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PIL5, a Phytochrome-Interacting Basic Helix-Loop-Helix Protein, Is a Key Negative Regulator of Seed Germination in *Arabidopsis thaliana* [W]

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The first decision made by an angiosperm seed, whether to germinate or not, is based on integration of various environmental signals such as water and light. The phytochromes (Phys) act as red and far-red light (Pfr) photoreceptors to mediate light signaling through yet uncharacterized pathways. We report here that the PIF3-like 5 (PIL5) protein, a basic helix-loop-helix transcription factor, is a key negative regulator of phytochrome-mediated seed germination. PIL5 preferentially interacts with the Pfr forms of Phytochrome A (PhyA) and Phytochrome B (PhyB). Analyses of a *pil5* mutant in conjunction with *phyA* and *phyB* mutants, a *pif3 pil5* double mutant, and *PIL5* overexpression lines indicate that PIL5 is a negative factor in Phy-mediated promotion of seed germination, inhibition of hypocotyl negative gravitropism, and inhibition of hypocotyl elongation. Our data identify PIL5 as the first Phy-interacting protein that regulates seed germination.

INTRODUCTION

As sessile organisms, plants must carefully integrate various environmental signals for optimization of growth and development. Among these signals, light plays a prominent role not only as an energy source, but also as a key environmental signal regulating various aspects of the plant life cycle. At least three photoreceptor systems, consisting of cryptochromes (Crys), phytochromes (Phys), and phototropins (Phots), are responsible for sensing light conditions and initiating various plant light responses (Fankhauser and Staiger, 2002). These photoreceptor systems differ in their abilities to perceive specific wavelengths and initiate specific light responses (Sullivan and Deng, 2003). Phytochromes perceive the red and far-red regions of the spectrum (Quail et al., 1995; Neff et al., 2000), cryptochromes perceive the UV-A/blue regions (Ahmad and Cashmore, 1993; Lin et al., 1995), and phototropins perceive the blue region of the spectrum (Huala et al., 1997).

In *Arabidopsis thaliana*, five phytochromes (PhyA, PhyB, PhyC, PhyD, and PhyE), two cryptochromes (cry1 and cry2), and two phototropins (phot1 and phot2) constitute the three photoreceptor systems (Quail, 1998; Cashmore et al., 1999; Briggs and Christie, 2002). The five Arabidopsis phytochromes can be classified into the light-labile PhyA (type I) protein and the light-stable PhyB to PhyE (type II) proteins. Regardless of their light stability, all phytochromes exist as two interconvertible spectral forms: a red light–absorbing (Pr) form and a far-red–light-absorbing (Pfr) form. Phytochromes are translocated to the nucleus in response to light. The translocation to the nucleus, however, differs among the phytochromes. In the case of PhyA, translocation to the nucleus is irreversibly induced by both red and far-red light; by contrast, translocation of type II phytochromes is induced by red light and reversed by far-red light (Nagy et al., 2000; Kircher et al., 2002). The irreversible translocation of PhyA to the nucleus by both red and far-red light and the subsequent degradation of PhyA in red light is thought to make PhyA a far-red light receptor, whereas the translocation of type II phytochromes only by red light suggests that PhyB to phyE act as the red light receptors (Sakamoto and Nagatani, 1996; Kircher et al., 1999; Sharrock and Clack, 2002).

Once translocated to the nucleus, phytochromes trigger various transcription cascades that ultimately lead to regulation of various physiological processes such as seed germination, hypocotyl elongation, cotyledon opening and expansion, anthocyanin accumulation, and chloroplast development (Chory et al., 1996; Neff et al., 2000; Sullivan and Deng, 2003). Previous studies have shown that transcription factors constitute a large portion of the light-signaling components. Among the identified transcription factors, *COG1* encodes a DOF family member, *HY5* and *HYH* encode basic domain/leucine zipper family members, *LAF1* encodes a MYB family member, and *HFR1*, *PIF3*, *PIF4*, and *PIL1* encode basic helix-loop-helix (bHLH) family members (Chattopadhyay et al., 1998; Ni et al., 1998; Fairchild et al., 2000; Ballesteros et al., 2001; Holm et al., 2002; Huq and Quail, 2002; Kim et al., 2003; Park et al., 2003; Salter et al., 2003). The roles of these factors in light signaling are diverse, ranging from modulation of cell elongation to regulation of secondary metabolism. The phytochromes appear to regulate transcription cascades at least partly by directly interacting with some transcription factors, including PIF3 and PIF4 (Ni et al., 1999; Huq and Quail, 2002). Alternatively, PHY-mediated signaling

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regulates levels of these transcription factors either at the transcriptional level (*COG1*, *HFR1*, *HY5*, *PIL1*, and *PIF4*) or at the protein level (HY5, HYH, LAF1, and PIF3) (Oyama et al., 1997; Fairchild et al., 2000; Holm et al., 2002; Huq and Quail, 2002; Park et al., 2003, 2004; Salter et al., 2003; Seo et al., 2003; Yamashino et al., 2003; Bauer et al., 2004).

Molecular and genetic investigations have indicated that a set of bHLH transcription factors (PIL1, PIF3, PIF4, and HFR1) play important roles in PHY-mediated light signaling. Of these bHLH transcription factors, PIF3 was the first characterized bHLH transcription factor that preferentially binds to the Pfr forms of both PhyA and PhyB (Ni et al., 1999). Functionally, PIF3 negatively regulates PhyB-mediated inhibition of hypocotyl elongation, PhyB-mediated cotyledon expansion, and Phy-mediated cotyledon opening, whereas it positively regulates anthocyanin accumulation (Kim et al., 2003). Analyses of other bHLH transcription factors revealed that HFR1 is a positive regulator of PhyA-mediated inhibition of hypocotyl elongation, induction of CAB genes. and inhibition of hypocotyl negative gravitropism (Fairchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000), whereas PIF4 is a negative regulator of PhyB-mediated inhibition of hypocotyl elongation and inhibition of cotyledon expansion (Huq and Quail, 2002). Recent analysis of PIL1 also indicated that shade avoidance is also regulated by a bHLH transcription factor (Salter et al., 2003).

Database analysis indicated that six out of 147 bHLH proteins have PIL (PIF3-like) domains at their N termini (Yamashino et al., 2003); these PIL family members were designated PIL1, PIL2, PIF3, PIF4, PIL5, and PIL6. Expression analyses indicated that some of the PIL family members were regulated by light. For example, expression levels of *PIL1* were dramatically reduced in response to white light, whereas expression levels of *PIF4* and *PIL6* were increased under the same conditions (Yamashino et al., 2003). Because homologous proteins tend to have similar roles, it is tempting to speculate that the uncharacterized *PIL* family members might also play roles in Phy-mediated light signaling.

In this report, we functionally characterized the roles of *PIL5* in Phy-mediated light signaling. We showed that PIL5 localizes in the nucleus and preferentially interacts with the Pfr forms of both PhyA and PhyB. By analyzing *PIL5* overexpression lines and a *pil5* mutant in conjunction with *phyA* or *phyB* mutants, we showed that *PIL5* is a key negative component in Phy-mediated promotion of seed germination, PhyA-mediated inhibition of hypocotyl negative gravitropism, and Phy-mediated inhibition of hypocotyl elongation. Our analyses identify PIL5 as the first phytochrome-interacting protein shown to negatively regulate seed germination and inhibition of hypocotyl negative gravitropism.

RESULTS

PIL5 Is a New Member of the Nuclear Localized Phy-Interacting bHLH Transcription Factors

PIL5 is a bHLH transcription factor that has a PIL domain at its N terminus (Yamashino et al., 2003). Because two factors in this family (PIF3 and PIF4) were previously shown to directly interact with phytochromes (Ni et al., 1999; Huq and Quail, 2002), we examined whether PIL5 could also interact with phytochromes. As shown in Figure 1A, glutathione *S*-transferase (GST)-tagged PIL5 preferentially interacted with the Pfr forms of PhyA and PhyB. In a control experiment, GST-tagged PIF3 also preferentially interacted with the Pfr form of both PhyA and PhyB, whereas GST alone did not (Figure 1B).

