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Journal: The Plant journal : for cell and molecular biology

Year: 2009 Nov

Volume: 60

Issue: 3

Pages: 449-61

DOI: 10.1111/j.1365-313X.2009.03971.x

Phytochrome Interacting Factors 4 and 5 redundantly limit seedling de-etiolation in continuous far-red light.

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Running title: PIF4 and PIF5 control de-etiolation in FR light.

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Key words: phytochrome, phyA, phytochrome interacting factor (PIF), photomorphogenesis, Arabidopsis, priming

Abstract

Phytochromes are red/far red photosensors regulating numerous developmental programs in plants. Among them phytochrome A (phyA) is essential to enable seedling de-etiolation in continuous far-red (FR) light a condition mimicking the environment under a dense canopy. The ecological relevance of this response is demonstrated by the high mortality rate of *phyA* mutants germinating in deep vegetational shade. phyA signaling involves a direct interaction of the photoreceptor with members of the bHLH transcription factor family, PIF1 and PIF3 (Phytochrome Interacting Factor). Here we investigated the involvement of PIF4 and PIF5 in phyA signaling and found that they redundantly control de-etiolation in FR light. The *pif4pif5* double mutant is hypersensitive to low fluence rates of FR light. This phenotype is dependent on FR light perception by phyA but does not rely on alterations of the phyA level. Our microarrays analysis shows that PIF4 and PIF5 are part of an inhibitory mechanism repressing the expression of some light-responsive genes in the dark and are also needed for full expression of several growth-related genes in the light. Unlike PIF1 and PIF3, PIF4 and PIF5 are not degraded in response to FR light indicating that they are light-regulated by a different mechanism. Our genetic analysis suggests that this is achieved through the sequestration of these PIFs by the closely related bHLH transcription factor HFR1 (long Hypocotyl in FR light).

Introduction

In natural conditions, light is a mix of different wavelengths whose quantity and quality depend on the environmental conditions. For instance, reduction of the red (R) to far red (FR) ratio in the incoming light is observed under a canopy and controls the shade avoidance program in shade-intolerant plants (Vandenbussche *et al.*, 2005). These different light parameters are perceived and decoded by a set of photoreceptors to optimize plant growth and development according to their environment. They control developmental programs such as germination, de-etiolation, flowering time as well as adaptive responses that maximize light capture such as phototropism. Phytochromes are red/far-red (R/FR) photosensors while several distinct photoreceptor families specifically sense blue light (Chen *et al.*, 2004; Rockwell and Lagarias, 2006; Bae and Choi, 2008). The model plant *Arabidopsis thaliana* possesses 5 phytochromes (phyA-E), which play redundant and specific functions throughout the plant life cycle. While seedlings grown in the dark are etiolated with long hypocotyls and closed cotyledons, seedlings grown in monochromatic R or FR light present short hypocotyls with opened and expanded cotyledons (Quail, 2002a). phyA and phyB are the main photoreceptors involved in seedling de-etiolation in R light, with phyA playing a predominant role in rapid light-regulated gene expression (Tepperman *et al.*, 2006). In contrast, phyA is the only photoreceptor involved in the so-called FR-High Irradiance Response (FR-HIR) developed during prolonged exposure to FR light. This light condition mimics the environment encountered by a seedling developing under a dense canopy. Indeed physiological experiments have demonstrated that phyA is important to promote seed germination and to mediate seedling survival under a canopy (Yanovsky *et al.*, 1995; Botto *et al.*, 1996).

The phytochromes exist into two interconvertible forms: the R-absorbing state called Pr is the inactive form while the FR-absorbing Pfr state is the active one. The phytochromes are synthesized in their inactive Pr form and are localized in the cytoplasm. Upon light perception, they are activated and translocated into the nucleus where they will modulate gene expression (Ma *et al.*, 2001; Tepperman *et al.*, 2001; Tepperman *et al.*, 2006;

Fankhauser and Chen, 2008). Importantly FR light conditions that lead to a FR-HIR will lead to a photoequilibrium with about 3% Pfr/Ptot. The exact mode of action of phyA under these conditions is however still not fully understood (Shinomura *et al.*, 2000). Recently, members of the bHLH transcription factors called PIFs for Phytochromes Interacting Factors have been described as a direct way to connect phytochromes and gene activation (Castillon *et al.*, 2007). These proteins have been involved in phytochrome-regulated processes such as seedling de-etiolation in R and FR light, germination and responses to shade signal (Castillon *et al.*, 2007). They interact with the active form of phyB (PIF1, PIF3-5, PIF7) or phyA (PIF1 and PIF3) through a domain located in the N-terminal part of the proteins respectively called APB (Active Phytochrome B) or APA (Active Phytochrome A) (Khanna *et al.*, 2004; Al-Sady *et al.*, 2006; Leivar *et al.*, 2008a; Shen *et al.*, 2008). Interestingly, all the PIFs described so far are stable in the dark. With the exception of PIF7, R-light perception induces phytochrome-dependent PIF phosphorylation and their subsequent degradation through the 26S proteasome (Bauer *et al.*, 2004; Al-Sady *et al.*, 2006; Nozue *et al.*, 2007; Shen *et al.*, 2007; de Lucas *et al.*, 2008; Lorrain *et al.*, 2008; Shen *et al.*, 2008). It has thus been proposed that PIFs are negative regulators of phytochrome-mediated responses, which are targeted for degradation upon light perception in a phytochrome-dependent process. For instance, phytochromes-induced degradation of PIF1 promotes germination and releases the inhibitory action of PIF1 on chlorophyll biosynthesis (Huq *et al.*, 2004; Oh *et al.*, 2004; Oh *et al.*, 2006; Moon *et al.*, 2008). Consistent with this idea multiple PIFs redundantly repress photomorphogenesis in the dark suggesting that the phytochromes activate this program by targeting PIFs to degradation (Leivar *et al.*, 2008b; Shin *et al.*, 2009; Stephenson *et al.*, 2009).

We previously showed that PIF4 and PIF5 are involved in responses to shade, a pathway mainly controlled by phyB (Lorrain *et al.*, 2008). Moreover PIF4 and PIF5 have been shown to act during the de-etiolation process in R light, another light condition during which phyB plays a predominant function (Fujimori *et al.*, 2004; Huq *et al.*, 2004; Khanna *et al.*, 2007). To test whether these two PIFs are also required for phyA-mediated light responses we studied the FR-HIR, which is exclusively controlled by phyA. Our

analysis showed that PIF4 and PIF5 redundantly control several light responses to continuous FR light with *pif4pif5* displaying a hypersensitive response to FR light. This phenotype was dependent on FR light perception by phyA but was not mediated by changes in phyA accumulation. Neither phosphorylation nor degradation of PIF4 and PIF5 were observed under these conditions suggesting that PIF4 and PIF5 light regulation is different in FR than in R light. Microarray experiments showed that in the dark *pif4pif5* expressed higher levels of numerous light-responsive genes. Moreover some of those genes were also expressed at higher levels in *pif4pif5* when the seedlings were exposed to FR light, which may explain the enhanced FR light response of the double mutant. Interestingly after 24 hours in FR light several gene products directly related to growth processes were expressed at lower levels in *pif4pif5*, which is consistent with the role of PIF4 and PIF5 in promoting hypocotyl elongation (Nozue *et al.*, 2007; Lorrain *et al.*, 2008; Niwa *et al.*, 2009).

Results

The *pif4pif5* double mutant is hypersensitive to FR light

It was previously shown that *pif4, pif5* and *pif4pif5* are hypersensitive to R light, with an additive phenotype in the double mutant (Fujimori *et al.*, 2004; Huq *et al.*, 2004; Khanna *et al.*, 2007; Lorrain *et al.*, 2008). On the contrary and in agreement with previous publications, *pif4* and *pif5* were indistinguishable from the wild type (WT) when grown in FR light (Figure 1a) (Fujimori *et al.*, 2004; Huq *et al.*, 2004; Khanna *et al.*, 2007). Nevertheless we noticed that plants over-expressing PIF4 or PIF5 either full length or deleted for their N terminus presented long hypocotyls in FR light (Supplemental Figure 1) (Lorrain *et al.*, 2008). There was a correlation between the protein level and hypocotyl length. This prompted us to re-evaluate the role of PIF4 and PIF5 in FR light using both single and double loss-of-function mutants. While the *pif4pif5* double mutant behaved as the WT under high fluence rates of FR, it presented a hypersensitive phenotype at lower fluence rates with shorter hypocotyls and cotyledon opening at fluence rates where the WT maintained closed cotyledons (Figure 1a, 1b).

This light hypersensitivity was also observed for inhibition of gravitropism, another response induced by FR light. When seedlings are grown in the dark, they grow straight against the gravity vector, which can be interpreted as a way to reach the surface of the soil. Negative gravitropism is inhibited by R or FR light, which may allow the plants to be more flexible to respond to phototropism (Iino, 2006). We quantified this response as previously described (Oh *et al.*, 2004). Basically seedlings that do not touch the agar plate are considered to have negative gravitropic hypocotyls. The *phyA* mutant is blind to FR light and almost all *phyA* seedlings grew straight in all tested light conditions (Figure 1c). On the contrary WT seedlings fell upon FR light perception, a response that is dependent on the fluence rate (Figure 1c). As observed for hypocotyl length and cotyledon opening, *pif4pif5* behaved as a WT at 5 µmol/m^2/s and was hypersensitive to FR light under lower fluence rates. Interestingly the single *pif4* and *pif5* mutants presented less negative gravitropic hypocotyls than the WT, this difference was

significant for *pif4* at both 0.5 and 0.05 µmol/m^2/s while for *pif5* the difference was only detected at 0.5 µmol/m^2/s.

