PIF3 is a repressor of chloroplast development

Patrick G. Stephenson[a], Christian Fankhauser[b], and Matthew J. Terry[a,1]

[a]School of Biological Sciences, University of Southampton, Boldrewood Campus, Southampton SO16 7PX, United Kingdom; and [b]Center for Integrative Genomics, University of Lausanne, CH-1015 Lausanne, Switzerland

Edited by J. Clark Lagarias, University of California, Davis, CA, and approved February 26, 2009 (received for review November 18, 2008)

The phytochrome-interacting factor PIF3 has been proposed to act as a positive regulator of chloroplast development. Here, we show that the *pif3* mutant has a phenotype that is similar to the *pif1* mutant, lacking the repressor of chloroplast development PIF1, and that a *pif1pif3* double mutant has an additive phenotype in all respects. The *pif* mutants showed elevated protochlorophyllide levels in the dark, and etioplasts of *pif* mutants contained smaller prolamellar bodies and more prothylakoid membranes than corresponding wild-type seedlings, similar to previous reports of constitutive photomorphogenic mutants. Consistent with this observation, *pif1*, *pif3*, and *pif1pif3* showed reduced hypocotyl elongation and increased cotyledon opening in the dark. Transfer of 4-d-old dark-grown seedlings to white light resulted in more chlorophyll synthesis in *pif* mutants over the first 2 h, and analysis of gene expression in dark-grown *pif* mutants indicated that key tetrapyrrole regulatory genes such as *HEMA1* encoding the rate-limiting step in tetrapyrrole synthesis were already elevated 2 d after germination. Circadian regulation of *HEMA1* in the dark also showed reduced amplitude and a shorter, variable period in the *pif* mutants, whereas expression of the core clock components *TOC1*, *CCA1*, and *LHY* was largely unaffected. Expression of both *PIF1* and *PIF3* was circadian regulated in dark-grown seedlings. PIF1 and PIF3 are proposed to be negative regulators that function to integrate light and circadian control in the regulation of chloroplast development.

chlorophyll synthesis | circadian regulation | phytochrome | etioplast | light signaling

Light is a major regulator of growth and development throughout the life cycle of the plant, and this myriad of complex responses is mediated by different photoreceptor families. Responses to blue light are predominantly controlled by the cryptochrome and phototropin photoreceptors, whereas the phytochromes are responsible for regulating growth and development in response to red (R) and far-red (FR) light (1). In *Arabidopsis*, there are 5 phytochromes (phyA–E) that regulate responses such as germination, seedling and chloroplast development, plant growth and architecture, and flowering. The mechanism by which the phytochromes regulate cellular processes is not yet understood, but remarkable progress has been made in recent years. Phytochromes are dimeric, photoreversible proteins that exist in the dark in the inactive Pr (R-absorbing) form and are converted by light to the active Pfr (FR-absorbing) form (2). After light absorption, phytochromes rapidly relocate to the nucleus, where they control the response to light through 2 main mechanisms. First, they act to exclude the E3-ubiquitin ligase, COP1, from the nucleus, thereby preventing the degradation of the positive signaling factors HY5, HFR1, and LAF1 (3). Second, phytochromes bind and target a family of bHLH proteins for degradation, thus relieving repression of light responses such as inhibition of hypocotyl elongation and germination (4).

The first of these bHLH proteins to be identified as a phytochrome-interacting protein was PIF3 (5), which binds to both phyA and phyB in a light-dependent manner (6), but through different motifs (7, 8). Activation of phytochrome results in PIF3 phosphorylation (8) and subsequent degradation (9, 10) in a mechanism that appears to be common to this class of signaling protein (11, 12). Although there seems to be broad agreement on what is known about the molecular events after phytochrome interaction with PIF3, there is less certainty about how PIF3 is functioning in photomorphogenesis. From the outset, PIF3 was proposed as a positive regulator of light signals, because the hypersensitive *poc1* mutant was initially described as a PIF3 overexpressor (13). Subsequent analysis of PIF3 loss-of-function mutants demonstrated that PIF3 promoted hypocotyl elongation, suggesting that PIF3 is a negative regulator of seedling growth (14). In contrast, PIF3 has been described as acting positively in the light regulation of chloroplast development (15), and this has led to the hypothesis that PIF3 has a dual function, acting early and positively as a transcription factor, but acting later to regulate phyB abundance and repress light-induced inhibition of hypocotyl elongation (16, 17). In contrast to the proposal for PIF3, other members of the PIF family appear to function predominantly as negative regulators (3, 4). This is clearly seen for PIF1 (PIL5), which negatively regulates phytochrome-mediated promotion of seed germination (18) through the repression of gibberellin biosynthesis genes (19) and repression of chlorophyll biosynthesis (20).

