Phytochromes promote seedling light responses by inhibiting four negatively-acting phytochrome-interacting factors

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PIF3 is a phytochrome-interacting basic helix–loop–helix transcription factor that negatively regulates light responses, including hypocotyl elongation, cotyledon opening, and hypocotyl negative gravitropism. However, the role of PIF3 in chlorophyll biosynthesis has not been clearly defined. Here, we show that PIF3 also negatively regulates chlorophyll biosynthesis by repressing biosynthetic genes in the dark. Consistent with the gene expression patterns, the etiolated *pif3* mutant accumulated a higher amount of protochlorophyllide and was bleached severely when transferred into light. The photobleaching phenotype of *pif3* could be suppressed by the *gun5* mutation and mimicked by overexpression of *GUN5*. When 4 negative phytochrome-interacting protein genes (*PIF1*, *PIF3*, *PIF4*, and *PIF5*) were mutated, the resulting quadruple mutant seedlings displayed constitutive photomorphogenic phenotypes, including short hypocotyls, open cotyledons, and disrupted hypocotyl gravitropism in the dark. Microarray analysis further confirmed that the dark-grown quadruple mutant has a gene expression pattern similar to that of red light-grown WT. Together, our data indicate that 4 phytochrome-interacting proteins are required for skotomorphogenesis and phytochromes activate photomorphogenesis by inhibiting these factors.

PIF1 | PIF3 | PIF4 | PIF5 | chlorophyll

The phytochrome-interacting basic helix–loop–helix (bHLH) transcription factor PIF3 regulates seedling light responses either negatively (1, 2) or positively (3). It is a member of bHLH subfamily 15, consisting of 15 bHLH proteins (4). Eight of these proteins [PIF1 (also known as PIL5), PIF3, PIF4, PIF5 (also known as PIL6), PIL1, HFR1, PIF7, and SPT] have been shown to regulate various light responses (5, 6). However, not all of them interact with phytochromes. Only 6 of them (PIF1, PIF3, PIF4, PIF5, PIF6, and PIF7) directly bind to the active form of phytochromes (7). Primary characterizations of these bHLH proteins indicate that these factors regulate various light responses. Similar to PIF3, PIF4 and PIF5 negatively regulate various seedling light responses (8, 9). In contrast, PIF1 negatively regulates seed germination and chlorophyll biosynthesis (10, 11). During vegetative stages, PIF4 and PIF5 negatively regulate phytochrome-mediated inhibition of shade avoidance (12). PIF7 plays more subtle roles in hypocotyl elongation, whereas the biological role of PIF6 is currently unclear (6, 13). However, the presence of potentially redundant multiple factors make it difficult to assign a specific function to any of these proteins.

Phytochromes inhibit PIF3 by destabilizing this protein in the nucleus. In the dark, phytochromes and PIF3 are localized in different subcellular compartments: phytochromes in the cytosol and PIF3 in the nucleus (2, 14). However, when light activates phytochromes, they move into the nucleus, bind to PIF3, and form nuclear speckles (15–17). The binding between phytochrome and PIF3 leads to the phosphorylation of PIF3 (18), but the specific kinase has not yet been identified. PIF3 can be phosphorylated by phytochrome itself through its intrinsic kinase activity or it can be phosphorylated by other kinases that recognize the phytochrome–PIF3 complex. PIF3 is further ubiquitinated and degraded by the 26S proteasome (19). Similar to PIF3, other factors (PIF1, PIF4, and PIF5) are also destabilized by active phytochromes (20–22). This removal of these PIF proteins may be the mechanism by which phytochromes activate light responses (23).

For phytochromes to activate light responses by removing PIF3, PIF3 has to be a negative component in light responses. This inhibitory mechanism, however, is contraindicated by findings suggesting that PIF3 positively regulates chlorophyll biosynthesis (3). PIF3 may positively regulate chlorophyll biosynthesis independent of phytochromes. Alternatively, because these experiments were performed with etiolated seedlings that were transferred to light, PIF3 may negatively regulate chlorophyll biosynthesis under standard dark or continuous light conditions.

In the present work, we show that PIF3 represses the expression of chlorophyll and photosynthetic genes. Consistent with the expression pattern of PIF3, the etiolated *pif3* mutant accumulates a higher amount of protochlorophyllide and undergoes photobleaching when transferred into the light. We further investigated the roles of PIF1, PIF3, PIF4, and PIF5 by generating and analyzing double, triple, and quadruple mutants. We found that these 4 negatively acting phytochrome-interacting proteins are required for skotomorphogenesis and phytochromes promote photomorphogenesis by removing these interacting factors.

