light at 24 ℃ ± 0.5 ℃.

For quantitation of hypocotyl lengths, digital photographs of seedlings were taken and at least 30 seedlings were measured using the publicly available software ImageJ (http://rsbweb.nih.gov/ij/). The seed germination assays and chlorophyll and carotenoid measurements were performed as described (Oh et al., 2004; Shen et al., 2005; Toledo-Ortiz et al., 2010). Experiments were repeated at least three times.

Quantitative β-galactosidase assay

HEC1 and HEC2 ORFs were amplified using PCR and then cloned into pGBT9 and pGAD424 vectors (Clonetech Laboratories Inc., Mountain View, CA) using the restriction sites included in the PCR primers. Prey constructs of full length PIF1 and PIF3 were constructed in pGAD424. The specific amino acid mutations in HEC2 were introduced using a site-directed mutagensis kit (Stratagene, La Jolla, CA). Procedures for

the yeast two-hybrid quantitative interaction assays were performed according to the manufacturers instructions (Matchmaker Two-Hybrid System, Clonetech Laboratories Inc., Mountain View, CA).

In vitro and *in vivo* co-immunoprecipitation assays

HEC2 ORF was cloned to into pET17b as a fusion protein with gal4 activation domain (GAD) using restriction sites designed within the primers. The construct was verified by sequencing. PIF1 and PIF3 constructs are as described (Huq et al., 2004; Toledo-ortiz et al., 2003). HEC2, PIF1 and PIF3 were co-translated using the TnT system (Promega, Madison, WI) and in vitro co-immunoprecipitation assays were carried out as previously described (Toledo-ortiz et al., 2003; Huq and Quail, 2002). For *in vivo* co-immunoprecipitation assays, HEC2-GFP expressing transgenic line was crossed into TAP-PIF1 expressed from the endogenous *PIF1* promoter (Bu, Zhu, et al., 2011), and homozygous transgenic plants were selected using antibiotic selection. The *in vivo* co-immunoprecipitation assays were carried out as previously described (Shen et al., 2008). Briefly, total proteins were extracted from ~0.4 g dark-grown seedlings with 1 mL native extraction buffer (100 mM NaH_2PO_4, pH 7.8, 100 mM NaCl, 0.05% NP-40, 1X Protease inhibitor cocktail [F. Hoffmann-La Roche Ltd, Basel, Switzerland], 2 mM PMSF, 10 μM MG132, 25 mM β-GP, 10 mM NaF, 2 mM Na orthovanadate and 100 nM calyculin A) and cleared by centrifugation at 16,000 g for 15 min at 4℃. Anti-GFP antibody was incubated with Dynabead (Invitrogen Inc., Carlsbad, CA) (20 mL/mg antibody) for 30 min at 4℃. The beads were washed twice with the extraction buffer to remove the unbound antibody. The bound antibody-beads were added to a total of 1 mg total protein extracts and rotated for another 3 h at 4℃ in the dark. The beads were collected using a magnet, washed three times with wash buffer, dissolved in 1X SDS-Loading buffer and heated at 65℃ for 5 min. The immunoprecipitated samples were separated on an 8% SDS-PAGE gel, blotted onto PVDF membrane, and probed with anti-myc antibody to detect TAP-PIF1.

Construction of vectors and generation of transgenic plants

DNA sequence from the nucleotide 59 to 359 of *HEC2* did not show any significant identity (≥20 bp) to any other *Arabidopsis* sequence; therefore this region has been used to construct RNAi vectors for *HEC2*. The above region was amplified by PCR and cloned into pENTRY vector (Invitrogen Inc., Carlsbad, CA). Sequence was verified and recombined into pB7GWIWG2 (II) vector (Karimi et al., 2005) produce binary plasmid for HEC2 RNAi. To construct overexpression and GFP fusion vectors, full-length *HEC2* open reading frame was cloned into pENTRY vector and recombined with pB7WG2 (for overexpression) and pB7FWG2 (for GFP fusion) (Karimi et al., 2005). A stop codon was included in the overexpression vector, but not in the GFP fusion vector to allow C-terminal fusion protein expression. These constructs were then transformed into wt using the *Agrobacterium* mediated transformation protocol as described (Clough and Bent, 1998). Single locus transgenic plants were selected based on antibiotic resistance and several homozygous lines were produced for analyses for each construct.

Histochemical GUS analysis and subcellular localization of HEC2

Histochemical GUS analysis was performed on intact seedlings. Transgenic plant samples were incubated with X-gluc buffer 2 hours at 37℃. These were rinsed, cleared of chlorophyll, by 75% (v/v) ethanol. The stained tissues were photographed under a Leica S6D stereo microscope with a Leica DFC 320 color camera (Leica Instrument, Nusslosh, Germany). For subcellular localization of HEC2, 4 days dark grown *35S::HEC2::GFP* homozygous lines were carefully transferred to glass slides under dime light. The GFP signal was examined under a Zeiss Axiovert 200 M microscope (Carl Zeiss AG, Oberkochen, Germany). After the fluorescent signal and the bright field signal were captured, 50µl 0.005µg/ml DAPI in DAPI solution (1XPBS, 50% glycerol and 0.001% Triton X-100) was added on top of the seedling with 5 minutes incubation. The DAPI stained nucleus signal was captured under UV light.

RNA isolation and quantitative RT-PCR assays

Total RNA was isolated from imbibed seeds using the Sigma plant RNA isolation kit as described (Oh et al., 2009). Total RNA was reverse transcribed using SuperScript™ III (Invitrogen, Carlsbad, CA) as per manufacturer's protocol. The qRT-PCR assays used the Power SYBR Green RT-PCR Reagents Kit (Applied Biosystems Inc., Foster City, CA). Primer sequences used for qRT-PCR and RT-PCR assays are listed (Table 4.1). *PP2A* (At1g13320) was used as a control for normalization of the expression data.

Electrophoretic Mobility Shift Assays (EMSA)

EMSAs were conducted according to (Moon et al., 2008). For the experiment, PIF1 and HEC2 recombinant proteins were produced using the TnT kit (Promega, Madison, WI) and incubated with a *PORC* promoter fragment containing the G-box motif labelled with ^{32}P-dCTP as described (Moon et al., 2008). A total of 30,000 cpm was used per lane. The samples were separated on 5% native PAGE gel. The gel was fixed, dried, and exposed to PhosphorImager cassette.

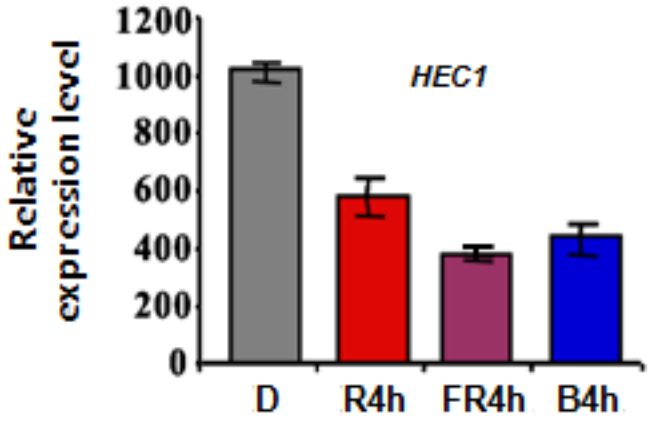

Figure 4.1: Light regulation of *HEC1* expression obtained from GENEVESTIGATOR web site (Zimmermann et al., 2004).

D, four days dark grown seedlings; R4h, four days dark grown seedlings with extra 4 hrs continuous red light treatment; FR4h, four days dark grown seedlings with extra 4 hrs continuous far red light treatment; B4h, four days dark grown seedlings with extra 4 hrs blue light treatment.

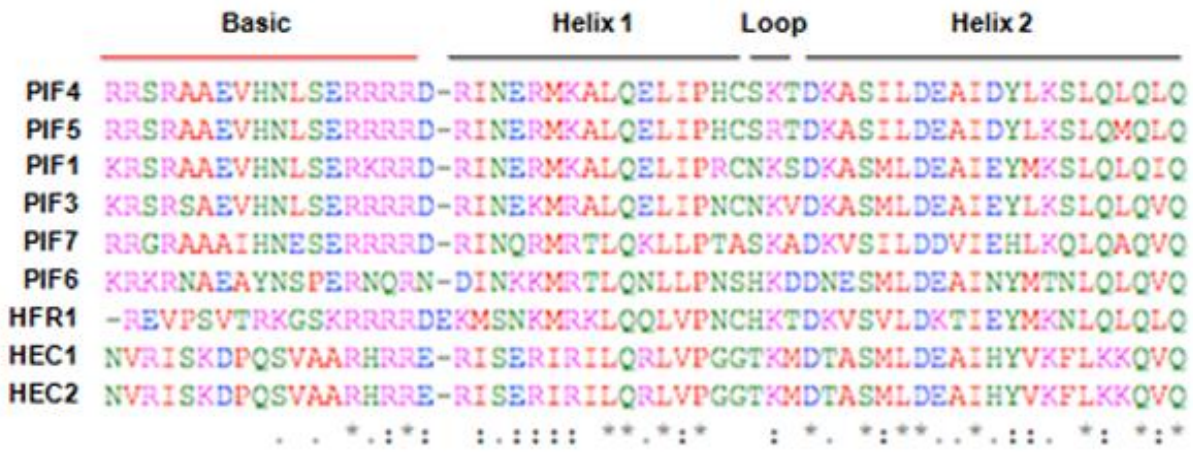

Figure 4.2: Sequence alignment of the bHLH domain of PIFs, HFR1 and HECATE proteins.

The red line represents the basic domain. The black lines represent the concensus helix-loop-helix domain. "*" indicates identical amino acid residues; ":" indicates different but highly conserved amino acids; and "." indicates different amino acids that are somewhat similar.

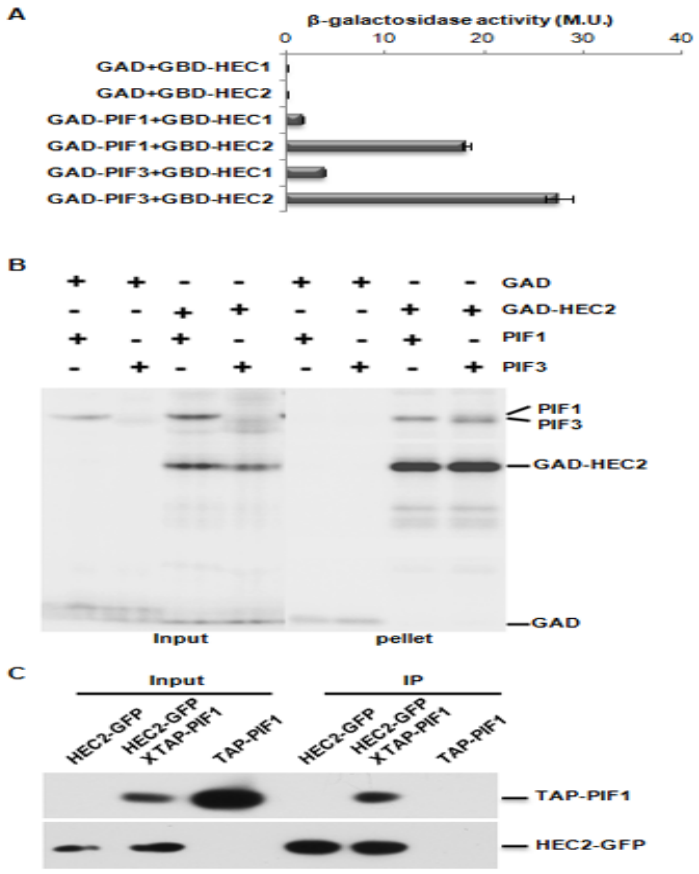

Figure 4.3: HECATE proteins interact with PIFs.

A) HEC1 and HEC2 interact with PIF1 and PIF3 in a quantitative yeast-two-hybrid assay. LacZ assays were performed in triplicate and the data represent mean ± SE. β-Galactosidase units are Miller units (M.U.). GAD, gal4 activation domain, and GBD, gal4 DNA binding domain. **B)** HEC2 heterodimerizes with PIF1 and PIF3 *in vitro*. Full-length HEC2 protein fused to GAD was used for this co-immunoprecipitation assay as described (Toledo-Ortiz et al., 2003; Shen et al., 2005; Huq and Quail, 2002). All proteins were synthesized as ^{35}S-methionine labeled products in TnT® T7 Quick Coupled Transcription/Translation System (Promega, Cat.# L1170, Madison, WI, USA). **C)** PIF1 interacts with HEC2 in *in vivo* co-immunoprecipitation assays. Total protein was extracted from four day-old dark-grown seedlings. Co-immunoprecipitations were carried out using the anti-GFP antibody and the immunoprecipitated samples were probed with anti-myc antibody.

