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PIF3 is a repressor of chloroplast development

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Abstract

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 pro-thylakoid membranes than corresponding wild-type seedlings, similar to previous reports of constitutive photomorphogenic mutants. Consistent with this observation, *pif1*, *pif3* and *pif1pif3* all showed reduced hypocotyl elongation and increased cotyledon opening in the dark. Transfer of 4d-old dark-grown seedlings to white light resulted in more chlorophyll synthesis in *pif* mutants over the first 2h 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 2d after germination. Circadian regulation of *HEMA1* in the dark also showed reduced amplitude and a shorter, variable period in the *pif* mutants while 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. It is proposed that PIF1 and PIF3 are negative regulators that function to integrate light and circadian control in the regulation of chloroplast development.

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Introduction

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, while the phytochromes are responsible for regulating growth and development in response to red (R) and far-red (FR) light [1]. In Arabidopsis there are five phytochromes (phyA-E) that between them 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]. Following light absorption phytochromes rapidly relocate to the nucleus where they control the response to light through two main mechanisms. Firstly, they act to exclude the E3-ubiquitin ligase, COP1, from the nucleus thereby preventing the degradation of the positive signalling factors HY5, HFR1 and LAF1 [3]. Secondly, phytochromes bind to 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 signalling protein [11,12]. Although there seems to be broad agreement on what is known about the molecular events following 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 as 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

lead to the hypothesis that PIF3 has a dual function, acting early and positively as a transcription factor, while 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] as well as acting to repress chlorophyll biosynthesis [20].

Given the controversy in the role of PIF3 we have re-evaluated 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* 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 new *pif1* allele designated *pif1-101* (see *SI text* and Fig S6). The PIF1 protein has previously 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 1A). All lines showed an increase in Pchlide. This increase was clearly detectable 2½ d after germination andat all time points the response of the *pif1pif3* double mutant appeared additive to that of *pif1* and *pif3*. Analysis of Pchlide in the *pif1-2* and *pif3-3* alleles resulted in almost identical results (Fig S7A). Since 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 germinated following 2h 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 S8A, 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 as all genotypes showed at least 95% germination by day one in these experiments (data not shown).

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 1B). 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 pre-treatment as seedlings germinated completely in the dark showed the same response (Fig S8B). 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 to WT with the *pif1pif3* double mutant again showing an additive phenotype (Fig 1C). This was also true in the absence of the WL pre-treatment (Fig S8C). Finally, one distinctive feature of constitutively photomophorphogenic seedlings such as *cop1* is that they show a partially-developed chloroplast in the dark that is characterised by a reduced prolamellar body (PLB) and increased prothylakoid membranes [22]. We therefore examined etioplasts in dark-grown *pif* mutant seedlings (Fig 2A-D). After 4d dark WT etioplasts showed a characteristic, highly regular PLB with little pro-thylakoid development (Fig 2A). In contrast both *pif1* and *pif3* etioplasts showed increased membrane development and PLBs that were generally reduced in size (Fig 2B,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 2D). In some cases no PLB was observed at all in *pif1pif3* double mutant seedlings, although full-size PLBs were detected occasionally (data not shown).

Greening of _pif1_ and _pif3_ is dependent on the time of transfer to white light

When grown in the dark for 4d before transfer to WL *pif1pif3* double mutants failed to green over the next 24h with *pif1* and *pif3* showing only moderate greening during this period (Fig 3A, S9). An identical result was observed with the *pif1-2* and *pif3-3* alleles (Fig S7B,C) and has been observed previously for *pif1* [20]. Interestingly, detailed examination of the time course following transfer to WL showed that at 2h *pif1*, *pif3* and the *pif1pif3* double mutant had more chlorophyll than WT, but that this was already reversed after 4h WL (Fig 3B). In contrast to the situation after 4d dark, seedlings transferred to WL after 2d dark were able to green with the *pif1pif3* double mutant accumulating the most chlorophyll (Fig 3C). The *pif1-2* and *pif3-3* alleles were also able to green more efficiently under these conditions (Fig S7B,C) and this ability was gradually lost as seedlings aged at transfer to WL (Fig 3C, Fig S7C). The timecourse of chlorophyll loss following transfer to WL and the effect of increasing the dark period on subsequent greening ability is consistent with the loss of chlorophyll in the *pif* mutants being due to photo-oxidative 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 3D, as the fluence rate increased the relative loss of chlorophyll in the *pif* mutants also increased compared to WT, consistent with photo-oxidation being the primary cause of chlorophyll deficiency in the *pif* mutants.

