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Antagonistic Basic Helix-Loop-Helix/bZIP Transcription Factors Form Transcriptional Modules That Integrate Light and Reactive Oxygen Species Signaling in *Arabidopsis*[W]

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The critical developmental switch from heterotrophic to autotrophic growth of plants involves light signaling transduction and the production of reactive oxygen species (ROS). ROS function as signaling molecules that regulate multiple developmental processes, including cell death. However, the relationship between light and ROS signaling remains unclear. Here, we identify transcriptional modules composed of the basic helix-loop-helix and bZIP transcription factors PHYTOCHROME-INTERACTING FACTOR1 (PIF1), PIF3, ELONGATED HYPOCOTYL5 (HY5), and HY5 HOMOLOGY (HYH) that bridge light and ROS signaling to regulate cell death and photooxidative response. We show that *pif* mutants release more singlet oxygen and exhibit more extensive cell death than the wild type during *Arabidopsis thaliana* deetiolation. Genome-wide expression profiling indicates that PIF1 represses numerous ROS and stress-related genes. Molecular and biochemical analyses reveal that PIF1/PIF3 and HY5/HYH physically interact and coordinately regulate the expression of five ROS-responsive genes by directly binding to their promoters. Furthermore, PIF1/PIF3 and HY5/HYH function antagonistically during the seedling greening process. In addition, phytochromes, cryptochromes, and CONSTITUTIVE PHOTOMORPHOGENIC1 act upstream to regulate ROS signaling. Together, this study reveals that the PIF1/PIF3-HY5/HYH transcriptional modules mediate crosstalk between light and ROS signaling and sheds light on a new mechanism by which plants adapt to the light environments.

INTRODUCTION

Besides being the primary energy source for photosynthesis, light provides signals that regulate diverse aspects of plant growth and development (Chen et al., 2004). In the dark, seedlings undergo skotomorphogenesis and exhibit long hypocotyls and closed cotyledons with undifferentiated chloroplasts. Light-triggered seedling deetiolation (photomorphogenesis) is a particularly important process, as it allows plants to establish autotrophic growth, which is essential for survival. When light is perceived by photoreceptors, including the red/far-red light-absorbing phytochromes (phyA to phyE) and blue/UV-A light-absorbing cryptochromes (cry1 and cry2), the light signals are sequentially transduced to a series of downstream intermediates, leading to changes in transcriptional programs and, eventually, to physiological changes, including reduced hypocotyl growth, opening of cotyledons, and chloroplast development (Quail, 2002; Chen et al., 2004).

Genetic and molecular studies have uncovered dozens of intermediates that relay the light signal. A group of CONSTITUTIVE PHOTOMORPHOGENIC (COP)/DEETIOLATED/FUSCA proteins acts as central repressors of photomorphogenesis downstream of both phytochromes and cryptochromes (Wei and Deng, 1996). COP1, a RING (for Really Interesting New Gene) finger protein, possesses E3 ubiquitin ligase activity toward a number of photomorphogenesis-promoting factors, facilitating their targeted degradation through the 26S proteasome pathway (Yi and Deng, 2005). The transcription factors ELONGATED HYPOCOTYL5 (HY5) and its close homolog, HY5 HOMOLOG (HYH), and PHYTOCHROME-INTERACTING FACTORs (PIFs) are known to mediate two distinct signaling branches of the photomorphogenic response in *Arabidopsis thaliana* (Castillon et al., 2007; Lau and Deng, 2010). HY5 and HYH are a pair of bZIP transcription factors that play pivotal roles in positively regulating seedling deetiolation (Oyama et al., 1997; Holm et al., 2002). Genome-wide gene expression and chromatin immunoprecipitation (ChIP)–based sequencing analyses revealed that HY5 directly binds to thousands of genes and regulates the expression of a broad range of genes (Lee et al., 2007; Zhang et al., 2011). Consistent with their biological function, HY5 and HYH are degraded in the dark by the COP1-mediated degradation pathway but are stabilized in the light (Osterlund et al., 2000; Holm et al., 2002). HY5 has also been reported to mediate plant responses to hormones, cold, and UV-B (Ulm et al., 2004; Lau and Deng, 2010; Catalá et al., 2011), indicating that HY5 serves as a master modulator during plant growth and development. PIFs are a small subfamily of basic helix-loop-helix (bHLH) transcription factors that play multiple functions in processes such as seed germination, seedling deetiolation, and shade avoidance responses (Castillon et al., 2007; Leivar et al., 2008; Shin et al., 2009; Stephenson et al., 2009; Leivar and Quail, 2011). Contrary to HY5/HYH, PIFs accumulate in the dark to promote skotomorphogenesis, whereas light induces the rapid phosphorylation and degradation of PIFs, through the activity of phyB and phyA (Shen et al.,

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2005; Al-Sady et al., 2006; de Lucas et al., 2008; Henriques et al., 2009). Molecular studies showed that PIFs directly regulate the expression of downstream genes by binding to a G-box motif (CACGTG) present in their promoters (Martínez-García et al., 2000; Huq et al., 2004; Shin et al., 2007). PIF1 and PIF3 are also involved in facilitating the seedling greening process, in part by negatively regulating the chlorophyll biosynthetic pathway in the dark (Huq et al., 2004; Moon et al., 2008; Shin et al., 2009; Stephenson et al., 2009).

Reactive oxygen species (ROS) are common byproducts of normal metabolism in cells from bacteria to mammals and act as important signaling molecules that regulate multiple developmental processes, such as root growth, stress tolerance, senescence, pathogen defense, and hormonal responses (Apel and Hirt, 2004; Mittler et al., 2004; Gechev et al., 2006; Miller et al., 2008). ROS are produced in cellular compartments, particularly when plants are subjected to environmental stress conditions. For instance, excess light irradiation results in the generation of ROS, including hydrogen oxygen, superoxide, and singlet oxygen, in chloroplasts, and this leads to photooxidative damage in plant cells or even to cell death (Li et al., 2009). Consequently, the ability of plants to use light energy for photosynthesis is limited (op den Camp et al., 2003; Gechev et al., 2006). Singlet oxygen has a short lifetime and is a strong electrophilic reagent that reacts with many biological molecules, including DNA, proteins, and lipids (Triantaphylidès and Havaux, 2009). The intensity, duration, and localization of different ROS signals are determined by the interplay between the ROS-producing and ROS-scavenging pathways of cells (Mittler et al., 2004). Thus, plants have evolved mechanisms that tightly regulate the ROS gene network to modulate the steady state level of ROS (Miller et al., 2008). Some important signaling components of the ROS gene network, such as transcription factors and protein kinases, have been identified by genetic and molecular approaches (Mittler et al., 2004; Miller et al., 2008). Studies also suggested that ROS signaling is integrated with many other signaling networks in plants; however, the mechanisms that mediate these interactions are poorly understood (Mittler et al., 2011). Although light is known to affect ROS production, the manner by which light signals regulate ROS production and ROS signaling remains unknown.

Here, we demonstrate that PIF1 and PIF3 prevent singlet oxygen production and cell death during seedling deetiolation in *Arabidopsis*. PIF1 regulates the genome-wide expression of ROS-responsive genes. We show that PIF1/PIF3 and HY5/HYH physically interact to form transcriptional modules that directly bind to the G-box motif in the promoters of five representative ROS signaling genes and regulate their expression coordinately. In addition, the functions of PIF1 and PIF3 largely require the presence of HY5 and HYH. Furthermore, phyA, phyB, cry1, and cry2 photoreceptors and COP1 are also involved in regulating ROS signaling. Our study demonstrates that key components of the light signaling pathway modulate the ROS signaling network and thus affect plant fitness in excess light.

RESULTS

PIF1 and PIF3 Redundantly Promote Seedling Greening and Prevent Singlet Oxygen Production

Previous studies documented that loss of either *PIF1* or *PIF3* caused overaccumulation of protochlorophyllide (Pchlide), a precursor of chlorophyll, in dark-grown seedlings and resulted in photobleaching upon transfer to light (Huq et al., 2004; Moon et al., 2008; Shin et al., 2009; Stephenson et al., 2009). The etiolated seedlings of the *pif5* mutant also showed the photobleaching phenotype after light exposure (Shin et al., 2009). To examine whether these PIF proteins have redundant functions in regulating seedling greening, we examined the phenotypes of the *pif1 pif3* double and *pifq* quadruple mutant (loss of *PIF1*, *3*, *4*, and *5*) (Leivar et al., 2008). The levels of Pchlide of dark-grown seedlings were determined by monitoring the fluorescence emission of the samples with a fluorescence spectrophotometer (Tang et al., 2012). We found that *pifq* possessed the highest levels of Pchlide and that *pif1 pif3* also accumulated more Pchlide than either of the single mutant parents and the Columbia (Col) wild type (see Supplemental Figure 1A online). We then tested the greening ability by subjecting 4-d-old dark-grown seedlings to increasing periods of growth in moderate light (60 μmol m^{-2} s^{-1}) followed by 2 d of growth in weak light (10 μmol m^{-2} s^{-1}). In the absence of moderate light treatment, ~80 and 60% seedlings of *pif1 pif3* and *pifq*, respectively, turned green normally, whereas *pif1* and *pif3* seedlings were indistinguishable from those of the wild type (Figure 1A). When the etiolated seedlings were exposed to moderate light for 5 min, ~68 and 98% of seedlings of *pif3* and *pif1 pif3*, respectively, were photobleached, while all of the *pifq* seedlings died. Moderate light treatments for 15 min to 6 h caused photobleaching in ~40 and 85% of *pif1* and *pif3* seedlings, respectively (Figure 1A). These data indicate that PIF3 and PIF1 play major and redundant roles in promoting seedling greening and that the etiolated seedlings are extremely sensitive to the onset of relatively high light illumination. In the following experiments, all light treatments were performed in 60 μmol m^{-2} s^{-1} light.

