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HFR1 Sequesters PIF1 to Govern the Transcriptional Network Underlying Light-Initiated Seed Germination in *Arabidopsis*[C][W][OPEN]

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Seed germination is the first step for seed plants to initiate a new life cycle. Light plays a predominant role in promoting seed germination, where the initial phase is mediated by photoreceptor phytochrome B (phyB). Previous studies showed that PHYTOCHROME-INTERACTING FACTOR1 (PIF1) represses seed germination downstream of phyB. Here, we identify a positive regulator of phyB-dependent seed germination, LONG HYPOCOTYL IN FAR-RED1 (HFR1). HFR1 blocks PIF1 transcriptional activity by forming a heterodimer with PIF1 that prevents PIF1 from binding to DNA. Our whole-genomic analysis shows that HFR1 and PIF1 oppositely mediate the light-regulated transcriptome in imbibed seeds. Through the HFR1–PIF1 module, light regulates expression of numerous genes involved in cell wall loosening, cell division, and hormone pathways to initiate seed germination. The functionally antagonistic HFR1–PIF1 pair constructs a fail-safe mechanism for fine-tuning seed germination during low-level illumination, ensuring a rapid response to favorable environmental changes. This study identifies the HFR1–PIF1 pair as a central module directing the whole genomic transcriptional network to rapidly initiate light-induced seed germination.

INTRODUCTION

Seeds play an essential role in the successful colonization of land for angiosperms. Upon maturation, the plant embryo stops growth and seed dormancy is established. Dormant seeds can survive long periods, even in severe environments, and are able to remain viable until the environment becomes favorable for germination. Seed germination is a crucial process in the life cycle of seed plants, because it determines the time when a new life cycle is initiated (Weitbrecht et al., 2011). The *Arabidopsis thaliana* seed is composed of a seed coat (testa), a single endosperm layer, and the embryo (Finch-Savage and Leubner-Metzger, 2006; Weitbrecht et al., 2011). Seed germination involves the sequential phases of testa rupture, endosperm rupture, and embryo radicle protrusion and elongation (Finch-Savage and Leubner-Metzger, 2006; Holdsworth et al., 2008). A wide range of environmental factors affects seed germination, including temperature, moisture, light, and nutrient availability (Finch-Savage and Leubner-Metzger, 2006; Cho et al., 2012).

As an important environmental signal, light regulates diverse developmental processes in plants. Phytochromes (PHYs; including phyA-E) are the red/far-red light receptors (Quail, 2002; Chen et al., 2004). There are two reversible phytochrome conformers, the inactive red light-absorbing Pr form and active far-red light-absorbing Pfr form. The phytochromes are synthesized in the Pr form in darkness. Upon red light irradiation, the Pr form of phytochrome is converted to the Pfr form to initiate red light–regulated plant development (Quail, 2002; Chen et al., 2004). PhyA and phyB are the main receptors of light-induced seed germination and other PHYs (phyC to E) also play minor roles during the process (Lee et al., 2012). PhyA mediates far-red light (FR), whereas PhyB mediates red light (R)–induced seed germination processes. During the initial phase of seed imbibition, the protein level of phyA is very low, therefore, seed germination is dependent on the red light activation of phyB (phyB-dependent germination) (Shinomura et al., 1994; Seo et al., 2009). After prolonged incubation in the dark (e.g., 48 h), phyA accumulates to high levels and mediates canopy light (FR) induced-seed germination, promoting germination to very low fluence response and FR high irradiation response (Shinomura et al., 1996; Kneissl et al., 2009; Seo et al., 2009; Lee et al., 2012). Recent study showed that canopy light suppresses phyB-dependent germination in the endosperm and activates phyA-dependent germination in the embryo, which involves spatial abscisic acid (ABA) signaling responses (Lee et al., 2012). The different roles of phyA and phyB are important for the plant's ability to adapt to different light conditions during germination.

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In the nucleus, there are two groups of functionally opposing transcription regulators, the negative regulators PHYTOCHROME-INTERACTING FACTORs (PIFs) and the positive regulators LONG HYPOCOTYL5, LONG HYPOCOTYL IN FAR-RED1 (HFR1) and LONG AFTER FAR-RED LIGHT1, to mediate red/far-red light-induced transcription regulatory networks (Leivar et al., 2008; Leivar and Quail, 2010). PIFs are basic helix-loop-helix (bHLH) proteins, belonging to the bHLH subfamily 15 of *Arabidopsis* (Leivar and Quail, 2010). PIFs function as repressors of photomorphogenesis and are degraded via the 26S proteasome upon interaction with the Pfr form of phytochromes (Castillon et al., 2007; Leivar and Quail, 2010). Among them, PIF1 (PIL5) was found to repress light-induced seed germination (Oh et al., 2004). In the dark, PIF1 is stabilized and exerts repression on seed germination partially by indirectly inhibiting the gibberellic acid (GA) pathway while activating the ABA pathway (Oh et al., 2006, 2007; Kim et al., 2008). Among the positive regulators of photomorphogenesis, overexpression of HFR1 lacking its N terminus (HFR1-Δ105) exhibited constitutive germination in the dark (Yang et al., 2003), suggesting the possible roles of HFR1 in seed germination. HFR1 also belongs to the bHLH subfamily 15 but is an atypical bHLH transcription regulator and does not directly bind to DNA (Fairchild et al., 2000). The HFR1 protein accumulates in the light, but in the dark, it is targeted by the E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENESIS 1 (Jang et al., 2005; Yang et al., 2005).

Unlike the extensive molecular studies focused on photomorphogenesis, the molecular mechanisms underlying light-regulated seed germination, aside from PIF1-repressed seed germination, is largely unknown. Specifically, three key questions remain unanswered: What are the positive regulators in seed germination, what is the relationship between the positive and negative regulators in regulating seed germination, and what is the central module for directing the light-initiated seed germination transcriptional network? Here, we identified that HFR1 is a positive regulator in phyB-dependent seed germination. Genetic evidence shows that HFR1 functions upstream of PIF1. Furthermore, HFR1 directly interacts with and sequesters PIF1 to prevent PIF1 from binding to its target genes. Transcriptome analysis indicates that the HFR1–PIF1 module mediates the light-regulated seed germination transcriptional network. In summary, the HFR1–PIF1 regulatory module in seeds enacts a fail-safe mechanism that enables a rapid response to low illumination to allow efficient germination.

RESULTS

HFR1 Is a Positive Regulator of phyB-Dependent Seed Germination

To investigate the role of HFR1 in seed germination, we examined the phenotypes of mutant *hfr1-201* (*hfr1*) in phyB-dependent seed germination. In phyB-dependent seed germination assays, the seeds were first exposed to 1 h white light (WL), starting from seed surface sterilization and followed by 5 min of FR irradiation to inactivate phyB. After that, the seeds were illuminated with red light (R) for 5 min to activate phyB and then kept in the dark (R condition). Under this condition, the germination frequencies of wild-type seeds increased gradually to almost 100% when incubated in the dark for 72 h (Figure 1A). However, the germination frequency of *hfr1* mutant seeds was dramatically less and fewer than 10% seeds germinated after 72 h (Figure 1A). After a 5-d incubation in the dark following the R pulse, the wild-type seeds germinated completely but most of the *hfr1* seeds failed to germinate (Figure 1A). Furthermore, overexpressing green fluorescent protein (GFP)-tagged HFR1 in the *hfr1* background (HFR1-GFP/*hfr1*) rescued the seed germination defect of *hfr1* and restored the germination frequency to wild-type levels. These results indicate that HFR1 positively regulates phyB-dependent seed germination.

Besides *hfr1*, we examined two additional independent *hfr1* alleles from the Arabidopsis Biological Resource Center and named *hfr1-1* and *hfr1-2*, respectively (Supplemental Figure 1A online). Both of these two alleles had a lower *HFR1* transcript level and showed longer hypocotyls under continuous FR light (Supplemental Figure 1B and 1C online), consistent with *hfr1-201* and previously reported *hfr1* mutants (Kim et al., 2002; Yang et al., 2005). We then checked the germination phenotypes of *hfr1-1* and *hfr1-2* in a phyB-dependent seed germination assay. The results showed that both *hfr1-1* and *hfr1-2* seeds exhibited defective germination in R condition, similar to *hfr1* (Supplemental Figure 1D online). These results further demonstrate the important roles of HFR1 in phyB-dependent seed germination.

HFR1 Promotes Seed Germination via PIF1

Previous studies showed that PIF1 represses phyB-dependent seed germination because *pif1* mutant seeds constitutively germinated in the dark, and overexpressing PIF1 transgenic plants showed germination defects under R condition (Oh et al., 2004). To gain insight into the relationship between HFR1 and PIF1, we performed a genetic analysis of HFR1 and PIF1. We generated a *pif1 hfr1* double mutant and examined the seed germination phenotypes of all the single and double mutants. Under the R condition, *pif1* germinated slightly earlier than the wild type and *hfr1* showed obvious germination defects as expected (Figure 1B). However, the *pif1 hfr1* double mutant seeds displayed a phenotype similar to *pif1* but not *hfr1* (Figure 1B). Furthermore, overexpression of both PIF1 (PIF1–Myc) and HFR1 (HFR–GFP) exhibited impaired seed germination under R condition, a phenotype also similar to PIF1–Myc (Figure 1C). Moreover, under the dark (D) condition, in which the seeds are irradiated with only FR to inactivate phyB, both *pif1* and *pif1 hfr1* germinated even without the R activation (Figure 1D). These results indicate that PIF1 functions downstream of HFR1 in mediating phyB-dependent seed germination.