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

Chloroplasts in the *pif3* mutant were generally more similar to WT in appearance than for *pif1* or *pif1pif3* (Fig 2K) 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 6h timepoints from 1¼ d after germination using real-time RT-PCR. *HEMA1* expression was strongly induced in all *pif* mutants relative to WT at 2d and 3d after germination with the response severely diminished or lost at days 4 and 5 (Fig 4A). The response in the *pif1pif3* double mutant was again equivalent to both single mutants combined. We also analysed two 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 3d after germination, high expression after 2d and little induction if any after 4d (Fig 4B). Analysis of *GUN4* expression in the *pif1-2* and *pif3-3* alleles gave similar results when measured 3d and 4d after germination (Fig S7D). Examination of glutamyl tRNA reductase protein levels showed an increase in *pif1*, *pif3* and the *pif1pif3* double mutant by 2d dark (Fig 4C).

The profile of the relative induction of *HEMA1* in the *pif* mutants is quite unusual with sharp peaks 2d and 3d after germination, but no induction at 2½ d. To understand the basis for this we plotted the level of *HEMA1* mRNA relative to *YLS8* for WT and the *pif* mutants independently (Fig 4D). This analysis revealed two main observations. Firstly, *HEMA1* expression was generally higher at early time points in *pif1*, *pif3* and *pif1pif3* compared to WT. Secondly, and most strikingly, *HEMA1* expression was out of circadian phase in the *pif* mutants compared to WT seedlings. While expression of *HEMA1* oscillated with a period close to 24h in WT seedlings, *pif1*, *pif3* and *pif1pif3* all showed a reduction in the amplitude of oscillation and a period of oscillation that was variable ranging from about 12h to 22h for *pif1pif3* (Fig 4D). 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 S10A-C). No major changes in expression were observed for all three genes indicating that the clock is still functional under these

conditions. 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 S10D) 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 4E).

***pif* mutants still show light induction of tetrapyrrole biosynthesis genes**

Since PIF3 has been proposed to function positively in the light induction of nuclear-encoded chloroplast genes we followed gene expression after transfer to 24h WL, a time at which chlorophyll levels are severely reduced in the mutants. Although as noted before expression was higher in the dark for *HEMA1*, *CHLH* and *GUN4* in all *pif* mutant lines, all three genes were light induced to a similar degree and the final expression level of these genes in the light was still higher in *pif1*, *pif3* and *pif1pif3* than in WT (Fig 4F, S11). We also tested whether *pif* mutants could respond to monochromatic lights sources and over shorter time periods. As shown in Fig S7, induction of *GUN4* and *CHLH* was still apparent in *pif1-2* and *pif3-3* after 4h FR and 8h 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 1A) and initial rates of chlorophyll synthesis were also greater (Fig 3B,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 previously 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 [16,17]. Our data suggest that

for the earliest stages of chloroplast development and for the target genes we have analysed (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 overexpression of PIF3 is not sufficient for 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 signalling protein.

The reason for the previous misinterpretation of the *pif3* mutant phenotype is that seedlings transferred to WL after 4d dark showed a reduced level of chlorophyll compared to WT (Fig 3). This response, which is identical for *pif1* and exaggerated in a *pif1pif3* double mutant, is most likely due to photo-oxidative destruction of chlorophyll. Our results are entirely consistent with this explanation as the loss of chlorophyll is dependent on the length of the dark period prior to 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. [29,30]) and over accumulation of Pchlide is well established as leading to photo-oxidative damage [29], for example in the FR block of greening response [31].