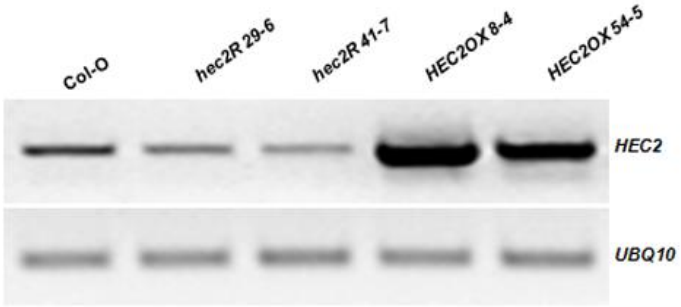

Figure 4.4: *HEC2* mRNA level in wild type, *HEC2* RNAi and overexpression lines.

Semi-quantitative RT-PCR assays were performed using total RNA isolated from 4 day-old dark grown seedlings. Full length *HEC2* gene specific primers were used to amplify *HEC2*. There are two folds down-regulation of *HEC2* in two *HEC2* RNAi lines and at least five folds up-regulation of *HEC2* in two *HEC2* overexpression lines. *UBQ10* was used as a control.

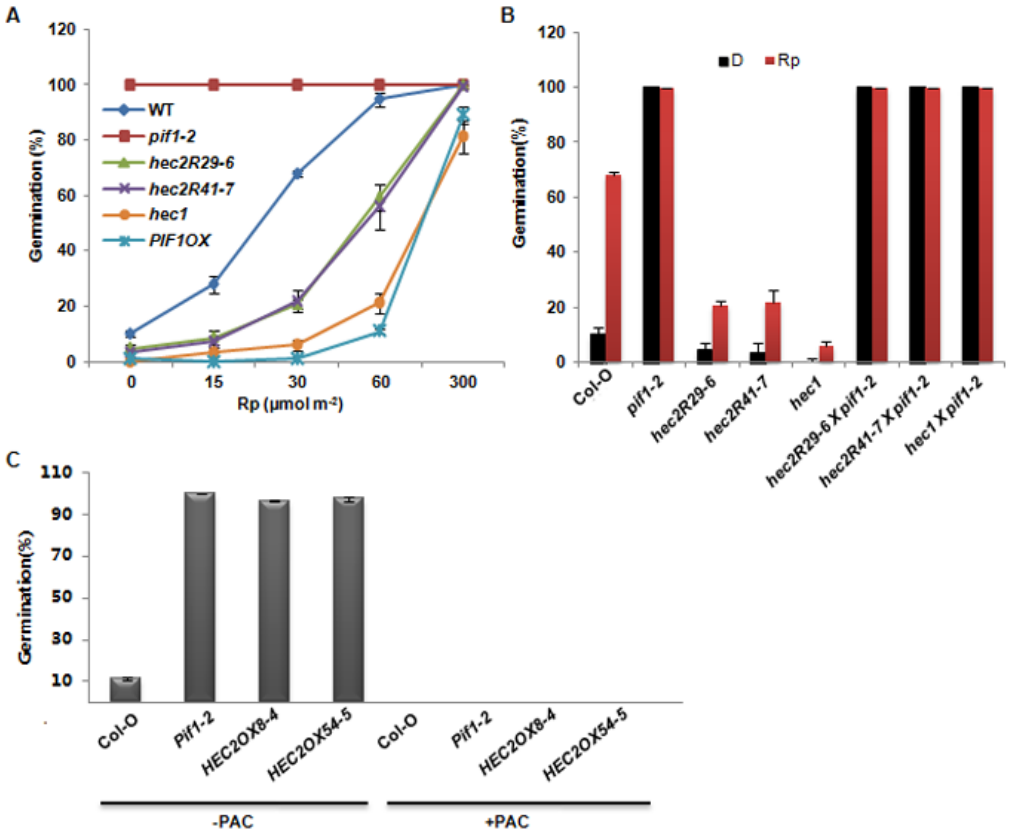

Figure 4.5: hec1 and hec2 promote seed germination in *Arabidopsis* in a pif1-dependent manner.

A) *hec1* and *hec2* RNAi lines showed reduced seed germination in response to light. Seed germination assays were performed as described (Oh et. al., 2004). All the plates were scored for radical emergence and percent of seeds germinated was plotted against the amount of R light exposed. **B)** Reduced seed germination of *hec1* and *hec2* RNAi lines is eliminated in the *pif1* background. The seed germination assays were performed as described in (A). After FR pulse, the seeds were either kept in dark or exposed to Rp (30 µmol) followed by dark incubation for 4 days. **C)** The seed germination phenotype of the *HEC2* overexpression lines is similar to *pif1* and is eliminated by GA biosynthetic inhibitor, paclobutrazol (PAC). The seed germination assays were performed as described in (A). The seeds were plated on GM-suc with (10 mM) and without paclobutrazol (PAC).

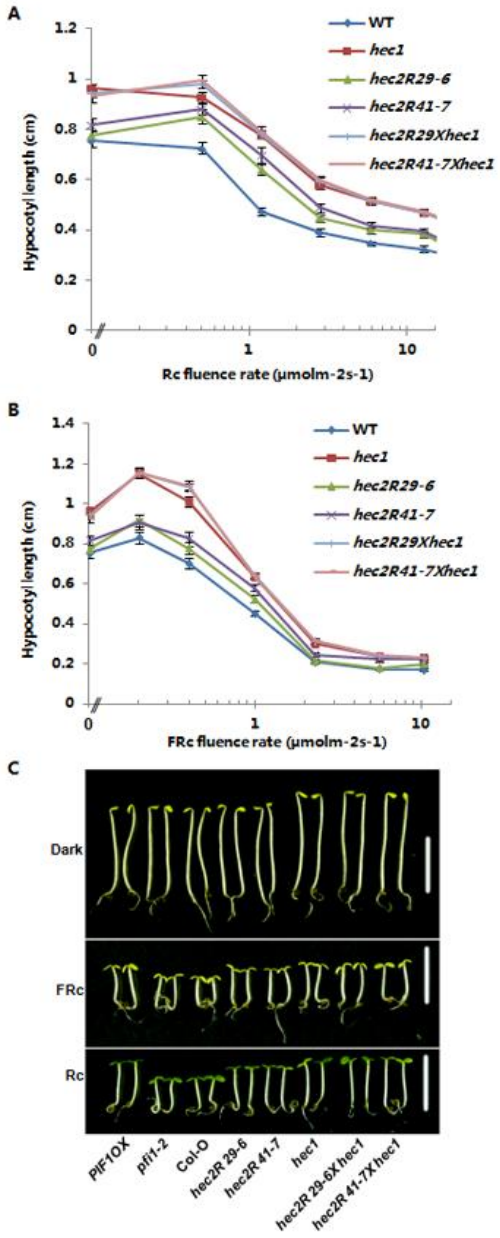

Figure 4.6: HEC1 and HEC2 promote seedling deetiolation in *Arabidopsis*.

Fluence-rate response curves of mean hypocotyl lengths of wild type Col-0, *hec1*, two independent *hec2* RNAi lines and one *hec1hec2* RNAi double mutant line grown for four days under either Rc **(A)** or FRc **(B)** or dark. **C)** Photographs of wild type Col-O, *pif1*, *hec1*, *PIF1* overexpression line, two independent *hec2* RNAi lines and two *hec1hec2* RNAi double mutant lines grown under dark (D), red (Rc, 8.7 $mmolm^{-2}s^{-1}$) and far-red light (FRc, 0.5 $mmolm^{-2}s^{-1}$) conditions for four days. White bar = 5 mm.

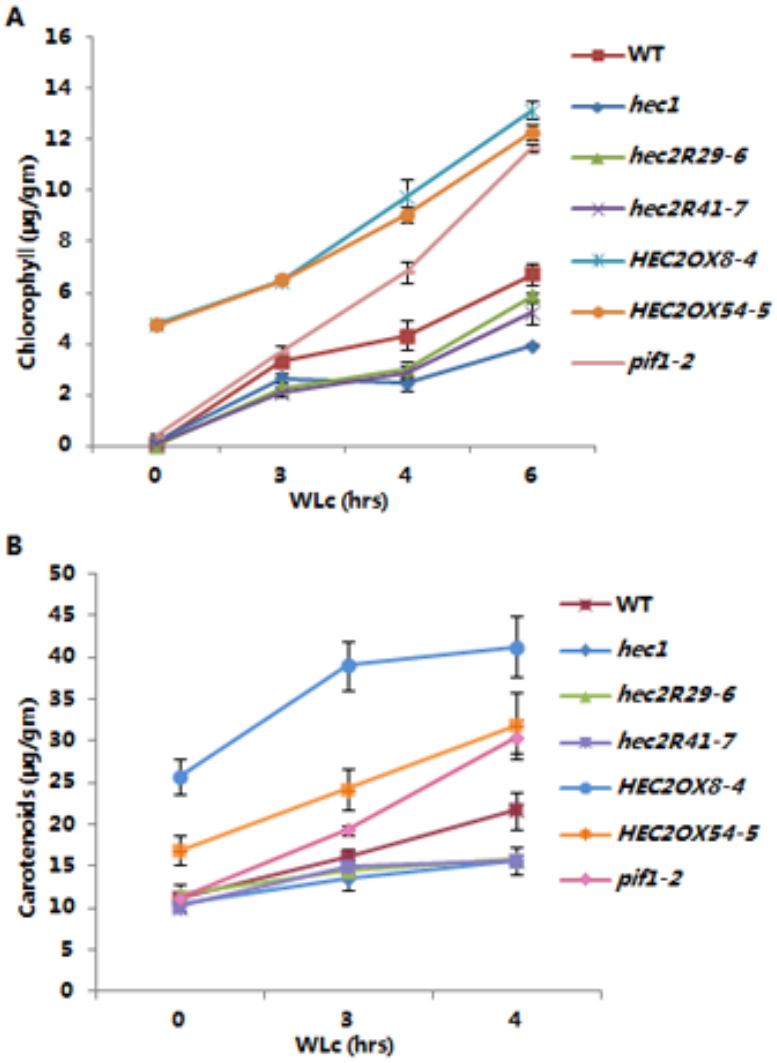

Figure 4.7: HEC1 and HEC2 promote chlorophyll and carotenoid biosynthesis in *Arabidopsis*.

hec1, two independent *hec2RNAi*, two independent *HEC2* overexpression and one *HEC2-GFP* overexpression lines were grown with wild type and *pif1-2* mutants for 2.5 days in the dark and then transferred to 80 µmolm^{-2}s^{-1} of white light for various times as indicated. Total chlorophyll **(A)** and carotenoid **(B)** contents were determined as described in Huq et al., (2004) or Toledo-Ort ź et al., (2010), respectively.

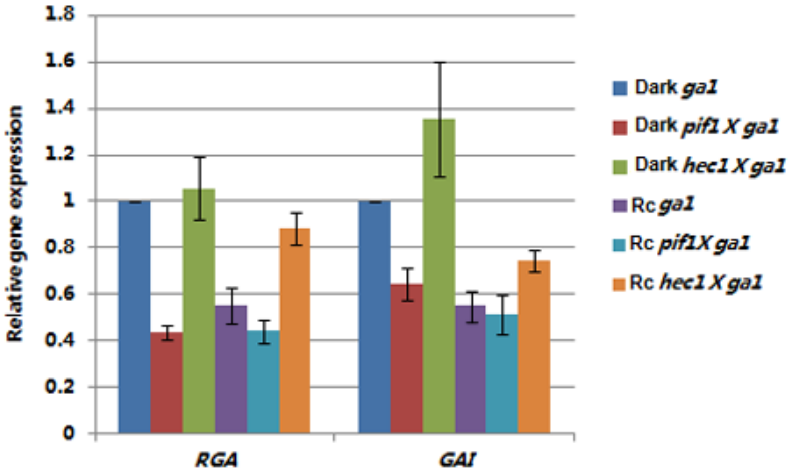

Figure 4.8: HEC1 and HEC2 oppositely control PIF1 target genes involved in Gibberellic Acid (GA) signaling pathway.

qRT-PCR data showing relative expression of *RGA* and *GAI* in *ga1*, *pif1 X ga1*, *hec1 X ga1* lines under phyB-dependent germination conditions described in Oh et al., (2007). The relative expression levels of the tested genes were normalized versus that of *PP2A.* (N=3 biological repeats, each with 3 technical replicates, ± SE).