Because both PIF1 and HFR1 are core transcription regulators in photomorphogenesis, we asked whether the genetic relationship between PIF1 and HFR1 in photomorphogenesis is the same as in seed germination. Thus, we examined the photomorphogenesis phenotypes of these mutants and found that all of the mutants showed no difference from the wild type in both darkness and in R (Supplemental Figure 2A and 2B online). In FR, *hfr1* exhibited longer hypocotyls than the wild type as previously reported and *pif1* showed no difference from the wild type (Supplemental Figure 2C online). Interestingly, *pif1 hfr1* seedlings displayed long hypocotyls, comparable to *hfr1*

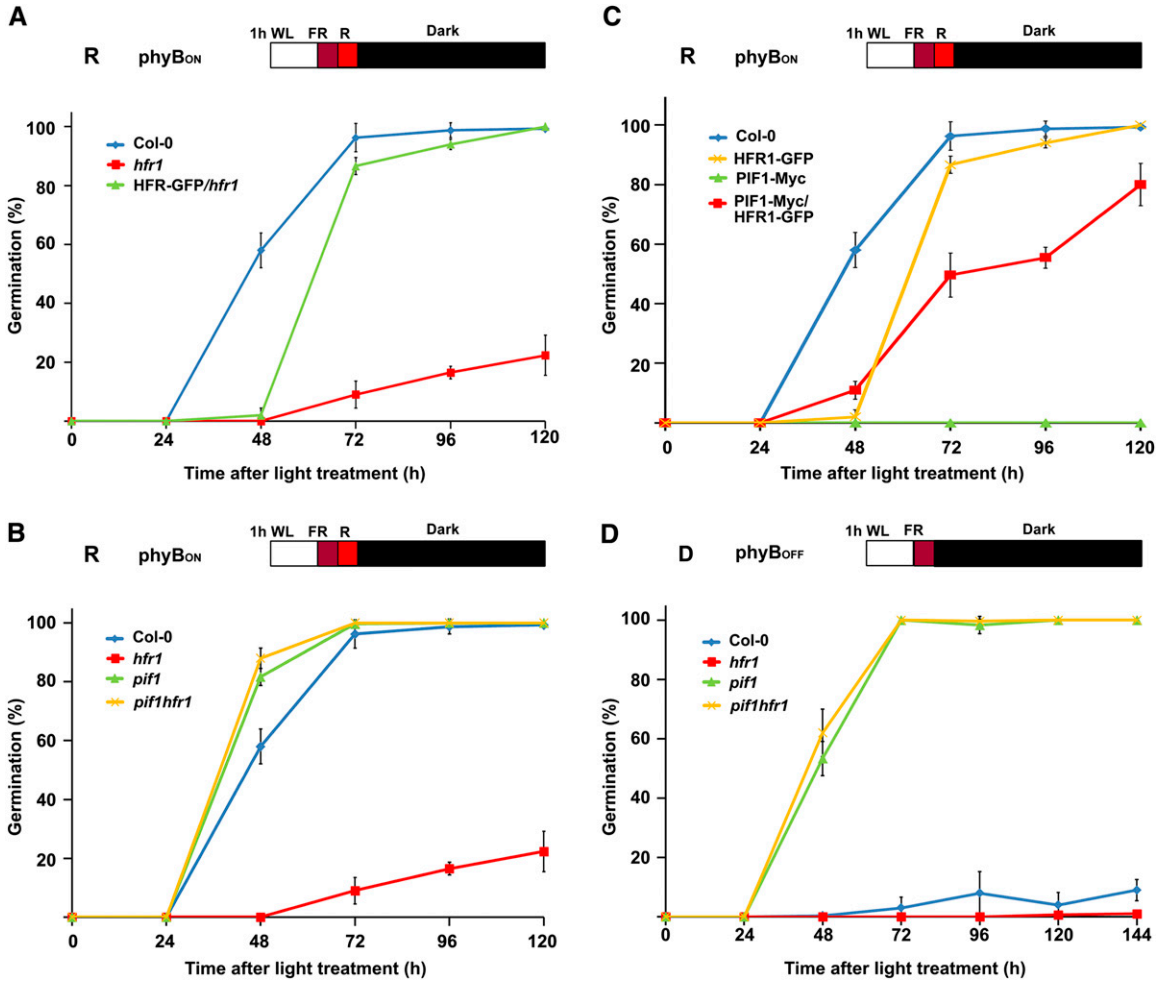

Figure 1. HFR1 Positively Regulates phyB-Dependent Seed Germination via PIF1.

(A) Germination frequencies of Columbia-0 (Col-0), *hfr1*, and HFR1–GFP/*hfr1* seeds under R condition. The top diagram indicates the light irradiation treatment in the experiments. Under R condition, the imbibed seeds were kept under WL for 1 h starting from seed surface sterilization and were subsequently irradiated for 5 min with FR followed by 5 min of R ($phyB_{ON}$, phyB in active form). Seeds were incubated in darkness and germination frequencies were recorded every 24 h after light treatment. Mean ± SD, $n = 3$.

(B) Germination frequencies of Col-0, *hfr1*, *pif1*, and *pif1 hfr1* seeds under R condition. Imbibed seeds were treated as in **(A)**. Germination frequencies were recorded every 24 h after light treatment. Mean ± SD, $n = 3$.

(C) Germination frequencies of Col-0, HFR1–GFP, PIF1–Myc, and PIF1–Myc/HFR1–GFP seeds under R condition. Imbibed seeds were treated as in **(A)**. Germination frequencies were recorded every 24 h after light treatment. Mean ± SD, $n = 3$.

(D) Germination frequencies of Col-0, *hfr1*, *pif1*, and *pif1 hfr1* seeds under D condition. Under D condition, the imbibed seeds were kept under WL for 1 h starting from seed surface sterilization and were subsequently irradiated for 5 min with FR ($phyB_{OFF}$, phyB in inactive form). Seeds were incubated in the dark, and germination frequencies were recorded every 24 h after light treatment. Mean ± SD, $n = 3$.

(Supplemental Figure 2C online), suggesting that HFR1 probably functions downstream of PIF1 in FR-regulated photomorphogenesis, which is different from their relationship in seed germination. Thus, the functional relationship between HFR1 and PIF1 is dependent on the developmental process and PIF1 acting downstream of HFR1 is specific to seed germination.

HFR1 Directly Interacts with PIF1

It was previously reported that HFR1 interacts with PIFs in a yeast two-hybrid screening assay (Bu et al., 2011). Therefore, we tested whether HFR1 interacts with PIF1 in vitro and in vivo. Our results showed that HFR1 strongly interacted with PIF1 in a yeast two-hybrid assay (Figure 2A). To investigate which domain is responsible for the HFR1–PIF1 interaction, we further divided the PIF1 protein into an N-terminal (PIF1N) region that contains the phytochrome interacting domain and a C-terminal (PIF1C) region that contains the bHLH domain for DNA binding activity.. As shown in Figure 2A, PIF1C displayed an even stronger interaction with HFR1, but there was no interaction between PIF1N and HFR1. Therefore, HFR1 interacts with PIF1 by directly binding to the C terminus of PIF1,

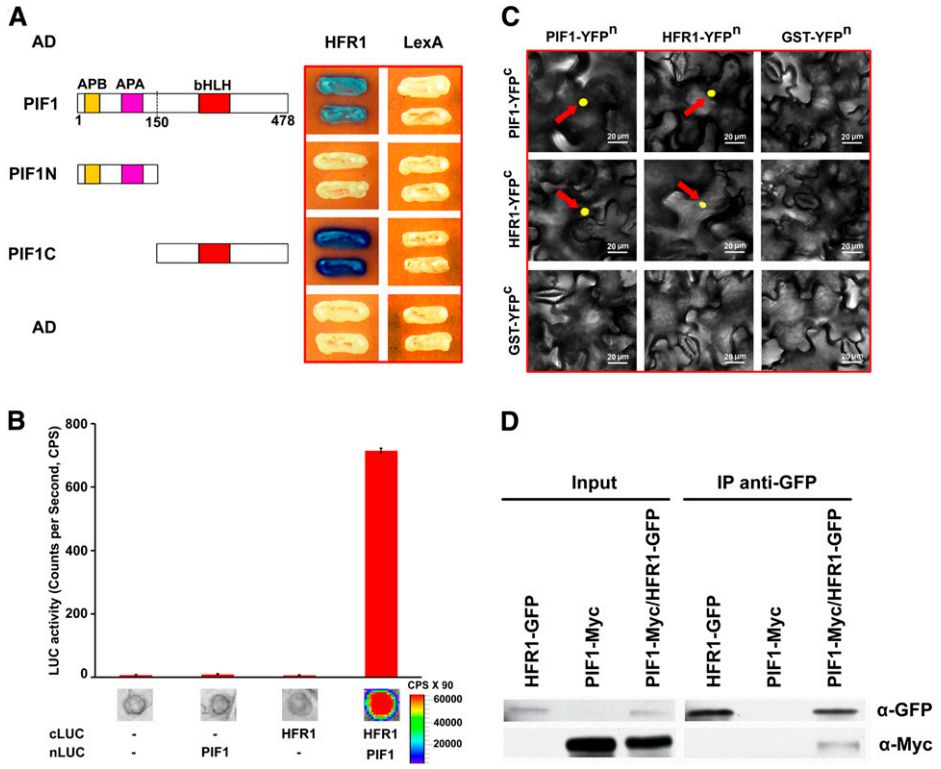

Figure 2. HFR1 Directly Interacts with PIF1.

(A) Yeast two-hybrid assay shows that HFR1 directly interacts with the C-terminal DNA binding domain (bHLH) of PIF1. Left diagrams indicate the various fragments of PIF1 fused with the GAL4 activation domain as the bait constructs. Full-length HFR1 fused with LexA DNA binding domain was the prey construct. Empty vectors were used as negative controls. APB, active phyB binding domain; APA, active phyA binding domain. The numbers indicate the amino acid residues in PIF1.

(B) LCI assay of the interaction of HFR1 with PIF1 in tobacco leaf cells. Full-length HFR1 and PIF1 were fused to the split cLUC or nLUC fragments of firefly (*Photinus pyralis*) LUC. Empty vectors were used as negative controls. Mean ± SD, $n = 5$.