Given the controversy in the role of PIF3, we have reevaluated the function of PIF3 in chloroplast development through careful examination of the phenotype of *pif3* and a *pif1pif3* double mutant. Our results show that PIF3 acts similarly and additively to PIF1 to repress chloroplast development and chlorophyll synthesis in the dark. Interestingly, the *pif1pif3* double mutant showed a broader range of constitutively photomorphogenic phenotypes, in keeping with roles for the PIF proteins as global repressors of photomorphogenesis.

Results

***pif1* and *pif3* Accumulate Protochlorophyllide in the Dark.** To further understand the role of PIF3 in early seedling development, we constructed a *pif1pif3* double mutant using an independently isolated *pif3* T-DNA insertion allele that is identical to *pif3-1* (14) and a previously undescribed *pif1* allele designated *pif1-101* (see *SI Text* and Fig. S1). The PIF1 protein has been shown to repress chloroplast development and protochlorophyllide (Pchlide) synthesis in the dark (20). To test whether PIF3 might be acting similarly, we followed accumulation of Pchlide in *pif1*, *pif3*, and the *pif1pif3* double mutant (Fig. 1*A*). All lines showed an increase in Pchlide. This increase was clearly detectable 2.5 d after germination, and at all time points the response of the *pif1pif3* double mutant appeared additive to that of *pif1* and *pif3*. Analysis of Pchlide levels in the *pif1-2* and *pif3-3* alleles confirmed our results (Fig. S2*A*). Because the *pif3-3* allele contains no detectable transcript (15) or protein (17), this result is consistent with the phenotype of *pif3* mutants being due to loss of PIF3 function. In our experiments, seeds were routinely

Author contributions: C.F. and M.J.T. designed research; P.G.S. performed research; C.F. contributed new reagents/analytic tools; P.G.S., C.F., and M.J.T. analyzed data; and M.J.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: mjt@soton.ac.uk .

This article contains supporting information online at www.pnas.org/cgi/content/full/0811684106/DCSupplemental.

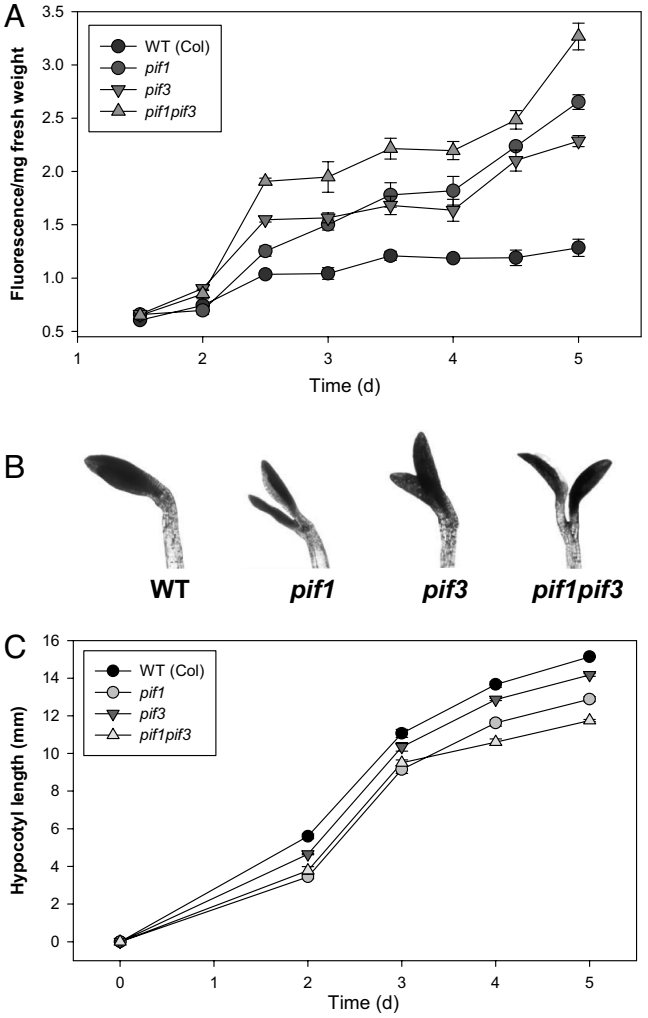

Fig. 1. Dark-grown phenotype of *pif* mutant seedlings. (*A*) Protochlorophyllide accumulation in WT and *pif* mutant seedlings in darkness. (*B*) Cotyledons of WT and *pif* mutant seedlings after 4 d in the dark. (*C*) Hypocotyl growth of WT and *pif* mutant seedlings in darkness. Values shown in *A* and *C* are the mean ± SE of 4 and 3 independent experiments, respectively. Photographs shown in *B* are representative and at the same scale.

germinated after 2 h of white light (WL), a treatment reported to have no longer-term effects on seedling growth in the dark (21). Nevertheless, we checked whether this short pretreatment contributed to the *pif* mutant response. As shown in Fig. S3*A*, Pchlide was also elevated in the *pif1pif3* double mutant even after germination in complete darkness. The increase in Pchlide was not simply due to an increased rate of germination, because all genotypes showed at least 95% germination by day 1 in these experiments.