It was reported that the seedling photobleaching phenotype during the dark-to-light transition is largely caused by ROS (Reinbothe et al., 1996). We then examined the cellular ROS levels of *pif1*, *pif3*, *pifq*, and the wild type by detecting the fluorescence of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), a ROS-sensitive dye, in the cotyledons (Tang et al., 2012). When 4-d-old dark-grown seedlings were illuminated with light for 24 h, H_2DCFDA fluorescence (Figure 1B, shown in green) was strong in *pif1* and further increased in *pif3* and *pifq* compared with the wild type, whereas chlorophyll autofluorescence was only observed in the wild-type cotyledons (Zhong et al., 2009; Figure 1B). Free Pchlide is a potent photosensitizer that generates singlet oxygen upon illumination (op den Camp et al., 2003). We thus investigated singlet oxygen production by these mutants using the fluorescent probe Singlet Oxygen Sensor Green (SOSG) (Flors et al., 2006). As shown in Figure 1C, the cotyledons of *pif1*, *pif3*, and *pifq* displayed SOSG fluorescence, whereas the wild type did not, indicating that the mutants release singlet oxygen. Superoxide and hydrogen peroxide are often simultaneously generated in cellular compartments. Surprisingly, the levels of both superoxide and hydrogen peroxide, as determined by nitroblue tetrazolium (NBT) and diaminobenzidine (DAB) staining, respectively, were reduced in *pif1* and completely absent in *pif3* and *pifq*, whereas they were abundant in the wild type (see Supplemental Figures 1B and 1C online). These results imply that PIF1 and PIF3

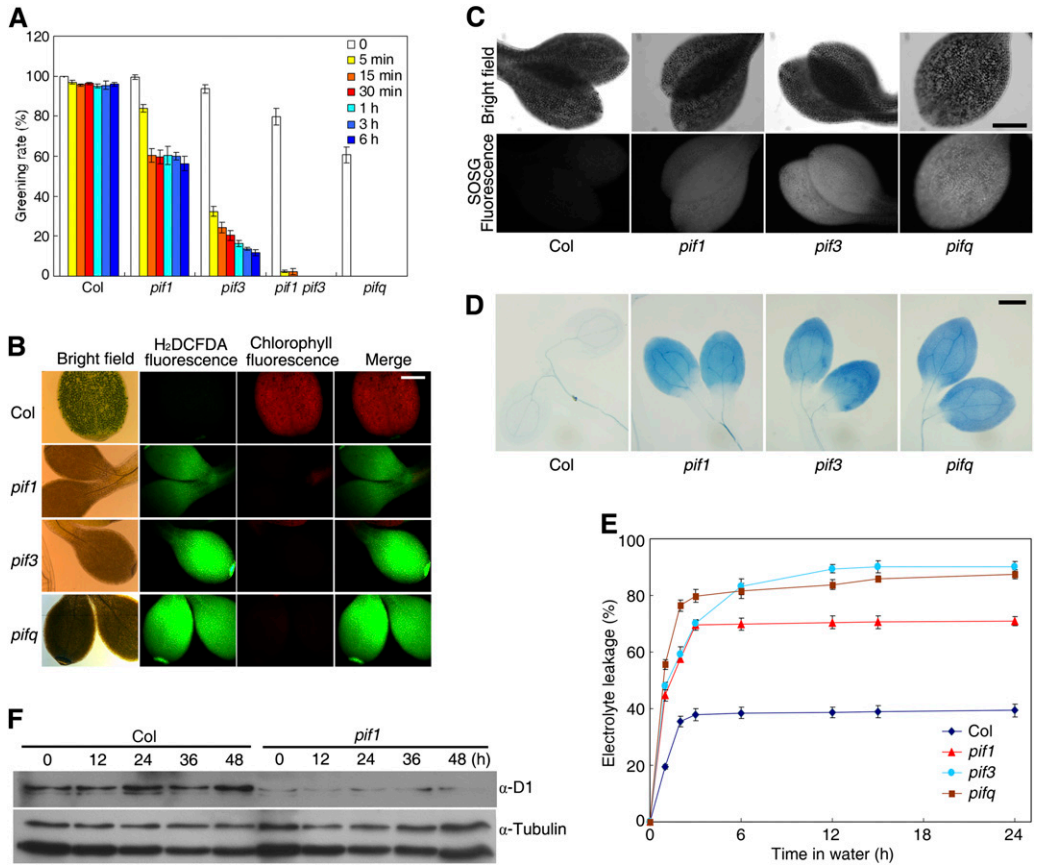

Figure 1. PIF1 and PIF3 Redundantly Promote Seedling Greening and Prevent Singlet Oxygen Production and Cell Death.

(A) Percentage of greening cotyledons in the wild type (Col) and various *pif* mutants under different light treatments. Four-day-old dark-grown seedlings were first exposed to growth light (60 μmol m^{-2} s^{-1}) for the indicated periods of time and then transferred to weak light (10 μmol m^{-2} s^{-1}) for 2 d. Data are mean ± SD, $n = 3$.

(B) to **(D)** Four-day-old etiolated seedlings were exposed to light (60 μmol m^{-2} s^{-1}) for 24 h **(B)** or 6 h (**[C]** and **[D]**). Bars = 200 μm.

(B) Cellular ROS levels in the cotyledons of the wild type (Col) and various *pif* mutants. H_2DCFDA fluorescence (green) indicates ROS, and chlorophyll autofluorescence is shown in red.

(C) Singlet oxygen production in the cotyledons as determined by SOSG fluorescence.

(D) Trypan blue staining of cotyledons of the wild type (Col) and *pif1*, *pif3*, and *pifq*.

(E) Electrolyte leakage of the *pif* mutants and wild-type seedlings. Four-day-old etiolated seedlings were exposed to light (60 μmol m^{-2} s^{-1}) for 12 h and immersed in water, and electrolyte leakage was measured periodically. Data are mean ± SD, $n = 3$.

(F) Turnover of D1 protein in the *pif1* mutant compared with the wild type. Immunoblot of a tubulin protein serves as a control. Seedlings were grown in darkness for 4 d before exposure to light (60 μmol m^{-2} s^{-1}) for the indicated periods of time.

specifically prevent singlet oxygen production during seedling deetiolation.

PIF1 and PIF3 Prevent Cell Death during Deetiolation

To determine whether photooxidative damage resulted in cell death in the *pif* mutants, seedlings were stained with trypan blue, which marks dead or dying cells. We found that the cotyledons of *pif1*, *pif3*, and *pifq* mutants showed a prominent increase in trypan blue staining; however, such stress symptoms were weakly detected in the wild-type control (Figure 1D). To quantify the extent of cell death in these mutants, we analyzed the cell death–induced electrolyte leakage of etiolated seedlings subjected to various periods of light treatment. In agreement with the trypan blue staining results, electrolyte leakage during the first 3 h of light irradiation was significantly greater in *pif1* and *pif3* than in the wild type and was greatest in the *pifq* mutant (Figure 1E). After 6 h, the electrolyte leakage of the *pif3* mutant was similar to that of *pifq* (Figure 1E). A previous study suggested that quenching of singlet oxygen is primarily linked to the rapid turnover of D1 protein of the photosystem II reaction center (Telfer et al., 1994). As expected, the steady state level of D1 protein in the *pif1* seedlings was significantly reduced relative to that of wild-type seedlings (Figure 1F). Taken together, our results indicate that PIF1 and PIF3, and possibly also PIF5, repress cell death during seedling deetiolation.

PIF1 Regulates Genome-Wide ROS-Responsive Gene Expression

To obtain insight into the regulation of PIFs at the whole-genome level, we performed a microarray study to compare global gene expression changes between *pif1* and Col of 4-d-old etiolated seedlings treated with light for 3 h. Compared with the wild type, 1466 genes were induced, whereas 1409 were downregulated twofold or more in *pif1* (see Supplemental Data Set 1 online). A functional classification of differentially expressed genes was performed using GO::TermFinder (Boyle et al., 2004). Among the upregulated genes, categories of response to abiotic, biotic stimulus/stress, hydrogen peroxide, high light, oxidative stress, defense, and hormones were significantly overrepresented (Figure 2A; see Supplemental Data Set 2 online for the complete list). Notably, the frequency of genes involved in the response to high light and hydrogen peroxide was 11 times greater in *pif1* than that found in the whole genome. These genes encode transcription factors, such as ethylene-responsive transcription factors (ERF13, ERF4, and ERF1) and WRKY transcription factors (WRKY51, WRKY67, and WRKY46), various regulatory proteins (zat zinc finger proteins, ZAT10 and ZAT12, sigma factor binding protein, SIB1), heat shock protein (HSP17, HSP90, and HSP21), and mitogen-activated protein kinases (MAPKKK19, MKK9, and MPK3), which are important components of ROS signaling pathways (Apel and Hirt, 2004; Mittler et al., 2004; Miller et al., 2008). We also observed differentially expressed genes involved in jasmonate signaling (*JAZ7*, *JAZ8*, and *JAZ5*), defense (*BAP1*, *PDF1.2*, and *RPS6*), and antioxidation (*APX2*, *AOX1*, and *MDAR2*).

The repressed genes are mostly localized to the plastid and cytoplasm. Remarkably, the enrichment of plastid-localized proteins was more than 3 times that found in the whole genome (Figure 2B). These genes are involved in various aspects of photosynthesis and chloroplast development, such as the light reaction, light harvesting, chlorophyll biosynthesis, electron transport, and response to light, consistent with the photooxidative phenotype of the *pif1* mutant (see Supplemental Data Sets 1 and 3 online).

A comparative analysis with previous light-regulated transcriptomes (Charron et al., 2009) revealed that 210 of the genes induced in *pif1*, and 734 of the genes repressed in *pif1*, were regulated by light (Figure 2C). PIF1 is capable of directly binding to the promoters of downstream targets through a G-box motif (Oh et al., 2009). Interestingly, 341 (23.2%) and 307 (21.8%) of the induced or repressed genes, respectively, possess at least one putative G-box motif within the 2-kb promoter regions (Figure 2D). These data suggest that PIF1 may directly regulate a wide range of ROS-responsive downstream genes by binding to their promoters.

PIF1 and PIF3 Bind to and Regulate ROS-Responsive Genes

To test the above hypothesis, two genes with prominent roles in ROS signaling, *ZAT10* and *ASCORBATE PEROXIDASE2* (*APX2*) (Miller et al., 2008), two singlet oxygen-responsive genes, *SIGMA FACTOR BINDING PROTEIN1* (*SIB1*) and *ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR4* (*ERF4*) (Laloi et al., 2007), and an oxidative stress–induced gene, *NDB2* [encoding

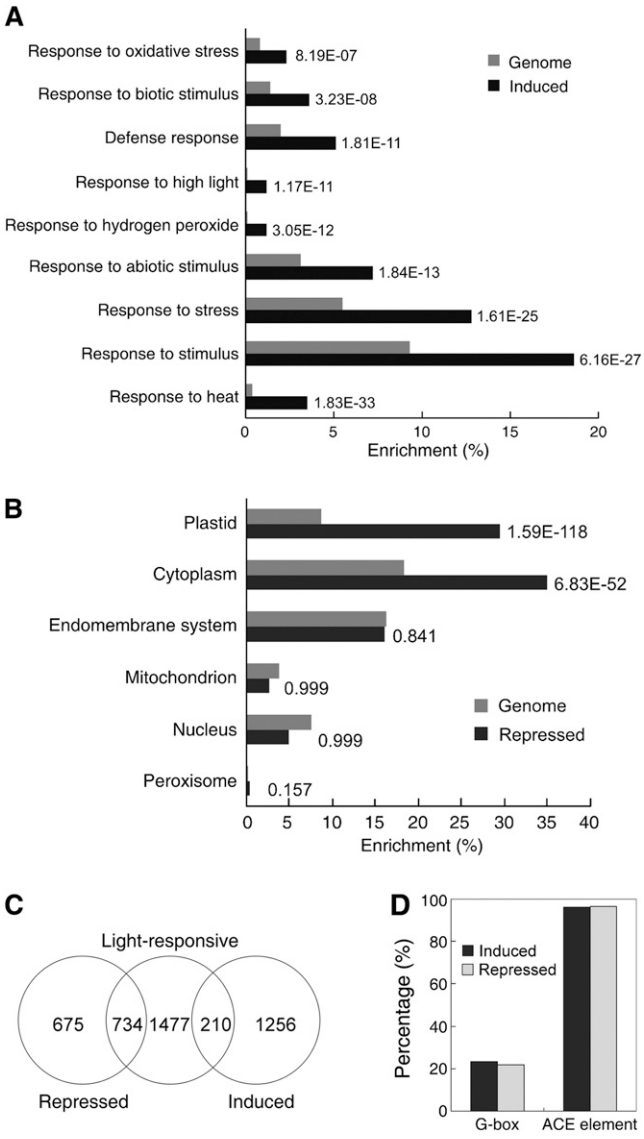

Figure 2. Microarray Analysis of PIF1-Regulated Genes.