Results

PIF3 Negatively Regulates both Chlorophyll Biosynthetic and Photosynthetic Genes in the Dark. To clarify the role of PIF3, we determined the expression of chlorophyll biosynthetic genes in dark-grown seedlings. We found that PIF3 negatively regulates the expression of 2 key chlorophyll biosynthetic genes and 2 photosynthetic genes. The *pif3* mutant seedlings expressed 4-fold higher levels of *HEMA1*, the gene that encodes glutamyl-tRNA reductase, the first committed enzyme in the tetrapyrrole biosynthetic pathway (Fig. 1*A*). The *pif3* mutant also expressed higher levels of *GUN5*, the gene that encodes the ChlH subunit

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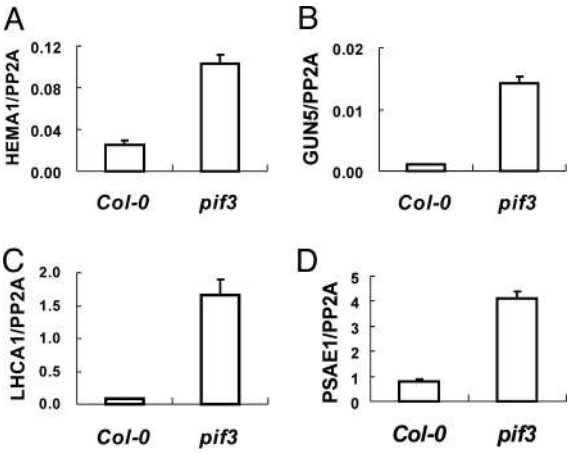

Fig. 1. PIF3 negatively regulates chlorophyll biosynthetic genes and photosynthetic genes. WT (Col-0) and *pif3* mutant (pif3) seedlings were grown for 2 days in the dark. (*A* and *B*) Relative expression levels of *HEMA1* (*A*) and *GUN5* (*B*) were determined by quantitative RT-PCR. (*C* and *D*) Relative expression levels of *LHCA1* (*C*) and *psaE1* (*D*) are shown. Error bars indicate SD ($n = 3$).

of Mg^{2+}-chelatase, the first enzyme in the chlorophyll biosynthetic branch in the tetrapyrrole pathway (Fig. 1*B*) (24). Similarly, many other chlorophyll biosynthetic genes were also overexpressed in the *pif3* mutant (Fig. S1). The negative role of PIF3 was not restricted to chlorophyll biosynthetic genes. We determined the expression of 2 photosynthetic genes (*LHCA1* and *psaE1*), both of which showed higher expression in the *pif3* mutant (Fig. 1 *C* and *D*). Together, our data indicate that PIF3 negatively regulates both chlorophyll biosynthetic and photosynthetic genes.

Etiolated *pif3* Mutant Seedlings Overaccumulate Protochlorophyllide. Etiolated seedlings that accumulate abnormally high levels of chlorophyll intermediates are bleached when transferred into the light (11). This phenomenon, called photobleaching, is a hallmark of the overaccumulation of chlorophyll intermediates. Because the etiolated *pif3* mutant expresses higher levels of chlorophyll biosynthetic genes, we assessed whether the *pif3* mutant is bleached more severely when transferred into the light. We grew both WT and mutants for several days in the dark and transferred then into white light. When grown for 5 days in the dark, WT seedlings were bleached ≈20–30% after the transfer. However, the *pif3* mutant seedlings grown for >2 days in the dark were bleached when transferred, with almost all grown for 5 days in the dark before transfer to light being completely bleached (Fig. 2 *A* and *B*). Unlike the *pif3* mutant, the *PIF3-OX* line was not bleached even when grown for 5 days in the dark. Although the exact degree of photobleaching varied from experiment to experiment, the *pif3* mutant was always bleached more severely than WT and the *PIF3-OX* was always bleached less severely than WT. The severe photobleaching of the *pif3* mutant suggests that it accumulates a higher level of chlorophyll intermediates than WT.

To determine whether the etiolated *pif3* mutant accumulates higher amounts of chlorophyll intermediates, such as protochlorophyllide, we determined the level of protochlorophyllide in etiolated seedlings and the level of chlorophyll after transfer into the light. Consistent with the photobleaching phenotype, we found that the etiolated *pif3* mutant seedlings accumulated an ≈2-fold higher amount of protochlorophyllide than the etiolated WT seedlings (Fig. 2*C*). When transferred to white light, protochlorophyllide was converted into chlorophyll in both seedlings (Fig. 2*D*).

