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Phytochrome interacting factors 4 and 5 control seedling growth in changing light conditions by directly controlling auxin signaling.

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Abstract

Plant growth is strongly influenced by the presence of neighbors competing for light resources. In response to vegetational shading shade-intolerant plants such as Arabidopsis display a suite of developmental responses known as the shade avoidance syndrome (SAS). The phytochrome B (phyB) photoreceptor is the major light sensor mediating this adaptive response. The control of the SAS occurs in part with phyB directly controlling protein abundance of Phytochrome Interacting Factors 4 and 5 (PIF4 and PIF5). The shade avoidance response also requires rapid biosynthesis of auxin and its transport to promote elongation growth. The identification of genome-wide PIF5 binding sites during shade avoidance reveals that this bHLH transcription factor regulates the expression of a subset of previously identified SAS genes. Moreover our study suggests that PIF4 and PIF5 regulate elongation growth by directly controlling the expression of genes coding for auxin biosynthesis and auxin signaling components.

Introduction

Many plants are sensitive to shade from the neighboring vegetation and display a developmental response known as the shade-avoidance syndrome (SAS) to adapt to this potentially threatening situation. These responses include elongation of hypocotyls, stems and petioles, elevated leaf angles (hyponasty), reduced leaf blade development and early flowering (Ballare 2009, Franklin 2008, Franklin and Quail 2010, Morelli and Ruberti 2000, Vandenbussche *et al.* 2005). Light filtered through vegetation has a specific spectral signature with a reduction of the red to far-red ratio (R/FR) due to selective absorption of red and blue light but not far-red by photosynthetic pigments. In direct sunlight the R/FR ratio is above 1 while under deep shade it can drop below 0.1 (Ballare 2009, Franklin 2008). Under vegetational shading plants experience reduced Photosynthetically Active Radiation (PAR) and R/FR ratio. Given that about 50% of far-red light is reflected from leaves, plants growing in the proximity of neighbors will also experience a reduction of the R/FR ratio but maintain access to normal PAR (Ballare 1999). Many plants respond to such "neighbor threat" by displaying responses similar to the SAS (Ballare 1999, Keller *et al.* 2011).

The red and far-red sensing phytochromes play a predominant role in the control of the SAS particularly under "neighbor threat" conditions when the low R/FR ratio occurs without PAR reduction. (Ballare 2009, Franklin and Quail 2010, Kami *et al.* 2010). In Arabidopsis phyB is the major sensor of low R/FR although phyD and phyE contribute to the response (Franklin and Quail 2010). Phytochromes are synthesized in the inactive red-light absorbing Pr conformer

that is primarily cytosolic. Upon light absorption it converts to the active Pfr form (far-red absorption maximum) that accumulates in the nucleus where it leads to rapid changes in gene expression (Nagatani 2004, Fankhauser and Chen 2008, Franklin and Quail 2010, Kami *et al.* 2010). Transfer of plants from sun to shade alters the Pfr/Ptot ratio and leads to rapid phytochrome-mediated modifications in gene expression (Devlin *et al.* 2003, Salter *et al.* 2003, Sessa *et al.* 2005, Tao *et al.* 2008). Under direct shading which includes a reduction in blue light additional photoreceptors, most notably the cryptochromes contribute to the SAS (Keller *et al.* 2011, Keuskamp *et al.* 2011, Sellaro *et al.* 2010, Zhang *et al.* 2011).

Multiple hormones are involved in the establishment of the SAS (Franklin 2008, Martinez-Garcia *et al.* 2010, Morelli and Ruberti 2000, Vandenbussche *et al.* 2005). Both TAA1-dependent auxin biosynthesis and auxin transport are essential to induce hypocotyl elongation by a reduction in the R/FR ratio (Keuskamp *et al.* 2010, Steindler *et al.* 1999, Tao *et al.* 2008). Moreover, gibberellins (GA), brassinosteroids (BR) cytokinins and ethylene also contribute to a normal SAS (Pierik *et al.* 2004, Carabelli *et al.* 2007, Djakovic-Petrovic *et al.* 2007, Keuskamp *et al.* 2011, Kozuka *et al.* 2010, Pierik *et al.* 2004).

PIF4 and PIF5, two members of the Phytochrome Interacting Factor (PIF) family of bHLH proteins are good candidates for a direct link between phytochrome regulation by shade and gene expression because their protein stability is directly controlled by the R/FR ratio (Keller *et al.* 2011, Lorrain *et al.* 2008). However the SAS is only partially affected in *pif4pif5* double mutants indicating

that additional factors mediate the SAS (Cole *et al.* 2011, Keller *et al.* 2011, Lorrain *et al.* 2008). Additional transcription factors including several target genes of PIF5 have been implicated in the control of SAS (Crocco *et al.* 2010, Hornitschek *et al.* 2009, Roig-Villanova *et al.* 2007, Salter *et al.* 2003, Sessa *et al.* 2005, Sorin *et al.* 2009, Steindler *et al.* 1999, Kunihiro *et al.* 2011). Among them HFR1, PIL1 and PAR1 act as negative regulators of the SAS (Roig-Villanova *et al.* 2007, Salter *et al.* 2003, Sessa *et al.* 2005). This negative regulation occurs at least in part via the inhibition of PIF4 and PIF5 suggesting the existence of complex regulatory networks controlling SAS including circadian regulation of the process (Salter *et al.* 2003, Sessa *et al.* 2005, Hornitschek *et al.* 2009, Hao *et al.* 2012, Sellaro *et al.* 2012).

This link between auxin that is essential for the response to low R/FR and the transcriptional network described above remains poorly understood. PAR1 over-expression inhibits shade-induced expression of auxin response genes (Roig-Villanova *et al.* 2007) while the positive regulator of SAS, ATHB2 controls auxin sensitivity through unknown mechanisms (Steindler *et al.* 1999, Kunihiro *et al.* 2011). Interestingly, PIF4 controls hypocotyl elongation in response to elevated temperature by direct regulation of the *TAA1* auxin biosynthesis gene (Franklin *et al.* 2011). Moreover it was shown that *pif4pif5* mutants display an altered sensitivity to auxin and altered expression of numerous “auxin genes”, however whether this is due to direct regulation of auxin signaling genes by those PIFs remains unknown (Nozue *et al.* 2011).

In order to better understand the mechanisms underlying PIF4 and PIF5-mediated growth responses we combined ChIP followed by sequencing (ChIP-seq) to identify chromatin-binding sites of PIF5 with gene expression studies. We identify a small set of shade-induced genes whose regulation depends on PIF4 and PIF5. Both transcription factors bind to promoter sequences of most of these genes indicating that they are likely direct targets of these PIFs. Our study reveals that PIF4 and PIF5 also influence gene expression in high R/FR particularly in low PAR. Finally, our work suggests that PIF4 and PIF5 impact auxin-mediated growth by directly controlling the expression of *YUC* genes that code for enzymes controlling a rate limiting step in auxin biosynthesis and of *IAA/AUX* auxin signaling genes.

Results

Identifying genome-wide PIF5 binding sites

PIF4 and PIF5 control the SAS and directly regulate the expression of several shade marker genes (Lorrain *et al.* 2008, Hornitschek *et al.* 2009). In order to obtain a global view of the importance of PIF5 during shade avoidance we performed a ChIP experiment followed by Ultra High Throughput sequencing (ChIP-seq) using a PIF5-HA line that was subjected to a 2 hour low R/FR treatment (Lorrain *et al.* 2008). We generated DNA libraries, one for the chromatin (input) and one for the enriched chromatin fragments following immunoprecipitation (IP). 1103 PIF5 binding sites were detected using Model-based Analysis of ChIP-seq (Zhang *et al.* 2008). For further analysis we considered peaks located in proximity of genes defined as follows: from -3000 bp of the transcript to 500 bp downstream of the transcript. This list comprises 962 peaks and identifies 1218 Arabidopsis Genome Initiative loci (supplementary table 1). As an example the reads located on 3 closely spaced G-boxes present 5' of the *PIL1* gene are presented (Figure 1A). We previously showed that these G-boxes are required for PIF5-mediated expression of a *PIL1* reporter in cell cultures (Hornitschek *et al.* 2009). In Arabidopsis seedlings, shade-induced expression of the *PIL1* reporter required these 3 G-boxes (Figure 2). We conclude that PIF5 binding to the G-boxes of the *PIL1* promoter is important for shade-regulated expression of this gene. Moreover, this experiment suggests that genes requiring PIFs for normal expression and possessing a nearby PIF5 binding site are likely direct targets of this transcription factor.

