PIF4 and PIF5 Transcription Factors Link Blue Light and Auxin to Regulate the Phototropic Response in *Arabidopsis*[C][W][OPEN]

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Both blue light (BL) and auxin are essential for phototropism in *Arabidopsis thaliana*. However, the mechanisms by which light is molecularly linked to auxin during phototropism remain elusive. Here, we report that PHYTOCHROME INTERACTING FACTOR4 (PIF4) and PIF5 act downstream of the BL sensor PHOTOTROPIN1 (PHOT1) to negatively modulate phototropism in *Arabidopsis*. We also reveal that PIF4 and PIF5 negatively regulate auxin signaling. Furthermore, we demonstrate that PIF4 directly activates the expression of the *AUXIN/INDOLE-3-ACETIC ACID* (*IAA*) genes *IAA19* and *IAA29* by binding to the G-box (CACGTG) motifs in their promoters. Our genetic assays demonstrate that IAA19 and IAA29, which physically interact with AUXIN RESPONSE FACTOR7 (ARF7), are sufficient for PIF4 to negatively regulate auxin signaling and phototropism. This study identifies a key step of phototropic signaling in *Arabidopsis* by showing that PIF4 and PIF5 link light and auxin.

INTRODUCTION

Phototropism is a rapid and visually striking adaptation response by which plant shoots grow toward a light source (Whippo and Hangarter, 2006; Holland et al., 2009). In *Arabidopsis thaliana*, blue light (BL), the predominant signal for phototropism, is perceived by the phototropin photoreceptors PHOTOTROPIN1 (PHOT1) and PHOT2, with PHOT1 acting as the main phototropin (Huala et al., 1997; Christie et al., 1998; Briggs et al., 2001). The PHOT1-interacting protein NONPHOTOTROPIC HYPOCOTYL3 (NPH3), which acts as a substrate adapter in a CULLIN3-based E3 ubiquitin ligase that ubiquitinates PHOT1 (Roberts et al., 2011), is required for all PHOT1-dependent phototropic responses of *Arabidopsis* (Motchoulski and Liscum, 1999).

Initial studies on phototropism led to the discovery of the first plant hormone, auxin (Whippo and Hangarter, 2006). The central importance of auxin signaling and polar auxin transport in phototropism has been demonstrated genetically (Harper et al., 2000; Friml et al., 2002; Blakeslee et al., 2004; Tatematsu et al., 2004; Stone et al., 2008; Christie et al., 2011; Ding et al., 2011). The auxin response factor (ARF) transcription factors and the auxin/indole-3-acetic acid (Aux/IAA) family of auxin response repressors are encoded by two large gene families, with 23 and 29 members, respectively, in *Arabidopsis* (Remington et al., 2004). Among them, ARF7 (also named NPH4) and IAA19 (also named MSG2)

antagonistically regulate phototropism (Harper et al., 2000; Tatematsu et al., 2004). Moreover, lateral auxin redistribution, which is mediated by the PIN-FORMED and ATP BINDING CASSETTE B19 (ABCB19) auxin efflux carriers, is important for phototropism (Friml et al., 2002; Blakeslee et al., 2004; Nagashima et al., 2008; Ding et al., 2011). Recently, ABCB19 was identified as a substrate target of the photoreceptor kinase PHOT1 during *Arabidopsis* phototropism (Christie et al., 2011). However, the main links between light and auxin in phototropic signaling remain unclear.

Phytochrome interacting basic helix-loop-helix factors (PIFs) play a central role in light signaling during *Arabidopsis* photomorphogenesis (Leivar and Quail, 2011). In the nucleus, light-activated phytochromes interact with and trigger the degradation of several PIFs (Ni et al., 1998; Huq and Quail, 2002; Shen et al., 2005, 2007; Al-Sady et al., 2006). Recent studies revealed that PIFs also regulate seed germination (Oh et al., 2004), high-temperature response (Koini et al., 2009; Franklin et al., 2011; Kumar et al., 2012; Sun et al., 2012), shade avoidance (Lorrain et al., 2008; Li et al., 2012), and the circadian clock (Nusinow et al., 2011). Intriguingly, however, it remains to be determined whether PIFs play a regulatory role in phototropism.

Although it is well known that directional BL triggers the auxin-mediated phototropic response, it is unclear how light and auxin are linked in this process (Whippo and Hangarter, 2006). Here, we identify the bHLH transcription factors PIF4 and PIF5 as a missing link between light and auxin in the modulation of *Arabidopsis* phototropism and show that they directly regulate the expression of specific auxin response repressors, IAA19 and IAA29.

RESULTS

PIF4 and PIF5 Negatively Regulate Phototropism

To assess the potential role of PIF proteins in *Arabidopsis* phototropism, we examined the hypocotyl phototropism of the overexpressors

and knockout mutants of *PIF4* and *PIF5*. Significantly, we found that the reported *PIF4* or *PIF5* overexpressors (de Lucas et al., 2008) displayed a severely reduced phototropic response upon unilateral BL illumination (Figures 1A and 1B). Consistent with this, the *pif4*, *pif5*, and *pif4 pif5* double mutants, which were previously reported (de Lucas et al., 2008) or identified as null mutants in this study (see Supplemental Figure 1 online), showed an increased phototropic response (Figures 1A and 1B). The more pronounced phototropic response in the *pif4 pif5* double mutant (Figure 1B) demonstrated the redundant action of PIF4 and PIF5 in phototropism. Furthermore, phototropism time-course experiments revealed that PIF4 and PIF5 indeed negatively regulate hypocotyl phototropism in *Arabidopsis* (see Supplemental Figure 2 online). Considering that *35S-PIF4* and *pif4 pif5* seedlings exhibited contrasting hypocotyl growth phenotypes under normal growth conditions (de Lucas et al., 2008), we examined their hypocotyl growth rate under BL. For these experiments, 3-d-old dark-grown seedlings of *35S-PIF4* and *pif4 pif5* were transferred to continuous BL and hypocotyl growth rate was measured at different durations. The results indicated that, during the time windows of 0 to 8 h and 8 to 16 h after transfer to BL, the hypocotyl growth rate of *35S-PIF4* and *pif4 pif5* seedlings was comparable to that of the wild-type seedlings; in the time window of 16 to 24 h after transfer to BL, *35S-PIF4* seedlings showed a greater hypocotyl growth rate than that of the wild type and the *pif4 pif5* double mutant (see Supplemental Figure 3 online). Given that in our standard phototropic assays, the hypocotyl bending was examined within 8 h after 3-d-old dark-grown seedlings were transferred to BL (Liscum and Briggs, 1995), these observations indicate that it is very unlikely that the effect of PIF4 and PIF5 on hypocotyl phototropic response is due to their actions on the general hypocotyl elongation.

To examine the genetic interaction between PIF4, PIF5, and the main phototropic receptor, PHOT1, during phototropism, we identified a T-DNA insertional mutant, *phot1*, which shows markedly reduced expression of *PHOT1* (see Supplemental Figure 1 online). Using the single mutants identified in this study (see Supplemental Figure 1 online), we generated a *phot1 pif4 pif5* triple mutant to investigate whether PHOT1 is required for the enhanced phototropic phenotype of *pif4 pif5*. The *phot1* mutant and *phot1 pif4 pif5* triple mutant showed similar nonphototropic responses, whereas the *pif4 pif5* double mutant exhibited an enhanced phototropic phenotype (Figure 1C). These results suggest that PIF4 and PIF5 act genetically downstream of PHOT1-mediated BL perception during phototropic signaling.