Another characteristic response to FR light is the accumulation of anthocyanins. As a positive control we analyzed the *cop1* mutant, which is de-etiolated in the dark and is hypersensitive to light. After 5 days in constant FR light, anthocyanins were extracted and quantified. The lowest fluence rate at which light induced detectable changes in anthocyanin content in the WT, *pif4*, *pif5* and *pif4pif5* mutants was 0.5 µmol/m^2/s (Figure 1d). On the contrary the *cop1* mutant presented higher level of anthocyanins in the dark and for all fluence rate tested. No significant differences were observed between the WT and the double mutant suggesting that either *pif4pif5* is not affected for anthocyanin accumulation or that these changes are too subtle to be detected at the low fluence rate, where the double mutant is phenotypically different from the WT.

pif4pif5 hypersensitivity to FR light is dependent on FR perception by phyA but not on phyA level

Since it has been proposed that *pif* hypersensitivity to R light could be due to over-accumulation of phyB (Khanna *et al.*, 2007; Al-Sady *et al.*, 2008; Leivar *et al.*, 2008a) we checked whether FR hypersensitivity of *pif4pif5* correlated with changes in phyA transcript/protein levels. The level of *PHYA* mRNA in etiolated seedlings or etiolated seedlings treated with FR light was not enhanced in *pif4pif5* seedlings (Figure 6). Since light affects the stability of phyA protein we monitored phyA accumulation. We quantified phyA levels in etiolated seedlings and seedlings grown under two fluence rates of FR light, as well as in etiolated seedlings transferred to R light to induce rapid phyA degradation (Hennig *et al.*, 1999). phyA levels were not significantly different in *pif4*, *pif5* and *pif4pif5* as compared to WT in 5-day-old seedlings subjected to constant irradiation with FR light either at 0.05 µmol/m^2/s (when *pif4pif5* seedlings had a clear phenotype) or at 5 µmol/m^2/s (Figure 2a). If anything there was a slightly reduced level of phyA in *pif4pif5* at 5 µmol/m^2/s, however this should result in hyposensitivity rather than hypersensitivity to FR light. The kinetics of phyA degradation in etiolated seedlings

transferred to R light was also unaffected in the *pif* mutants (Figure 2b). This indicates that the *pif4pif5* mutations do not affect the light-regulated stability of phyA and argues against the idea that the FR hypersensitivity displayed by *pif4pif5* is due to phyA over accumulation.

To verify whether the hypersensitivity of *pif4pif5* to FR light requires phyA-mediated light perception we generated and analyzed a *phyApif4pif5* triple mutant. The *phyApif4pif5* mutant behaved as *phyA* mutants at all FR fluence rates tested (Figure 3). Especially *phyApif4pif5* was blind to FR light with long hypocotyl and closed cotyledons (Figure 3a, b). Furthermore, while the WT and *pif4pif5* seedlings respond to FR with a decrease in negative gravitropic hypocotyls, 90% of *phyApif4pif5* seedlings stayed straight, as did the *phyA* seedlings. In conclusion, *pif4pif5* hypersensitivity to FR light was not due to a higher level of phyA but required phyA-mediated light sensing, indicating that PIF4 and PIF5 are part of signaling mechanism acting downstream of phyA light perception.

PIF4 and PIF5 control the expression of a set of FR-expressed genes in the dark

FR light perception induces massive modifications of gene expression in etiolated seedlings (Ma *et al.*, 2001; Tepperman *et al.*, 2001). To get a better understanding of the *pif4pif5* phenotype we analyzed gene expression in the mutant in darkness and after transfer into FR light using the Affimetrix ATH1 gene chip. We extracted RNAs in 3-day-old-etiolated seedlings that were kept in the dark or subjected to 1 or 24 hours of 0.5 $\mu mol/m^2/s$ FR, a fluence rate sufficient to reveal a phenotype in the *pif4pif5* double mutant. Using an ANOVA approach, we looked for genes whose expression was statistically different between the *pif4pif5* mutant and the WT at any of the 3 time points (dark, 1 and 24 hours in FR). This analysis identified 242 genes (FDR < 0.05) with 137 upregulated, 104 downregulated and 2 genes that are upregulated in one condition and downregulated in another one (*At4g14690*: *ELIP2*, *At5g16030*) (Supplemental Table 1). Roughly, the same number of genes was affected in the 3 conditions tested (113 in the dark, 132 and 142 after respectively 1 and 24 hours in FR light, Supplemental Table 1).

In the dark and after one hour in FR, most of those genes were upregulated in *pif4pif5* mutants (78 and 79% respectively), suggesting that PIF4 and PIF5 are mainly acting as negative regulators of gene expression in these conditions (Figure 4a). However the opposite trend was observed after 24h in FR where 73% of the genes affected by *pif4pif5* mutant were downregulated (Figure 4a). Thus, PIF4 and PIF5 appear to be necessary for full expression of the light program after prolonged exposure to FR light.

Enrichment in Gene Ontology (GO) terms was used to identify biological processes affected in the *pif4pif5* mutant. Genes associated with photosynthesis were strongly enriched among *pif4pif5* upregulated genes (Figure 4b, Supplemental Table 2). For instance we identified genes encoding components of the photosynthetic apparatus (e.g. *LHCA1*, *PSAE-1*, *CAB3*) as well as genes involved in tetrapyrrole synthesis (e.g. *PORC*, *HEMA1*, *CHLH/GUN5 GUN4*) (supplemental table 1). Thus, PIF4 and PIF5 function as inhibitors of the mechanisms optimizing the light capture, which is similar to the recently described functions of PIF1 (Moon *et al.*, 2008). On the other hand, fewer terms were enriched among the genes downregulated in the double mutant. Those terms highlight genes associated with auxin pathways (*IAA19, IAA20*) (Figure 4b, data not shown). We also observed an enrichment of genes associated with response to hormone described in (Nemhauser *et al.*, 2006) mostly after prolonged exposure to FR light (data not shown). This result is consistent with the reduced hypocotyl growth and the hypersensitive phenotype displayed by the *pif4pif5* mutant (Figure 1).

Interestingly, 113 genes were already affected in dark-grown *pif4pif5* mutants although we could not observe any phenotype under those conditions (Figure 1). This is consistent with the current view that PIFs proteins are acting in the dark independently of phytochrome activation (Huq *et al.*, 2004; Oh *et al.*, 2004; Oh *et al.*, 2006; Khanna *et al.*, 2007; Leivar *et al.*, 2008b; Moon *et al.*, 2008; Shen *et al.*, 2008; Shin *et al.*, 2009; Stephenson *et al.*, 2009). The analysis of those genes revealed that etiolated *pif4pif5* are more related to the WT after 24 hours in FR light than the WT in the dark (Figure 5a). This is consistent with the fact that 75% of the genes affected in dark-grown *pif4pif5* mutant are light-responsive genes. Especially, we observed an important and tight gene

cluster with late-light-responsive genes (Figure 5b). They are strongly expressed after 24 hours in FR light in the WT and present the same pattern in the *pif4pif5* mutant except that their expression level is already high in the dark (Figure 5b, supplemental Figure 2). Most of these genes encode components ensuring the light capture such as the light harvesting complexes or subunits of the photosystems (Supplemental Figure 2). Thus, a subset of the *pif4pif5* double mutant transcriptome in the dark presents similarity to the one of seedlings grown in FR light.

We selected a number of genes to validate our microarrays data by performing quantitative RT-PCR (Q-PCR) with the WT, *pif4pif5* and *phyA*. Our analysis included both genes affected (e.g. *XTR7*) and not affected (e.g. *HY5*) by *pif4pif5* based on our microarrays data and *PIL1*, which is absent from the ATH1 chip. As expected none of the studied genes were significantly regulated by FR light in the *phyA* mutant. Consistent with our microarrays analysis the expression of some genes was already affected in etiolated *pif4pif5* mutants, such as *PIL1*, *XTR7* and *CAB1*, this latter gene being up regulated in the double mutants while the former were down regulated (Figure 6). These genes present different pattern of expression after transfer to FR light: *PIL1* and *XTR7* are downregulated while *CAB1* is a late upregulated gene (Figure 6). *pif4pif5* responded to the FR light in a similar way as the WT for *XTR7* and *CAB1* but with exaggerated responses (Figure 6). For instance, the light-repression of *XTR7* was exacerbated in *pif4pif5* where this gene is most significantly different from the WT after 24 hours in FR (Figure 6). This is interesting given that *XTR7* codes for a putative cell wall remodeling enzyme. *PIL1* was transiently downregulated by FR light, the down-regulation was similar in the WT and *pif4pif5*, but the subsequent increase upon prolonged exposure to FR light was very much attenuated in *pif4pif5* (Figure 6). We also tested several early light-induced genes (e.g. *HY5*) and confirmed that none of them was affected in the *pif4pif5* mutant (Figure 6, supplemental Figure 3). Thus, our Q-PCR data confirm our microarrays analysis and indicate that *pif4pif5* mutants were only affected in the expression of a subset of genes controlled by FR light. These include many photosynthetic genes, which were already upregulated in etiolated *pif4pif5* mutants (e.g. *CAB1*) and genes that may be directly connected to the growth process (e.g. *XTR7*).

