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PIL5, a Phytochrome-Interacting bHLH Protein, Regulates Gibberellin Responsiveness by Binding Directly to the *GAI* and *RGA* Promoters in *Arabidopsis* Seeds [W]

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Previous work showed that PHYTOCHROME-INTERACTING FACTOR3-LIKE5 (PIL5), a light-labile basic helix-loop-helix protein, inhibits seed germination by repressing *GIBBERELLIN 3β-HYDROXYLASE1* (*GA3ox1*) and *GA3ox2* and activating a gibberellic acid (GA) catabolic gene (*GA2ox2*). However, we show persistent light-dependent and PIL5-inhibited germination behavior in the absence of both de novo GA biosynthesis and deactivation by GA2ox2, suggesting that PIL5 regulates not only GA metabolism but also GA responsiveness. PIL5 increases the expression of two GA repressor (DELLA) genes, *GA-INSENSITIVE* (*GAI*) and *REPRESSOR OF GA1-3* (*RGA*/*RGA1*), in darkness. The hypersensitivity of *gai-t6 rga-28* to red light and the suppression of germination defects of a *rga-28 PIL5* overexpression line show the significant role of this transcriptional regulation in seed germination. PIL5 also increases abscisic acid (ABA) levels by activating ABA biosynthetic genes and repressing an ABA catabolic gene. PIL5 binds directly to *GAI* and *RGA* promoters but not to GA and ABA metabolic gene promoters. Together, our results show that light signals perceived by phytochromes cause a reduction in the PIL5 protein level, which in turn regulates the transcription of two DELLA genes directly and that of GA and ABA metabolic genes indirectly.

INTRODUCTION

In plants, germination determines the growth habitat and growth season, and the proper decision to germinate is critical for plant survival. When deciding whether or not to germinate, plant seeds monitor various environmental factors, including moisture, temperature, and light, and translate these conditions into signals transmitted by plant hormones such as abscisic acid (ABA), gibberellic acid (GA), brassinosteroid, and ethylene (Finch-Savage and Leubner-Metzger, 2006). Therefore, elucidation of the process by which a plant translates environmental conditions into plant hormonal signals is critical to our understanding of how a plant seed makes the decision to germinate.

The roles of ABA and GA in seed germination are relatively well understood. ABA is involved in establishing and maintaining seed dormancy, whereas GA is involved in breaking seed dormancy and promoting germination (Finch-Savage and Leubner-Metzger, 2006). The importance of these plant hormones for seed germination can be seen in the phenotypes of plants defective for their metabolism or signaling cascades. ABA biosynthetic mutants such as *abscisic acid-deficient1* (*aba1*) and *aba2* display greatly reduced seed dormancy (Debeaujon and Koornneef, 2000; Rook et al., 2001), whereas ABA catabolic mutants such as *cyp707a2* show increased seed dormancy (Kushiro et al., 2004), and ABA-positive signaling mutants such as *abscisic acid-insensitive3* (*abi3*), *abi4*, and *abi5* are less sensitive to ABA-mediated inhibition of seed germination (Finkelstein, 1994). Similarly, GA biosynthetic mutants such as *gibberellic acid-requiring1* (*ga1*) do not germinate even under favorable conditions, and various GA signaling mutants, such as *GA-insensitive* (*gai*), *sleepy1* (*sly1*), and *spindly* (*spy*), affect seed germination (Koornneef et al., 1985; Jacobsen and Olszewski, 1993; Steber et al., 1998).

A number of GA signaling components have been identified, including the DELLA proteins. These DELLA domain–containing members of the GRAS family of proteins have been shown to repress various GA responses, including the promotion of seed germination (Sun and Gubler, 2004). DELLA proteins are regulated by changes in their protein stability. When the bioactive GA level is low, DELLA proteins are relatively stable and GA responses are repressed. By contrast, when the bioactive GA level is high, the DELLA proteins are ubiquitinated by E3 ligases ($SCF^{SLY1\ SNE}$ in *Arabidopsis thaliana* and SCF^{GID2} in rice [*Oryza sativa*]) and rapidly degraded by the 26S proteasome (Silverstone et al., 2001; McGinnis et al., 2003; Sasaki et al., 2003; Dill et al.,

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2004; Strader et al., 2004; Tyler et al., 2004). In *Arabidopsis*, five DELLA proteins (GAI, REPRESSOR OF GA1-3 [RGA], RGA-LIKE1 [RGL1], RGL2, and RGL3) repress overlapping but distinct GA responses (Cheng et al., 2004; Tyler et al., 2004; Yu et al., 2004; Tyler, 2006). Among these DELLA proteins, RGL2 is known to repress seed germination, as shown by germination of *ga1* mutants in the presence of an *rgl2* loss-of-function mutation (Lee et al., 2002). However, a recent study showed that the *ga1 rgl2* double loss-of-function mutant still requires light to germinate; this light requirement was abolished only in the *ga1 gai rga rgl2* quadruple loss-of-function mutant (Cao et al., 2005). These results suggest that light promotes seed germination by inhibiting RGA, GAI, and RGL2 in *Arabidopsis*. However, it is not yet known whether light inhibits these DELLA proteins in germinating seeds solely by increasing bioactive GA levels or by other additional mechanisms.