(A) Enrichment of selected categories of GO biological process in genes induced in *pif1*. The numbers on the right are P values calculated based on their relative abundance in the wild-type genome. For a complete list of significant GO terms, see Supplemental Data Set 2 online.

(B) Enrichment of selected categories of GO cellular component in genes repressed in *pif1*. The numbers on the right are P values calculated based on their relative abundance in the wild-type genome. For a complete list of significant GO terms, see Supplemental Data Set 3 online.

(C) Venn diagram showing the overlap of differentially regulated genes in *pif1* identified in this study with previously reported light-responsive genes (Charron et al., 2009).

(D) Distribution of the putative G-box motif (CACGTG) and ACE element (ACGT) in the 2-kb promoter regions of PIF1-regulated genes.

a NAD(P)H dehydrogenase] (Ho et al., 2008), were selected for further analysis. Quantitative RT-PCR (qRT-PCR) analysis showed that these genes were upregulated in *pif1* and *pif3* compared with the wild type after more than 1 h of light exposure (60 μmol m^{-2} s^{-1} for 1 to 3 h; Figure 3A), suggesting that PIF1 and PIF3 repress ROS-responsive gene expression in the light. The expression of *APX2* and *ERF4* was even increased in the *pif3* mutant relative to the wild type when seedlings were exposed to light for half an hour. By contrast, no obvious expression difference between the mutants and wild type was observed in the dark-grown seedlings (Figure 3A). Furthermore, we found that after low light (10 μmol m^{-2} s^{-1}) irradiation, the expression of *APX2* and *ERF4* was also upregulated in the *pif3* and *pif1 pif3* mutants compared with the wild-type seedlings (see Supplemental Figure 2 online).

We conducted electrophoretic mobility shift assays (EMSAs) to test if PIF1 could bind to the DNA of the downstream genes in vitro. GST-PIF1 (PIF1 fused with glutathione *S*-transferase) recombinant proteins were expressed in *Escherichia coli* and incubated with ^{32}P-labeled oligonucleotide fragments that contain putative G-box sequences of the target promoters. As shown in Figures 3B and 3C, GST-PIF1, but not GST alone, caused a mobility shift of the promoter fragments of *APX2*, *ZAT10*, *SIB1*, and *ERF4* (lane 2). Moreover, the amount of shifted band was significantly decreased by the addition of excess unlabeled wild-type DNA (lane 3) but not by that of the mutant competitors in *NDB2* (CACGTG changed to CTTGTG; Figure 3C).

To analyze the protein-DNA binding in vivo, we performed ChIP experiments using transgenic seedlings expressing *Pro35S*:*TAP-PIF1* (Moon et al., 2008) or *Pro35S*:*Myc-PIF3* (see Supplemental Figure 3 online). After precipitation with anti-Myc antibody, the DNA fragments were quantified by real-time PCR using primers spanning a region of the promoter that contains the G-box motif (region 1) and primers spanning a region that is upstream of the G-box motif (region 2) (Figure 3D). We found that the occupancy of PIF1 at region 1 of *APX2*, *ZAT10*, *SIB1*, *ERF4*, and *NDB2* was remarkably higher than at region 2 (Figure 3E). Similarly, PIF3 was strongly recruited to region 1 of those genes (Figure 3F). Hence, PIF1 and PIF3 directly associate with these key ROS-responsive genes.

HY5 and HYH Directly Bind to ROS-Responsive Genes

Previous studies documented that the ACE element (ACGT) and its special form G-box motif are putative binding sites of HY5 (Lee et al., 2007; Zhang et al., 2011). We found that the ACE element is overrepresented in the promoters of PIF1-regulated genes (Figure 2D). We then asked whether HY5 and its close homolog HYH could also bind to DNA of the ROS-responsive genes. GST-HY5 and GST-HYH recombinant fusion proteins were incubated with probes of *ERF4*, *SIB1*, *APX2*, *ZAT10*, and *NDB2* genes in the absence or presence of cold competitors. As shown in Figure 4A, both GST-HY5 and GST-HYH bound strongly to these genes and caused bands to shift upwards on the gel, although the affinity of HYH for the promoter fragment of *APX2* was weak. These bands were much fainter or completely absent in the presence of excess amounts of unlabeled wild-type oligonucleotides but not in the presence of unlabeled oligonucleotides containing the G-box mutation (*ZAT10* and *NDB2* are shown), demonstrating that HY5 and HYH bind to the promoters of these genes directly via the G-box in vitro. To substantiate the binding in vivo, we performed ChIP assays of DNA isolated from Col wild-type seedlings using an anti-HY5 antibody. We found that the region 1 fragments of *APX2*, *ZAT10*, *SIB1*, *ERF4*, and *NDB2* were significantly enriched compared with region 2 fragments after precipitation with the HY5 antibody (Figure 4B), indicating that HY5, like PIF1 and PIF3, associates with the promoters of ROS-regulated genes in plant cells.

PIF1 and PIF3 Physically Interact with HY5 and HYH

The ability of PIF1, PIF3, HY5, and HYH to bind the same *cis*-element of the target genes prompted us to test whether PIF1/PIF3 and HY5/HYH could interact with each other. We thus performed an in vitro pull-down assay using His-fused PIF1 (His-PIF1) or His-PIF3, and GST-tagged HY5 (GST-HY5) or GST-HYH recombinant fusion proteins. Our results showed that His-PIF1 and His-PIF3, but not proteins extracted from *E. coli* expressing His empty vector, were able to coprecipitate GST-HY5 and GST-HYH (Figure 5A). Using a yeast two-hybrid assay, we found that a LexA DNA binding domain fusion of the C-terminal fragment of PIF1 (LexA-PIF1C) interacted with the C terminus of PIF1 or HY5 tagged with B42 activation domain (AD-PIF1C or AD-HY5C) (see Supplemental Figure 4 online). The full-length and N terminus of PIF1 showed strong transcriptional activation activity, as previously reported (Shen et al., 2008). This result indicates that the C-terminal portions of PIF1 and HY5, which contain the bHLH and bZIP domain, respectively, are responsible for mediating their interaction.

We next performed coimmunoprecipitation assays using proteins isolated from *Pro35S*:*TAP-PIF1* or *Pro35S*:*Myc-PIF3* transgenic plants or from the wild type to substantiate the interaction in vivo. As shown in Figure 5B, the HY5 antibody was able to immunoprecipitate TAP-PIF1 or Myc-PIF3 fusion proteins, as detected by the Myc antibody, both in dark-grown seedlings and in plants transferred to light for 30 min. Next, a bimolecular fluorescence complementation (BiFC) assay was conducted by transiently coexpressing the N terminus of yellow fluorescent protein (YFP^n)– and the C terminus of YFP (YFP^c)–fused constructs in *Arabidopsis* protoplasts (Walter et al., 2004). Coexpression of HY5-YFP^n and PIF3-YFP^c, or HY5-YFP^n and PIF1-YFP^c, reconstituted a functional YFP in the nucleus with strong fluorescence in plants grown in darkness, but weak fluorescence after exposure to light (Figure 5C). Similarly, a firefly luciferase (LUC) complementation imaging (LCI) assay further showed that coexpression of HY5-LUC^n and LUC^c-PIF1, or PIF3-LUC^n and LUC^c-HY5, caused high levels of LUC activity (see Supplemental Figure 5 online). PIF3-LUC^n and LUC^c-PIF1 showed strong interaction, as previously documented (Bu et al., 2011). Furthermore, a fluorescence resonance energy transfer (FRET) analysis was conducted by coexpressing PIF1-YFP and HY5-CFP in the protoplasts. The sharp drop in fluorescence intensity of the acceptor PIF1-YFP due to photobleaching was compensated for by the increase in the intensity of the donor HY5-CFP, and the FRET efficiency was ~0.2 to ~0.3, whereas the FRET efficiency of the control where HY5-CFP was cotransformed with YFP alone was below 0.03 (see Supplemental Figure 6 online). Collectively, these data demonstrate that PIF1 and PIF3 physically

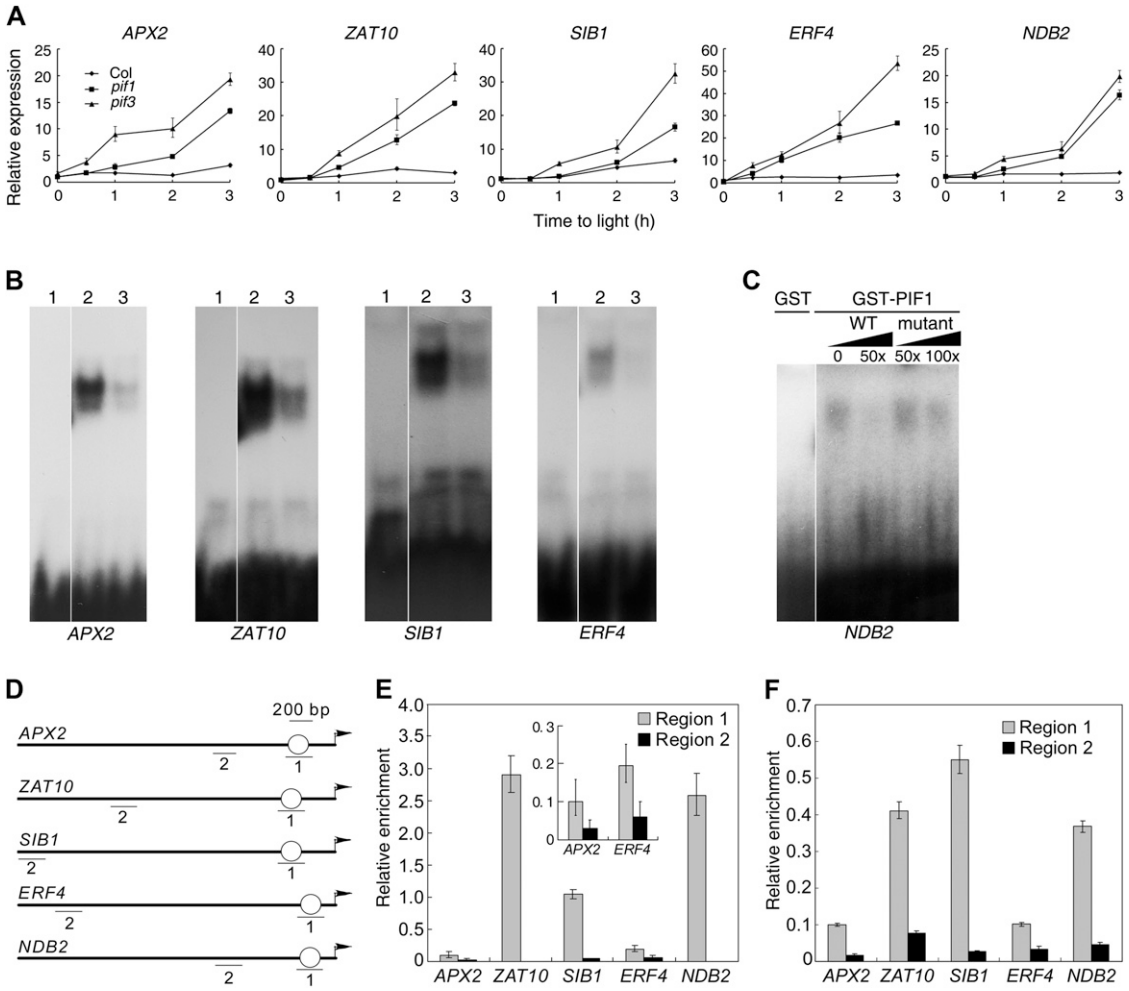

Figure 3. PIF1 and PIF3 Directly Inhibit ROS-Responsive Gene Expression in the Light.