Most genes in our list contained a binding site in promoter regions with a higher frequency towards the transcriptional start site (TSS) and fewer peaks within the transcript or immediately 3' of it (Figure 1B). PIF5 was previously shown to directly bind to the G-box DNA motif (5'-CACGTG-3') (Hornitschek *et al.* 2009). We thus analyzed PIF5 binding peaks, defined as 200 bp centered to the peak summit, for the presence of this sequence and the E-box (5'-CANNTG-3'), a degenerated G-box that is also bound by bHLH transcription factors. Almost all PIF5 peaks contained an E-box (96%) the majority of which being a G-box (55%) (Figure 1C). Using motif-based sequence analysis tools (http://meme.sdsc.edu/meme/intro.html) we confirmed that the G-box is highly over-represented in PIF5 peaks. G-boxes were enriched in the center of PIF5 peaks suggesting that they mediate DNA binding (Figure 1D).

In order to compare *in vivo* binding sites of PIF5 with its DNA-binding specificity we used protein-binding microarrays (PBM) (Godoy *et al.* 2011). We included PIF4, the closest homologue of PIF5 and HFR1 in our analysis. PIF5 and PIF4 showed a strong preference for the G-box which is the sequence that was most enriched in PIF5 peaks determined by ChIP (Figures 1, 3 and S1). In addition, binding of PIF5 to the G-boxes *in vitro* was influenced by the nucleotides immediately 5' and 3' of the G-box while this was not the case for PIF4 (Figure 3). Moreover this experiment demonstrated that HFR1 did not possess sequence-specific DNA binding capacity (Figure 3). Our data does not exclude that HFR1 heterodimers with other bHLH factors could bind to DNA. However taken together with recent publications it is most likely that HFR1 works by preventing

other bHLH factors from binding to DNA (Figure 3) (Galstyan *et al.* 2011, Hornitschek *et al.* 2009).

Gene Ontology (GO) enrichment analysis was performed on genes close to PIF5 peaks in order to identify biological processes that may be regulated by PIF5 (supplementary table 2). The terms response to light stimulus, response to red or far-red light, response to radiation and shade avoidance were enriched. Response to hormone stimulus and especially response to auxin stimulus were also strongly enriched. Transcription factor activity and interestingly auxin responsive SAUR, basic helix-loop-helix and AUX/IAA proteins were also over-represented. This first analysis suggested that PIF5 might regulate light responses by directly controlling the expression of hormonal pathways, in particular auxin.

Shade-regulated gene expression in *pif4pif5*

The wild type, *pif5*, *pif4pif5* and the *PIF5-HA* line used for ChIP-seq were subjected to a 2 hour low R/FR treatment or maintained in the high R/FR light to determine the importance of PIF4 and PIF5 in shade-regulated gene expression using Affymetrix® Arabidopsis ATH1 chip. To identify genes misregulated by the treatment in *pif4pif5*, we used a linear model to compute the interaction between the genotype (*pif4pif5 vs* wild-type) and the treatment (low R/FR *vs* high R/FR). We identified 77 genes with significant interaction (FDR < 5%, Figure 4A), meaning that genes in this list showed a significantly different fold-change by the treatment in *pif4pif5* than in the wild type (supplementary table 3). The expression of these genes is presented as a heatmap that also includes their

expression in *pif5* and *PIF5-HA* (Figure 4A). Hierarchical clustering of the expression of these 77 genes identified 2 major groups and 5 smaller ones (3-7) that we will not further discuss here. The expression of the majority of these genes was robustly regulated by shade in *pif4pif5*. This was particularly obvious for group 1 representing the largest set of genes. Genes belonging to group 1 presented similar expression level in low R/FR in the wild type and in *pif4pif5*. They are present in this list because their expression was reduced in high R/FR grown *pif4pif5* leading to greater shade induction. In contrast genes belonging to group 2 showed reduced induction by low R/FR in *pif4pif5*. This small group contains genes previously identified as dependent on PIF4 and PIF5 for regulation such as *HFR1* and *ATHB2* (Figure 4) (Lorrain *et al.* 2008). Considering previously published data *PIL1*, that is not included in the ATH1 chip, would also be part of this group (Hornitschek *et al.* 2009, Lorrain *et al.* 2008). Genes identified in this category include *PIL2*, *CKX5* and *FHL*. GO enrichment analysis was performed in order to identify biological processes that may be misregulated in *pif4pif5* during the response to low R/FR (supplementary table 2). Interestingly several GO terms identified among genes close to PIF5 peaks were also found in this list. In particular the most over-represented term was response to auxin stimulus and AUX/IAA proteins was also over-represented in the list of genes with a misregulated expression by shade in *pif4pif5*. This analysis provided further support for a role of these PIFs in auxin-mediated growth responses.

Direct target genes likely show altered expression in the mutant and binding of the transcription factor to their promoter (e.g. *PIL1*) (Figures 1 and 2)

(Hornitschek *et al.* 2009). We thus compared the list of genes of the interaction list with genes that have a PIF5 binding site in their promoter (supplementary tables 1 and 3). We found that 39% of the genes of the interaction present a PIF5 binding peak in their vicinity. Interestingly these putative direct target genes are not evenly distributed in the different groups. Especially 8 out of the 9 genes of group 2 (including *PIL1*) show a PIF5 binding site in their promoter. This data suggests that most genes that are not properly upregulated by shade in *pif4pif5* (group 2) are direct targets of this transcription factors (Figure 4).

To confirm these genome-wide data we conducted additional gene expression and ChIP analysis on selected genes (Figure 4C and D). We present data for representatives of groups 1 and 2 of the interaction list, which contained a PIF5 binding peak determined by ChIP-seq. This experiment confirmed that genes of group 2 (*CKX5* and *FHL*) were primarily misexpressed in response to a low R/FR treatment in *pif4pif5*. In contrast genes belonging to group 1 (*YUC8* and *IAA29*) showed a slightly reduced expression in *pif4pif5* exposed to low R/FR but had strongly reduced expression in high R/FR (Figure 4C). Moreover by analyzing the expression of these genes in *pif4* and *pif5* single mutants we noticed that PIF5 played a predominant function in the expression of group 2 genes in low R/FR, while the expression of group 1 genes was reduced both in *pif4* and *pif5* grown in high R/FR (Figure 4C). ChIP experiments were conducted with the *PIF5-HA* line and seedlings expressing *PIF4-citrine-HA* under the control of the *PIF4* promoter (hereafter referred to as *PIF4-HA*). Using chromatin from seedlings exposed to a 2 hour low R/FR treatment, we confirmed binding of PIF5 to 10 (out of 10) genes selected based on the presence of a PIF5 binding site and misexpression in

response to shade (interaction list) (Figures 1, 4, S2). Moreover PIF4-HA also bound to the promoter of all tested genes (*FHL*, *CKX5, IAA29* and *YUC8*, Figure 4D). This suggests that both PIF4 and PIF5 directly control the expression of shade-regulated genes including genes coding for auxin biosynthesis and signaling (Figure 4D).

PIF4 and PIF5 regulate gene expression in low PAR

Our gene expression analysis identified numerous genes misexpressed in *pif4pif5* in our high R/FR conditions (Figure 4A). These conditions correspond to relatively low PAR, which prompted us to analyze the implication of PIF4 and PIF5 in low PAR more carefully. Using a FDR < 0.05 we found 521 genes whose expression was different between *pif4pif5* and the wild type (Figure 5) (supplementary table 4). Close to 80% of these genes showed reduced expression in *pif4pif5* suggesting that PIF4 and PIF5 primarily promote gene expression. Hierarchical cluster analysis identified four main expression classes (Figure 5A). Among the genes that were downregulated in *pif4pif5* only a subset was expressed at a higher level in *PIF5-HA* than in the wild type (compare groups I and II). A third cluster contained genes that were downregulated in *pif4pif5* and even further downregulated in *PIF5-HA*. Finally, the last group contained genes with a higher expression in *pif4pif5* than in the wild type. Generally speaking, the *pif5* expression phenotype was intermediate between the one of the WT and *pif4pif5* (Figure 5A). Analysis of GO term enriched among these 521 genes include response to hormone stimulus, response to auxin, response to light stimulus and response to radiation, terms that were all

previously identified in our GO analysis of genes nearby PIF5 peaks in low R/FR-grown seedlings.

We hypothesized that PIF4 and PIF5 might play a particularly important role in low light intensity, which prompted us to analyzed seedling growth of the WT, *pif4*, *pif5*, *pif4pif5* and the *pif1pif3pif4pif5* (*pifq*) mutants under several intensities of PAR (Figure 5B). Interestingly, while the *pif* mutants showed no significant defect in hypocotyl elongation under high PAR, phenotype strength increased with decreasing PAR (Figure 5B). Similar to the gene expression phenotype *pif5* showed a phenotype intermediate between the WT and *pif4pif5*. Finally the *pifq* hypocotyl elongation phenotype was stronger than the one of *pif4pif5* only at the lowest fluence rate tested (Figure 5B).