(C) BiFC assay showing the interaction of HFR1 with PIF1 in tobacco leaf cells. Full-length HFR1 and PIF1 were fused to the split N- or C-terminal (YFP^n or YFP^c) fragments of YFP. GST fused to YFP^n or YFP^c fragments were used as negative controls. The images show the merging of YFP fluorescence and bright light images. Red arrow indicates the position of YFP speckle. Bar = 20 μm.

(D) In vivo Co-IP assay of PIF1 with HFR1. Five-day-old etiolated seedlings of transgenic plants HFR1–GFP, PIF1–Myc, or PIF1–Myc/HFR1–GFP were immunoprecipitated using an anti-GFP antibody and immunoblotted using anti-Myc or anti-GFP antibodies.

suggesting that the interaction might affect the DNA binding activity of PIF1.

Moreover, we performed transient luciferase complementation (LCI) and bimolecular fluorescence complementation (BiFC) in tobacco (*Nicotiana benthamiana*) leaves to examine the HFR1–PIF1 interaction in planta. Coexpressing HFR1 fused to the C terminus of luciferase (cLUC) and PIF1 fused to the N terminus of luciferase (nLUC) in tobacco leaves exhibited a high luciferase (LUC) signal compared with the negative controls (Figure 2B), indicating a strong interaction between HFR1 and PIF1 in planta. For the BiFC assay, we generated different combinations of yellow florescent protein (YFP): N-terminal (YFP^n) and C-terminal (YFP^c) fusions with PIF1 and HFR1. Strong YFP fluorescence signals were observed in the nucleus when HFR1 and PIF1 were cotransformed (Figure 2C), indicating that HFR1 and PIF1 can form both homo- and heterodimers in the nucleus. Finally, coimmunoprecipitation (Co-IP) assays using double-transgenic lines carrying HFR1–GFP and PIF1–Myc confirmed the interaction between HFR1 and PIF1 in *Arabidopsis* (Figure 2D).

HFR1 Prevents PIF1 from Binding to Its Target Genes and Antagonistically Regulates PIF1-Mediated Gene Expression

To determine whether the interaction between HFR1 and PIF1C inhibits the DNA binding activity of PIF1, we performed an electrophoretic mobility assay (EMSA), which tests PIF1–DNA binding biochemically. It was previously reported that PIF1 can bind to the G-box motif in the *RGA* promoter (Oh et al., 2007). We confirmed that PIF1 protein binds specifically to the G-box containing region of the *RGA* promoter, as demonstrated in a competition experiment using cold competitor probes (Figure 3A). To test whether HFR1 could interfere with PIF1–DNA binding, both HFR1 and PIF1 proteins were added to the assay. Incubation of PIF1 with increasing levels of HFR1 significantly reduced the DNA binding of PIF1 (Figure 3A). Furthermore, when

we added a similar amount of mutated HFR1 protein, which interferes with the dimerization properties of the HLH domain in HFR1 (Hornitschek et al., 2009; Galstyan et al., 2011), the PIF1–DNA binding activity was slightly affected. These results suggest that the interaction between HFR1 and PIF1 inhibits the ability of PIF1 to bind DNA in vitro.

To further investigate the sequestration of PIF1 byHFR1 in vivo, we performed a chromatin immunoprecipitation followed by a quantitative real-time PCR assay (ChIP-qPCR) in using imbibed seeds of transgenic *Arabidopsis*. In the assay, the promoter fragments of known PIF1-targeted genes *SOM*, *PIL1*, and *PIL2* (Oh et al., 2009) were significantly enriched in PIF1–Myc seeds (Figure 3B). However, in homozygous PIF1–Myc/HFR1–GFP seeds, these enrichments were dramatically decreased (Figure 3B). We also detected the association of PIF1 with the promoter of *GA3ox1*, which was shown to be not a direct target of PIF1 (Oh et al., 2009), and no visible enrichments were found (Figure 3B). An alternative possibility is that PIF1–Myc protein levels were decreased in PIF1–Myc/HFR1–GFP double-transgenic plants, resulting in the decreased association between PIF1 and the target promoter regions. To examine this possibility, we measured PIF1 protein levels in PIF1–Myc and PIF1–Myc/HFR1–GFP transgenic plants, and the results showed that there was no visible difference between the single- and double-transgenic

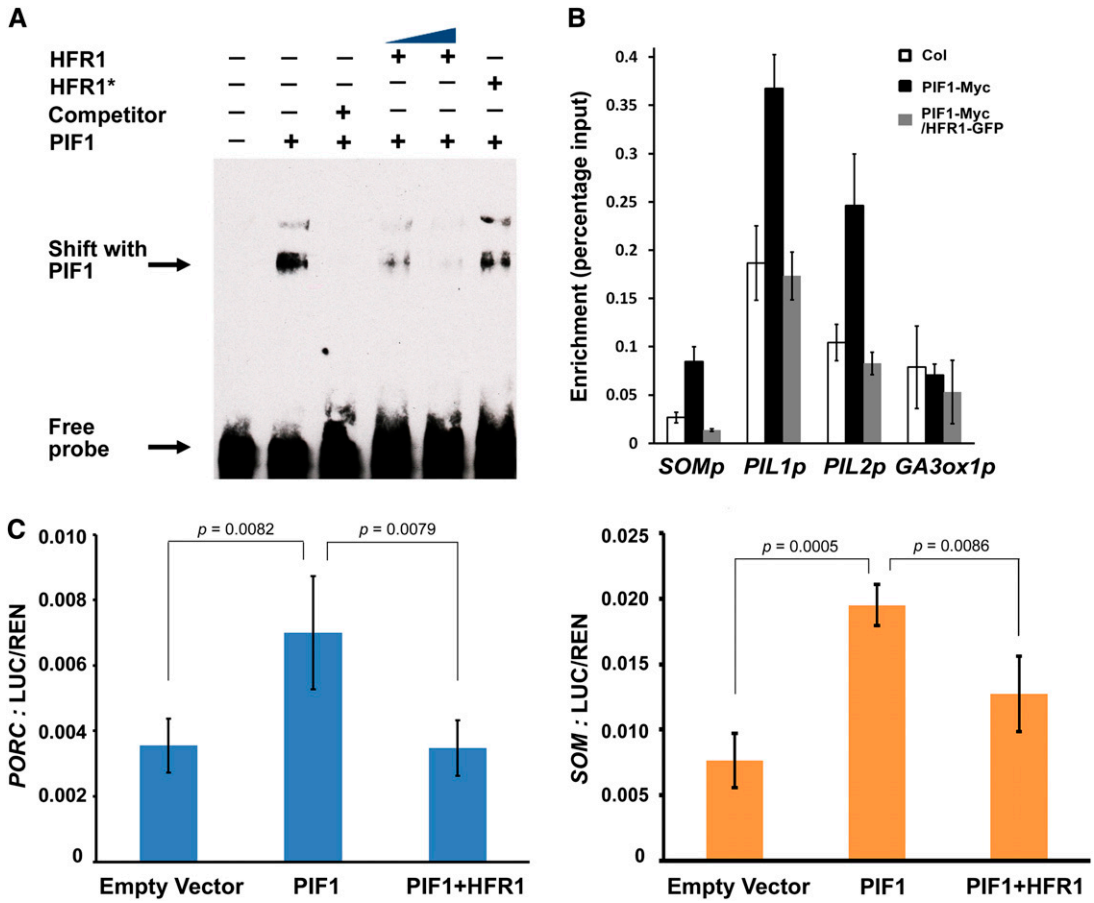

Figure 3. HFR1 Prevents PIF1 from Binding to Its Target Genes and Suppresses PIF1-Mediated Transcriptional Regulation.

(A) EMSA shows that HFR1 inhibits the DNA binding ability of PIF1 in vitro. A biotin-labeled DNA probe of the *RGA* promoter sequence containing a double G-box was used as a PIF1-targeted DNA sequence. An unlabeled probe (1000-fold excess over labeled probe) was used as a cold competitor. HFR1* is a modified version of the HFR1 protein that has a substitution of two conserved residues in the HLH domain (Val172 Leu173 to Asp172 Glu173) that would interfere with the dimerization properties of the HLH domain.

(B) A ChIP-qPCR assay shows that HFR1 prevents PIF1 from binding to its target genes in vivo. An anti-Myc antibody was used to precipitate the PIF1–Myc protein in extracts from imbibed seeds of PIF1–Myc, PIF1–Myc/ HFR1–GFP, and Col-0 (a negative control). This was followed by quantitative PCR detection of the promoter fragments of *SOM*, *PIL1*, *PIL2*, and *GA3OX1* (a negative control) genes. Mean ± SD, $n = 3$.

(C) Transient dual LUC reporter gene assay shows HFR1 represses the transcriptional activity of PIF1 in tobacco leaf cells. A reporter construct (*PORC*:LUC or *SOM*:LUC) was cotransformed with empty vectors (negative control), a PIF1 construct only, or both PIF1 and HFR1 constructs in tobacco leaves. Relative expressions of *PORC*:LUC and *SOM*:LUC were normalized to *35S*:*Renilla reniformis* luciferase (REN) (the internal transfection control). Mean ± SD, $n = 3$. Statistical significance was determined using Student's *t* tests.

[See online article for color version of this figure.]

lines in PIF1–Myc protein levels of dark-grown seedlings and responses to red light irradiation (Supplemental Figure 3 online). These results indicate that HFR1 prevented PIF1 from binding to its target genes in vivo.