Genetic interaction between *HFR1, PIF4* and *PIF5*

In response to R and white light the levels of PIF4 and PIF5 is rapidly downregulated in a phytochrome-regulated manner which contributes to the reduced hypocotyl elongation in light-grown seedlings (Nozue *et al.*, 2007; Shen *et al.*, 2007; de Lucas *et al.*, 2008; Lorrain *et al.*, 2008). We thus tested whether a similar regulation of PIF4 and PIF5 occurred when seedlings were transferred to FR light. Consistent with our previous observation the levels of PIF4-HA and PIF5-HA in seedlings constitutively expressing those constructs were not affected by prolonged treatments with different FR fluence rates (supplemental Figure 4) (Lorrain *et al.*, 2008). In order to get insight into the mode of PIF4 and PIF5 regulation during the FR-HIR we tested the genetic interaction between *pif4pif5* and *hfr1* (*long Hypocotyl in Far Red light*). *HFR1* codes for PIF-related protein required for a full de-etiolation response in FR light (Fairchild *et al.*, 2000; Fankhauser and Chory, 2000; Soh *et al.*, 2000). We obtained *hfr1pif4pif5* by crossing and compared its phenotype to both parents. Consistent with previous publications, *hfr1* had a long hypocotyl and less negative gravitropic hypocotyls than the WT (Figure 7) (Fairchild *et al.*, 2000; Fankhauser and Chory, 2000; Soh *et al.*, 2000). *pif4pif5* was completely epistatic over *hfr1* for hypocotyl elongation and negative gravitropism at all fluence rate tested (Figure 7). Interestingly, while *pif4pif5* looked WT when grown at 5 µmol/m^2/s FR light, it completely rescued the *hfr1* phenotype revealing an effect of the *pif4pif5* mutation in a sensitized genetic background (Figure 7).

We further characterized this genetic interaction at the level of gene expression by performing Q-PCR analyses of etiolated seedlings transferred into FR light for 0, 1 and 24 hours (at 0.5 µmol/m^2/s when the hypersensitive phenotype of *pif4pif5* is obvious and at 5 µmol/m^2/s when the *pif4pif5* effect is only apparent in the *hfr1* background). We tested *PIL1* and *XTR7* whose expression is PIF4/PIF5-dependent and *HY5* which is not regulated in PIF4/PIF5-dependent manner (Figure 6) (Lorrain *et al.*, 2008; Hornitschek *et al.*, 2009). The expression pattern of these 3 genes in the WT was the same in response to 0.5 or 5 µmol/m^2/s except that the light regulation had more amplitude at the higher

fluence rate (Figure 8). In accordance with our previous analysis *HY5* expression remained unchanged in all the tested genotypes (Figure 6 and 8). While *pif4pif5* did not display a morphological phenotype when grown at 5 µmol/m^2/s FR light, it still presented a reduced expression of *XTR7* and *PIL1* as compared to the WT, which was particularly obvious after 24hr in FR (Figures 1 and 8b). Concurrently with its morphological phenotypes, the *hfr1* mutant presented only small gene expression differences with the WT at low fluence rates of FR light while its effects were much more striking at higher fluence rates and especially after 24hr (Figures 7 and 8a). As for the morphological analyses, *pif4pif5* was epistatic over *hfr1* for *XTR7* and *PIL1* expression (Figures 7 and 8). One possible interpretation of those results is that PIF4 and PIF5 activity is downregulated by HFR1 in FR-light-grown seedlings (see discussion).

Discussion

Light perception induces massive changes in gene expression leading to the transition from skotomorphogenesis to photomorphogenesis (Quail, 2002b; Jiao *et al.*, 2007). The PIF proteins represent a direct link between phytochromes activation and gene expression since they are transcription factors interacting with the phytochromes. There is a good relationship between the preferential binding of PIFs to phyA and/or phyB and the involvement of those PIFs in phyA and/or phyB signaling pathways. For instance PIF1 and PIF3 have the strongest affinity for phyA *in vitro* and they were also the only ones known to be involved in responses to FR light. (Zhu *et al.*, 2000; Kim *et al.*, 2003; Huq *et al.*, 2004; Oh *et al.*, 2004; Al-Sady *et al.*, 2006; Jiao *et al.*, 2007; Shin *et al.*, 2007; Shen *et al.*, 2008). PIF4 and PIF5 interact with phyB and participate to the phyB signaling pathways in response to red light or to shade conditions (Huq and Quail, 2002; Fujimori *et al.*, 2004; Lorrain *et al.*, 2008). In contrast, *pif4* and *pif5* single mutants present a WT phenotype in FR light which is in good agreement with the weak affinity of this pair of bHLHs for phyA (Huq and Quail, 2002; Shen *et al.*, 2007). However our work uncovered a function for PIF4 and PIF5 in the phyA-mediated de-etiolation process in FR light. Interestingly for this light response the PIF4 and PIF5 mode of action does not involve PIF-mediated changes to phytochrome levels (Figure 2) and apparently does not require direct interaction between the PIFs and phyA. Based on microarrays analysis we propose that both a priming to FR responses and a reduction in the expression of growth-related genes could explain the observed phenotype. Finally our work suggests a novel mechanism by which light limits the activity of PIF4 and PIF5.

PIF4 and PIF5 repress the photomorphogenetic gene expression program in the dark

Interestingly with the exception of PIF7 light perception targets PIFs to degradation suggesting that they act as negative components of the light program and that phytochromes have to inactivate them. As predicted from this model several PIFs act in the dark to repress light-grown development (Huq *et al.*, 2004; Oh *et al.*, 2004; Leivar *et al.*, 2008b; Moon *et al.*, 2008; Shin *et al.*, 2009; Stephenson *et al.*, 2009). Although the

pif4pif5 double mutant does not present a morphological phenotype in the dark, transcriptomic analyses show that it already affects gene expression (Figures 4 and 5). Close to 80% of the genes affected in *pif4pif5* are upregulated in the mutant (Figure 4A). and as reported for a *pif1pif3pif4pif5* quadruple mutant a large fraction of the upregulated genes in etiolated *pif4pif5* mutants are required for photosynthesis (Figure 4) (Shin *et al.*, 2009). Thus, our data contributes to an emerging theme in the control of photomorphogenesis indicating that two mechanisms repress this developmental transition in the dark (Leivar *et al.*, 2008b; Shin *et al.*, 2009; Stephenson *et al.*, 2009). In addition to COP1-mediated degradation of positive regulators of light-grown development (e.g. HY5 and HFR1), plants have a second control level through the redundant inhibitory action of the PIFs (Bae and Choi, 2008; Josse and Halliday, 2008). Removal of one or several PIFs will thus prime the seedlings to respond to light. Since different wavelength induce qualitatively similar gene expression, the hypersensitive phenotype of the *pif* mutants in different light qualities could result from priming in the dark (Ma *et al.*, 2001). Moreover in the *pif4pif5* double mutant the remaining PIFs might be degraded more rapidly in response to light than in the wild type thus leading to an enhanced light sensitivity.

PIF4 and PIF5 are growth-promoting factors after prolonged exposure to FR light

In addition to the "priming" mechanism proposed above, the gene expression profile in seedlings grown for 24 hours in FR light also suggests that other mechanisms explain the *pif4pif5* phenotype. While in etiolated seedlings most deregulated genes are over-expressed in *pif4pif5*, this tendency is totally reversed after 24 hours exposure to FR light with 75% of the affected genes being less expressed in the double mutant. This suggests that during prolonged exposure to FR, PIF4 and PIF5 can act directly or indirectly as transcriptional activator. Consistent with this idea two direct targets of PIF4 (*PIL1* and *XTR7*) are most strongly downregulated in *pif4pif5* mutants grown in FR light for a prolonged period (Figure 6) (de Lucas *et al.*, 2008). Interestingly, *XTR7* codes for a xyloglucan endotransglycosylase-related protein that is presumably implicated in the cell elongation process. The reduced expression of this gene in *pif4pif5* could contribute to

the reduced hypocotyl length of the mutant grown in low FR light (Figure 6). Similarly, the reduced expression of *IAA19/MSG2* in *pif4pif5* after 24 hours in FR light but not in the dark is consistent with the light-specific gravitropic phenotype of the *pif4pif5* mutant. (Supplemental Table 1) (Figure 1). MSG2 has been implicated in gravitropic and phototropic responses (Tatematsu *et al.*, 2004). More generally the gene expression profile after 24 hours in FR light shows an over-representation of genes related to hormonal pathways and auxin in particular (Figure 5, data not shown). The majority of those genes are differentially expressed only after a long treatment in FR light, which is consistent with the altered growth pattern of FR-light grown but not etiolated *pif4pif5* mutants. This role of PIF4 and PIF5 in promoting elongation growth during de-etiolation in FR light is analogous to the one proposed for those two bHLH transcription during the shade avoidance response (Lorrain *et al.*, 2008). Finally it is consistent with the elongated hypocotyls observed in PIF-over-expressing seedlings grown in continuous FR light (Supplemental Figure 1). It should be pointed out that during the de-etiolation process phyA acts in two discrete modes known as the FR-HIR and the very low fluence response (VLFR) (Casal *et al.*, 1997; Wang and Deng, 2003). Future studies are needed to determine if PIF4 and PIF5 act in both phyA branches.