Because PIF3 belongs to the bHLH subgroup 15, we tested

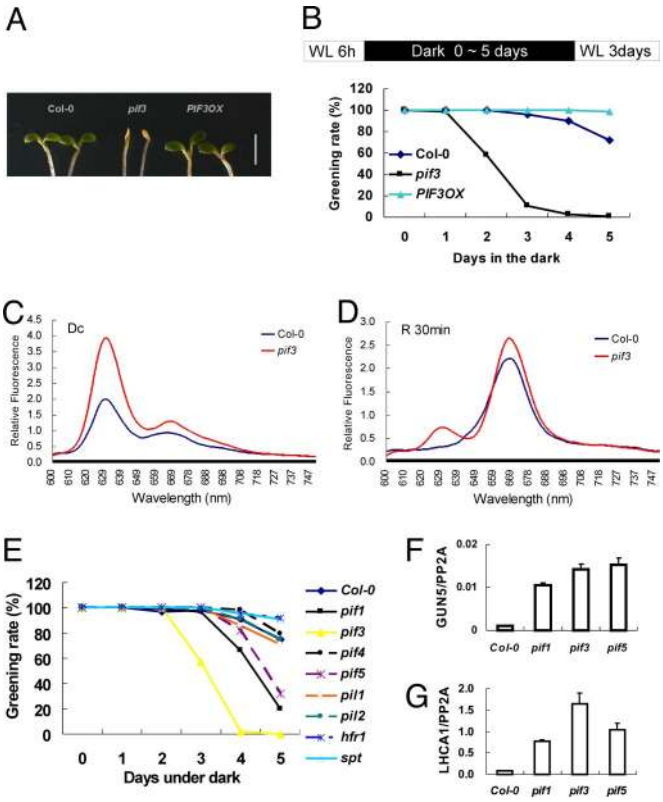

Fig. 2. The *pif3* mutant accumulates protochlorophyllide in the dark and undergoes photobleaching if transferred to light. (*A*) A representative picture showing 5-day-old etiolated seedlings that were transferred to white light for 3 days. (Scale bar: 0.5 cm.) (*B*) Quantification of photobleaching. Seedlings grown in the dark for various periods of time were transferred to white light and grown for 3 days, and the number of green seedlings was counted. The degree of photobleaching was expressed as percentage of green seedlings. A total of 100 seedlings was used for each time point. (*C*) Relative fluorescence of protochlorophyllide of 4-day-old etiolated WT (Col-0, blue line) and *pif3* mutants (red line). (*D*) Relative fluorescence of protochlorophyllide and chlorophyll of etiolated Col-0 and the *pif3* mutant after exposure to white light for 30 min. (*E*) Photobleaching of various *pif* mutants. (*F* and *G*) Relative expression of *GUN5* (*F*) and *LHCA1* (*G*) was determined by quantitative RT-PCR.

whether other members of this subgroup also play roles in chlorophyll biosynthesis. We found that the *pif3* mutant showed the severest photobleaching (Fig. 2*E*) and *pif1* and *pif5* mutants showed more severe photobleaching than WT. Unlike these 3 mutants, however, 5 other mutants (*pil1*, *pif4*, *pif6*, *hfr1*, and *spt*) did not show any significant photobleaching, indicating that these members of bHLH subgroup 15 do not negatively regulate chlorophyll biosynthesis in etiolated seedlings. We also assessed whether PIF1 and PIF5 regulate the expression of the *GUN5* and *LHCA1* genes in the etiolated seedlings. Similar to the *pif3* mutant, both the *GUN5* and *LHCA1* genes were up-regulated in the *pif1* and *pif5* mutants (Fig. 2 *F* and *G*). Together, these findings indicate that PIF1, PIF3, and PIF5 negatively regulate chlorophyll and photosynthesis genes in etiolated seedlings.

Ectopic Expression of a Chlorophyll Biosynthetic Gene Causes Photobleaching. We generated *GUN5-OX* lines to investigate whether the overexpression of chlorophyll biosynthetic genes is sufficient to cause photobleaching. *GUN5* was overexpressed from a *35S* promoter. Interestingly, most transgenic lines developed partially or completely white leaves, as if they were photobleached (Fig. 3*A*). Most of these plants eventually dried out and died, but some survived and produced a few seeds. When the harvested

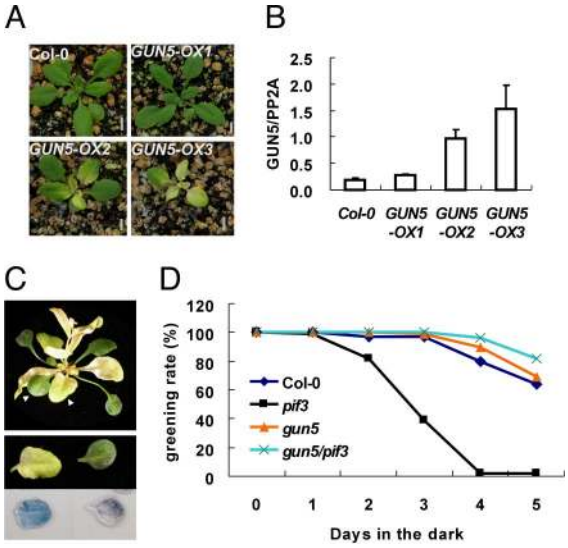

Fig. 3. Overexpression of *GUN5* causes spontaneous photobleaching. (*A*) Representative pictures of WT (Col-0) and 3 *GUN5* overexpression lines (*GUN5-OX1*, *GUN5-OX2*, and *GUN5–OX3*). (*B*) Relative expression of each transgenic gene determined by quantitative RT-PCR. Only *GUN5-OX2* and *GUN5-OX3* overexpressed the *GUN5* gene. (*C*) Trypan blue staining of *GUN5-OX3* leaves. (*D*) Quantification of photobleaching. Seedlings grown in the dark for various periods of time were transferred to white light for 3 days, and the number of green seedlings was counted. The degree of photobleaching was expressed as percentage of green seedlings. A total of 100 seedlings was used for each time point.