In order to determine whether the hypocotyl elongation phenotype correlated with gene expression, we analyzed the expression of several genes under high and low PAR conditions by RT-Q-PCR. We concentrated on genes with the GO term auxin as this term was strongly overrepresented and auxin has been implicated in growth. The expression of these genes was lower in high than low PAR correlating with the shorter hypocotyls of seedlings grown in high PAR conditions (Figure 5C). Moreover, we found a good correlation between gene expression and hypocotyl length, as differences in gene expression between Col and *pif4pif5* are smaller in high compared to low PAR (Figure 5C). In order to determine whether these genes were bound by PIF4 and PIF5 in low PAR we conducted ChIP experiments. PIF4-HA and PIF5-HA bound to the promoters of genes involved in auxin biosynthesis and signaling (*IAA29, YUC8*) suggesting that

they also control growth by directly regulating auxin synthesis and signaling in low PAR but high R/FR (Figure 5D). Binding of both transcription factors was also observed in the promoter of the shade marker genes *PIL1* and *HFR1*, which also show higher expression in low compared to high PAR (Figure 5D).

PIF4 and PIF5 control growth by directly regulating auxin signaling

Our gene expression, ChIP and physiological experiments suggested that PIF4 and PIF5 control hypocotyl elongation by controlling auxin biosynthesis and/or signaling (Figures 4 and 5). We thus determined auxin levels in the aerial parts of young seedlings maintained in high R/FR or transferred for an hour into low R/FR because it was previously shown that such a treatment leads to an increase in auxin concentration (Tao *et al.* 2008). We confirmed that a low R/FR treatment increased auxin concentration in the WT. Interestingly the shade-mediated increase was much reduced both in *pif4pif5* and in *PIF5-HA* (Figure 6). The auxin concentration in high R/FR was normal in *pif4pif5* while in *PIF5-HA* it was reduced (Figure 6).

In order to analyze auxin sensitivity of *pif4pif5*, we compared hypocotyl elongation of the mutant and the wild type grown in presence of different concentrations of picloram. This experiment showed that the auxin sensitivity of *pif4pif5* was altered particularly under low PAR (Figure 7A and 7B). In addition we tested the effect of picloram on gene expression and compared it with the effect of shade. *HFR1* was upregulated by shade but not picloram while *IAA29* expression was induced by both treatments in *pif4pif5* and the WT (Figure 7C). However, the expression of *IAA29* in *pif4pif5* never reached WT levels when

seedlings were treated by picloram or shade (Figure 7C). Collectively our data suggest that PIF4 and PIF5 control hypocotyl growth at least partially by directly controlling the expression of genes involved in auxin biosynthesis and signaling.

Discussion

To get a broader view of the role of PIF5 and PIF4 during the SAS, we analyzed their contribution to gene expression in seedlings treated with low R/FR and identified PIF5 binding sites genome-wide. ChIP-seq revealed a large number of genes in proximity of which we found PIF5 binding sites, a number that is comparable to those identified in genome-wide ChIP experiments for other transcription factors involved in light signaling (Lee *et al.* 2007, Oh *et al.* 2009, Ouyang *et al.* 2011). Binding sites were abundant 5' of transcription start sites (TSS) with a further enrichment within the first 500nt directly upstream of the TSS. A similar binding pattern was reported for other members of the bHLH family (Oh *et al.* 2009, Morohashi and Grotewold 2009). PIF5 peaks were strongly enriched in E- and G-boxes (96/55% of peaks), another feature shared with PIF1 (Oh *et al.* 2009). By comparing the sequences bound by PIF5 *in vitro* with our ChIP-seq data we conclude that most PIF5 binding on chromatin reflects direct binding to DNA (Figure 1, 3 and S1). Although *in vitro* PIF5 exclusively binds to G-boxes with high affinity our ChIP data shows that a sizable fraction of PIF5 ChIP peaks do not contain a G-box (Figures 1 and 3). Several hypotheses can explain this apparent paradox and future experiments are needed to understand this difference.

Interestingly, *in vitro* binding experiments show that although PIF4 and PIF5 have a preference for G-boxes, as was previously reported for several members of this family, PIF4 robustly binds to a wider range of sequences than PIF5 (Figure 3) (Martinez-Garcia *et al.* 2000, Huq and Quail 2002, Huq *et al.* 2004). All

the genes we tested for PIF5 binding *in vivo* were also bound by PIF4 indicating that *in vivo* PIF4 and PIF5 share an overlapping set of binding sites (Figures 4 and 5). This is consistent with the additive phenotype of *pif4* and *pif5* that was reported in several situations including during shade avoidance (Lorrain *et al.* 2008, Lorrain *et al.* 2009, Nozue *et al.* 2007) (Figures 4 and 5). However, hypocotyl elongation in response to temperature involves PIF4 and not PIF5, which is difficult to explain based on the similar expression patterns of those genes (Foreman *et al.* 2011, Koini *et al.* 2009, Nozue *et al.* 2007, Stavang *et al.* 2009). The greater number of E-box variants efficiently bound by PIF4 may provide an explanation for the specific functions of PIF4 (Figure 3). The fact that over-expression of PIF4 leads to a stronger and more pleiotropic phenotype than over-expression of PIF5 is consistent with this hypothesis (Lorrain *et al.* 2008).

Gene expression analyses were conducted to identify those requiring PIF4/PIF5 for normal regulation by a low R/FR treatment (Figure 4A). Among these genes a small group required PIF4/PIF5 for robust low R/FR-induced expression (Figure 4A). With the exception of one gene all members of this group also show PIF5 binding 5' of their TSS (Figure 4). Given that our subsequent ChIP analysis also showed binding of PIF4 to the promoters of all tested group 2 genes, they represent likely direct targets of both PIF4 and PIF5 (Figure 4). This group includes previously identified PIF5 targets *PIL1*, *HFR1* and *ATHB2* and we show that PIF4 also binds to promoter regions of these genes (Figure 4) (Hornitschek *et al.* 2009, Kunihiro *et al.* 2011). This was confirmed for *PIL1* where the 3 G-boxes present in the PIF5 binding peak are essential for shade-induced expression in seedlings (Figure 2). These data show that *PIL1* is a direct target of

PIF4 and PIF5 because the transcription factors and the sequence to which they bind are both needed for robust shade-induced expression.

While some group 2 genes promote the SAS (*ATHB2*) others (*HFR1, PIL1*) play a negative role in shade avoidance (Hornitschek *et al.* 2009, Salter *et al.* 2003, Sessa *et al.* 2005, Sorin *et al.* 2009). The other members of this group are *PIL2*, *FHL*, *CKX5*, *ATMGL*, a B-box type zinc finger protein (At5g54470 or BBX29) and an unknown protein in the promoter of which we found no PIF5 peak (Figures 4 and supplementary table 1). FHL mediates import of phytochrome A (phyA) into the nucleus (Hiltbrunner *et al.* 2006, Rosler *et al.* 2007). phyA plays a negative role in the SAS (Salter *et al.* 2003), moreover the levels of FHY1 and FHL are limiting thus controlling the extent of phyA import into the nucleus (Rausenberger *et al.* 2011). The shade-induced up-regulation of *FHL* may thus contribute to phyA-mediated inhibition of the SAS by promoting its import into the nucleus. BBX29 belongs to the Arabidopsis B-box family which includes members with a role in light signaling, in particular BBX21 that negatively regulates shade-avoidance (Khanna *et al.* 2009, Crocco *et al.* 2010). CKX5 is involved in cytokinin catabolism and CKX6, a close homologue of CKX5, regulates the SAS (Carabelli *et al.* 2007). CKX6 does not control hypocotyl elongation but limits leaf primordia growth in plants subjected to a shade treatment (Carabelli *et al.* 2007). *CKX6* expression is upregulated by shade and auxin linking cytokinin-mediated responses to shade and auxin (Carabelli *et al.* 2007). By analogy with the role of CKX6 it is conceivable that CKX5 also acts as a negative regulator of the SAS (Figure 4). *PIL2* is a member of the PIF family that also shows shade-induced gene expression, however its function is poorly

understood (Salter *et al.* 2003, Yamashino *et al.* 2003). Finally, ATMGL is involved in methionine catabolism and its role in shade avoidance is currently unknown (Rebeille *et al.* 2006). Collectively these data indicate that PIF4 and PIF5 directly control the expression of several genes acting as negative regulators of the shade avoidance response (Figures 4 and 5). The relatively normal expression of many shade-regulated genes in *pif4pif5* and the reduced induction of several negative regulators of the SAS in *pif4pif5* may explain why a low R/FR signal still induces hypocotyl growth in *pif4pif5* (Figure 4) (Cole *et al.* 2011, Hornitschek *et al.* 2009, Lorrain *et al.* 2008).