HFR1 inhibits PIF4 and PIF5 functions in high fluence rates of FR light

The *pif4pif5* phenotype suggests that PIF4 and PIF5 limit the expression of the light program under weak FR fluence rates but that their activity has been inhibited at higher fluence rates (Figure 1). In red light, phyB interacts with PIFs and targets them to degradation, which can release PIFs inhibitory action (Castillon *et al.*, 2007). The same mechanisms operates in FR light for PIF1 and PIF3 which both interact with phyA leading to their degradation (Bauer *et al.*, 2004; Park *et al.*, 2004; Shen *et al.*, 2005; Oh *et al.*, 2006; Shen *et al.*, 2008). On the other hand, while PIF4 and PIF5 are phosphorylated and degraded in response to R light we did not detect such modifications in FR light using plants expressing HA-tagged proteins from a constitutive promoter (supplemental Figure 3) (Nozue *et al.*, 2007; Shen *et al.*, 2007; de Lucas *et al.*, 2008; Lorrain *et al.*, 2008). However the study of the endogenous proteins levels may reveal additional levels

of light regulation. Furthermore since no interaction between phyA and PIF4 and PIF5 has been described, the hypothesis that in FR light phyA directly inhibits PIF4 and PIF5 action as proposed for PIF1 is unlikely (Shen *et al.*, 2007)

We thus investigated alternative mechanisms that may contribute to the inactivation of PIF4 and PIF5 in seedlings grown in continuous FR light. HFR1 appeared to be a good candidate for a number of reasons. The *hfr1* mutant has the opposite phenotype from *pif4pif5* (Fairchild *et al.*, 2000; Fankhauser and Chory, 2000; Soh *et al.*, 2000). Moreover HFR1 is a PIF-class bHLH transcription factor that can directly interact with other members of the PIF family (Fairchild *et al.*, 2000; Hornitschek *et al.*, 2009). HFR1 is degraded in the dark and its accumulation in the light correlates with the fluence rate (Duek *et al.*, 2004; Jang *et al.*, 2005; Yang *et al.*, 2005b). This suggested that at higher fluence rates HFR1 may interact with PIF4 and PIF5 and inactivate them. Indeed, we have shown that heterodimerization of HFR1 with PIF4 and PIF5 inhibits their DNA-binding capacity (Hornitschek *et al.*, 2009). A clear prediction from this model is that *pif4pif5* should be epistatic over *hfr1* because in the absence of PIF4 and PIF5 the inhibitory activity of HFR1 should no longer be detectable. Our data are consistent with this hypothesis (Figures 7, 8), An alternative explanation for our findings is the reduced expression of *HFR1* in the *pif4pif5* double mutant (data not shown) (Lorrain *et al.*, 2008). However in FR-light-grown seedlings *HFR1* is still expressed at 45% of the wild-type levels indicating that HFR1-mediated inhibition of PIF DNA binding explains our data at least partly. Thus under high fluence rates of FR light PIF4 and PIF5 inactivation depends on dimerization with HFR1, which is induced by PIF4 and PIF5. This represents a typical negative feedback loop which is also operates during shade avoidance (Sessa *et al.*, 2005; Hornitschek *et al.*, 2009).

Our model predicts that modifications to the balance between PIF4, PIF5 and HFR1 should induce hypersensitivity (more PIF4 and PIF5 as compared to HFR1 level) or hyposensitivity (less PIF4 and PIF5 as compared to HFR1) to FR light. Indeed, PIF4 or PIF5 over-expression induces an *hfr1*-like phenotype for hypocotyl elongation in FR (supplemental Figure 1). On the other hand, hypersensitivity to FR light is observed in

lines over-accumulating HFR1 (*spa1* mutant or HFR1 over-expressing plants) (Yang *et al.*, 2003; Duek and Fankhauser, 2005; Jang *et al.*, 2005; Yang *et al.*, 2005a; Yang *et al.*, 2005b). Moreover over-expression of HFR1 versions deleted for their N-terminus part, which are stable in the dark, induce a de-etiolation phenotype in the dark reminiscent of the quadruple *pif* mutants (Yang *et al.*, 2003; Jang *et al.*, 2005; Leivar *et al.*, 2008b). This suggests that HFR1 is also capable of inhibiting other PIF proteins. However during de-etiolation in far-red light our genetic analysis indicates that HFR1 primarily acts via PIF4 and PIF5 (Figures 7, 8). The role of HFR1 is expected to be most important when the protein is present at high levels such as during the FR-HIR or during the shade avoidance response (Figures 7, 8) (Hornitschek *et al.*, 2009).

To conclude we propose that the appropriate activation of the light program depends on the fine-tuning between positive and negative regulators. Positive regulators such as HY5 and HFR1 are degraded in the dark and stabilized in the light, while negative regulators such as the PIFs are stable in the dark and inhibit the inappropriate activation of light-responsive genes. Light-perception by the phytochromes releases this dual repressive program by inhibiting COP1 and by limiting the activity of the PIFs (Bae and Choi, 2008; Josse and Halliday, 2008; Leivar *et al.*, 2008b). Inhibition of the PIFs is induced by phytochrome-mediated and proteasome-dependent degradation of those proteins in R light and for PIF1 and PIF3 in FR light as well. The work presented here and in another manuscript suggests an additional mechanism of PIF inactivation which operates in FR-rich environments (Figures 7, 8) (Hornitschek *et al.*, 2009). Under these conditions PIF4 and PIF5 are inactivated by forming non-DNA binding heterodimers with HFR1. Interestingly this mode of light regulation couples the two light-induced processes described above given that HFR1 stabilization depends on inactivation of COP1.

Acknowledgments

We thank Patricia Hornitschek for sharing scientific results, Céline Wyser for technical help, Manuel Bueno (DAFL) for advice regarding Q-PCR, Alexandra Paillusson, Otto Hagenbuechle and Keith Harshmann for micro-array experiments (DAFL) and Keara Franklin (University of Leicester) for her comments on the manuscript. We are grateful to Trudie Allen (University of Leicester) who pointed our attention to the FR hypersensitivity of the *pif4pif5* mutant. We thank Akira Nagatani and Karen Schumacher for providing phyA and DET3 antibodies respectively. This work was supported by a grant from the Swiss National Science Foundation (3100A0-112638); SystemsX.ch project “Plant Growth in a changing environment” and the University of Lausanne.

Experimental procedures

Plant material and growth conditions

Seedlings were grown as described previously (Duek *et al.*, 2004). All mutants were in the Columbia background (Col) and the *pif4pif5* double mutant has been described previously (Lorrain *et al.*, 2008). We obtained the triple mutants *phyApif4pif5* and *hfr1pif4pif5* by crossing *pif4pif5* respectively with *phyA-211 and hfr1-101*. *phyA-211* and *hfr1-101* genotyping was performed as described (Duek and Fankhauser, 2003).

Characterization of the mutants.

Characterization of the mutants including western blotting was performed as previously described (Lorrain *et al.*, 2008). The negative gravitropism assay is described in (Oh *et al.*, 2004) except that germination was induced by a 3 hours red treatment (50 µmol/m^2/s). Plates were then returned to the dark for 21 hours before being subjected to the different light conditions. Quantitative western blots were performed as described in (Trupkin *et al.*, 2007).

RNA extraction and Q-PCR

Approximately 100 seeds were plated on half MS and kept in the dark and cold for 3 days. Germination was induced by a 3 hours red light (50µmol/m^2/s) and seedlings were kept in the dark for 3 days before being subjected to 1 or 24 hrs of 0.5 or 5µmol.m^2.s^1 of FR light. Seedlings were flash-frozen in liquid nitrogen, ground using a tissulyser (Qiagen) 2 times 30s, 30Hz without extraction buffer, then twice again in the presence of RLT extraction buffer (Qiagen). RNAs were extracted using the RNeasy plant mini kit (Qiagen) following the manufacturer's recommendations except that 3 washes were done with the RLP buffer instead of 2. Samples were treated with DNAseI (Qiagen) on the column during RNAs extraction First strand cDNA synthesis was performed with 750ng of RNA using the invitrogen SuperScript II and random hexamers. 1µL of 10 times-diluted cDNAs was used for Q-PCR on ABI machine (7900) using PowerSYBRgreen as reporter (Applied Biosystem). Samples were assembled in 384-wells plates with Tecan

robot. 5 house-keeping genes were tested using geNorm (Vandesompele *et al.*, 2002) and 2 of them, *YLS8* (*At5g08290*) and *UBC* (*At5g25760*), were further used as normalization for the experiments. Data from 3 technical and 3 biological replicates were then analyzed using Q-base (Hellemans *et al.*, 2007). The primers used for Q-PCR are given in the supplemental table 2 except for *HY5* (Sibout *et al.*, 2006).

Microarray

Three series of samples with wild-type and *pif4pif5* double mutant were harvested and processed as described above. All RNA quantities were assessed by NanoDrop®ND-1000 spectrophotometer and the quality of RNA was controlled on Agilent 2100 bioanalyzer chips. For each sample, 100ng of total RNA were amplified and labeled using the Message Amp II-biotin Enhanced reagents (Ambion; catalog #AM1791). *Arabidopsis* ATH1 Genome Array (Affymetrix, Santa Clara, CA, USA) arrays were hybridized with 11 μg of labeled, amplified cRNA , washed, stained and scanned according to the protocol described in Affymetrix GeneChip® Expression Analysis Manual (Fluidics protocol EukGeWS2v5_450). Data analysis is presented in the supplemental information.

Figure legend

Figure 1. The *pif4pif5* double mutant is hypersensitive to FR light.

(a) Phenotype of seedlings grown under constant FR light. Seedlings were grown 5 days in the dark or in different fluence rates of continous FR light (FR 0.05=0.05 µmol/m^2/s; FR0.5=0.5 µmol/m^2/s: FR5=5 µmol/m^2/s). Pictures of representative seedlings are shown.

(b) Hypocotyl elongation of the seedlings shown in A. The growth conditions are the same as described in A. Data are mean +/- 2SE, n=20.

(c) Negative gravitropic hypocotyls of the seedlings grown in different light conditions. Hypocotyls are called negative gravitropic when they do not touch the agar plate as described in (Oh *et al.*, 2004). Data represent the mean of biological triplicates, error bars= SE.

