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Inhibition of the shade avoidance response by formation of non-DNA binding bHLH heterodimers

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In shade-intolerant plants such as *Arabidopsis*, a reduction in the red/far-red (R/FR) ratio, indicative of competition from other plants, triggers a suite of responses known as the shade avoidance syndrome (SAS). The phytochrome photoreceptors measure the R/FR ratio and control the SAS. The phytochrome-interacting factors 4 and 5 (PIF4 and PIF5) are stabilized in the shade and are required for a full SAS, whereas the related bHLH factor HFR1 (long hypocotyl in FR light) is transcriptionally induced by shade and inhibits this response. Here we show that HFR1 interacts with PIF4 and PIF5 and limits their capacity to induce the expression of shade marker genes and to promote elongation growth. HFR1 directly inhibits these PIFs by forming non-DNA-binding heterodimers with PIF4 and PIF5. Our data indicate that PIF4 and PIF5 promote SAS by directly binding to G-boxes present in the promoter of shade marker genes, but their action is limited later in the shade when HFR1 accumulates and forms non-DNA-binding heterodimers. This negative feedback loop is important to limit the response of plants to shade.

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Introduction

Light is a source of energy for plants, but also is an important source of information about the surrounding environment. As plants are sessile photosynthetic organisms, it is of major importance that they adapt their growth habit to changing light conditions. One well-studied phenomenon is the shade avoidance response. In high vegetative density, the red/far-red (R/FR) ratio decreases because red light is absorbed by photoactive pigments of neighbouring plants, whereas FR light is mainly transmitted and reflected (Ballare, 1999; Vandenbussche *et al*, 2005; Franklin, 2008). This change of light quality is detected by the phytochrome family of R/FR photoreceptors (phyA-phyE in *Arabidopsis*) and leads to the shade avoidance syndrome (SAS; Franklin and Whitelam, 2005). To reach direct sunlight, several morphological changes take place. SAS includes elongation growth of stems and petioles at the expense of development of leaf blades and storage organs. In addition, plants have elevated leaf angles (hyponasty), increased apical dominance leading to reduced lateral branching and accelerated flowering (Ballare, 1999; Franklin and Whitelam, 2005; Vandenbussche *et al*, 2005). Although SAS can negatively impact biomass production and seed yield, it is of major adaptive significance in natural environments (Franklin and Whitelam, 2005; Izaguirre *et al*, 2006; Moreno *et al*, 2009). Moreover, the study of SAS is of direct relevance for agriculture where high-density planting is common practice.

By monitoring the changes in the R/FR ratio, phytochrome photoreceptors function as primary regulators of SAS (Franklin and Whitelam, 2005). In *Arabidopsis*, phyB has a predominant function although phyD and phyE also contribute to this adaptive response (Devlin *et al*, 1998, 1999). A drop in the R/FR ratio leads to rapid changes in the level of numerous transcripts, including several encoding transcription factors (Carabelli *et al*, 1996; Devlin *et al*, 2003; Salter *et al*, 2003; Sessa *et al*, 2005; Sorin *et al*, 2009). Moreover, numerous genes coding for hormone signalling components or metabolic enzymes are rapidly induced by shade (Devlin *et al*, 2003). Several hormones, including auxin, GA, brassinosteroids and ethylene have been functionally linked to shade-regulated growth processes (Morelli and Ruberti, 2002; Tanaka *et al*, 2002; Hisamatsu *et al*, 2005; Vandenbussche *et al*, 2005; Carabelli *et al*, 2007; Kurepin *et al*, 2007a, b; Alabadi and Blazquez, 2009). The hormone-light connection has most extensively been studied for auxin (Morelli and Ruberti, 2002; Tanaka *et al*, 2002; Carabelli *et al*, 2007; Roig-Villanova *et al*, 2007). Both auxin transport and biosynthesis have been shown to be required for an effective SAS (Morelli and Ruberti, 2002; Tanaka *et al*, 2002; Kanyuka *et al*, 2003; Carabelli *et al*, 2007; Tao *et al*, 2008). In particular, a reduction in the R/FR ratio leads to a rapid increase of auxin biosynthesis. This upregulation critically depends on the TAA1 aminotransferase that catalyses the first step in a newly described auxin biosynthetic pathway (Stepanova *et al*, 2008; Tao *et al*, 2008). Although numerous aspects of SAS strictly depend on TAA1, several early shade marker genes are still normally upregulated in the *sav3/taa1* mutant (Tao *et al*, 2008).

PIF4 and PIF5 (phytochrome-interacting factors 4 and 5) represent a direct link between the phytochromes and the regulation of shade marker genes (Lorrain *et al*, 2008). In high R/FR condition, PIF4 and PIF5 are degraded presumably upon interaction with the Pfr conformer of the photoreceptor (Nozue *et al*, 2007; Shen *et al*, 2007; de Lucas *et al*, 2008; Lorrain *et al*, 2008). Following transfer into the shade, the phytochrome photo-equilibrium shifts towards Pr, which has reduced affinity for the PIFs, and thus stabilizes these proteins leading to the expression of shade-induced genes (Lorrain

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et al, 2008). Interestingly, several early shade marker genes are inhibitors of SAS, showing that this system includes a negative feedback loop that prevents an excessive response (Sessa *et al*, 2005; Roig-Villanova *et al*, 2007). For example, *PAR1* and *PAR2* (phytochrome rapidly regulated 1 and 2) coding for small bHLH proteins are involved in the downregulation of genes involved in auxin responses (Roig-Villanova *et al*, 2007). *HFR1* that codes for a bHLH protein related to PIF4 and PIF5 also has an important role in preventing an excessive response to shade (Sessa *et al*, 2005). Although HFR1 belongs to the bHLH family, several sequence features distinguish its basic domain. This leads to the proposal that HFR1 either does not bind to the canonical E-box or does not bind to DNA at all. However, its molecular mode of action remains unknown (Fairchild *et al*, 2000).

In this study, we examined the mode of HFR1 action, focusing on responses to shade. Using a combination of genetic and biochemical experiments, we show that HFR1 prevents an exaggerated shade avoidance response by forming non-DNA-binding heterodimers with PIF4 and PIF5.

Results

Genetic relationship between PIF4, PIF5 and HFR1 during shade avoidance

The bHLH transcription factors, PIF4 and PIF5, are necessary for a complete shade avoidance response, whereas the related bHLH protein HFR1 is involved in a negative mechanism that prevents an excessive shade avoidance response (Sessa *et al*, 2005; Lorrain *et al*, 2008). To gain mechanistic insight into the regulatory network of these bHLH class transcription factors, we first studied the genetic interaction between mutants defective for these proteins. We generated all possible mutant combinations between *hfr1*, *pif4* and *pif5*, and studied their growth under high and low R/FR (to simulate shade) keeping photosynthetically active radiation (PAR) constant. We concentrated our analysis on *hfr1*, *pif4pif5* and the *hfr1pif4pif5* triple mutant, because *pif4pif5* has a more severe phenotype than each single mutant (Lorrain *et al*, 2008). *hfr1pif4* and *hfr1pif5* double mutants essentially showed intermediate phenotypes between the two parental lines (data not shown). All tested genotypes were grown for 4 days in high R/FR condition then either kept in the same condition or transferred to low R/FR condition for additional 4.5 days. The wild type, *hfr1*, *pif4pif5* and *hfr1pif4pif5* responded to low R/FR condition with elongation of the hypocotyls (Figure 1A). As previously reported, the *pif* mutants had the opposite phenotype of *hfr1* in low R/FR shade-mimicking conditions; *pif4pif5* had a shorter hypocotyl and *hfr1* displayed an elongated hypocotyl compared with the wild type. An analysis of the triple mutant showed that the *pif4pif5* was largely epistatic over the *hfr1* mutant phenotype. This experiment confirmed that a reduction in the R/FR ratio still induced elongation of the hypocotyl in *pif4pif5*, indicating that, in addition to PIF4 and PIF5, other pathways also promote SAS (Figure 1; Lorrain *et al*, 2008). The recently discovered TAA1 aminotransferase and members of the homeodomain leucine zipper class II transcription factors are good candidates for this (see Discussion section for details; Tao *et al*, 2008; Sorin *et al*, 2009).