seeds were planted into soil without any antibiotics, the progenies were a mix of green WT and transgenic plants with partially or completely white leaves. This pattern of inheritance was repeated over a few generations, and we were unable to obtain homozygous plants for the majority of transgenic lines. We were able to generate 1 homozygous transgenic line (*GUN5-OX1*) that did not develop any white leaves. We determined the expression level of *GUN5* transgene in this *GUN5-OX1* line and 2 heterozygous lines (*GUN5-OX2* and *GUN5-OX3*). We found that *GUN5-OX1* had a similar level of *GUN5* mRNA as WT, whereas the *GUN5-OX2* and *GUN5–OX3* lines had higher levels of *GUN5* than WT (Fig. 3*B*). These findings indicate that only *GUN5-OX2* and *GUN5-OX3* are *GUN5*-overexpressing lines and that the overexpression of *GUN5* causes spontaneous whitening of leaves, which may be caused by photobleaching.

Consistent with this photobleaching hypothesis, we found that the white leaves in the *GUN5* transgenic plants consisted of partly-dead cells. When stained with trypan blue, the white leaves were stained blue, whereas a relatively green cotyledon from the same plant was stained only partially (Fig. 3*C*). Because trypan blue stains dead cells, these results indicate that the white leaves in the *GUN5-OX* plants were caused by cell death. These results suggest that the proper regulation of chlorophyll biosynthesis is critical for the survival of plants under white light.

Analysis of *gun5 pif3* double mutants provides further evidence for the role of chlorophyll biosynthetic genes in photobleaching. When etiolated *gun5* mutant and WT seedlings were transferred to white light, they were barely bleached, whereas the *pif3* single mutant was bleached severely. In contrast to the *pif3* single mutant, the degree of bleaching of the *pif3 gun5* double mutant was similar to that of the *gun5* single mutant (Fig. 3*D*). These findings collectively indicate that photobleaching of the *pif3* mutant is likely caused by overexpression of chlorophyll biosynthetic genes such as *GUN5*.

PIF1, PIF3, PIF4, and PIF5 Regulate Overlapping but Distinct Sets of Light Responses. The negative role of PIF3 in chlorophyll biosynthesis suggests that this protein and its 3 homologs (PIF1, PIF4, and PIF5) play mainly negative roles in phytochrome-mediated light signaling. To further investigate the roles of these 4 factors, we generated a series of double, triple, and quadruple mutants of the *pif1*, *pif3*, *pif4*, and *pif5* genes and analyzed their light responses.

Hypocotyl elongation was additively regulated by the 4 factors. In the dark, each single mutant had similar hypocotyl length as WT. Double and triple mutants also had similar or only marginally shorter hypocotyls. In contrast, the *pif* quadruple mutant (*pifQ*) had ≈40% shorter hypocotyls than WT (Fig. 4*A*). These results suggest that PIF3 and its 3 homologs play redundant roles in the elongation of hypocotyls in the dark. In red light, hypocotyl elongation is regulated mainly by PIF3 and PIF4, but also weakly by PIF5. The *pif3*, *pif4*, and *pif5* single mutants each had shorter hypocotyls, as reported (Fig. 4*B*). Further analysis of double, triple, and quadruple mutants shows that the *pif3 pif4* double mutant had greatly-shortened hypocotyls and that the *pif3 pif4 pif5* triple mutant had slightly further shortened hypocotyls. These findings suggest that PIF3, PIF4, and PIF5 redundantly regulate hypocotyl elongation in red light, whereas PIF1 plays a minimal role in red light conditions.

PIF3 and its 3 homologs also negatively regulate hypocotyl-negative gravitropism. As reported (10), the *pif1* and *pif3* single mutants each displayed disrupted hypocotyl-negative gravitropism, whereas the *pif4* and *pif5* single mutants showed normal hypocotyl-negative gravitropism (Fig. 4*C*). The *pif1 pif3* double mutant displayed further disruption as reported (10). In contrast to any double or triple mutants, the *pifQ* mutant was completely agravitropic, indicating that hypocotyl-negative gravitropism is regulated redundantly, mainly by PIF1 and PIF3 but also weakly by PIF4 and PIF5. Cotyledon opening was also regulated mainly by PIF1 and PIF3 but also weakly by PIF4 and PIF5. As shown in Fig. 4*D*, the *pif1 pif3*, the *pif1 pif3 pif4*, the *pif1 pif3 pif5*, and the *pif1 pif4 pif5* showed the partial cotyledon opening in the dark. Unlike these double and triple mutants, however, the *pifQ* mutant showed complete cotyledon opening in the dark. Together, these results indicate that these 4 factors are required for skotomorphogenesis. It should be noted, however, that light further decreased the hypocotyl lengths of the *pifQ* mutant. The presence of this remaining light response further suggests that phytochromes regulate hypocotyl elongation not just through these 4 factors but through other factors as well.