The *pif1pif3* double shows a constitutively photomorphogenic phenotype

One interesting phenotype 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 post imbibition (Fig S8). 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 previously 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 PIF 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, it remains to be seen whether the *pif1pif3* double mutant still requires the presence of seed Pfr (produced during seed set) to reveal the response. 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. It was previously suggested that the increased in tetrapyrrole synthesis in *pif1* was due to a subtle downregulation of the ferrochelatase gene (*FCII*) and a concomitant upregulation of the heme oxgenase *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 as *HO3* has exceptionally low expression in seedlings and its loss has no impact on chromophore synthesis in the presence of HO1 [37], it is unlikely that these changes make more than a minor contribution compared to 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-gown *pif1* [35] and *pif3* [15] and their follow-up analyses were all performed using seedlings that had been grown for 4d in the dark. As is clear from our current studies (Fig 4, S7D) 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 previously been shown to be circadian regulated in the light [25], but this is the first demonstration of circadian regulation for *GUN4.* The altered clock regulation of *HEMA1* was not due to a major defect in the circadian clock as 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 previously 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 2d 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 following 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 favour 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] it has previously been shown 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 a 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 previously 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, since 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.

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Experimental Procedures

Plant growth conditions

Arabidopsis (*Arabidopsis thaliana* L.) seeds were imbibed at 4°C for 2d in darkness, followed by 2h WL (110 $\mu mol.m^{-2}.s^{-1}$) 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 averaged for one biological repeat. For Pchlide measurements 100 µg seedling material was extracted twice in acetone:0.1M NH_4OH, 90:10 (v/v) as described previously [36]. Chlorophyll was also measured as described previously [27]. Cotyledon samples for transmission electron microscopy were prepared and examined as previously [36]. Numerous plastids in at least two 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 RT-PCR methods were exactly as described previously [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 used for comparison between genotypes. For primers see *SI Text*. Protein extraction and immunoblotting were conducted exactly as described previously [27,36] with 50 seedlings extracted in 100 μl SDS extraction buffer.

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Figure legends

Fig. 1. Dark-grown phenotype of *pif* mutant seedlings. (A) Pchlide accumulation in WT and *pif* mutant seedlings in darkness. (B) Cotyledons of WT and *pif* mutant seedlings after 4d 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.

Fig. 2. Plastid ultrastructure in *pif* mutant seedlings. Transmission electron micrographs of plastids from WT (A,E,I), *pif1* (B,F,J), *pif3* (C,G,K) and *pif1pif3* (D,H,L) seedlings. Seedlings were grown for 4d dark (A-D), 2d dark followed by 1d WL (110 $\mu mol.m^{-2}.s^{-1}$) (E-H) or 4d dark followed by 1d WL (I-L). Bar corresponds to 500 nm (A-D) or 1 μm (E-L).

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

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 is 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 following 2d dark. One of two repeat experiments with similar results is shown and equal protein loading was confirmed by staining of duplicate gels (data not shown). (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 following either 3d dark (filled symbols) or 2d dark + 1d WL (110 $\mu mol.m^{-2}.s^{-1}$; open symbols). Vertical bars indicate the level of light induction. Values shown are the mean ± SE of ≥3 independent experiments.