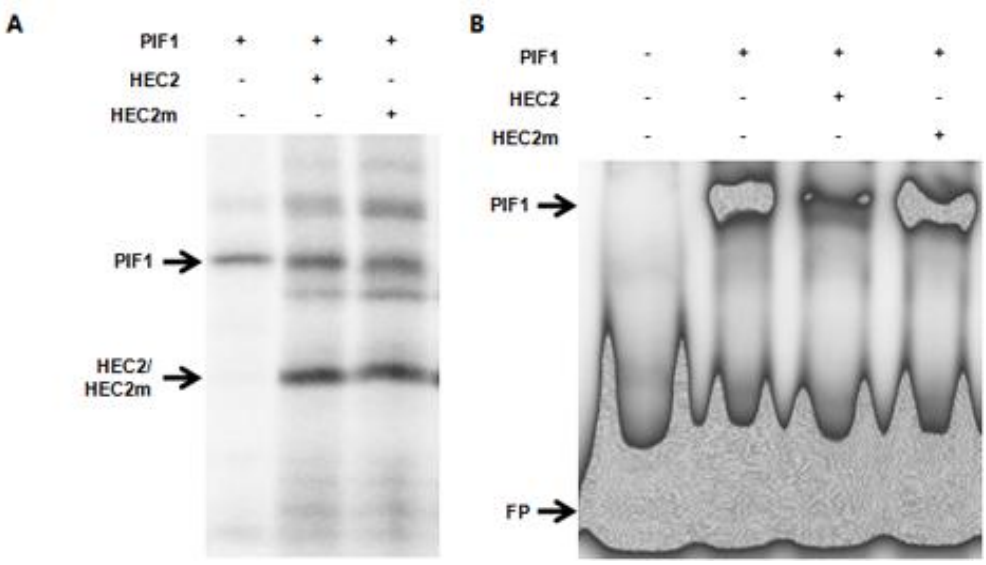

Figure 4.9: HEC2 inhibits the DNA binding ability of PIF1 to its target promoter.

A) Electrophoretic mobility shift assays (EMSA) showing PIF1 binding to *PORC* G-box is inhibited by HEC2. A total of 30,000 cpm of labeled probe were used in each lane. EMSA conditions are described in Moon et al. (2008). **B)** An SDS/PAGE gel photograph shows the amount of protein used for EMSA assays shown in A. PIF1 and wild type and mutant forms of HEC2 clones were co-expressed in TnT, and the TnT mix was used for EMSA as shown in A.

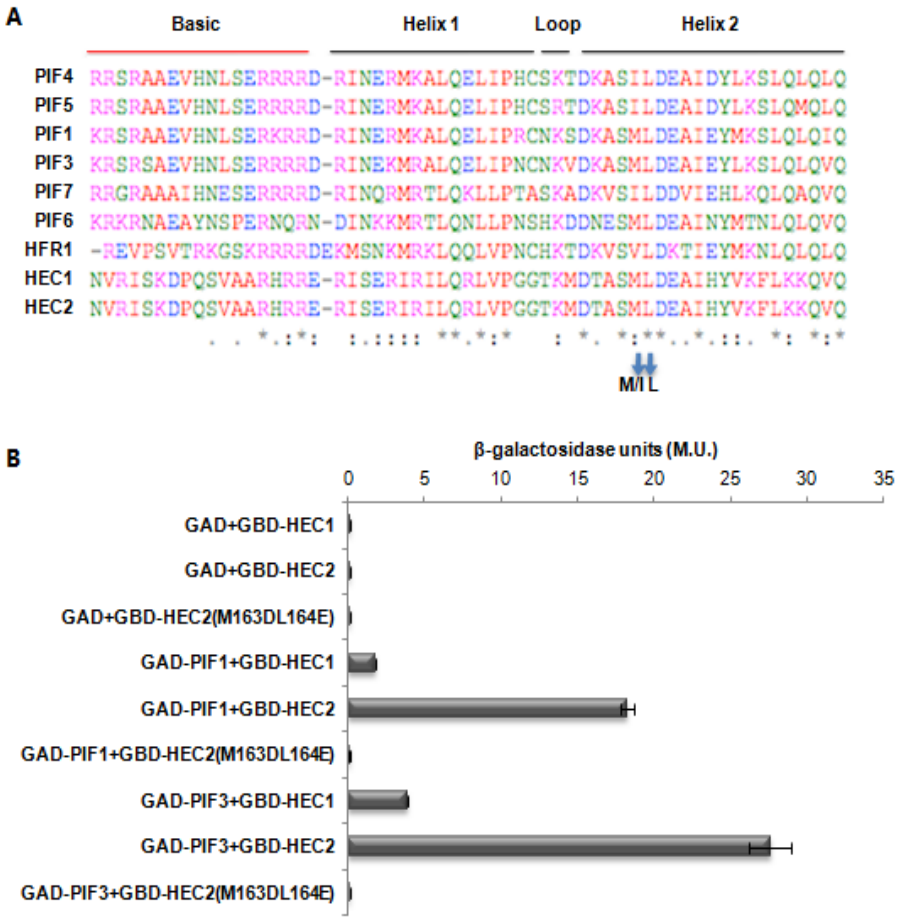

Figure 4.10: Yeast two-hybrid interactions assays between wild type and mutant HEC2 with PIF1 and PIF3.

A) Sequence alignments of the HLH domain in *Arabidopsis* bHLH proteins. The blue arrow pointed out two conserved amino acids, Met/Ile and Leu, in PIFs and HECs responsible for protein-protein interaction. **B)** HEC1 and HEC2, not mutated HEC2 (M163DL164E), interact with PIF1 and PIF3 in a quantitative yeast-two-hybrid assay. LacZ assays were performed in triplicate and the data represent mean $\pm$ SE. β-Galactosidase units are Miller units (M.U.). GAD, gal4 activation domain, and GBD, gal4 DNA binding domain.

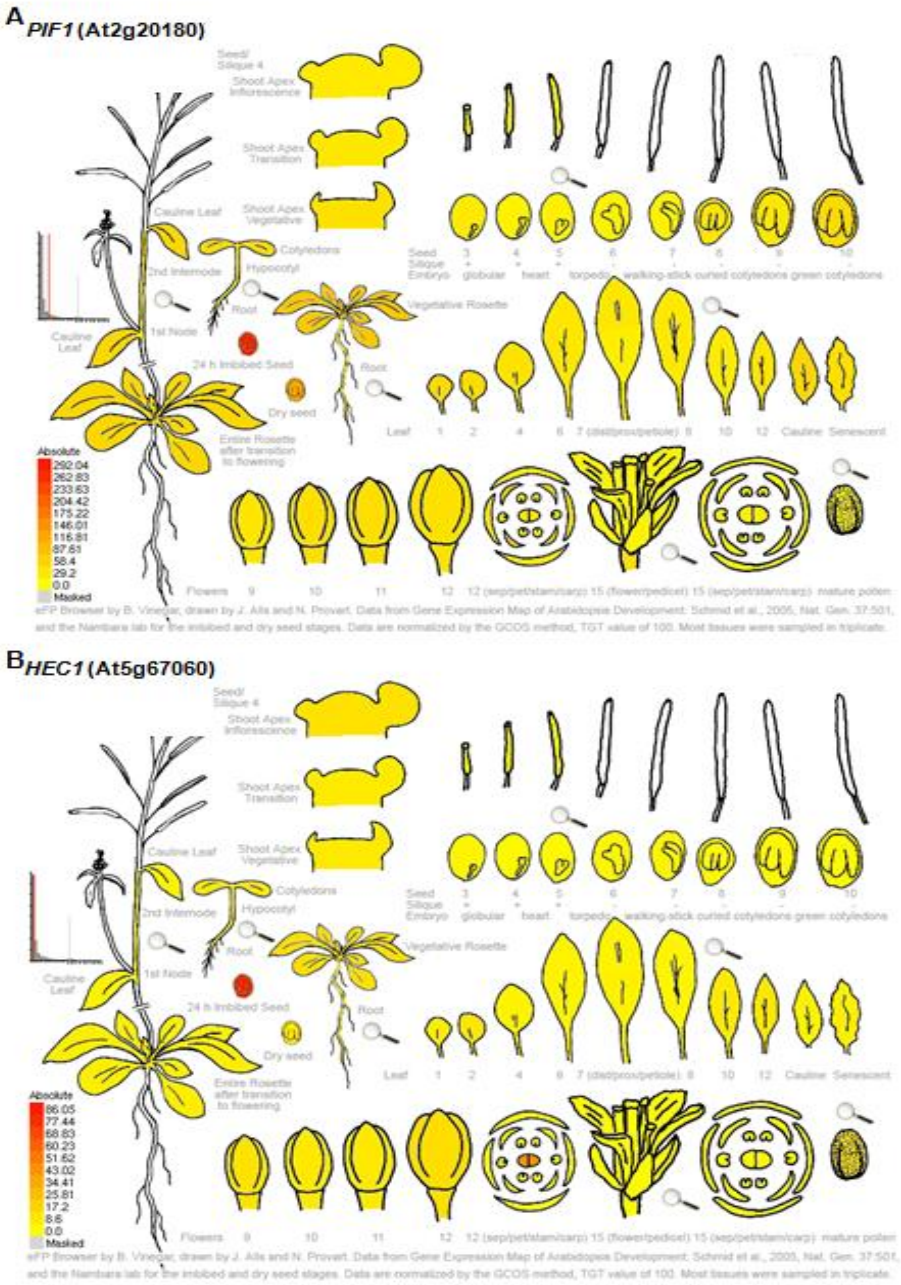

Figure 4.11: Co-expression analyses of *PIF1* and *HEC1* in *Arabidopsis*.

Digital expression patterns for *PIF1* (At2g20180) and *HEC1* (At5g67060) in various tissues were obtained from eFP browser (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) of multiple microarray data. Red represents the highest expression and yellow color represents the lowest expression. Probe for *HEC2* is absent in the microarray chips.

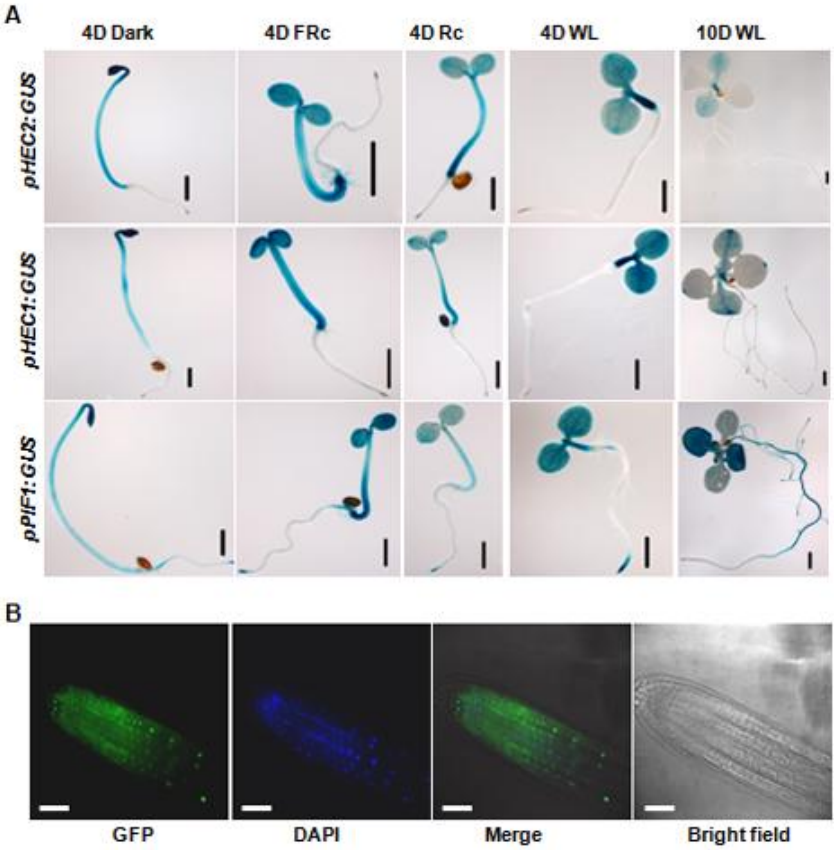

Figure 4.12: Co-expression analyses of *PIF1* and *HEC1/2* from promoter:GUS fusion transgenic plants.