***pif1* and *pif3* Mutants Have a Constitutively Photomorphogenic Phenotype.** In addition to the effects on Pchlide accumulation, we also observed that dark-grown *pif* mutant seedlings had open cotyledons and had lost their apical hook (Fig. 1*B*). This response was observed in the majority, but not all, of the single mutant seedlings but was more consistent and stronger in the *pif1pif3* double mutant. Moreover, it was not due to the WL pretreatment because seedlings germinated completely in the dark showed the same response (Fig. S3*B*). To test whether *pif* mutant seedlings showed the full constitutive photomorphogenic phenotype, we also measured hypocotyl lengths of dark-grown seedlings. In all cases, *pif* mutants were shorter in the dark compared with WT, with the *pif1pif3* double mutant again showing an additive phenotype (Fig. 1*C*). This was also true in the absence of the WL pretreatment (Fig. S3*C*). Finally, one distinctive feature of constitutively photomorphogenic seedlings such as *cop1* is that they show a partially developed chloroplast in the dark that is characterized by a reduced prolamellar body (PLB) and increased prothylakoid membranes (22). We therefore examined etioplasts in dark-grown *pif* mutant seedlings (Fig. 2 *A–D*). After 4 d in the dark, WT etioplasts showed a characteristic, highly regular PLB with little prothylakoid development (Fig. 2*A*). In contrast, both *pif1* and *pif3* etioplasts showed increased membrane development and PLBs that were generally reduced in size (Fig. 2 *B* and *C*). The most significant differences to WT were seen with the *pif1pif3* double mutant, where PLB size was severely reduced and prothylakoid membranes were extensive, although no membrane stacking was observed (Fig. 2*D*). In some cases, no PLB was observed in *pif1pif3* double mutant seedlings, although full-size PLBs were detected occasionally.

Greening of *pif1* and *pif3* Is Dependent on the Time of Transfer to White Light. When grown in the dark for 4 d before transfer to WL, *pif1pif3* double mutants failed to green over the next 24 h, with *pif1* and *pif3* showing only moderate greening during this period (Fig. 3*A* and Fig. S4). An identical result was observed with the *pif1-2* and *pif3-3* alleles (Fig. S2 *B* and *C*) and has been observed for *pif1* (20). Interestingly, detailed examination of the time course after transfer to WL showed that at 2 h *pif1*, *pif3*, and the *pif1pif3* double mutant had more chlorophyll than WT but that this was already reversed after 4 h of WL (Fig. 3*B*). In contrast to the situation after 4 d in the dark, seedlings transferred to WL after 2 d in the dark were able to green, with the *pif1pif3* double mutant accumulating the most chlorophyll (Fig. 3*C*). The *pif1-2* and *pif3-3* alleles were also able to green more efficiently under these conditions (Fig. S2 *B* and *C*), and this ability was gradually lost as seedlings aged at transfer to WL (Fig. 3*C* and Fig. S2*C*). The time course of chlorophyll loss after transfer to WL and the effect of increasing the dark period on subsequent greening ability are consistent with the loss of chlorophyll in the *pif* mutants being due to photooxidative destruction rather than reduced synthesis. To test this, we examined the effect of different WL fluences on chlorophyll levels in WT and *pif* mutant seedlings. As shown in Fig. 3*D*, as the fluence rate increased, the relative loss of chlorophyll in the *pif* mutants also increased compared with WT, consistent with photooxidation being the primary cause of chlorophyll deficiency in the *pif* mutants.

We examined chloroplast ultrastructure in WT and *pif* mutant seedlings after transfer to WL after 2 or 4 d in the dark (Fig. 2 *E–L*). Wild-type chloroplasts were already well developed 24 h after transfer from 2 d in the dark, with some thylakoid stacking evident at this stage, although in some cases a residual PLB was observed (Fig. 2*E*). Consistent with the chlorophyll data, there was no evidence of any repression of chloroplast development in *pif1*, *pif3*, and *pif1pif3* mutants, and in all cases there appeared to be more thylakoid stacking than in WT (Fig. 2 *F–H*). No residual PLBs were observed in *pif1*, *pif3*, or *pif1pif3* under these conditions. The situation after transfer to WL from 4 d in the dark was more complex. Development of WT chloroplasts was similar to that seen after transfer from 2 d in the dark (Fig. 2*I*). In contrast, *pif1* and particularly *pif1pif3* double mutants contained chloroplasts with poorly defined membrane structure and no evidence of granal stacking (Fig. 2 *J* and *L*). The appearance of the chloroplasts was reminiscent of chloroplasts damaged through pigment-induced photooxidative stress (e.g., ref. 23) and was not due to problems of fixation, because other structures in these sections were well defined (for example, the mitochondrion