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

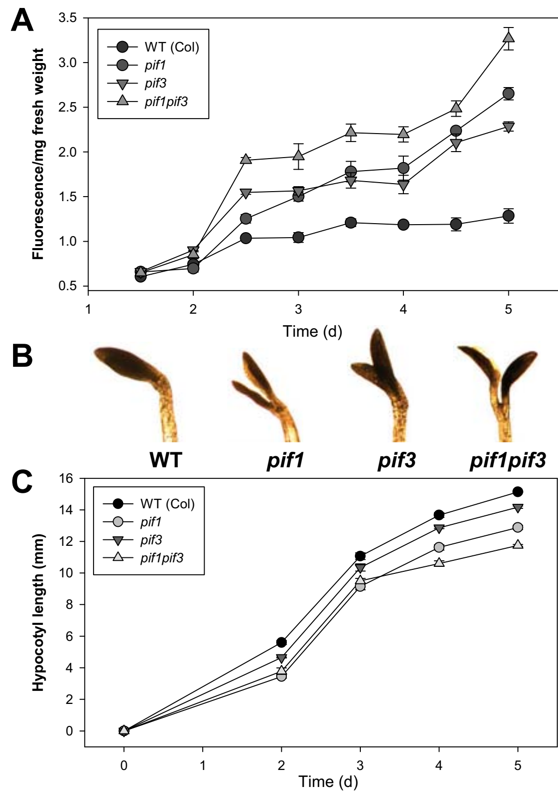
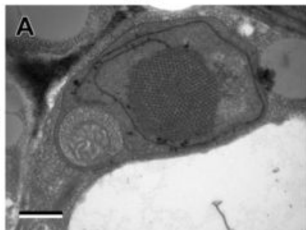