Changes in the R/FR ratio have profound effects on gene expression (Devlin *et al*, 2003; Salter *et al*, 2003; Sessa *et al*, 2005). We analysed the expression of the early shade marker genes *PIL1*, coding for a PIF-related transcription factor (Salter *et al*, 2003), and *XTR7*, coding for a xyloglucan endotransglycosylase-related protein, by quantitative PCR (Q-PCR). We chose *XTR7* because its levels respond rapidly to shade and it encodes a protein that is presumably directly related to the cell elongation process (Devlin *et al*, 2003; Salter *et al*, 2003). Moreover, chromatin immunoprecipitation (ChIP) experiments demonstrated that *PIL1* and *XTR7* are direct targets of PIF4 and PIF5 (de Lucas *et al*, 2008; see

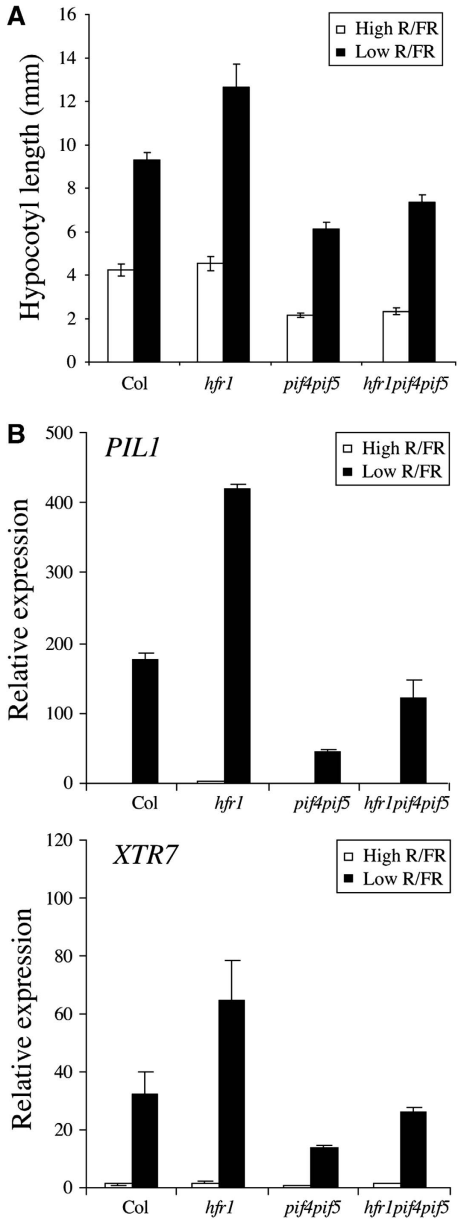

Figure 1 The *pif4pif5* mutations are largely epistatic over *hfr1* in long-term shade conditions. Seedlings were grown for 8.5 days in high R/FR (white bars) or for 4 days in high R/FR followed by 4.5 days in low R/FR (black bars). (**A**) Hypocotyl length measurements, data are represented as the mean, error bars represent $2 \times$ s.e. values, $n = 15$. (**B**) Gene expression of *PIL1* and *XTR7* was determined by Q–PCR analysis. Biological triplicates were performed with technical triplicates for each sample. Values were normalized with *EF1α* and *GAPC-2*. Relative expressions to Col-0 in high R/FR are shown. Error bars represent s.e. values of biological triplicates.

below). Both genes were expressed at low levels in high R/FR condition in all genotypes. In response to low R/FR condition, the expression of *PIL1* and *XTR7* was induced in all tested genotypes. In *pif4pif5* prolonged growth in low R/FR condition led to a reduced induction of their expression, whereas in the *hfr1* mutant this induction was more pronounced than in the wild type (Figure 1B). Interestingly, as for the growth response, the gene expression response of *hfr1pif4pif5* was more similar to that of *pif4pif5* than *hfr1* (Figure 1B). *HFR1* expression is reduced in low R/FR-grown *pif4pif5* (Lorrain *et al*, 2008). In our conditions, *HFR1* was expressed at about 50% of the wild-type levels (Supplementary Figure S1). The reduced expression of *HFR1* in *pif4pif5* can contribute, but not fully explain the epistatic relationship observed here (see Discussion section for details).

Simulated shade leads to very rapid changes in the expression of shade marker genes (Devlin *et al*, 2003; Salter *et al*, 2003; Sessa *et al*, 2005). This response is gated by the circadian clock (Devlin *et al*, 2003; Salter *et al*, 2003). We thus analysed shade-induced changes in gene expression in seedlings that were synchronized by growth in a 12:12 day-night cycle. We followed the expression of *PIL1* and *XTR7* directly upon transfer from high to low R/FR conditions. Both *PIL1* and *XTR7* were rapidly induced in the wild type and *hfr1* mutant. In the *hfr1* mutant, the induction was more pronounced but the most striking feature was the previously reported reduced downregulation of expression of these genes after prolonged exposure to low R/FR condition (Figure 2; Sessa *et al*, 2005). The effect of HFR1 on shade-regulated gene expression is not as pronounced as what was reported previously (Sessa *et al*, 2005). This is most probably because of the different protocols used to study shade avoidance. In our study, we maintained constant PAR and only changed the R/FR ratio, whereas in a previous study, simulated shade conditions were obtained with a combination of red, blue and FR LED lights, which led both to changes in PAR and R/FR ratio (Sessa *et al*, 2005). A direct comparison of the two protocols showed that they induce a different SAS for gene expression and hypocotyl elongation (data not shown). Consistent with our previous observations, the expression of *PIL1* and *XTR7* was markedly reduced in low R/FR-grown *pif4pif5* double mutants (Figure 2; Lorrain *et al*, 2008). Importantly, the *hfr1pif4pif5* triple mutant had essentially the same phenotype as *pif4pif5* (Figure 2). Interestingly, the shade-induced expression of *IAA19* and *IAA29*, which depends on the TAA1 pathway, is still robustly induced in *pif4pif5* (Supplementary Figure S2). However, the level of these genes was reduced in high R/FR-grown *pif4pif5* and *hfr1pif4pif5* (Supplementary Figure S2). Finally, same effects on shade-induced gene expression were observed when these genotypes were grown in continuous light before a change in light quality (Supplementary Figure S3). These genetic data led us to hypothesize that HFR1 may inhibit PIF4 and PIF5 during the response to shade and thus limit the shade avoidance response particularly after a prolonged exposure to low R/FR.