BL Stimulates the Expression of *PIF4* and *PIF5*

To understand how PIF4 and PIF5 integrate information from BL to regulate phototropism, we examined the effect of BL on the expression of the two *PIF* genes. For these experiments, 3-d-old dark-grown seedlings of the wild type and *phot1 phot2* double mutant were exposed to BL for different amounts of time and the expression levels of *PIF4* and *PIF5* were quantified with quantitative real-time PCR (qRT-PCR) assays. As shown in Figures 2A and 2B, transcript levels of *PIF4* and *PIF5* were markedly elevated at 60 min upon BL treatment and reached a maximum at 120 min (Figures 2A and 2B), indicating that BL indeed stimulates the
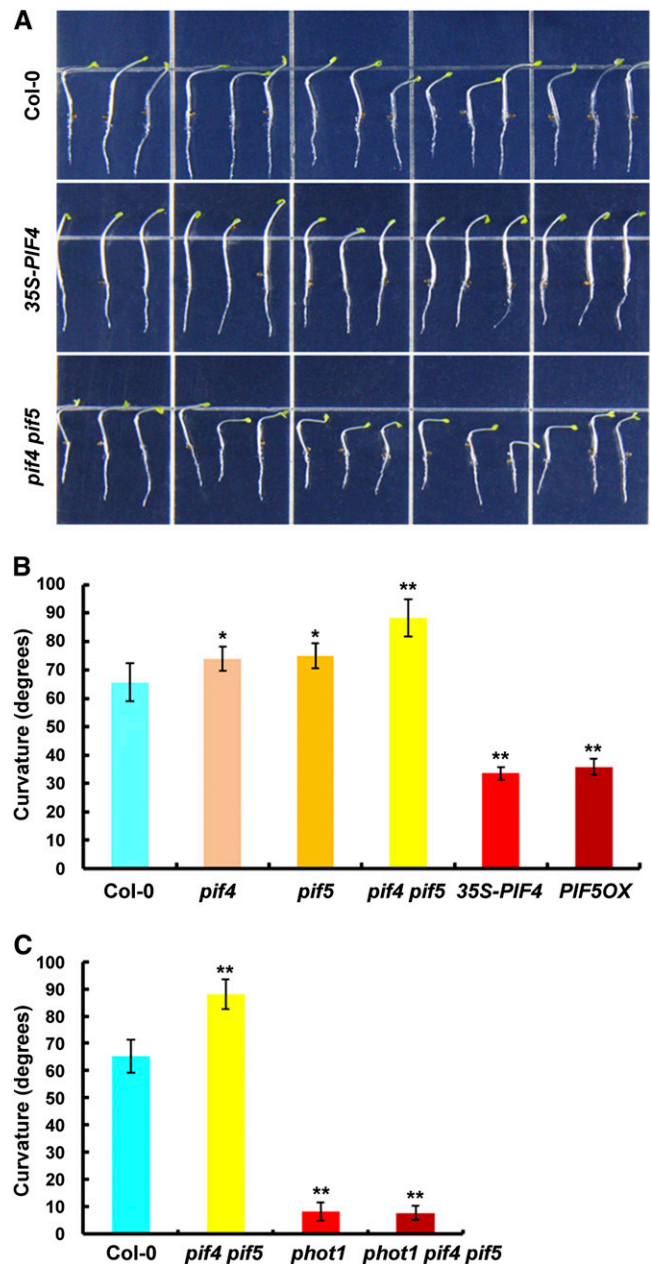

Figure 1. PIF4 and PIF5 Negatively Regulate Phototropism and Act Genetically Downstream of phot1 in Phototropic Signaling.

(A) Photos showing the phototropic phenotypes of Col-0, *35S-PIF4*, and *pif4 pif5* seedlings.
(B) and **(C)** Quantitative analysis of hypocotyl phototropic curvature of the indicated genotypes.
Three-day-old dark-grown seedlings of the indicated genotypes were illuminated with BL (2 µmol m^{-2} s^{-1}) for 8 h. These experiments were repeated five times, yielding similar results. Error bars represent SD (Student's *t* test compared with Col-0, *P < 0.05 and **P < 0.01).
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expression of *PIF4* and *PIF5* in wild-type seedlings. Significantly, we noted that the BL-mediated stimulation of *PIF4* and *PIF5* expression was substantially enhanced in the *phot1 phot2* double mutant (Figures 2A and 2B), demonstrating that PHOT1 and PHOT2 have a repressive role in BL-induced transcriptional expression of *PIF4* and *PIF5*. Considering that the phytochrome family of photoreceptors also perceive and respond to BL (Lau and Deng, 2010; Leivar and Quail, 2011), we compared BL-induced expression of the two *PIF* genes in the wild type and the *phyA* mutant (Ruckle et al., 2007). As shown in Supplemental Figure 4 online, the BL-mediated induction of *PIF4* and *PIF5* expression was substantially reduced by the *phyA* mutation, indicating a positive role for PhyA in BL-mediated induction of *PIF4* and *PIF5* expression. These results support that the phototropin photoreceptors and the phytochrome photoreceptors play distinct roles in BL-mediated induction of *PIF4* and *PIF5* expression.

To test whether BL also regulates the expression of PIF4 at the protein level, *35S:PIF4-HA* seedlings, which express a fusion of PIF4 to the hemagglutinin (HA) antigen (PIF4-HA) (de Lucas et al., 2008), were treated with continuous BL or a BL pulse and the accumulation of PIF4-HA was examined with immunoblotting (see

Methods). The results indicated that while continuous BL substantially increased the accumulation levels of PIF4-HA, a BL pulse had minor, if any, effects on the accumulation of this fusion protein at the time points investigated (see Supplemental Figure 4 online).

To spatially determine the BL-induced expression domains of *PIF4* and *PIF5*, we generated *PIF4 promoter:β-glucuronidase* (*pPIF4:GUS*) and *pPIF5:GUS* transgenic lines. Interestingly, GUS staining assays revealed that the BL-induced expression domains of *pPIF4:GUS* and *pPIF5:GUS* were restricted to the hypocotyl apex, where phototropic perception occurs (Figure 2C). These data suggest that *PIF4* and *PIF5* are BL-responsive genes that possibly form part of a complex negative-feedback regulatory loop that fine-tunes phototropism.

PIF4 and PIF5 Repress Auxin Signaling That Is Important for Phototropism

To determine whether the PIF4- and PIF5-mediated regulation of phototropism is associated with the auxin pathway, we visualized the differential auxin response in the hypocotyls of wild-type and *35S-PIF4* seedlings after unilateral BL illumination using an
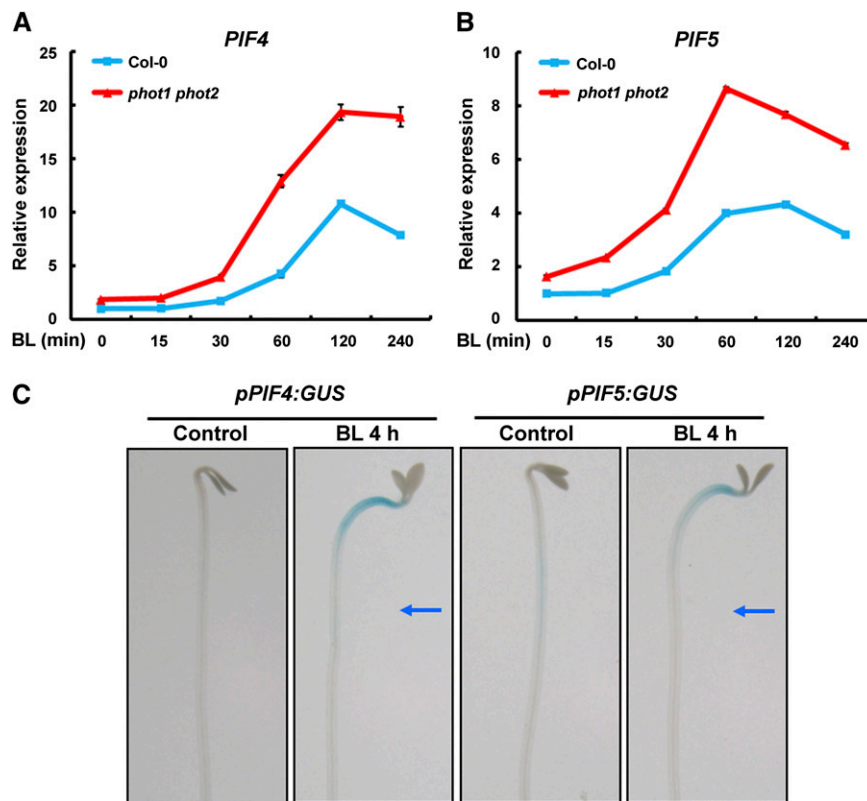

Figure 2. BL Stimulates the Transcriptional Expression of *PIF4* and *PIF5*.