To examine whether the sequestration of PIF1 by HFR1 affects the transcriptional activity of PIF1 in plants, we then used a transient transformation LUC reporter gene assay in tobacco leaves. In this assay, the promoters of PIF1-targeted genes, *PORC* or *SOM* (Kim et al., 2008; Moon et al., 2008), drove LUC gene expression as reporters for PIF1 transcriptional activity. Effector constructs for PIF1 and HFR1 proteins were expressed under the control of the cauliflower mosaic virus 35S promoter and cotransformed with the reporter construct. We found that the LUC expression of either the *PORC* or *SOM* promoters was specifically activated by PIF1, but when HFR1 was coexpressed with PIF1, this activation was dramatically decreased (Figure 3C). This suggests that HFR1 inhibits the transcriptional activity of PIF1. We further checked the expression levels of some PIF1-regulated genes in *pif1*, *hfr1*, and *pif1 hfr1* imbibed seeds. We observed that the expression of *PIL2*, *SOM*, and *ABA1*, PIF1-activated genes (Oh et al., 2006, 2009), was decreased in *pif1* but was activated in *hfr1* (Supplemental Figure 4 online). Conversely, *CP1* and *GA3ox2*, PIF1-repressed genes (Oh et al., 2006, 2009), were activated in *pif1* but suppressed in *hfr1* (Supplemental Figure 4 online). The gene expression levels in *pif1 hfr1* double mutants exhibited similar pattern with *pif1* but not *hfr1* (Supplemental Figure 4 online), which was consistent with the germination phenotypes (Figure 1B and 1D). Taken together, these results suggest that HFR1 represses the transcriptional activity of PIF1 and antagonistically regulates PIF1-mediated gene expression.

HFR1 and PIF1 Are the Major Transcription Regulators Responsible for Light-Directed Transcriptome Changes in Seed Germination

To further understand the functions of HFR1 and PIF1 in phyB-dependent seed germination, we examined the HFR1-, PIF1-, and R-regulated transcriptome changes by mRNA deep sequencing analysis in imbibed seeds (Supplemental Data Set 1 online). We performed transcriptomic analyses of Col-0 (the wild type), *pif1* and *hfr1* seeds under D and R conditions as in the germination assay and collected the samples with 12 h of incubation in the dark after light treatments. We identified 2069 genes that displayed statistically significantly twofold (SSTF) changes under R in wild-type imbibed seeds (the wild type/R versus the wild type/D) and are hereafter referred to as light-regulated genes (see Supplemental Data Set 2 online). We then compared the expression profiles of *pif1* with wild type in imbibed seeds and identified 1122 SSTF genes in D condition (*pif1*/D versus wild type/D) and 44 SSTF genes in R condition (*pif1*/R versus wild type/R) (Supplemental Figure 5A and Supplemental Data Set 2 online). These results are consistent with previous microarray data, which indicates that the extremely low abundance of PIF1 in R causes the limited abundance of SSTF genes under this condition (Oh et al., 2009). We combined the *pif1*-regulated genes in both R and D condition and the 1133 genes are hereafter referred to as *pif1*-regulated genes (Supplemental Figure 5A online). When analyzing the *hfr1*-regulated genes in R condition (*hfr1*/R versus wild type/R) and D condition (*hfr1*/D versus wild type/D), we found that over 85% of *hfr1*-regulated genes in D condition were also similarly regulated in R condition. Furthermore, *hfr1*-regulated genes in R condition were much more abundant compared with D condition (Supplemental Figure 5B and Supplemental Data Set 2 online). To keep the data analysis consistent with *pif1*-regulated genes, we combined the *hfr1*-regulated genes in both D and R conditions and obtained 2943 *hfr1*-regulated genes (Supplemental Figure 5A online).

A heat map of *hfr1*-, *pif1*-, and light-regulated genes revealed very similar transcriptome changes by *pif1* and light, while *hfr1* modified the transcriptome in an opposite manner as did *pif1* and light (Figure 4A). We further analyzed the correlations between light- and *pif1*- or *hfr1*-regulated genes and found that most of the light-regulated genes were also similarly regulated by *pif1*, with an extremely high correlation ($R = 0.93$) in the *pif1* and light coregulated genes (Figure 4B). When comparing light- with *hfr1*-regulated genes, most genes were regulated oppositely, with a very high correlation ($R = 0.89$) in light and *hfr1* coregulated genes (Figure 4C). In order to identify the key genes regulated by light through the HFR1–PIF1 module with high confidence, we compared the three subsets and found that light, *hfr1*, and *pif1* commonly regulate a set of 843 genes (Figure 4D). A cluster analysis of these commonly regulated genes revealed that *pif1* and light coregulated genes displayed highly similar expression patterns, while *hfr1* and light coregulated genes exhibited very opposite expression patterns (Figure 4E). These data suggest an essential but antagonistic role for PIF1 and HFR1 in mediating the expression of these light-regulated genes, in which HFR1 functions in the same way as light and PIF1 opposes this regulation. This genome-wide analysis further supports our genetic and molecular data demonstrating the essential role of PIF1 and HFR1 in light-induced seed germination.

HFR1 and PIF1 Antagonistically Regulate a Wide Range of Cellular Activities and Biological Processes

When comparing the similarities between *pif1*- and *hfr1*-regulated genes, we found that ~80% of *pif1*-regulated genes were also regulated by *hfr1* (Supplemental Figure 6A online). After performing a cluster analysis of the *pif1* and *hfr1* coregulated genes, we found that *pif1* and *hfr1* modulate these genes in an opposing manner (Supplemental Figure 6B online). In addition, our correlation results showed that most of the genes were oppositely regulated by *pif1* and *hfr1*, with a high correlation ($R = 0.79$) in their coregulated genes (Supplemental Figure 6C online). Among the 896 *pif1* and *hfr1* coregulated genes, 843 genes were also regulated by light (Supplemental Figure 6D online). We further analyzed the regulation of these 843 genes coregulated by light, *pif1*, and *hfr1* and found that >95% (801 genes) were modulated in the same way by light and *pif1* but in the opposite way by *hfr1* (Supplemental Figure 6E online). We referred to these 801 coregulated genes as members of the light-HFR1–PIF1 pathway, and divided these genes into two groups: PIF1-activated and light/HFR1-repressed genes (*pif1* downregulated genes, 42.2%) and PIF1-repressed and light/HFR1-activated genes (*pif1* upregulated genes, 57.8%) (Supplemental Figure 6B online).

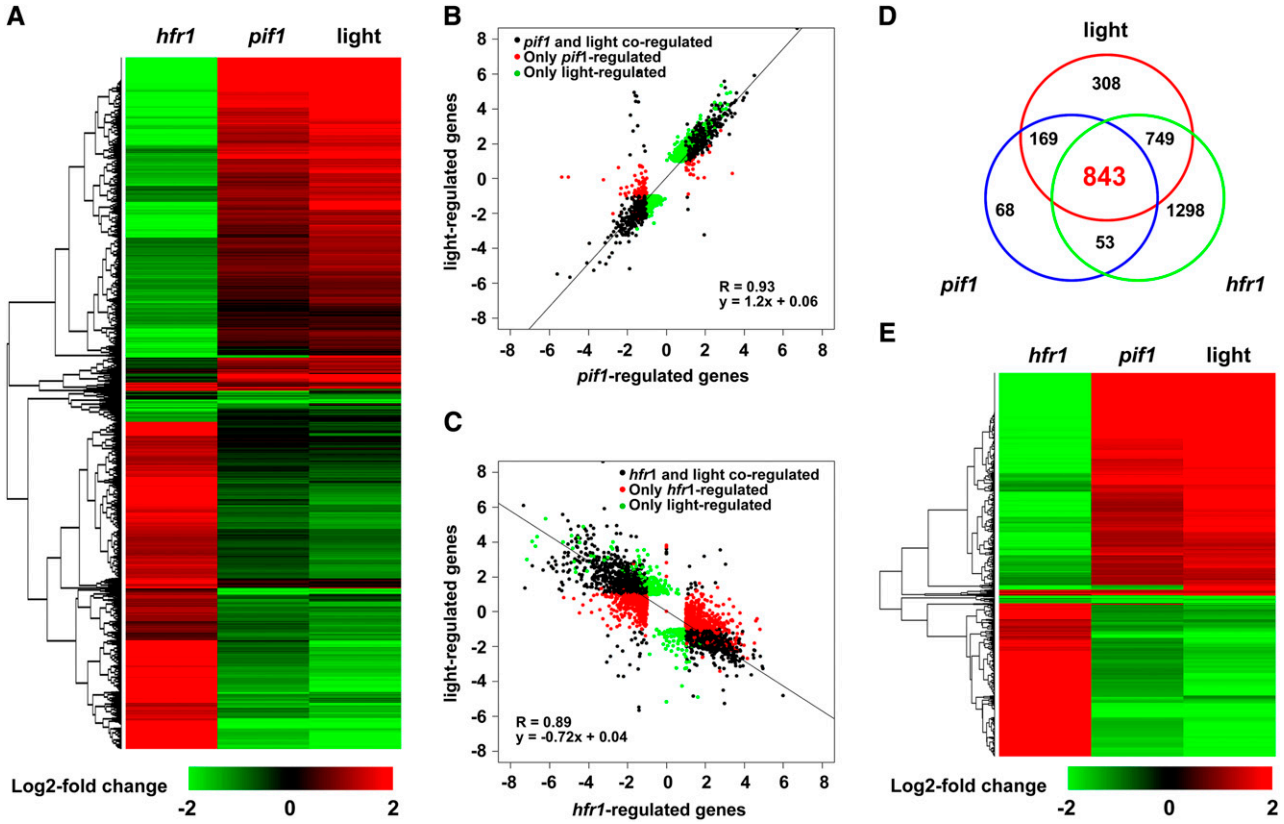

Figure 4. HFR1 and PIF1 Are the Major Transcription Regulators for Light-Regulated Transcriptome Changes in Imbibed Seeds.

(A) Cluster analysis of *hfr1*-, *pif1*-, and light- regulated genes. The genes in at least one of the subsets were analyzed. The bar represents the log2 of the ratio.

(B) and **(C)** Correlation by scatterplot of log2 fold change values between *pif1*- and light-regulated genes **(B)** or between *hfr1*- and light-regulated genes **(C)**.

(D) Venn diagram shows the overlaps between light-, *pif1*-, and *hfr1*-regulated genes.

(E) Cluster analysis of coregulated genes by light, *pif1*, and *hfr1*. The scale bar represents the $\log_2$ of the ratio.