Seed germination is affected by various environmental factors, including moisture, temperature, and light. Among them, light plays a prominent role in germination (Borthwick et al., 1952). The effect of light on seed germination depends on plant species; light can inhibit germination in cactus, is neutral to it in cultivated rice, and promotes germination in *Arabidopsis* and lettuce (*Lactuca sativa*) (Borthwick et al., 1952; Shinomura et al., 1994; Milberg et al., 2000). It is not clear why different seeds show differential light responses, but seed mass tends to be negatively correlated with the requirement of light for germination (Milberg et al., 2000).

Among light spectra, red and far-red wavelengths regulate seed germination through phytochromes (Shinomura et al., 1994). The *Arabidopsis* genome contains five phytochromes, PHYA to PHYE (Quail, 1998), which regulate shared but distinct physiological processes in response to different light spectra and fluences. For hypocotyl elongation, PHYA mediates the very low fluence response and the far-red high irradiance response (FR-HIR), while PHYB mediates the low fluence response (LFR) (Mazzella et al., 1997). For flowering, PHYA promotes flowering, while PHYB inhibits flowering (Lin, 2000). The other phytochromes play more minor roles in these processes; PHYC and PHYD mediate LFR for inhibition of hypocotyl elongation, while PHYD and PHYE mediate LFR for inhibition of petiole elongation (Aukerman et al., 1997; Devlin et al., 1998; Monte et al., 2003). For germination, PHYA promotes germination in response to the very low fluence response and FR-HIR, while PHYB promotes germination in response to red light LFR (Shinomura et al., 1994, 1996). However, since PHYA is not present in dry *Arabidopsis* seeds, PHYA is functional only after imbibition. PHYE has also been shown to mediate LFR and FR-HIR for seed germination (Hennig et al., 2002).

Light perceived by phytochromes is known to promote seed germination partly by increasing GA biosynthesis, as inferred from the ability of exogenous GA treatment to trigger seed germination in the absence of light and/or phytochromes (Hilhorst and Karssen, 1988). Gene expression analysis revealed that phytochromes increase bioactive GA levels in germinating seeds by activating the transcription of GA3-oxidase genes (*GA3ox1* and *GA3ox2*) and repressing the transcription of a GA2-oxidase gene (*GA2ox2*) (Yamaguchi et al., 1998; Oh et al., 2006). Since the GA3-oxidase converts GA precursors (GA_9 and GA_{20}) to bioactive GAs (GA_4 and GA_1) and the GA2-oxidase catabolizes bioactive GAs to biologically inactive GAs (GA_{34} and GA_8), the reciprocal transcriptional regulation of these genes by phytochromes leads to increased bioactive GA levels (Oh et al., 2006), which promote seed germination by activating the degradation of DELLA proteins (Tyler et al., 2004).

PHYTOCHROME-INTERACTING FACTOR3-LIKE5 (PIL5/PIF1/bHLH015), a phytochrome-interacting basic helix-loop-helix (bHLH) protein, is one of the major components linking light signals to GA metabolism. PIL5 inhibits seed germination by lowering bioactive GA levels in the absence of light, at least partly by repressing the transcription of two *GA3ox* genes while activating one *GA2ox2* gene (Oh et al., 2004, 2006). The ability of PIL5 to link light signals and GA metabolism in seeds is mediated by its light-dependent protein stability (Oh et al., 2006). When activated by light, phytochromes accelerate the degradation of PIL5 in seeds, releasing the transcriptional repression of the GA3-oxidase genes and the transcriptional activation of the GA2-oxidase gene, leading to increases in the levels of bioactive GA in seeds. Phytochromes also accelerate the degradation of PIL5 in seedlings (Shen et al., 2005), but its role in GA metabolism is not clear in seedlings. Another known component is SPT/bHLH024, which represses transcription of the two GA3-oxidase genes and integrates cold and light signaling with GA metabolism (Penfield et al., 2005). The relationship between SPT and phytochromes is not yet known.

Although PIL5 links light signals to GA metabolism by regulating the transcription of GA metabolic genes, it is not yet known whether PIL5 directly regulates the transcription of the *GA3ox* genes and/or the *GA2ox2* gene, and it is not yet known whether PIL5-mediated light signaling promotes seed germination solely by regulating GA metabolism. Here, we show that PIL5-mediated light signaling regulates not only GA metabolism but also GA signaling and ABA metabolism. Using chromatin immunoprecipitation (ChIP) analysis, we further show that two DELLA genes, *RGA* and *GAI*, are direct target genes of PIL5, whereas the GA and ABA metabolic genes are regulated indirectly by PIL5. These data show that phytochrome-perceived light signals are translated into changes in the level of the PIL5 protein, which in turn directly regulates the transcription of two DELLA genes and indirectly regulates the transcription of GA and ABA metabolic genes.