The predicted subcellular localization of PIL5 is in the nucleus (http://www.psort.org). To experimentally confirm this predicted subcellular localization, we fused the coding region of *PIL5* to the β-glucuronidase (*GUS*) reporter gene and transfected this construct into onion epidermal cells by particle bombardment

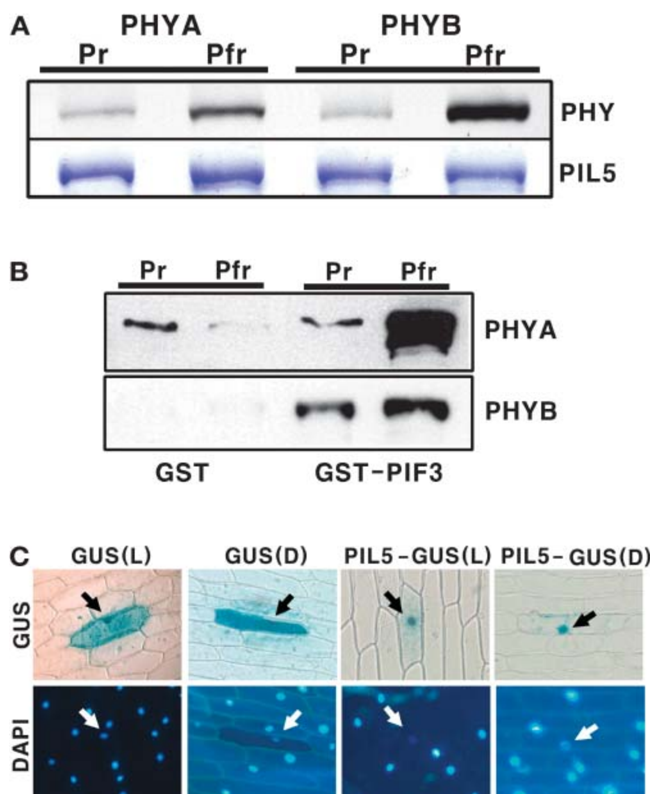

Figure 1. PIL5 Is a New Member of the Nuclear Localized Phytochrome-Interacting bHLH Protein Family.

(A) In vitro binding assay between phytochromes (PhyA and PhyB) and PIL5. PhyA and PhyB were preirradiated with red (20 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or far-red (3.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) light for 15 min before incubation with PIL5 at 4°C in the dark. After incubation, glutathione sepharose bead-bound proteins were pelleted and analyzed by protein gel blotting using anti-PhyA and PhyB antibodies. Coomassie blue–stained PIL5 was used as the loading control.

(B) Positive and negative controls of the in vitro binding assay. GST-tagged PIF3 (positive control) interacted with both PhyA and PhyB, whereas GST alone (negative control) did not.

(C) PIL5 is localized in the nucleus. A *GUS*-fused *PIL5* gene (*PIL5-GUS*) was introduced into onion epidermal cells by particle bombardment. The cells were incubated for 16 h either in the dark (D) or under white light (L) and localization of PIL5-GUS was visualized by GUS assay. Nuclei of onion cells were visualized by DAPI staining.

method. As shown in Figure 1C, the resulting PIL5-GUS fusion protein was localized in the nucleus and its localization was not changed by different light conditions. Taken together, these results indicate that PIL5 is a new member of the nuclear localized phytochrome-interacting bHLH proteins.

PIL5 Is a Negative Component in Phy-Mediated Inhibition of Hypocotyl Elongation

To examine the functional role of PIL5 in Phy-mediated light signaling, a T-DNA–inserted *pil5* mutant (*pil5-1*, salk_072677) was analyzed. The T-DNA was inserted in the second exon, resulting in deletion of the C-terminal 335 amino acids of PIL5, including the bHLH domain (Figure 2A). RT-PCR analysis confirmed that there was no full length *PIL5* gene expression in the *pil5* mutants. Phenotype analysis in the F2 population indicated that a recessive mutant phenotype is cosegregated with a T-DNA (Figure 2B).

Previous analyses had shown that PIF3 acts as a negative component in PhyB- but not PhyA-mediated inhibition of the hypocotyl elongation process (Kim et al., 2003). To investigate whether PIL5 plays a similar role in the hypocotyl elongation process, we measured the hypocotyl lengths of *pil5* mutants grown in the dark, under 12-h red light cycle (12R:12D cycle) or under 12-h far-red light cycle (12FR:12D cycle). As shown in Figure 2C, there were no significant length differences in hypocotyls of wild-type and *pil5* seedlings grown in the dark or under red light. However, under far-red light, the *pil5* mutants had shorter hypocotyls than wild type. Hypocotyl length of *pil5 phyA* double mutant indicated that *phyA* is epistatic to *pil5* (Figure 2D). These results suggest that PIL5 is a negative component in PhyA- but not PhyB-mediated inhibition of hypocotyl elongation.

Because PIF3 and PIL5 are homologous proteins, it is possible that they could be functionally redundant, and thus the phenotypes observed in each mutant might not fully reflect their respective roles. To examine the role of PIL5 in the absence of PIF3, we generated a *pil5 pif3* double mutant by crossing *pif3-1* and *pil5-1* plants. The hypocotyl lengths of the double mutants were measured after growing under various light conditions. All plants had similar hypocotyl lengths in the dark (Figure 2E). When grown under red light, the *pil5 pif3* double mutant had a short hypocotyl length similar to that of the *pif3* single mutant, whereas the *pil5 pif3* double mutant grown under far-red light had short hypocotyl length similar to that of the *pil5* single mutant. These results suggest that the hypocotyl phenotypes of *pil5* and *pif3* under specific monochromatic light conditions are not attributable to reciprocal compensation.

Although the mutant analyses suggested that PIL5 is a negative component in PhyA- but not PhyB-mediated inhibition of the hypocotyl elongation process, this does not necessarily indicate that PIL5 cannot function in PhyB-mediated inhibition of hypocotyl elongation. To determine whether PIL5 can also regulate hypocotyl elongation under red light, we generated *PIL5* overexpression lines that expressed the full-length *PIL5* coding sequence under the control of the 35S promoter of *Cauliflower mosaic virus* (Figure 2F). Two independent overexpression lines (*PIL5OX1* and *PIL5OX2*) expressing high levels of the *PIL5* transcript were used for further analyses. Figure 2F shows that the *PIL5* overexpressing transgenic seedlings had longer hypocotyls than wild-type seedlings under both red and far-red light, suggesting that PIL5 could act as a negative component in both PhyA- and PhyB-mediated inhibition of hypocotyl elongation. This is in contrast with the case of the *PIF3* overexpressing transgenic seedlings, which had longer hypocotyls under red light, but wild-type-like hypocotyls under far-red light (Kim et al., 2003). This different response to far-red light in the *PIL5OX* and *PIF3OX* plants was not attributable to differing experimental conditions because these results were reproducibly observed even on the same agar plate (data not shown). Taken together, these data imply that PIF3 and PIL5 behave differently in PhyA-mediated inhibition of the hypocotyl elongation.

PIL5 Is a Negative Component in Phy-Mediated Promotion of Seed Germination

During our measurements of hypocotyl lengths, we noticed that the *PIL5* overexpression lines did not germinate well if the plates were kept in the dark. Because all seeds germinated well under red and white light conditions, this phenomenon suggested that PIL5 affects seed germination only under certain light conditions. Previous reports indicated that PhyA and PhyB play key roles in regulating seed germination under different light conditions (Shinomura et al., 1994, 1996; Botto et al., 1996; Poppe and Schafer, 1997). Because PIL5 is a Phy-interacting protein, we tested whether phytochromes regulate seed germination through PIL5.

For the PhyB-related germination assay, seeds were illuminated by either red light (6 mmol·m^{-2}) or far-red light (0.95 mmol·m^{-2}) after 1 h of seed surface sterilization and then kept in the dark for 6 d. Six days later, the germinated seedlings were counted. As a control, plates were kept under continuous white light and scored for germination. As shown in Figure 3, wild-type seeds germinated even in the absence of any extra light treatment, suggesting that the light signaling occurring either in the dry seeds or during the 1-h surface sterilization provided a sufficient inductive light signal for germination. In the absence of any extra red light treatment, seeds of the *pil5* mutant, the *pif3* mutant, and the *pil5 pif3* double mutant also germinated well, but seeds of *PIL5OX1* and *PIL5OX2* did not germinate. Decreasing handling time for the surface sterilization did not change the germination pattern. In fact, the germination patterns did not change even when dry seeds were scattered directly (without surface sterilization) on wet filter paper under a green light and immediately transferred to darkness (data not shown), suggesting that the 1-h seed-handling time did not change the germination pattern. Furthermore, treatment with extra red light (6 mmol·m^{-2}) did not alter the germination patterns. Together, these data suggest that PIL5 negatively regulated seed germination, and that 6 mmol·m^{-2} of extra red light was not sufficient to overcome the inhibitory action of overexpressed PIL5.