We then performed gene ontology (GO) analysis and functionally clustered the results by using the DAVID (for Database for Annotation, Visualization, and Integrated Discovery) resource (Huang et al., 2009). This analysis revealed that the light-HFR1-PIF1–coregulated genes are preferentially associated with hormone, cell wall, cell growth and DNA replication functions (Figure 5A). Further analysis revealed that PIF1-activated and light/HFR1-repressed (PIF1-activated) and PIF1-repressed and light/HFR1-activated genes (PIF1-repressed) genes are associated with different GO terms. PIF1-activated genes are enriched with hormone, lipid storage and seed development genes, while PIF1-repressed genes are enriched with cell wall, cell growth, and DNA replication genes (Figure 5A).

We further classified the light-HFR1–PIF1 pathway coregulated genes into eight GO functional categories. Our results show that environmental responses and development related genes are enriched in PIF1-activated genes compared with PIF1-repressed genes. By contrast, in PIF1-repressed genes, the cell wall and cell growth related genes are largely enriched (Figure 5B). Based on prior knowledge, the enrichments of these genes imply possible molecular mechanisms for the light-HFR1–PIF1–regulated seed germination pathway (Figure 5C). A large number of genes that are involved in cellular processes and hormone responses are regulated by the light-HFR1–PIF1 pathway and likely mediate the internal developmental transitions during seed germination (Figure 5C). The light-HFR1–PIF1 pathway also regulates many genes involved in environmental responses, which are likely responsible for allowing the emerging seedling to adapt to external environmental changes during seed germination (Figure 5C). Thus, the light-HFR1–PIF1 pathway integrates both internal and external factors to regulate seed germination.

Light-HFR1–PIF1 Pathway Regulates Hormone-Related, Cell Cycle Initiation, and Cell Wall–Loosening Genes to Initiate Seed Germination

GA and ABA are two key hormones that have been studied extensively in seed germination (Finch-Savage and Leubner-Metzger, 2006; Holdsworth et al., 2008). Previous studies showed

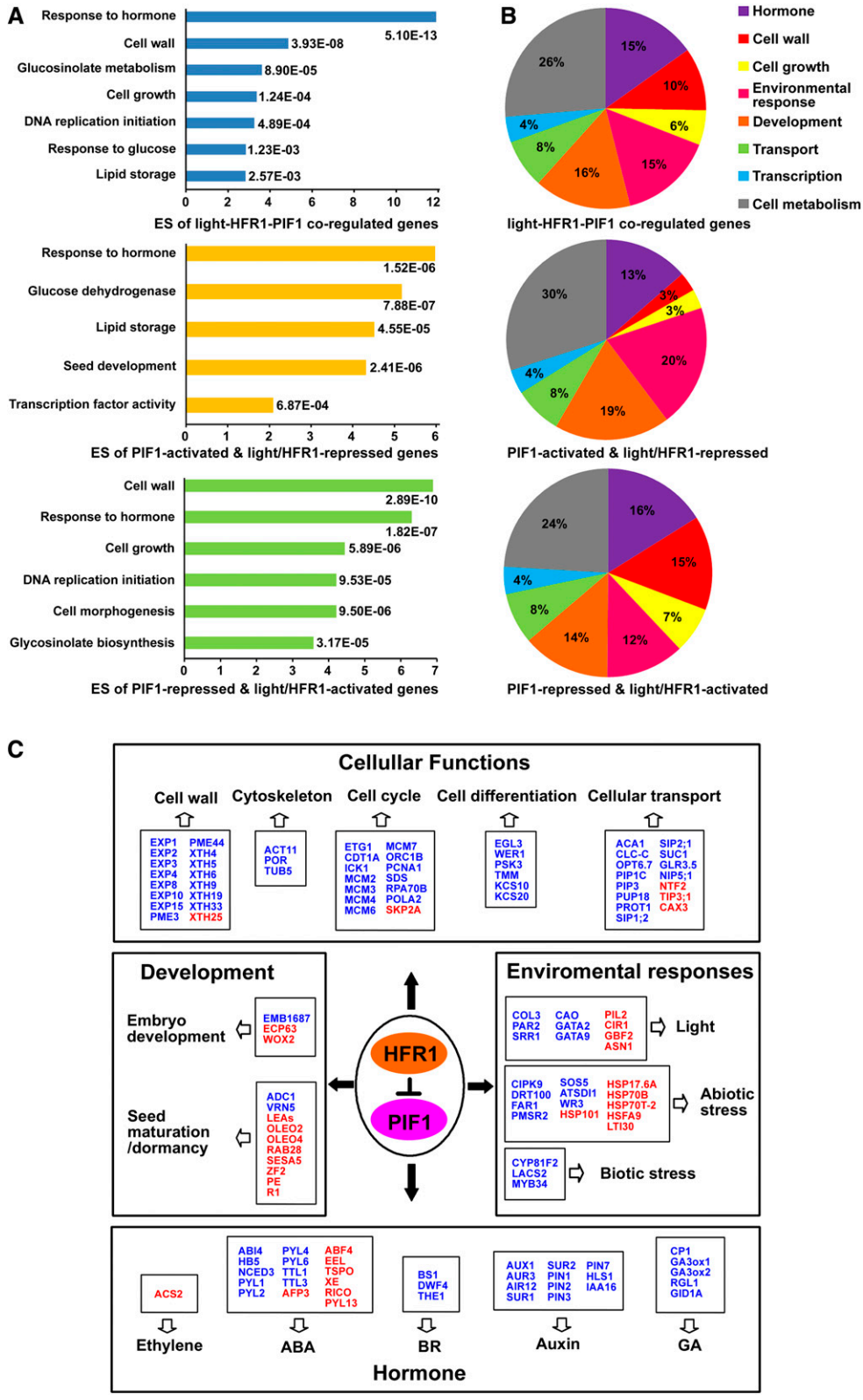

Figure 5. GO Analysis of Genes Regulated by Light through the HFR1–PIF1 Module.

that PIF1 indirectly inhibits GA biosynthesis genes through repressing *GA3ox1* and *GA3ox2* expression (Oh et al., 2006). From our transcriptome analysis, we confirmed that *GA3ox1* and *GA3ox2* expression is repressed by PIF1 but also antagonistically activated by HFR1 (Figure 5C). Also, *ABI4* is repressed by PIF1 (Figure 5C), which is consistent with previous results (Oh et al., 2009). We also found ABA receptor *PYL* genes are regulated by HFR1–PIF1 (Figure 5C), but their functions in seed germination are unknown. Further investigation of these genes would be of great interest. In addition, our results indicate that auxin may play an important role in seed germination as well. Our data show that the auxin efflux carriers *PIN1*, *PIN2*, *PIN3*, and *PIN7*, auxin influx transporter *AUX1*, and many auxin-responsive genes are regulated by the light-HFR1–PIF1 pathway (Figure 5C and Supplemental Data Set 2 online). Quantitative RT-PCR (qRT-PCR) confirmed that the expression of *PIN1*, *PIN2*, *PIN3*, *PIN7*, and *AUX1* was activated by both light and HFR1 but repressed by PIF1 (Figure 6A).

Arabidopsis seed germination involves sequential testa rupture, endosperm rupture and embryo radicle protrusion (Finch-Savage and Leubner-Metzger, 2006; Holdsworth et al., 2008). Because the endosperm functions as a mechanical barrier and inhibits the growth potential of the embryo, the weakening of the endosperm is a critical regulation point for germination (Finch-Savage and Leubner-Metzger, 2006; Holdsworth et al., 2008). In several species, the induction of cell wall remodeling enzymes plays an important role in this process (Holdsworth et al., 2008). Xyloglucan endotransglycosylase/hydrolase (XTH) and expansin (EXP) are two groups of key enzymes important in cell wall loosening and cell expansion (Li et al., 2003; Van Sandt et al., 2007). Previous studies showed that some of the XTHs and EXPs are associated with the endosperm (Holdsworth et al., 2008). For example, *EXP2* expression is endosperm specific and is used as a marker for endosperm tissue (Penfield et al., 2006). Moreover, *XTH15* (*XTR7*) was reported to be oppositely regulated by HFR1 and PIF4/5 in the shade avoidance response (Hornitschek et al., 2009). Here, we identified many members of *XTH*s and *EXP*s families that are suppressed by PIF1 but stimulated by light and HFR1 (Figure 5C and Supplemental Data Set 2 online). Quantitative RT-PCR confirmed the regulation patterns of *EXP1*, *EXP2*, *EXP3*, *EXP9*, *EXP10*, *EXP14*, and *EXP15* as well as *XTH4*, *XTH5*, *XTH8*, *XTH9*, *XTH16*, *XTH19*, and *XTH33* by the light-HFR1–PIF1 pathway (Figure 6B). These results suggest that upon exposure to light, likely through the HFR1–PIF1 module, cell wall–loosening enzymes are activated to weaken the endosperm, functioning to initiate germination.

Interestingly, we also found that some cell cycle–related genes, most of them involved in the transition from the G1 to S phase, are repressed by PIF1 but activated by HFR1 (Figure 5C). This finding is consistent with the previous report that most cells in dry seeds remain in G1 phase (Ogawa et al., 2003). Preinitiation complex, the DNA replication licensing complex, is established during the early G_1 to S transition. The key factors involved in assembling this complex include the origin recognition complex binding to the DNA, then the cell division cycle 6 and DNA replication factor CDT1 binding to the origin recognition complex and finally the minichromosome maintenance2–7 (MCM2–7) subunits loading onto the complex. The establishment of the preinitiation complex results in the initiation of DNA replication (Labib et al., 2000; Shultz et al., 2007). In our RNA-seq results, we found that *ORC1B*, *CDT1A*, and all of the *MCM*s are PIF1-repressed and light/HFR1-activated genes (Figures 5C and 6C). These data indicate that the light-HFR1–PIF1 pathway plays an essential role in cell cycle regulation during seed germination, especially the transition from the G1 to S phase, which likely results in promoting cell division and embryo growth.