RESULTS

Light Regulates GA Responsiveness through PIL5 in Germinating Seeds

Previous studies have shown that light regulates bioactive GA levels through PIL5 by reciprocally regulating the transcription of two GA biosynthetic genes (*GA3ox1* and *GA3ox2*) and one GA catabolic gene (*GA2ox2*) in germinating seeds (Oh et al., 2006). To investigate whether light mediates seed germination solely by regulating GA metabolic genes, we examined the light-dependent germination frequency of *ga1* mutant and *pil5 ga1* double mutant seeds on media containing various concentrations of GA_3. Unlike the endogenously produced GA_4, GA_3 contains a C1-C2 double bond; thus, it is thought be protected

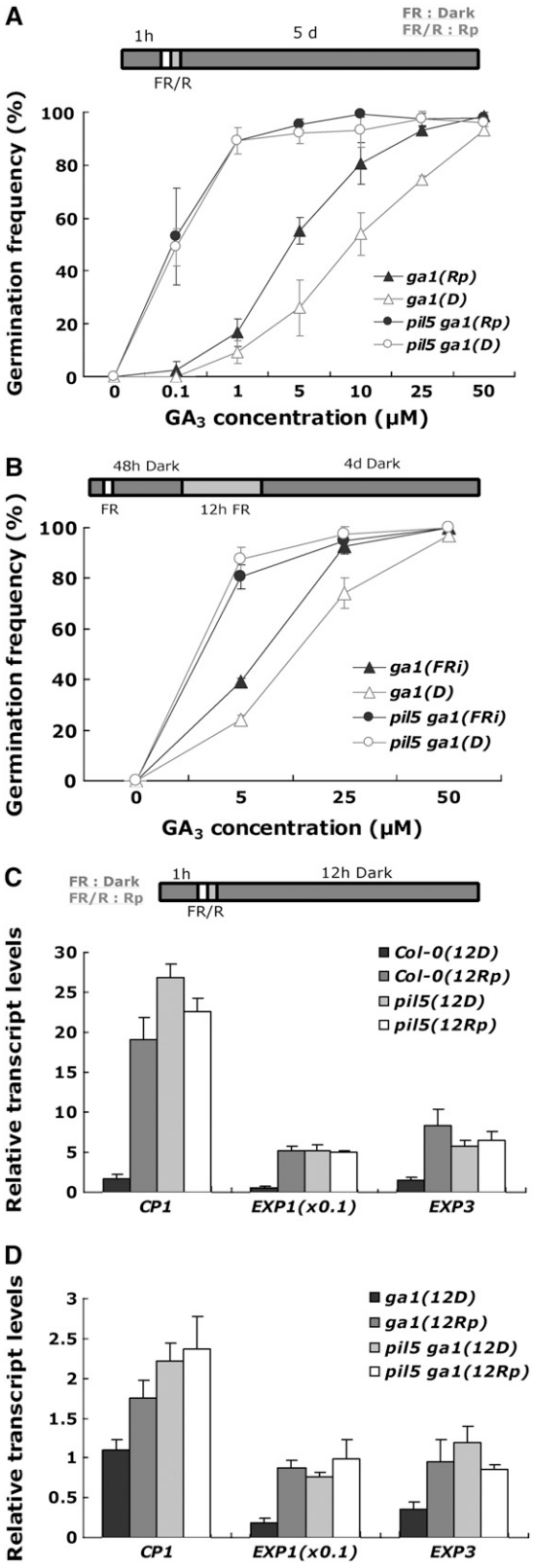

Figure 1. Both Light and the *pil5* Mutation Increase GA Responsiveness in Seeds.

from deactivation by GA2-oxidase (Nakayama et al., 1990). In fact, we confirmed that GA_3 was not metabolized by recombinant GA2ox2 protein produced in *Escherichia coli*, whereas the majority of GA_4 was 2-hydroxylated in the same enzyme assay conditions (S. Yamaguchi, unpublished data). Therefore, if light regulates germination only through *GA3ox1*, *GA3ox2*, and *GA2ox2*, the *ga1* mutant lacking synthesis of de novo bioactive GA should respond identically to exogenously supplied GA_3 irrespective of light conditions.