(A) and **(B)** BL-induced expression of *PIF4* **(A)** and *PIF5* **(B)** revealed by qRT-PCR assays. Three-day-old dark-grown seedlings of the indicated genotypes were illuminated with BL (2 µmol m^{-2} s^{-1}) for different periods of time before tissues were collected for RNA extraction. The experiments were repeated three times, yielding similar results. Error bars represent SD.

(C) Expression patterns of *pPIF4:GUS* and *pPIF5:GUS* in response to unilateral BL illumination. Three-day-old dark-grown *pPIF4:GUS* and *pPIF5:GUS* seedlings were illuminated with unilateral BL (2 µmol m^{-2} s^{-1}) for 4 h before a GUS staining assay was performed. Arrows indicate direction of BL illumination. The experiments were repeated three times, yielding similar results.

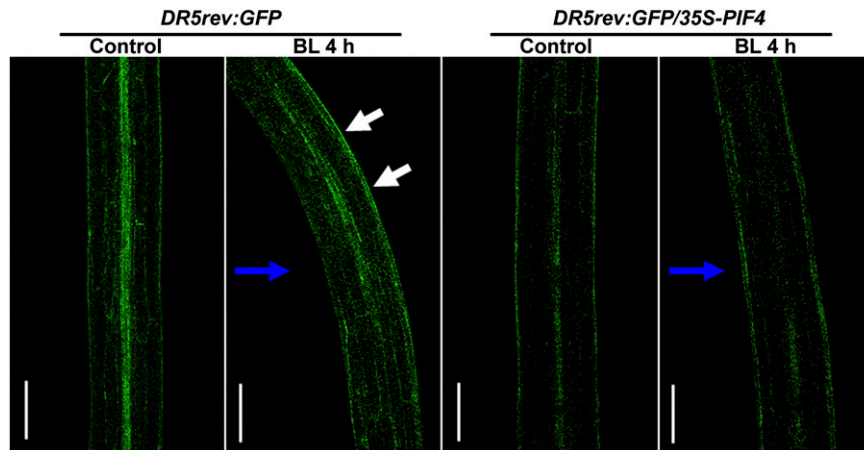[See online article for color version of this figure.]

Figure 3. PIF4-Mediated Modulation of Phototropism Is Associated with Auxin.

Expression analysis of *DR5rev:GFP* in 3-d-old dark-grown wild-type and *35S-PIF4* hypocotyls without or with 4 h of unilateral BL illumination (2 µmol m^{-2} s^{-1}). The experiments were repeated three times, yielding similar results. The blue arrows indicate the direction of BL illumination. The white arrows indicate the increased auxin response. Bars = 50 µm.

auxin-responsive marker, *DR5rev:GFP* (Sun et al., 2009). In line with previous reports that auxin accumulates at the shaded side of hypocotyls upon unilateral illumination (Friml et al., 2002; Esmon et al., 2006; Ding et al., 2011), our results showed increased *DR5* activity in the shaded side of wild-type *Arabidopsis* hypocotyls after

4 h of unilateral BL illumination (Figure 3). However, no asymmetric *DR5* activity was detected in the hypocotyls of *35S-PIF4* (Figure 3). Taken together, these results indicate that PIF4 represses the establishment of the differential auxin response, which is considered to be required for phototropic hypocotyl bending.
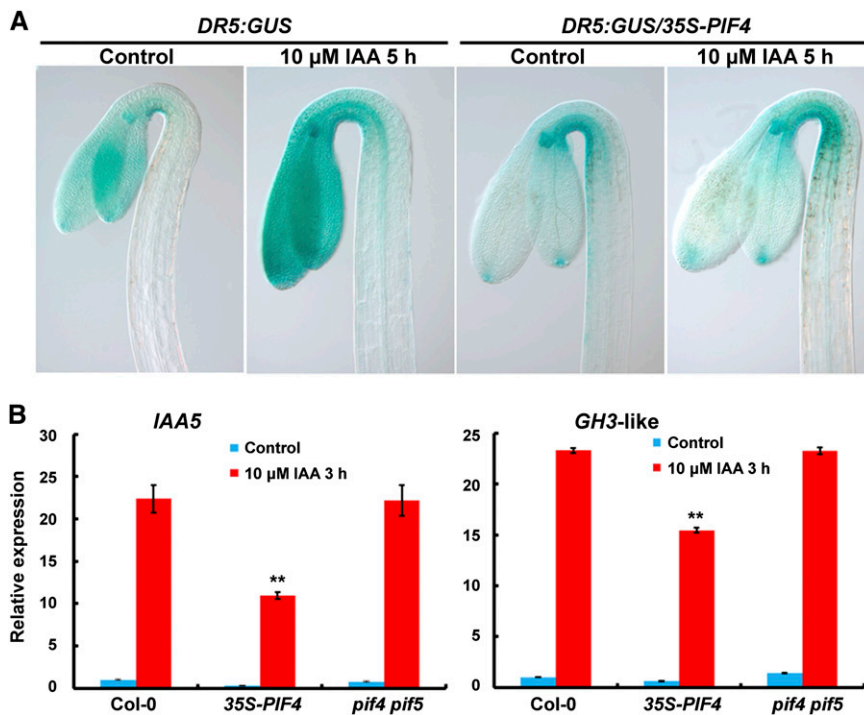

Figure 4. PIF4 and PIF5 Negatively Regulate Auxin Responses.

