Title: PIF3 is a repressor of chloroplast development.
Authors: Stephenson PG, Fankhauser C, Terry MJ
Journal: Proceedings of the National Academy of Sciences of the United States of America
Year: 2009 May 5
Volume: 106
Issue: 18
Pages: 7654-9
DOI: 10.1073/pnas.0811684106
PIF3 is a repressor of chloroplast development

Patrick G. Stephenson¹, Christian Fankhauser², Matthew J. Terry¹

¹School of Biological Sciences, University of Southampton, Boldrewood Campus, Southampton
SO16 7PX, UK

²Center for Integrative Genomics, University of Lausanne, CH-1015 Lausanne, Switzerland

Address for correspondence: Dr Matthew Terry
School of Biological Sciences,
University of Southampton,
Boldrewood Campus,
Southampton SO16 7PX, UK

Tel: +44 2380 592030
Fax: +44 2380 594459
E-mail: mjt@soton.ac.uk

Text pages: 19
Figures: 5
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.
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 and at 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 photomorphogenic 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 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 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-grown 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+/Ca²⁺ 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.

Acknowledgments:

Thanks to 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. Thanks also to Patricia Hornitschek, Paula Duek and Séverine Lorrain for generating the mutants used in this study. PGS was supported by a studentship from the Biotechnology and Biological Sciences Research Council and work in the CF laboratory was supported by Swiss National Science Foundation grant n° 3100A0-112638.

Experimental Procedures

Plant growth conditions
Arabidopsis (Arabidopsis thaliana L.) seeds were imbibed at 4°C for 2d in darkness, followed by 2h 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 averaged for one biological repeat. For Pchlide measurements 100 µg seedling material was extracted twice in acetone:0.1M NH₄OH, 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.

References


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 µmol.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 µmol.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 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.
Fig. 5. Model for regulation of tetrapyrrole synthesis genes and chloroplast development in *pif* mutant seedlings.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5