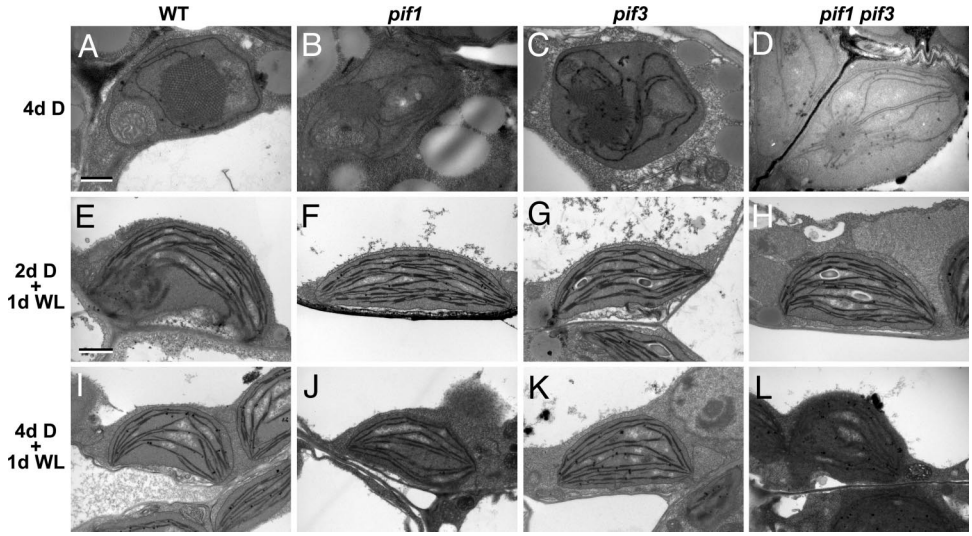

Fig. 2. Plastid ultrastructure in *pif* mutant seedlings. Transmission electron micrographs of plastids from WT (*A*, *E*, and *I*), *pif1* (*B*, *F*, and *J*), *pif3* (*C*, *G*, and *K*), and *pif1pif3* (*D*, *H*, and *L*) seedlings. Seedlings were grown for 4 d in the dark (*A–D*), 2 d in the dark followed by 1 d of WL (110 μmol·m^{-2}·s^{-1}) (*E–H*), or 4 d in the dark followed by 1 d of WL (*I–L*). [Scale bars: 500 nm (*A–D*) and 1 μm (*E–L*).]

to the right of the chloroplast in Fig. 2*L*). Chloroplasts in the *pif3* mutant were generally more similar to WT in appearance than those for *pif1* or *pif1pif3* (Fig. 2*K*), consistent with the higher levels of chlorophyll in *pif3* at this time point.

***pif1* and *pif3* Affect the Expression of Tetrapyrrole Biosynthesis Genes in the Dark.** To understand the basis of the increase in Pchlide, we followed the expression of *HEMA1* encoding glutamyl tRNA reductase, the rate-limiting step in tetrapyrrole synthesis (24). Expression was measured at 6 h time points from 1¼ d after germination using real-time PCR. *HEMA1* expression was strongly induced in all *pif* mutants relative to WT at 2 and 3 d after germination, with the response severely diminished or lost at days 4 and 5 (Fig. 4*A*). The response in the *pif1pif3* double mutant was again equivalent to both single mutants combined. We also analyzed 2 additional genes shown to be key regulatory targets in the tetrapyrrole pathway, *CHLH* encoding the H subunit of Mg chelatase and the chelatase regulator *GUN4* (25–27). Both genes showed a similar pattern, with the strongest peak 3 d after germination, high expression after 2 d, and little induction if any after 4 d (Fig. 4*B*). Analysis of *GUN4* expression in the *pif1-2* and *pif3-3* alleles gave similar results when measured 3 and 4 d after germination (Fig. S2*D*). Examination of glutamyl tRNA reductase protein levels showed an increase in *pif1*, *pif3*, and the *pif1pif3* double mutant by 2 d in the dark (Fig. 4*C*).