To further test whether inhibition of seed germination by *PIL5* overexpression could be overcome by increased red light fluence, we first treated the samples with far-red light to convert the phytochromes to the Pr form, and then treated the samples with various fluences of red light (0 to 1200 mmol·m^{-2}) and scored for germination. As shown in Figure 4A, increased red

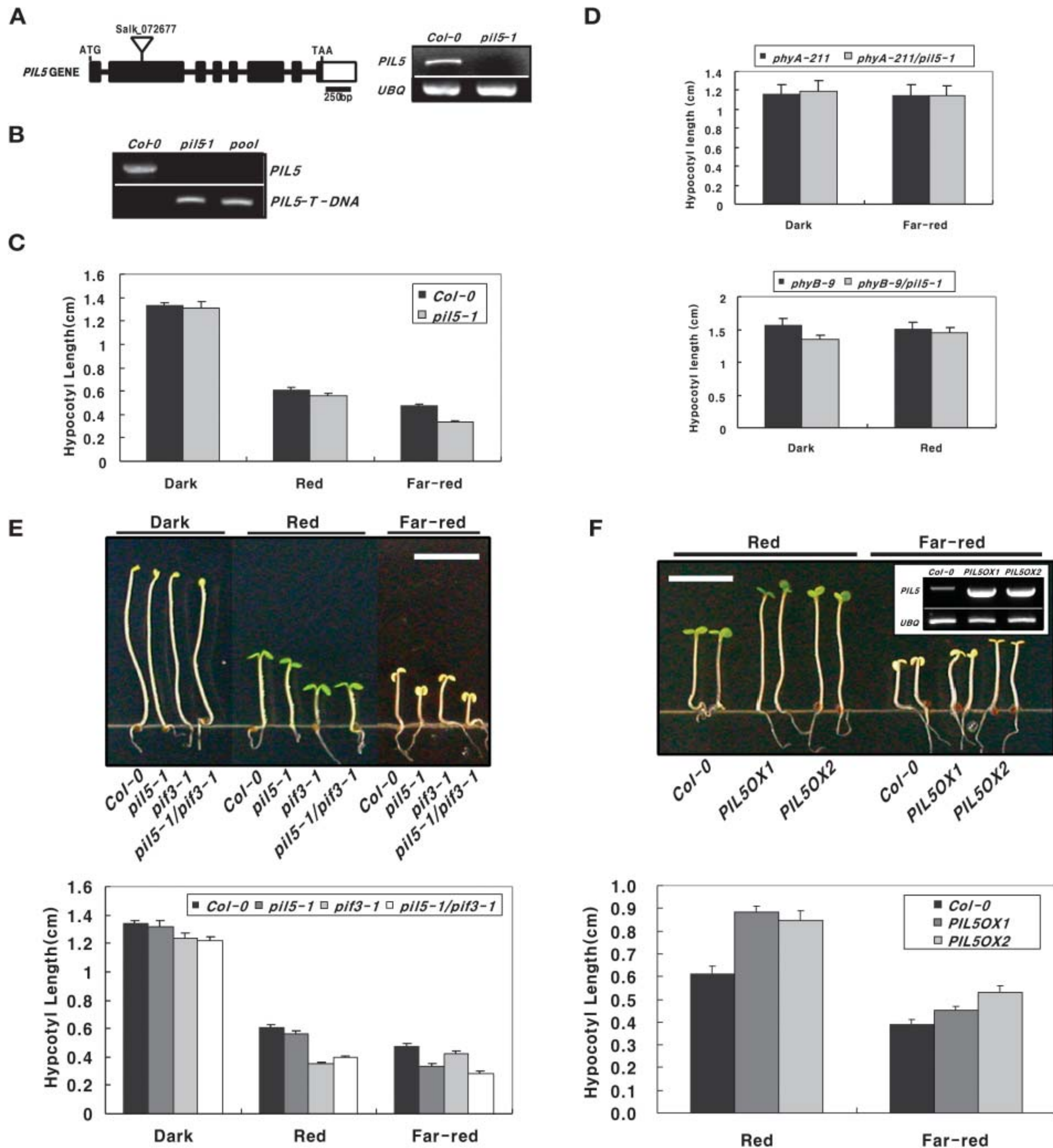

Figure 2. PIL5 Is a Negative Component of Phy-Mediated Inhibition of Hypocotyl Elongation.

(A) Genomic structure of *PIL5* and a T-DNA–insertion site (*pil5-1,* salk_072677). No expression of *PIL5* was detected in the *pil5-1* mutant by RT-PCR. Expression of *UBQ* was used as a control.

(B) Cosegregation analysis. An ability of *pil5-1* mutant seed to germinate after far-red treatment was used for the analysis. Among 300 F2 seeds from a cross between *pil5-1* and *Col-0*, 76 seeds were germinated after far-red light treatment (0.95 mmol·m^{-2}). Genomic DNAs from these germinated seedlings (*pool*), *Col-0*, and *pil5-1* were used to amplify a *PIL5* fragment (*PIL5*) and PIL5-T-DNA hybrid fragment (*PIL5-T-DNA*). All germinated seedlings from this F2 population were homozygous *pil5* mutants.

(C) Hypocotyl lengths of *pil5-1* mutants under 12-h red (20 μmol·m^{-2}·s^{-1})/12-h dark cycle and 12-h far-red light (3.2 μmol·m^{-2}·s^{-1})/12-h dark cycle.

(D) Hypocotyl length of *pil5-1 phyA-211* and *pil5-1 phyB-9* double mutants under red and far-red cycle.

(E) Hypocotyl lengths of *pil5-1 pif3-1* double mutants under red and far-red light cycle.

(F) Hypocotyl lengths of *PIL5OX1* and *PIL5OX2* under red and far-red light cycle. An inset shows the overexpression of *PIL5* in two *PIL5OX* lines. RNAs from 4-d-old light-grown seedlings were used for the RT-PCR analysis. Expression of *UBQ* was used as a control. White bar = 5 mm. Error bars = SD.

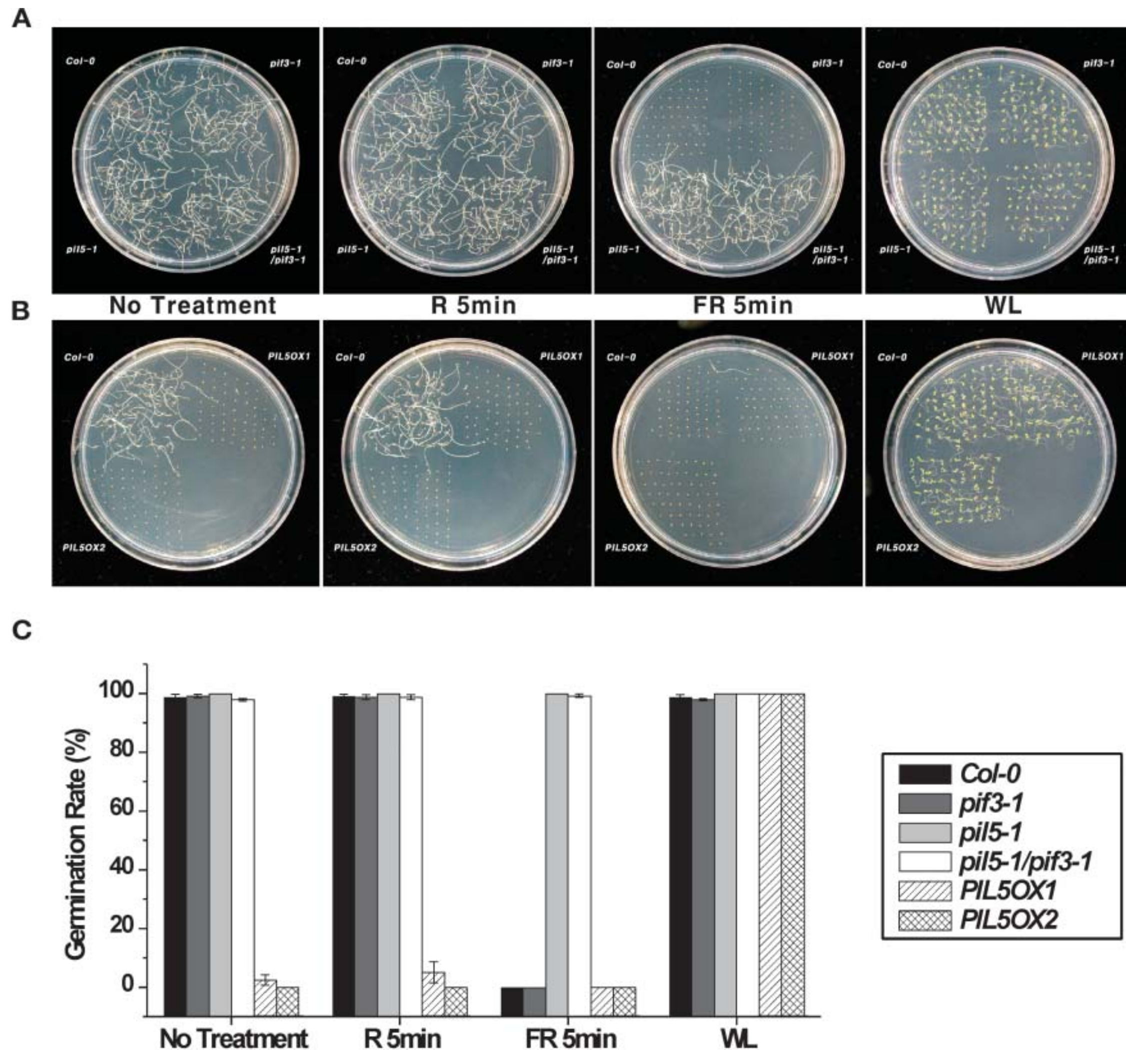

Figure 3. PIL5 Is a Negative Component in PhyB-Mediated Promotion of Seed Germination.