It was previously reported that PIFs preferentially bind to variants of the E-box element (CANNTG), especially to the G-box (CACGTG) and the PBE-box (CACATG) (Oh et al., 2009; Hornitschek et al., 2012; Zhang et al., 2013). Bioinformatic analysis of the promoters of light-HFR1–PIF1–regulated *PIN*s, *EXP*s and *XTH*s genes revealed that most of them contained at least one G-box or PBE-box element. We then performed ChIP-qPCR analysis to examine whether PIF1 binds to the promoters of these genes in seeds. Our results showed that PIF1 was associated with the promoters of *PIN3*, *EXP9*, *XTH4*, and *XTH33*, and the PIF1–DNA associations were dramatically suppressed by HFR1 (Figure 6D). Taken together, our data suggest that light, probably through HFR1–PIF1, activates cell wall–loosening and cell cycle initiation genes to promote seed germination.

HFR1 Optimizes Seed Sensitivity to R during Seed Germination

To investigate the responses of seed germination to different durations of light, we used 5 min of FR to inactivate phyB and then irradiated the seeds with progressively increasing durations of R (from 5 s to 24 h). After the light irradiation, the seeds were then incubated in darkness for 5 d, and germination frequencies were scored. Our results showed that wild-type seeds germinated completely with as little as 5 s of R irradiation, *pif1* germinated independent of light irradiation, whereas PIF1–Myc required at least 30 min of R to induce germination (Figure 7A). Moreover, the germination frequency of *hfr1* was extremely low compared with the wild type under a short duration of R, with only 10% germination after 5 s of R irradiation (Figure 7A). To reach complete germination, *hfr1* seeds required at least 12 h of prolonged R irradiation compared with 5 s with wild-type seeds (Figure 7A).

Furthermore, we examined the stages of seed germination in R condition after 2 d of dark incubation. We divided the initial

Figure 5. (continued).

(A) DAVID functional clustering enrichment score (ES) of the highly enriched GO terms in light through HFR1–PIF1–regulated (light-HFR1–PIF1) genes. These genes are divided into two groups: PIF1-activated and light/HFR1-repressed genes and PIF1-repressed and light/HFR1-activated genes.

(B) GO functional categorization of light-HFR1–PIF1 genes and the two subsets as indicated.

(C) Representative light-HFR1–PIF1 genes with known functions in various cellular functions, development, environmental responses, and hormone pathways. Genes repressed and activated by PIF1 are in blue and red, respectively.

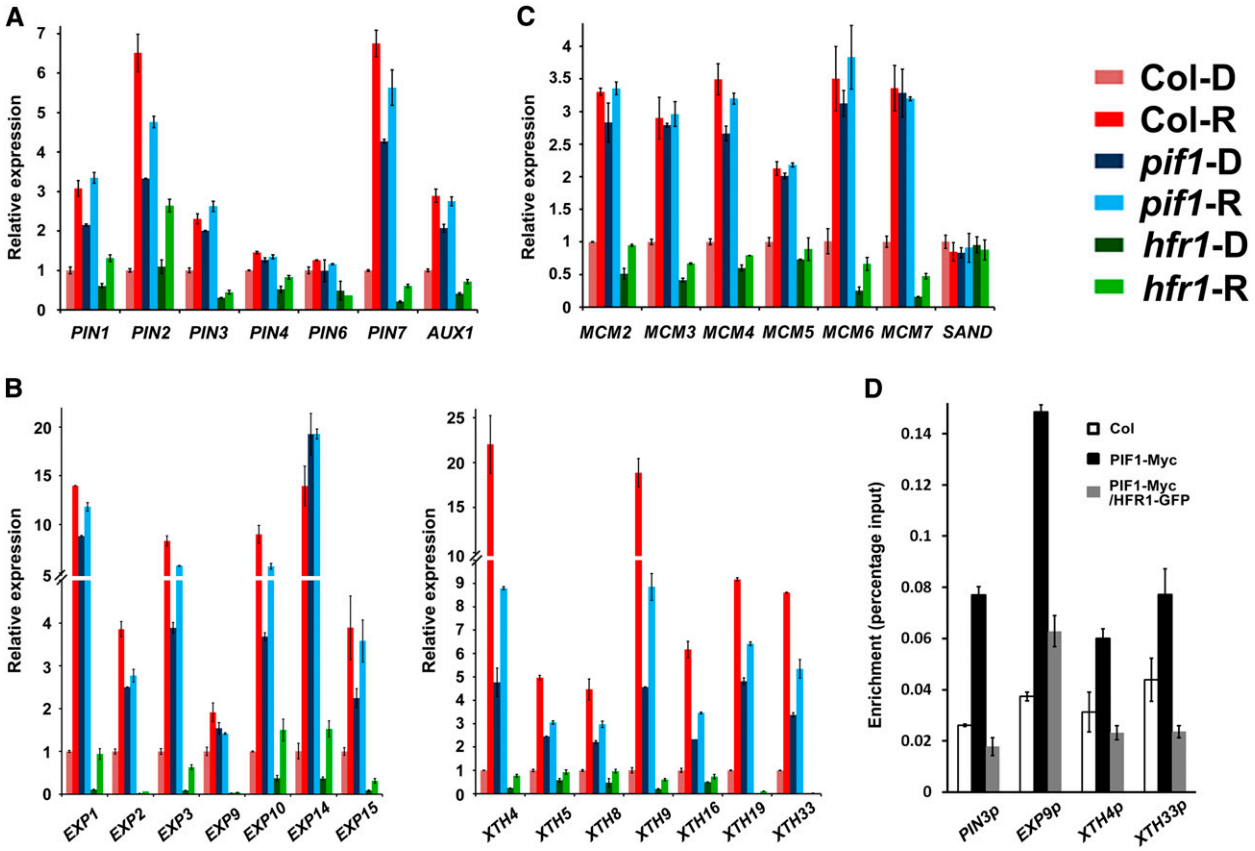

Figure 6. Light, through the HFR1–PIF1 Module, Regulates Genes Involved in Polar Auxin Transport, Cell Wall Loosening, and Initiation of DNA Replication.

(A) to **(C)** qRT-PCR results showing the expression of *PIN* **(A)**, *EXP* and *XTH* **(B)**, and *MCM* **(C)** genes in imbibed seeds. The imbibed seeds were incubated for 1 h under white light and were then irradiated with 5 min of far-red light followed with (R) or without (D) 5 min of red light. Seeds were incubated in dark for 12 h before extracting mRNA. The expression of the housekeeping gene *SAND* (At2g28390; for Sp100, AIRE-1, NucP41/75, DEAF-1) was used as a control. Mean ± SD, $n = 3$.

(D) ChIP-qPCR assay shows that PIF1 directly associates with the G-box–containing promoter fragments of *PIN3*, *EXP9*, *XTH4*, and *XTH33*. In addition, HFR1 sequesters PIF1 and prevents it from binding to these genes in vivo. Mean ± SD, $n = 3$.

germination phase into three stages: stage I represents no germination, stage II represents endosperm breakage with no radical protrusion, and stage III represents radical protrusion. The results showed that after 2-d dark incubation, most of the wild-type and *pif1* seeds were germinated (stage III) or started to germinate (stage II) (Figure 7B). However, a majority of the *hfr1* and PIF1–Myc seeds remained in stage I (Figure 7B). These results indicate an essential role of HFR1 in maintaining a high sensitivity for seeds in response to a short duration of light, ensuring rapid initiation of germination during favorable environmental changes.

DISCUSSION

As sessile organisms, plants must be highly sensitive to environmental fluctuations for optimizing their survival. The germination and subsequent development of seeds is essential for the colonization by land plants because they usually are dispersed in a dormant state, which ensures that they can survive in various environments. Once the environmental conditions become favorable, seeds break dormancy and germinate. Light is a major environmental factor affecting seed germination. The ability of seeds to monitor and respond to light is vital for plant survival. If the seed is not sensitive enough to dim light and fails to germinate, it will miss the opportunity to begin a new life cycle.

However, until now, the molecular mechanism underlying the rapid response of seeds to light irradiation was largely unknown. One key reason is because the main positive transcription regulators that promote light-induced seed germination were unknown. In this study, we found that light-directed whole transcriptomic changes are almost completely reversed by mutation of a single gene, *HFR1*, indicating the essential roles of HFR1 in mediating light-induced seed germination. Genetic analysis identified that HFR1 functions upstream of PIF1, a known key transcription regulator in suppressing seed germination. Biochemical studies showed that HFR1 directly interacts with PIF1 to suppress the transcriptional activity of PIF1. Because our mRNA deep sequencing results contained