The profile of the relative induction of *HEMA1* in the *pif* mutants is quite unusual, with sharp peaks 2 and 3 d after germination but no induction at 2½ d. To understand the basis for this, we plotted the normalized level of *HEMA1* mRNA (relative to *YLS8*) for WT and the *pif* mutants independently (Fig. 4*D*). This analysis revealed 2 main observations. First, *HEMA1* expression was generally higher at early time points in *pif1*, *pif3*, and *pif1pif3* compared with WT. Second, and most strikingly, *HEMA1* expression was out of circadian phase in the *pif* mutants compared with WT seedlings. Although expression of *HEMA1* oscillated with a period close to 24 h in WT seedlings, *pif1*, *pif3*, and *pif1pif3* showed a reduction in the amplitude of oscillation and a period of oscillation that was variable ranging from ≈12 to 22 h for *pif1pif3* (Fig. 4*D*). To test whether the circadian clock is functioning normally in dark-grown *pif* mutant seedlings, we examined the expression of the central clock genes *CCA1*, *TOC1*, and *LHY* in the same samples (Fig. S5 *A–C*). No major changes in expression were observed for all 3 genes, indicating that the clock is still functional in etiolated *pif* mutants. We also examined the expression of another circadian-regulated gene, *CAX1*, that is not involved in chloroplast development. Circadian expression of this output gene was unaffected in the *pif* mutants (Fig. S5*D*), indicating that *pif1* and *pif3* might specifically affect circadian regulation of chloroplast-related genes. Finally, we investigated the circadian regulation of *PIF1* and *PIF3* at this developmental stage. *PIF1* and *PIF3* showed a robust circadian rhythm in dark-grown seedlings, with a similar phase to that of *HEMA1* (Fig. 4*E*).

***pif* Mutants Still Show Light Induction of Tetrapyrrole Biosynthesis Genes.** Because PIF3 has been proposed to function positively in the light induction of nuclear-encoded chloroplast genes, we followed gene expression after transfer to 24 h of WL, a time at which chlorophyll levels are severely reduced in the mutants. Although, as noted previously, expression was higher in the dark for *HEMA1*, *CHLH*, and *GUN4* in all *pif* mutant lines, all 3 genes were light induced to a similar degree, and the final expression levels of these genes in the light were still higher in *pif1*, *pif3*, and *pif1pif3* than in WT (Fig. 4*F* and Fig. S6). We also tested whether *pif* mutants could respond to monochromatic lights sources and over shorter time periods. As shown in Fig. S2, induction of *GUN4* was still apparent in *pif1-2* and *pif3-3* after 4 h FR and 8 h R light treatments.

Discussion

PIF3 Is a Negative Regulator of Chloroplast Development. The data presented here are consistent with PIF3 functioning as a repressor of chloroplast development in the dark. Pchlide synthesis was higher in *pif3* than WT seedlings (Fig. 1*A*), and initial rates of chlorophyll synthesis were also greater (Fig. 3 *B* and *C*). *pif3* seedlings also showed more advanced development of etioplasts and chloroplasts (Fig. 2). In these respects, the *pif3* mutant behaved identically to the *pif1* mutant, which has been identified as a negative regulator of chloroplast development (20). Consistent with these observations, the *pif1pif3* double mutant showed an additive phenotype. Previously, the *pif3* mutant had been described as showing inhibition of chloroplast development (15), and the hypothesis that PIF3 acts positively early in signal transduction (and negatively in the longer term) is still current

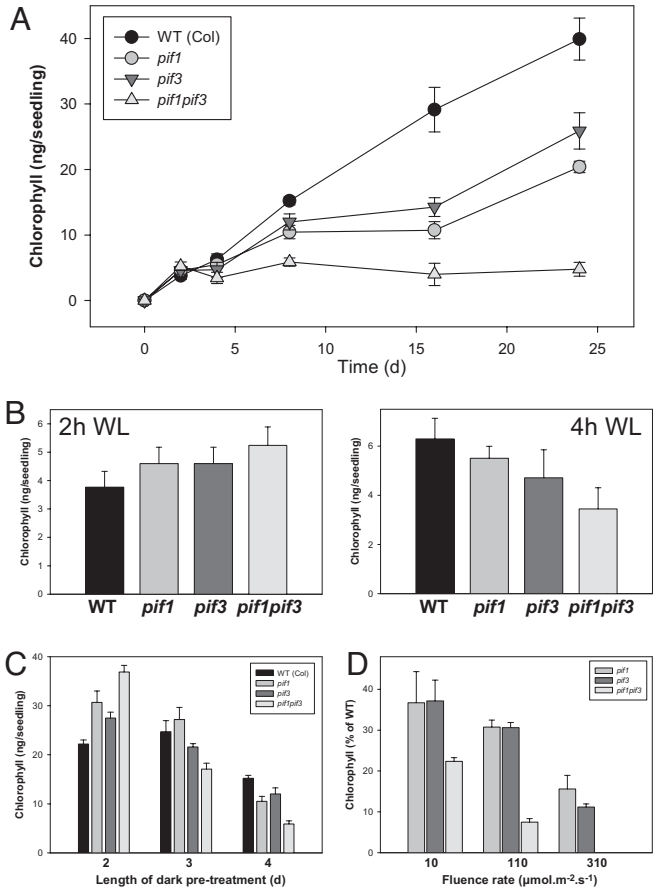

Fig. 3. Light-grown phenotype of *pif* mutant seedlings. (*A*) Chlorophyll accumulation in WT and *pif* mutant seedlings after transfer to WL (110 μmol·m^{-2}·s^{-1}) after 4 d in the dark. (*B*) Chlorophyll levels in WT and *pif* mutant seedlings after 4 d in the dark and either 2 or 4 h of WL. (*C* and *D*) Chlorophyll levels in WT and *pif* mutant seedlings after 8 h of WL after different dark periods (*C*) or after 4 d in the dark and transfer to 1 d of WL of different fluence rates (*D*). Values shown are the mean ± SE of 4 independent experiments.