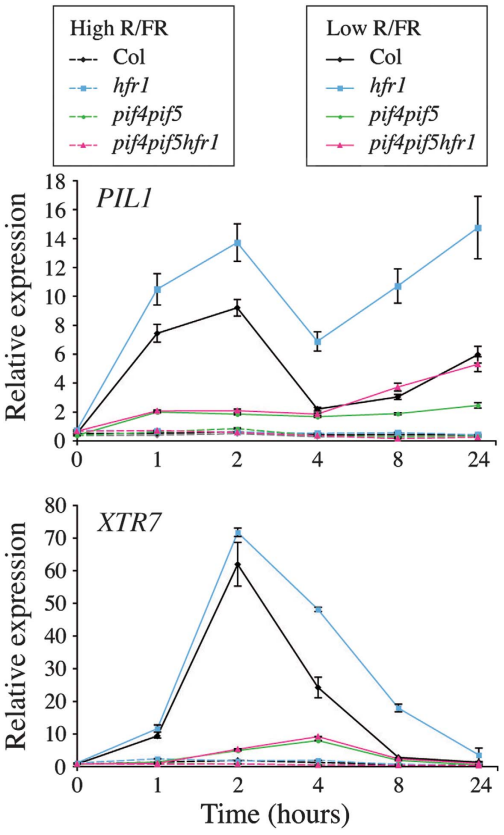

Figure 2 The *pif4pif5* mutations are epistatic over *hfr1* in early responses to shade. Seedlings were grown for 6 days in high R/FR (12 h light–12 h dark) and then either kept in high R/FR ratios or shifted to low R/FR ratios. The expression of *PIL1* and *XTR7* was analysed by Q–PCR. Three technical replicas were performed for each sample. Values were normalized with *EF1α* and *GAPC-2*. Relative expressions to Col-0 (point 0) are shown. Error bars represent s.e. values of technical triplicates.

HFR1 interacts with PIF4 and PIF5

bHLH class transcription factors function as homo- and/or heterodimers. Moreover, it has been reported previously that HFR1 interacts with PIF3 *in vitro* and in the yeast two-hybrid assay (Fairchild *et al*, 2000). We thus tested whether HFR1 interacted with PIF4 and PIF5 by co-immunoprecipitation of *in vitro*-transcribed and -translated proteins. As a control for specificity, we included a modified version of HFR1 (HFR1*), which contains a substitution of two conserved residues in the HLH domain (Val 172 Leu 173 to Asp 172 Glu 173) (Supplementary Figure S4A). On the basis of a previous study, these substitutions are expected to interfere with the dimerization properties of the HLH domain (Voronova and Baltimore, 1990). Homology modelling of the wild type and mutant versions of HFR1 supported this prediction (data not shown). Co-immunoprecipitation showed that HFR1 interacted with PIF4 and PIF5, whereas no specific binding of HFR1* to PIF4 or PIF5 was detected (Figure 3A and B). These data show that HFR1 specifically interacted with PIF4 and PIF5, and that this interaction critically depended on two residues in the HLH domain (Figure 3A and B).

To confirm this interaction in plant cells, we used the Bimolecular Fluorescence Complementation (BIFC) assay in transiently transformed onion epidermal cells. The N- and C-terminal halves of YFP were fused to the C-terminus of PIF4, PIF5, HFR1 and HFR1*. As a transformation control, these cells were co-transformed with a soluble DsRed construct, and DsRed-positive cells were monitored for YFP fluorescence. HFR1 interacted with PIF4 and PIF5 in this assay and

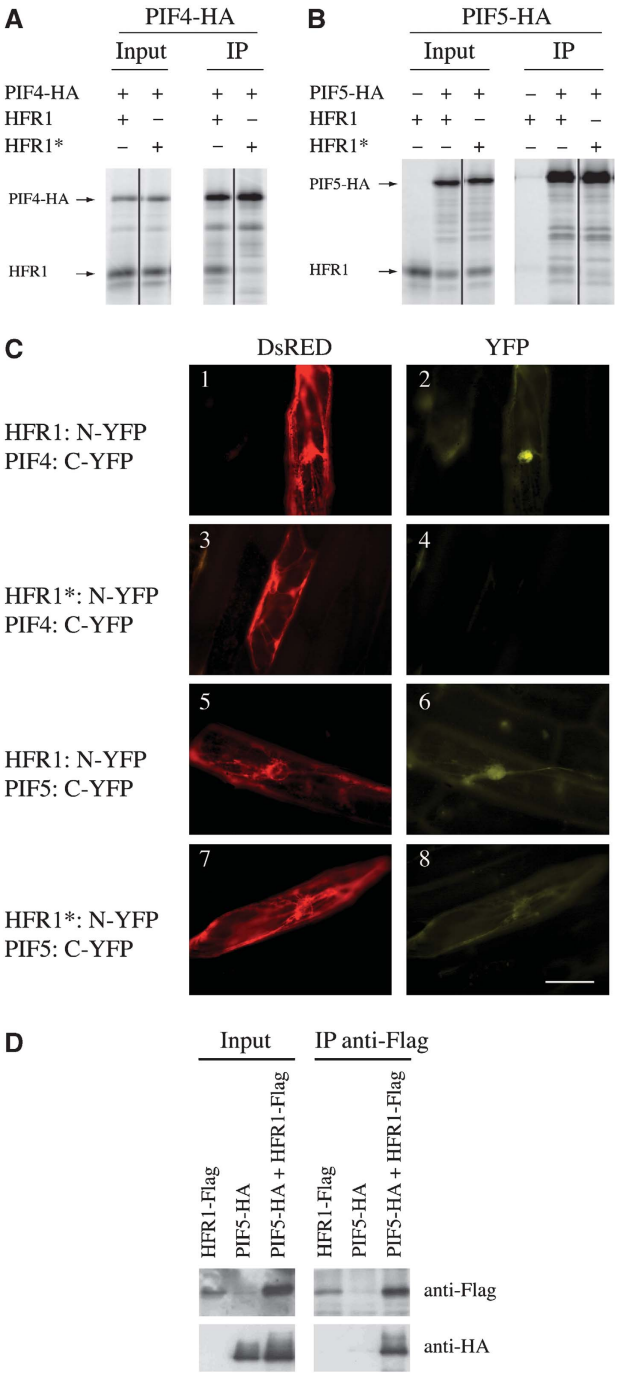

Figure 3 HFR1 interacts with PIF4 and PIF5. (**A**, **B**) Co-immunoprecipitation of *in vitro*-transcribed and -translated proteins (^{35}S-Met labelled). The HA tag was used for immunoprecipitation of PIF4 (A) or PIF5 (B) using HA-antibodies coupled to agarose beads. Proteins were separated by SDS–PAGE and visualized by autoradiography (immunoprecipitation,IP). The lanes come from the same gel and intervening lanes have been removed (indicated by a dividing line) (**C**) Bimolecular fluorescence complementation (BiFC) with HFR1/HFR1* with PIF4 or PIF5 in plant cells. Onion cells were co-bombarded with N– and C–YFP fusion proteins. 1/3/5/7 dsRED signal of transfected cells; 2/4/6/8 YFP channel; Scale bar = 100 μm. (**D**) Co-immunoprecipitation of HFR1–Flag and PIF5–HA. 35S::HFR1–3 × Flag (HFR1–Flag), 35S::PIF5–3 × HA (PIF5–HA) and seedlings expressing both transgenes (HFR1–Flag and PIF5–HA) were grown for 3 days in the dark. After 2 h 30 min in low R/FR condition, proteins were extracted and co-immunoprecipitated using anti-Flag antibodies. Proteins were separated by SDS–PAGE, western blotted and detected using antibodies raised against HA and Flag.

as expected the YFP fluorescence was detected in the nuclei of transformed cells (Figure 3C). In contrast, cells transformed with HFR1* and either PIF4 or PIF5 were not YFP positive, again indicating that two conserved residues of the HFR1 HLH domain are important for dimerization (Figure 3C). Finally, co-immunoprecipitation using double transgenic lines carrying PIF5-HA and HFR1-Flag showed the interaction of HFR1 with PIF5 in *Arabidopsis* plants (Figure 3D).