In contrast to seedling light responses, these 4 factors do not redundantly regulate light responses associated with seed germination or flowering. Among analyzed light responses, germination is regulated mainly by PIF1 but not by other factors. As reported (10), the *pif1* single mutant germinated almost completely even if phytochromes were not activated (Fig. S2*A*). Among the higher-order mutants, only those including *pif1* showed light-independent germination, indicating that PIF1 plays a major role in seed germination. These 4 factors also do not regulate flowering, in that all 4 single mutants and the *pifQ* mutant flowered similar to WT (Fig. S2*B*). Thus, PIF1, PIF3, PIF4, and PIF5 likely play a major role in seedling light responses and vegetative shade avoidance response (12), but they have limited or no roles in light responses associated with seed germination or flowering.

The Etiolated *pifQ* Mutant Has Gene Expression Profiles Similar to Those of Light-Grown Seedlings. To investigate the role of these 4 factors at the genome level, we performed microarray analysis with the *pifQ* mutant. Our microarray analysis showed that the dark-grown *pifQ* mutant has similar gene expression patterns as the red light-grown WT. Red light altered the expression of ≈10% of the genes [2,279 differentially-expressed genes (DEGs)] present in WT. We found that 403 DEGs were differentially expressed between dark-grown *pifQ* mutant [pifQ(D)] and dark-grown WT. Among these, 196 genes were

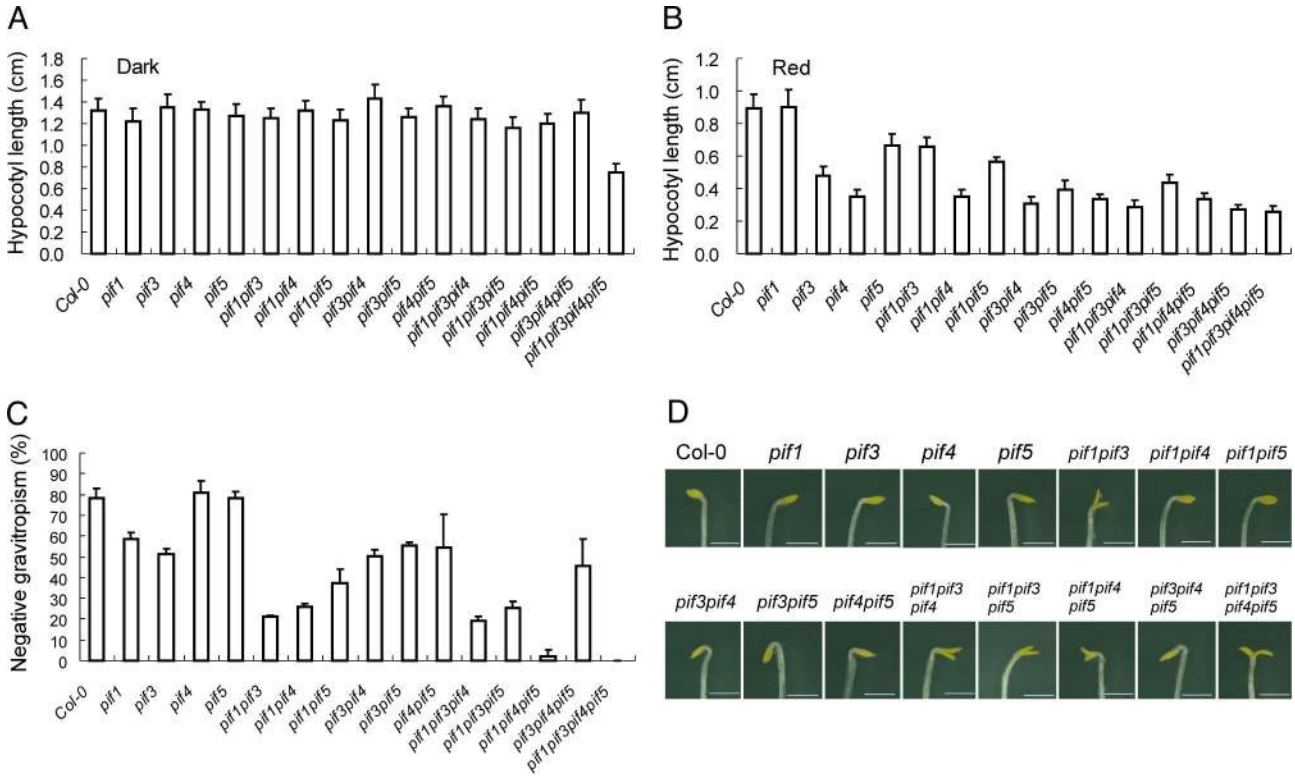

Fig. 4. The *pif1 pif3 pif4 pif5* quadruple mutant (*pifQ*) displays constitutive photomorphogenic phenotypes in the dark. (*A*) Hypocotyl lengths of dark-grown single, double, triple, and quadruple mutants. Seedlings were grown in the dark for 4 days. Hypocotyl lengths of 30 seedlings were measured. (*B*) Hypocotyl length of red light-grown mutants. (*C*) Quantification of hypocotyl-negative gravitropism of dark-grown mutants. The degree of hypocotyl negative gravitropism was assessed as the number of seedlings that grew upright. (*D*) Representative pictures showing open cotyledons in the dark-grown mutants.