(A) Germination patterns of the *pil5-1* mutant, the *pil3-1* mutant, and the *pil5-1 pif3-1* double mutant. No treatment, no extra light illumination after 1-h imbibition; R 5min, red light (6 mmol·m$^{-2)}$ illumination for 5 min after 1-h imbibition; FR 5min, far-red light (0.96 mmol·m$^{-2)}$ illumination for 5 min after 1-h imbibition. WL, continuous white light.

(B) Germination patterns of the *PIL5OX* transgenic lines.

(C) Quantification of the germination rates of the various mutants under different light conditions. Error bars = SD.

light fluence caused an increased germination rate in *PIL5OX1* and *PIL5OX2* plants. In the case of wild type, treatment with 0.1 mmol·m^{-2} red light was sufficient to induce germination, whereas a much higher red light fluence was required for germination of the two *PIL5OX* lines. Unlike the red light treatment, seeds of the wild type and the *PIL5OX* lines did not germinate if far-red light was applied after seed sterilization, whereas seeds of the *pil5* mutant and the *pil5 pif3* double mutant germinated regardless of far-red treatment. Changing the fluence of far-red from 0 to 57.6 mmol·m^{-2} did not alter the germination rate of the *pil5* mutant and the *pil5 pif3* double mutant (Figure 4B), suggesting that the ability of the *pil5* mutant to germinate even after the far-red light treatment was not attributable to insufficient irradiation of far-red light.

To further investigate whether there is a functional relationship between PIL5 and PhyB, we generated *pil5 phyB (pil5-1 phyB-9)* and *pil5 phyA (pil5-1 phyA-211)* double mutants and examined their germination rates. As shown in Figure 5, wild-type, *phyA* mutant, *pil5* mutant, and *pil5 phyA* double-mutant seeds germinated well in the absence or the presence of extra red light, whereas *phyB* mutant seeds did not germinate under the same conditions. The lack of seed germination in the *phyB* mutant suggests that the germination observed in the absence of any extra light treatment depended on the presence of functional PhyB. Under the same conditions, the *pil5 phyB* double mutant germinated only half compared with the *pil5* single mutant. These results are consistent with the hypothesis that PIL5 acts as a negative factor in PhyB-mediated seed germination. In addition,

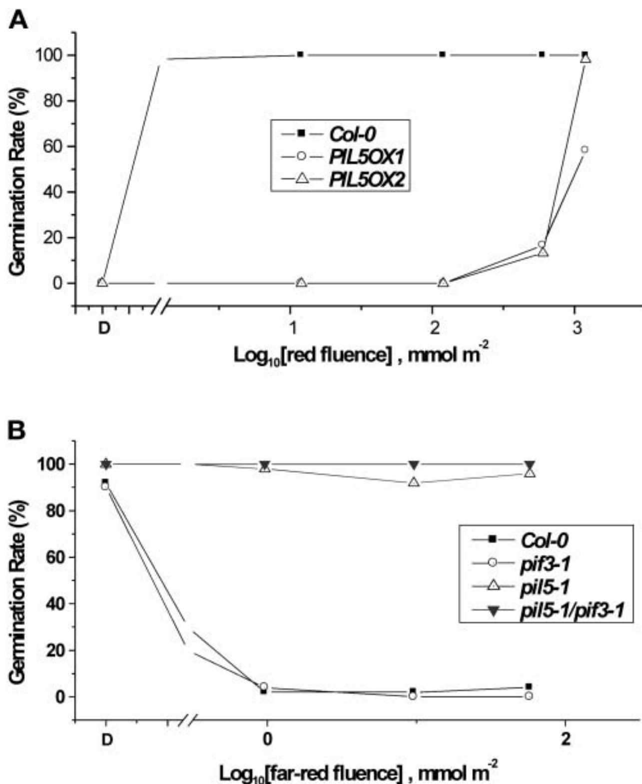

Figure 4. Effect of Fluence on Seed Germination.

(A) Increased red light fluence overcame the inhibition of seed germination observed in the *PIL5OX* lines. After 1-h imbibition, various fluences of red light (0 to 1200 mmol·m^{-2}) were applied to far-red light pretreated seeds, and the samples were then incubated in the dark for 6 d. D indicates 0 mmol·m^{-2}.

(B) Increased far-red light fluence did not inhibit seed germination of *pil5* mutants and *pil5 pif3* double mutants. After 1-h imbibition, various fluences of far-red light (0 to 57.6 mmol·m^{-2}) were applied to the samples, which were then incubated for 6 d in the dark. D indicates 0 mmol·m^{-2}.

the partial decrease of germination rate in the *pil5 phyB* double mutant suggests that factors other than PIL5 must be present for proper progression of PhyB-mediated germination.

When treated with a short pulse of far-red light after seed surface sterilization, wild-type and *phyA* mutant seeds did not germinate, but *pil5* mutant and *pil5 phyA* double-mutant seeds germinated well (Figure 5A). Unlike the *pil5* single mutant, only half of the *pil5 phyB* double-mutant seeds germinated (Figure 5B). The difference in germination rates between far-red–treated *pil5* and *pil5 phyB* seeds suggests that in dry seeds, PhyB signaling could promote germination in the absence of PIL5.

PhyA was shown to induce seed germination when PhyA protein was allowed to accumulate during prolonged incubation of imbibed seeds in the dark (Shinomura et al., 1996). For assays of PhyA-related germination, seeds were treated with far-red light 1 h after seed surface sterilization and incubated in the dark. After 56 h, seeds were irradiated with various fluences of far-red light and allowed to germinate for 5 d in the dark. As shown in Figure 6A, a portion of the wild-type and *pif3* mutant seeds germinated after far-red light treatment (96 mmol·m^{-2}), whereas *phyA* mutant seeds did not. Under the same conditions, the *phyB* mutant seeds germinated better than did the wild type. These results are consistent with previous reports that induction of seed germination by far-red light under these assay conditions is mediated by PhyA, whereas the Pr form of PhyB inhibits this PhyA-mediated germination (Shinomura et al., 1994; Hennig et al., 2001).

Under the PhyA-related germination assay conditions, the *pil5*, *pil5 pif3*, and *pil5 phyA* mutants germinated well regardless of far-red treatment, which is consistent with the PhyB-related germination assay data (Figures 3 and 5). However, unlike in the PhyB-related germination assay, the germination rates of the *phyB* and *pil5 phyB* mutants increased with enhanced far-red fluence. Because this increased germination occurred in the absence of PIL5, these results suggest that factors other than PIL5 also regulate PhyA-mediated seed germination.

Because the *pil5* mutant germinated even in the absence of PhyA activation, it was difficult to assess the role of PIL5 in PhyA-mediated seed germination. To determine the role of PIL5 in PhyA-mediated seed germination, we determined the germination rates of the *PIL5OX* mutants in response to increases in far-red fluence. As shown in Figure 6B, the *PIL5OX* mutants required much higher fluences of far-red light for germination, as compared with wild type, suggesting that PIL5 inhibits seed germination in PhyA-mediated promotion of seed germination. Taken together, our data suggest that PIL5 is a negative factor in both PhyA- and PhyB-mediated seed germination, and further indicates that other factors are involved in these processes.

PIL5 Regulates Phy-Mediated Inhibition of Hypocotyl Negative Gravitropism

When Arabidopsis seedlings are grown in the dark, hypocotyls orient in a negative gravitropic direction. Under red and far-red light, however, hypocotyl negative gravitropism is inhibited and hypocotyls become randomly oriented. This inhibition of hypocotyl negative gravitropism under red and far-red light is mediated by phytochromes (Robson and Smith, 1996). Under far-red light, *phyA* mutants grow in an upward direction, indicating that PhyA is required for inhibition of negative gravitropism. By contrast, *phyB* single mutants orient randomly under red light, suggesting that other phytochromes are involved in the inhibition of hypocotyl negative gravitropism under red light. Analysis of *phyA phyB* double mutants later showed that both PhyA and PhyB must be present for inhibition of hypocotyl negative gravitropism under red light (Robson and Smith, 1996).