HFR1 inhibits PIF5-mediated expression of PIL1

The facts that HFR1 and PIF4/PIF5 had an opposite effect on the expression of shade marker genes and that these proteins dimerized raised the possibility that HFR1 may inhibit PIF-mediated gene expression by forming heterodimers. To test this possibility, we used a transient expression system with *Arabidopsis* cell cultures. We used 2 kb of the *PIL1* promoter containing three G-boxes fused the glucuronidase gene (*GUS*) as a reporter (Figure 4A). Effector constructs for PIF5, HFR1 and HFR1* were expressed under the control of the 2 × *CaMV 35S* promoter and co-bombarded with the reporter construct and a transformation reference plasmid (2 × *CaMV 35S* promoter:*LUC*) (Figure 4A). Transformation with PIF5 resulted in a strong stimulation of the *PIL1* reporter activity, which depended on the presence of the G-boxes in the promoter sequence (Figure 4B). This result is consistent with our genetic data indicating that PIF5 is a positive regulator of *PIL1* expression (Figures 1 and 2; Lorrain *et al*, 2008). Transformation with HFR1 or HFR1* alone had a minor effect on reporter expression (Figure 4C). The co-expression of PIF5 and HFR1 limited PIF5-mediated *PIL1* expression. Importantly, co-transformation with HFR1* did not affect the transactivation activity of PIF5 (Figure 4C), strongly suggesting that HFR1 inhibits PIF5-mediated transcription by forming heterodimers. In agreement with this finding, transgenic lines carrying HFR1* under the control of the 35S promoter did not complement the *hfr1* phenotype, whereas wild-type HFR1 slightly overcomplemented the *hfr1* phenotype (Supplementary Figure S4). These data confirm the functional importance of the HFR1 dimerization capacity.

HFR1 prevents PIF4 and PIF5 from binding a G-box sequence

Several possibilities could explain how HFR1 inhibits PIF-mediated expression of shade marker genes. HFR1 PIF heterodimers may be unable to bind DNA and/or such dimers could have reduced transactivation activity. Given that the basic domain of HFR1 is unusual and has been suggested to be incompatible with binding to a G-box (Fairchild *et al*, 2000), we first tested whether HFR1 PIF dimers are capable of binding to a piece of the *PIL1* promoter containing a G-box using homology modelling. Our analysis predicted binding of the PIF5 homodimer to the CACGTG G-box present in the *PIL1* promoter. The basic region of PIF5 made direct contact with the major groove of the DNA molecule at the level of the G-box centre (Supplementary Figure S5). Several important interactions occurred between the PIF5 protein and the *PIL1* promoter. Glu 266 made hydrogen bonds to the adenine ring that faces the T base of the CACGTG G-box, whereas Arg 270 interacted with the backbone and the guanine ring of the first G of the G-box. In addition, Arg 267 and Arg 269 made ionic interactions with the backbone of both the central CG bases

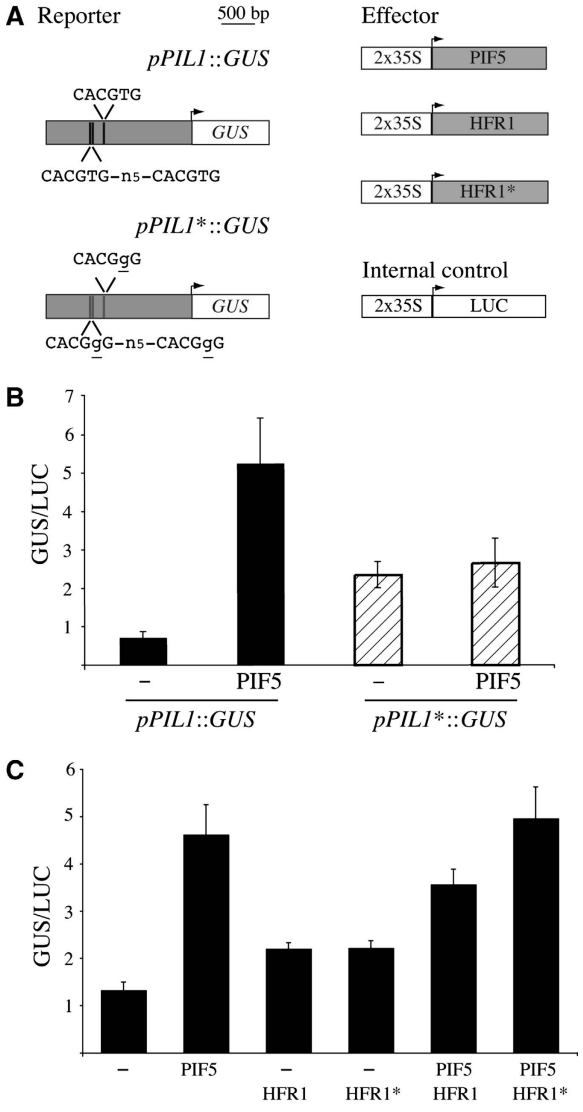

Figure 4 HFR1 inhibits PIF5 transactivation activity in *Arabidopsis* cells. (**A**) Schematic presentation of the constructs including the positions of the 3 G-boxes present in the *PIL1* promoter. (**B**) *Arabidopsis* cells were co-bombarded with the *pPIL1*::*GUS* or *pPIL1**::*GUS* (*PIL1* promoter in which the 3 G-boxes are mutated) and either a vector control or PIF5. The transactivation activity of the effectors is given with the GUS values normalized to luciferase activity (the internal transfection control). Values are represented as mean of three different transfections ± s.e. (**C**) *Arabidopsis* cells were co-bombarded with the *pPIL1*::*GUS* construct and combinations of the different effector constructs as indicated in the figure. The transactivation activity is calculated as in (B).