(A) Relative expression of various genes by qRT-PCR. Four-day-old etiolated seedlings were kept in darkness or transferred to light (60 μmol m^{-2} s^{-1}) for up to 3 h. Data are mean ± SD, $n = 3$.

(B) EMSA of the binding of promoter fragments of the indicated genes to GST-PIF1 or GST-PIF3. Lane 1, GST protein only; lanes 2 and 3, GST-PIF1 fusion protein without (lane 2) or with (lane 3) cold competitor DNA. Signals at the bottom indicate free probes.

(C) EMSA of the binding of the *NDB2* promoter fragment to GST-PIF1. WT, wild-type cold competitor; mutant, cold competitor with a mutation in the G-box. The numbers indicate the amount of excess cold competitor added to the reaction mix.

(D) Promoter diagrams of genes that function downstream of PIF1 and PIF3. Arrows indicate the translation start sites of the genes. Circles denote the position of the G-box motif. "1" and "2" indicate the approximate positions of primers used for ChIP amplification.

(E) and **(F)** ChIP assays showing the enrichment of regions 1 and 2 of DNA isolated from *Pro35S:TAP-PIF1* **(E)** and *Pro35S:Myc-PIF3* **(F)** plants following precipitation with an anti-Myc antibody. Seedlings were grown in darkness for 4 d and then were irradiated (60 μmol m^{-2} s^{-1}) for 30 min. Data are mean ± SD, $n = 3$. Inset in **(E)** is the enlargement for *APX2* and *ERF4* genes.

interact with HY5 in the nucleus to form heterodimers both in dark and light conditions.

PIF1/PIF3 and HY5/HYH Coregulate ROS-Responsive Genes

The observation that PIF1/PIF3 and HY5/HYH physically interact and bind to common targets suggests that they might coregulate downstream genes. To address this possibility, we introduced *hy5* and/or *hyh* mutations into the *pif1* mutant background through genetic crossing, and double or triple homozygous mutants were used for analyses. When 4-d-old etiolated seedlings were transferred to light for a series of time (up to 3 h), the expression of *APX2*, *ZAT10*, *SIB1*, and *ERF4* was lower in *hy5* than in the wild type after 1 h of light exposure. However, the transcript levels of *APX2*, *ZAT10*, *SIB1*, *ERF4*, and *NDB2* were drastically reduced in the *hy5 pif1* double mutant and reduced even further in the *hy5 hyh pif1* triple mutant compared with those in the *pif1* single mutant background (Figure 6A), indicating that the repression activity of PIF1 is partly dependent on both HY5 and HYH in vivo. We

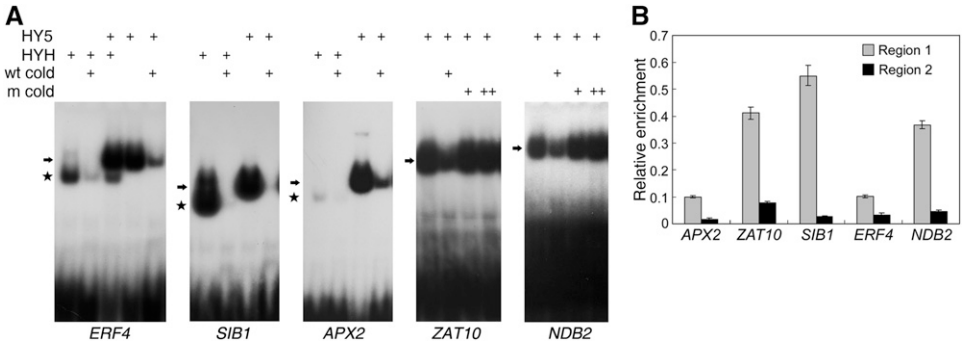

Figure 4. HY5 Binds to the Promoter Regions of ROS-Responsive Genes in Vitro and in Vivo.

(A) EMSA of the binding of various promoter fragments to GST-HY5 or GST-HYH recombinant proteins. Arrows indicate HY5-DNA complexes; stars denote HYH-DNA complexes. wt cold, unlabeled wild-type competitor DNA; m cold, unlabeled competitor DNA with mutations (CttGTG) in the G-box motif. For cold DNA, "+" and "++" indicate a 50- and 100-fold excess, respectively.

(B) ChIP assays showing enrichment of regions 1 and 2 in DNA isolated from Col wild-type plants following precipitation with an anti-HY5 antibody. Regions 1 and 2 are defined in Figure 3D. Seedlings were grown in darkness for 4 d and then exposed to light (60 μmol m^{-2} s^{-1}) for 30 min. Data are mean ± SD, $n = 3$.

found that after light exposure, HY5 protein level was gradually increased, while the level of PIF1 was drastically reduced (Shen et al., 2005; see Supplemental Figures 7A and 7B online). To determine whether the two types of proteins are sensitive to light quantity during dark-to-light transition, we conducted immunoblotting with 4-d-old etiolated seedlings exposed to various intensities of white light. The data indicate that the stability of HY5 and PIF1 is modulated in a light quantity–dependent manner (see Supplemental Figures 7C and 7D online). Consistently, PIF1 was found to be sensitive to the intensity of monomeric red and far-red light (Shen et al., 2005, 2008). As the direct targets of PIF1/PIF3 and HY5/HYH, the transcript levels of *APX2*, *ZAT10*, *SIB1*, *ERF4*, and *NDB2* were gradually increased by increasing light intensities (see Supplemental Figure 7E online).

Next, we transiently expressed HY5 and/or PIF1/PIF3, together with a LUC reporter gene driven by the *ERF4* promoter (the 2.0-kb region upstream of the ATG start site), in *Arabidopsis* protoplasts. As shown in Figure 6B, HY5 greatly promoted LUC

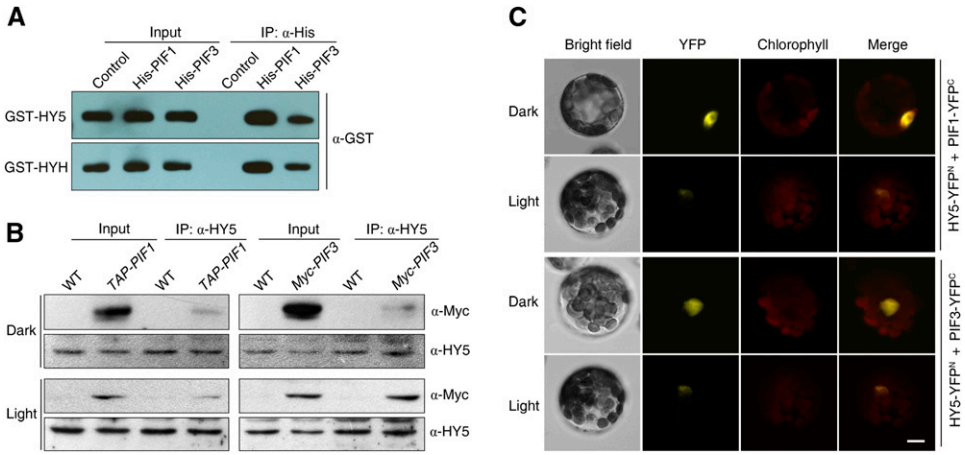

Figure 5. PIF1 and PIF3 Physically Interact with HY5 and HYH.

(A) In vitro pull-down assay of His-PIF1 or His-PIF3 and GST-HY5 or GST-HYH. His-PIF1 or His-PIF3 fusion proteins were incubated with GST-HY5 or GST-HYH and pulled down by nickel-nitrilotriacetic acid agarose. The precipitated fractions were probed with an anti-GST antibody. Control, proteins extracted from *E. coli* expressing His empty vector. IP, immunoprecipitation.

(B) In vivo coimmunoprecipitation assay between TAP-PIF1 or Myc-PIF3 with HY5. *Pro35S*:*TAP-PIF1*, *Pro35S*:*Myc-PIF3*, or Col wild-type (WT) seedlings were grown in darkness for 4 d and then either kept in the dark or transferred to light (60 μmol m^{-2} s^{-1}) for an additional 30 min. After precipitation with the anti-HY5 antibody, proteins were immunoblotted with anti-HY5 or anti-Myc antibodies.

(C) BiFC analysis of interactions between HY5, PIF1, and PIF3 in the nuclei of *Arabidopsis* protoplasts. After cotransformation, the protoplasts were incubated in darkness for 16 h and then kept in darkness or exposed to light (10 μmol m^{-2} s^{-1}) for 1 h before observation. Chlorophyll autofluorescence is shown in red. YFP^n and YFP^c, the N-terminal or C-terminal fragment of YFP, respectively. Bar = 5 μm.

reporter gene expression, whereas PIF1 inhibited the expression of LUC. Remarkably, coexpression of PIF1 or PIF3 drastically suppressed the activation activity of HY5 on the *ProERF4:LUC* reporter, suggesting that HY5 and PIF1/PIF3 associate with the promoter and act together to fine-tune *ERF4* expression. Consistently, HY5 and PIF1 were able to form a heterodimer and bind to the promoter of *APX2* and *ERF4* in vitro (see Supplemental Figure 8 online).

To determine whether the binding of PIF1 to the promoters of the target genes requires HY5, we performed ChIP assays using *Pro35S:TAP-PIF1* transgenic plants in the wild-type and *hy5* mutant backgrounds. Our data showed that relatively less target DNA was pulled down by the Myc antibody in the *hy5* mutant than in the wild type both in dark-grown and light-treated seedlings (Figure 6C), suggesting that the DNA binding activity of PIF1, in part, requires the presence of HY5. Similarly, the binding activity of HY5 to the targets was also partly dependent on PIF1 and PIF3, as mutations in both *PIF1* and *PIF3* diminished the enrichment of downstream genes when pulled-down samples were analyzed with the HY5 antibody (Figure 6D). These data further indicate that HY5 and PIF1 are able to bind to these ROS-related genes both in darkness and after light irradiation.