up-regulated, whereas 194 were down-regulated in the pifQ(D), indicating that these 4 factors regulate genes both positively and negatively. When we compared the 2 DEG sets, we found that ≈82% of DEGs of pifQ(D)/Col-0(D) were differentially expressed between Col-0(R) and Col-0(D), whereas ≈15% of DEGs of Col-0(R)/Col-0(D) were differentially expressed between pifQ(D) and Col-0(D) (Fig. S3*A*), indicating that a subset of red light-regulated genes is differentially expressed in the dark-grown *pifQ* mutant. The small number of shared DEGs was likely caused by lower fold changes in the pifQ(D), because the expression of combined DEGs showed a relatively high correlation coefficient between samples ($r = 0.72$) (Fig. S3*B*). Together, the results indicate that the dark-grown *pifQ* mutants and red light-grown WT have similar gene expression patterns.

To investigate which classes of genes are enriched in the shared DEGs, we performed GO analysis using BiNGO (25). We found that the shared DEGs were enriched for genes encoding proteins localized in plastids and thylakoids (Fig. S3*C*). Consistent with this finding, genes involved in photosynthesis were also highly enriched, suggesting that a major role of these 4 factors is the repression of genes involved in chlorophyll biosynthesis and photosynthesis. Genes encoding proteins involved in other metabolic processes, including carbohydrate and lipid metabolic processes and the generation of precursor metabolites and energy, were also enriched in the shared DEGs. Beyond metabolic processes, genes involved in stress responses were also enriched in the shared DEGs. Genes in the shared DEGs can be found in Table S1, and genes in the enriched GO biological processes can be found in Table S2.

To further investigate how chlorophyll biosynthetic and photosynthetic genes are expressed in the dark-grown *pifQ* mutant, we analyzed their expression patterns in more detail. Lists of all chlorophyll biosynthetic and light harvesting-related photosynthetic genes were obtained from a previous publication (26) and The *Arabidopsis* Information Resource (www.arabidopsis.org), respectively. The majority of chlorophyll biosynthetic genes were up-regulated by red light in WT. Consistent with the negative roles of these 4 factors, most of these genes were also overexpressed in the dark-grown *pifQ* mutant (Fig. 5*A* and Table S3). Similarly, light-harvesting-related photosynthetic genes were overexpressed in both the dark-grown *pifQ* mutant and red light-grown WT (Fig. 5*B* and Table S4). Together, these results indicate that a major role of these 4 factors is the repression of chlorophyll biosynthetic and photosynthetic genes in the dark.

Discussion

PIF3 Negatively Regulates Chlorophyll Biosynthesis. We found that PIF3 negatively regulates chlorophyll biosynthesis by repressing metabolic genes in the dark. Consistent with its negative role, the *pif3* mutant expresses higher levels of chlorophyll biosynthetic and photosynthetic genes and accumulates a higher level of protochlorophyllide in the dark. Because of the increase in protochlorophyllide, the etiolated *pif3* mutant undergoes rapid photobleaching when transferred to light. The photobleaching of etiolated *pif3* can be further mimicked by the overexpression of a chlorophyll biosynthetic gene, *GUN5*. Together, these results indicate that PIF3 negatively regulates chlorophyll biosynthetic and photosynthetic genes in the dark and further suggests that the photobleaching of seedlings transferred to light is likely the cause of the altered gene expression patterns reported.

The negative role of PIF3 is similar to the reported negative role of PIF1 in chlorophyll biosynthesis (11). Similar to the *pif3* mutant, the *pif1* mutant was shown to accumulate higher amounts of protochlorophyllide and undergo photobleaching

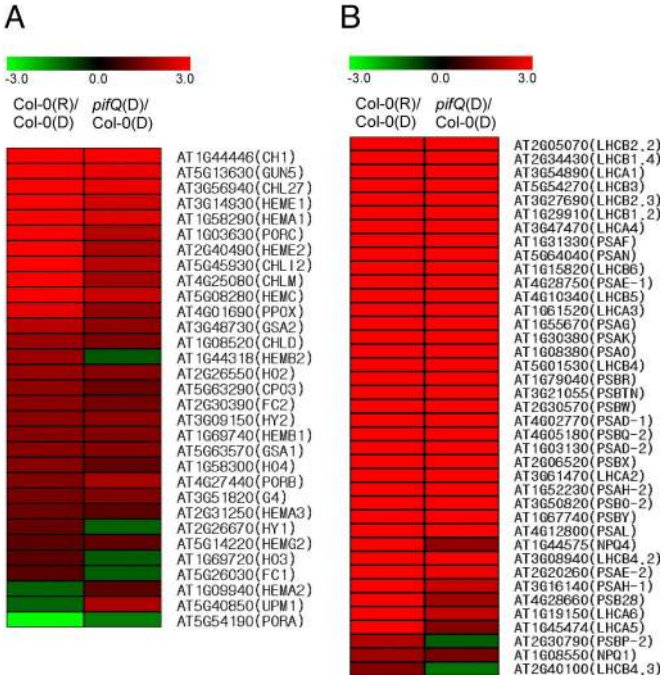

Fig. 5. Chlorophyll biosynthetic and photosynthetic genes are highly overexpressed in the dark-grown *pifQ* mutant. The hierarchical cluster showing similar overexpression of all chlorophyll biosynthetic genes (*A*) and photosynthetic genes (*B*).