(16, 17). Our data suggest that for the earliest stages of chloroplast development and for the target genes that we have analyzed (Fig. 4) that this is not the case. As discussed later, there are possible explanations for the previously reported loss of induction of chloroplast genes in *pif3* (15, 17), but the observations that the overexpression of PIF3 is not sufficient for the induction of phytochrome-regulated genes and that DNA binding of PIF3 in the dark is required (17) are certainly consistent with a role for PIF3 as a repressor. Moreover, the phytochrome-interacting PIF proteins have generally been shown to be acting as repressors not activators of photomorphogenic responses (3, 4, 28), and our results are therefore consistent with a common molecular mechanism for this class of signaling protein.

The reason for the previous misinterpretation of the *pif3* mutant phenotype is that seedlings transferred to WL after 4 d in the dark showed a reduced level of chlorophyll compared with WT (Fig. 3). This response, which is identical for *pif1* and exaggerated in a *pif1pif3* double mutant, is most likely due to photooxidative destruction of chlorophyll. Our results are entirely consistent with this explanation, because the loss of chlorophyll is dependent on the length of the dark period before transfer (and therefore the degree of excess Pchlide production), the fluence rate of WL, and the time of WL exposure. Misregulation of the tetrapyrrole synthesis pathway commonly leads to a photobleaching phenotype (e.g., refs. 29 and 30), and overac-