(A) Expression patterns of *DR5:GUS* in 3-d-old dark-grown wild type and *35S-PIF4* seedlings untreated or treated with 10 µM IAA for 5 h before GUS staining assay. **(B)** qRT-PCR assays for auxin-responsive gene expression in Col-0, *35S-PIF4*, and *pif4 pif5* seedlings. Three-day-old dark-grown seedlings of the indicated genotypes were treated with 10 µM IAA for 3 h before tissues were collected for RNA extraction. Values represent the relative expression level compared with that of untreated Col-0 seedlings. The experiments were repeated three times, yielding similar results. Error bars represent SD. Asterisks indicate significant differences between Col-0 and *35S-PIF4* plants after treatment according to Student's *t* test (** P < 0.01).
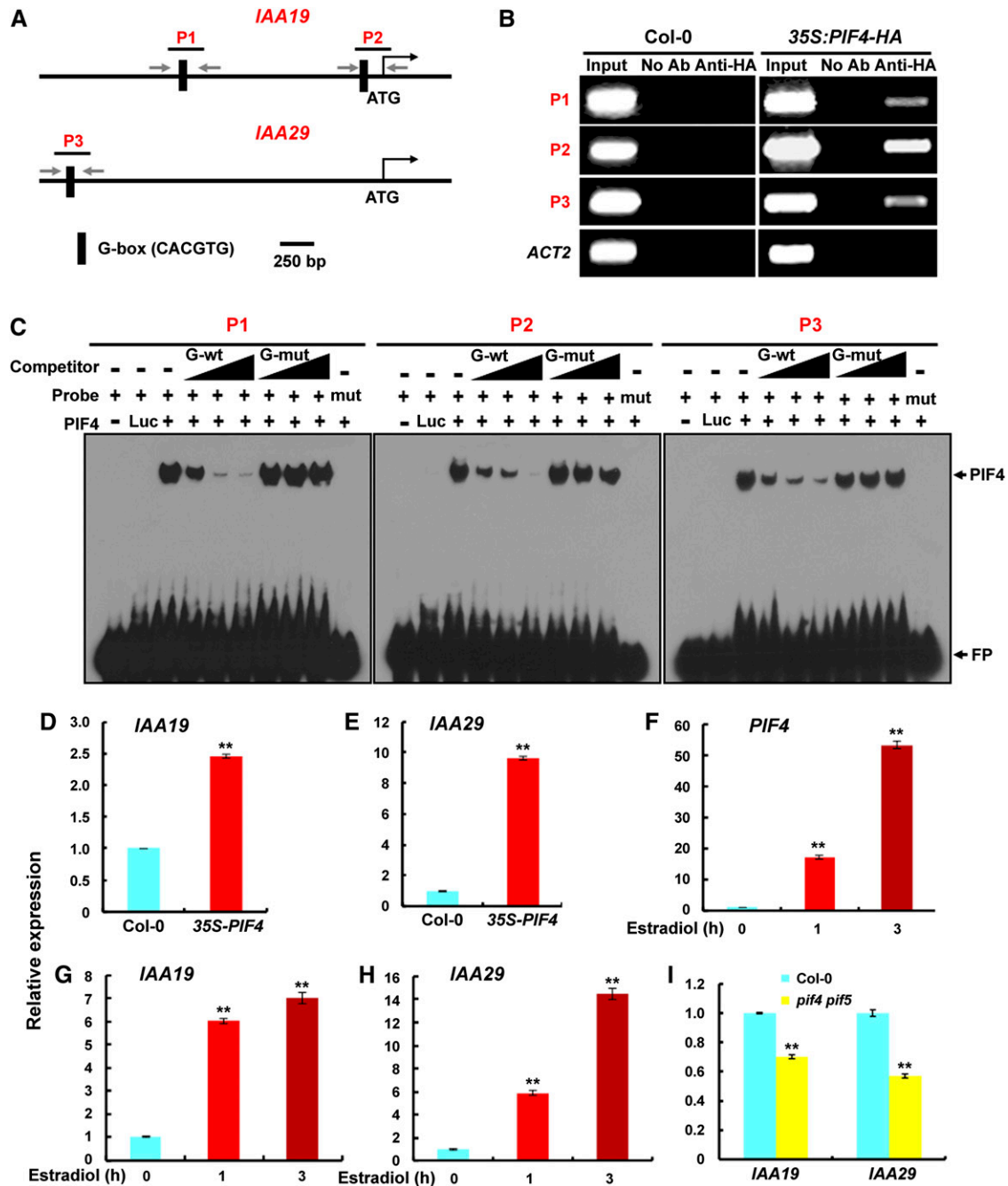
Figure 5. PIF4 Directly Activates Expression of *IAA19* and *IAA29*.

(A) Illustration of the *IAA19* and *IAA29* promoter regions showing the presence of G-box DNA motifs. Arrows indicate the positions of primers used in the ChIP-PCR experiment. P1-3, the DNA fragments with G-box motif.

(B) The amplified products from ChIP assays. ChIP assays were performed using the 8-d-old Col-0 and *35S:PIF4-HA* seedlings expressing the PIF4-HA fusion protein. DNA was amplified using primers specific for the *IAA19* and *IAA29* promoter regions containing the G-box elements or a control region in the *ACT2* promoter.

(C) EMSA showing that PIF4 binds to the G-box motifs present in the *IAA19* and *IAA29* promoters in vitro. The *IAA19* and *IAA29* promoter fragments containing the G-box motifs, as indicated in **(A)**, were incubated with in vitro TNT-expressed PIF4. Competition for PIF4 binding was performed with 10×, 20×, and 50× unlabeled *IAA19* and *IAA29* probes (G-wt) or G-box–mutated probes (G-mut). FP, free probe. Luc indicates in vitro–expressed luciferase proteins, which were used as controls. Mut indicates mutated probes.

(D) and **(E)** qRT-PCR analysis of *IAA19* and *IAA29* expression in the 6-d-old light-grown wild-type and *35S-PIF4* seedlings.

Our recent work demonstrated that the total free IAA levels of whole seedlings were elevated in *35S-PIF4* due to the PIF4-mediated activation of auxin biosynthesis (Sun et al., 2012). However, we showed here that the expression of *DR5:GUS* was reduced in the cotyledons of *35S-PIF4* compared with that in the wild type (Figure 4A). Thus, PIF4 may directly or indirectly repress auxin signaling. Indeed, three lines of evidence lend support to this hypothesis. First, we found that the response to auxin, as visualized by the auxin-induced expression of *DR5:GUS*, was largely reduced in *35S-PIF4* plants (Figures 4A and below), as it was in the auxin signaling mutants *msg2-1* and *arf7-1* (see Supplemental Figure 5 online). Second, qRT-PCR analyses revealed that the auxin-induced expression levels of *IAA5* and *GH3*-like, which are auxin-responsive marker genes, were reduced in the *35S-PIF4* seedlings (Figure 4B). Third, in line with a recent observation (Nozue et al., 2011), we showed that in 2,4-D–induced hypocotyl elongation assays, *pif4 pif5* seedlings were more sensitive than wild-type seedlings, whereas *35S-PIF4* seedlings were less sensitive than wild-type seedlings (see Supplemental Figure 6 online). These observations are consistent with recent transcriptome analyses showing that the expression of auxin-responsive genes was affected in the *pif4 pif5* double mutant (Nozue et al., 2011). Together, these data support that PIF4 and PIF5 negatively modulate auxin signaling and that this modulation is associated with the regulatory role of PIF4 and PIF5 on phototropism.

PIF4 Binds to the Promoters of *IAA19* and *IAA29* and Activates Their Expression

Given the above results that PIF4 and PIF5 negatively regulate auxin signaling and the knowledge that the *Aux/IAA* genes are negative regulators of auxin signaling (Mockaitis and Estelle, 2008), we hypothesized that PIF4 and PIF5 might activate the transcriptional expression of some *Aux/IAA* genes to repress auxin signaling. At the molecular level, PIFs bind to the G-box motifs (CACGTG) of their target promoters (Moon et al., 2008; Sun et al., 2012). Sequence analysis revealed that several *Aux/IAA* family genes in *Arabidopsis*, including *IAA19* and *IAA29* (Figure 5A), contain G-box (CACGTG) motifs in their promoters. To determine whether PIF4 associates with the promoters of *Aux/IAA* genes, we performed chromatin immunoprecipitation (ChIP) assays using the previously reported transgenic line *35S:PIF4-HA* (de Lucas et al., 2008) and anti-HA antibody (Abcam). Significantly, our assays showed that, among the G-box–containing and non-G-box–containing *Aux/IAA* family genes analyzed, only the *IAA19* and *IAA29* promoter regions were greatly

enriched in the ChIP assays (Figure 5B; see Supplemental Figure 7 online). These results demonstrate that PIF4 specifically associates with the promoters of *IAA19* and *IAA29*.