(d) Anthocyanin accumulation in the seedlings grown as in A except that the media contained sucrose to induce anthocyanin accumulation. Anthocyanins were extracted and quantified from 30 seedlings grown in the dark or in constant FR light during 5 days. Data are mean +/- 2SE from three biological replicas.

Figure 2. The *pif4pif5* mutant contains wild-type levels of phyA.

(a) phyA accumulation in seedlings grown in the dark or in constant FR light. Total proteins were extracted from five-day-old seedlings, separated on SDS-PAGE, western-blotted and probed with anti-phyA or anti-DET3 (control) antibodies. Quantifications were performed with the Li-cor system and error bars represent standard error across technical triplicates. Results are expressed relatively to Col sample in the dark. One representative western-blot is presented in the lower panel.

(b) phyA accumulation in response to red light. Total proteins were extracted at the indicated times from three-day-old etiolated seedlings subjected to constant red light (50 µmol/m^2/s). Western-blot and quantification were performed as described in A.

Figure 3. *phyA* is completely epistatic over *pif4pif5*

(a) Phenotype of Col, *phyA*, *pif4pif5* and *phyApif4pif5* seedlings under different light conditions. Seedlings were gown for 5 days in the dark or in different fluence rates of continuous FR light. Representative seedlings are shown.

(b) Hypocotyl elongation of the seedlings shown in A. The growth conditions are the same as described in A. Data are mean +/- 2SE, n=20.

(c) Negative gravitropic hypocotyls of the seedlings grown in different light conditions. Hypocotyls are called negative gravitropic when they do not touch the agar plate as described in (Oh *et al.*, 2004). Column represent the mean of biological triplicates, error bars= SE.

Figure 4. The *pif4pif5* mutant is affected for gene expression in darkness and after transfer to FR light.

(a) Venn diagram showing the repartition of the *pif4pif5*-affected genes (either upregulated or downregulated) in function of the different conditions (dark, 1 or 24 hours in FR). Affected genes between *pif4pif5* and the WT were determined based on FDR<0.05.

(b) Selected GO annotations in the different categories of genes affected by the *pif4pif5* mutations.

Figure 5. *pif4pif5*-affected genes in the dark are mostly FR late-responsive genes.

Two-way complete linkage clustering was performed on the 113 genes affected by the *pif4pif5* mutant in the dark using Pearson's correlation distance metric. Prior to clustering, RMA normalized expression values of each genes were mean centered and variance normalized.

(a) Hierarchical clustering analysis of the samples. The color box represents the average normalized expression for the 113 genes in any of the 18 samples.

(b) Heatmap and hierarchical clustering of the 113 genes. The black bar on the right indicates the cluster detailed in the supplementary material.

Figure 6. The *pif4pif5* double mutant is affected for a set of FR-responsive genes.

Expression of FR-responsive genes was determined by Q-PCR in the WT, *phyA* and *pif4pif5* mutants in three-day-old etiolated seedlings either in the dark or after 1 or 24 hours in constant FR light (0.5 µmol/m^2/s). Three biological repeats were performed, with three technical replicates for each. Relative expression was determined after normalization with two reference genes using the Q-base software, comparing each sample to one Col-0 sample in the dark. Each column represents the mean relative expression for the three biological repeats, error bars represent SE.

Figure 7. *pif4pif5* is epistatic over *hfr1*.

(a) Phenotype of Col, *hfr1*, *pif4pif5* and *hfr1pif4pif5* seedlings under different light conditions. Seedlings were gown for 5 days in the dark or in different fluence rates of continuous FR light. Representative seedlings are shown.

(b) Hypocotyl elongation of the seedlings shown in A. The growth conditions are the same as described in A. Data are mean +/- 2SE, n=20.

(c) Negative gravitropic hypocotyls of the seedlings grown in different light conditions. Hypocotyls are called negative gravitropic when they do not touch the agar plate as described in (Oh *et al.*, 2004). Column represent the mean of biological triplicates, error bars= SE.

Figure 8. *pif4pif5* is epistatic over *hfr1*for gene expression.

Expression of FR-responsive genes was determined by Q-PCR in the different genotypes in three-day-old etiolated seedlings either in the dark or after 1 or 24 hours in constant FR light. Three biological repeats were performed, with three technical replicates for each. Relative expression was determined after normalization with two reference genes using the Q-base software, comparing each sample to one Col-0 sample in the dark. Each column represents the mean relative expression for the three biological repeats, error bars represent SE.

(a) Gene expression after transfer to 0.5 µmol/m^2/s of FR light.

(b) Gene expression after transfer to 5 µmol/m^2/s of FR light.

Supplemental Figure 1. Increased hypocotyl elongation is observed in *PIF4* and *PIF5* over expressing lines grown in FR light.

Supplemental Figure 2. *pif4pif5*-affected genes in the dark are mostly FR late-responsive genes.

Supplemental Figure 3. The *pif4pif5* double mutant presents a WT expression of *PKS1* in response to FR light.

Supplemental Figure 4. PIF4 and PIF5 protein level is unaffected in response to FR light.

Supplemental Table 1: Genes affected in the *pif4pif5* double mutant.

Supplemental Table 2: Gene Ontology (GO) terms enriched in the *pif4pif5*-affected genes.

Supplemental Table 3: List of primers used in this study.

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Figure 1.

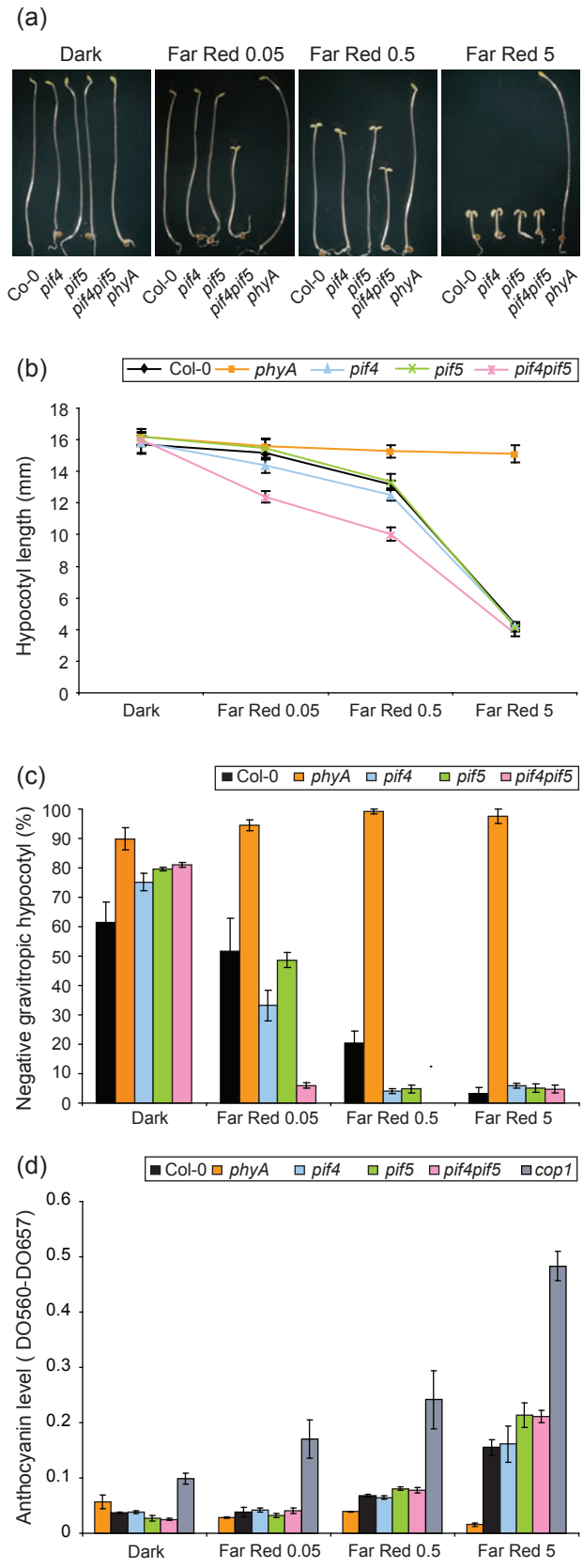
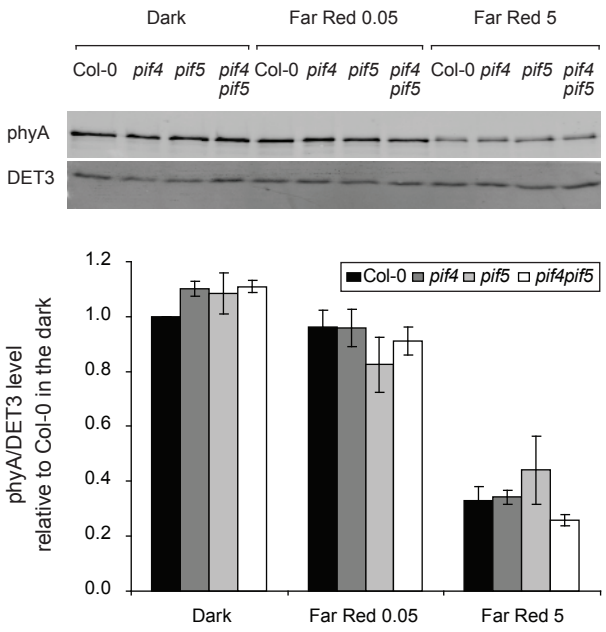

(a)
Dark
Far Red 0.05
Far Red 0.5
Far Red 5
Co-0
pif4
pif5
pif4pif5
phyA
(b)
Col-0
phyA
pif4
pif5
pif4pif5
Hypocotyl length (mm)
Dark
Far Red 0.05
Far Red 0.5
Far Red 5
(c)
Negative gravitropic hypocotyl (%)
(d)
cop1
Anthocyanin level (DO560-DO657)

Figure 2.