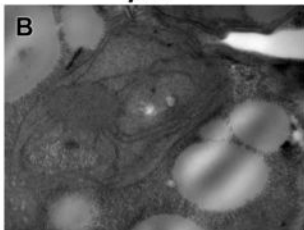
Figure 1

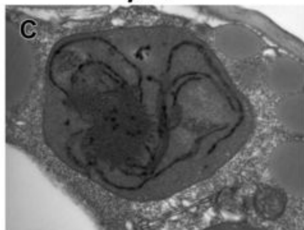
Figure 2

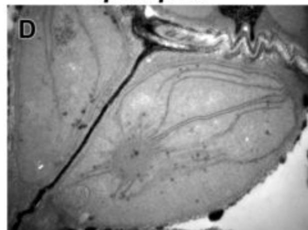
WT | *pif1* | *pif3* | *pif1 pif3*

4d D

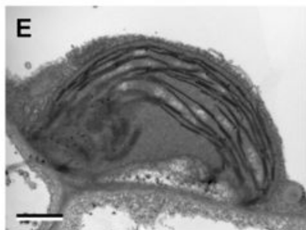


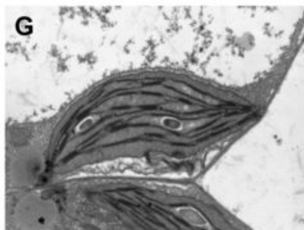


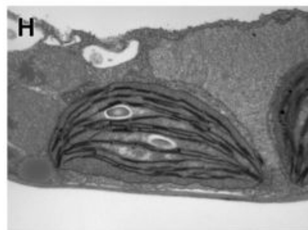




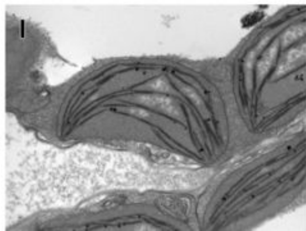
2d D + 1d WL

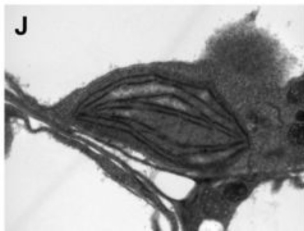


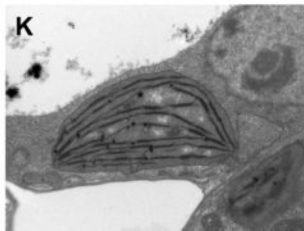


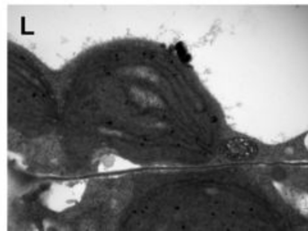


4d D + 1d WL









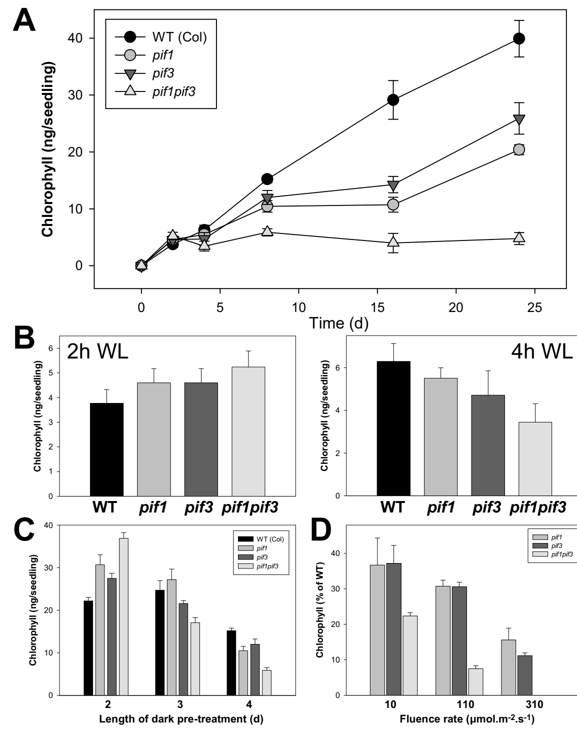


Figure 3

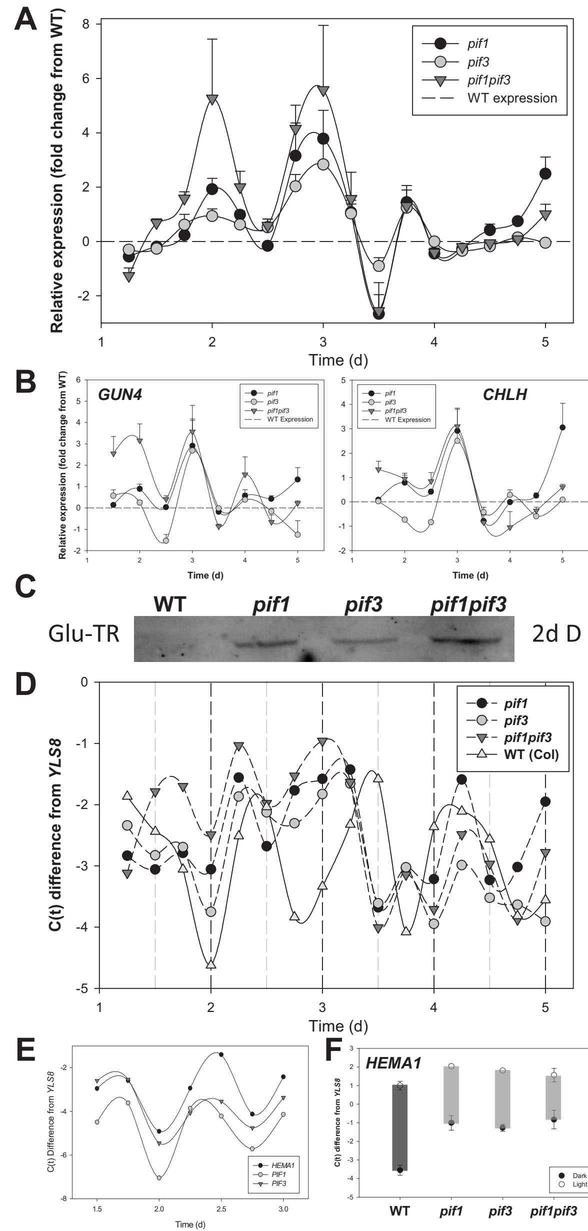


Figure 4

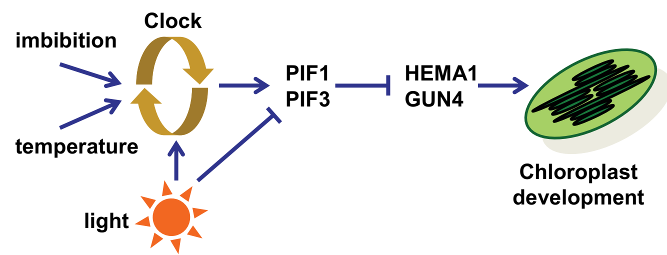


Figure 5