To further confirm the binding of PIF4 to the *IAA19* and *IAA29* promoters, we performed electrophoretic mobility shift assays (EMSAs) using in vitro–expressed PIF4. As shown in Figure 5C, PIF4 bound to the G-box (CACGTG)–containing DNA fragments present in the promoter regions of *IAA19* and *IAA29*. Furthermore, binding could be effectively competed for by the addition of excess amounts of unlabeled G-box–containing DNA probes (Figure 5C). As controls, we showed that unlabeled DNA probes consisting of *IAA19* and *IAA29* containing G-box–mutated (CACGGG) motifs failed to compete for the binding of PIF4 to the G-box–containing DNA fragments (Figure 5C). Parallel experiments indicated that PIF4 failed to bind labeled *IAA19* and *IAA29* probes containing G-box–mutated (CACGGG) motifs (Figure 5C). Together, these results reveal that PIF4 directly binds to the G-box motifs (CACGTG) of the *IAA19* and *IAA29* promoters.

Next, we examined whether PIF4 regulates the transcriptional expression of *IAA19* and *IAA29* in planta. qRT-PCR analyses showed that the transcript levels of *IAA19* and *IAA29*, but not of other *Aux/IAA* genes tested, were significantly elevated in the *35S-PIF4* seedlings relative to wild-type seedlings (Figures 5D and 5E; see Supplemental Figure 8A online). Moreover, the transcriptional expression of *IAA19* and *IAA29*, but not of the other *Aux/IAA* genes tested, was rapidly activated by the induced overexpression of *PIF4* (Figures 5F to 5H; see Supplemental Figure 8B online) in the chemical-inducible *pMDC7:PIF4* transgenic plants (Sun et al., 2012). Furthermore, the transcript levels of *IAA19* and *IAA29* were consistently markedly reduced in the *pif4 pif5* double mutants compared with those in the wild type (Figure 5I).

Taken together, we conclude that PIF4 activates the transcriptional expression of *IAA19* and *IAA29* through binding to their promoters. This finding provides a useful clue for deciphering the biological function of PIF4 and PIF5 in modulating auxin signaling and phototropism.

IAA19 and IAA29 Are Required for PIF4-Mediated Modulation of Auxin Signaling and Phototropism

To determine whether IAA19 and IAA29 are necessary and sufficient to mediate the PIF4- and PIF5-mediated modulation of auxin signaling and phototropism, we identified the *iaa19* and *iaa29* loss-of-function mutants from the SIGnAL T-DNA collection (Alonso et al., 2003), which showed no expression of the corresponding genes (see Supplemental Figure 1 online). Using these mutants, we generated the *DR5:GUS/35S-PIF4/iaa19 iaa29* line.

Figure 5. (continued).

(F) to **(H)** qRT-PCR analysis of *PIF4*, *IAA19*, and *IAA29* expression in 6-d-old *pMDC7:PIF4* seedlings treated with 10 μM estradiol for various periods of time.

(I) qRT-PCR assay showing that the expression of *IAA19* and *IAA29* was reduced in 3-d-old dark-grown *pif4 pif5* double mutant seedling compared with that in the wild type.

For **(D)** to **(I)**, the experiments were repeated three times, yielding similar results. Error bars represent sd. Asterisks indicate significant differences from Col-0, according to Student's *t* test, **P < 0.01.
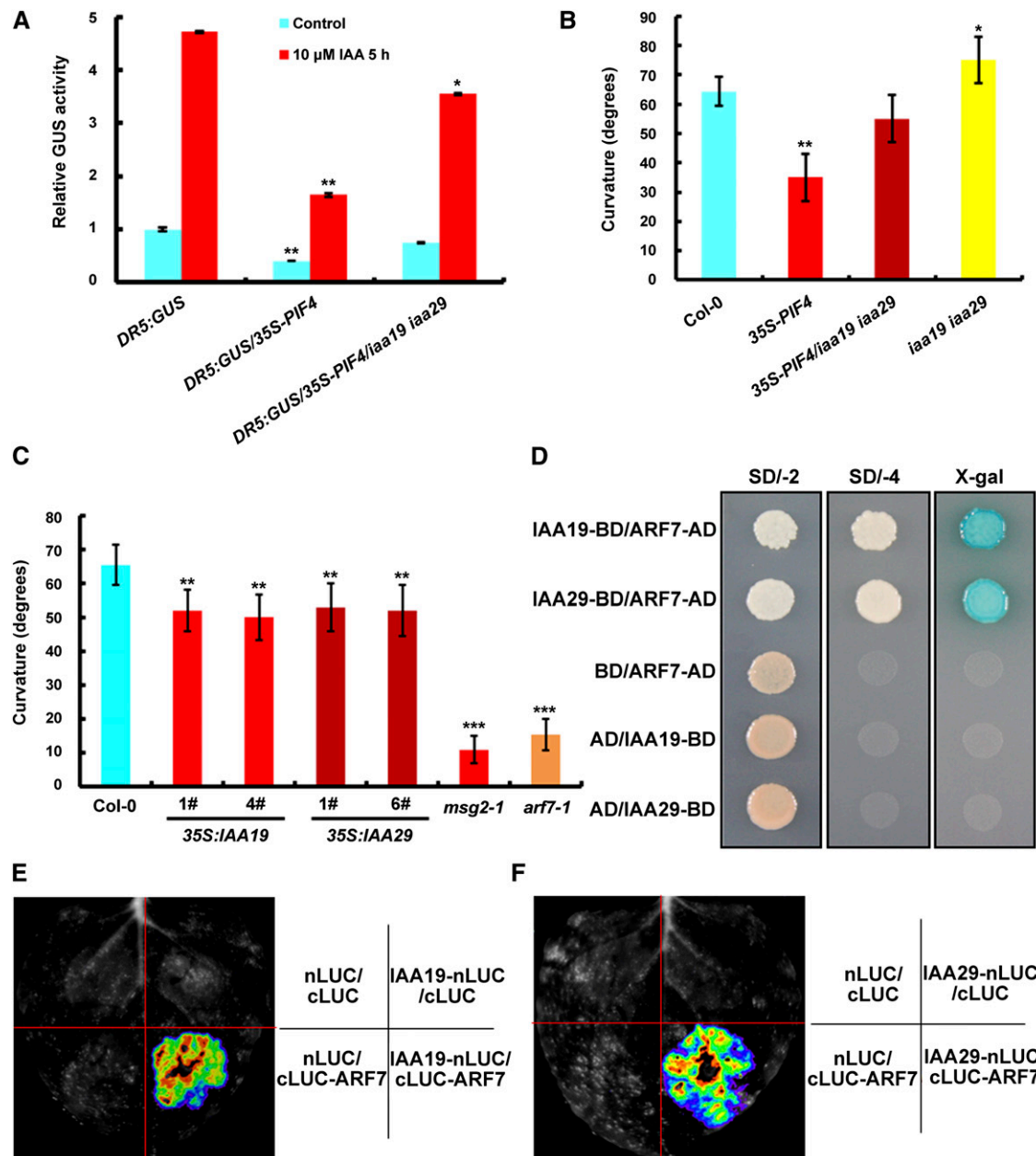
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Figure 6. IAA19 and IAA29 Are Required for PIF4-Mediated Modulation of Auxin Signaling and Phototropism.