(a)


Dark
Far Red 0.05
Far Red 5
Col-0
pif4
pif5
pif4 pif5
phyA
DET3
phyA/DET3 level relative to Col-0 in the dark
Col-0 pif4 pif5 pif4pif5
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Dark
Far Red 0.05
Far Red 5

(b)

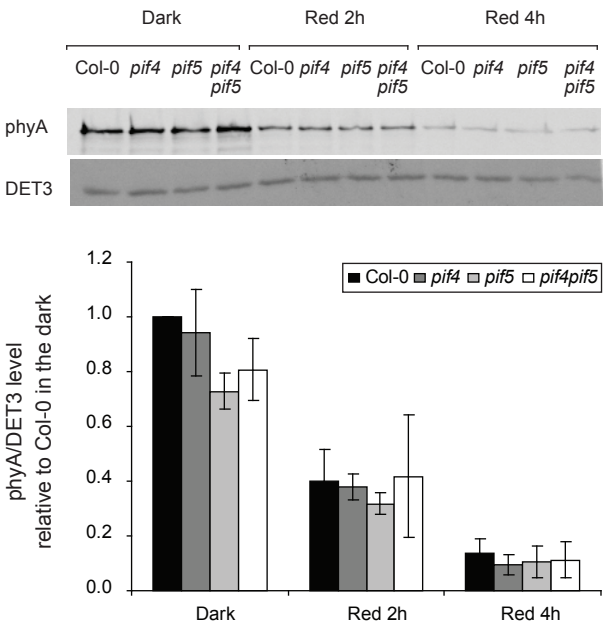

Dark
Red 2h
Red 4h
Col-0
pif4
pif5
pif4 pif5
phyA
DET3
phyA/DET3 level relative to Col-0 in the dark
Col-0 pif4 pif5 pif4pif5
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Dark
Red 2h
Red 4h

Figure 3.

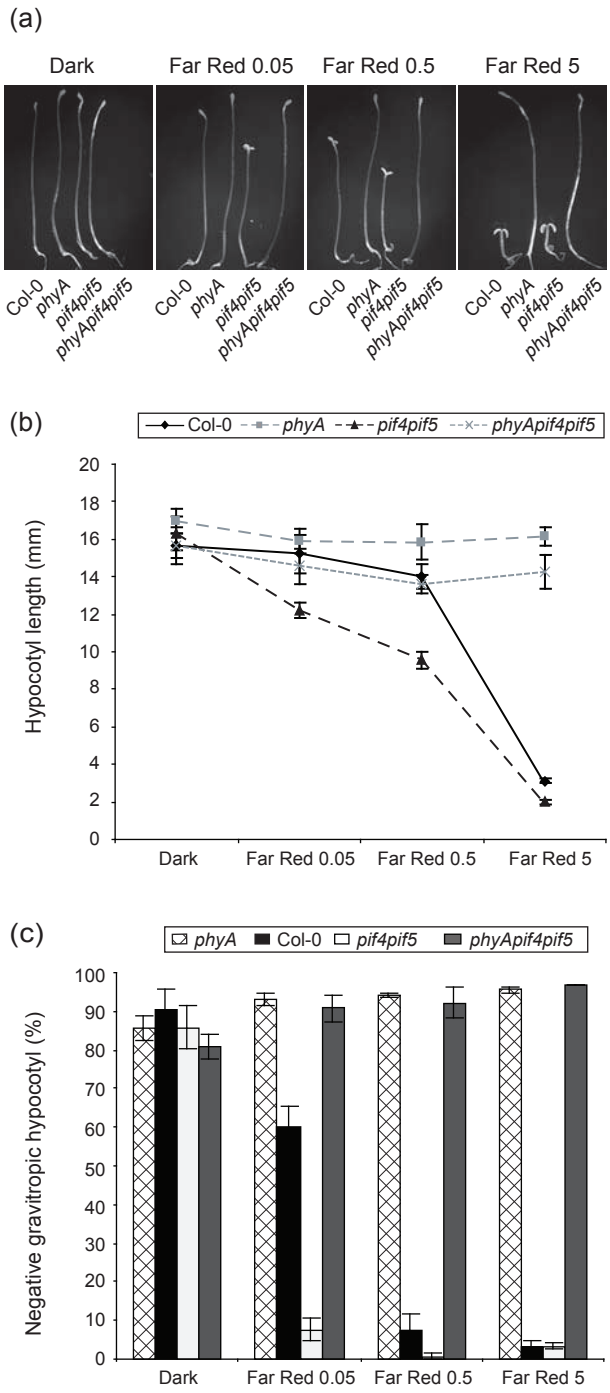
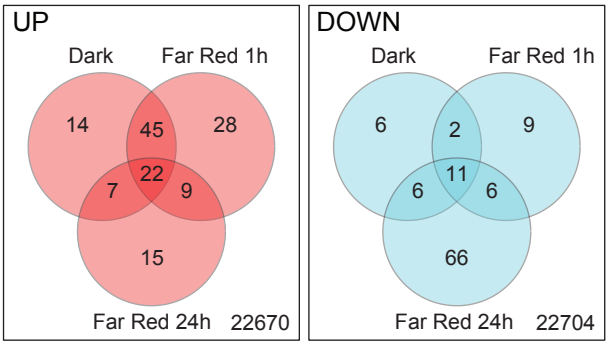

(a)
Dark
Far Red 0.05
Far Red 0.5
Far Red 5
Col-0
phyA
pif4pif5
phyApif4pif5
(b)
Col-0
phyA
pif4pif5
phyApif4pif5
Hypocotyl length (mm)
0
2
4
6
8
10
12
14
16
18
20
Dark
Far Red 0.05
Far Red 0.5
Far Red 5
(c)
phyA
Col-0
pif4pif5
phyApif4pif5
Negative gravitropic hypocotyl (%)
0
10
20
30
40
50
60
70
80
90
100
Dark
Far Red 0.05
Far Red 0.5
Far Red 5

Figure 4.

(a)


UP
Dark
Far Red 1h
14
45
28
22
7
9
15
Far Red 24h
22670
DOWN
Dark
Far Red 1h
6
2
9
11
6
6
66
Far Red 24h
22704

(b)

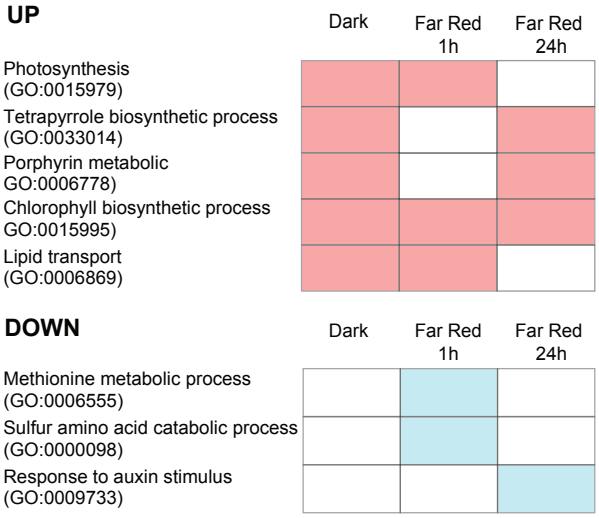

UP
Dark
Far Red 1h
Far Red 24h
Photosynthesis (GO:0015979)
Tetrapyrrole biosynthetic process (GO:0033014)
Porphyrin metabolic GO:0006778)
Chlorophyll biosynthetic process GO:0015995)
Lipid transport (GO:0006869)
DOWN
Dark
Far Red 1h
Far Red 24h
Methionine metabolic process (GO:0006555)
Sulfur amino acid catabolic process (GO:0000098)
Response to auxin stimulus (GO:0009733)

Figure 5.

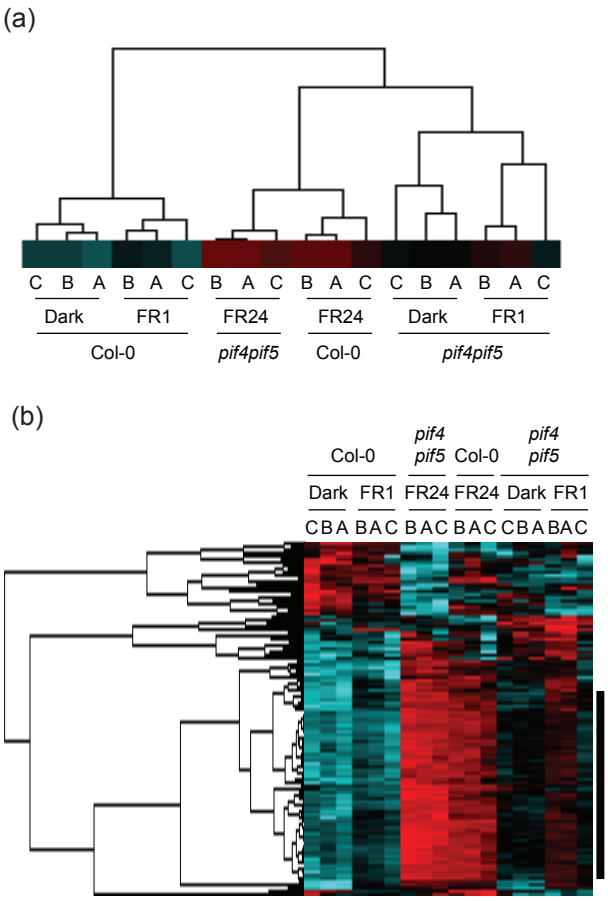

(a)
C B A B A C B A C B A C C B A B A C
Dark FR1 FR24 FR24 Dark FR1
Col-0 pif4pif5 Col-0 pif4pif5
(b)
Col-0 pif4 pif5 Col-0 pif4 pif5
Dark FR1 FR24 FR24 Dark FR1
C B A B A C B A C B A C C B A B A C

Figure 6.