when transferred to light. However, a previous report (27) showed that PIF1 did not up-regulate chlorophyll biosynthetic genes including *GUN5*. Rather, PIF1 was suggested to increase chlorophyll biosynthesis by inhibiting the expression of *HO3*, encoding a heme oxygenase, thereby disrupting the feedback inhibition of chlorophyll biosynthesis by heme. However, we found that both PIF1 and PIF5 negatively regulate *GUN5*, a key chlorophyll biosynthetic gene (Fig. 2*F*). Our microarray analysis using the quadruple mutant further showed that the majority of chlorophyll biosynthetic genes are negatively regulated by these factors. The different expression patterns in 2 experiments may be caused by differences in experimental conditions; that is, we assessed gene expression patterns in 2-day-old etiolated seedlings, whereas the previous study analyzed gene expression in 4-day-old etiolated seedlings (27).

The *pif1 pif3 pif4 pif5* Quadruple Mutant Is Constitutively Photomorphogenic (cop). The redundant negative roles of PIF3 and its 3 homologs in phytochrome signaling were further confirmed by the quadruple loss-of-function mutant, *pifQ*. This mutant displayed cop phenotypes, including short hypocotyls, open cotyledons, and agravitropic hypocotyls in the dark. Consistent with its photomorphogenic phenotypes, most DEGs of dark-grown *pifQ* mutant were also differentially expressed in red light-grown WT seedlings. The expression patterns of most chlorophyll biosynthetic and photosynthetic genes were similarly up-regulated in dark-grown *pifQ* and red light-grown WT. These results indicate that PIF3 and its homologs are required for skotomorphogenesis in the dark. Because these factors are destabilized by light, these results further suggest that phytochromes promote photomorphogenesis by destabilizing these factors.

These cop phenotypes of the *pifQ* mutant are not likely caused by an elevated level of phytochrome B. A previous report suggested that PIF3 and its homologs regulate hypocotyl elongation by altering phytochrome B protein levels under a prolonged red light growth condition, rather than being signaling intermediates that directly regulate hypocotyl elongation (6). According to our data, the *pifQ* mutant displays constitutive photomorphogenic phenotypes in the dark (Fig. 4), and PHYB overexpression does not cause shorter hypocotyls in the dark, indicating that PIF3 and its homologs can regulate hypocotyl elongation even in the absence of active phytochromes. Our data, however, do not exclude the possibility that these factors exert their roles partly by altering the level of phytochrome B protein under prolonged red light condition. Further investigations will clarify how much of this effect is directly attributable to the PIFs and how much is an indirect effect acting through the level of phytochrome B protein.

A few phytochrome signaling components also display cop phenotypes when they are mutated or overexpressed. A point mutation in the GAF domain of phytochromes ($PHYB^{Y276H}$, $PHYA^{Y242H}$) has been found to result in light-independent constitutively-active phytochromes (28). Overexpression of these mutant phytochromes confers the cop phenotypes, including light-independent seed germination, short hypocotyls, and open cotyledons. Because phytochromes promote the degradation of these 4 PIFs (12, 15, 19, 20), the transgenic line expressing light-independent phytochromes may cause cop phenotypes by greatly reducing the levels of these PIF proteins in the dark.

In addition, mutations in the *COP/DET/FUS* genes, including *COP1*, cause cop phenotypes. Most of those COP/DET/FUS proteins play roles in ubiquitin-dependent proteolysis. For example, COP1 acts as an ubiquitin E3 ligase that degrades various proteins including HY5, HFR1, and LAF1, positive light signaling components (29). COP1 has been shown to be necessary for the stability of PIF3 protein in the dark (15). If COP1 is also needed to stabilize other PIFs in the dark, the cop phenotypes of the *cop1* mutant could be caused by the greatly-reduced levels of these PIFs. Unlike light-independent phytochromes, however, the *cop1* mutant seeds still require light for germination, indicating that the *cop1* and *pifQ* mutants are not identical. This finding may indicate that COP1 does not regulate PIF1 protein level in the dark or that cop phenotypes of the *cop1* mutant are independent of PIFs.