Surprisingly, the germination frequency of *ga1* mutant seeds showed light dependence in the presence of low to moderate concentrations of exogenous GA_3. Under PHYB-dependent germination conditions (1 h of imbibition followed by a pulse of red or far-red light), the germination frequency of the *ga1* mutant reached virtually 100% in the presence of 50 μM GA_3, irrespective of light conditions (Figure 1A). However, at concentrations of <50 μM GA_3, the germination frequency was higher in *ga1* mutant seeds irradiated with red light versus those irradiated with far-red light. Although less prominent, the germination frequency of the *ga1* mutant also showed a similar light dependence in the presence of 2,2-dimethyl-GA_4 (Beale and MacMillan, 1981) (see Supplemental Figure 1 online). By contrast, the *pil5 ga1* double mutant germinated almost 100% in response to even 5 μM GA_3, irrespective of light conditions, suggesting that PIL5-mediated light signaling regulates GA responsiveness under PHYB-dependent germination conditions even in the absence of de novo GA biosynthesis and deactivation by GA2-oxidase. Similar results were obtained under PHYA-dependent germination conditions (inactivation of PHYB with a far-red light pulse, followed by seed imbibition for 48 h and induction of germination by prolonged far-red light irradiation [FRi]). Under these conditions, 50 μM GA_3 was sufficient to induce 100% germination in the *ga1* mutant irrespective of FRi, whereas *ga1* mutant seeds exposed to lower concentrations of GA_3 germinated better when PHYA

(A) PHYB-dependent germination frequency of *ga1* and *pil5 ga1* mutants in the presence of various concentrations of GA_3. The diagram at top depicts the light treatment scheme for the experiments. Rp, seeds were irradiated with red light followed by far-red light and then subjected to dark incubation for 5 d; D, seeds were irradiated with far-red light prior to dark incubation for 5 d.

(B) PHYA-dependent germination frequency of *ga1* and *pil5 ga1* mutants in the presence of various concentrations of GA_3. FRi, seeds were allowed to imbibe for 48 h in the dark and then irradiated with far-red light for 12 h; D, seeds were allowed to imbibe for 48 h in the dark but not subjected to irradiation.

(C) Relative expression levels of GA-inducible genes in ecotype Columbia (Col-0) and *pil5* mutant seeds. The relative expression levels of the GA-inducible genes were normalized versus the expression level of *PP2A*. The diagram at top depicts the light treatment scheme for the experiments. 12D, seeds were irradiated with far-red light and then dark incubated for 12 h; 12Rp, seeds were irradiated with red light followed by far-red light and then dark incubated for 12 h. The relative level of *EXP1* was multiplied by 0.1 (×0.1) to allow it to be presented in the same graph.

(D) Relative expression levels of GA-inducible genes in *ga1* and *pil5 ga1* mutant seeds. The light treatment scheme and notations are as described for **(C)**. Similar results were obtained from three independent experiments, and a representative result is shown along with SD values.

was activated by FRi (Figure 1B). Furthermore, the *pil5 ga1* double mutant germinated better than the *ga1* mutant at lower concentrations of GA_3 and did not show light dependence. Together, these results suggest that both PHYA and PHYB promote seed germination not only by regulating known GA metabolic genes but also by regulating other genes that affect GA responsiveness through PIL5.

The notion that light regulates GA signaling by mechanisms other than modulation of GA metabolism prompted us to examine whether red light or the *pil5* mutation could trigger GA responses without de novo GA biosynthesis. We determined the expression of three well-known GA-inducible genes, *CYSTEINE PROTEINASE1* (*CP1*), *EXPANSIN1* (*EXP1*), and *EXP3* (Ogawa et al., 2003), in *ga1* and *pil5 ga1* seeds. Consistent with a previous report that red light irradiation and the *pil5* mutation caused increased bioactive GA levels (Oh et al., 2006), we found that red light irradiation of wild-type seeds and the *pil5* mutation caused highly increased expression of the tested genes (Figure 1C). Red light also increased the expression of the tested genes in *ga1* mutant seeds (Figure 1D), whereas the expression levels of the tested genes were high in *pil5 ga1* double mutant seeds irrespective of light conditions. These results indicate that light triggers GA signaling through PIL5 without de novo GA biosynthesis. The degree of induction by red light, however, was lower in the *ga1* mutant than in the wild type, suggesting that both GA biosynthesis and GA signaling contribute to the expression of these GA-inducible genes in seeds.

Light Does Not Regulate the Protein Stabilities of GAI and RGA in the Absence of GA Biosynthesis