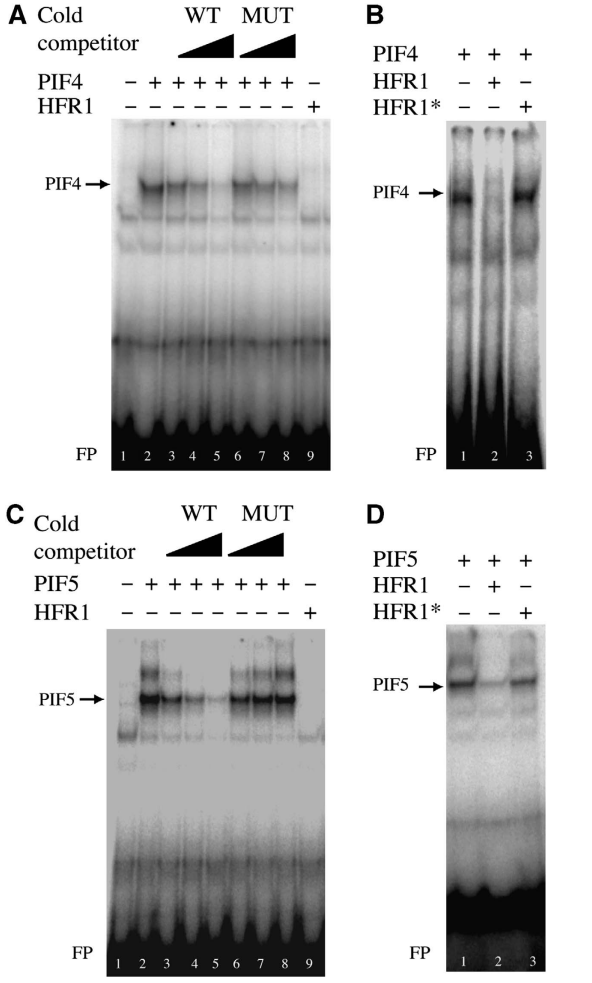

Figure 5 HFR1 prevents PIF4 and PIF5 from binding to the G-box DNA sequence. Electromobility shift assays (EMSA) in (**A**–**D**) were performed using *in vitro*-transcribed and -translated proteins, and a ^{32}P-radiolabelled DNA probe of the *PIL1* promoter sequence containing a double G-box. (A and C) The DNA probe (lane 1–9) was incubated with TNT master mix (lane 1) or PIF4 (A)/PIF5 (C) with increasing amounts of unlabelled probe (lane 3–5) or mutated unlabelled probe (lane 6–8). Lane 9 contains HFR1. (B, D) Lane 1: PIF4 or PIF5 alone; Lane 2: PIF4 or PIF5 with HFR1; Lane 3: PIF4 or PIF5 with HFR1*. The arrow indicates the specific PIF–DNA complex. FP, free probe.

of the G-box, and the PIF5 Glu 266 side chain and the backbone of the DNA strand facing the CACGTG G-box, respectively. A structural model of the HFR1 DNA complex suggested that compared with PIF5 DNA, several key protein DNA interactions were either lost or were unfavorable in HFR1 DNA. In the HFR1 homodimer or HFR1 PIF5 heterodimer, residues Glu 266 and Arg 270 in PIF5 are replaced by Arg 143 and Asp 147 in HFR1, respectively (Supplementary Figure S5). These drastic modifications inverse the charges of corresponding residues and strongly diminish the possibility of interaction taking place between the protein and the G-box. In the model structures of the HFR1 homo- and heterodimer complexes with DNA, Asp 147 did not make any contact with the promoter, whereas Arg 143 made interactions with the backbone and the guanine ring of the first G base of the G-box. This modified scheme of interactions between PIF5 DNA and HFR1 DNA suggested that the HFR1 PIF5 heterodimer does not form a stable interaction with the G-box. Identical conclusions were reached by analysing PIF4 PIF4 homodimers and PIF4 HFR1 heterodimers (data not shown).

To test these predictions biochemically, we performed Electrophoretic Mobility Shift Assays (EMSA) with a fragment of the *PIL1* promoter containing the two closely spaced G-boxes (Figure 4A) and *in vitro*-transcribed and -translated HFR1, HFR1*, PIF4 and PIF5. PIF4 specifically bound to the G-box in the *PIL1* promoter, as demonstrated with competition experiments using wild-type and G-box mutant probes (Figure 5A; Huq and Quail, 2002). Similar data were obtained

for PIF5 except that two complexes of different sizes could be detected raising the possibility that PIF5 could simultaneously bind to both G-boxes in the DNA probe (Figure 5C). Finally, confirming our *in silico* predictions, HFR1 was not able to bind to the *PIL1* promoter (Figure 5A and C; lane 9). To test whether HFR1 could interfere with PIF4 and PIF5 DNA binding, HFR1 and either PIF4 or PIF5 were co-produced by *in vitro* transcription/translation reactions and used for EMSA assays. These experiments showed that HFR1 inhibited the capacity of PIF4 and PIF5 to bind DNA (Figure 5B and D). Importantly, when PIF4 or PIF5 were co-produced with HFR1*, the non-heterodimerizing HFR1 variant did not interfere with PIF DNA binding (Figure 5B and D). Equal protein production of the different bHLH proteins was verified by labelling the *in vitro* transcription translation reactions with ^{35}S Met (Supplementary Figure S6). Our biochemical experiments thus confirmed that HFR1 inhibits PIF4 and PIF5 from binding to the G-boxes in the *PIL1* promoter by forming non-DNA-binding heterodimers with these transcription factors. Importantly, these G-boxes are required for PIF5-mediated *pPIL1*::*GUS* expression (Figure 4B).

PIF5 directly binds to the G-box of shade marker genes in vivo

Our data suggest that PIF4 and PIF5 regulate shade marker gene expression by directly binding to G-boxes present in those promoters (Figure 4). PIF4 has been shown to bind to the promoter of *PIL1* and *XTR7* (de Lucas *et al*, 2008). We analysed the binding of PIF5 to promoters of *HFR1*, *XTR7* and *PIL1* by ChIP using plants constitutively expressing PIF5-HA (Lorrain *et al*, 2008). For controls, we used wild-type Col plants and HFR1-HA-expressing plants. ChIP performed with an anti-HA epitope antibody was followed by Q-PCR to compare the binding to a part of the promoter containing a G-box with a part of the same gene devoid of a G-box. We observed significant binding of PIF5-HA, but not HFR1-HA (up to 1% of the input DNA on the *HFR1* promoter) specifically to the G-box-containing fragment of *HFR1*, *PIL1* and *XTR7* (Figure 6). The fraction of DNA co-immunoprecipitated with PIF5-HA was consistently higher for *HFR1* than *XTR7* (Figure 6; data not shown). However, in all three genes tested, the difference between PIF5-HA and HFR1-HA was very large (Figure 6). Consistent with our *in vitro* experiments, these data indicate that PIF5-HA, but not HFR1-HA, directly bound to the G-box present in the promoter regions of *HFR1*, *PIL1* and *XTR7* (Figures 5 and 6).