A) Tissue-specific expression of *pPIF1:GUS*, *pHEC1:GUS* and *pHEC2:GUS*. Homozygous transgenic seedlings were grown either in dark or R/FR/WL for various times as indicated and GUS assays were performed as described Shen, et al,. (2007). Black bar = 10mm. **B)** HEC2 is localized to the nucleus. Photographs of HEC2-GFP fusion constructs expressed in stable transgenic wild type plants are shown. From the left, the first image shows GFP staining for HEC2-GFP driven by constitutively active 35S promoters, the second image shows DAPI staining for nuclei, the third image shows superimposition of the GFP and DAPI signals, and the fourth image shows the bright field photograph. White bar = 0.05mm.

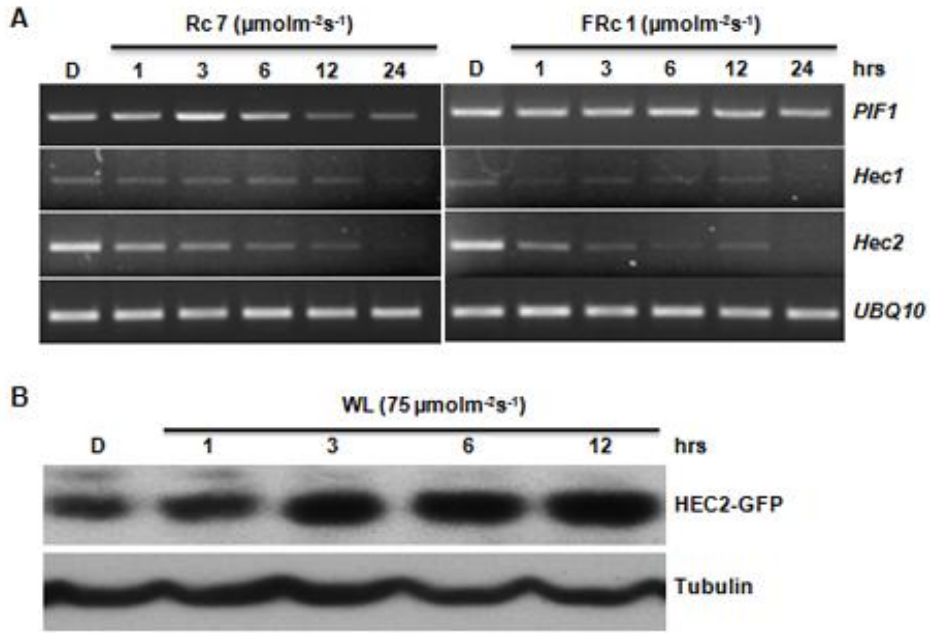

Figure 4.13: Light regulations of *PIF1*, *HEC1* and *HEC2* expressions at the mRNA levels and HEC2-GFP at the protein level.

A) Light down regulates *PIF1*, *HEC1* and *HEC2* gene expressions. Four days old col-0 seedlings were treated with 7 µmolm^{-2}s^{-1} red light or 1 µmolm^{-2}s^{-1} far red light for the durations indicated, or kept in darkness. Total RNA was extracted and reverse transcribed. *PIF1*, *HEC1* and *HEC2* gene expressions at each time point was detected by semi-quantitative RT-PCR. *UBQ10* was used as control. **B)** HEC2-GFP is modestly stabilized under light. Transgenic seedlings expressing *HEC2-GFP* using the constitutively active 35S promoter were grown in the dark for four days and then exposed to white light (75 µmol m^{-2} s^{-1}) for the durations indicated. Total protein was extracted from these samples, separated on SDS-PAGE gel and probed with anti-GFP antibody to detect HEC2-GFP fusion protein. An anti-Tubulin antibody was used as a control.

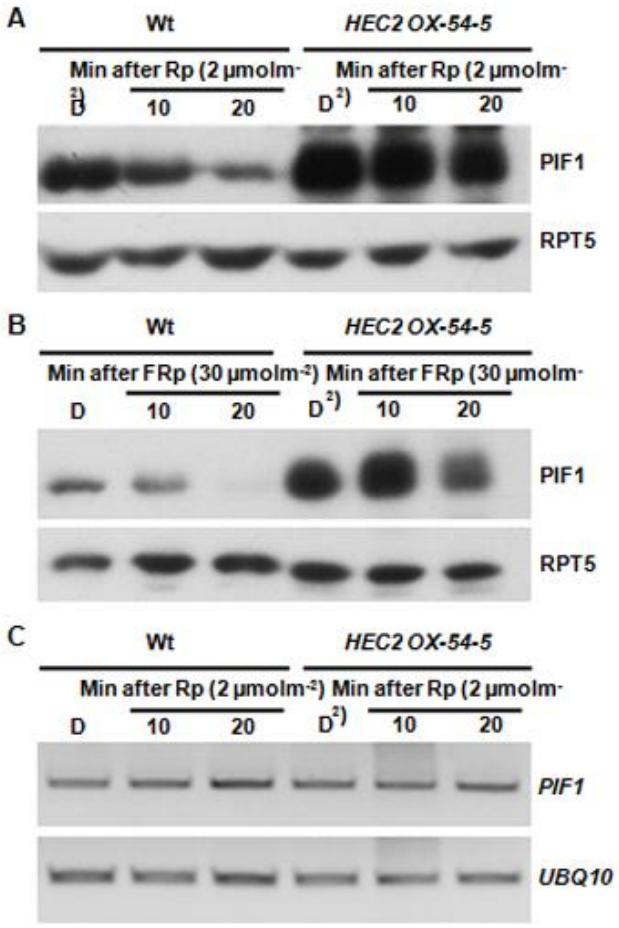

Figure 4.14: HEC2 reduces the light-induced degradation of PIF1.

Four day-old dark-grown seedlings were either kept in the dark or dark-grown seedlings exposed to Rp (2 mmolm^{-2}) **(A)** or FRp (30 mmolm^{-2}) **(B)** light and then incubated in the dark for the durations indicated before being harvested for protein extraction. Total protein from each sample was separated on an 8% polyacrylamide gel, transferred to PVDF membrane and probed with anti-PIF1 antibody. A similar blot was probed with anti-RPT5 antibody. The bands corresponding to PIF1 and RPT5 are labeled. **C)** *PIF1* mRNA level is not altered in the *HEC2* overexpression line. Semi-quantitative RT-PCR assays were performed using total RNA isolated from wild type and *HEC2* overexpression seedlings grown under the same conditions as described in A.

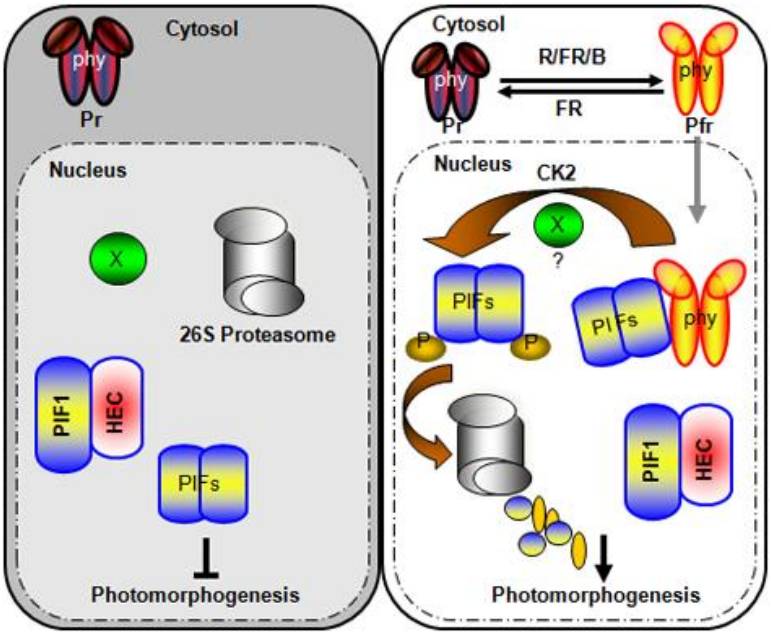

Figure 4.15: Simplified model of regulation of PIF function by HECATE proteins.

(Left) In the dark, phys are localized to the cytosol, while PIFs and HECs are constitutively localized to the nucleus. PIF1 and HEC1/2 interact with each other and form homo and heterodimer combinations. PIF homodimers negatively regulate photomorphogenesis, while PIF-HEC heterodimers inactivate the PIF functions to promote photomorphogenesis. (Right) Light signals induce photo-conversion of the Pr form to the active Pfr form of phys and promote nuclear migration of the Pfr form. The photoactivated phys interact with PIFs in the nucleus and induce phosphorylation of PIF1 and other PIFs either directly or indirectly. The phosphorylated forms of PIFs are then poly-ubiquitinated by an ubiquitin ligase, and subsequently degraded by the 26S proteasome. HECs are stable under light and interact with PIFs to remove the residual PIF activity under light. The combined removal of PIF function by HEC-mediated inactivation and light-induced proteolytic degradation of PIFs relieve the negative regulation, thus promoting photomorphogenesis. CK2 is a Ser/Thr kinase that phosphorylates PIF1 and promotes degradation of PIF1. X, indicates an unknown factor that might be involved in the light-induced phosphorylation of PIFs. P, phosphorylated form. This figure is adapted and modified from Shen et al., (2008).

Table 4.1: Primer sequences used in experiments described in text.

Gene	Forward	Reverse
Cloning		
HEC1 Y2H	AGAGAATTCATGGATTCTGACATAATGAAC	CCTGTCGACTCATCTAAGAATCTGTGCATTG
HEC1 Promoter	CACCAGTCTTAAATGTGATTTTGTAC	AGAGAAAGATATGGAGAAGCTGA
HEC2 Y2H	AGAGAATTCATGGATAACTCCGACATTCTAATG	CCTGTCGACTCATCTAAGAATCTGTGCATTTC
HEC2 RNAi	CACCGAACACTTCTCTAACTCAAACC	CTTTGGTGGCTTTACGGATTCC
HEC2 OX	CACCATGGATAACTCCGACATTCTAATG	CCTGTCGACTCATCTAAGAATCTGTGCATTTC
HEC2 YFP	CACCATGGATAACTCCGACATTCTAATG	TCTAAGAATCTGTGCATTTCC
HEC2 Promoter	CACCGAGAGAGCAGCGAAACGTCATCG	CCTCCTTTTTTGTGGAATTTATAG
qRT Primers		
PORC	GGGCAAAACAGTTCAATGA	GGAAAAAGAAGCCGAAACAG
PSY	GACACCCGAAAGGCGAAAGG	CAGCGAGAGCAGCATCAAGC

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Chapter V: Identification of factors in the degradation of PIF1 through the luminescent imaging system

Abstract

Plants perceive light to regulate seed germination and seedling de-etiolation through a family of photoreceptors called phytochromes. Phytochromes transduce the light signals to trigger a cascade of downstream gene regulations via a subfamily of bHLH proteins called PIFs. As the repressors in light signaling pathways, most PIFs are phosphorylated and degraded through the 26S ubiquitin proteasome degradation pathway in response to light. The factors involved in the phosphorylation and degradation of PIFs have not been identified yet. Here I used EMS mutagenesis and the luminescent imaging system to identify six mutants defective in the degradation of one of the PIFs, PIF1. All six mutants showed decreased degradation of PIF1 with light treatment in both luminescent imaging and Western blot assays. The level of stability in *140B* and *141C* were similar to a PIF1 mutant (PIF1-3M) that lacks interactions between PIF1 and phyA/phyB under light. *38A* showed phenotypes defective in the light signaling pathways with normal growth of cotyledons and roots but elongated hypocotyls under both red and far-red light. *38A* has been mapped onto the upper arm of the chromosome one, about a 4,000 kb region. This study will help identify the factors directly involved in the regulation of PIF1 protein level. At a broad level, the characterization of the mutants I found will help answer the key questions in the light signaling pathways, such as what are the kinase(s) and the E3 ligase(s) responsible for the light-induced degradation of PIFs.

Keywords

Phytochromes, PIF1, phosphorylation, degradation, 26S ubiquitin-proteasome system, luminescent imaging, EMS mutant screening.