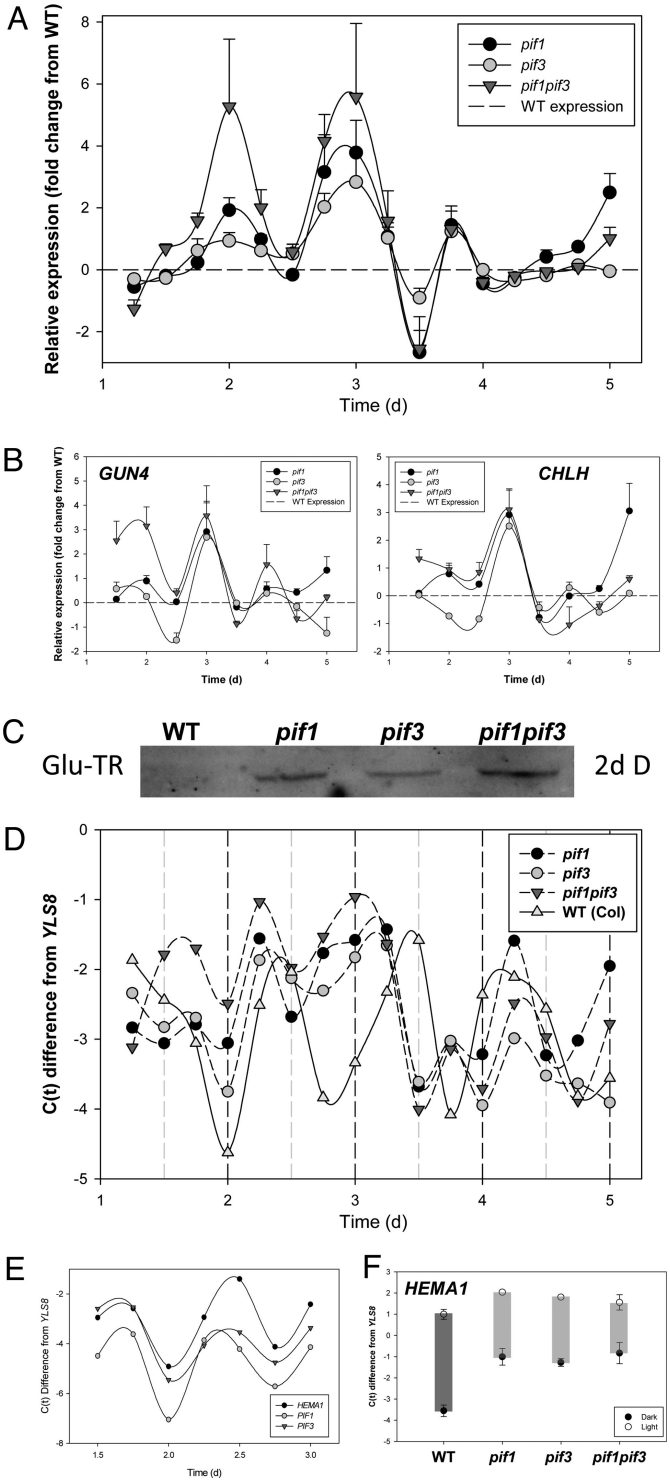

Fig. 4. Expression of tetrapyrrole synthesis genes in *pif* mutant seedlings. (*A*) Real-time PCR data showing expression of *HEMA1* in dark-grown *pif* mutant seedlings. Data are presented as the fold difference from WT after normalizing to the control gene *YLS8*. (*B*) *GUN4* and *CHLH* expression as for (*A*). (*C*) Glu-TR protein levels in WT and *pif* mutant seedlings after 2 d in the dark. One of 2 repeat experiments with similar results is shown, and equal protein loading was confirmed by staining duplicate gels. (*D*) Expression of *HEMA1* in dark-grown WT and *pif* mutant seedlings replotted from (*A*). (*E*) Expression of *PIF1*, *PIF3*, and *HEMA1* in dark-grown WT seedlings. (*F*) Expression of *HEMA1* in WT and *pif* mutants after either 3 d in the dark (filled symbols) or 2 d in the dark + 1 d of WL (110 μmol·m^{-2}·s^{-1}; open symbols). Vertical bars indicate the level of light induction. Values shown are the mean ± SE of ≥3 independent experiments.

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cumulation of Pchlide is well established as leading to photooxidative damage (29), for example, in the FR block of greening response (31).

The *pif1pif3* Double Mutant Shows a Constitutively Photomorphogenic Phenotype. One interesting phenotype that we observed for the *pif1pif3* double mutant was that it showed a moderate constitutive photomorphogenic response in dark-grown seedlings (Fig. 1). This response was seen even when seeds were kept in complete darkness postimbibition (Fig. S3). Further investigation demonstrated that both *pif1* and *pif3* single mutants showed a similar, but less pronounced, response. A shorter hypocotyl in the dark has been seen for *pif3* (14, 18) and *pif1* (32), and a similar phenotype with expanded cotyledons, hook opening, and hypocotyl inhibition was recently observed for *pif1*, *pif3*, and a *pif1pif3* double mutant (28). In this case, the authors reported a synergistic interaction between PIF1 and PIF3 in contrast to the additive phenotype reported here. A constitutive photomorphogenic phenotype of the *pif1pif3* double mutant is expected based on the stronger, dominant negative phenotype of overexpressed truncated PIF1 (12). Presumably, in this case, the PIF1 protein is interfering with the function of additional PIFs, including PIF4 and PIF5 (28). Interestingly, constitutive activation of phytochromes in the dark also results in this phenotype, which could result from Pfr-mediated degradation of multiple PIFs (33). However, whether the *pif1pif3* double mutant still requires the presence of seed Pfr (produced during seed set) to reveal the response remains to be seen. In our assays, we saw all aspects of the phenotype in seedlings that had only seen light during seed plating, before the seeds had fully imbibed, and Leivar et al. (28) were unable to block the *pif1* and *pif1pif3* response, even with a FR light treatment immediately after plating.

PIF1 and PIF3 Repress the Expression of key Chlorophyll Synthesis Genes. The rate-limiting step for Pchlide (and chlorophyll) synthesis is the enzyme glutamyl tRNA reductase (24). Light regulation of this step is mediated through changes in expression of the *HEMA1* gene (34), and *HEMA1* is one of a small group of highly regulated tetrapyrrole genes including *CHLH* and *GUN4* (25, 27). The substantial increase in *HEMA1* expression and consequent increase in glutamyl tRNA reductase protein can fully account for the observed increase in Pchlide levels in the *pif1* and *pif3* mutants. The increase in tetrapyrrole synthesis in *pif1* was previously suggested to be due to a subtle down-regulation of the ferrochelatase gene (*FCII*) and a concomitant up-regulation of the heme oxygenase *HO3*, resulting in less free heme and less inhibition of glutamyl tRNA reductase activity (35), the opposite of the phenotype of the phytochrome-chromophore-deficient mutants in which the heme branch of the pathway is almost completely blocked (36). We have not tested these genes directly, but because *HO3* has exceptionally low expression in seedlings and its loss has no impact on chromophore synthesis in the presence of HO1 (37), these changes are unlikely to make more than a minor contribution compared with the substantial increase in levels of the rate-limiting enzyme of the pathway. One reason that previous studies did not observe the changes seen here is that microarrays using dark-grown *pif1* (35) and *pif3* (15) and their follow-up analyses were performed using seedlings that had been grown for 4 d in the dark. As is clear from our current studies (Fig. 4 and Fig. S2*D*) differences between WT and the *pif* mutants are minor at this time.

PIF1 and PIF3 may Function in the Output from the Circadian Clock. We observed that both the *pif1* and *pif3* mutations affected circadian regulation of *HEMA1*, *CHLH*, and *GUN4*. *HEMA1* and *CHLH* have been shown to be circadian regulated in the light (25), but circadian regulation for *GUN4* has not been reported previously.