During our experiments, we noticed that the hypocotyls of the *pil5 pif3* double mutants oriented randomly in the dark. Because phytochromes regulate hypocotyl negative gravitropism, we tested whether phytochromes regulate this process through PIL5. Hypocotyl growth orientation away from the vertical on vertical-grown agar plates is traditionally used as an indicator of hypocotyl negative gravitropism (Liscum and Hangarter, 1993; Poppe et al., 1996; Robson and Smith, 1996). However, we found that it was difficult to illuminate the spotted seeds with exact fluence rates when plates were in a vertical position. To

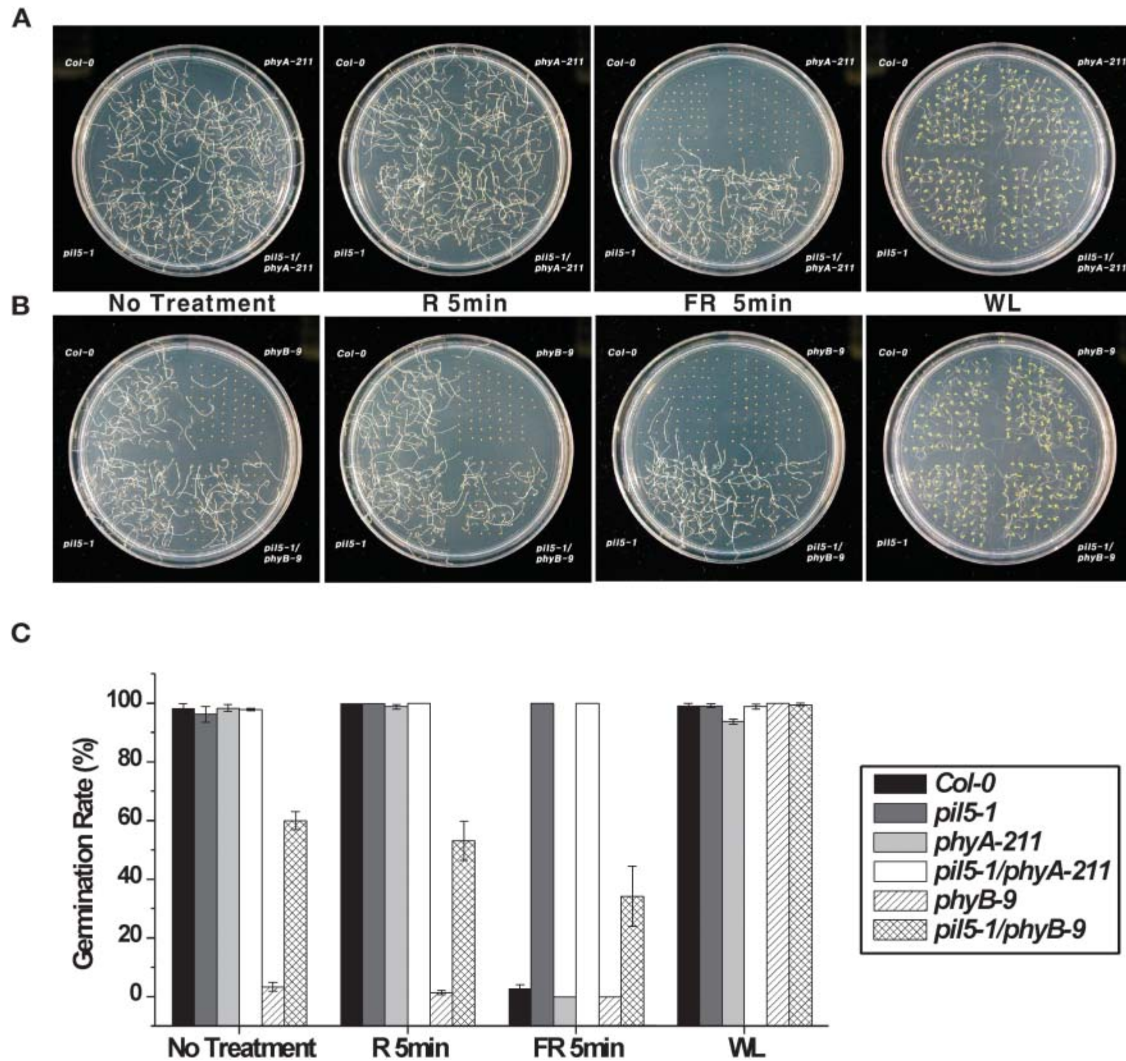

Figure 5. Germination Patterns of *pil5 phyA* and *pil5 phyB*.

(A) Germination patterns of *phyA* and *pil5 phyA.* No treatment, no extra-light illumination after 1-h imbibition; R 5 min, red light (6 mmol·m^{-2}) illumination for 5 min after 1-h imbibition; FR 5 min, far-red light (0.96 mmol·m^{-2}) illumination for 5 min after 1-h imbibition; WL, continuous white light.

(B) Germination patterns of *phyB* and *pil5 phyB*.

(C) Quantification of the germination rates of the various mutants under different light conditions. Error bars = SD.

overcome this, we grew seeds horizontally, and then counted the number of seedlings lying flat on the agar as an indicator of hypocotyl negative gravitropism. All seedlings were able to grow upwardly under white light (Figure 7A). Because phototropism is a main determinant of hypocotyl growth orientation under white light, the result indicated that none of the seedlings have an intrinsic problem to grow upwardly. When wild-type seedlings were grown in the dark, the majority of their hypocotyls were oriented in an upward direction (Figure 7B). However, when wild-type seedlings were grown under red or far-red light, the majority of the seedlings grew flat on the agar plates (Figure 7C). Consistent with the previously defined roles of PhyA and PhyB in hypocotyl negative gravitropism, the *phyA* mutants grew upward under far-red light, whereas the *phyB* mutants grew flat under red light (Figure 7C). These results indicate that counting flat-grown seedlings on the horizontal orientation is qualitatively similar to measuring deviation from the vertical in vertical-grown seedlings as an indicator of hypocotyl negative gravitropism.

Inhibition of hypocotyl negative gravitropism was observed in the dark in the *pil5* mutant, the *pif3* mutant, and the *pil5 pif3* double mutant, although the degrees of inhibition differed. Compared with wild-type seedlings, higher percentages of *pil5* and *pif3* mutant seedlings grew flat in the dark, suggesting that both *PIF3* and *PIL5* play roles in hypocotyl negative gravitropism in darkness. The roles of *PIF3* and *PIL5* in this process were additive; in the *pil5 pif3* double mutant, almost 80% of seedlings grew flat in the dark, whereas ~40% of seedlings grew flat in each of the single mutants (Figures 7B and 7C). Interestingly, this

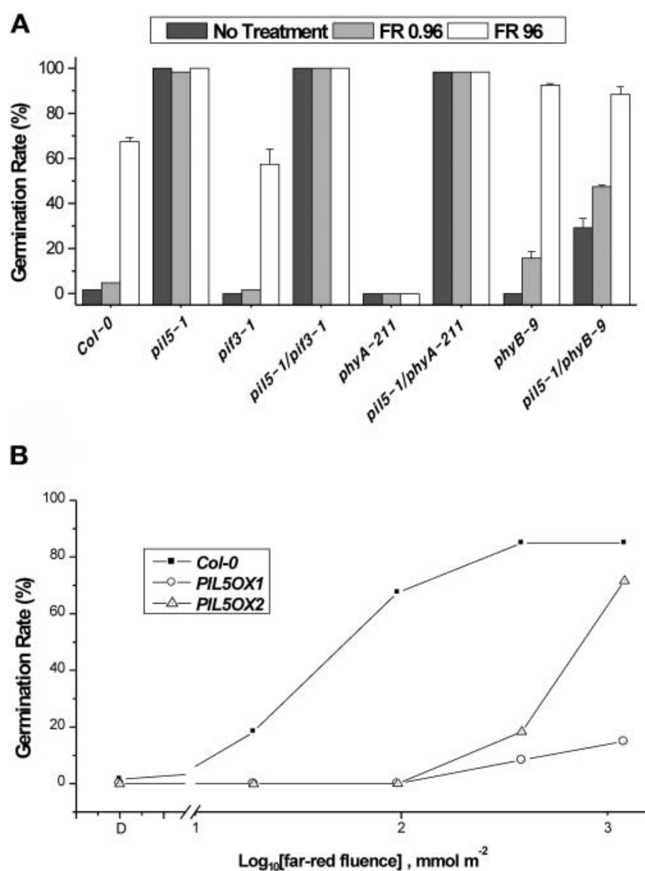

Figure 6. PIL5 Regulates PhyA-Mediated Promotion of Seed Germination.

(A) Germination rates of wild-type, *pil5, pif3, pil5 pif3, phyA, pil5 phyA, phyB*, and *pil5 phyB* mutants. Seeds were preilluminated with far-red light (0.96 mmol·m^{-2}) after 1-h imbibition and incubated for 56 h in the dark. Then, seeds were illuminated with far-red light (0.96 or 96 mmol·m^{-2}) and incubated further for 5 d in the dark.

(B) Far-red fluence rate response of wild-type and *PIL5OX* lines. The *PILOX* lines are hyposensitive to far-red light for germination. D indicates 0 mmol·m^{-2}. Error bars = SD.

hypocotyl negative gravitropism in the dark was influenced by phyB. As shown in Figure 7C, the *phyB* mutant was slightly more negatively gravitropic in the dark compared with the wild type or the *phyA* mutant. Similarly, the *pil5 phyB* double mutant was more negatively gravitropic compared with the *pil5* single mutant. Unlike the *phyB* mutant, the *phyA* mutant and the *pil5 phyA* double mutant showed similar degrees of negative gravitropism, as compared with the wild-type and *pil5* single-mutant seeds, respectively. This regulation of hypocotyl negative gravitropism by PhyB in the dark is reminiscent of the regulation of germination by PhyB in the absence of any extra light treatment (Figure 5B). Although the phenotypes of the *phyB* mutant and the *pil5 phyB* double mutant suggest that PIL5 is a negative component in PhyB-mediated inhibition of hypocotyl negative gravitropism in the dark, further investigation will be required to clarify the role of PIL5 in this process.