PIF1/PIF3 and HY5/HYH Function Antagonistically

We next investigated how HY5 and HYH affect the function of PIF1 and PIF3. Mutation in *HY5* partly inhibited the phenotypes of high Pchlide levels and low greening rates of *pif1*, and to a lesser extent of *pif3*. Relative to *hy5*, the *hyh* single mutant had minor effects on these phenotypes (Figures 7A and 7B). As shown in Figure 7C, we observed that H_2DCFDA fluorescence was barely detected in the *hy5* mutant seedlings after light treatment. The high level of H_2DCFDA fluorescence in the *pif1* mutant (Figure 1) was largely suppressed by the *hy5* mutation in the *hy5 pif1* double mutant, while the fluorescence in the *hy5 hyh pif1* triple mutants was almost identical to that in the wild type. Compared with the *pif3* single mutant (Figure 1), H_2DCFDA fluorescence was also greatly reduced in the *hy5 pif3* and *hy5 hyh pif3* mutants. Consistent with this, singlet oxygen generation was largely blocked in

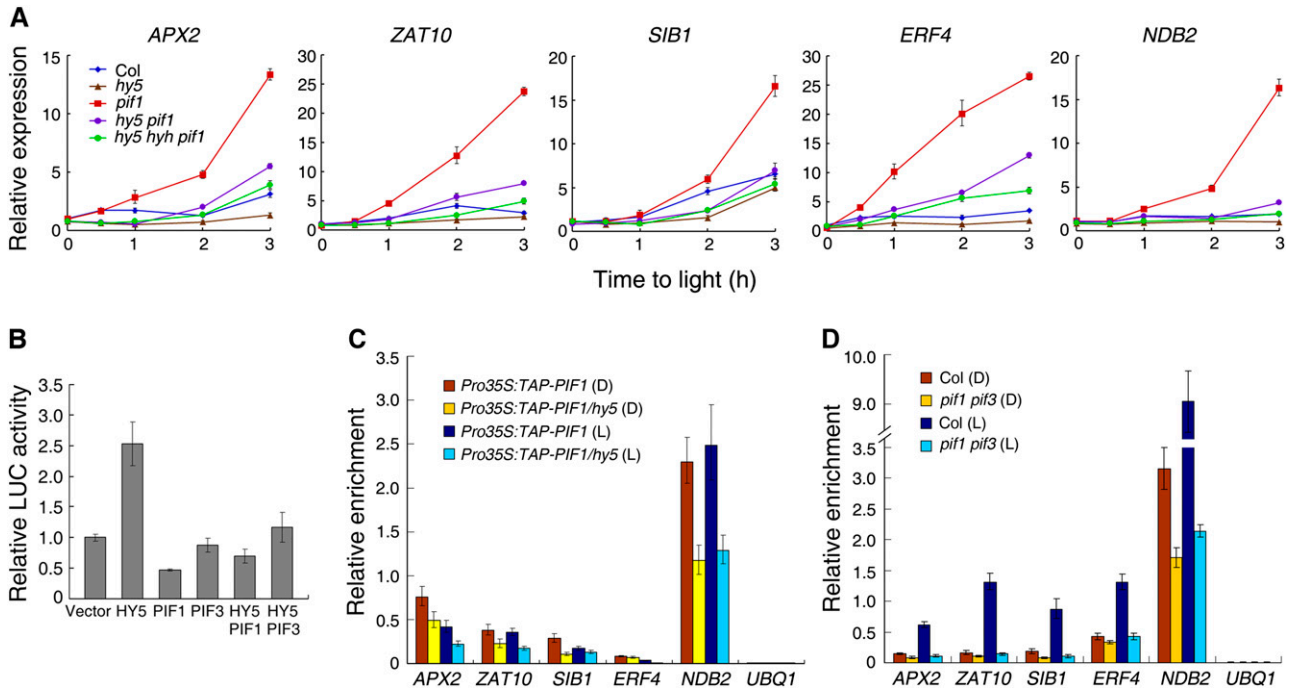

Figure 6. PIF1/PIF3 and HY5/HYH Coregulate ROS-Responsive Genes.

(A) qRT-PCR showing the relative expression of various ROS-responsive genes. Four-day-old etiolated seedlings were kept in darkness or transferred to light (60 μmol m^{-2} s^{-1}) for up to 3 h. Data are mean ± SD, n = 3.

(B) The relative activity of the *ProERF4:LUC* reporter in *Arabidopsis* protoplasts cotransformed with the indicated effector constructs. The relative LUC activities were normalized to the *Pro35S:GUS* internal control. Protoplast transformation, incubation, and protein extraction were performed in darkness. Mean ± SD, n = 3.

(C) and **(D)** Seedlings were grown in darkness (D) for 4 d or irradiated with light (L; 60 μmol m^{-2} s^{-1}) for 30 min. Data are mean ± SD, n = 3. The enrichment of *UBQ1* serves as a negative control.

(C) Relative enrichment of region 1 fragments (shown in Figure 3D) in DNA isolated from *hy5* and Col wild-type plants harboring *Pro35S:TAP-PIF1* and coimmunoprecipitated with the anti-Myc antibody.

(D) Relative enrichment of region 1 fragments (shown in Figure 3D) in DNA isolated from *pif1 pif3* and Col wild-type plants coimmunoprecipitated with HY5 antibody.

the *hy5 pif1* and *hy5 pif3* double mutants relative to *pif1* and *pif3* (Figures 1 and 7D). Accordingly, trypan blue staining showed that the extent of cell death of the *hy5 pif1* and *hy5 pif3* double mutants was greatly reduced and close to the levels in the wild type (Figure 7E). Moreover, the electrolyte leakage of *hy5 pif1* and *hy5 hyh pif1* plants dropped to levels similar to those of the wild type (Figure 7F).

We further examined how the overexpression of *PIF1*, *PIF3*, or *HY5* affects seedling greening and downstream gene expression. We found that the expression of ROS-responsive genes, including *ZAT10*, *SIB1*, *ERF4*, and *NDB2*, was decreased in the *Pro35S:TAP-PIF1* and *Pro35S:Myc-PIF3* transgenic lines, while the levels of *APX2*, *ZAT10*, *SIB1*, and *ERF4* were increased in the *Pro35S:HA-HY5* overexpression plants compared with the wild-type seedlings (see Supplemental Figure 9A online). Consistently, *PIF1* and *PIF3* overexpression lines have higher greening rate than the wild type when dark-grown seedlings were exposed to high light (250 μmol m^{-2} s^{-1}). By contrast, *HY5* overexpression plants showed reduced greening ability compared with the control (see Supplemental Figure 9B online). We further examined the responses of PIF1 and PIF3 overexpression in the *hy5* mutant background and found that their phenotypes were even stronger than the single mutant/transgenic line, consistent with their antagonistic role (see Supplemental Figures 9C and 9D online). Together, these observations demonstrate that HY5/HYH and PIF1/PIF3 antagonistically regulate singlet oxygen production and cell death during the seedling greening process. Interestingly, we further observed that HY5 and PIF1/PIF3 had a slight regulatory effect on each other at the protein level (see Supplemental Figure 10 online).

Phytochromes and Cryptochromes Positively Regulate and COP1 Negatively Regulates ROS Signaling

We speculated that the phytochrome and cryptochrome photoreceptors and COP1 might also contribute to the regulation of ROS signaling, as they are the upstream components in the light signaling pathway. To test this hypothesis, we grew seedlings of the *phyA*, *phyB*, *phyA phyB*, *cry1*, *cry2*, and *cop1* mutants and also of the Col wild type in darkness for 4 d followed by 3 h of light exposure. qRT-PCR results showed that the transcript levels of *APX2*, *ZAT10*, *SIB1*, *ERF4*, and *NDB2* were remarkably reduced in all of the photoreceptor mutants but were drastically increased in *cop1* (Figure 8A), indicating that phyA, phyB, cry1, and cry2 promote ROS-responsive gene expression, whereas COP1 represses it. Surprisingly, the etiolated seedlings of *phyA*, *phyB*, *phyA phyB*, *cry1*, and *cry2* exhibited low Pchlide levels relative to the wild type (see Supplemental Figure 11A online). Accordingly, the photoreceptor mutants had higher greening rates than the wild type when seedlings were grown in the dark for 4 d or longer and then exposed to light (see Supplemental Figure 11B online). A previous study showed that the *cop1* mutant accumulated extremely high levels of Pchlide and was unable to turn green after light exposure (Zhong et al., 2009). Consistently, the photoreceptor mutants accumulated lower levels of singlet oxygen and underwent less cell death than the wild type. By contrast, the *cop1* mutant displayed higher levels of singlet oxygen and cell death than the wild type (Figures 8B to 8D). These results indicate that the phyA, phyB, cry1, and cry2 photoreceptors and COP1 have opposite effects on ROS signaling and cell death.

DISCUSSION

Key Light Signaling Components Regulate Singlet Oxygen Production and Cell Death

During the past decades, numerous studies have established the role of light signaling networks in regulating diverse plant growth and developmental processes (Quail, 2002; Chen et al., 2004; Lau and Deng, 2010). Recently, the light signaling pathways have been shown to interact with phytohormone signaling pathways to mediate distinct responses (Alabadí and Blázquez, 2009). In this study, we provide insight into the functional diversity of the key components in light signaling pathways that contribute either positively or negatively to singlet oxygen production and cell death. We show that the first 5 min of exposure to relatively high levels of light (60 μmol m^{-2} s^{-1}) are critical for the survival of etiolated seedlings and that PIF1, PIF3, and possibly also PIF5 are required for survival during the onset of illumination (Figure 1). In addition, the survival rate of plants exposed to light correlates inversely with the levels of Pchlide, in agreement with our previous report (Tang et al., 2012). Pchlide is a potent photosensitizer in the chloroplast that leads to photooxidative damage in plants (op den Camp et al., 2003). Accordingly, the *pif* mutants displayed severe cell death, as determined by trypan blue staining as well as electrolyte leakage, during the dark-to-light transition (Figure 1). We demonstrate that PIF proteins, including PIF1, PIF3, and PIF5, specifically and redundantly prevent singlet oxygen production during seedling deetiolation, with PIF1 and PIF3 playing the major roles. However, the cotyledons of these mutants accumulated reduced amounts of superoxide and hydrogen peroxide, possibly due to the antagonistic effect on other ROS by singlet oxygen (Laloi et al., 2007). This study also shows that the *pif1* and *pif3* phenotypes largely depend on the presence of functional HY5 and HYH, supporting a negative role of HY5/HYH in regulating greening and plant survival (Figure 7). Thus, PIF1/PIF3 and HY5/HYH have opposite effects on the regulation of ROS and cell death.

The phyA, phyB, cry1, and cry2 photoreceptors promote Pchlide synthesis in the dark and induce ROS signaling in the light (Figure 8). We speculate that the phytochromes and cryptochromes might also transduce signals in the cytosol to regulate tetrapyrrole biosynthesis, even in darkness, although the underlying mechanism needs to be determined. cry1 has been shown to trigger blue light–dependent, singlet oxygen–mediated programmed cell death, as the *cry1* mutant suppressed the cell death phenotype of *fluorescent* (*flu*) after the dark-to-blue light transition (Danon et al., 2006). The *cop1* mutants accumulate a high level of singlet oxygen and almost die after transition from dark to light (Figure 8), consistent with a previous report showing high levels of Pchlide accumulation in *cop1* (Zhong et al., 2009). These phenotypes of *cop1* are similar to those observed for the *pifq* mutant, in agreement with their constitutive photomorphogenic response in darkness (Leivar et al., 2008; Shin et al., 2009).