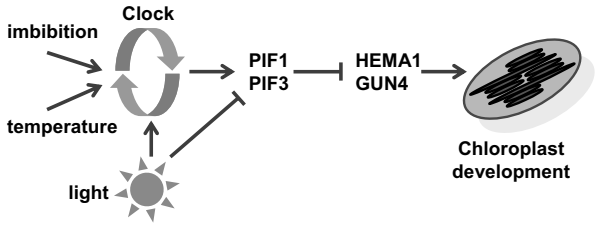

Fig. 5. Model for regulation of tetrapyrrole synthesis genes and chloroplast development in *pif* mutant seedlings.

The altered clock regulation of *HEMA1* was not due to a major defect in the circadian clock, because the *pif* mutants did not have a strong effect on the expression of the core clock components *CCA1*, *LHY*, and *TOC1*. The control output gene *CAX1*, a H^+/Ca^{2+} antiporter (38) unrelated to chloroplast function, was also unaffected, suggesting that PIF1 and PIF3 function specifically in circadian control of genes involved in chloroplast development. A circadian clock has been shown to be functional in dark-grown *Arabidopsis* seedlings, with entrainment initiated through changes in temperature or imbibition (39), and can be observed just 2 d after imbibition (39) or even earlier (40). Moreover, the importance of this clock in controlling chloroplast development is supported by the observation that a range of clock mutants fail to green normally after transfer to WL (40). We therefore propose that PIF1 and PIF3 function in circadian control of chloroplast development as shown in the model in Fig. 5. Furthermore, we favor a role for the PIF proteins in the output from the clock. Although phytochrome has a major role in the entrainment of the circadian clock by light (41), PIF3 does not play a significant role in controlling light input or function of the clock (15, 42, 43). Although we cannot completely rule out a role in entrainment, the apparent specificity of the response for chloroplast development genes suggests otherwise.

In our experiments, *PIF1* and *PIF3* showed robust circadian regulation in dark-grown seedlings, suggesting that clock regulation of PIF function is via circadian control of expression. Analysis of multiple circadian microarray experiments suggests that *PIF1*, but not *PIF3*, expression is under circadian control (44). However, a low-amplitude circadian rhythm has also been observed for *PIF3* using a *PIF3:LUC*$^+$ reporter construct (43). Within the resolution of our experiments, the *PIF* genes appear to cycle in the same circadian phase as *HEMA1*, precluding a simple mechanism of circadian-regulated *PIF* repression of *HEMA1* expression. However, a small difference in phase could still permit such a mechanism. Alternatively, because both PIF1 and PIF3 have been shown to interact directly with TOC1 (45), a model in which the clock controls PIF function through direct protein interaction is also plausible.

In summary, our results show that both PIF1 and PIF3 are negative regulators of chloroplast development that function to integrate light and circadian control of this critical process. Exactly how they achieve this will be the focus of future studies.

Experimental Procedures

Plant Growth Conditions. *Arabidopsis* (*Arabidopsis thaliana* L.) seeds were imbibed at 4 °C for 2 d in darkness, followed by 2 h of WL (110 μmol·m^{-2}·s^{-1}), and returned to darkness at 23 °C, indicating the start of the respective experiment (unless otherwise stated).

Phenotypic Analyses. For hypocotyl measurements, 15 *Arabidopsis* seedlings were measured, and the longest 10 were averaged for 1 biological repeat. For Pchlide measurements, 100 μg of seedling material was extracted twice in acetone/0.1 M NH_4OH, 90:10 (vol/vol) as described in ref. 36. Chlorophyll was measured as described in ref. 27. Cotyledon samples for transmission electron microscopy were prepared and examined as described in ref. 36. Numerous plastids in at least 2 independent samples were viewed for each

genotype and experimental condition and photographs were taken of representative plastids.

Gene Expression Analyses. RNA extraction and real-time PCR methods were exactly as described in ref. 27, with one exception (see *SI Text*). To assess the expression of genes between genotypes at different time points, the absolute $C(t)$ value of the *YLS8* control gene was subtracted from the absolute value of the experimental gene for each biological replicate, and the average $C(t)$ value for all biological replicates was used for comparison between genotypes. For primers, see *SI Text*. Protein extraction and immunoblotting were conducted exactly as described in refs. 27 and 36, with 50 seedlings extracted in 100 μL of SDS extraction buffer.

ACKNOWLEDGMENTS. We thank Anton Page (Biomedical Imaging Unit, University of Southampton) for help with electron microscopy and Dieter Söll (Yale University) for the polyclonal antibody to glutamyl tRNA reductase. We also thank Patricia Hornitschek, Paula Duek, and Séverine Lorrain for generating the mutants used in this study. P.G.S. was supported by a studentship from the Biotechnology and Biological Sciences Research Council, and work in the C.F. laboratory was supported by Swiss National Science Foundation Grant 3100A0-112638.

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