When seedlings were grown under red light, the hypocotyls of all mutant seedlings grew flat, comparable to the growth of wild-type seedlings. By contrast, when grown under far-red light, most of the mutant seedlings grew flat except the *phyA* and *pil5 phyA* mutants. The majority of the *phyA* mutant seedlings were negatively gravitropic under far-red light, as reported previously (Robson and Smith, 1996), whereas the *pil5 phyA* double mutant showed fewer negatively gravitropic seedlings than the *phyA* mutant. As in PhyA-mediated seed germination, however, it was difficult to assess the role of PIL5 in PhyA-mediated inhibition of hypocotyl negative gravitropism from the *pil5* mutant phenotype alone. To investigate that PIL5 is a negative regulator of PhyA-mediated inhibition of hypocotyl negative gravitropism, we measured the hypocotyl negative gravitropism of the *PIL5OX* line under red (2 μmol·m^{-2}·s^{-1}) and far-red (0.32 μmol·m^{-2}·s^{-1}) light. As shown in Figure 7D, overexpression of *PIL5* caused hyposensitivity to far-red light, although it did not affect sensitivity to red light. Although further investigation will be warranted to clarify the role of PIL5 in PhyB signaling, our results suggest that PhyA inhibits hypocotyl negative gravitropism partly through PIL5.

DISCUSSION

We report here that PIL5 is the first phytochrome-interacting protein that regulates seed germination and hypocotyl negative gravitropism. By analyzing *pil5* mutants in conjunction with either *phyA* or *phyB* mutants and the *PIL5* overexpression lines, we demonstrated that PIL5 is a negative component in various Phy-mediated light-signaling processes, including inhibition of hypocotyl elongation, promotion of seed germination, and inhibition of hypocotyl negative gravitropism.

PIL5 Is a Negative Component in Phy-Mediated Inhibition of Hypocotyl Elongation

Ectopic expression of *PIL5* caused a long hypocotyl phenotype both under red and far-red light, which indicates that ectopically expressed *PIL5* can regulate both PhyA- and PhyB-mediated inhibition of hypocotyl elongation. However, unlike the *PIL5* overexpression lines, the *pil5* mutant exhibited shortened hypocotyl phenotypes only under far-red light, suggesting that PhyA-mediated inhibition of hypocotyl elongation is a main target of endogenous PIL5. A few explanations may account for the discrepancy of hypocotyl phenotypes between the *pil5* mutant and the *PIL5OX* lines. First, it is possible that lack of PIL5 function under red light might correlate with lack of PIL5 expression. However, expression analysis showed that the *PIL5* gene is expressed both in red and far-red light conditions (see Supplemental Figure 3 online), indicating that the lack of hypocotyl phenotype under red light is not because of the absence of *PIL5* transcripts. However, although *PIL5* mRNA is expressed, we cannot exclude the possibility that PIL5 protein is not present under red light conditions. A second hypothesis is that endogenous PIL5 may play a role under red light conditions, but its phenotypic contribution is too weak to be experimentally determined. In this situation, the hypocotyl length of *pil5* mutants under red light could not be experimentally differentiated from that of wild type, whereas the hypocotyl phenotypes of the

PIL5OX lines were evident because of the dramatically increased expression of PIL5. A third hypothesis is that the function of PIL5 under red light may be compensated for by other factors, such as different PIL family members. Because a *pif3* mutant showed a short hypocotyl phenotype under red light (Kim et al., 2003), we first tested whether PIF3 provides a compensatory function under red light. We found that the hypocotyl length of the *pil5 pif3* double mutant was identical to that of the *pif3* single mutant under red light, indicating that PIF3 does not compensate for PIL5 in PhyB-mediated inhibition of hypocotyl elongation. However, other factors have been shown to regulate hypocotyl elongation under red light (Oyama et al., 1997; Genoud et al., 1998; Choi et al., 1999; Fankhauser et al., 1999; Reed et al., 2000; Huq and Quail, 2002); hence, more analyses will be needed to determine whether the discrepancy in hypocotyl phenotypes between the *pil5* mutant and the *PIL5OX* lines under red light is attributable to other compensatory factors.

PIL5 Is the First Identified Phy-Interacting Protein That Regulates Seed Germination

Phytochromes regulate various light responses including seed germination, photomorphogenesis, flowering, and hypocotyl negative gravitropism (Robson and Smith, 1996; Neff et al., 2000; Sullivan and Deng, 2003). Molecular and genetic investigations have identified many components involved in the various Phy-regulated light responses. However, no signaling component has previously been identified as a component in Phy-mediated promotion of seed germination.

Our analyses place *PIL5* as the first Phy-interacting protein that regulates Phy-mediated seed germination. Under our PhyB-related germination assay conditions, the *phyB* mutant did not germinate in the absence of light or in the presence of red and far-red light treatments, whereas the wild-type seeds germinated well in the absence of any light and under red light treatment. In the case of the wild type, but not the *phyB* mutant, germination was seen even when dry seeds were scattered on wet filter paper under a green light and immediately transferred to darkness. This *phyB* mutant phenotype indicates that the germination of wild-type seeds in the absence of any extra light treatment depends on the presence of the Pfr form of PhyB in the dry seed. Under the same conditions, the *pil5* mutant germinated well under all light conditions, whereas the *PIL5OX* lines required much higher red light fluences for germination. Because PIL5 is a Phy-interacting protein, these results suggest that PIL5 functions as a negative

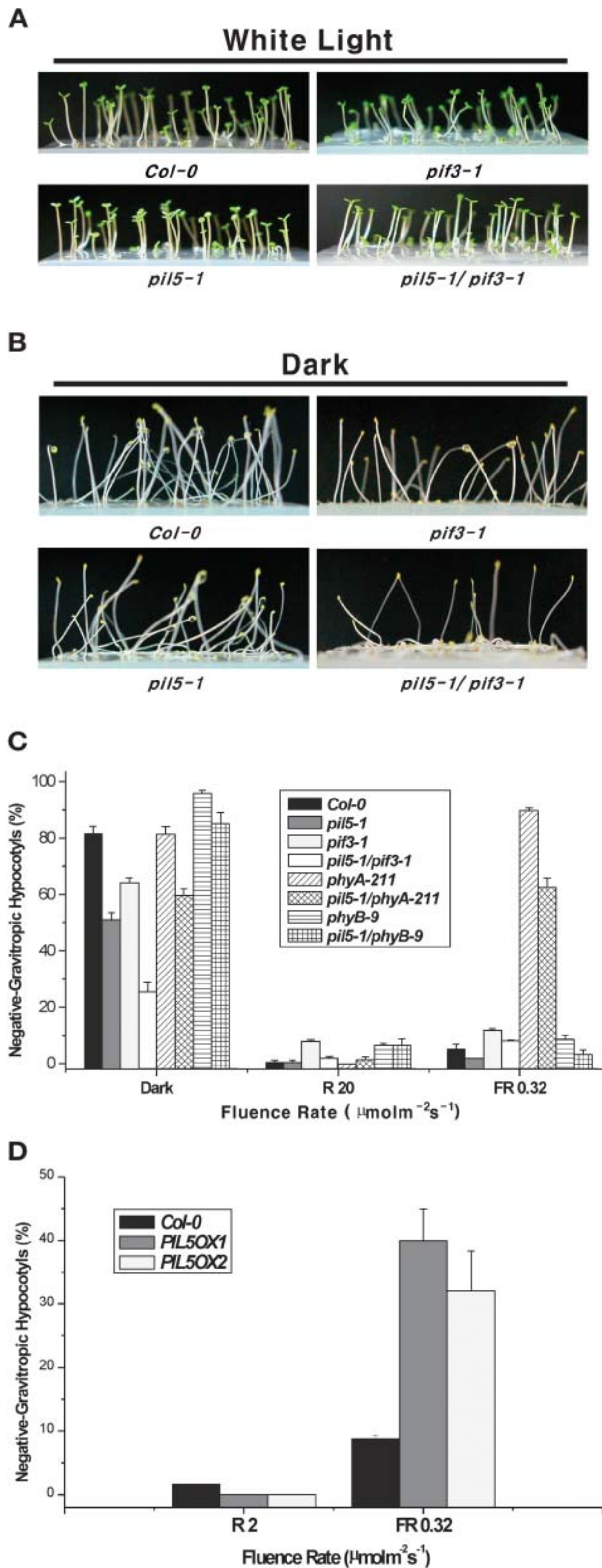

Figure 7. PIL5 Regulates PhyA-Mediated Inhibition of Hypocotyl Negative Gravitropism.

(A) Hypocotyl negative gravitropic growth patterns of wild-type, *pil5, pif3*, and *pil5 pif3* mutants under continuous white light (8 to 9 μmol·m^{-2}·s^{-1}).

(B) Hypocotyl negative gravitropic growth patterns of wild-type, *pil5, pif3*, and *pil5 pif3* mutants in the dark.

(C) Hypocotyl negative gravitropism of wild-type, *pil5, pif3, pil5 pif3, phyA, pil5 phyA, phyB*, and *pil5 phyB* mutants in the dark, under red (20 μmol·m^{-2}·s^{-1}), or far-red light (0.32 μmol·m^{-2}·s^{-1}).