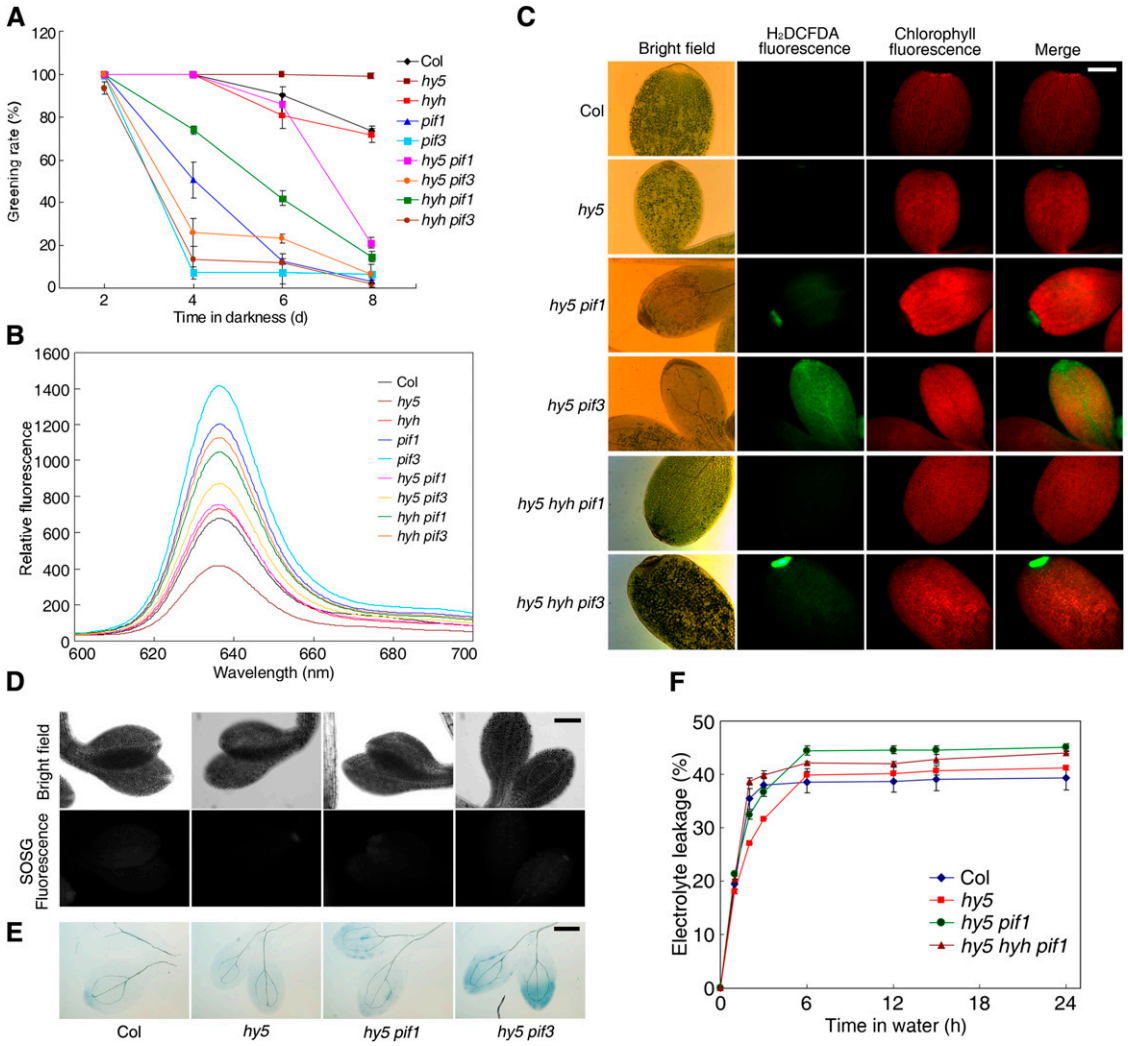

Figure 7. Genetic Interaction between HY5/HYH and PIF1/PIF3.

(A) Seedling greening rate. Two- to eight-day-old etiolated seedlings were transferred to light (60 μmol m^{-2} s^{-1}) for an additional 2 d. Mean ± SD, $n = 3$.
(B) Pchlide accumulation of 4-d-old dark-grown seedlings in the indicated mutants and the wild type.
(C) H_2DCFDA fluorescence showing cellular ROS production of 4-d-old dark-grown seedlings after 24 h of light exposure (60 μmol m^{-2} s^{-1}).
(D) and **(E)** SOSG fluorescence imaging **(D)** and trypan blue staining **(E)** of 4-d-old etiolated seedlings after light illumination (60 μmol m^{-2} s^{-1}) for 6 h. Bars = 200 μm.
(F) Relative electrolyte leakage showing the extent of cell death. Four-day-old etiolated seedlings were exposed to light (60 μmol m^{-2} s^{-1}) for 12 h and immersed in water, and electrolyte leakage was measured periodically. Data are mean ± SD, $n = 3$.

Therefore, light signaling networks not only remodel the morphological structures of plants (e.g., inhibit hypocotyl elongation and promote the unfolding of cotyledons), but also trigger cellular and metabolic changes that ensure plant survival and development.

ROS regulate many processes in plants; however, little is known about the specific ROS responses that underlie particular stimuli (Mittler et al., 2004, 2011; Miller et al., 2008). The *flu* mutant was previously shown to specifically generate singlet oxygen in etiolated seedlings irradiated with light or in plants grown under a dark–light cycle (op den Camp et al., 2003; Wagner et al., 2004). Our data demonstrate that PIF1 and PIF3 represent two components that predominantly regulate the production of singlet oxygen. However, the molecular mechanisms by which PIF1/PIF3 and FLU mediate singlet oxygen generation and signaling might be diverse, since the former proteins are transcription factors in the nucleus, whereas the latter one is localized in the plastid and its biochemical function is not well understood. It will be interesting to test whether (and how) PIF1/PIF3 and FLU could coact to regulate singlet oxygen signaling.

Interaction between bZIP and bHLH Transcription Factors

Direct interactions between PIF1 and PIF3 and between HY5 and HYH have been documented previously (Holm et al., 2002;

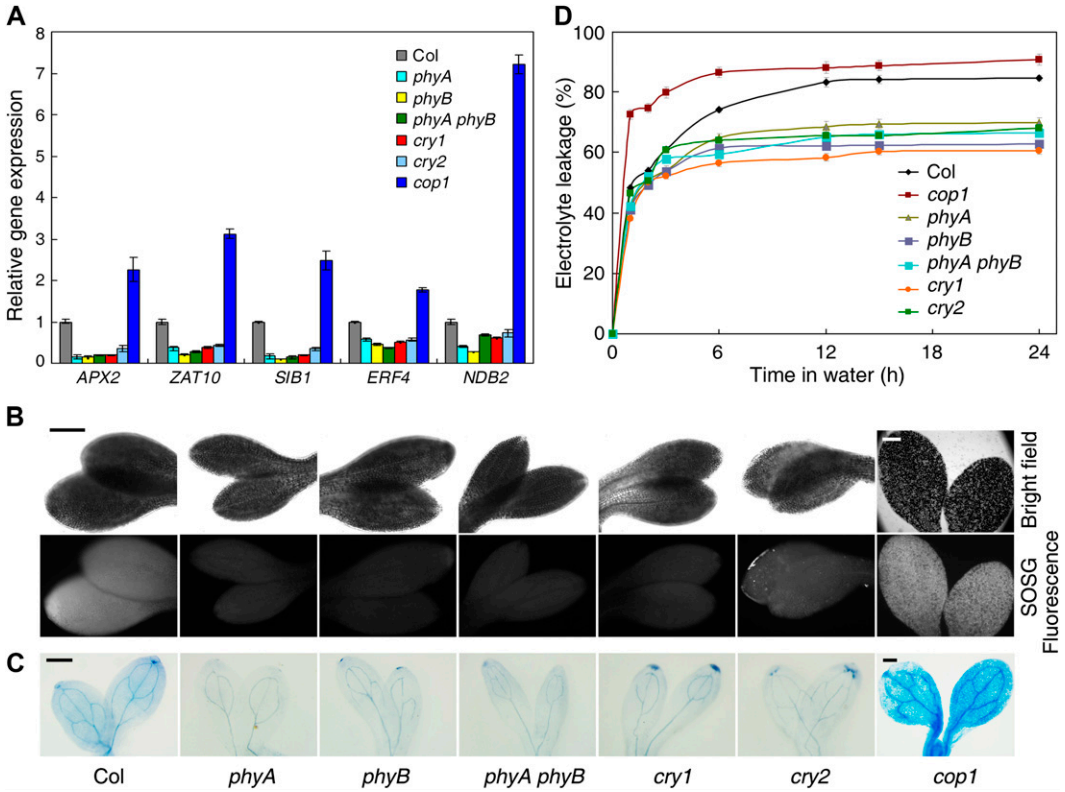

Figure 8. The Opposite Role of Photoreceptors and COP1 in Regulating ROS Production and Signaling.

(A) qRT-PCR showing the expression of the indicated ROS-responsive genes. Seedlings were grown in darkness for 4 d and transferred to light (60 μmol m^{-2} s^{-1}) for 3 h. Data are from three biological replicates; bars indicate SD.

(B) and **(C)** SOSG fluorescence imaging **(B)** and trypan blue staining **(C)** of 6-d-old wild-type and mutant etiolated seedlings after light illumination (60 μmol m^{-2} s^{-1}) for 6 h. Bars = 200 μm.

(D) Electrolyte leakage levels in the indicated photoreceptor and *cop1* mutants. Data are mean ± SD, $n = 4$.

Bu et al., 2011). Although HY5 and HYH are unstable in the dark, a small amount of HY5 is detectable in darkness (Hardtke et al., 2000; Osterlund et al., 2000; this study). Similarly, PIF1 and PIF3 proteins were detectable after 2 h of light exposure, despite their rapid degradation in the presence of light (Shen et al., 2005; Al-Sady et al., 2006; this study). Evidence from yeast two-hybrid, pull-down, coimmunoprecipitation, and LCI approaches consistently demonstrated that PIF1/PIF3 (bHLH transcription factors) and HY5/HYH (bZIP transcription factors) directly interact with each other in vitro and in vivo (Figure 5; see Supplemental Figures 4 and 5 online). Our BiFC and FRET assays further indicate that these transcription factors coexist and interact in the same nucleus at the same time in darkness or after light transition (Figure 5; see Supplemental Figure 6 online). From a structural point of view, the bHLH and bZIP proteins share some similar features: The short basic domain at the N-terminal region makes contact with the major groove of the DNA, and the HLH and ZIP domains mediate dimerization (Sibéril et al., 2001; Jones, 2004). Furthermore, both types of transcription factors are able to bind to the G-box *cis*-element (The bZIP Research Group, 2002; Toledo-Ortiz et al., 2003). These structural similarities between bHLH and bZIP might allow PIF and HY5 heterodimerization and the binding of their basic regions to the same site of the G-box. Consistent with this, the C-terminal regions containing the bHLH domain of PIF1 and the bZIP domain of HY5 are responsible for mediating their interaction in yeast cells (see Supplemental Figure 4 online), and PIF1 and HY5 formed heterodimers and bound DNA fragments of *APX2* and *ERF4* (see Supplemental Figure 8 online). The possibility that they form heterotetramers cannot be excluded. The formation of analogous complexes of bZIP and bHLH transcription factors have been observed in yeast, and these complexes were found to bind to a *cis*-element (TCACGTG) similar to the G-box motif (Kuras et al., 1997; Blaiseau and Thomas, 1998), indicating that the interaction and DNA binding activities of bZIP/bHLH proteins are likely conserved among organisms.

In addition, homo- or heterodimerization of the components of HY5/HYH and PIF1/PIF3 might also offer regulatory flexibility and diversity in the interactions with target genes in response to diverse signals. A previous study reported that PIF3 regulates anthocyanin biosynthesis in an HY5-dependent manner, although the authors failed to detect a direct interaction between PIF3 and HY5 (Shin et al., 2007). Moreover, ChIP-based sequencing analyses showed that some of the direct targets of

HY5 overlap with those of PIF1, whereas others are distinct (Lee et al., 2007; Oh et al., 2009). Other transcription repressors or activators might also be involved in this process by interacting with PIFs and HY5 proteins. We recently found that FAR-RED ELONGATED HYPOCOTYL3 (FHY3) and FAR-RED-IMPAIRED RESPONSE1 (FAR1) participate in regulating Pchlide synthesis and seedling greening by interacting and cooperating with PIF1 (Tang et al., 2012). HY5 feedback regulates phyA signaling homeostasis by interacting with FHY3 and FAR1 (Li et al., 2010). FHY3 and FAR1 likely possess a similar function as HY5 in mediating ROS production and cell death. The discovery of more interacting factors will enable us to better understand the regulatory complexity underlying the actions of these bHLH and bZIP transcription factors in response to developmental and environmental cues.