Our gene expression analysis showed that numerous genes are misexpressed in *pif4pif5* grown in high R/FR conditions (Figures 4 and 5). Combined with our ChIP analysis we conclude that PIF4 and PIF5 are likely direct regulators of the expression of a number of these genes in high R/FR conditions (Figure 5). Importantly our high R/FR conditions correspond to relatively low PAR a condition in which PIF4/PIF5 were previously shown to control growth (Keller *et al.* 2011). Interestingly we show that by increasing PAR we can correct both hypocotyl length and gene expression in *pif4pif5* (Figure 5). We made similar observation during de-etiolation in far-red light (Lorrain *et al.* 2009). Many of the genes showing reduced expression in *pif4pif5* in high R/FR are strongly induced by shade in the mutant suggesting that another transcriptional regulator controls their expression in response to low R/FR (Figure 4). Other members of the PIF family are candidates for such a function given that they bind to similar DNA sequences and can act additively (Figure 3)(Martinez-Garcia *et al.* 2000, Huq and Quail 2002, Huq *et al.* 2004, Leivar *et al.* 2008, Shin *et al.* 2009).

Both the analysis of genes bound by PIF5 and genes misregulated in the *pif4pif5* mutant show a strong over-representation of the GO term response to auxin stimulus and response to hormone stimulus (supplementary table 2). This is remarkable in view of the strong links between auxin and shade avoidance (Keller *et al.* 2011, Keuskamp *et al.* 2010, Kozuka *et al.* 2010, Morelli and Ruberti 2000, Roig-Villanova *et al.* 2007, Tao *et al.* 2008). These results are also in agreement with the recent findings linking PIF4 and PIF5 to auxin-mediated growth responses (Franklin *et al.* 2011, Nozue *et al.* 2011). By comparing the genes misregulated in our high R/FR conditions with the genes whose expression correlates with growth and requires PIF4 and PIF5 for normal expression we found a large overlap (Nozue *et al.* 2011) (Figure 5, supplementary table 5). Taken together with previous studies our data suggest that PIF4 and PIF5 modulate elongation growth responses by directly regulating auxin-controlled responses at multiple levels.

In warm conditions PIF4 binds to the promoter and controls the expression of *TAA1* and *CYP79B2*, two genes coding for auxin biosynthetic enzymes (Franklin *et al.* 2011). Although TAA1/SAV3 is essential for the SAS, its expression is not induced by shade rendering it unlikely that PIF4 and/or PIF5 control shade-induced growth by regulating *TAA1* expression (Tao *et al.* 2008). However, we found members of the YUCCA family that act downstream of TAA1 in auxin biosynthesis have PIF5 binding sites in their promoter (*YUC5*, *YUC8* and *YUC9*) (supplementary table 1) (Mashiguchi *et al.* 2011, Stepanova *et al.* 2011, Won *et al.* 2011). YUCCA proteins are rate limiting for auxin biosynthesis and increasing

their expression leads to hypocotyl elongation (Mashiguchi *et al.* 2011, Won *et al.* 2011, Zhao *et al.* 2001). The increased expression of several *YUCCA* genes in response to low R/FR may thus contribute to shade-induced hypocotyl elongation. We show that both PIF4 and PIF5 bind to the promoter of *YUC8* and that the gene displays reduced expression in *pif4* and *pif5* mutants suggesting that PIF4 and PIF5 might directly control auxin biosynthesis. We thus determined auxin content in *pif4pif5* seedlings grown in high R/FR with or without a one-hour low R/FR shade treatment. Despite the reduced *YUC8* expression in *pif4pif5* grown in high R/FR we found a wild-type auxin content in aerial parts of these seedlings (Figures 4, 5 and 6). More local auxin content measurements may reveal differences between *pif4pif5* and the wild type and thus explain the shorter hypocotyl of these seedlings grown in high R/FR but low PAR. Of note, the *PIF5-HA* line that was used for ChIP-seq had the lowest auxin content of all lines despite having the longest hypocotyls (Figure 6) (Lorrain *et al.* 2008). This indicates that despite a promoting effect of auxin on hypocotyl growth the auxin content in aerial parts does not simply correlate with hypocotyl length. Another unanticipated finding was that in *pif4pif5* the low R/FR-induced increase in auxin was strongly reduced although this mutant shows hypocotyl elongation in response to low R/FR both in long-term and short-term measurements (Cole *et al.* 2011, Lorrain *et al.* 2008). Again more localized auxin measurements may help with the interpretation of these results.

We provide evidence for a direct link between PIF4 and PIF5 and auxin signaling by showing that PIF4 and PIF5 bind to the promoter region of *IAA29* a gene that shows reduced levels in *pif4*, *pif5* and *pif4pif5* (Figures 4 and 5). *IAA29*

expression can be induced by the addition of picloram to *pif4pif5*, however both in response to shade and in response to picloram *IAA29* expression does not reach wild-type levels in the mutant (Figure 7). In addition we analyzed hypocotyl elongation in response to picloram and consistent with a previous study found that auxin sensitivity in *pif4pif5* was altered (Figure 7) (Nozue *et al.* 2011). Importantly auxin sensitivity was most altered in low PAR conditions, where we also found greater gene expression defects in *pif4pif5* (Figures 5 and 7). We thus suggest that PIFs modulate plant growth by directly controlling the expression of auxin signaling genes. Moreover, we propose that PIF-mediated control of auxin-driven growth might involve different mechanisms (transport, signaling, synthesis) in different situations (this work) (Franklin *et al.* 2011).

Materials and Methods

Plant material and growth conditions

Seedlings were grown as described in (Hornitschek *et al.* 2009) except that PAR intensity was 40 μ mol m^{-2} s^{-1}. The *pif4*, *pif5* and *pif4pif5* mutants as well as the transgenic lines were in the Columbia background (Col-0) and were described in (Lorrain *et al.* 2008).

Cloning procedure and generation of transgenic lines

The generation of new transgenic lines is described in detail in the supplementary materials and methods.

GUS staining and MUG assay

GUS staining and quantitative determination of GUS activity (MUG assays were performed according to standard procedures and described in detail in the supplementary materials and methods.

Picloram treatment and quantification of IAA

Picloram (SIGMA-Aldrich) was dissolved in DMSO at 400mM. Seeds were sown on a nylon mesh on ½ strength MS plates that were kept vertical during the experiment. On day 4, nylon meshes were transferred to new ½ strength MS plates containing different concentrations of picloram. Seedlings were grown 4 more days on those plates before being photographed and measured using the ImageJ software. For gene expression analysis in response to picloram, 50 seeds were sown on a nylon mesh on ½ strength MS petri dishes and grown 7 days in

constant light conditions. At day 7, seedlings were transferred in 1ml of liquid ½ strength MS with 5 μ M picloram or 0.01% DMSO as a control for additional 2 hours.

Seedlings were pooled in quintuplicates, weighted and frozen in liquid nitrogen for quantification of free IAA according to (Andersen *et al.* 2008).

Identification of PIF4, PIF5 and HFR1 binding sites *in vitro*

Given the expected size of the DNA-motif recognized by PIF4, PIF5 and HFR1 a 10 nucleotides design in PBM was chosen. In this case, we used the same PBM design as in (Berger and Bulyk 2009). Protein incubation was as in (Godoy *et al.* 2011) but in these cases we employed soluble protein extracts from recombinant *E. coli* cultures expressing MBP-PIF4, MBP-PIF5 and MBP-HFR1 recombinant proteins. Synthesis of double-stranded microarray and immunological detection of DNA-protein complexes were as in (Godoy *et al.* 2011).

Analysis of gene expression

RNA extraction and RT-QPCR experiment were performed as described in (Lorrain *et al.* 2009) except that results were analyzed using the qbase^{PLUS} software (http://www.biogazelle.com/products). Primer sequence is given in Supplementary Table 6.

For microarray analysis samples were amplified, labeled and hybridized on Affymetrix® Arabidopsis ATH1 Genome arrays as described previously (Lorrain *et al.* 2009). Subsequent data analysis was performed using the statistical

language R (http://www.R-project.org) and various Bioconductor packages (http://www.Bioconductor.org). Normalized expression signals were calculated using RMA, and differential hybridized features were identified using LIMMA, as before (Lorrain *et al.* 2009). We used a statistical model where the four conditions were included as factors and then extracted the comparisons of interest as contrasts: (i) *pif4pif5* double mutant versus the wild type in high R/FR (ii) *pif4pif5* double mutant versus the wild type in low R/FR, (iii) interaction between high and low R/FR factor and mutant/wild-type factor. P values from each comparison were adjusted separately for multiple testing with the Benjamini and Hochberg method to control the false discovery rate (FDR). Genes depicted as heat map were mean centered and analyzed by average linkage hierarchical clustering (Cluster 3.0) and subsequently visualized using Java TreeView. Gene Ontology (GO) terms belonging to the GO Biological Process or Interpro database were tested for enrichment using DAVID. Microarray and ChIP-seq data can be obtained from the Gene Expression Omnibus (GEO) database (GSE35062).

ChIP sequencing

The ChIP experiment was performed as described in (Hornitschek *et al.* 2009). A detailed description of the ChIP-seq procedure can be found in the supplementary materials and methods.