Introduction

Light is one of the most important factors for plant growth and development. As a sessile photosynthetic organism, plants need fine regulation of their response to any environmental changes, such as light quality and quantity. At the seed stage, most plant seeds are induced to germinate by the small amount of light that penetrates through soil. When the young seedlings reach the surface of the soil, they are exposed to more light that changes the morphology of the seedlings to have short hypocotyls, erect and expanded cotyledons, elongated roots and increased chlorophyll biosynthesis. The initiation of the transition from skotomorphogenesis (etiolated seedling) to photomorphogenesis (de-etiolated seedling) is mainly mediated by a class of photoreceptors, called phytochromes (phys) (Bae and Choi, 2008; Leivar and Quail, 2011). There are five phys in *Arabidopsis*, phyA-phyD (Whitelam and Halliday, 1999; Salisbury et al., 2007). The cytosolic biologically inactive form of phys (Pr) can be changed into the active form (Pfr) and transported into nucleus by red light stimuli (Nagatani, 2004; Schäfer and Bowler, 2002; Nagy and Schäfer, 2002). The conformational changes and the trans-localization of the phys can trigger a series of changes in gene expression (Jiao et al., 2007). The phy-mediated gene expression regulation is mediated by a subfamily of basic helix-loop-helix (bHLH) transcription factors called Phytochrome Interacting Factors (PIFs) (Duek and Fankhauser, 2005; Toledo-ortiz et al., 2003; Leivar and Quail, 2011). PIFs act as repressors in the phy-mediated light signaling to prevent photomophogenesis in darkness. The light-induced phosphorylation and degradation of PIFs are necessary for the switch from skotomorphogenesis to photomorphogenesis and are dependent on the interaction of PIFs with phys, at least phyA and/or phyB (Shen et al., 2005; Al-Sady, Ni, Kircher, Schäfer, and Quail, 2006a; Castillon et al., 2007; Shen, Zhu, Castillon, Majee, Downie, and Huq, 2008a; Lorrain et al., 2008; Leivar and Quail, 2011).

The phosphorylation followed by the degradation of the components associated with phys via the ubiquitin-proteasome system is one of the major post-translational regulations of the factors in the light signaling pathways. The mechanisms of dark-induced degradation of positive regulators in the light signaling have been broadly studied (Hoecker, 2005; Henriques et al., 2009). In far red light signaling, the positive regulator LONG

HYPOCOTYL5 (HY5) is degraded in the dark but stabilized under light to promote photomorphogenesis (Osterlund et al., 2000). The unphosphorylated form of HY5 is recognized and degraded in the dark, and the degradation is CONSTITUTIVE PHOTOMORPHOGENESIS1 (COP1) mediated (Hardtke et al., 2000). Similarly, another positive regular, LONG HYPOCOTYL IN FAR-RED1 (HFR1) is degraded in the dark through COP1 (Duek et al., 2004). Unlike the well characterized posttranslational regulation of the positive regulators, little is known about the light-induced degradation of the repressors in light signaling pathways, such as PIFs. All the PIFs except PIF7 are phosphorylated and degraded in response to light (Al-Sady, Ni, Kircher, Schäfer, and Quail, 2006a; Shen et al., 2007; Leivar et al., 2008; Shen, Zhu, Castillon, Majee, Downie, and Huq, 2008a). Recently, casein kinase II has been reported to be involved in the phosphorylation of PIF1, but not in a light-inducible manner (Bu, Zhu, Dennis, et al., 2011; Bu, Zhu, and Huq, 2011). At the protein degradation level, a protein called HEMERA has been shown to be necessary for the nuclear speckle formation of PIF1 and PIF3. HEMERA is predicted to be structurally similar to RAD23 which functions as a polyubiquitylated protein shuffler for degradation (Chen et al., 2010). However, neither the putative protein kinase(s) responsible for the light-induced phosphorylation of PIFs nor the putative E3 ligase(s) for the subsequent ubiquitination and degradation have been identified.

Another type of post-translational regulation for the bHLH proteins such as PIFs is heterodimerization with non-DNA binding HLH proteins to inhibit the DNA binding and transcriptional activation activity of PIFs. In mammals, the interaction of HLH/bHLH proteins and their antagonistic functions have been well studied. A classic example is the human inhibitor of DNA binding (Ids) proteins, which lack the DNA binding motif, function as a negative regulator of bHLH proteins, such as MYOD and NUEROD, through heterodimerization (Benezra et al., 1990; Fairman et al., 1993; Perk et al., 2005). There are 162 bHLH proteins in *Arabidopsis*. Among these, at least 27 of them are non-DNA binding proteins through the domain structure prediction (Toledo-ortiz et al., 2003). Several cases demonstrating that HLH proteins block the DNA binding activity of transcription factors including bHLH proteins have been discovered in plants (Husbands et al., 2007; Hornitschek et al., 2009; Zhang et al., 2009). The first example is bHLH48.

It interacts with LATERAL ORGAN BOUNDARIES (LOB) and blocks LOB binding to DNA, in consequence, regulates the function of LOB at lateral organ boundaries (Husbands et al., 2007). Later on, two studies showed that the antagonistic function of HLH/bHLH also mediates the shade avoidance response and brassinosteroid signaling (Hornitschek et al., 2009; Zhang et al., 2009). We have also identified two HLH proteins, HEC1 and HEC2. Both lack the DNA binding motif and can block the DNA binding of PIF1, which results in decreased transcriptional activation of PIF1 target genes. The side effect of the interaction between HEC1/HEC2 and PIF1 is that the heterodimers may not be recognized by the proteasome machinery. As a result, PIF1 within the heterodimers version is stable and available for homodimerization if necessary, which provides another level of fine tuned protein regulation (Zhu, L., unpublished data). So the association/dissociation of PIFs with other HLH proteins may regulate their binding to E3 ligases and therefore affect their stability.

Although a number of mutants have been identified by genetic screens based on the visible morphological phenotypes, factors involved in the light-induced degradation of PIFs are still unknown. Therefore, new screens must be devised that can detect the rapid and minor posttranslational modifications of PIFs, such as phosphorylation, ubiquitination, degradation and sequestration by HLH proteins mentioned above.

Screening mutants by bioluminescent imaging has been used to identify mutants in circadian clock regulation and abiotic stress signaling (Millar et al., 1992, 1995; Chinnusamy et al., 2002; Lee et al., 2002; Onai et al., 2004). Here I mutagenized *35S: LUC (firefly)-PIF1* transgenic plants by ethyl methanesulfonate (EMS) and monitored the LUC-PIF1 fusion protein level through the intensity of luminescent signal. The main focus of this study is to identify the mutants that have more stable PIF1 protein. In consequence, I can identify the factors involved in the phosphorylation and the degradation of PIF1. So far, I have six best mutant lines that showed stable PIF1 both in luminescent imaging and Western blot assays. This study not only helps identify mutants in PIF degradation pathway, but also demonstrates a new way of mutant screening based on protein level.

Results

The strategy of EMS mutagenesis and mapping by luciferase imaging

About 40,000 well dried *35S:LUC-PIF1* transgenic homozygous seeds have been mutagenized. The mutated seeds were equally distributed into 150 2x2 pots, which represented 150 M1 bulks. Each bulk progeny has been harvested together. One hundred seeds from each bulk were plated to check the luminescent signal under NightOwl camera (Berthold Technologies GmbH & Co. KG, Germany). ~15,000 M2 seedlings have been investigated for the stability of LUC-PIF1 through the luminescent imaging method. All the mutants were back crossed with parental *35S:LUC-PIF1* three times and the homozygous lines were established and sent for SHOREmap (Schneeberger et al., 2009; Schneeberger and Weigel, 2011) to identify the mutation in the mutants. At the same time, the back crossed homozygous lines were crossed with the homozygous seedlings of *35S:LUC-PIF1* ^{x} *Ler* outcross with five times. The F2 seeds from each cross established the mapping population for each individual mutant. The genomic DNA of F2 seedlings containing high luminescent signal were extracted for PCR. Both SSLP and Indel markers are used to map the mutations (Fig. 1).

Six best mutagenic lines showed slow degradation of LUC-PIF1

Among the 15,000 M2 seedlings, thirteen lines inherited the mutation (s) to the M3 generation. The thirteen homozygous mutants were sequenced for *PIF1* gene. Six out of thirteen have the mutation(s) in the PhyA Binding domain (APA) and /or PhyB Binding domain (APB) of *PIF1*, suggesting that these might be seed contamination. In the rest seven lines, one line showed similar phenotype to *phyB* mutant at the adult stage. Sequencing of *PHYB* showed that the mutant contains two mutations in *PHYB* gene, which introduced an early stop codon and results in a truncated version of PHYB.

Among the remaining six best lines, all of them showed stronger luminescent signal after fifteen minutes of continuous white light treatment compared with *35S:LUC-PIF* parental line (Fig. 2). LUC-PIF1 has been reported to have fifteen minutes half life after 3000μmol·m^{-2} red light treatment (Shen, Zhu, Castillon, Majee, Downie, and Huq, 2008b). In my experiment, based on the luminescent signal captured by NightOwl camera (Berthold Technologies GmbH & Co. KG, Germany), LUC-PIF1 fusion protein is

degraded fully after fifteen minutes of continuous white light in *35S:LUC-PIF1* transgenic seedlings. LUC-PIF1 is more stable in all six EMS mutant lines than the *35S:LUC-PIF1* transgenic line. Some of the lines, such as *38A*, *140B* and *141C* showed the same signal level as *PIF1-3M* transgenic line, which has been shown to have very stable PIF1 protein under lights caused by the lack of key amino acids necessary for interactions with phyA and phyB (Shen, Zhu, Castillon, Majee, Downie, and Huq, 2008b).

The loci responsible for the mutants are involved in the degradation of PIF1, not the light induced phosphorylation of PIF1

The LUC-PIF fusion protein level has been verified by Western blot. All six mutants showed slower degradation of LUC-PIF1 than *35S:LUC-PIF1* control after 3000 $\mu molm^{-2}$ Rp (Fig. 3). *20G*, *38A* and *57Q* represent the class, which only showed slight inhibition of degradation of LUC-PIF1. *127C, 140B* and *141C* represent the second class, which has more stabilized LUC-PIF1 protein under the same condition. In the *127C* mutant, the LUC-PIF1 fusion protein is more stable at the early time point. Combined luminescent imaging and Western blot data indicate that *140B* and *141C* are the most promising mutant lines containing the mutation(s) in the genes involved in degradation of PIF1.

The Western blot data also showed that even the mutants have slower degradation of LUC-PIF1, all LUC-PIF1 in the mutants are still phosphorylated after Rp with the band shift (Fig.3). These data also suggest that all the mutants identified so far are responsible for the degradation of PIF1 and most likely not the light-induced phosphorylation of PIF1.

No severe growth defects were found in the mutants

The stabilized PIF1 under light condition might block photomorphogenesis. In consequence, the mutants might contain light green color in the leaves of adult plants, and have longer hypocotyls than wild type. But all the six mutants do not have any severe growth defects. They are normal at the adult stage (data not shown). At the seedling stage, only two lines, *20G* and *38A*, have long hypocotyls (Fig. 4). *20G* showed short hypocotyls in the dark, small cotyledons and short root growth under continuous red light, which suggest a possible hormone defect instead of light signaling pathway defect in this mutant. Opposite to *20G, 38A* has normal growth in the dark, regular cotyledon size and root growth under red light, which indicate defects in light signaling pathways.

The native PIF1 is stable under all light conditions in *38A/Col-0* mutant

Based on the strong hypocotyl phenotype of *38A*, we out-crossed *38A* to Col-0 wild type and selected a homozygous line to eliminate *35S:LUC-PIF1* background. The *38A/Col-0* showed strong long hypocotyl phenotype under continuous red light similar to the original *38A* (Fig.4). This result suggested that the mutation(s) affect the level of native PIF1 as well.

To directly test this possibility, we checked the native PIF1 protein level in *38A/Col-0* under all Rp, FRp and BLp treatment. Under all three light conditions, the native PIF1 is more stable in *38A/Col-0* compared with Col-0 at least at the early time point (Fig. 5A & 5C). These results suggest that the stabilized native PIF1 might be the cause of the long hypocotyl phenotype in 38A/Col-0.

To exclude the possibility that the increased PIF1 proteins in the *38A/Col-0* is due to a higher transcription level of *PIF1* gene in the mutant than in the Col-0 wild type, we checked the *PIF1* gene expression by semi quantitative RT-PCR under the same red light conditions. *PIF1* gene expression is the same in the wild type and the *38A/Col-0* mutant both in the dark and under red light treatment (Fig. 5B), suggesting that the defect is posttranslational.