The PIF1/PIF3-HY5/HYH Transcriptional Modules Define a Key Link between Light and ROS Signaling

PIF1/PIF3 and HY5/HYH have been studied individually as regulators of photomorphogenic responses. Here, we reveal that transcriptional modules composed of PIF1/PIF3 and HY5/HYH act as a key molecular node that directly connects light to ROS signaling during plant postgermination development. First, by analyzing genome-wide gene expression changes in the *pif1* mutants, we found that a large number of ROS-related genes was induced during the first 3 h of light illumination of etiolated seedlings. Strikingly, many of the singlet oxygen–responsive genes (Laloi et al., 2007) were present in the group of genes induced in *pif1*. Similarly, when 4-d-old etiolated seedlings were exposed to weak red light for 1 h, 42 out of 139 upregulated genes (more than twofold change) in *pif3* were found also to be induced in the *flu* mutant (Monte et al., 2004; Laloi et al., 2007). Second, the EMSA and/or ChIP results demonstrate that PIF1, PIF3, HY5, and HYH directly bind to the promoters of five ROS-responsive genes, including *APX2*, *ZAT10*, *SIB1*, *ERF4*, and *NDB2*, through the G-box motif (Figures 3 and 4). Third, nearly 23% and almost all of the upregulated genes contain the putative G-box motif and ACE elements in their promoters, respectively, suggesting that they are the potential direct targets of PIF1/PIF3 and HY5/HYH (Figure 2D). Moreover, qRT-PCR data show that PIF1 and PIF3 repress the expression of these ROS-responsive genes in light and that the repression also partly requires both HY5 and HYH (Figure 6A).

The observations that PIF1 and HY5 bound to the target DNAs and the binding ability was partly dependent on each other in vivo (Figures 6C and 6D) and that they formed both DNA-bound homodimers and heterodimers (see Supplemental Figure 8 online) suggest that homodimerization and heterodimerization between PIF1/PIF3 and HY5/HYH may exist together in plant cells in darkness and after light transition. In the dark, PIF1/PIF3 are abundant, and their homodimers repress ROS-responsive gene expression in the nucleus and meanwhile inhibit Pchlide synthesis and singlet oxygen production in the plastid by regulating the biosynthesis of chlorophyll intermediates. Because of the opposite effect of PIF1/PIF3 and HY5/HYH on downstream gene expression, the heterodimers of the bZIP and bHLH factors likely function as inactive forms, which maintain the ROS-related transcripts at a basal level (mild repression) (Figure 6B). Most strikingly, the protein levels of PIF1/PIF3 and HY5/HYH are sensitively degraded or stabilized, respectively, by light (Henriques et al., 2009). The heterodimeric states are hence dynamically regulated by the environmental light conditions. After short periods or low fluence of light exposure, where plants encounter less light stress, PIF1/PIF3 are partly degraded and relatively high amounts of the PIF-HY5/HYH heterodimers are formed, such that the downstream ROS-related genes are inhibited. However, extended or high-light exposure leads to almost complete degradation of PIF1/PIF3 and abolishes the PIF-HY5/HYH heterodimeric states, which consequently relieve their repressive effect. Under this circumstance, accumulated HY5/HYH form homodimers that predominantly elevate the transcripts of ROS-responsive genes and activate their network (Figure 9). Consistent with this notion, the protein stability of HY5/HYH and PIF1/PIF3 and the expression of downstream ROS-responsive genes are coordinately regulated by the exposure length and intensity of light (Figures 3A and 6A; see Supplemental Figure 7 online).

Therefore, we propose that the PIF1/PIF3-HY5/HYH transcription modules serve as rheostats to fine-tune the flow of the ROS signaling pathway. Since etiolated seedlings are sensitive to excess light and the heterotrophic-to-autotrophic switch is critical for plant survival, the integration and involvement of the transcriptional modules might be central for the regulation of ROS-mediated photoprotective machinery against high light stress during seedling deetiolation. Repression of ROS pathway under low-light conditions by the bHLH/bZIP transcription modules

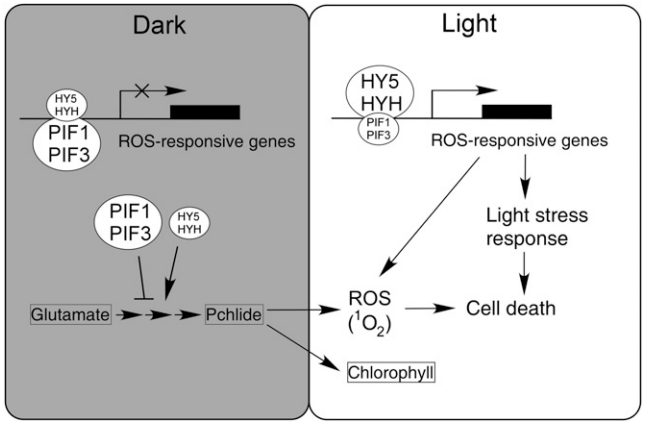

Figure 9. A Model for the Function of PIF1/PIF3 and HY5/HYH in Integrating Light and ROS Signaling.

In the dark, PIF1 and PIF3 accumulate while HY5 and HYH are largely degraded, leading to less Pchlide accumulation. Meanwhile, HY5/HYH and PIF1/PIF3 interact and bind to the promoter regions of ROS-responsive genes, resulting in the inhibition of their gene expression. Upon light irradiation, photosensitized Pchlide generates singlet oxygen ($^1O_2$), which causes photooxidative damage and cell death in plants; on the other hand, light promotes the stabilization of HY5/HYH and the rapid turnover of PIF1/PIF3, which in turn activates the expression of ROS-responsive genes and the ROS signaling, thus allowing plants to adjust ROS level and to cope with cell death under unfavorable light stress conditions. Light also promotes the formation of chlorophyll from Pchlide. Arrows, positive effect; bar, negative regulation.

could have adaptive value, as it would save energy and nutrient resources. The efficiency of the plants to activate this pathway in response to light is further supported by studies showing that PIF3 acts transiently and mainly mediates phytochrome-induced signaling during the dark-to-light transition (Bauer et al., 2004; Monte et al., 2004). It will be interesting to investigate whether this regulatory mechanism is conserved for plants during day/night cycles. The ROS-related genes are likely simultaneously activated by the loss of PIF1/PIF3 together with the generation of singlet oxygen. Accordingly, when etiolated seedlings were exposed to light, singlet oxygen generation gradually increased in conjunction with increasing lengths of light illumination, with higher level found in *pif1 pif3* compared with the wild type. The transcript levels of *APX2*, *ZAT10*, and *SIB1* increased in a similar pattern to that of singlet oxygen (see Supplemental Figure 12 online). However, upon activation, the ROS network may adjust ROS levels through detoxifying and quenching systems, such as antioxidant molecules and enzymes (Triantaphylidès and Havaux, 2009).

Although studies suggested that ROS signaling is highly integrated with hormonal signaling networks, such as gibberellic acid, abscisic acid, salicylic acid, and auxin, to regulate various developmental processes and adaptive responses in plants, unraveling the components that connect ROS with other signaling pathways has been a challenge in the field (Mittler et al., 2011). A previous study reported that gibberellic acid signaling contributes to the fine-tuning of ROS levels by stimulating the degradation of DELLA proteins, which regulate the transcript levels of antioxidant enzymes (Achard et al., 2008). Light signaling can also interact with the ROS pathway, and the PIF1/PIF3-HY5/HYH transcriptional modules represent a key convergence point between these signaling pathways. Moreover, PIF1, PIF3, PIF4, and HY5 have independently been shown to be involved in integrating light with hormone signaling (Chen et al., 2008a; de Lucas et al., 2008; Feng et al., 2008; Oh et al., 2009; Catalá et al., 2011). It is speculated that the transcriptional module described in this study might constitute a common molecular point of crosstalk between light and other cellular pathways.

METHODS

Plant Materials and Growth Conditions

The *pif1-2* (Huq et al., 2004), *pif3* (Salk_030753; Kim et al., 2003), *hy5-215* (Oyama et al., 1997), *hyh* (cs849765; Kleine et al., 2007), *pifq* (Leivar et al., 2008), *phyA-211* (Reed et al., 1994), *phyB-9* (Reed et al., 1993), *cry1-304* (Mockler et al., 1999), *cry2-1* (Guo et al., 1998), and *cop1-4* (McNellis et al., 1994) mutants and the *Pro35S:Myc-PIF3*, *Pro35S:TAP-PIF1* (Moon et al., 2008), and *Pro35S:HA-HY5* (Lee et al., 2007) transgenic lines were derived from the *Arabidopsis thaliana* Col ecotype. Double and triple mutants were generated by genetic crossing and homozygous lines were confirmed by PCR genotyping and/or antibiotic selection. The original mutations in the transgenic lines were removed by crossing. Seedlings were grown on Murashige and Skoog medium containing 1% Suc and 0.8% agar. For light treatment, etiolated seedlings were transferred to moderate growth light (60 μmol m^{-2} s^{-1}) for various periods of time or subjected to certain conditions, as described in the text.

Greening Rate and Pchlide Determination

Greening rate was determined by counting the number of dark-green cotyledons from 50 to 80 seedlings of each genotype. For Pchlide measurement, dark-grown seedlings were homogenized in 500 μL of ice-cold 80% acetone and incubated in darkness overnight. Samples were excited at 440 nm and scanned from 600 to 700 nm using a fluorescence spectrophotometer (Hitachi).

Fluorescence Imaging of ROS and Singlet Oxygen

ROS fluorescence determination was performed as previously described (Tang et al., 2012). Imaging of singlet oxygen production was performed as described (Flors et al., 2006). Briefly, dark-grown seedlings were immersed in a solution of 10 μM SOSG (Invitrogen) in 50 mM phosphate buffer, pH 7.5, for 2 h in darkness and then transferred to light for 3 h. Following excitation of SOSG by UV light, fluorescence images were acquired with a charge-coupled device camera (Olympus) with a GFPA interference filter in the objective. Fluorescence intensity was determined by Image J software (http://rsb.info.nih.gov/ij/), and the background was subtracted.

Trypan Blue, DAB, and NBT Staining

For all histochemistry studies, etiolated seedlings were exposed to white light for the indicated period of time before staining. Trypan blue and DAB staining were performed as described (Yang et al., 2007). For NBT staining, seedlings were submerged in solution (1 mg/mL NBT, 10 mM NaN_3, and 10 mM potassium phosphate, pH 7.8) and stained for 30 min at room temperature. Samples were then boiled in 95% ethanol for 10 min and stored in 60% glycerol. After staining, all samples were mounted on slides and photographed through a dissecting microscope.

Electrolyte Leakage Measurement

Cell death was quantified by electrolyte leakage, using a method adapted from Laloi et al. (2007). Dark-grown seedlings were transferred to light for 12 h and then immersed in 5 mL of distilled water in a glass tube for up to 24 h. The conductivity of the solutions was determined at different time points with a conductivity meter (HANNA Instruments). The maximum electrolyte content was obtained by boiling the samples for 25 min at 100°C. The electrolyte leakage rate was expressed as percentage of the maximum content.