(A) The *iaa19 iaa29* mutations suppress the reduced auxin responsiveness of *35S-PIF4* seedlings, as determined by a *DR5:GUS* expression analysis. Six-day-old seedlings were either mock treated with water or treated with 10 μM IAA for 5 h, and then GUS activity was measured using a 4-methylumbelliferyl-β-D-glucuronide assay. The experiments were repeated three times with similar results. Data represent one experiment. Error bars represent SD. Asterisks indicate significant differences between the wild type and other genotypes for control and treatment (Student's *t* test, *P < 0.05 and **P < 0.01).
(B) The *iaa19 iaa29* mutations were able to rescue the phototropic defect of *35S-PIF4* seedlings.
(C) Overexpression of *IAA19* or *IAA29* reduces the phototropic response to BL illumination.
For **(B)** and **(C)**, 3-d-old dark-grown seedlings of different genotypes were exposed to 8 h of unilateral BL illumination (2 μmol m^{-2} s^{-1}) and then photographs were taken for quantitative analysis of hypocotyl phototropic curvature. Data represent the mean response of a minimum of 30 seedlings for each genotype. The experiments were repeated three times, yielding similar results. Error bars represent SD. Asterisks indicate significant differences between the wild-type and other genotypes (Student's *t* test, *P < 0.05, **P < 0.01, and **P < 0.001).
(D) Interactions of IAA19 and IAA29 with ARF7 in the Y2H system. The transformants were streaked on SD/-Trp/-Leu (SD/-2) medium to examine growth. Protein–protein interactions were assessed on SD/-Ade/-His/-Trp/-Leu (SD/-4) medium and further confirmed by monitoring β-galactosidase activity.
(E) and **(F)** LCI assays showing that ARF7 interacts with IAA19 and IAA29 in *N. benthamiana*. *N. benthamiana* leaves were infiltrated with *Agrobacterium* strains containing the indicated construct pairs. The data were collected 72 h after infiltration.
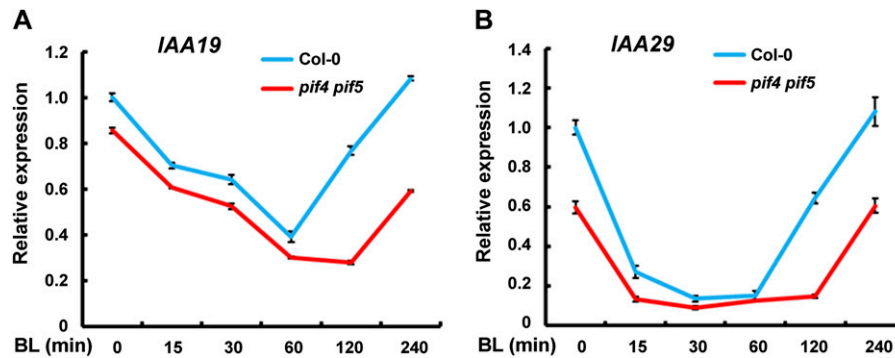
Figure 7. BL-Regulated Expression of *IAA19* and *IAA29* in the Col-0 and *pif4 pif5* Seedlings.

Three-day-old dark-grown seedlings of the indicated genotypes were illuminated with BL (2 μmol m^{-2} s^{-1}) for different periods of time before tissues were collected for RNA extraction and qRT-PCR assays. Values represent the expression levels of *IAA19* **(A)** and *IAA29* **(B)** relative to those of untreated Col-0 seedlings. These experiments were repeated three times, yielding similar results. Error bars represent SD.

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As described above, the auxin induction of *DR5:GUS* expression was largely abolished in the *35S-PIF4* line compared with that in the wild type (Figures 4A and 6A). Significantly, we showed that the auxin induction of *DR5:GUS* expression in the *35S-PIF4/iaa19 iaa29* lines was comparable to that in the wild type (Figure 6A), demonstrating that IAA19 and IAA29 are necessary for PIF4-mediated reduction of auxin response.

Whereas the *35S-PIF4* seedlings showed a marked reduction in phototropism, the *35S-PIF4/iaa19 iaa29* line exhibited a normal phototropic response (Figure 6B), suggesting that IAA19 and IAA29 are also required for the PIF4 function in phototropism. Taken together, our genetic data strongly support the notion that IAA19 and IAA29 are required for PIF4-mediated modulation of auxin signaling and phototropism.

IAA19 and IAA29 Negatively Regulate Phototropism

Previously, an analysis of the gain-of-function mutant *massugu2* (*msg2*) showed that IAA19 negatively regulates phototropism (Tatematsu et al., 2004). However, it is unknown whether IAA29 also negatively regulates phototropism. To test this experimentally, we generated transgenic plants overexpressing *IAA19* and *IAA29*, respectively. qRT-PCR analysis confirmed that the expression levels of *IAA19* or *IAA29* were indeed increased in these transgenic plants (see Supplemental Figure 9 online). As expected, the *35S:IAA19* and *35S:IAA29* plants displayed reduced hypocotyl phototropic responses compared with the wild type (Figure 6C). Consistent with this, the *iaa19 iaa29* double mutants displayed an increased phototropic response (Figure 6B). These results showed that IAA19 and IAA29 negatively regulate hypocotyl phototropism in *Arabidopsis*.

IAA19 and IAA29 Physically Interact with ARF7

Previous studies demonstrated that the transcriptional activator ARF7 is a positive regulator of phototropism in *Arabidopsis* (Harper et al., 2000) and that IAA19 physically interacts with and inhibits the activity of ARF7 (Tatematsu et al., 2004). Thus, we speculated that IAA29 might also physically interact with ARF7

and hinder its activity. Indeed, similar to the findings for IAA19 (Tatematsu et al., 2004), we showed that IAA29 binds to the C-terminal domain of ARF7 in a yeast two-hybrid assay (Figure 6D).

Interaction of ARF7 with IAA19 and IAA29 was confirmed with firefly luciferase (LUC) complementation imaging (LCI) assays (Song et al., 2011). For these experiments, *ARF7* was fused to the 3′ part of *LUC* to generate the cLUC-ARF7 construct, whereas *IAA19* was fused to the 5′ part of *LUC* to generate the IAA19-nLUC construct. When cLUC-ARF7 and IAA19-nLUC were co-infiltrated into *Nicotiana benthamiana* leaves, strong LUC activity was detected (Figure 6E), indicating that ARF7 interacts with IAA19 in vivo. Parallel experiments indicated that ARF7 also interacts with IAA29 in vivo (Figure 6F).

BL Regulates the Expression of *IAA19* and *IAA29*

The findings that IAA19 and IAA29 are involved in phototropic response raised the possibility that BL may regulate their expression. To test this hypothesis, 3-d-old dark-grown seedlings were exposed to BL for different lengths of time, and the transcript levels of *IAA19* and *IAA29* were examined by qRT-PCR. As shown in Figures 7A and 7B, the *IAA19* and *IAA29* expression showed a transient repression at 15 min upon BL illumination and reached a minimum at 60 min; their expression then showed a marked increase in the duration of the experiments. Importantly, the BL-induced expression levels of *IAA19* and *IAA29* were generally lower in the *pif4 pif5* double mutant than in the wild type (Figures 7A and 7B), indicating a positive effect of PIF4 and PIF5 on BL-mediated regulation of *IAA19* and *IAA29* expression. Parallel experiments revealed that the expression of *ARF7* is not regulated by BL or PIF4/PIF5 (see Supplemental Figure 10 online).

Given that auxin induces the expression of *IAA19* in an ARF7-dependent manner (Tatematsu et al., 2004), we asked whether ARF7 also plays a role in BL-mediated regulation of *IAA19* and *IAA29* expression. To address this question, 3-d-old dark-grown seedlings were illuminated with BL for different times, and the expression levels of *IAA19* and *IAA29* were compared between the wild type and *arf7-1*, a T-DNA insertion mutant that disrupts the expression of the *ARF7* gene (Okushima et al., 2005). Our

qRT-PCR assays revealed that the BL-induced expression levels of *IAA19* and *IAA29* were substantially lower in *arf7-1* than in the wild type (see Supplemental Figure 11 online), indicating that ARF7 is also required for BL-induced expression of *IAA19* and *IAA29*.