***38A* contains a mutation in a gene localized on chromosome one**

Because of the strong long hypocotyl phenotype of *38A*, it was mapped using the mutant phenotype from the F2 mapping population created by crossing *38A/Col-0* with Ler. The mutation in *38A* is localized on the upper arm of the chromosome one. It was narrowed down to four BACs, around 400kb (Fig. 6).

There are 56 candidate genes within the 400kb region (data not shown). Twenty of them are unknown genes without any conserved domains. Nine are transcription factors, containing three ARF proteins, two myb-like transcription factor family proteins, one bHLH protein (bHLH80), one bZIP family transcription factor, and one TCP family transcription factor. Three known proteins are in this 400kb region, only one of them has been well characterized, which is a member of 14-3-3 protein family which interacts with BZR1 transcription factor in the brassinosteroid signaling (Gampala et al., 2007).

Sequencing of several target genes and T-DNA insertional mutant analyses failed to identify the mutant gene in *38A*. Further efforts are necessary to clone the gene.

Discussion

The PIF proteins are one of the best characterized families of negative regulators in phy-mediated light signaling pathways. The identification of PIFs provided a simple linear biochemical pathway for light signal integration and transduction, where phytochromes directly interact with and transduce the photo signals to transcription factors in response to light and regulate downstream target gene expression (Leivar and Quail, 2011). All PIFs except PIF7 are rapidly phosphorylated and ubiquitinated *in vivo* prior to their degradation in response to light (Shen et al., 2005; Al-Sady, Ni, Kircher, Schäfer, and Quail, 2006b; Shen et al., 2007). Structure-function relationship studies showed that phytochrome interaction with PIFs is necessary for the light-induced phosphorylation and subsequent degradation of PIFs through the 26S proteasome pathway (Shen, Zhu, Castillon, Majee, Downie, and Huq, 2008a; Al-Sady, Ni, Kircher, Schäfer, and Quail, 2006b). Although major research was focused on identification, phytochrome interactions and early steps in PIF degradation, little is known about the factors necessary for degradation of PIFs.

Genetic and biochemical approaches have identified three classes of mutants that showed stable PIFs under light. The first class is the photoreceptors, phytochromes. Different phytochromes induce degradation of different PIFs with differential kinetics under R and/or FR light conditions (Shen, Zhu, Castillon, Majee, Downie, and Huq, 2008a; Al-Sady, Ni, Kircher, Schäfer, and Quail, 2006b). The kinetics of degradation of different PIFs largely reflect their affinities toward phyA and/or phyB (Shen, Zhu, Castillon, Majee, Downie, and Huq, 2008b; Al-Sady, Ni, Kircher, Schäfer, and Quail, 2006b). This is not surprising as phytochromes are the photoreceptors that perceive light signals to induce degradation of PIFs. The second class only contains one mutant, called *hemera*. HEMERA is necessary for the light-induced degradation of PIF1 and PIF3 (Chen et al., 2010). The homolog of HEMERA in yeast, RAD23, is a multiubiquitin-binding protein, functions in carrying polyubiquitylated proteins to the 26S proteasome for degradation. Using candidate gene approach, we identified a kinase, Casein Kinase 2 (CK2) that

phosphorylates PIF1 independent of light, and CK2-mediated phosphorylation enhances the light-induced degradation of PIF1 (Bu, Zhu, Dennis, et al., 2011). How CK2-mediated phosphorylation of PIF1 promotes light-induced degradation of PIF1 is still unknown (Bu, Zhu, and Huq, 2011). Therefore, neither the protein kinase phosphorylating PIFs nor the E3 ligase responsible for the light-induced degradation of PIFs has been identified yet.

We have taken an unbiased noninvasive LUC imaging based genetic screen to identify mutants affecting PIF stability in response to light. This approach has several advantages over conventional hypocotyl length-based genetic screens. First, the noninvasive nature of this method allows propagation of the seedlings to the next generation after imaging. The noninvasive feature also provides the opportunity to monitor the luminescent signal in every step of the mapping procedure. Second, because of the rapid degradation of PIF1, the LUC reporter system can detect very low levels of PIF1 protein in a real time manner that can't be detected using Western blots. Third, the luminescent signal is quantitative and the data can be used in the mapping process to distinguish homozygous and heterozygous mutants. The luminescent imaging method can be used universally for studying posttranslational regulation of any proteins. Fourth and most importantly, this method allows identification of mutants based on protein level as opposed to morphological phenotypes. Our screen has identified six extragenic mutants that are involved in the degradation of PIF1. However, only one, *38A*, showed strong defect in hypocotyl lengths under light. The *140B* and *141C* mutants have the most stable LUC-PIF1 protein level in both luminescent imaging and Western blot assays. Both mutants do not have strong phenotypes either at the seedling stage or at the adult stage. Both of these mutants would have been eliminated based on conventional hypocotyl-based genetic screens. Identification of these mutants highlights the power of the luminescent imaging screening method compared to the traditional screening methods employed in light signaling field.

Combining phenotypic analyses with the powerful LUC imaging technique allowed us to categorize the mutants that showed robust phenotypes under monochromatic light conditions as well as LUC-PIF1 stability vs only LUC-PIF1 stability without any discernible phenotypes. *38A* mutant showed hyposensitive phenotypes under both

continuous Rc and FRc conditions (Fig. 5.4). The *38A* mutant does not contain any mutation in either *PIF1* or phytochrome genes. These data suggest that *38A* represents a novel positive regulator in the phy-mediated light signaling pathways. In addition, the mutants that did not display any hypocotyl phenotypes (e.g., xyz) also represent a novel class of mutants that displayed molecular phenotypes without any visible phenotypes. Why these mutants don't show any visible phenotype even if they have stable PIF1 is unknown. It is possible that these mutants are defective in degradation of both positive and negatively acting factors involved in light signaling pathways, resulting in balancing out the effects. Alternatively, they might also have defects in other pathways that oppose light signaling pathways. Further characterization of these mutants including *38A* will contribute to better understanding of the networks of phy-mediated light signaling pathways.

Material and Methods

EMS mutagenesis

Around 40,000 seeds of LUC-PIF1 transgenic line have been mutagenized with Ethyl Methane Sulfonate (EMS). Briefly, the seeds were washed in 0.1% tween20 for 15 minutes, slowly rotated in 0.3% EMS in a 50ml falcon tube for 14 hours, washed by 50ml distilled water 3 times, washed by another 50ml distilled water with 2 hours rotation, and re-suspended in 10ml 0.1% agar. The seeds were equally distributed onto 150 2x2 pots by using Eppendorf repeat pipette. Each pot contained 1ml 0.1% agar with seeds. The seeds were then stratified at 4℃ for 4 days, and grown in the greenhouse to maturity. Seeds were harvested into ~150 M2 families for screening.

Luciferase imaging

Approximately 100 seeds from each bulk were grown on a 100×100×15mm square Petri dish for 3 days in the dark, exposed to WL light for 15 minutes, and then Luciferin solution (1 mM Luciferin + 0.01% Triton X-100) was sprayed on the seedlings. The plates remained in the dark for another 5 minutes before being imaged by the NightOWL camera (Berthold Technologies GmbH & Co. KG, Germany). The luminescent signal picture and the regular picture taken under white light have been combined together by

the software Photoshop. The pseudo colors, red for the luminescent signal, green for the regular picture, were added using the same software.

Plant growth condition and phenotypic assay

Plants were grown under constant white light at 22 ℃ for the regular growth and maturity. Seeds were surface sterilized and plated on Murashige-Skoog (MS) growth medium (GM) containing 0.9% agar without sucrose (GM-Suc) as described (Shen et al., 2005). Seeds were stratified at 4 ℃ in the dark for 4 days, and exposed to 3 hours white light at room temperature to induce germination before placing them in the dark for additional 21 hours. The plates were then either placed in the dark or under specific wavelengths of light for an additional 3 days. Two seedlings from each condition were picked up and lined up on a 0.9% agar plate for photographying.

Protein extraction and Western blot

Protein extraction and Western blotting were performed essentially as described (Shen, Zhu, Castillon, Majee, Downie, and Huq, 2008b). Four day-old dark-grown seedlings were either kept in the dark or exposed to pulses of R, FR or BL light followed by incubation in the dark for various times as indicated on each figure before protein extraction. To detect LUC-PIF1 proteins in transgenic plants, 4 days old dark grown seedlings, about 0.2g tissue were ground in 0.8ml of boiling denaturing buffer (100 mM MOPS, pH 7.6, 5% SDS, 10% Glycerol, 4 mM EDTA, 40 mM β-mercaptoethanol, 2 mM PMSF, 1X protease inhibitor for plant cell and tissue extracts [Cat. No. P9599, Sigma-Aldrich]) and boiled for 5 minutes. To detect native PIF1 in wild type plants, the extraction buffer was changed to 100 mM Tris-HCl pH 6.8, 20% glycerol, 5% SDS, 80 μM MG132, 20 mM DTT, 1 mM bromophenol blue, 2 mM PMSF, and 1X protease inhibitor for plant cell and tissue extracts (Cat. No. P9599, Sigma-Aldrich). The rest of the procedure was kept the same. Total proteins were separated on 6% SDS-PAGE gels for LUC-PIF1 detection and 8% SDS-PAGE gels for native PIF1 detection, blotted onto PVDF membrane and probed with anti-LUC, anti-PIF1 or anti-RPT5 antibodies.

RNA isolation and RT-PCR

Total RNA was isolated from 4 day-old seedlings using the SpectrumTM Plant Total RNA Kit (Cat. No. STRN50, Sigma-Aldrich) and reverse transcribed using SuperScript ® III (Cat. No. 18080-085, Invitrogen) as per manufacturer's protocol. The PIF1 was amplified using the forward primer 5'-GATCCCGGGCTGAGAGGGGATTTTAATAACGGTAG-3' and the reverse primer

EH137 with 58℃ annealing temperature by 30 cycles. The control gene UBQ was amplified using the forward primer 5'-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3'

and the reverse primer 5'-CGACTTGTCATTAGAAAGAAAGAGATAACAGG-3' with 62℃ annealing temperature by 23 cycles.

Crude genomic DNA extraction

One to two young leaves were cut from young seedlings and ground. 400µl Thompson Buffer (0.2M Tris·Cl PH 7.5, 0.25M NaCl, 25mM EDTA, and 0.5% SDS) were added and vortexed for 15 seconds. After 15 minutes of micro-centrifuge at 16,000g, the supernatants were transferred into new tubes. The same amount of Isopropanol and the supernatant was pippetted into each tube. The mixture was incubated in the room temperature for 2 minutes and followed by 5 minutes of micro-centrifuge at 16,000g. The supernatants were discarded and the pellets were washed by 70% ethonal. The pellets were air dried and resuspended into 50µl Elution Buffer (10mM Tris·Cl PH 8.0) for PCR.