Supplemental data

Supplementary materials and methods.

Supplementary Figure S1. PIF5 binds with different frequency to various E-box sequences *in vivo*.

Supplementary Figure S2. Additional ChIP PCR experiments on selected genes.

Supplementary table 1. List of genes with a PIF5 peak identified by ChIP sequ.

Supplementary table 2. Lists of GO anaylsis.

Supplementary table 3. List of genes showing an altered regulation of gene expression in *pif4pif5* in response to low R/FR.

Supplementary table 4. List of genes with altered expression in *pif4pif5* grown in high R/FR.

Supplementary table 5. Comparison of PIF4/5-regulated gene expression performed in (Nozue *et al.* 2011) with our analysis (table 4).

Supplementary table 6. List of primers used in this study.

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Figure legends

Figure1: PIF5-HA preferentially binds to promoters containing E- and G-boxes.

(A) Sequence read distribution in the genomic region containing *PIL1*. Reads are enriched on top of G-boxes (green dot) located in the *PIL1* promoter. Tags mapping to the + and – strands are labeled in yellow and blue respectively. The *PIL1* coding sequence (CDS) is marked with a red bar.

(B) Distribution of PIF5-HA binding loci relative to the transcriptional start site. PIF5-HA binding sites, which map within CDSs, were plotted relative to 2.5 kb (horizontal bar). Only PIF5-HA binding sites assigned to one gene were considered.

(C) Percentage of PIF5-HA binding loci containing at least one G- or E-box. Note that loci containing a G-box may also contain additional E-boxes.

(D) Distribution of G-boxes within 200 bp sequence covered by peaks. The x-axis represents the relative distance in bp to the center of peaks.

Figure 2: The shade-induced expression of *PIL1* is dependent on G-boxes.

Seedlings were grown for 7 days in constant high R/FR before being transferred 5 hours to low R/FR or kept in high R/FR (control). Transgenic lines carrying *PILpro1::GUS* or *PIL1*pro::GUS* (*PIL1* promoter containing point mutations in all 3 G-boxes) were used.

(A) GUS staining of *PILpro1::GUS* and *PIL1*pro::GUS* lines.

(B) Quantification of *PILpro1::GUS* and *PIL1*pro::GUS* reporter gene activity using MUG assay. Results of two independent transgenic lines are presented. Data are means ±2 SE from three biological replicas.

Figure 3: Identification of PIF4 and PIF5 binding sites *in vitro*.

(A) Position weight matrix representation of the first scoring 8-mers corresponding to PIF4 and PIF5.

(B) Enrichment scores (E-scores) of all the possible G-box-containing 8-mers for the two proteins tested.

(C) Box-plot of E-scores of G-box-related variants including both single-site mutations and E-boxes for PIF4 (blue) and PIF5 (green). Boxes represent quartiles 25% to 75%, and black line represents the median of the distribution (quartile 50%). Bars indicate quartiles 1 to 25% (above) and 75 to 100% (below), and dots denote outliers of the distribution.

(D) Box-plot of E-scores of G-box-related variants including both single-site mutations and E-boxes corresponding to HFR1, as in (C). HFR1 did not show significant binding to any of the elements represented in the PBM.

Figure 4: Genes displaying an altered regulation by a low R/FR treatment in *pif4pif5*.

(A) Hierarchical clustering of relative expression levels across all samples for 77 genes significantly (adj. P-value < 0.05) dependent on an interactive effect of the genotype (*pif4pif5 vs* wild type) and the condition (low *vs* high R/FR ratio).

(B) Gene expression from the microarray experiment for a representative gene of group 1 and 2.

(C) Gene expression determined by RT-Q-PCR in response to 2 hours of low R/FR. Col, *pif4, pif5* and *pif4pif5* seedlings were grown 7 days in constant light conditions before being moved for 2 hours to low R/FR or kept in high R/FR.

Expression levels were normalized to *YLS8* and *UBC* and expressed relative to the Col value in high R/FR. Error bars represent standard error to the mean of three biological replicates.

(D) Chromatin Immunoprecipitation (ChIP) of PIF4-HA or PIF5-HA grown for 7 days in constant light followed by a 2 hour low R/FR treatment. Immunoprecipitated DNA was quantified by Q-PCR using primers in the promoter region containing a G-box or control region. Data are average of technical triplicates of the Q-PCR. Error bars represent the S.D. calculated on the Ct scale. Error bars are $2^{\text{mean} \pm \text{SD}}$. Data from one representative ChIP experiment are shown.

Figure 5. PIF4 and PIF5 are involved in responses to low light intensities.

(A) Hierarchical clustering of genes differently expressed between *pif4pif5* and Col-0 grown in high R/FR light.

(B) Hypocotyl length in constant white light. Seedlings were grown 4 days under different constant white light conditions before hypocotyls were measured. Representative seedlings are shown for Col and *pif4pif5* in the left panel. Data are means +- 2SE (n=23-30).

(C) Gene expression determined by RT-Q-PCR after 7 days growth in constant low or high light intensity (40 or 130 µmoles m^{-2} s^{-1}). Expression levels were normalized to *YLS8* and *UBC* and expressed relative to the Col value in PAR 40. Error bars represent standard error to the mean of three biological replicates.

(D) Chromatin Immunoprecipitation (ChIP) of PIF4 or PIF5 in high R/FR. Col, *PIF4-HA* and *PIF5-HA* lines were grown for 7 days in constant light (40 µmoles m^{-2} s^{-1}) immunoprecipitated DNA was quantified by Q-PCR using primers in the

promoter region containing a G-box (black bar) or control region (gray bar). Data are average of technical triplicates of the Q-PCR. Error bars represent the S.D. calculated on the Ct scale. Error bars are $2^{mean \pm SD}$. Data from one representative ChIP experiment are shown.

Figure 6: *pif4pif5* is affected in auxin accumulation in response to shade.

WT, *pif4pif5* and *PIF5-HA* seedlings were grown 7 days in constant high R/FR light. Free IAA was measured after 1 hour of high or low R/FR treatment. Data are means +- 2SE (n=5).

Figure 7: *pif4pif5* is affected for responses to the auxin analog, picloram.

(A) Hypocotyl length in response to picloram of seedlings grown under PAR 40 µmoles m^{-2} s^{-1} or PAR 130 molesE m^{-2} s^{-1}. Seedlings were grown 4 days in constant white light conditions (40 or 130 µmoles m^{-2} s^{-1}) before being transferred on plates containing different concentrations of picloram (PIC). They were grown 4 more days in constant white light (40 or 130 µmoles m^{-2} s^{-1}). Data are means +- 2SE (n=36-43).

(B) Relative hypocotyl length of the data presented in panel (A), defined as the hypocotyl length relative to growth in the absence of picloram for each genotype.

(C) Gene expression in response to picloram. Col and *pif4pif5* seedlings were grown 7 days in constant white light (PAR= 40 or 130 μ moles m^{-2} s^{-1}) before being treated for 2 hours with 5 μ M picloram (PIC) or moved under low R/FR conditions (PAR=40 μ E m^{-2} s^{-1}) for 2 hours. Expression levels determined by

RT-Q-PCR were normalized to *YLS8* and *UBC* and expressed relative to the Col control grown in PAR 40 μ E m^{-2} s^{-1} without picloram treatment (DMSO). Error bars represent standard error to the mean of three biological replicates.

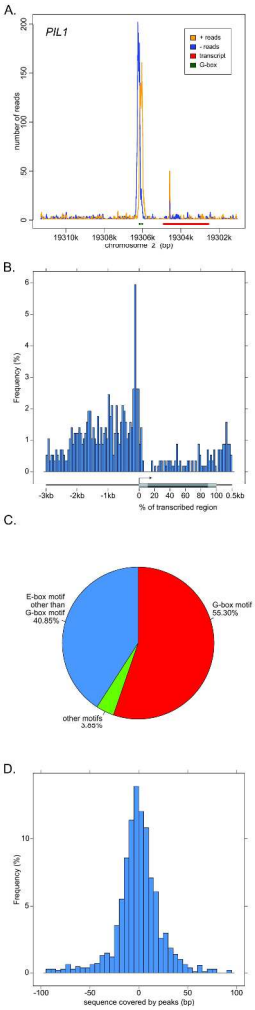

Figure1: PIF5-HA preferentially binds to promoters containing E- and G-boxes.

(A) Sequence read distribution in the genomic region containing *PIL1*. Reads are enriched on top of G-boxes (indicated with a green dot) located in the *PIL1* promoter sequence. Tags mapping to the + and – strands are labeled in yellow and blue respectively. The *PIL1* coding sequence (CDS) is marked with a red bar.
(B) Distribution of PIF5-HA binding loci relative to the transcriptional start site. PIF5-HA binding sites, which map within CDSs, were plotted relative to 2.5 kb (horizontal bar). Only PIF5-HA binding sites assigned to one gene were considered.
(C) Percentage of PIF5-HA binding loci containing at least one G- or E-box.
(D) Distribution of G-boxes within 200 bp sequence covered by peaks. The x-axis represents the relative distance in bp to the center of peaks.