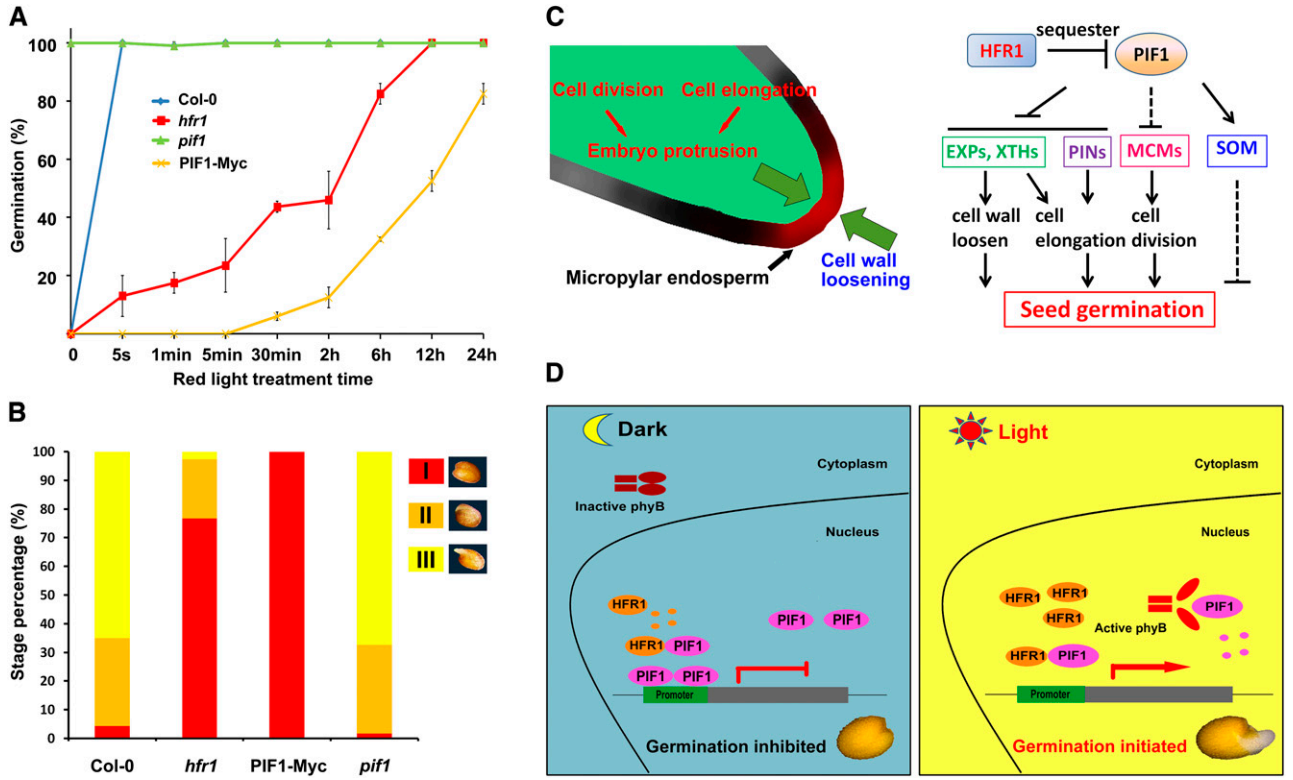

Figure 7. HFR1–PIF1 Pair Enacts a Fail-Safe Mechanism to Ensure High Sensitivity to Low Quantity of Light and Synchronicity in Seed Germination.

(A) Germination frequencies of Col-0, *hfr1*, *pif1*, and PIF1–Myc seeds with various red light irradiant time treatments after 5 min of far-red light treatment. The seeds were first exposed to 1 h of WL, starting from seed surface sterilization and followed by 5 min of FR irradiation to inactivate phyB. After that, the seeds were illuminated with the indicated durations of red light and then kept in the dark. Seeds were incubated in the dark for 5 d after light treatment, and germination frequencies were recorded. Mean ± SD, $n = 3$.

(B) Germination stage percentage of seeds under R condition. The seeds were incubated in darkness for 2 d after light treatments, and germination stages were observed and scored under the stereomicroscope. Germination stages I to III are represented as photos of seeds in corresponding stages. Stage I represents no germination, Stage II represents endosperm breakage with no radicle protrusion, and Stage III represents radical protrusion.

(C) The left diagram shows the micropylar endosperm and embryo radicle of an *Arabidopsis* seed. During germination, cell wall–loosening enzymes play an important role in endosperm rupture, and radicle protrusion occurs as a result of cell division and cell elongation of the embryo radicle. The right schematic diagram shows how light, through the HFR1–PIF1 pair, regulates the key components involved in the phyB-dependent seed germination process.

(D) Proposed working model of HFR1–PIF1 in phyB-dependent seed germination. In the dark, phyB is inactive in the cytoplasm, and PIF1 accumulates to high levels in the nucleus. The excess abundance of PIF1 overrides the inhibition of HFR1, resulting in dominant repression of seed germination by PIF1. Upon low-level irradiation, phyB is activated and translocated into the nucleus, where it interacts with PIF1, causing the degradation of PIF1. Meanwhile, the abundance of HFR1 is likely increased by light and sequesters PIF1 to further inhibit the suppression of seed germination by PIF1. With this mechanism, seeds may be able to rapidly respond to low light exposure to initiate seed germination without delay.

>10 million mapped reads and three biological replicates for each sample, we illustrate a very high quality transcriptional network for light-initiated seed germination. Our deep mRNA-seq results were validated by using qRT-PCR to confirm the expression of a variety of previously reported genes involved in light-mediated seed germination. Comparing the three sets of transcriptome regulated by light, HFR1 and PIF1 revealed that HFR1 and PIF1 oppositely mediate the light-regulated transcriptome in imbibed seeds. Our genomic analysis affirmed that the functionally antagonistic HFR1–PIF1 pair is the core module for directing the transcriptional network in light-induced seed germination.

By functionally analyzing the light-HFR1–PIF1–regulated genes, we confirmed the involvement of GA and ABA pathways in modulating seed germination, as previously reported (Finch-Savage and Leubner-Metzger, 2006; Holdsworth et al., 2008). In addition, our results suggest that the auxin efflux carriers also play important roles in promoting seed germination. Previous studies showed that three auxin transport proteins (PIN1, PIN2, and PIN7) are involved in phyA-dependent germination and *pin7* showed germination defects upon FR (Ibarra et al., 2013). Also, auxin influx transport AUX1 functions in ABA-dependent repression of embryonic axis elongation in the early seedling development (Belin et al., 2009). Our results further suggest that

the auxin efflux carriers are also involved in promoting phyB-dependent seed germination, demonstrating the important roles of auxin in seed germination.

More importantly, with help of this high-quality transcriptomic map, we identified key cellular processes required for light-promoted seed germination. Our results showed that light, through the HFR1–PIF1 module, stimulates the gene expression of *EXP*s and *XTH*s, the two key groups of cell wall–loosening enzymes. At the same time, through the HFR1–PIF1 module, light activates *MCM*s to initiate cell division and *PIN*s to promote cell elongation in the embryo. By cell wall weakening and radicle protrusion, the seeds are able to break through the mechanical barrier of the endosperm and initiate germination (Figure 7C). By identifying the core transcription regulators, we revealed key components and interactions of the molecular mechanism of light-initiated seed germination. In the dark, PIF1 accumulates to high levels and predominately suppresses seed germination. Light induces PIF1 degradation and likely accumulation of HFR1 (Jang et al., 2005; Yang et al., 2005; Oh et al., 2006; Shen et al., 2008). This causes a reduction of PIF1 levels, while the high level of HFR1 protein directly binds to the remaining PIF1, which prevents PIF1 from binding to its target genes. This mechanism allows the seeds to rapidly change the transcriptome in the presence of light and causes the initiation of seed germination (Figure 7D).

The bHLH proteins are widely distributed in *Arabidopsis* and play important roles in transcriptional regulation. Basic domains were identified in most of the bHLH proteins, and are responsible for the binding to specific DNA sequences. However, atypical bHLH proteins were found to lack the DNA binding domain (Toledo-Ortiz et al., 2003). Previous studies showed that some atypical bHLH proteins dimerize with bHLH proteins to form HLH/bHLH complexes to regulate many developmental processes (Fairchild et al., 2000; Hornitschek et al., 2009; Galstyan et al., 2011). PIF1 is a bHLH protein, which was shown to bind specifically to G-box DNA sequences to activate or repress target gene expression (Oh et al., 2009). HFR1 is an atypical bHLH protein lacking a DNA binding domain (Fairchild et al., 2000). Here, we found that HFR1 interacts with PIF1 directly in vitro and in vivo to sequester PIF1 from binding to its target genes. The antagonistic HFR1–PIF1 pair plays a central role in directing the transcriptional regulatory network for light-initiated seed germination. HFR1 was also shown to form heterodimers with the bHLH transcription regulators PIF4 and PIF5 to repress the shade avoidance syndrome (Hornitschek et al., 2009). Therefore, HFR1 might form heterodimers with different bHLH proteins to regulate various developmental processes.

Multiple types of regulations for key transcription regulators provide more efficient and precise regulation during signal transduction. Previous studies showed that PIF3 and PIF4, which act as convergent nodes of endogenous and exogenous modulating factors, are regulated at multiple regulatory levels (de Lucas et al., 2008; Feng et al., 2008; Zhong et al., 2012). PIF1 is a master transcription regulator in repressing light-induced seed germination and its protein is degraded upon light (Oh et al., 2004, 2006). In our study, we propose that the transcriptional activity of PIF1 is also regulated in addition to its protein level. Both our in vitro and in vivo data show that the interaction between HFR1 and PIF1 inhibits PIF1–DNA binding activity directly, and the transcriptional activity of PIF1 is largely reduced by HFR1. Therefore, upon low-level irradiation during which PIF1 is not rapidly degraded (Shen et al., 2008), HFR1 is able to effectively suppress the transcriptional activity of the remaining PIF1 to enable seeds to germinate rapidly and robustly. However, in the absence of HFR1, the repression of seed germination by PIF1 cannot be removed quickly enough to ensure a rapid response to light. As a result, the seeds germinated at a very low rate and much more light irradiation is required to acquire full germination.

METHODS

Plant Material and Growth Conditions

The wild-type *Arabidopsis thaliana* seedlings used in this study are Col-0 ecotype. *pif1* (SALK_131872C) (Penfield et al., 2005; Zhong et al., 2009), *hfr1-201*(Kim et al., 2002; Yang et al., 2005), PIF1–Myc (Oh et al., 2004), and HFR1–GFP/*hfr1-201* (Yang et al., 2005) are previously reported. *hfr1-1* (SALK_037727C) and *hfr1-2* (SALK_049497C) were obtained from the Arabidopsis Biological Resource Center. Double mutants were generated by crossing, and homozygous lines were genotyped. Plants were grown under long-day photoperiod WL at 22°C.