(D) Hypocotyl negative gravitropism of *PIL5OX* lines either under red (2 μmol·m^{-2}·s^{-1}) or far-red light (0.32 μmol·m^{-2}·s^{-1}).

factor in PhyB-mediated promotion of the seed germination process.

Analyses of the *pil5 phyA* and *pil5 phyB* double mutants further suggested the presence of other components in PhyB-mediated seed germination. The germination rate of the *phyA* mutant was identical to that of wild-type seeds, whereas the germination rate of the *pil5 phyA* double mutant was identical to that of the *pil5* single mutant under various light conditions, indicating that PhyA does not affect germination in the presence or absence of *PIL5* under our PhyB-related germination assay conditions. By contrast, the *phyB* mutant did not germinate under any tested light conditions except under white light. Unlike the *phyB* single mutant, the *pil5 phyB* double mutant germinated under all light conditions, but the germination rates were lower than those of the *pil5* single mutant. If PIL5 is a sole factor involved in the PhyB-mediated germination process, the germination rates of the *pil5* and *pil5 phyB* mutants would be expected to be identical. Thus, the different germination rates in these mutants imply that factors other than PIL5 must be involved in the PhyB-mediated germination process.

Because PIL5 also interacts with PhyA, we tested whether PIL5 regulated PhyA-mediated promotion of seed germination. Under our PhyA-related germination assay conditions, far-red light treatment did not induce seed germination in the *phyA* mutant, whereas this treatment induced germination in the wild-type seeds, indicating that under these conditions, germination depends on PhyA. Under these same experimental conditions, the *pil5*, *pil5 pif3*, and *pil5 phyA* mutants germinated well regardless of far-red light treatment. Because these mutants germinated even in the absence of any light treatment (Figure 6A), it is difficult to assess the role of PIL5 in PhyA-mediated germination based on the mutant germination patterns. However, the *PIL5OX* lines required much higher far-red light fluences for germination. Because PIL5 interacts with PhyA, this result suggests that PIL5 functions as a negative factor in the PhyA-mediated promotion of seed germination.

Our analyses of the *pil5 phyB* double mutants further suggested that other components are present in PhyA-mediated seed germination. When far-red fluence was increased, the germination rate of the *pil5 phyB* double mutant increased, indicating that PhyA can induce seed germination in the absence of PIL5. If PIL5 were the sole factor involved in PhyA-mediated germination, the germination rates of the *pil5 phyB* double mutant should not be increased by enhanced far-red fluence. Thus, these results support the hypothesis that PhyA regulates seed germination not only through PIL5, but also through other factors.

Thus, our analyses suggest the presence of other components both in PhyA- and PhyB-mediated seed germination. Because homologous proteins tend to regulate similar processes, it is tempting to speculate that some of the other factors regulating seed germination could be additional PIL proteins. Among the six PIL proteins, PIL1, PIF3, and PIF4 have been characterized in relation to light signaling. Our analysis of the *pif3* mutant indicated that PIF3 is not involved in seed germination. In the case of PIL1 and PIF4, their roles in seed germination have not been experimentally determined. The PIL family is a part of the bHLH family, further suggesting that other bHLH proteins might also regulate seed germination. One candidate bHLH protein is HFR1. A previous study reported that overexpression of an N-terminal–deleted *HFR1* (*ΔN105HFR1*) caused partially constitutive photomorphogenic phenotypes including very short hypocotyls, opened cotyledons, and seed germination in the dark (Yang et al., 2003). However, because *ΔN105HFR1* retains a potential DNA-binding bHLH domain and a domain facilitating dimerization with other bHLH proteins, it is not clear whether the phenotypes shown in the *ΔN105HFR1* overexpression lines are attributable to the genuine function of HFR1, especially considering that *hfr1* mutation and *HFR1* overexpression did not affect seed germination. Because the lack of phenotypes in the *hfr1* mutant could be attributed to compensation by other factors, further analysis will be needed to clarify the possible role of HFR1 in seed germination.

PIL5 Regulates Phy-Mediated Inhibition of Hypocotyl Negative Gravitropism

In Arabidopsis, hypocotyls grow in an upward direction in the dark and under white light. Under monochromatic red or far-red light, however, hypocotyls grow in random directions, indicating that phytochromes inhibit hypocotyl negative gravitropism (Liscum and Hangarter, 1993; Robson and Smith, 1996). The physiological significance of this regulation is not clear. One possibility is that the inhibition of hypocotyl negative gravitropism might be a shade-escaping response that allows hypocotyls to elongate away from a shaded area and toward an open area.

Our analyses place PIL5 as the first Phy-interacting protein that also functions as a negative component in PhyA-mediated inhibition of hypocotyl negative gravitropism. When grown in the dark, the majority of wild-type seedlings showed negative gravitropism. Under the same conditions, the *pil5* and *pif3* single mutants showed decreased negative gravitropism. The *pil5 pif3* double mutant showed further decreased hypocotyl negative gravitropism in the dark, suggesting that the roles of PIL5 and PIF3 were additive. As in the germination assay, however, the meaning of data gathered under the dark conditions should be interpreted carefully. First, dry seeds have been exposed to white light at least once from the time of seed harvesting to inception of the experiment. Second, germination is generally induced by strong white light before the seeds being transferred to darkness. Thus, it is necessary to include photoreceptor mutants to determine the role of photoreceptors in the dark-grown seedlings. Similar to the case in germination, hypocotyl negative gravitropism in the dark is also influenced by PhyB. As shown in Figure 7C, the *phyB* mutant seedlings, but not the *phyA* mutant seedlings, showed slightly stronger negative gravitropism as compared with wild type in the dark. In addition, the degree of hypocotyl negative gravitropism in the *pil5 phyB* double mutant was comparable to that of wild type. Taken together, these results suggested that PhyB influences negative gravitropism under the tested dark conditions. Because both PIL5 and PIF3 are Phy-interacting proteins, the stronger inhibition of hypocotyl negative gravitropism in the *pil5*, *pif3*, and *pil5 pif3* mutants compared with the wild type suggest that these two proteins regulate hypocotyl negative gravitropism as components of PhyB signaling. Our analyses of hypocotyl negative gravitropism under red light, however, did

not clarify the role of PIL5 in PhyB signaling. As shown in Figure 7C, all plants including the *phyB* mutant itself showed normal inhibition of hypocotyl negative gravitropism under red light. Overexpression of *PIL5* also did not alter the hypocotyl negative gravitropism under red light. Thus, further investigation will be required to clarify the exact role of PIL5 in PhyB-mediated inhibition of hypocotyl negative gravitropism.

Unlike the role of PIL5 in PhyB signaling, our analyses suggested that PIL5 regulates PhyA-mediated inhibition of hypocotyl negative gravitropism. Under far-red light, the *phyA* mutant showed inhibited hypocotyl negative gravitropism, whereas wild type showed strong hypocotyl negative gravitropism, which is consistent with the previous report that PhyA is necessary for this process under far-red light (Robson and Smith, 1996). Because virtually all wild-type seedlings already grew flat under far-red light, it is difficult to determine the role of PIL5 using the *pil5* mutant alone. However, the *pil5 phyA* double mutant showed the stronger inhibited hypocotyl negative gravitropism compared with *phyA* mutant, suggesting a negative role of PIL5 on hypocotyl negative gravitropism. Consistent with the negative role of PIL5 in this process, overexpression of *PIL5* caused stronger hypocotyl negative gravitropism under far-red light. Taken together, these results suggest that PhyA inhibits hypocotyl negative gravitropism partly through inhibition of PIL5.

A Set of bHLH Proteins Regulates Various Aspects of Phy-Mediated Light Responses

Molecular and genetic investigations identified five bHLH transcription factors that have shared, but distinct functions in various Phy-mediated light responses. PIF3 is the first identified bHLH transcription factor that interacts with phytochromes and negatively regulates the inhibition of hypocotyl elongation and cotyledon expansion under red light, and positively regulates anthocyanin accumulation under far-red light (Ni et al., 1999; Kim et al., 2003). Later, PIF4 was identified as a phytochrome-interacting factor that also negatively regulates PhyB-mediated inhibition of hypocotyl elongation and cotyledon expansion (Huq and Quail, 2002). Genetic screening of far-red insensitive mutants by three different research groups then isolated the third such bHLH transcription factor, HFR1/REP1/RSF1 (Fairchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000). Functionally, HFR1 positively regulates inhibition of hypocotyl elongation, *CAB* gene expression, and inhibition of hypocotyl negative gravitropism under far-red light. PIL1 was the fourth characterized bHLH protein, and was shown to regulate shade avoidance (Salter et al., 2003).