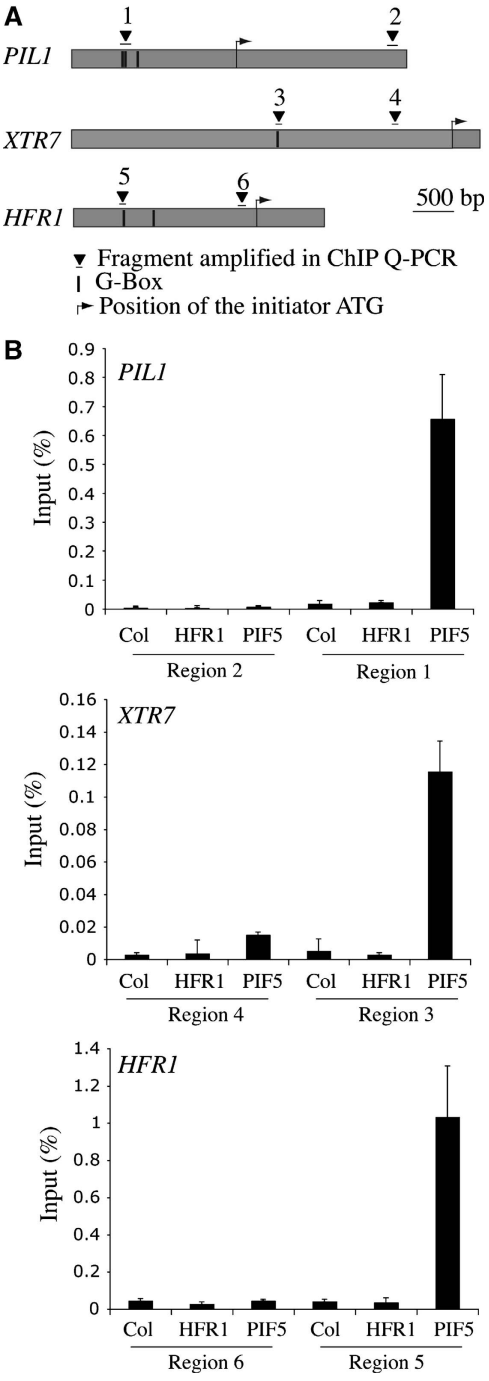

Figure 6 PIF5–HA, but not HFR1–HA, binds to the promoter of shade-induced genes *in vivo*. Chromatin immunoprecipitation (ChIP) from 12-day-old Col, 35S::HFR1–3 × HA (HFR1) and 35S::PIF5–3 × HA (PIF5) seedlings. (**A**) Schematic representation of the *PIL1*, *XTR7* and *HFR1* genes, including the regions amplified after ChIP and the position of G-boxes. (**B**) Immunoprecipitated DNA was quantified by Q–PCR using primers in the promoter region containing G-boxes (region 1, 3 and 5) or control regions without G-boxes (region 2, 4 and 6). Data are average of technical triplicates of the Q–PCR (values ± s.d.). Data from one representative ChIP experiment are shown.

Discussion

For shade-intolerant plants such as *Arabidopsis*, a drop in the R/FR ratio signals the presence of competitors that absorb red and blue light with their photosynthetic pigments. In response to this signal, shaded plants adapt their morphology to reach direct sunlight. However, SAS includes a negative feedback loop (consisting of HFR1, PAR1 and PAR2) to prevent an exaggerated growth response (Sessa *et al*, 2005; Roig-Villanova *et al*, 2007). The mechanism of action of these three bHLH transcription factors was unknown. Our data provide a mechanistic understanding of the network of positively and negatively acting bHLH transcription factors involved in the response of plants to a signal from neighbours indicative of competition for light. Depending on the light conditions, phytochromes use two distinct mechanisms to control PIF activity. In conditions typical of sunlight, PIF4 and PIF5 are rapidly degraded, whereas in conditions typical of shade PIF4 and PIF5 remain stable but the HFR1 inhibitor is

induced in a phytochrome-regulated manner (Fairchild *et al*, 2000; Duek and Fankhauser, 2003; Sessa *et al*, 2005; Nozue *et al*, 2007; Lorrain *et al*, 2008). This second mechanism is much slower than phytochrome-induced degradation (and potentially reversible), which thus leads to distinct windows of opportunity for PIF activity depending on the light condition.

The positive regulators of shade-induced growth, PIF4 and PIF5, are rapidly stabilized in response to a reduction of the R/FR ratio (Lorrain *et al*, 2008). This contributes to the rapid induction of shade marker genes and elongation growth responses (Figures 1 and 2; Lorrain *et al*, 2008). Expression of these marker genes presumably depends directly on the binding of PIF4 and PIF5 to G-boxes present in their promoters (Figures 4 and 6; de Lucas *et al*, 2008). Here we show that HFR1 can dimerize with these PIFs and that these heterodimers are unable to bind to G-boxes present in the *PIL1* promoter (Figures 3 and 5). Consistent with this data, co-expression of HFR1 and PIF5 in *Arabidopsis* cells inhibits PIF5-mediated expression of *pPIL1*::*GUS* (Figure 4). bHLH class transcription factors are known to dimerize through their HLH domain (Voronova and Baltimore, 1990). We demonstrate the functional importance of HFR1's HLH domain in several ways. First, a substitution of two amino acids in the HLH domain, which was shown to prevent dimerization of other HLH proteins (Voronova and Baltimore, 1990), also prevented HFR1 from binding to PIF4 and PIF5 (Figure 3). Importantly, this variant of HFR1 (HFR1*) was unable to prevent PIF4 and PIF5 from binding to DNA *in vitro* and PIF5 from promoting the expression from the *PIL1* promoter in *Arabidopsis* cells (Figures 4 and 5). Finally, HFR1* was inactive *in vivo* as it could not complement the *hfr1* phenotype (Supplementary Figure S4). Taken together our results strongly support a model in which HFR1 inhibits the shade avoidance response by forming non-DNA-binding heterodimers with PIF4 and PIF5. This model predicts that HFR1 acts through PIF4 and PIF5, and thus that *pif4pif5* should be epistatic over *hfr1*, which is largely consistent with our genetic analysis (Figures 1 and 2). This is particularly clear for the rapid light effects on gene expression (Figure 2 and Supplementary Figure S3), whereas after a prolonged treatment in the shade, *pif4pif5* is not fully epistatic over *hfr1* (Figure 1). One possible interpretation of this result is that HFR1 could also inactivate additional PIF proteins, such as PIF3, which was shown to interact with HFR1 *in vitro* (Fairchild *et al*, 2000). An alternative explanation for the genetic interactions reported here would be that in *pif4pif5* mutants *HFR1* is no longer expressed. Consistent with our previous results, HFR1 expression is reduced in the *pif4pif5* double mutant, however, it was still at 50% of the wild-type level in the double mutant (Supplementary Figure S1; Lorrain *et al*, 2008). We thus conclude that it is unlikely that this reduction in *HFR1* expression in *pif4pif5* plants fully explains the genetic interactions observed by us.

Interestingly, *HFR1* is also induced in a PIF-dependent manner when plants perceive low R/FR and PIF5 binds directly to the *HFR1* promoter (Figure 6 and Supplementary Figure S1; Lorrain *et al*, 2008). Thus a negative regulator of the shade avoidance response is an early responsive gene, which is typical for negative feedback loops. The pattern of *HFR1* expression may, at least in part, explain the transient upregulation of many shade marker genes. In the early phase of the response to shade, the response is dominated by the stabilization of PIF4 and PIF5, whereas at later stages the increased expression of *HFR1* limits their activity. This model is fully consistent with the greater influence of HFR1 on the later stages of low R/FR-regulated gene expression (Figure 2; Sessa *et al*, 2005). It should, however, also be noted that the transient upregulation of shade maker genes is also partly due to gating of the shade avoidance response by the circadian clock (Salter *et al*, 2003). Interestingly, *PIF4* and *PIF5* expressions are under circadian regulation, which may directly contribute to gating of SAS (Nozue *et al*, 2007).