Germination Frequency and Hypocotyl Length Measurements

Surface-sterilized seeds were plated on Murashige and Skoog (MS) medium (4.4 g/L MS salts, 1% Suc, pH 5.7, and 8 g/L agar). Including surface sterilization and plating, seeds were exposed for 1 h of WL and then exposed to 5 min of FR to inactivate phyB (D condition, $phyB_{OFF}$). Seeds were then irradiated with 5 min of R (10 $\mu molm^{-2}s^{-1}$) to activate phyB (R condition, $phyB_{ON}$) unless specified otherwise. Seeds were then incubated for the indicated times in darkness and germination frequencies were determined. In a parallel effort, we grew all the plants and harvested the seeds side by side. After harvesting, the seeds were dried at room temperature for 6 to 8 weeks and were used for subsequent experiments. At least 80 seeds were used for each experimental treatment, and three biological replicates were used for statistical analysis.

Hypocotyl lengths were measured from the point at which the cotyledons join the hypocotyl to the hypocotyl-root junction using the ImageJ software (http://rsbweb.nih.gov/ij/). Seedlings were grown for 4 d on MS medium under the indicated light conditions. More than 20 seedlings were measured for each set of experiments.

RNA Extractions and qRT-PCR

Seeds were treated using the D and R conditions as in the germination assay and then incubated in the dark for 12 h before collecting the samples. The imbibed seeds were ground to powder in liquid nitrogen, and total RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma) with On-Column DNase I Digestion treatment. Spectrophotometric and gel electrophoretic analysis were performed to detect RNA quality. To synthesize cDNA, 2 μg of RNA was used for the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time PCR was performed by using SYBR Green Mix (Takara) in CFX96 Real-Time system (Bio-Rad). The gene expression results were normalized by *PP2A*. All quantitative PCR experiments were independently performed in triplicate, and representative results were shown. Primers used are listed in Supplemental Table 1 online.

Bioinformatic Analysis of Transcriptomes

For whole genomic transcriptome analysis, total RNA was extracted as described above, and an mRNA-seq library was prepared by using an

mRNA Seq Kit (Illumina). More information can be found at the Yale Center for Genome Analysis (http://medicine.yale.edu/keck/ycga/index.aspx).

All raw tags from each sample were mapped to the *Arabidopsis* genome The Arabidopsis Information Resource 10 and its corresponding annotated gene profile (downloaded at http://www.Arabidopsis.org/). Up to two mismatches were allowed during the mapping. Tags mapped to a unique location were used for the downstream analysis, whereas those mapped to multiple loci were discarded to avoid ambiguity. Cuffdiff (http://cufflinks.cbcb.umd.edu/manual.html) was applied to detect differentially expressed genes in mutant compared with wild-type condition. We picked significantly expressed genes using the following conditions: larger than twofold change and $P \leq 0.001$. All statistical tests, regression modeling, and clustering were done in R project for statistical computing.

Functional classification was performed by using the DAVID functional annotation clustering tool (http://david.abcc.ncifcrf.gov/home.jsp) (Huang et al., 2009). The functional clusters enrichment analysis was calculated by comparing the whole *Arabidopsis* genome, and the highest classification stringency was chosen for clustering.

Yeast Two-Hybrid Assay

The yeast assay was done in the yeast strain EGY48 by using the AD and LexA system (BD Clontech). The AD and LexA fusion plasmids were cotransformed with different combinations into yeast cells that already contained the reporter plasmid *p8op*:LacZ (BD Clontech). Transformants were grown on proper dropout plates containing 5-bromo-4-chloro-3-indolylb-D-galactopyranoside for blue color development as described in the Yeast Protocols Handbook (BD Clontech).

BiFC, LCI, and Dual-LUC Transient Expression Assays

BiFC assays were performed as previously described (Feng et al., 2008). Briefly, the *Agrobacterium tumefaciens* strain GV2260 carrying various YFP^n and YFP^c construct combinations was infiltrated in tobacco (*Nicotiana benthamiana*) leaves. YFP fluorescence signals were detected by using the LSM 510 Meta confocal laser scanning microscope (Carl Zeiss).

LCI assays were performed as previously described (Chen et al., 2008). Briefly, *Agrobacterium* strain GV2260 carrying various nLUC and cLUC construct combinations was infiltrated into tobacco leaves. LUC activities were detected using Xenogen IVIS Spectrum and quantified using Living Image software (Caliper).

Dual-LUC Transient expression assays were performed using the Dual-Luciferase Reporter Assay System (Promega) kit. Briefly, *Agrobacterium* strain GV2260 carrying the LUC reporter (*pGreen-SOM*:LUC or *pGreen-PORC*:LUC) and various effector (empty, PIF1, or PIF1+HFR1) constructs was infiltrated in tobacco leaves. The firefly (*Photinus pyralis*) LUC and (REN) activities of infiltrated leaves were measured on a GLOMAX 20/20 luminometer (Promega). Final transcriptional activity was calculated as LUC/REN. Five biological repeats were measured per sample.

Co-IP Assay

For co-IP assays, 200 to 300 mg of *Arabidopsis* tissues was homogenized in 500 μL of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Tween 20, and 1× Roche protease inhibitor cocktail). Then the samples were centrifuged at 16,000*g* in cold room twice, and the supernatant was collected in a new tube. The total protein concentration was determined using the Bio-Rad Protein Assay. After precleaning by using 20 μL of protein A–agarose beads (Millipore), 2 μL of anti-GFP antibody (BD Clontech) was added into 500 μg of total soluble protein, and the solution was incubated at 4°C for 2 h with gentle rotation. Then, 10 μL of protein A-agarose beads (Millipore) was added and incubated at 4°C for 2 h with gentle rotation for the immunoprecipitation. After incubation, the beads were washed twice with lysis buffer containing 200 mM NaCl and then twice with lysis buffer containing 150 mM NaCl. Finally, the washed beads were collected for immunobloting by spinning down. For protein gel blot, anti-Myc (Abcam) antibody was used at a dilution of 1:1000, and anti-GFP (BD Clontech) antibody was used at a dilution of 1:1000.

ChIPs

ChIP assays were performed as described (Gendrel et al., 2005). In the assay, anti-Myc polyclonal antibody (Abcam) was used for immunoprecipitation. Col-0 with the same immunoprecipitation conditions was used as a negative control. After ChIP, the enrichment for specific DNA fragments was examined by quantitative PCR.

EMSAs

EMSA assays were performed using biotin-labeled probes and the Light shift Chemiluminescent EMSA kit (Pierce). The *RGA* promoter sequences containing a double G-box were used to generate the biotin-labeled and unlabeled probes. A total of 450 ng of His-PIF1 protein only or with increasing amounts of His-HFR1 (200 to 600 ng) protein or with 600 ng of mutated His-HFR1* protein was incubated together with 20 nM biotin-labeled probes in 20-μL reaction mixtures (10 mM Tris-HCl, 150 mM KCl, 1 mM DTT, 50 ng/μL poly dI-dC, 2.5% glycerol, 0.05% NP-40, 100 μM $ZnCl_2$, and 0.5 μg/μL BSA) for 20 min at room temperature. For the cold competitor (lane 3 shown in Figure 3A), 20 μM unlabeled probes was added into the reaction mixture. The reaction mixtures were separated on 6% native polyacrylamide gels. The labeled probes were detected according to the instructions provided with the EMSA kit. HFR1* contains a substitution of two conserved residues in the HLH domain (Val172-Leu173 to Asp172 Glu173), which are the same point mutant sequences as previously reported (Hornitschek et al., 2009).

Accession Numbers

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative or GenBank/EMBL data libraries under the following accession numbers: PIF1 (AT2g20180), HFR1 (AT1g02340), SOM (At1g03790), PIL1 (AT2g46970), PIL2 (At3g62090), PORC (AT1g03630), CP1 (At4g36880), ABA1 (AT5g67030); GA3OX1 (AT1g15550), GA3OX2 (AT1g80340), EXP1 (AT1g69530), EXP2 (AT5g05290), EXP3 (AT2g37640), EXP9 (AT5g02260), EXP10 (AT1g26770), EXP14 (AT5g56320), EXP15 (AT2g03090), XTH4 (AT2g06850), XTH5 (AT5g13870), XTH8 (AT1g11545), XTH9 (AT4g03210), XTH16 (AT3g23730), XTH19 (AT4g30290), XTH33 (AT1g10550), PIN1 (AT1g73590), PIN2 (AT5g57090), PIN3 (AT1g70940), PIN7 (AT1g23080), AUX1 (AT2g38120), MCM2 (AT1g44900), MCM3 (AT5g46280), MCM4 (AT2g16440), MCM5 (AT2g07690), MCM6 (AT5g44635), MCM7 (AT4g02060), SAND (AT2g28390), and PP2A (AT1g13320). Germplasm was used as: *pif1* (SALK_131872C), *hfr1-1* (SALK_037727C), and *hfr1-2* (SALK_049497C).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Three Independent *hfr1* Alleles Showed Similar Defects in phyB-Dependent Seed Germination.

Supplemental Figure 2. The Genetic Relationship That HFR1 Functions Upstream of PIF1 Is Specific to Seed Germination.

Supplemental Figure 3. PIF1–Myc Protein Levels Are Not Changed in PIF1–Myc Single and PIF1–Myc/HFR–GFP Double-Transgenic Plants.

Supplemental Figure 4. HFR1 Antagonistically Regulates PIF1-Regulated Genes in Imbibed Seeds.

Supplemental Figure 5. Analysis of PIF1- and HFR1-Regulated Transcriptome Changes in Imbibed Seeds.

Supplemental Figure 6. HFR1 Functions Antagonistically with PIF1 in Seed Germination.

Supplemental Table 1. List of Primers Used in This Study.

Supplemental Data Set 1. Summary of the mRNA Sequencing Data Mapping Results.

Supplemental Data Set 2. List of Light-, HFR1-, and PIF1-Regulated Genes Identified in the mRNA Sequencing Analysis.

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AUTHOR CONTRIBUTIONS

H.S., S.Z., and X.W.D. designed the research. H.S., S.Z., and X.M. performed the experiments. N.L., H.S., and S.Z. conducted the bioinformatics analysis. C.D.N. constructed some clones and revised the article. S.Z., H.S., and X.W.D. analyzed the data. H.S., S.Z., and X.W.D wrote the article.

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