To elucidate the molecular mechanism(s) by which PIL5 regulates GA responsiveness, we investigated whether light regulates the stability of the DELLA proteins. We generated transgenic plants expressing GAI-myc and RGA-myc proteins under the control of the cauliflower mosaic virus 35S promoter. The germination frequencies of the transgenic plants were lower than those of wild-type plants, suggesting that both recombinant proteins were functional (data not shown). We then determined the protein stabilities of GAI-myc and RGA-myc under PHYB-dependent seed germination conditions. When the transgenic seeds were irradiated with red light, the amounts of GAI-myc and RGA-myc decreased (Figure 2A), indicating that red light triggered the degradation of these DELLA proteins in seeds. However, the light-induced degradation of GAI-myc or RGA-myc was inhibited in the presence of paclobutrazol, indicating that the degradation was dependent on de novo GA biosynthesis (Figure 2B). Consistent with this notion, treatment of the transgenic seeds with exogenous GA triggered degradation of the GAI-myc and RGA-myc proteins (Figure 2B). These results indicate that phytochromes activate the degradation of DELLA proteins by increasing de novo GA biosynthesis but do not directly regulate the stabilities of DELLA proteins in the absence of de novo GA biosynthesis. Since the enhancement of GA responsiveness by red light was observed even in the *ga1* mutant, these results further suggest that the direct light-dependent destabilization of DELLA proteins is not responsible for the light-induced enhancement of GA responsiveness.

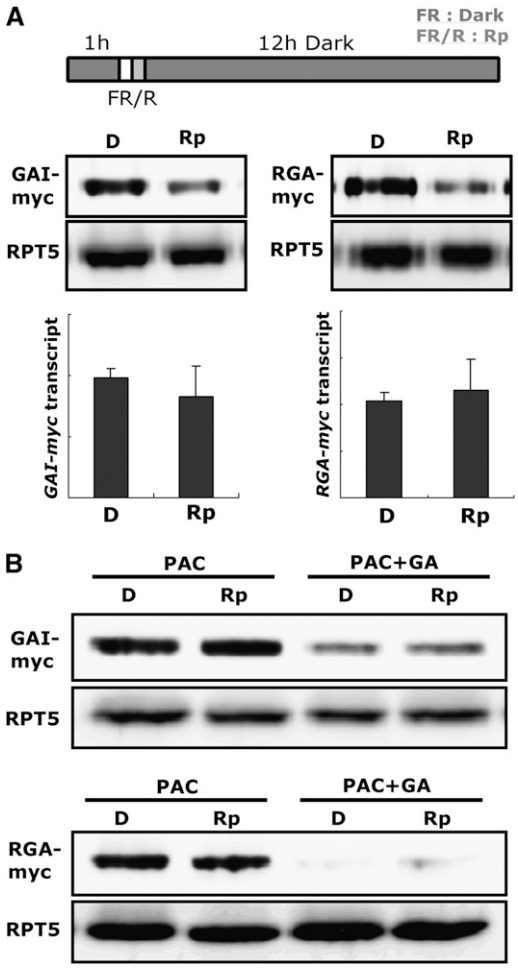

Figure 2. Light Does Not Regulate the Protein Stabilities of GAI and RGA in the Absence of GA Biosynthesis.

(A) Protein levels of myc-tagged GAI and RGA in seeds subjected to dark incubation for 12 h after either far-red light irradiation or red light irradiation followed by far-red light irradiation. RPT5 was used as a loading control. Myc-tagged GAI and RGA were expressed under the control of the cauliflower mosaic virus 35S promoter. The diagram at top indicates the light treatment. The other panels indicate the transcript levels of *GAI-myc* and *RGA-myc* transgenes in different light conditions. Abbreviations are as described for Figure 1C.

(B) Protein level of myc-tagged GAI and myc-tagged RGA in seeds allowed to imbibe in medium containing 100 μM paclobutrazol (PAC) or 100 μM paclobutrazol plus 100 μM GA_{4+7} (PAC+GA). The light treatment scheme and notations are as described for **(A)**.

PIL5 Regulates Seed Germination Partly through the Transcriptional Regulation of *GAI* and *RGA*

Next, we examined whether PIL5 regulates the expression levels of various GA signaling genes. We extracted total RNA from *ga1* and *pil5 ga1* mutant seeds that were irradiated with a far-red light pulse alone or with a far-red pulse followed by a red light pulse. We used *ga1* mutant seeds instead of Col-0 seeds to avoid the expression change associated with newly synthesized bioactive

GAs. The expression patterns of various GA metabolic and signaling genes were determined by quantitative RT-PCR. Consistent with our previous report, both red light irradiation and the *pil5* mutation caused increased expression of *GA3ox1* and *GA3ox2* but decreased expression of *GA2ox2* (Figure 3A) (Oh et al., 2006).