DISCUSSION

In this article, we discover that the PIF4 and PIF5 transcription factors play an important role in BL-mediated phototropic response. We show that BL stimulates the transcriptional expression of *PIF4* and *PIF5*. We also show that PIF4 and PIF5 bind to the promoter regions of *IAA19* and *IAA29* and positively regulate their expression. PIF4- and PIF5-mediated regulation of *IAA19* and *IAA29* expression therefore represents a signaling hub by which plants integrate environmental light stimuli into endogenous auxin signaling to modulate phototropism.

PIF4 and PIF5 Are Critical Components of BL-Mediated Phototropic Signaling

The findings that BL-induced phototropism in the hypocotyl can be enhanced by red light (RL)–mediated phytochrome signaling (Whippo and Hangarter, 2004) and that *Arabidopsis* PhyA physically interacts with PHOT1 at the plasma membrane (Jaedicke et al., 2012) suggest the existence of crosstalk between the two light signaling pathways. Here, we provide several lines of evidence showing that PIF4 and PIF5, which are well-known molecular components of phytochrome signaling (Leivar and Quail, 2011), also play an important role in BL-mediated phototropic signaling. First, reduced expression of *PIF4* and/or *PIF5* leads to enhanced phototropic response, whereas overexpression of *PIF4* or *PIF5* leads to reduced phototropic response (Figures 1A and 1B), indicating a negative role of PIF4 and PIF5 in regulating phototropism. Second, genetic analysis reveals that the action of PIF4 and PIF5 in regulating phototropic response is downstream of that of the BL receptor PHOT1 (Figure 1C). Third, BL stimulates the transcriptional expression of *PIF4* and *PIF5* in a PhyA-dependent manner (Figure 2; see Supplemental Figure 4 online), and this stimulation is negatively regulated by the BL receptors PHOT1 and PHOT2 (Figure 2). Together, these data support that PIF4 and PIF5 act downstream of PHOT1 and PHOT2 and negatively regulate BL-mediated phototropic response. Considering that the PIF transcription factors were originally identified as molecular components of RL-mediated phytochrome signaling (reviewed in Castillon et al., 2007; Lau and Deng, 2010; Leivar and Quail, 2011), these results support the notion that PIF4 and PIF5 serve as integrators of RL and BL signaling in regulating plant growth and development.

The finding that PIF4 and PIF5 are involved in BL-mediated phototropic signaling raised several significant questions needed to be addressed in future studies. For example, how is the BL-mediated signal information relayed from phototropins to the PIF transcription factors? It is well established that during phytochrome signaling, the RL signaling information is propagated to the transcriptional network through direct, physical interaction of the PIF transcription factors with the Phy photoreceptors (reviewed in Castillon et al., 2007; Lau and Deng, 2010; Leivar and Quail,
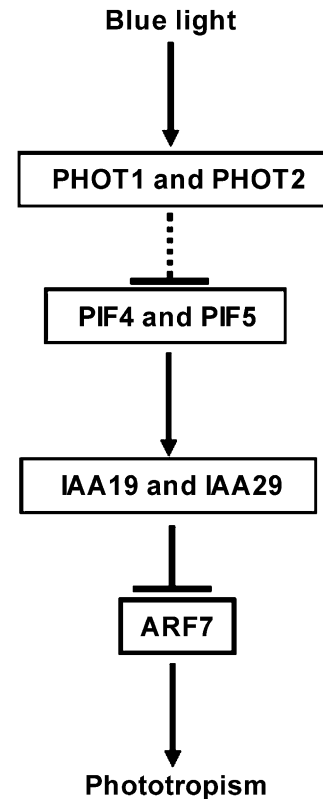

Blue light

PHOT1 and PHOT2

PIF4 and PIF5

IAA19 and IAA29

ARF7

Phototropism

Figure 8. A Molecular Framework of Phototropic Signaling.

PIF4 and PIF5 directly activate the expression of auxin response repressors *IAA19* and *IAA29*, which negatively regulate auxin response by repressing ARF7 activity. Thus, PIF4 and PIF5 play a negative role in the BL-induced hypocotyl phototropic response. Moreover, PHOT1 and PHOT2 have an inhibitory role in the transcriptional expression of PIF4 and PIF5.

2011). Therefore, it will be interesting in future studies to explore the possible interaction of PIF4 and PIF5 with the BL receptors PHOT1 and PHOT2. The finding that the PIF4 and PIF5 transcriptional factors are involved in phototropism also opens an avenue to investigate the transcriptional regulatory networks of BL-mediated phototropic signaling, which is important to understand the molecular and cellular mechanisms of phototropism but remain largely unexplored to date.

PIF4 and PIF5 Repress Auxin Signaling by Directly Activating *IAA19* and *IAA29* Expression

We also provide several lines of evidence that PIF4 and PIF5 repress auxin signaling during phototropic response. First, the *35S-PIF4* plants show reduced auxin-responsive gene expression (Figures 4A and 4B), suggesting that PIF4 and PIF5 negatively regulate auxin signaling. Second, PIF4 specifically binds to the promoters of *IAA19* and *IAA29* (Figures 5A to 5C) and activates the expression of the two auxin signaling repressors (Figures 5D to 5I). Third, genetic analyses revealed that IAA19 and IAA29 are required for the PIF4-mediated negative regulation of auxin signaling and phototropism (Figures 6A and 6B). Finally, we demonstrate that

both IAA19 and IAA29 physically interact with ARF7 (Figure 6D), which has been shown to be an important positive regulator of phototropism (Harper et al., 2000). Taken together, these data demonstrate that PIF4 and PIF5 repress auxin signaling through directly activating the transcriptional expression of *IAA19* and *IAA29* and thereby negatively modulate *Arabidopsis* phototropism (Figure 8). In support of our view, it was recently reported that PIF5 directly regulates the expression of *IAA29* (Hornitschek et al., 2012).

In contrast with this study showing that PIF4 represses auxin signaling during phototropic response, it was recently shown by us and others that PIF4 activates auxin biosynthesis during high-temperature-induced hypocotyl growth (Franklin et al., 2011; Sun et al., 2012). These seemingly paradoxical observations suggest that the molecular mechanisms by which PIF4 regulates the auxin pathway are complex. It is reasonable to speculate that, in response to different environmental cues, plants employ PIF4 to differentially modulate the auxin pathway to achieve adaptive growth. Indeed, we noticed that in *35S-PIF4* seedlings, while the expression of *DR5:GUS* is increased at the base of the hypocotyls (Sun et al., 2012), the expression of this auxin-responsive marker was reduced in the cotyledons (Figure 4A).

The finding that PIF4 and PIF5 negatively modulate auxin-mediated phototropism through directly activating *IAA19* and *IAA29* transcription is reminiscent of the case in which a primary cytokinin response transcription factor, ARR1, directly activates the expression of *IAA3* (also named *SHY2*) to negatively regulate the auxin responses during root meristem development in *Arabidopsis* (Dello Ioio et al., 2008). These observations demonstrate that the simple Aux/IAA regulatory modules act as central signaling hubs to integrate environmental and endogenous cues into specific developmental programs.

METHODS

Plant Materials and Growth Conditions

The transgenic and mutant lines used in this study were previously described: *35S-PIF4*, *35S:PIF4-HA*, PIF5OX (*35S:PIF5-HA*), *pif4-2*, and *pif4 pif5* (*pif4-101 pif5-1*) (de Lucas et al., 2008); *msg2-1* (Tatematsu et al., 2004); *arf7-1* (Okushima et al., 2005); *phot1 phot2* (*phot1-5 phot2-1*) (Kinoshita et al., 2001); *phyA* (SALK_ 014575) (Ruckle et al., 2007); *DR5: GUS* and *DR5rev:GFP* (Sun et al., 2009); *pMDC7:PIF4* and *DR5:GUS/35S-PIF4* (Sun et al., 2012). The *pif4* (SALK_140393), *pif5* (SALK_087012), *iaa19* (SALK_034924), *iaa29* (SALK_091933), and *phot1* (SALK_088841) lines were identified from the SIGnAL T-DNA collection (Alonso et al., 2003). The *phot1 pif4 pif5* triple mutants were generated by genetic crossing using the *phot1* (SALK_088841), *pif4* (SALK_140393), and *pif5* (SALK_087012) single mutants.