Plasmid Construction

To obtain the open reading frames and/or the N- or C-terminal fragments of *PIF1*, *PIF3*, *HY5*, and *HYH*, PCR was performed using the primers listed in Supplemental Data Set 4 online and *pfu* DNA polymerase, and the fragments were cloned into the pEASY-Blunt vector (TransGen), resulting in pEASY-PIF1, pEASY-PIF3, pEASY-HY5, pEASY-HYH, pEASY-PIF1N (1 to 271 amino acids), pEASY-PIF1C (272 to 478 amino acids), pEASY-HY5N (1 to 77 amino acids), and pEASY-HY5C (78 to 168 amino acids). All clones were validated by sequencing.

To generate constructs for the yeast two-hybrid assay, pEASY-PIF1C was digested with *Eco*RI and *Sal*I, and the PIF1 C-terminal fragment was inserted into the pLexA vector (Clontech) cut by *Eco*RI and *Xho*I, to give rise to pLexA-PIF1C. The pEASY-PIF1, pEASY-PIF1N, and pEASY-PIF1C plasmids were digested with *Eco*RI and *Sal*I, and the corresponding fragments were cloned into the pB42AD vector (Clontech) digested with *Eco*RI and *Xho*I, to generate pAD-PIF1, pAD-PIF1N, and pAD-PIF1C, respectively. To construct pAD-HY5N and pAD-HY5C, pEASY-HY5N and pEASY-HY5C, respectively, were cut with *Eco*RI and *Xho*I, and inserted into pB42AD.

To construct vectors for expressing recombinant protein, the corresponding fragments from pEASY-PIF1 (cut with *Eco*RI and *Sal*I), pEASY-PIF3 (cut with *Eco*RI and *Xho*I), pEASY-HY5 (cut with *Eco*RI and *Xho*I), or pEASY-HYH (cut with *Eco*RI and *Sal*I) were cloned into the pGEX-5X-1 vector (GE Healthcare) and digested with *Eco*RI and *Xho*I, resulting in

pGEX-PIF1, pGEX-PIF3, pGEX-HY5, and pGEX-HYH, respectively. The same fragments from pEASY-PIF1 or pEASY-PIF3 were also inserted into the pET-28a vector (Novagen) digested with *Eco*RI and *Xho*I, to generate pHis-PIF1 and pHis-PIF3, respectively.

To make vectors for the BiFC experiment, the fragments from pEASY-PIF3 or pEASY-HY5 cut with *Eco*RI and *Xho*I were cloned into the pUC-SPYNE vector (Walter et al., 2004) digested with *Eco*RI and *Xho*I, to generate pSPYNE-PIF3 and pSPYNE-HY5, respectively. The corresponding fragments from pEASY-PIF1 (cut with *Eco*RI and *Sal*I) or pEASY-HY5 (cut with *Eco*RI and *Xho*I) were cloned into the pUC-SPYCE vector digested with *Eco*RI and *Xho*I, to give rise to pSPYCE-PIF1 and pSPYCE-HY5, respectively.

To generate plasmids for LCI analysis, the fragments from pEASY-PIF3 or pEASY-HY5 cut with *Kpn*I and *Xho*I were cloned into the pUC19-nLUC vector (Chen et al., 2008b) and digested with *Kpn*I and *Sal*I, to generate pLUCn-PIF3 and pLUCn-HY5, respectively. The corresponding fragments from pEASY-PIF1 (cut with *Kpn*I and *Sal*I) or pEASY-HY5 (cut with *Kpn*I and *Xho*I) were cloned into the pUC19-cLUC vector digested with *Kpn*I and *Sal*I, to give rise to pLUCc-PIF1 and pLUCc-HY5, respectively.

To construct plasmids for the FRET assay, the HY5 fragment was PCR amplified from pEASY-HY5 digested with *Bgl*II and *Kpn*I and inserted into pSAT6-CFP cut with *Bgl*II and *Kpn*I, to give rise to pCFP-HY5. The PIF1 open reading frame was amplified using pEASY-PIF1 as template, digested with *Sal*I and *Kpn*I, and cloned into the *Sal*I-*Kpn*I site of pSAT6-YFP to generate pYFP-PIF1.

For the transient expression assay, the coding fragment from pEASY-HY5 (digested with *Eco*RI and *Xho*I), pEASY-PIF1 (*Eco*RI and *Sal*I), or pEASY-PIF3 (*Eco*RI and *Xho*I) was inserted into the *Mfe*I-*Xho*I site of pUC-3HA, resulting in the effectors *Pro35S*:*HY5*, *Pro35S*:*PIF1*, and *Pro35S*:*PIF3*, respectively. The promoter fragment (~1.0 kb upstream of the ATG start site) of *ERF4* was PCR amplified from Col genomic DNA and cloned into pEASY to generate pEASY-ERF4p. The LUC open reading frame was released from pGEM-LUC (Lin et al., 2007) and inserted into the *Bam*HI-*Sac*I site of pUC-CPYNE to generate pUC-35sLUC. pEASY-ERF4p was then cut with *Hin*dIII and *Bam*HI, and the promoter fragment was inserted into the pUC-35sLUC vector digested with *Hin*dIII and *Bam*HI, to generate *ProERF4p*:*LUC*.

qRT-PCR

Four-day-old dark-grown seedlings were transferred to white light or kept in darkness for up to 3 h. Plant total RNA was extracted using an RNAprep Pure plant kit (Tiangen), and the first-strand cDNA was synthesized by reverse transcriptase (Invitrogen). Real-time PCR was performed using the SYBR Premix ExTaq kit (Takara) following the manufacturer's instructions. The expression levels were normalized to the expression of a *UBIQUITIN* (*UBQ*) gene. Primers are listed in Supplemental Data Set 4 online.

ChIP

The Col wild-type, *Pro35S*:*TAP-PIF1*, and *Pro35S*:*Myc-PIF3* transgenic plants were used in a ChIP assay, following a previously described procedure (Lin et al., 2007). Briefly, the seedlings were cross-linked with 1% formaldehyde and ground to powder under liquid nitrogen. After isolation and sonication, the chromatin complexes were incubated with anti-Myc (Abcam) or anti-HY5 polyclonal antibodies. The precipitated DNA fragments were recovered and quantified by quantitative PCR with the primers shown in Supplemental Data Set 4 online.

Yeast Two-Hybrid Assay

The yeast two-hybrid assay was performed as previously described (Lin et al., 2007). Briefly, the respective combinations of AD and LexA fusions were cotransformed with the *LexAop*:*LacZ* (Clontech) reporter construct into yeast strain EGY48. Transformants were grown on SD/-Trp-Ura-His dropout plates containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for blue color development.

Preparation of Recombinant Proteins

GST and GST-PIF1, GST-PIF3, GST-HY5, GST-HYH, His-PIF1, and His-PIF3 recombinant fusion proteins were induced by isopropyl β-D-1-thiogalactopyranoside and expressed in the *Escherichia coli* BL21 (DE3) strain. The proteins were then purified using Glutathione Sepharose 4B beads (GE Healthcare; for GST fusion proteins) or nickel-nitrilotriacetic acid agarose (Qiagen; for His fusion proteins), following the manufacturer's instructions.

EMSA

The EMSA analysis was performed as previously described (Tang et al., 2012). The oligonucleotide sequences of the probes are listed in Supplemental Data Set 4 online.

In Vitro Pull-Down, Coimmunoprecipitation, and Immunoblot Assays

The procedures for these assays were as described previously (Tang et al., 2012). Anti-GST (Abcam), anti-His (Abcam), anti-Myc (Abcam), and anti-D1 (Agrisera) were commercially available. Anti-HY5 and antitubulin antibodies were raised in rabbits (Jing et al., 2013).

BiFC Assay

Plasmids containing N- and C-terminal YFP fusions were cotransformed into *Arabidopsis* protoplasts as previously described (Walter et al., 2004). The protoplasts were incubated under darkness for 12 to 16 h before observation. YFP fluorescence was monitored using a Leica TCS SP5 confocal microscope.

LUC Activity Assay

For the transient reporter expression assay, the *ProERF4*:*LUC* reporter plasmid, effector constructs (*Pro35S*:*HY5*, *Pro35S*:*PIF1*, or *Pro35S*:*PIF3*), and the *Pro35S*:*GUS* internal control were cotransformed into *Arabidopsis* protoplasts. For the LCI assay, plastid combinations of various N- and C-terminal LUC fusions were cotransformed into protoplasts together with an internal control. The protoplasts were incubated under weak light for 12 to 16 h before harvesting. The LUC and GUS activities were determined with a luminometer/fluorometer (Promega) as described previously (Tang et al., 2012). The relative LUC activity was expressed as the ratio of LUC to GUS.

Microarray Analysis

The *pif1* mutant and Col wild-type seedlings were grown in darkness for 4 d and exposed to light for 3 h, and total RNA was isolated using the RNAprep Pure plant kit. Hybridization to the Agilent *Arabidopsis* Oligo Microarray (44k; Agilent Technologies) was performed according to the manufacturer's instructions. Three biological replicates were analyzed. Gene Ontology (GO) terms enriched in upregulated and downregulated genes were identified with GO::TermFinder (Boyle et al., 2004). Raw P values of GO term enrichment were corrected for multiple tests using false discovery rate (Benjamini and Hochberg, 1995).

Accession Numbers

Sequence data from this article can be found in the Genome Initiative or GenBank/EMBL data libraries under the following accession numbers: *PIF1* (At2g20180), *PIF3* (AT1G09530), *HY5* (At5g11260), *HYH* (At3g17609),

APX2 (At3g09640), *ERF4* (At3g15210), *SIB1* (At3g56710), *ZAT10* (At1g27730), *NDB2* (At4g05020), and *UBQ1* (At3g52590).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The *pif* Mutants Accumulate Increased Levels of Pchlide in Darkness but Have Reduced Superoxide and Hydrogen Peroxide Levels after Light Exposure.

Supplemental Figure 2. *APX2* and *ERF4* Expression under Weak Light Condition.

Supplemental Figure 3. Characterization of the *Pro35S:Myc-PIF3* Transgenic Plants.

Supplemental Figure 4. Interaction between PIF1 and HY5 in Yeast Cells.

Supplemental Figure 5. HY5 Interacts with PIF1 and PIF3 in a Luciferase Complementation Imaging Assay.

Supplemental Figure 6. Fluorescence Resonance Energy Transfer Assay between HY5 and PIF1 in the Nucleus.

Supplemental Figure 7. Light-Dependent Regulation.

Supplemental Figure 8. HY5 and PIF1 Form Homo- and Heterodimers That Bind to DNA in the EMSA Assay.

Supplemental Figure 9. Analysis of *PIF1*, *PIF3*, and *HY5* Overexpression Plants.

Supplemental Figure 10. The Mutual Regulation between PIF1 and HY5 at the mRNA and Protein Levels during Seedling Deetiolation.

Supplemental Figure 11. Greening Rate and Pchlide Levels of the Photoreceptor Mutants.

Supplemental Figure 12. Time-Course Analysis of Singlet Oxygen Production and ROS-Responsive Gene Expression.

Supplemental Data Set 1. List of PIF1-Regulated Genes Identified in the Microarray Analysis.

Supplemental Data Set 2. Overview of the Category of GO Biological Process Enriched of Genes Differentially Expressed in *pif1*.

Supplemental Data Set 3. Overview of the Category of GO Cellular Component Enriched in Genes Repressed in *pif1*.

Supplemental Data Set 4. List of Primers Used in This Study.

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AUTHOR CONTRIBUTIONS

D.C. and R.L. designed the research. D.C., G.X., W.T., Q.J., and Y.J. performed the research. D.C., Z.F., and R.L. analyzed the data. D.C. and R.L. wrote the article.

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