Among the examined GA signaling genes, we found that red light irradiation reduced the expression levels of two *DELLA* genes, *GAI* and *RGA*, by threefold to fourfold in *ga1* seeds (Figure 3A). The expression levels of the same genes were reduced in *pil5 ga1* seeds irrespective of light conditions, suggesting that PHYB regulates the expression of *GAI* and *RGA* through PIL5. By contrast, the expression levels of three other *DELLA* genes, *RGL1*, *RGL2*, and *RGL3*, were not affected by red light irradiation or the *pil5* mutation (Figure 3A). Among other signaling genes, expression of *SPY* was reduced slightly by red light and the *pil5* mutation, whereas that of *SLY1* was not affected by either condition. To further demonstrate that the light-mediated reductions in *RGA* expression levels led to reduced RGA protein levels, we performed protein gel blot analysis using an anti-RGA antibody (Figure 3C). Our results revealed that endogenous RGA protein levels were decreased by red light in the *ga1* mutant. In the *pil5 ga1* double mutant, the RGA level was low irrespective of light conditions, suggesting that the transcriptional repression of *RGA* and the subsequent decrease of RGA protein is mediated by PIL5. Since DELLA genes encode GA-negative signaling components (Sun and Gubler, 2004), repression of these genes and the subsequent decrease of these proteins by red light and the *pil5* mutation could explain the higher GA responsiveness in red light–irradiated *ga1* mutant seeds and *pil5 ga1* double mutants.

Since we also observed that activation of PHYA increased the GA responsiveness (Figure 1B), we examined whether the expression levels of *GAI* and *RGA* were also reduced by prolonged far-red light irradiation (i.e., PHYA-dependent germination conditions). Similar to our findings under the PHYB-dependent germination conditions, the expression levels of *GAI* and *RGA* were reduced by both prolonged FRi and the *pil5* mutation (Figure 3B), suggesting that PHYA also increases GA responsiveness by inhibiting the expression of *GAI* and *RGA* though PIL5.

The functional significance of the PIL5-mediated transcriptional regulation of *GAI* and *RGA* was further investigated by examining the germination frequencies of the following: *gai-t6* and *rga-28*, which are loss-of-function mutants caused by a Ds insertion and a T-DNA insertion, respectively (Peng et al., 1997; Tyler et al., 2004); *gai-1*, which is a dominant gain-of-function mutant caused by a GA-insensitive active gai mutant protein (Koornneef et al., 1985; Peng et al., 1997); and the *gai-t6 rga-28*, *PIL5OX3 rga-28*, and *pil5 gai-1* double mutants. The germination frequencies of the various mutants were determined in response to varying red light fluences under PHYB-dependent germination conditions. Our results revealed that the *gai-t6* and *rga-28* single loss-of-function mutants germinated similarly to wild-type plants in response to varying red light fluences (Figure 4A). By contrast, the *gai-t6 rga-28* double mutant germinated much better in response to increasing red light fluences. At 0 μmol·m^{-2} red light fluence, the double mutant germinated ~15%, whereas the wild-type and single mutant seeds did not germinate under this light condition. At 5 μmol·m^{-2}, the double mutant germinated ~60%,

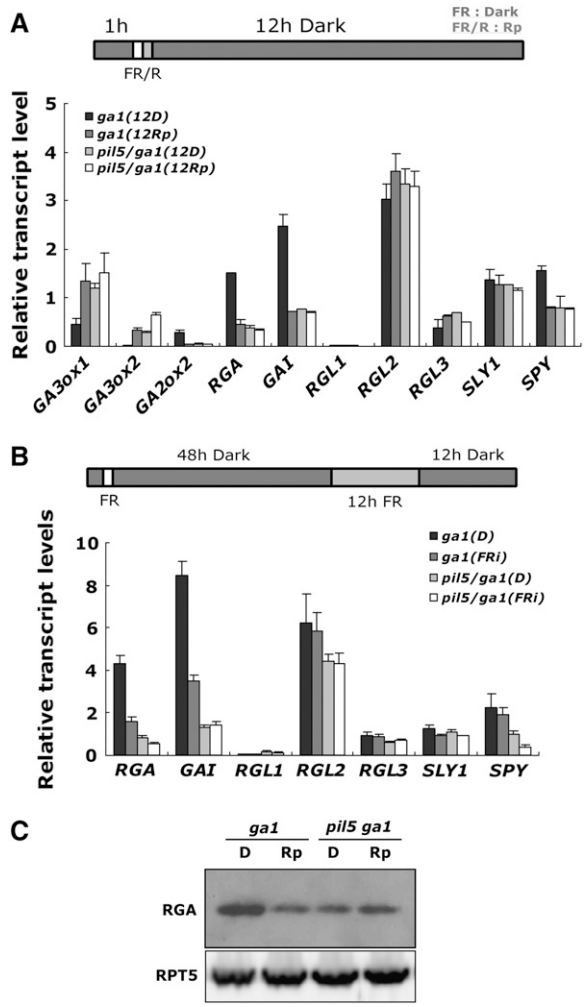

Figure 3. PIL5 Regulates the Expression of *GAI* and *RGA*.

(A) Relative expression levels of GA metabolic genes and GA signaling genes in *ga1* and *pil5 ga1* mutant seeds under PHYB-dependent germination conditions. The relative expression levels of the tested genes were normalized versus that of *PP2A*. Abbreviations in the light treatment diagram are as described for Figure 1C.