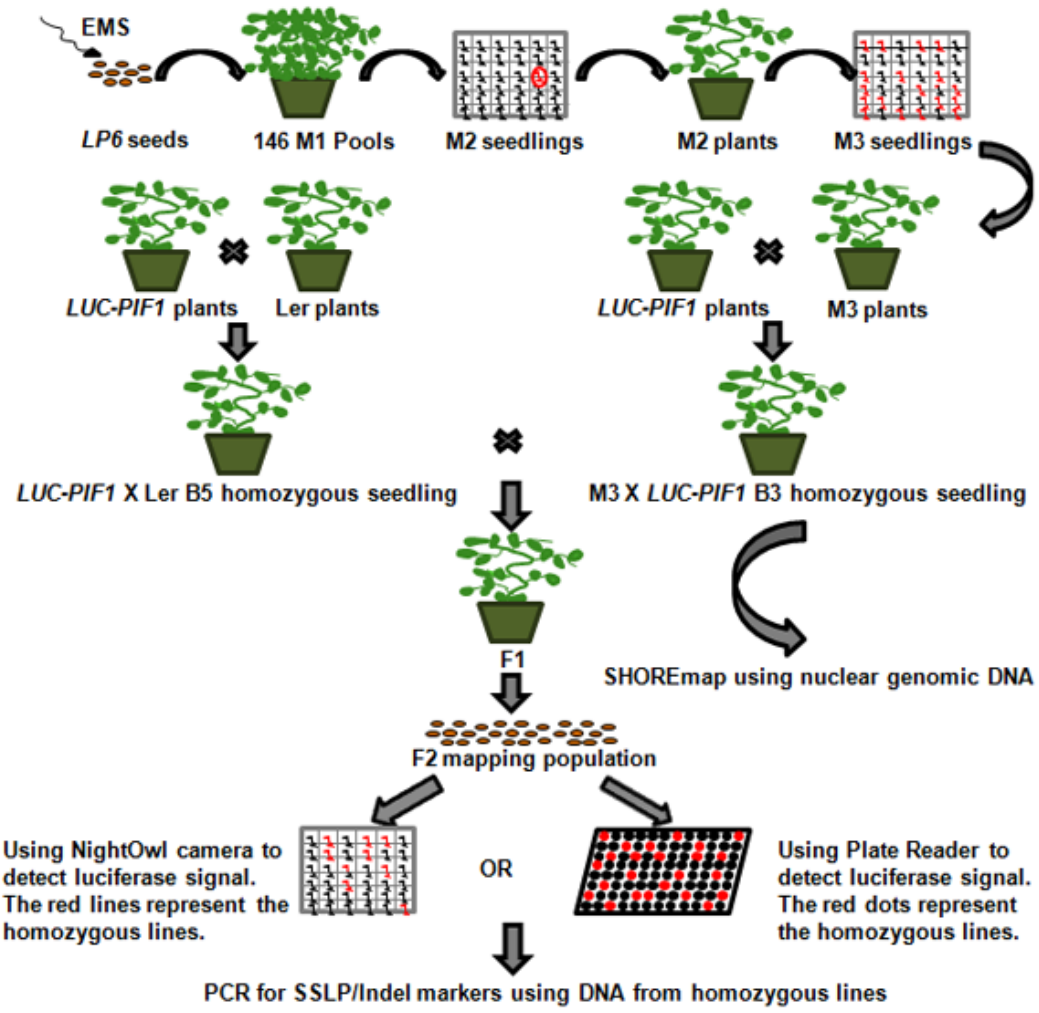

Figure 5.1: Schematic diagram of the EMS mutant screening by luciferase imaging.

Briefly, around 40,000 *LUC-PIF1* transgenic seeds were mutagenized with EMS, equally distributed into 150 pots for growth and harvested in 150 bulks. 100 seeds from each bulk were tested by luciferase imaging as described (Chinnusamy et al., 2002). The seedlings showing stable luminescent signal after 15 minutes of white light exposure were selected and back crossed with the parent line *LUC-PIF1* three times. The homozygous lines after three times backcrosses were outcrossed with the homozygous *LUC-PIF1* seedlings that have been introgressed into Ler by five times crossing. The F2 generation represents the mapping population used for rough mapping SSLP/Indel markers. At the same time, the backcrossed homozygous lines were sent out for sequencing and looking for mutation using SHOREmap method.

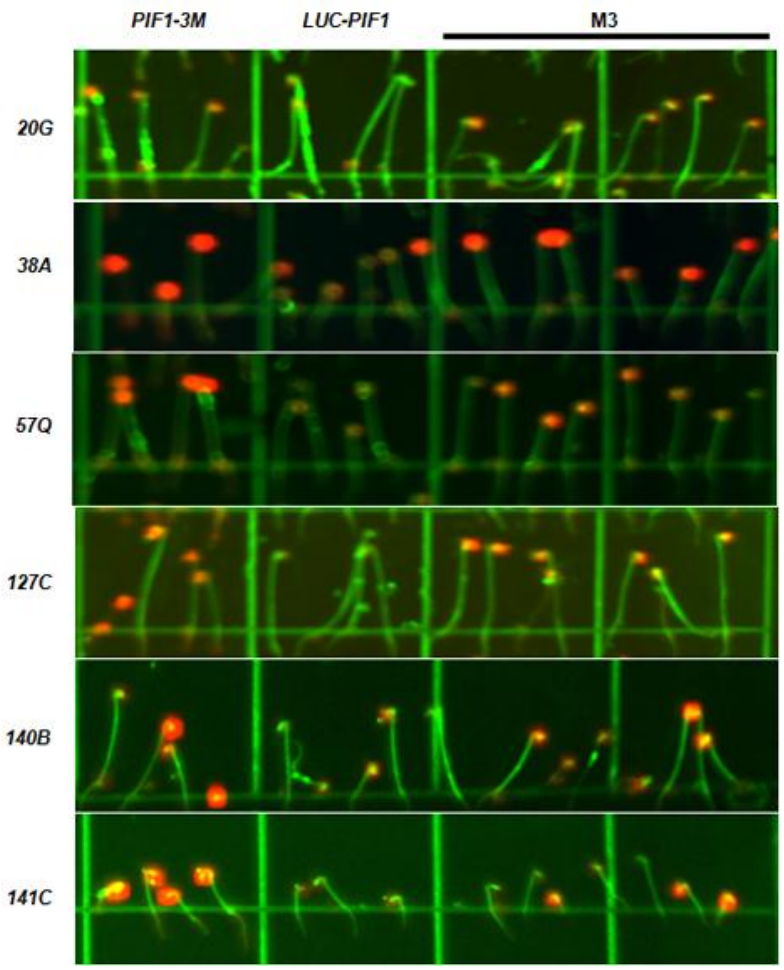

Figure 5.2: Luciferase imaging to identify six best lines showing stable LUC-PIF1 fusion protein level.

Seeds were plated on 100×100×15mm square plates with MS media and imbibed at 4℃ in darkness for four days. The plates were exposed to three hours of continues white light to stimulate germination and then kept in the dark at 21℃ for three days. The luciferase images were taken after 15 minutes of white light exposure followed by 5 minutes treatment with 1mM Luciferin plus 0.01% Triton X-100. Each plate contains *PIF1-3M* as a positive control and *LUC-PIF1* as a negative control.

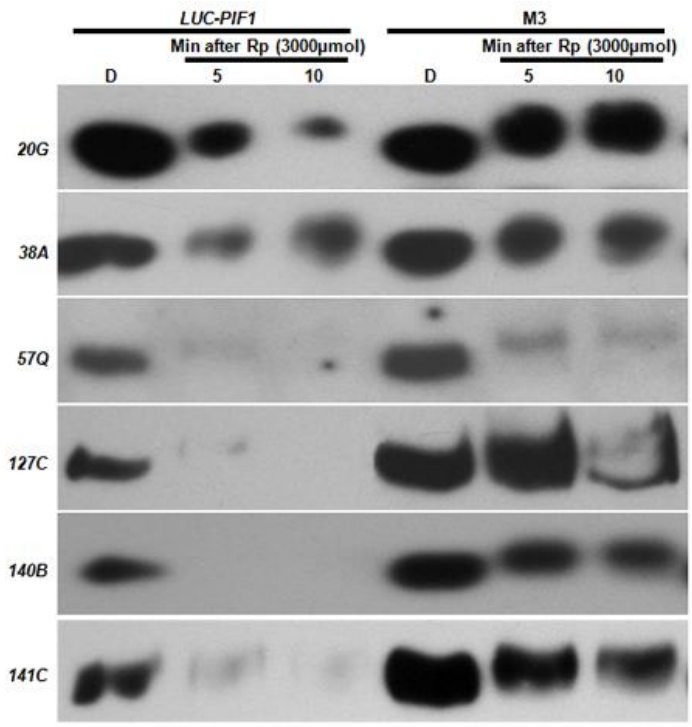

Figure 5.3: Western blots showing slower degradation kinetics of LUC-PIF fusion proteins in the mutant lines compared to the LUC-PIF1 control.

Four day-old dark grown seedling of the mutants and the *LUC-PIF1* were either kept in darkness or given 3000 µmolm^{-2} red light followed by different duration of dark incubation indicated above. The total protein of the mutants and the *LUC-PIF1* were extracted from each time point and loaded into 6% SDS-PAGE gel for Western blot. The LUC-PIF1 fusion protein was detected by the primary antibody against luciferase and visualized using chemiluminescence method.

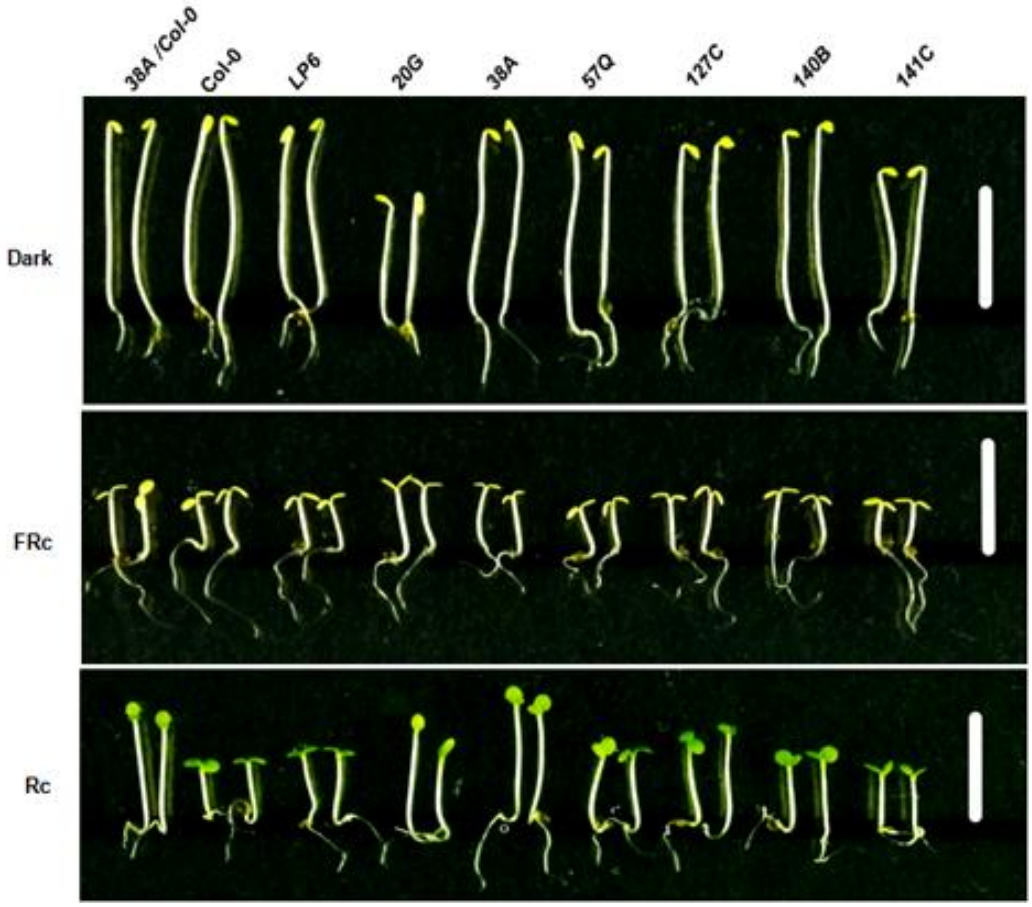

Figure 5.4: The seedling de-etiolation phenotypes of the mutant and the control lines.

Seeds from each line were plated on MS media and stratified at 4℃ in darkness for four days. Three hours of white light treatment was used to induce seed germination. The plates were then wrapped with aluminum foil and kept at 21℃ for 21 hours. Then they were grown under either 7~8μmolm^{-2} red light, or 1 μmolm^{-2} far-red light , or kept in darkness for additional three days. Bar=0.5 cm.