Arabidopsis thaliana seeds were surface sterilized with 10% bleach for 10 min and washed three times with sterile water. Sterilized seeds were then suspended in 0.2% agarose and plated on Murashige and Skoog medium. Plants were vernalized in darkness for 3 d at 4°C and then transferred to a phytotron set at 22°C with a 16-h-light/8-h-dark cycle.

Phototropic Hypocotyl Bending Analysis

Phototropic hypocotyl bending curvatures were measured as described previously for dark-grown *Arabidopsis* seedlings (Liscum and Briggs, 1995). Three-day-old dark-grown seedlings on vertical plates were unilaterally

illuminated with 2 µmol m^{-2} s^{-1} BL from a light-emitting diode (Wan et al., 2012). Images were recorded with a digital camera at specific time points. Hypocotyl bending curvatures were measured using ImageJ software. All the data points are the average of at least 30 seedlings. Similar results were seen in at least three different experiments.

Expression Analysis of *DR5rev:GFP*

Three-day-old dark-grown seedlings of *DR5rev:GFP* and *DR5rev:GFP* in the *35S-PIF4* background were illuminated with unilateral BL (2 µmol m^{-2} s^{-1}) for 4 h or not before observation. The GFP fluorescence of hypocotyls was imaged under a Leica confocal laser scanning microscope (Leica Microsystems).

Molecular Techniques

All molecular manipulations were performed according to standard methods (Sambrook and Russell, 2001). A 2.0-kb genomic fragment upstream of the *PIF4* or *PIF5* translation start codon was amplified by PCR and cloned into the *AscI-PacI* sites of the binary vector pMDC162 (Curtis and Grossniklaus, 2003), resulting in the transcriptional fusion of the *PIF4* or *PIF5* promoter with the GUS coding region. *IAA19* and *IAA29* cDNAs were amplified by PCR from the reverse transcription products. Then, these cDNAs were cloned using the pENTR Directional TOPO cloning kit and recombined with the binary vector pGWB2 (Nakagawa et al., 2007) to generate the *35S:IAA19* and *35S:IAA29* constructs. These constructs were then transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90), which was used for transformation of *Arabidopsis* plants by vacuum infiltration (Bechtold and Pelletier, 1998).

Gene Expression Analysis

For qRT-PCR analysis, seedlings were harvested and frozen in liquid nitrogen for RNA extraction. RNA extraction and qRT-PCR analysis were performed as previously described (Sun et al., 2009). See Supplemental Table 1 online for a list of primers used. GUS staining assays were performed as previously described (Sun et al., 2009).

Immunoblot Analysis

For PIF4-HA immunoblot analysis, 3-d-old dark-grown *35S:PIF4-HA* seedlings were treated with continuous BL or a BL pulse and then were harvested for protein extraction. PIF4-HA fusion proteins were visualized by immunoblots using the anti-HA antibody (Abmart). Ponceau S–stained membranes were shown as loading controls.

ChIP-PCR Assay

One gram of 8-d-old Columbia-0 (Col-0) and *35S:PIF4-HA* transgenic seedlings and the anti-HA antibody (Abcam) were used in ChIP experiments. ChIP assays were performed as previously described (Sun et al., 2012). The enrichment of DNA fragments was determined by PCR analysis.

EMSA

PIF4 and LUC were synthesized using the TNT® SP6 High-Yield Wheat Germ Protein Expression System (Promega) (Sun et al., 2012). The 60-bp *IAA19* and *IAA29* promoter probes containing G-box motifs were synthesized and labeled with biotin at the 3′ end (Invitrogen). Unlabeled competitor probes were generated from dimerized oligos of the *IAA19* and *IAA29* promoter regions containing the G-box motifs. EMSAs were performed as described. Probe sequences are shown in Supplemental Table 1 online.

Yeast Two-Hybrid Assay

Full-length coding sequences of *IAA19* and *IAA29* were PCR amplified from reverse transcription product with gene-specific primers (see Supplemental Table 1 online). The resulting PCR products were cloned into the *Eco*RI and *Bam*HI sites of the pGBKT7 vector. The 3′ part of *ARF7* was also PCR amplified from reverse transcription product with gene-specific primers (see Supplemental Table 1 online) and cloned into the *Nde*I and *Bam*HI sites of the pGADT7 vector. Yeast two-hybrid assays were based on the Matchmaker GAL4 two-hybrid system (Clontech). Constructs were cotransformed into the yeast strain *Saccharomyces cerevisiae* AH109. The presence of the transgenes was confirmed by growth on SD/-Leu/-Trp plates. To assess protein interactions, the transformed yeast was suspended in liquid SD/-Leu/-Trp medium and cultured to OD = 1.0. Five microliters of suspended yeast was spread on plates containing SD/-Ade/-His/-Leu/-Trp/X-α-Gal (4 mg/mL) medium. Interactions were observed after 3 d of incubation at 30°C. The experiments were repeated three times with similar results.

LCI Assay

The LCI assays were performed in *Nicotiana benthamiana* leaves as previously described (Song et al., 2011). Primers used for the vector construction are shown in Supplemental Table 1 online. The experiments were repeated three independent biological replicates.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: ACTIN7 (At5g09810), PIF4 (At2g43010), PIF5 (At3g59060), PHOT1 (At3g45780), PHOT2 (At5g58140), IAA19 (At3g15540), IAA29 (At4g32280), and ARF7 (At5g20730).

Supplemental Data

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

Supplemental Figure 1. Molecular Identification of the SALK Mutants Used in This Study.

Supplemental Figure 2. Kinetics of Phototropic Response of *35S-PIF4* and *pif4 pif5* Lines.

Supplemental Figure 3. Comparison of Hypocotyl Growth of Col-0, *35S-PIF4*, and *pif4 pif5* Seedlings.

Supplemental Figure 4. BL-Mediated Regulation of *PIF4* and *PIF5*.

Supplemental Figure 5. The *msg2-1* and *arf7-1* Mutants Show Reduced Responses to Exogenous IAA Treatment, as Visualized by *DR5*:*GUS* Expression.

Supplemental Figure 6. Auxin Responses of *pif4 pif5* and *35S-PIF4* Plants in Hypocotyl Elongation.

Supplemental Figure 7. ChIP Assays Showing That PIF4 Does Not Associate with the Promoters of Other *IAA* Genes.

Supplemental Figure 8. Expression Patterns of Several *Aux/IAA* Genes in the *35S-PIF4* and *pMDC7:PIF4* Seedlings.

Supplemental Figure 9. Expression Levels of *IAA19* and *IAA29* in the *35S:IAA19* and *35S:IAA29* Transgenic Plants.

Supplemental Figure 10. BL-Induced Expression of *ARF7* in Col-0 and the *pif4 pif5* Double Mutant.

Supplemental Figure 11. BL-Induced Expression of *IAA19* and *IAA29* in Col-0 and *arf7-1*.

Supplemental Table 1. List of the Primers and Probes Used in This Study.

AUTHOR CONTRIBUTIONS

J.S. and C.L. conceived and designed the experiments. J.S., L.Q., Y.L., and Q.Z. performed the experiments. J.S. and C.L. analyzed the data and wrote the article.

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