(B) Relative expression levels of GA signaling genes in *ga1* and *pil5 ga1* mutant seeds under PHYA-dependent germination conditions. The diagram at top depicts the light treatment scheme for the experiments. D, seeds were allowed to imbibe for 48 h in the dark and not subjected to irradiation; FRi, seeds were allowed to imbibe for 48 h in the dark and then subjected to far-red irradiation for 12 h. Similar results were obtained from three independent experiments, and a typical result is presented with SD values.

(C) Endogenous RGA protein levels in response to red light treatment in the *ga1* and *pil5 ga1* seeds. Abbreviations for the light treatment are as described for Figure 1C. The protein blot contained 160 μg of total protein from imbibed seeds and was probed with polyclonal anti-RGA antibodies from rat. RPT5 protein level was used to confirm equal loading. Experiments using two additional biological replicates showed similar results.

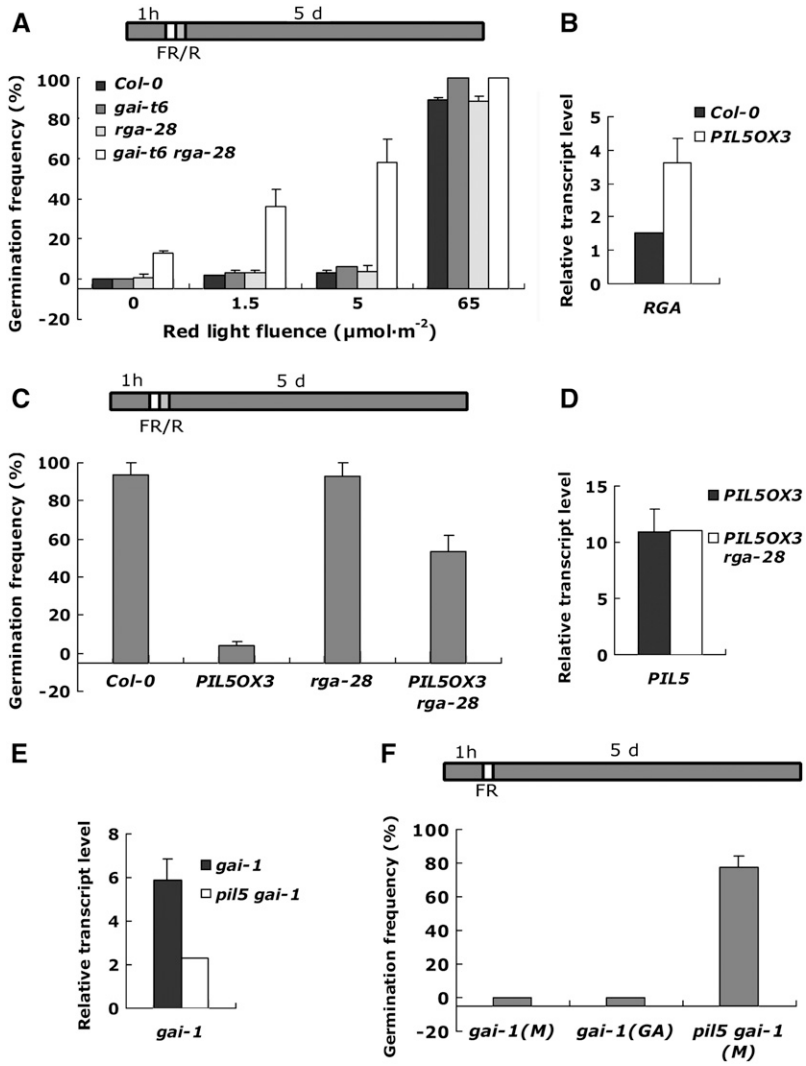

Figure 4. PIL5-Mediated Transcriptional Regulation of *GAI* and *RGA* Is Functionally Significant for the Regulation of Seed Germination by Light.

(A) Germination frequencies of Col-0, *gai-t6*, *rga-28*, and *gai-t6 rga-28* seeds irradiated with various fluences of red light. The diagram at top depicts the light treatment scheme for the experiments.

(B) Relative expression levels of *RGA* in Col-0 and *PIL5OX3* seeds 12 h after seeds were subjected to a red light pulse followed by a far-red light pulse.

(C) Germination frequencies of Col-0, *PIL5OX3*, *rga-28*, and *PIL5OX3 rga-28* seeds after red light irradiation. The diagram at top depicts the light treatment scheme for the experiments.

(D) Relative expression levels of *PIL5* in *PIL5OX3* and *PIL5OX3 rga-28* seeds.

(E) Relative expression levels of the *gai* gene in *gai-1* and *pil5 gai-1* mutant seeds 12 h after seeds were subjected to a red light pulse followed by a far-red light pulse.

(F) Germination frequencies of *gai-1* and *pil5 gai-1* seeds after far-red light irradiation. The diagram at top depicts the light treatment scheme for the experiments. M, mock medium; GA, 100 μM GA_{4+7} medium.