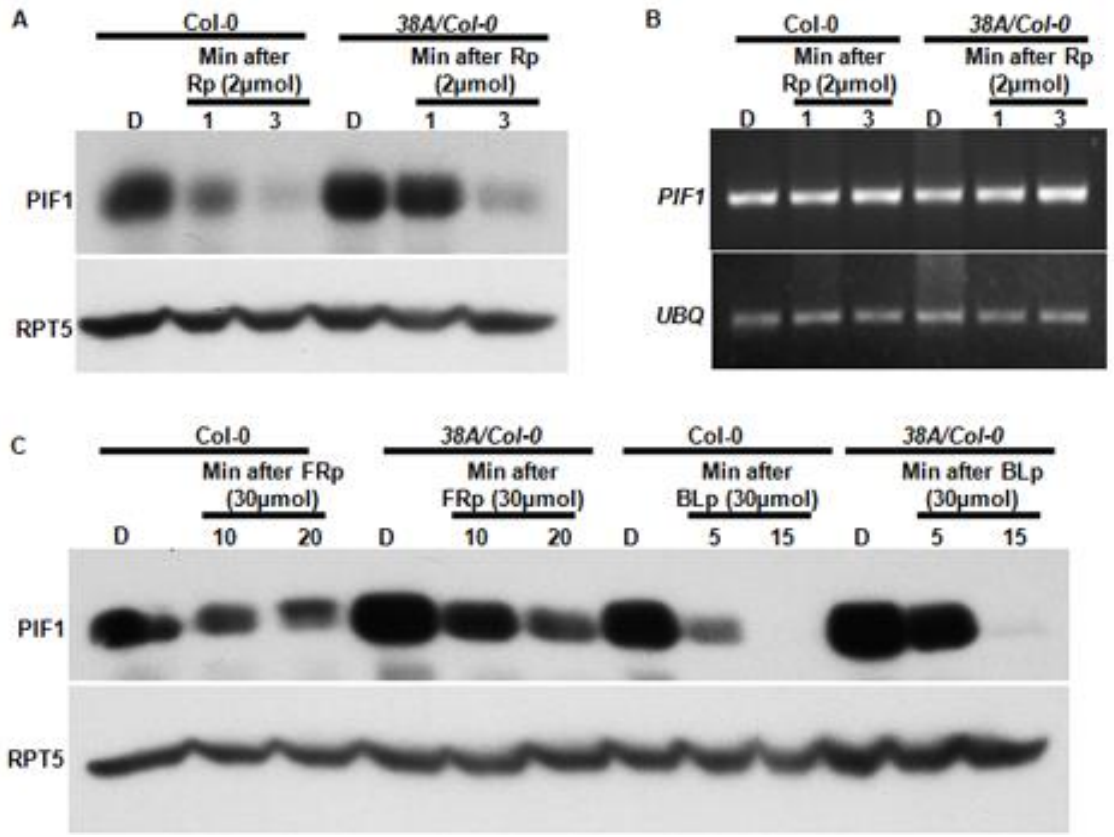

Figure 5.5: The native PIF1 shows slower degradation in the *38A/Col-0* mutant compared to Col-0 under Rp, FRp and BLp conditions.

A) The degradation kinetics of native PIF1 in *38A/Col-0* mutant compared with Col-0 under Rp. The total proteins of each time point were extracted and loaded into 8% SDS-PAGE gel for western blot. The native PIF1 antibody was used as primary antibody. B) PIF1 mRNA levels in Col-0 and the *38A/Col-0* mutant under the same Rp condition as described in A. Total RNAs were extracted from each time point and reverse transcribed by reverse transcriptase. The semi-quantitative PCR was performed to compare the *PIF1* mRNA level in each sample. *UBQ* was used as a control. C) The degradation kinetics of native PIF1 in *38A/Col-0* mutant compared to Col-0 under both FRp and BLp conditions. The Western blot procedure is described in A.

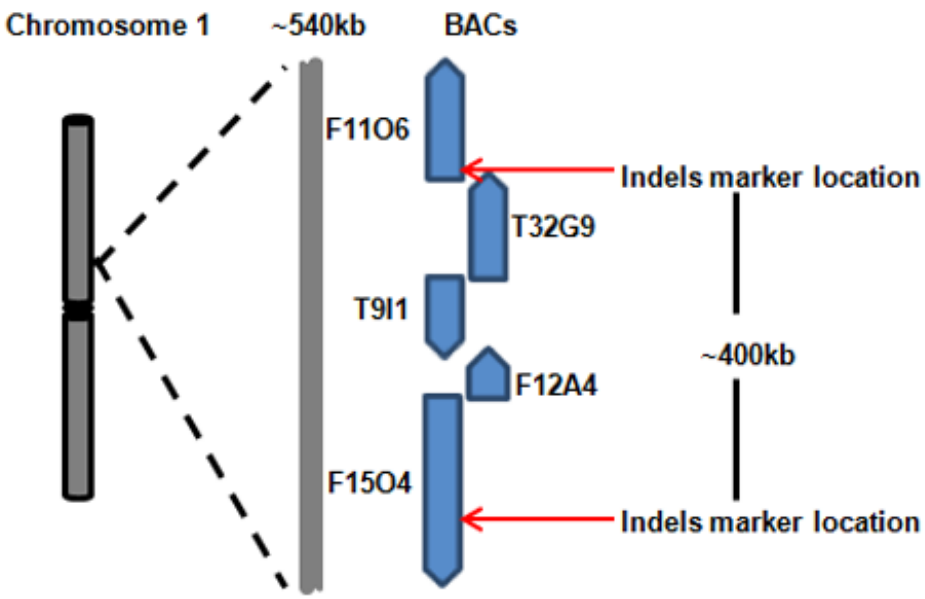

Figure 5.6: Diagram shows the map position of *38A* mutation on chromosome one.

The *38A* locus is in the upper arm of chromosome 1 about 400kb region. The mapping population is derived from *38A/col X Ler* F2 seedlings, and both SSLP and Indel markers were used to map the mutant.

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Chapter VI: Summary

Since 2005, many discoveries have been made to illuminate the light signaling pathways. Besides the well-known photoreceptor-mediated inhibition of cell elongation by light, an additional pathway, where light inhibits cell division through a flavin mononucleotide-binding protein and E3 ligase, has been discovered in rice (Sun et al., 2009). In the photoreceptor mediated light signaling pathways, the UV-B photoreceptor, UV Resistance Locus 8 (UVR8), has been identified in 2011 (Rizzini et al., 2011). In structure-function studies of phytochromes, the Tyr242 residue of phyA and Tyr276 residue of phyB in the universally conserved GAF domain, where the bilin chromophore is associated, plays a key role in the phytochrome mediated light signal perception and transduction. The Tyr to His mutation in both phyA and phyB results in chromophore-dependent constitutive activation of the photoreceptors resulting in photomorphogenesis in the dark (Su and Lagarias, 2007). In addition, the three-dimensional solution structure of the bilin-binding domain of phytochromes as Pfr form was solved using a cyanobacterial phytochrome (Ulijasz et al., 2010). Although the expression of *PHYA* has been shown to be transcriptionally repressed by light in a reversible manner (Cantón and Quail, 1999), a recent epigenetic study demonstrated the presence of activating and repressive histone marks that are responsible for the rapid and reversible light-mediated regulation of *PHYA* (Jang et al., 2011). However, DNA methylation and small RNA pathways are not involved in this process. Phytochrome proteins accumulate in the nucleus upon light treatment. Previously, Far-red elongated Hypocotyl1 (FHY1) and FHY1-like (FHL) have been shown to facilitate the nuclear transport of phyA (Desnos et al., 2001; Zhou et al., 2005), while the light-induced conformation change of phyB was proposed to expose cryptic nuclear localization signal for phyB nuclear import (Chen et al., 2005). However, recent study showed that the interactions between Phytochrome Interacting Factors (PIFs) and phyB mediate in part the light-dependent nuclear import of phyB (Pfeiffer et al., 2012).

The discovery of PIFs, a small group of basic helix-loop-helix (bHLH) transcription factors, heralded a new area in phytochrome research. There are 7 PIFs (PIF1, PIF3-8) in Arabidopsis interacting with five phys (phyA-phyE) in a light dependent manner (Castillon et al., 2007; Leivar and Quail, 2011). PIFs bind sequence-specifically to a G-box (CACGTG) DNA motif and function as transcription factors regulating gene expression (Oh et al., 2009; Leivar and Quail, 2011). PIF3 directly binds to the promoters of anthocyanin biosynthesis genes to positively regulate anthocyanin biosynthesis (Shin et al., 2007). PIF1 increases Abscisic Acid (ABA) level, decreases Gebberellic Acid (GA) level, and particularly binds to the promoters of two GA repressor genes, *GA-Insensitive (GAI)* and *Repressor of GA1-3 (RGA)* to inhibit seed germination (Oh et al., 2007). A genome wide study has shown that PIF1 inhibits seed germination not only through GA and ABA, but also either directly or indirectly regulating other hormone signals, such as cytokinin and auxin (Oh et al., 2009). PIF1 also has been shown to directly bind to *Protochlorophylide Ozidoreductase C (PORC)* in the chlorophyll biosynthesis pathway and *Phytoene Synthase (PSY)* in the carotenoid biosynthesis pathway (Moon et al., 2008; Toledo-Ortiz et al., 2010). In addition, PIF4 and PIF5 have been shown to interact with HFR1, a non-DNA binding HLH protein that regulates PIF4 and PIF5 activity in the shade avoidance syndrome (Hornitschek et al., 2009). The identification of direct targets of all the PIFs is expected to unravel a large regulatory network triggered by the phytochrome-mediated light signaling pathways that regulate plant growth and development.

Mutational studies have shown that PIFs and phys have antagonistic roles in regulating photomorphogenesis. Quadruple *pif* (*pifQ*) mutants displayed constitutive photomorphogenesis in the dark, suggesting that PIFs repress photomorphogenesis in the dark (leivar-2008, Shin et al, 2009). To eliminate this negative regulation and to promote photomorphogenesis in response to light, photoconverted active Pfr forms of phys interact with PIFs and induce their rapid degradation through the ubi/26S-proteasome pathway (Park et al., 2004; Shen et al., 2005; Oh et al., 2006; Leivar and Quail, 2011). The phyA and phyB binding domains of PIFs have been identified and characterized in

multiple PIFs (Al-Sady et al., 2006; Shen et al., 2005). The deletion of the active phyA binding domain (APA) and the active phyB binding domain (APB) in both PIF1 and PIF3 results in the reduced degradation of PIF1 and PIF3 protein under light. These data suggested that both phyA and phyB interactions with PIFs are necessary for the light-induced degradation of PIFs (Al-Sady et al., 2006; Shen, Zhu, Castillon, Majee, Downie, and Huq, 2008a). PIFs are rapidly phosphorylated and poly-ubiquitylated prior to their degradation in response to light (Al-Sady et al., 2006; Shen et al., 2007; Shen, Zhu, Castillon, Majee, Downie, and Huq, 2008a). In a recent study, Casein Kinase II (CKII) has been shown to enhance the light-induced degradation of PIF1 (Bu, Zhu, Dennis, et al., 2011; Bu, Zhu, and Huq, 2011). In addition, a protein called HEMERA (HMR) has been reported to be necessary for the light-induced degradation of PIF1 and PIF3. HMR is similar to the yeast multiubiquitin-binding protein, RAD23, which functions as a shuttle protein to bring poly-ubiquitylated proteins to the proteasome (Chen et al., 2010). Despite these progresses, neither the kinase necessary for the light-induced phosphorylation nor the E3 ligase necessary for the light-induced ubiquitylation of PIFs have been identified yet.

Based on the four specific aims in my dissertation, I contributed in several areas related to the phytochrome-mediated light signaling pathways. Using microarray analysis, we found that PIF1 regulates a discrete set of genes in darkness, which are involved in controlling the chlorophyll biosynthetic pathway. The direct target of PIF1, *PORC*, was identified by using the chromatin immunoprecipitation (ChIP) and DNA gel shift assays. Similar to other PIFs, the G-box (CACGTG) DNA sequence element present in the *PORC* promoter is the binding site for PIF1. Moreover, in transient assays, PIF1 activates transcription of *PORC* in a G-box dependent manner (Moon et al., 2008). As a phytochrome interacting factor, both phyA and phyB binding domains in PIF1 were identified. Both APA and APB motifs are localized at the amino terminus of PIF1, which overlap with the transcriptional activation domain of PIF1 (Shen, Zhu, Castillon, Majee, Downie, and Huq, 2008b). Besides the amino-terminal 150 amino acid region of PIF1, which contains APA and APB motifs, two clusters of Ser phosphorylation sites at the

carboxy-terminal end of PIF1 were shown to be involved in the light-induced degradation of PIF1. The mutant PIF1 with the Ser to Ala (S464AS465AS466A) mutations at the carboxyl-terminus displayed significantly reduced degradation compared to the wild type PIF1 in transgenic plants (Bu, Zhu, Dennis, et al., 2011; Bu, Zhu, and Huq, 2011). In addition to the light-induced turnover of PIFs, antagonistic HLH transcription factors were discovered that regulate PIF1 activity. Two HECATE proteins, HEC1 and HEC2, which belong to the HLH protein family lacking the DNA binding domain, heterodimerize with PIF1 and negatively regulate the function of PIF1. In consequence, HEC1 and HEC2 promote photomorphogenesis in response to light. To identify the kinase and the E3 ligase necessary for the light-induced degradation of PIF1, an EMS mutagenesis coupled with luminescent imaging system was used. Six mutants have been identified with different levels of defect in the degradation rate of PIF1 under light. One of the mutants encodes a novel protein that stabilizes PIF1 under light. The cloning and characterization of these mutants in future will provide key players in the light-induced degradation of PIF1 and possibly other PIFs.

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