Although our model is fully consistent with our results, the phenotype of the *pif4pif5* double mutant also shows that other important mechanism contribute to shade-induced growth (Figures 1, 2 and Supplementary Figure S3; Lorrain *et al*, 2008). Indeed, the *pif4pif5* double mutant still displays a robust induction of hypocotyl growth in response to a reduction in the R/FR ratio (Figure 1; Lorrain *et al*, 2008). We thus propose that in response to a drop in the R/FR ratio, multiple mechanisms are coordinately implemented to ensure a robust response. The rapid increase in TAA1-mediated auxin biosynthesis is certainly one of them (Tao *et al*, 2008). Interestingly, in the *sav3/taa1* mutant, several early shade marker genes, including *HFR1*, *ATHB2* and *RIP* are still normally induced, while the expression of these genes strongly depends on PIF4 and PIF5 (Figures 1 and 2; data not shown; Lorrain *et al*, 2008; Tao *et al*, 2008) In contrast, the induction by shade of several auxin-regulated transcripts that depend on the TAA1 pathway is only marginally affected in *pif4pif5* (Supplementary Figure S2). These results suggest that at least two pathways can be activated independently. Although the PIF4, PIF5 and HFR1 network that we describe largely explains the transcriptional regulation of shade-regulated genes, the mechanism by which TAA1 is activated by shade is currently unknown, but *TAA1* transcript levels do not increase in response to a drop in the R/FR ratio (Tao *et al*, 2008). Interestingly, both SAV3/TAA1 and PIF4 are not only required to promote growth in response to shade, but also in response to elevated temperatures (Tao *et al*, 2008; Koini *et al*, 2009).

The mechanism of HFR1 action that we describe here is comparable with the one that was recently described for the DELLA proteins that also inhibit PIF proteins through heterodimerization (Alabadi *et al*, 2008; Feng *et al*, 2008; de Lucas *et al*, 2008). Interestingly, DELLA proteins have also been implicated in the response of plants to shade (Djakovic-Petrovic *et al*, 2007). However, the interplay between DELLA and PIF proteins during shade avoidance is currently unknown. In both cases, the HLH domain of PIFs has been implicated as the site of dimerization, suggesting that depending on the conditions either HFR1 or the DELLA proteins will predominantly downregulate PIF activity. Our genetic data indicate that during the response to a drop in the R/FR ratio, HFR1 has a predominant role in preventing excessive PIF activity. Moreover, we have recently shown that PIF4 and PIF5 are also required during the de-etiolation phase of seedlings grown under continuous FR light (the FR-HIR) (Lorrain *et al*, 2009). The genetic interaction between *hfr1* and *pif4pif5* indicates that under these conditions as well HFR1 functions by inhibiting PIF4 and PIF5, because *pif4pif5* is fully epistatic over *hfr1* (Lorrain *et al*, 2009). The strong expression of *HFR1* during the FR-HIR and during shade

avoidance is consistent with a predominant function of HFR1 under these conditions, whereas in high R/FR *HFR1* expression is low and *hfr1* mutants have no obvious phenotype (Figure 1; Fairchild *et al*, 2000; Duek and Fankhauser, 2003; Sessa *et al*, 2005). The DELLA proteins may primarily inhibit PIF proteins under conditions in which HFR1 levels are low, such as in darkness and in high R/FR light. This hypothesis is consistent with the reduced growth of the hypocotyls in etiolated seedlings with a reduced GA content (Alabadi *et al*, 2008). Low GA stabilizes the DELLAs that could then inhibit PIF activity, which is required for normal etiolated development (Leivar *et al*, 2008; Shin *et al*, 2009; Stephenson *et al*, 2009).

bHLH proteins are capable of interacting with transcription factors from other families. In plants, this has been particularly well documented during the control of trichrome development in which bHLH and MYB class transcription factors form a regulatory complex involved in cell-fate determination (Zhao *et al*, 2008). Interestingly, HFR1 has recently been shown to interact with the R2R3MYB factor LAF1 (Jang *et al*, 2007). The heterodimerization of these two transcription factors leads to mutual stabilization of the two proteins. Surprisingly, however, genetic analysis suggests that LAF1 and HFR1 act largely independently during the FR-HIR (Jang *et al*, 2007). Moreover, it is currently unknown whether this protein interaction has any effect on the DNA-binding capacity of these transcription factors. Mechanistically more related to the PIF HFR1 regulatory network described here is the finding that *Arabidopsis* bHLH048 can inhibit DNA binding of an unrelated class of transcription factor. However, biological consequences of this interaction remain unknown (Husbands *et al*, 2007).

Previous studies in animals identified HLH proteins, such as ID (Inhibitor of DNA binding), which on dimerization with bHLH proteins lead to the formation of non-DNA-binding heterodimers (Norton, 2000). In contrast to ID proteins, HFR1 possesses a basic domain just N-terminal of the HLH domain but their mode of action seems to be analogous. Interestingly, ID proteins have recently been implicated in circadian processes in mice potentially acting though the bHLH proteins BMAL1 and CLOCK (Duffield *et al*, 2009). Given that HFR1 inhibits PIF4 and PIF5, which are also required for the circadian-regulated plant growth, there might be a related regulatory network of HLH proteins controlling circadian responses in plants and animals (Nozue *et al*, 2007; Duffield *et al*, 2009; Niwa *et al*, 2009). Small HLH proteins, PAR1 and PAR2, are negative regulators of the shade avoidance response, which may also act by preventing other bHLH proteins from binding to DNA (Roig-Villanova *et al*, 2007). Similarly, the regulator of hypocotyl growth, KIDARI, has also been proposed to act like ID proteins (Hyun and Lee, 2006). HFR1 and KIDARI regulate hypocotyl elongation in opposite ways and both proteins interact *in vitro*, raising the possibility that by sequestering HFR1, KIDARI may promote PIF-mediated growth. However, to the best of our knowledge HFR1 is the first plant bHLH protein for which there is a direct demonstration that it acts by inhibiting DNA binding of other bHLH proteins (PIF4 and PIF5). Future study will determine whether HFR1 can also interfere with other members of the PIF family by heterodimerization. The finding that a stabilized version of HFR1 leads to a constitutively photomorphogenic phenotype similar to the one reported for *pif1pif3pif4pif5* quadruple mutants is certainly consistent with this idea (Yang *et al*, 2003; Leivar *et al*, 2008; Shin *et al*, 2009; Stephenson *et al*, 2009).

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana*, ecotype Columbia, were surface sterilized and either plated on 1/2 strength MS, 0.7% phytagar (Murashige and Skoog medium, GIBCO 23118-037) or directly onto soil. After 3 days of growth at 4°C, plants were grown at 22°C in a Percival Scientific Model I-66L with or without supplementary FR ($\lambda_{max} = 739$ nm; Quantum Device, USA) diodes. Fluence rates were determined using an International light IL1400A photometer equipped with an SEL033 probe with appropriate light filters. The ratios of R/FR were: high = 17, low = 0.25. PAR was constant at 60 μmol m^{-2} s^{-1}. The double mutant *pif4pif5* has been described previously by Lorrain *et al* (2008). The triple mutant was obtained by crossing the *pif4pif5* double mutant with *hfr1-101* and genotyping was performed as described previously (Duek *et al*, 2004; Lorrain *et al*, 2008). Hypocotyl length measurement was achieved using ImageJ software.

To generate plants expressing tagged versions of PIF5 and HFR1, we transformed PIF5-HA-expressing plants (Lorrain *et al*, 2008) with a construct coding for HFR1 with a triple Flag tag under the control of the cauliflower mosaic virus promoter 35S. A Flag-tagged HFR1 was generated by PCR using the primers pSP05 and pPH24 with the full-length HFR1 cDNA as a template. The PCR product was digested with *Kpn*I and *Sac*I, and introduced into pSL35 (pBSIISK+ (Invitrogen) containing a triple Flag tag) to generate pSL30. HFR1-3 × Flag was then sub-cloned into the pCHF6 binary vector to generate pSL33. This construct was transformed into PIF5-3 × HA-overexpressing *Arabidopsis* plants by the *Agrobacterium* dipping method. Transformants with a 3:1 segregation ratio were self-fertilized, and homogenous progeny were selected. Primer sequences are given in Supplementary Table I.