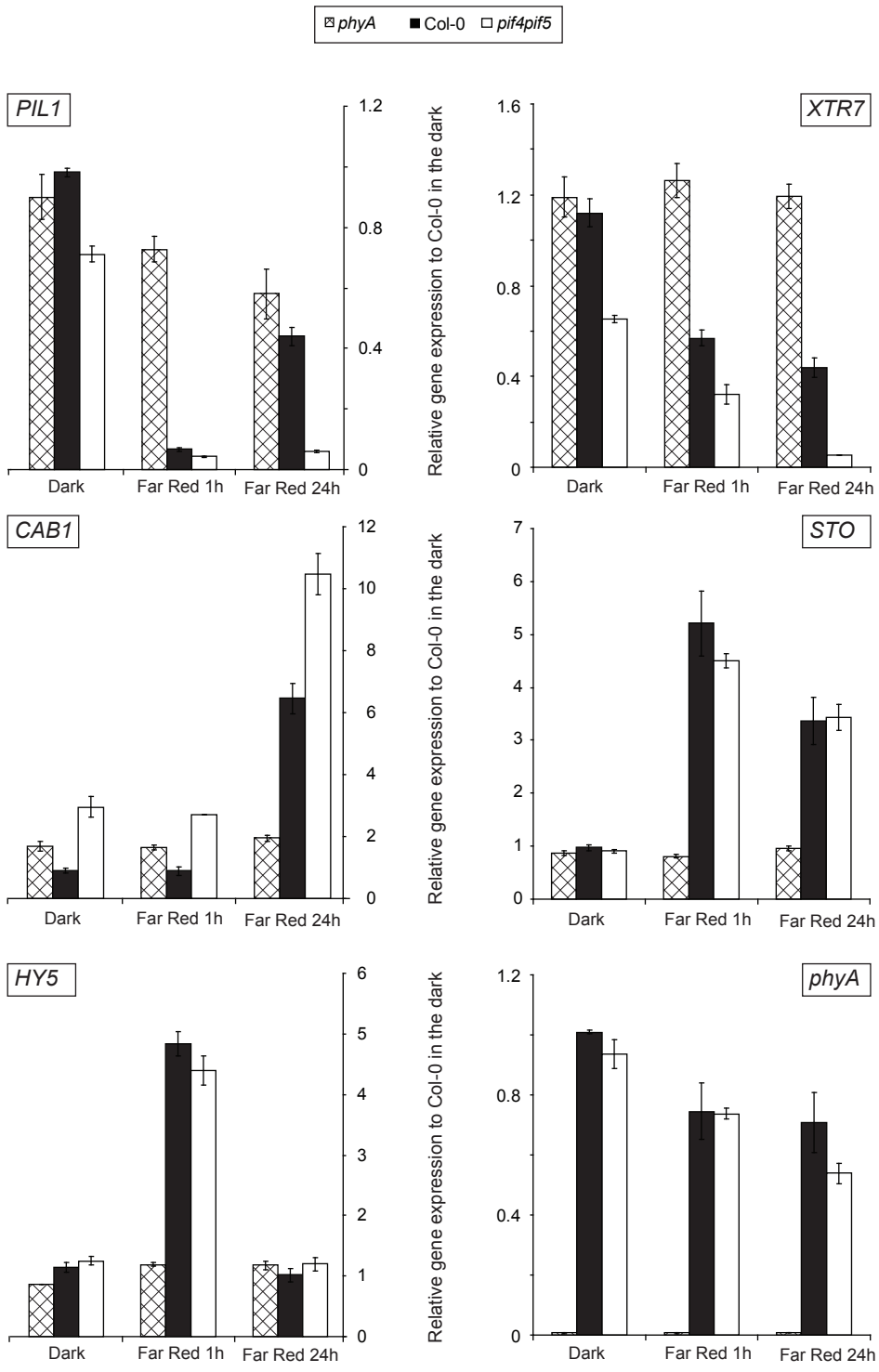

phyA
Col-0
pif4pif5
PIL1
XTR7
CAB1
STO
HY5
phyA
Relative gene expression to Col-0 in the dark
Dark
Far Red 1h
Far Red 24h

Figure 7.

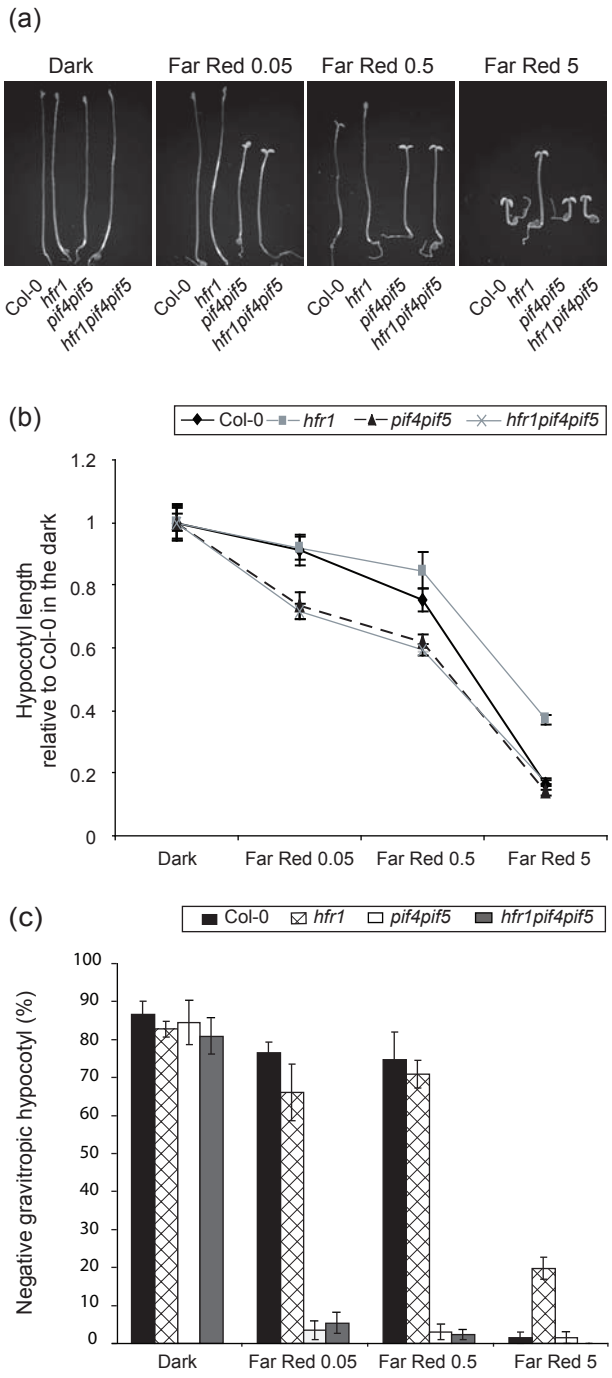
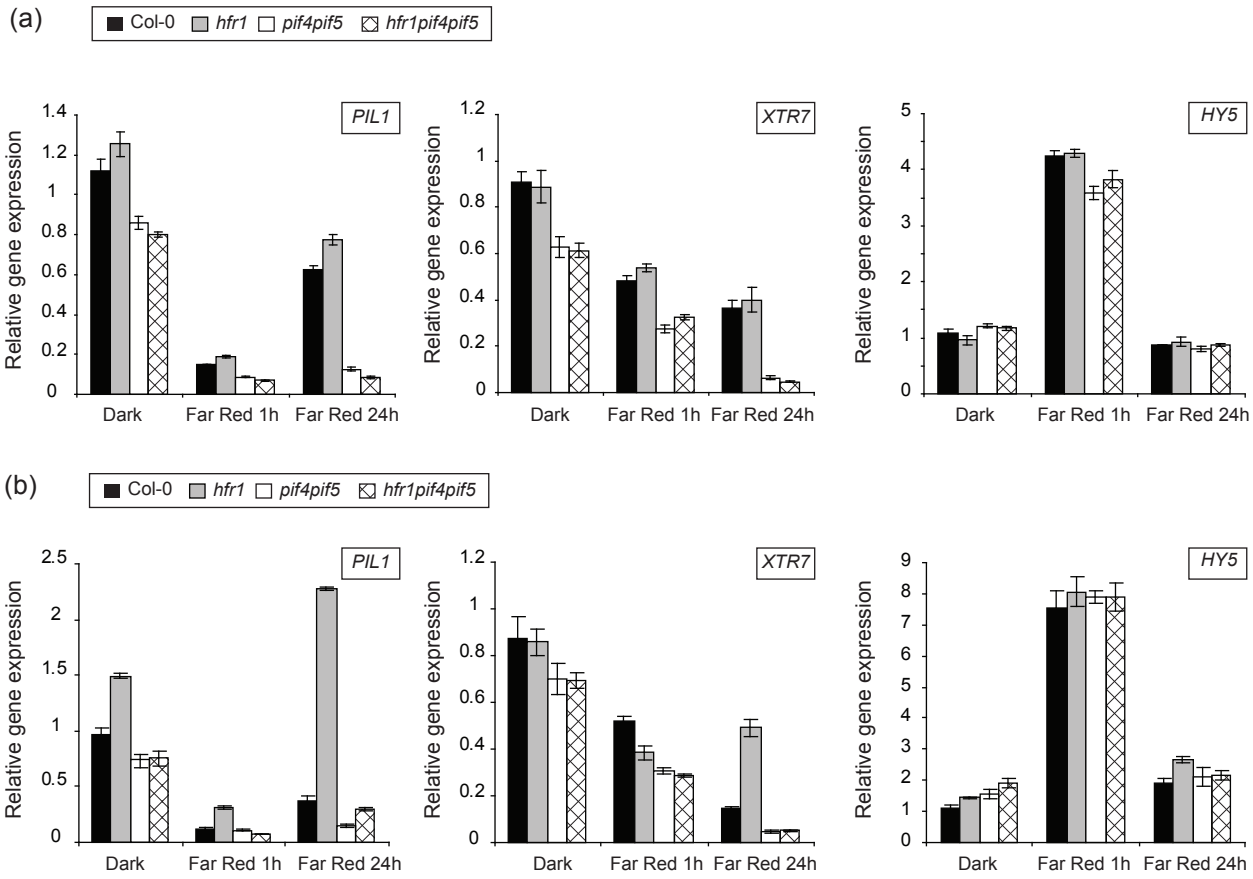
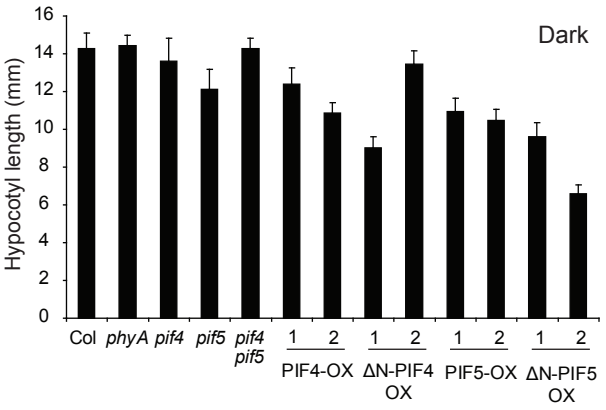