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Figure 2

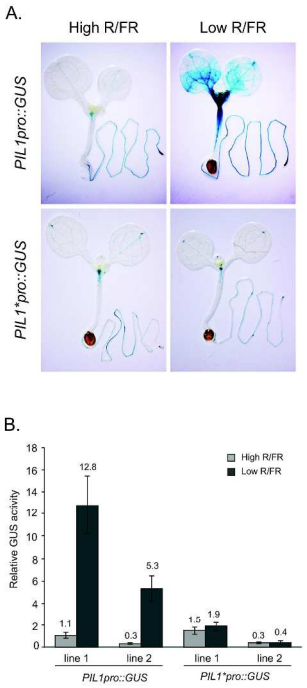


Figure 2: The shade-induced expression of *PIL1* is dependent on G-boxes.

Seedlings were grown for 7 days in constant high R/FR before being transferred 5 hours in low R/FR or kept in high R/FR as a control. Transgenic lines carrying *PILpro1::GUS* (wild type version of the *PIL1* promoter) or *PIL1*pro::GUS* (*PIL1* promoter containing point mutations in all 3 G-boxes) were used.

(A) GUS staining of *PILpro1::GUS* and *PIL1*pro::GUS* lines. Representative seedlings are shown.
(B) Quantification of *PIL1pro::GUS* and *PIL1*pro::GUS* reporter gene activity using MUG assay. Results of two independent transgenic lines are presented. Data are means ±2 SE from three biological replicas.

282x378mm (300 x 300 DPI)

Figure 3

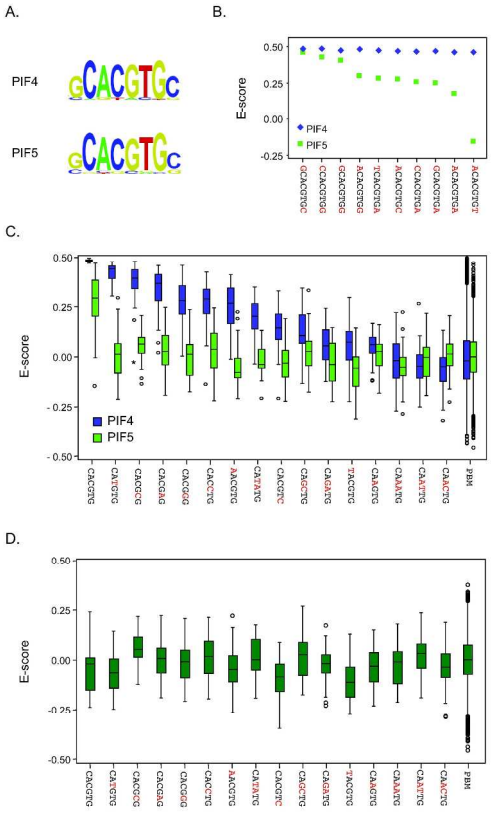
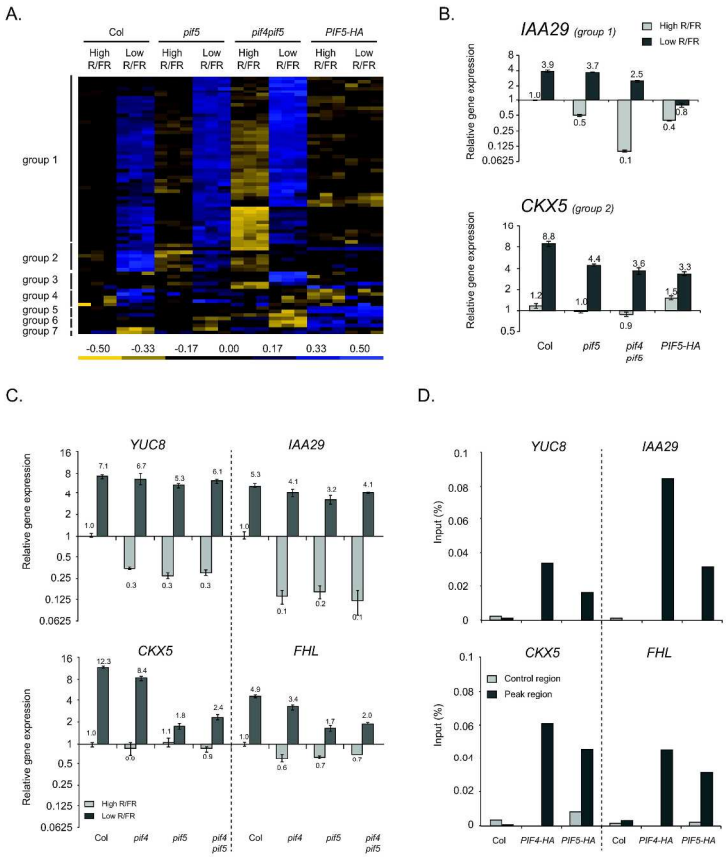


Figure 3: Identification of PIF4 and PIF5 binding sites *in vitro*.

(A) Position weight matrix representation of the first scoring 8-mers corresponding to PIF4 and PIF5.
(B) Enrichment scores (E-scores) of all the possible G-box-containing 8-mers for the two proteins tested. PIF4 did not show particular 5'- and 3'-end preferences, whereas binding of PIF5 to DNA resulted highly dependent on G and C at both ends.
(C) Box-plot of E-scores of G-box-related variants including both single-site mutations and E-boxes. Boxes represent quartiles 25% to 75%, and black line represents the median of the distribution (quartile 50%). Bars indicate quartiles 1 to 25% (above) and 75 to 100% (below), and dots denote outliers of the distribution. Boxes in blue correspond to PIF4 and green ones correspond to data from PIF5. Binding of PIF5 to DNA was restricted to canonical G-boxes, whereas PIF4 showed intermediate affinities for other G-related variants.
(D) Box-plot of E-scores of G-box-related variants including both single-site mutations and E-boxes corresponding to HFR1, as in (C). HFR1 did not show significant binding to any of the elements represented in the PBM.

278x468mm (300 x 300 DPI)

Figure 4



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(A) Hierarchical clustering of relative expression levels across all samples for 77 genes significantly (adj. P-value < 0.05) dependent on an interactive effect of the genotype (*pif4pif5* vs wild type) and the condition (low vs high R/FR ratio).

(B) Gene expression from the microarray experiment for a representative gene of group 1 and 2.

(C) Gene expression determined by RT-Q-PCR in response to 2 hours of low R/FR. Col, *pif4*, *pif5* and *pif4pif5* seedlings were grown 7 days in constant light conditions before being moved for 2 hours in low R/FR or kept in high R/FR. Expression levels were normalized to *YLS8* and *UBC* and expressed relative to the Col value in high R/FR. Error bars represent standard error to the mean of three biological replicates.

(D) Chromatin Immunoprecipitation (ChIP) of PIF4-HA or PIF5-HA after 2 hours in low R/FR. Col, PIF4-HA and PIF5-HA lines were grown for 7 days in constant light conditions before being shifted for 2 hours in low R/FR conditions. Immunoprecipitated DNA was quantified by Q-PCR using primers in the promoter region containing a G-box or control region (minimum 1 kb 3' or 5' from the peak region). Data are average of technical triplicates of the Q-PCR.
Data from one representative ChIP experiment are shown.

284x456mm (300 x 300 DPI)

Figure 5

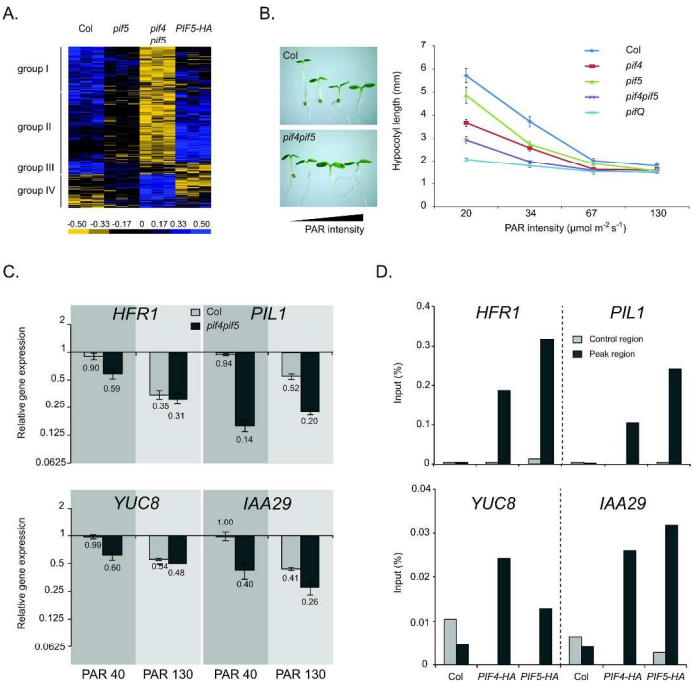

Figure 5. PIF4 and PIF5 are involved in responses to low light intensities.