In this report, we showed that PIL5 is a new member of the phytochrome-interacting factor family; it negatively regulates promotion of seed germination, inhibition of hypocotyl elongation, and inhibition of hypocotyl negative gravitropism. Database analyses indicated that these five bHLH proteins belong to bHLH subfamily 15 (Toledo-Ortiz et al., 2003), suggesting that bHLH subfamily 15 is closely associated with light signaling in Arabidopsis. However, not all bHLH proteins in subfamily 15 have roles in Phy-mediated light responses. For example, subfamily members ALCATRAZ and SPATULA regulate fruit dehiscence and carpel development, respectively (Alvarez and Smyth, 1999; Heisler et al., 2001; Rajani and Sundaresan, 2001). A systematic analysis of the remaining eight members in this subfamily should be undertaken to clarify the role of bHLH subfamily 15 in light responses. Furthermore, the determination of functional relationships among these bHLH proteins and other identified signaling components will be needed to elucidate light signaling networks.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana plants were grown in a growth room with a 16-h light/8-h-dark cycle at 22 to 24°C for general growth and seed harvesting. A T-DNA inserted *pil5* mutant (*pil5-1*) was obtained from the Arabidopsis Stock Center (Salk_072677) (Alonso et al., 2003). For *PIL5OX1* and *PIL5OX2* transgenic lines, full-length cDNA of *PIL5* (At2g20180) was amplified with a primer set (5′-AGAGTGATCAAAAATGCATCATTTT-GTCCCTGAC-3′, 5′-AGAGTGATCACCTTAACCTGTTGTGTGGTT-3′) and cloned into the GUS-deleted pBI121 vector for the expression under the control of the 35S promoter of *Cauliflower mosaic virus*. Among primary transgenic lines, two lines (*PIL5OX1* and *PIL5OX2*) were selected for further analysis. All plants used in the experiments (the *pil5-1*, the *PIL5OX*s, the *pif3-1*, the *phyA-211*, and the *phyB-9*) are Col-0 ecotype background. *PIL5* expression levels were determined by RT-PCR analysis. Primers used for the RT-PCR analysis of *PIL5* were 5′ primer (5′-GAGAGAATTCGGTGGCATGCATCATTTTGTCCCTGAC-3′) and 3′ primer (5′-AGAGTGATCACCTGTTGTTGATACACGTGC-3′).

In Vitro Binding Assay

Recombinant phytochrome proteins were purified from *Pichia* using an expression vector, pPIC3.5K (Invitrogen, Carlsbad, CA). Ten–amino acid streptavidin affinity tag (strep-tag) from pASK75 vector (Biometra, Tampa, FL) was attached to the 3′ end of the phytochrome genes. The primers, 5′-CGGGATCCACCATGGCTTCCTCAAGGCCTGCTTCC-3′ (forward, *Bam*HI) and 5′-TCGCGTCGACTTGTCCCATTGCTGTTG-GAGC-3′ (backward, *Sal*I), were used for the subcloning of oat PhyA genes into pPIC3.5K. The primers, 5′-CTCTACGTAACCATGGTTTCCG-GAGTCGGGG-3′ (forward, *Sna*BI) and 5′-TCGCAGCGCTATATGGCAT-CATCAGCATCATG-3′ (backward, *Eco*47III) were used for Arabidopsis PhyB. The pPIC3.5K constructs with phytochrome genes were transformed into *Pichia* cells using a Micropulser Electroporation apparatus (Bio-Rad, Hercules, CA). Recombinant phytochrome proteins were expressed in the *Pichia* expression system, according to the manufacturer's recommendations (Invitrogen), and purified using streptavidin affinity chromatography (Sigma-Genosys, Haverhill, UK). Phycocyanobilin was purified using Spirulina extracts (Sigma, St. Louis, MO) by methanolysis. Holophytochromes were prepared by adding chromophores in DMSO to apoproteins at a final concentration of 20 μM, and the mixture was incubated on ice for 1 h. From the harvested *Pichia* cells, crude extract was prepared by breaking cells in liquid nitrogen using a homogenizer (Nihonseiki Kaisha, Tokyo, Japan). The phytochrome samples were precipitated by adding 0.23 g/L ammonium sulfate, resuspended in a buffer (100 mM Tris, pH 7.8, and 1 mM EDTA), then chromophores were added to the samples for in vitro reconstitution. The direct addition of chromophores to ammonium sulfate fraction makes better reconstitution and removal of free chromophores, compared with the addition to purified apoproteins. After dialysis to remove free chromophores, the samples were loaded to streptavidin affinity chromatography and purified holophytochromes without any free chromophore.

For in vitro binding assay, recombinant oat PhyA and Arabidopsis PhyB with phycocyanobilin were preilluminated with red light (18 mmol·m^{-2}) or far-red light (2.88 mmol·m^{-2}) to convert them to Pfr or Pr form. For the assay, 4 μg of GST-fused PIL5 protein from pET50 vector (Novagene, Darmstadt, Germany; pET42 vector without 5′ His-tag site) were mixed with 2 μg of the preilluminated phytochromes and 30 μL of 50% glutathione-agarose bead slurry in a binding buffer (50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% [v/v] Triton X-100, 0.05% [w/v] na-deoxycholate, 10% [v/v] glycerol, BSA 100 μg/mL). After incubating 2 h at 4°C in the dark, glutathione-agarose beads were collected by spin down (30 s) and washed three times with 500 μL of the binding buffer each. The binding and washing were performed under the safety green light. After washing, bound proteins were solubilized by boiling in 50 μL of SDS sample buffer. The dissolved proteins in SDS sample buffer were resolved on 8% SDS-PAGE gel and analyzed by protein gel blot with antiphytochrome antibody.

Subcellular Localization Assay

To determine subcellular localization of PIL5, the coding region of *PIL5* was cloned into pBI221-GUS vector. This construct was transfected to onion epidermal cells by the particle bombardment and the bombarded epidermal cells were incubated for 16 h in the dark or under white light at 22°C. The location of PIL5-GUS was determined using X-gluc.

Germination Assay

For PhyB-related seed germination assay, triplicates of 60 seeds for each mutant were surface sterilized, plated on aqueous agar medium (0.6% phytoagar, pH 5.7) and imbibed for 1 h at 22°C. Seed surface-sterilization step was included for this 1-h imbibition time. After 1 h imbibition, red or far-red light (red light, 20 μmol·m^{-2}·s^{-1}; far-red light, 3.2 μmol·m^{-2}·s^{-1}) was illuminated for 5 min and incubated for 6 d in darkness. Germinated seed was determined by radicle formation.

For PhyA-related seed germination assay, triplicates of 60 seeds for each mutant were plated on aqueous agar medium and imbibed for 1 h at 22°C. As in the PhyB-related germination assay, 1-h imbibition was counted from the start of seed surface sterilization. After 1-h imbibition, far-red light (3.2 μmol·m^{-2}·s^{-1}) was illuminated for 5 min and incubated in the dark at 22°C for 56 h. After 56-h incubation in the dark, far-red light was illuminated for various times and incubated for 5 d in the dark. Germinated seed was determined by radicle formation.

Hypocotyl Negative Gravitropism Assay

For hypocotyl negative gravitropism assay, triplicates of 60 seeds for each mutant were plated on MS agar (half-strength MS, 0.8% phytoagar, and 0.05% Mes, pH 5.7) and cold treated for 4 d in the dark. After germination was induced by white light (80 to 90 μmol·m^{-2}·s^{-1}) for 6 h, plates were incubated for 4 d either in the dark, continuous red light (20 μmol·m^{-2}·s^{-1}), continuous far-red light (0.32 μmol·m^{-2}·s^{-1}), or continuous white light (8 to 9 μmol·m^{-2}·s^{-1}). The degree of hypocotyl negative gravitropism was determined by counting flat-grown seedlings with cotyledons that touched the agar surface.

Inhibition of Hypocotyl Elongation Assay

To measure hypocotyl length, seeds were sterilized, plated on MS agar (half-strength MS, 0.8% phytoagar, and 0.05% Mes, pH 5.7), and imbibed for 4 d at 4°C in the dark. After germination was induced by white light (6 h at 80 to 90 μmol·m^{-2}·s^{-1}), plates were incubated for 4 d either in the dark, in 12-h red light (20 μmol·m^{-2}·s^{-1})/12-h dark cycle, or in 12-h far-red light (3.2 μmol·m^{-2}·s^{-1})/12-h dark cycle. Of 50 seedlings, hypocotyl lengths of the longest 30 seedlings were measured for each sample.

Cosegregation Analysis

F2 seeds from a cross between the *pil5-1* mutant and *Col-0* were plated. After plating, seeds were illuminated with far-red light (0.95 mmol·m^{-2}) and kept for 6 d in the dark. Seventy-six out of 300 seeds were germinated. To determine if the germinated seedlings were all homozygous *pil5* mutants, the pooled genomic DNA of germinated seedlings was used to amplify with a *PIL5* gene–specific primer set (5′-GAGATTATGAACTT-CAGCAGCACG-3′, 5′-TGGAGCTTTTATGGCAGAACGG-3′) or with a PIL5-T-DNA fusion fragment-specific primer set (5′-TGGAGCTTT-TATGGCAGAACGG-3′, 5′-GCGTGGACCGCTTGCTGCAACT-3′).

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