Constitutive photomorphogenesis can also be induced by the overexpression of mutated versions of downstream components, such as Δ77HY5 (30), HFR1-ΔN105 (31), and PIF1 (C327) (32). The truncated forms of these factors are more stable than WT forms because of the deletions of domains that interact with COP1 (Δ77HY5 and HFR1-ΔN105) or phytochromes PIF1 (C327). Among them, PIF1 (C327) is likely to be dominant negative because it has DNA binding and dimerization domains but lacks a transcription activation domain. If the truncated form is dominant negative, it likely causes cop phenotypes by interfering with the functions of these 4 PIFs.

Although cop, the *pifQ* mutant still responds to light treatment. For example, when exposed to red or far-red light, the hypocotyl lengths of the *pifQ* mutant become shorter. In addition, microarray analysis showed that only 15% of red light regulated genes are differentially expressed in the dark-grown *pifQ* mutant. The lower overlap is partly caused by lower fold changes in the *pifQ* mutant ($r = 0.72$). *Arabidopsis* contains >20 phytochrome-interacting proteins, including other PIF3 homologs (e.g., PIF6 and PIF7) (6, 13). Thus, the residual light responses may be mediated by these other phytochrome-interacting proteins.

Materials and Methods

Plant Materials and Growth Conditions. *Arabidopsis thaliana* plants were grown in a growth room with a 16-h light/8-h dark cycle at 22–24 °C for general growth and seed harvesting. *pil1-2* (Salk_025372), *pif6* (*pil2*) (Salk_090239), *pif3-1* (Salk_030753), *pif1* (*pil5-1*) (Salk_072677), *pif5* (*pil6-1*) (Salk_087012), *hfr1* (Salk_037727), *spt* (WiscDsLox466B7), and *gun5* (CS806665)

were of Col-0 ecotype background. The *pif4* mutant (GT_3_1934) was backcrossed to Col-0 4 times, and homozygous lines were selected. The double, triple, and quadruple mutants were generated by crossing these single or double mutants.

Gene Expression Analysis. Surface-sterilized seeds were plated on wet filter paper on Murashige and Skoog (MS) agar plates [half-strength MS, 0.8% phytoagar, 0.05% Mes (pH 5.7), and 1% sucrose] and imbibed for 3 days at 4 °C in the dark. After germination was induced under white light (17 μmol·m^{-2}·s^{-1}) for 6 h, the seedlings were grown in various light conditions for various periods of time before sampling and total RNA purification. Quantitative real-time RT-PCR analysis was performed by using SyBR and the primers listed in Table S5.

Hypocotyl Length Measurement. Surface-sterilized seeds were plated on MS agar and imbibed for 3 days at 4 °C in the dark. After germination was induced, plates were incubated for 4 days either in the dark or in continuous red light (24 μmol·m^{-2}·s^{-1}). Of 50 seedlings, hypocotyl lengths of ≈30 seedlings were measured for each sample.

Photobleaching Assay. Seeds were plated on MS-agar plates containing 1% sucrose and imbibed for 3 days at 4 °C in the dark. After germination was induced, seedlings were grown in the dark for 0–5 days and moved into continuous white light for an additional 3 days. To determine the bleaching ratio, we divided the plants into those that were bleaching or greening and counted the number of plants in each group.

Protochlorophyllide Determination. Ten frozen etiolated seedlings or etiolated seedlings that were transferred to white light (17 μmol·m^{-2}·s^{-1}) for 30 min were ground with a Mixer-Mill (MM301; Retsch), and pigments were extracted by gently agitating the seedling powder in 1 mL of ice-cold 80% acetone for 1 h in the dark at 4 °C. Cell debris was removed by centrifugation at 4 °C for 10 min at 14,000 rpm (Micro 17R; Hanil). The room-temperature fluorescence emission spectra of the samples were obtained with a Hitachi F-4500 fluorescence spectrophotometer, with an excitation wavelength of 440 nm and a bandwidth of 5 nm. The fluorescence emission spectra were recorded between 600 and 800 nm.

Germination Assay. Seeds were surface-sterilized and plated on aqueous agar medium (0.6% phytoagar, pH 5.7). One hour later, the seeds were irradiated with far-red light (2.4 μmol·m^{-2}·s^{-1}) for 5 min and incubated in the dark for 5 days. Germinated seeds were determined by radical emergence. Triplicates of 50 seeds were used for each mutant.

Hypocotyl-Negative Gravitropism. Surface-sterilized seeds were plated on MS agar and imbibed for 3 days at 4 °C in the dark. After germination was induced, the plates were incubated for 4 days in the dark. The degree of hypocotyl-negative gravitropism was determined by counting the number of flat-grown seedlings with cotyledons that touched the agar surface. Triplicates of 50 seeds were used for each mutant.

Microarray Data Analysis. Seedlings were grown for 60 h in the dark or in continuous red light (24 μmol·m^{-2}·s^{-1}) before sampling. The Agilent *Arabidopsis* Genome 44k chip was used for microarray analyses. The analysis was performed by using the LIMMA package in the Bioconductor R project (33). Background correction was performed by using the normexp method implemented in LIMMA. The background-corrected intensity data were normalized by using the lowess method to remove the bias within each array. DEGs were defined as genes that had a false discovery rate of 5% and showed a 2-fold difference and signal intensity >64.

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