(A) Hierarchical clustering of genes differently expressed between *pif4pif5* and Col-0 grown in high R/FR light.

(B) Hypocotyl length in constant white light. Seedlings were grown 4 days under different constant white light conditions before hypocotyls were measured. Representative seedlings are shown for Col and *pif4pif5* in the left panel. Data are means +- 2SE (n=23-30).

(C) Gene expression determined by RT-Q-PCR after 7 days in constant low or high light intensities. WT and *pif4pif5* seedlings were grown under two constant light conditions (40 or 130 µmoles m-2 s-1). After 7 days, RNA was extracted and gene expression levels were analyzed by RT-Q-PCR. Expression levels were normalized to *YLS8* and *UBC* and expressed relative to the Col value in PAR 40. Error bars represent standard error to the mean of three biological replicates.

(D) Chromatin Immunoprecipitation (ChIP) of PIF4 or PIF5 in high R/FR. Col, PIF4-HA and PIF5-HA lines were grown for 7 days in constant light (40 µmoles m-2 s-1). Immunoprecipitated DNA was quantified by Q-PCR using primers in the promoter region containing a G-box (black bar) or control region (gray bar, minimum 1 kb 3' or 5' from the peak region). Data are average of technical triplicates of the Q-PCR. Data from one representative ChIP experiment are shown.

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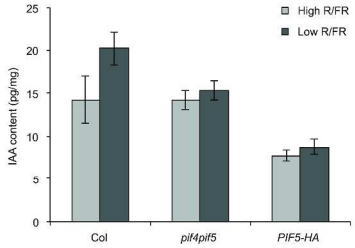
Figure 6



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WT, *pif4pif5* and *PIF5-HA* seedlings were grown 7 days in constant high R/FR light. Free IAA was measured after 1 hour of high or low R/FR treatment.

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Figure 7

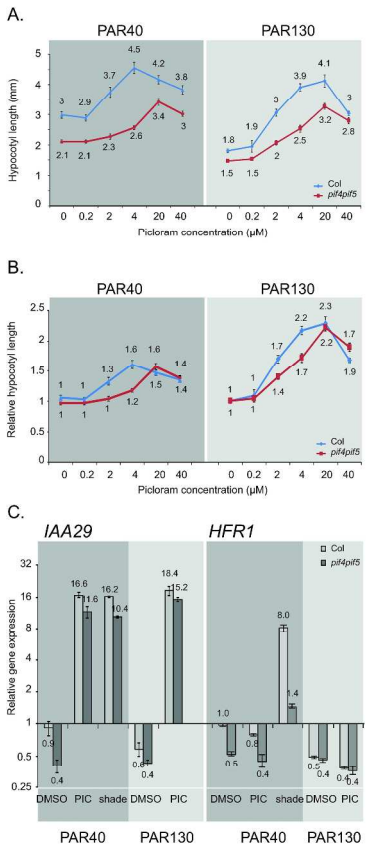

Figure 7: *pif4pif5* is affected for responses to the auxin analog, picloram

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(A) Hypocotyl length in response to picloram of seedlings grown under PAR 40 µmoles m-2 s-1 or PAR 130 moles m-2 s-1. Seedlings were grown 4 days in constant white light conditions (40 or 130 µmoles m-2 s-1) before being transferred on plates containing different concentrations of picloram (PIC). They were then grown 4 more days in constant white light (40 or 130 µmoles m-2 s-1). Data are means +- 2SE (n=36-43).

(B) Relative hypocotyl length of the data presented in panel (A), defined as the hypocotyl length relative to the control experiment (growth in the absence of picloram) for each genotype.

(C) Gene expression in response to picloram. Col and *pif4pif5* seedlings were grown 7 days in constant white light (PAR= 40 or 130 µ E m-2 s-1) before being treated for 2 hours with 5µM picloram (PIC) or moved under low R/FR conditions (PAR=40 µE m-2 s-1) for 2 hours. After RNA extraction, gene expressions were analyzed by RT-Q-PCR. Expression levels were normalized to *YLS8* and *UBC* and expressed relative to the Col control grown in PAR 40 µE m-2 s-1 without picloram treatment (called DMSO). Error bars represent standard error to the mean of three biological replicates.

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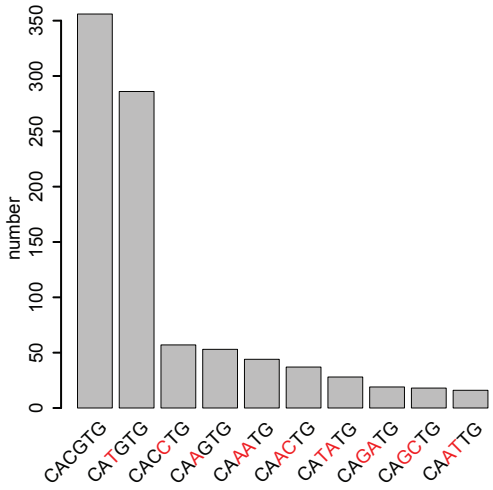
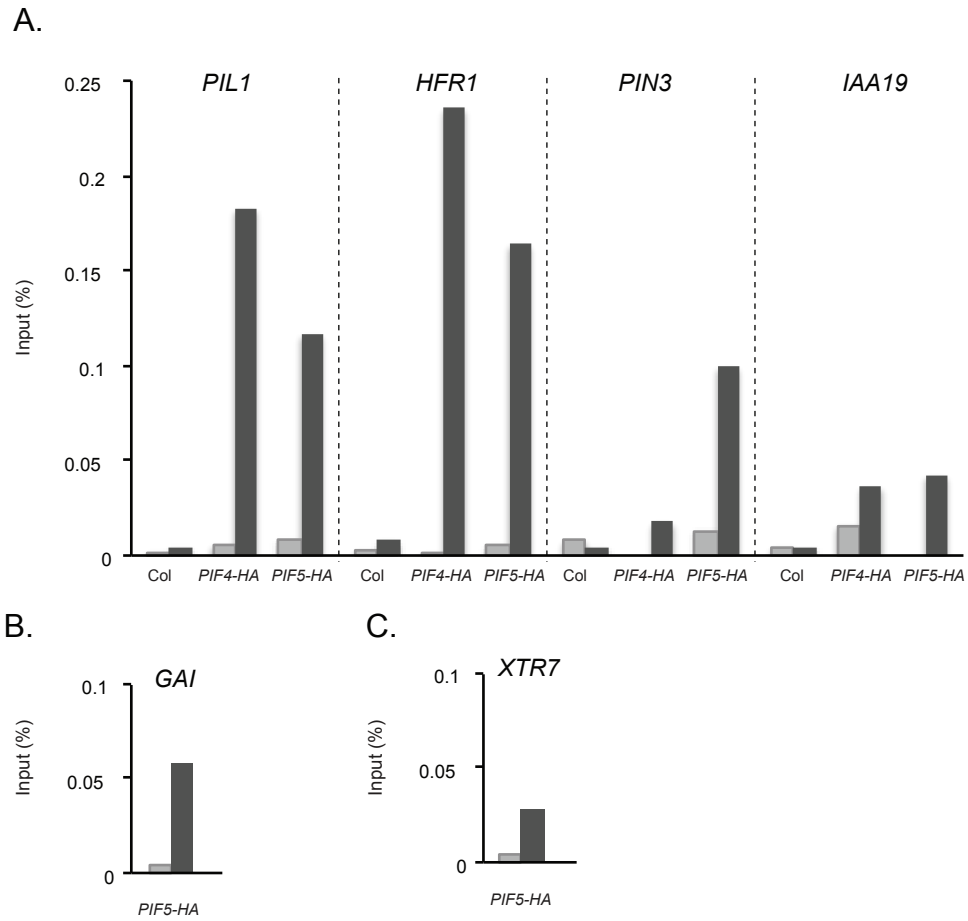
Figure S1



group name	nr.	complementary sequences
CACGTG	356	cacgtg; gtgcac
CATGTG	286	catgtg; gtacac; cacatg; gtgtac
CACCTG	57	cacctg; gtggac; caggtg; gtccac
CAAGTG	53	caagtg; gttcac; cacttg; gtgaac
CAAATG	44	caaatg; gtttac; catttg; gtaaac
CAACTG	37	caactg; gttgac; cagttg; gtcaac
CATATG	28	catatg; gtatac
CAGATG	19	cagatg; gtctac; catctg; gtagac
CAGCTG	18	cagctg; gtcgac
CAATTG	16	caattg; gttaac



Chromatin Immunoprecipitation (ChIP) of PIF5-HA after 2 hours in low R/FR. Immunoprecipitated DNA was quantified by Q-PCR using primers in the promoter region containing a G-box or control region (minimum 1 kb 3' or 5' from the peak region). Data are average of technical triplicates of the Q-PCR.