(a)
Dark
Far Red 0.05
Far Red 0.5
Far Red 5
Col-0
hfr1
pif4pif5
hfr1pif4pif5
(b)
Col-0
hfr1
pif4pif5
hfr1pif4pif5
Hypocotyl length relative to Col-0 in the dark
1.2
1
0.8
0.6
0.4
0.2
0
Dark
Far Red 0.05
Far Red 0.5
Far Red 5
(c)
Col-0
hfr1
pif4pif5
hfr1pif4pif5
Negative gravitropic hypocotyl (%)
100
90
80
70
60
50
40
30
20
10
0
Dark
Far Red 0.05
Far Red 0.5
Far Red 5

Figure 8.


(a)
Col-0
hfr1
pif4pif5
hfr1pif4pif5
PIL1
XTR7
HY5
Relative gene expression
Dark
Far Red 1h
Far Red 24h
(b)
Col-0
hfr1
pif4pif5
hfr1pif4pif5
PIL1
XTR7
HY5
Relative gene expression
Dark
Far Red 1h
Far Red 24h

Supplemental figure 1

(a)



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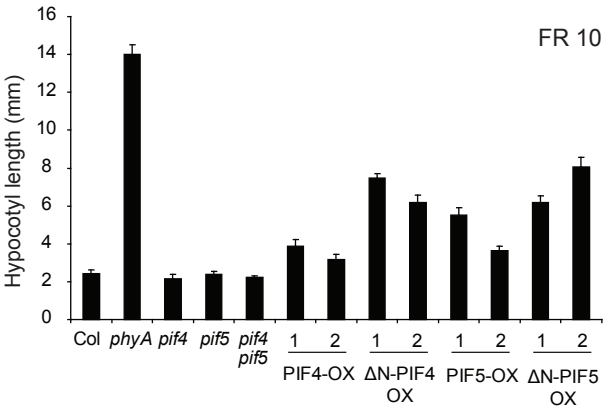
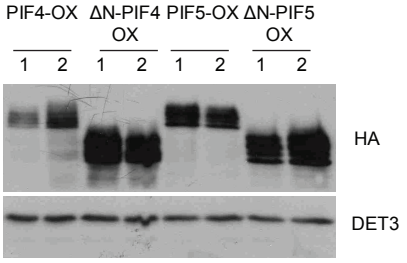
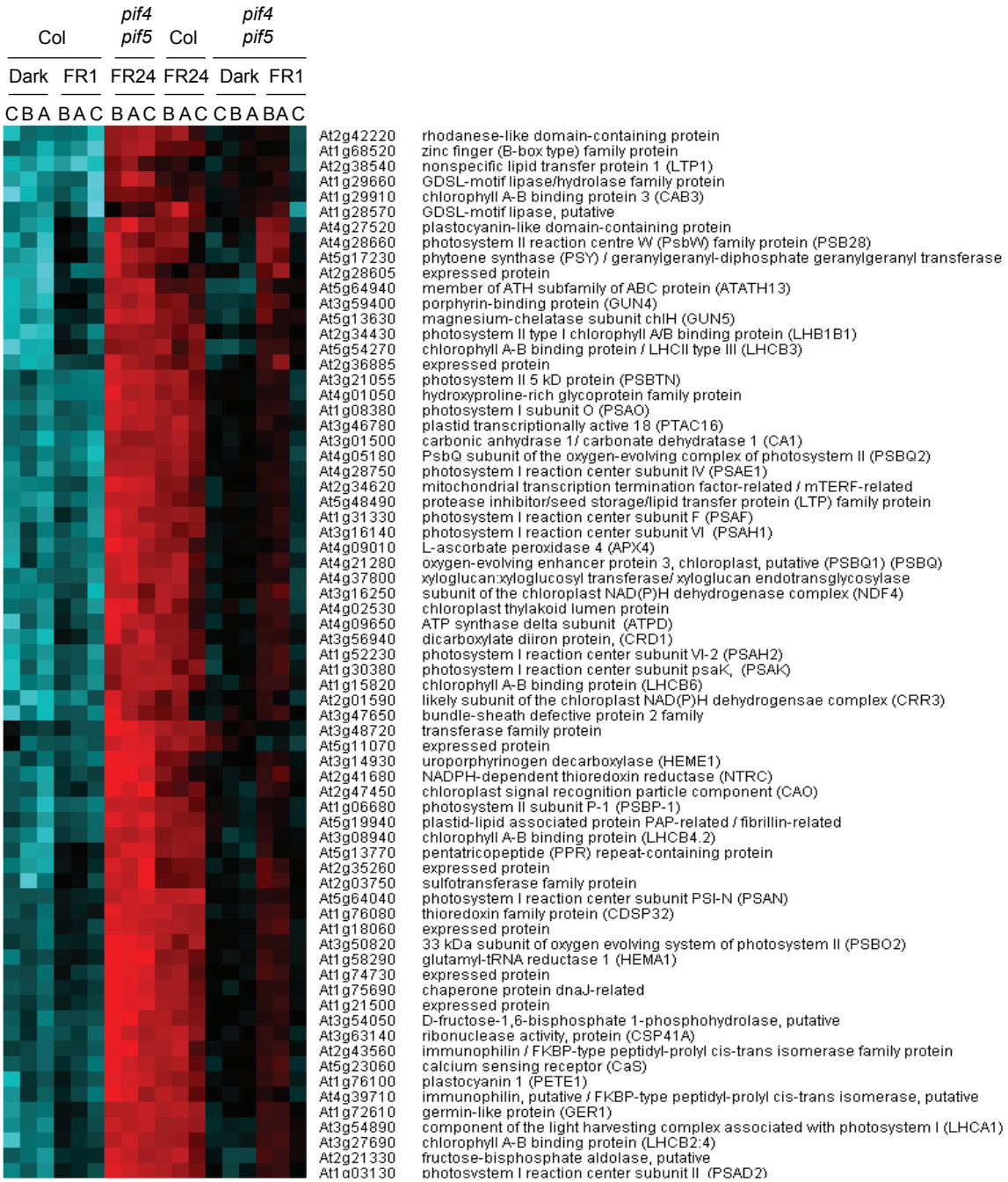


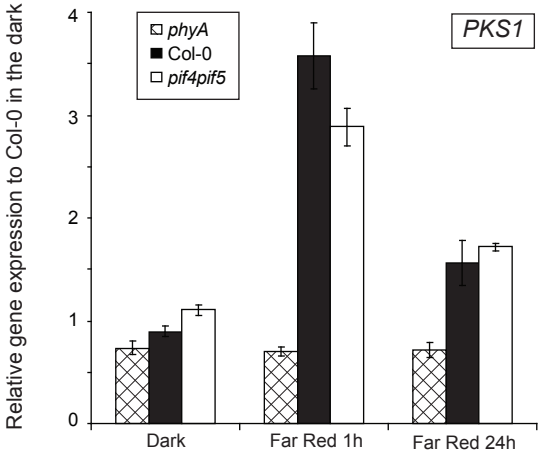
(c)



Supplemental Figure 2

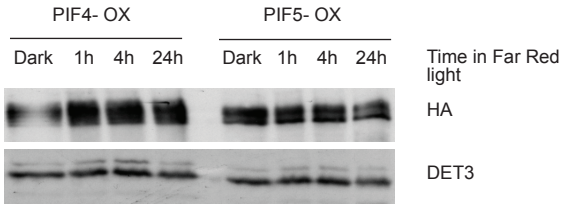


Supplemental Figure 3



Supplemental Figure 4

(a)



(b)