Analysis of gene expression

RNA extraction was performed using the kit Nucleo Spin for plant RNA from Machery-Nagel and reverse-transcribed using the Super-Script II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Q-PCR was performed with the Power SYBR Green PCR master mix from Applied Biosystems using the ABI Prism 7900HT Sequence Detection Systems according to the manufacturer's instructions. For the relative quantification of the genes, qBase, software for management and automated analysis of real-time Q-PCR data was used (http://medgen.ugent.be/qbase). Each reaction was performed in triplicate using a primer concentration of 300 nM. Q-PCR were performed using the primer pairs pPH49/pPH50 (*PIL1*: At2G46970); Mt121/Mt122 (*XTR7*: At4G14130); SL44/SL45 (*HFR1*: At1G02340); Mt123/Mt124 (*IAA19*: At3G15540); Mt157/Mt158 (*IAA29*: At4G32280), F_*EF1α*/R_*EF1α* (*EF1α*: At5G60390) and F_*GAPC-2*/R_*GAPC-2* (*GAPC-2*: A1G13440). Primer sequences are given in Supplementary Table I.

In vitro co-immunoprecipitation

Proteins were synthesized in the reticulocyte TNT *in vitro* transcription-translation system (Promega) and labelled with ^{35}S-methionine according to the manufacturer's instructions. The full-length *HFR1* cDNA was cloned with *Bam*HI linkers into pCMX-PL1. HFR1* was generated by site-directed mutagenesis using the primers, pPH20 and pPH21, using the QuikChange Site-Directed Mutagenesis Kit from Stratagene. PIF4-3 × HA was inserted using *Kpn*I and *Nhe*I into PCMX-PL1 and PIF5-3 × HA was inserted using *Kpn*I and *Nhe*I into PCMX-PL2. All constructs were verified by sequencing. Proteins were incubated with HA-antibodies coupled to agarose beads (anti-HA Affinity Matrix; Roche) in binding buffer (25 mM HEPES (pH 7.5), 100 mM KCl, 0.2 mM EDTA, 10% glycerol, 2.5 mM $MgCl_2$, 1 mM DTT, 0.5% NP-40 and Sigma protease inhibitor 10 ul/ml). The beads were washed five times using the binding buffer. Specifically bound proteins were eluted with Laemmli buffer. Immunoprecipitates and input fractions were separated on 10% SDS–PAGE gels and visualized using a phosphorimager.

Bimolecular fluorescence complementation assays

Genes were cloned under the control of the 35S promoter and fused to either the C- or N-terminal part of YFP. HFR1/HFR1* were cloned into the *Xba*I*Xho*I sites of pUC-SPYNE (Walter *et al*, 2004). PIF4 was cloned into *Xba*I*Xho*I sites of pUC-SPYCE and PIF5 was cloned into *Spe*I*Xho*I sites of pUC-SPYCE (Walter *et al*, 2004). The resulting constructs were mixed as indicated (800 ng each) and co-bombarded into onion cells. DNA precipitation and particle bombardment was performed using the Bio-Rad helium-driven particle accelerator (PDS-1000) according to the manufacturer's instructions. Onions were kept in the dark for 16 h at 22°C to allow the expression of the transfected DNA and reconstruction of the functional YFP. All fluorescence microscopy was performed using Leica DM6000B microscope.

In vivo co-immumoprecipitation

A total of 10 mg of seeds were plated in Petri dishes and stored in the dark for 3 days at 4°C. A germinating red light treatment was given at 22°C and the plates were returned to darkness for further 3 days. Plates were then transferred to white light with high R/FR ratio for 1 h and 30 min and then in white light with a low R/FR for additional 2 h and 30 min before protein extraction. Seedlings were ground in cold mortar with protein extraction buffer (50 mM TrisHCl (pH 7.5); 100 mM NaCl; 10% glycerol; 0.1% NP-40; 1 mM DTT; 1 × protease inhibitors (Sigma); 50 μM MG132). Soluble proteins were incubated with 40 μl of EZview red anti FlagM2 affinity gel (Sigma) beads for 1 h and 30 min at 4°C. After four washes in the protein extraction buffer, specifically bound proteins were eluted with Laemmli buffer. HRP-conjugated anti-HA antibodies (Roche) or Anti-Flag M2 antibodies (Sigma) and HRP-conjugated anti mouse antibodies (Promega) were used to detect proteins.

Transactivation assay

The transactivation assays were performed as previously described by de Lucas *et al* (2008). The effector constructs carry PIF5 or HFR1/HFR1* under the control of the $2 \times 35S$ promoter. The reporter construct carries the *GUS* gene driven by 2 kb of the *PIL1* promoter, which was amplified using the primers pPH017 and pPH09. The triple G-box mutant of *pPIL1* (p*PIL1**::GUS) was generated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit from Stratagene. The double G-Box was mutated using the primers pPH45 and pPH46, and the single G-Box using the primers pPH47 and pPH48. A $2 \times 35S$::luciferase construct was used as an internal control. Three independent experiments were carried out with three biological replica plates for each treatment.

Electrophoresis mobility shift assays

Proteins were synthesized using the TNT system (Promega). To produce PIF4 protein, full-length PIF4 cDNA was cloned into pCMX-PL1. Two PIF4 fragments (*Bcl*I/*Nco*I, *Nco*I/*Kpn*I) were inserted via a three-way ligation. Full-length PIF5 cDNA was cloned with *Kpn*I and *Nhe*I into pCMX-PL2. For the DNA probe, single-strand primers were 5′ labelled with radioactive γ-phosphate of ATP (γ^{32}P) using polynucleotide kinase. Forward and reverse primers, FGbox/RGbox or FGbox-Mt/RGbox-Mt, were annealed and purified using the Quick Spin Column (Roche). The binding reactions were performed according to Martinez-Garcia *et al* (2000). The binding complexes (45 000 c.p.m. per reaction) were resolved on a 6% polyacrylamide gel and visualized using a phosphorimager.

Chromatin immunoprecipitation and PCR amplification

Arabidopsis thaliana seeds (Col, 35S::HFR1-3 × HA (CF396) (Duek *et al*, 2004) and 35S::PIF5-3 × HA (Lorrain *et al*, 2008)) were plated on 1/2 strength MS, 0.7% phytagar. After 3 days at 4°C, seedlings were grown in long-day conditions at 22°C. 10-day-old seedlings were shifted for 2 h into low R/FR before fixation. ChIP assays were performed as described previously (Pruneda-Paz *et al*, 2009). Immunoprecipitation was performed with HA-antibodies coupled to agarose beads (Anti-HA Affinity Matrix; Roche) and immunocomplexes were eluted from the beads using elution buffer (0.1 M $NaHCO_3$, 1% SDS). DNA was purified with the GenElute PCR Clean up Kit from Sigma and used for the quantification of immunoprecipitated DNA by Q–PCR. Each Q–PCR reaction was performed in triplicate. The forward and reverse primer pairs used to amplify the region 1–6 are: *PIL1*-region 1 (pPH78-pPH79); *PIL1*-region 2 (pPL8F-pPL8R); *XTR7*-region 3 (pPH120-pPH121); *XTR7*-region 4 (pPH130-pPH131); *HFR1*-region 5 (pPH112-pPH113) and *HFR1*-region 6 (pPH126-pPH127). Primer sequences are given in Supplementary Table I.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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