(A / B) Col and/or PIF5-HA lines were grown for 7 days in constant light conditions before being shifted for 2 hours in low R/FR conditions.

(C) PIF5-HA lines were grown for 14 days in constant light conditions before being shifted for 2 hours in low R/FR conditions.

Supplementary material Hornitschek et al.,

Supplementary material and methods

Cloning procedure and generation of transgenic lines

Primers used in this study are listed in supplementary table 6. Fusions to the maltose-binding proteins (MBP) were generated by PCR. *HFR1, PIF4* and *PIF5* CDS were amplified respectively from the plasmids PH49, pCF402 and pCF404 using the primer pairs pPH153/154 (*HFR1*), pPH149/150 (*PIF4*) pPH151/152 (*PIF5*). Fragments were cloned *NotI/XhoI* into pMAL-c2 TEV V5. The promoter region from *PIL1* and *PIL1** were described previously in (Hornitschek et al., 2009). They were inserted into the pCB308 binary vector using *EcoRI* and *BamHI* sites. To generate the *PIF4pro:PIF4-citrine-3HA* (*PIF4-HA*) line, the *PIF4-3HA* CDS was amplified from the plasmid pCF402 (Lorrain *et al.* 2008) with the primers SL131 and SL135 and digested by *NheI* and *XhoI.* The digestion product was introduced into the pCF300 binary vector with the *BamHI-NheI* digested *PIF4pro* previously described to generate pAM02 (*PIF4pro:PIF4-3HA*). The citrine coding region was amplified by PCR using the primers SL136 and SL137, digested with *SalI* and *XhoI* and introduced into the *SalI*-digested pAM02 vector to generate the pSL90 vector (*PIF4pro:PIF4-citrine-3HA*). This construct was introduced in the *pif4-101* mutant background plants by the *Agrobacterium tumefaciens* dipping procedure.

GUS staining and MUG assay

Seedlings were grown for 7 days in constant light (high R/FR) and then either kept in high R/FR or shifted to low R/FR for the indicated times. For the GUS staining, seedlings were incubated at 37°C for 6 hours within a buffer containing 2mM 5-bromo-4-chloro-3-indolyl β-d-glucuronide (X-Gluc, Duchefa Biochimie BV), 2mM ferrocyanide, 2mM potassium ferricyanide and 50mM sodium phosphate. Stained seedlings were washed with 100% ethanol over night and then rinsed with 70% ethanol. Seedlings were observed and photographed with the stereomicroscope Nikon SMZ 1500. Biological triplicates were performed for each treatment of the MUG assay (4-methylumbelliferyl-beta-D-glucuronide). Seedlings were ground in liquid nitrogen, homogenized on ice in a buffer containing 25mM Tris (pH 7.8), 2mM EDTA, 2mM DTT, 10% glycerol, and 1% Triton X-100, and cleared by centrifugation at 12,000g for 5 min. The extract (25µl) was incubated with 500 µl MUG assay buffer (50mM $NaPO_4$ pH7, 1mM MUG, 10mM EDTA, 10mM β-mercaptoethanol, 0.1% sarkosyl, 0.1% Triton X-100) at 37°C for 2 hours. The reaction was stopped by adding 450µl of 0.2M Na_2CO_3.

ChIP sequencing

The ChIP experiment was performed as described in (Hornitschek *et al.* 2009)). The forward and the reverse primer pairs to amplify the peak and the control region are provided in supplemental S6. For the ChIP-Seq experiment 300 mg of seeds were plated on ½ strengh MS. UTH-sequencing of the ChIP samples were performed at the Lausanne Genomics Technologies Facility (GTF) (http://www.unil.ch/cig/page7861_en.html). For ChIP-Seq analysis 145 bp

(PIF5-HA ChIP sample) and 166 bp (input DNA control) fragments were used to generate 37 bp or 40 bp reads, respectively. The software Bowtie version 0.12.7 (bowtie -S -n 3 --best --strata --solexa1.3-quals -a -m 1) (Langmead *et al.* 2009) was used to map sequence reads to the Arabidopsis genome (TAIR8; www.arabidopsis.org). 8.6 million (for the IP sample) and 26.4 million (for the input sample) uniquely mapping reads were selected and sequence read enrichments were identified with Model-based Analysis of ChIP-Seq (MACS) version 1.4.0alpha2 (-p 1e-7 -slocal 500) (Zhang *et al.* 2008). Genome regions identified by MACS were analyzed with Mali Salmon's PeakSplitter software (version 0.1; -v 0.21; -c 20) to determine several peaks per sequence. Sequences covered by peaks were defined as 200 bp centered to the summit positions reported by MACS or PeakSplitter. Putative direct target genes were identified using a perl script of Vivian Praz (University of Lausanne), which compares the center of peaks with gene annotations. Peaks were assigned to genes if they located 3000 bp upstream, 500 bp downstream or within an annotated region. If several genes per peak fulfill the criteria, only the immediate neighboring genes up and downstream were assigned. Subsequent analyses were performed with R (version 2.12.2).

Supplementary Figure S1: PIF5 binds with different frequency to various E-box sequences in vivo. PIF5 binding sites that were assigned to a gene locus were chosen. For each binding site the most central E-box was detected. Sequences with two E-boxes with the same distance to the peak summit were discarded from the analysis. E-box sequences were then counted and sense and antisense sequences as well as their reverse complements were grouped.

Supplementary table 1. List of genes with a PIF5 peak identified by ChIP sequ in the *PIF5-HA* line transfered for 2 hours into low R/FR.

Supplementary table 2. Lists of GO anaylsis. Sheet a : GO analysis of genes set identified by PIF5-HA ChIP-seq; sheet b : GO analysis of genes showing a significant interaction between WT and *pif4pif5* when comparing gene expression in high versus low R/FR; sheet c: GO analysis of list and sublists for genes differentially expressed in high R/FR between WT and *pif4pif5*; sheet d: GO analysis of genes with close binding site for PIF5 and PIF1.

Supplementary table 3. List of genes showing an altered regulation of gene expression in *pif4pif5* when comparing seedlings grown in high versus low R/FR (interaction between the genetic backgrounds and environmental conditions).

Supplementary table 4. List of genes with altered expression in *pif4pif5* compared to wild-type seedlings grown in high R/FR.

Supplementary table 5. Comparison between the genes we identified as requiring PIF4 and PIF5 for normal expression in high R/FR (Figure 5) and the genes whose expression correlates with growth and requires PIF4 and PIF5 identified by (Nozue *et al.* 2011).

Supplementary table 6. List of primers used in this study

Reference

Hornitschek, P., Lorrain, S., Zoete, V., Michielin, O. and Fankhauser, C. (2009) Inhibition of the shade avoidance response by formation of non-DNA binding bHLH heterodimers. *EMBO J*, **28**, 3893-3902.

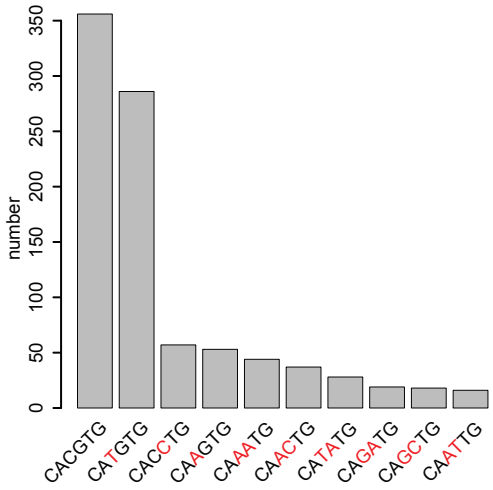
Langmead, B., Trapnell, C., Pop, M. and Salzberg, S.L. (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol*, **10**, R25.

Lorrain, S., Allen, T., Duek, P.D., Whitelam, G.C. and Fankhauser, C. (2008) Phytochrome-mediated inhibition of shade avoidance involves degradation of growth-promoting bHLH transcription factors. *Plant J*, **53**, 312-323.

Nozue, K., Harmer, S.L. and Maloof, J.N. (2011) Genomic analysis of circadian clock-, light-, and growth-correlated genes reveals PHYTOCHROME-INTERACTING FACTOR5 as a modulator of auxin signaling in Arabidopsis. *Plant Physiol*, **156**, 357-372.

Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W. and Liu, X.S. (2008) Model-based analysis of ChIP-Seq (MACS). *Genome Biol*, **9**, R137.

Figure S1



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CAAATG	44	caaatg; gtttac; catttg; gtaaac
CAACTG	37	caactg; gttgac; cagttg; gtcaac
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