Error bars indicate SD ($n = 3$).

while the wild type and the two single mutants germinated only ~5%. At a higher red light fluence (65 μmol·m^{-2}), all seeds germinated ~100%. These results clearly indicate that GAI and RGA negatively regulate seed germination.

If PIL5 regulates seed germination partly by regulating the transcription of *GAI* and *RGA*, then the germination frequency of *PIL5OX3 rga-28* double mutant seeds should be higher, because functional *RGA* would not be increased by PIL5 overexpression in these seeds. Similarly, the germination frequency of *pil5 gai-1* should be higher than that of the *gai-1* single mutant, because the level of *gai-1* would be lower in the *pil5 gai-1* double mutant than in the *gai-1* single mutant. Consistent with the lower expression of *RGA* in the *pil5* mutant (Figure 3A), overexpression of PIL5 led to increased expression of *RGA* (Figure 4B). Under

PHYB-dependent light conditions at 65 μmol·m^{-2} red light fluence, *PIL5OX3 rga-28* seeds germinated ~50%, *PIL5OX3* seeds failed to germinate, and wild-type and *rga-28* mutants seeds showed almost 100% germination (Figure 4C). Since the crossing of a transgenic plant with other plants could change the transgene expression level, we examined the expression level of *PIL5* in *PIL5OX3 rga-28* and found that it was similar to that in *PIL5OX3* plants (Figure 4D), indicating that the higher germination frequency was not due to reduced expression of the *PIL5* transgene in *PIL5OX3 rga-28* plants. These results suggest that PIL5 inhibits seed germination partly through transcriptional activation of *RGA*.

The functional significance of PIL5-mediated transcriptional regulation of the two *DELLA* genes was also determined in the *pil5 gai-1* double mutant. The expression of *gai-1* in the *pil5 gai-1* double mutant was lower than in the *gai-1* single mutant (Figure 4E). The lower expression of *gai-1* in the double mutant was associated with a higher germination frequency in the double mutant compared with the *gai-1* single mutant (Figure 4F). The *gai1-1* single mutant did not germinate in the absence of red light irradiation, whereas the *pil5 gai1-1* double mutant germinated well even without any red light irradiation. Addition of exogenous GA did not rescue the germination phenotype of *gai-1* mutant seeds (Figure 4F), suggesting that the higher germination frequency of *pil5 gai-1* double mutants was not due to increased synthesis of endogenous GA governed by the *pil5* mutation. Together, our results indicate that the PIL5-induced transcriptional regulation of the *GAI* and *RGA* genes plays an important role in regulating light-dependent seed germination.

PIL5 Binds Directly to *GAI* and *RGA* Gene Promoters through G-Box Elements in Vivo but Does Not Bind to the Promoters of GA Metabolic Genes

Expression analysis showed that PIL5 regulates both GA metabolic genes and GA signaling genes. Since PIL5 is a transcription factor, it could regulate these genes either directly or indirectly. To investigate whether PIL5 regulates these genes by directly binding to their promoters in vivo, we performed ChIP assays. Since a previous in vitro binding assay showed that PIL5 binds to the G-box element (CACGTG) (Huq et al., 2004), we examined the promoters of various GA metabolic and signaling genes for the presence of G-box elements. Sequence analysis indicated that the promoters of *GA3ox1*, *GA3ox2*, *GAI*, and *RGA* contain one or more G-box elements, whereas the promoters of *GA2ox2* and *SPY* do not (Figure 5A). Since E-box elements (CANNTG) serve as binding sites for many bHLH transcription factors (Chaudhary and Skinner, 1999), we also searched for E-box elements and found that all of the examined gene promoters contained multiple copies of the E-box element. Using PIL5OX3 seeds that express myc-tagged PIL5, we then performed ChIP assays on cross-linked and sonicated protein–DNA complexes precipitated with an anti-myc antibody. The amount of a given target DNA fragment present in the precipitated complex was measured by quantitative RT-PCR, and the enrichment of the target fragment was determined as the relative amount of precipitated DNA fragment versus input DNA. As a negative control, we performed the same ChIP assay using transgenic seeds expressing myc-tagged green fluorescent protein (GFP).

The relative amounts of precipitated promoter fragments of *GA3ox1*, *GA3ox2*, *GA2ox2*, and *SPY* were very low (<0.2%) when myc-tagged PIL5 was precipitated with the anti-myc antibody (Figure 5B). These values were not significantly different from those obtained with myc-tagged GFP (Figure 5C), suggesting that PIL5 does not bind the promoters of these genes. By contrast, the relative amounts of precipitated promoter fragments of *GAI* and *RGA* were much higher in experiments containing the myc-tagged PIL5 versus those containing myc-tagged GFP (Figure 5C). Some of the *GAI* and *RGA* promoter fragments were precipitated much more strongly (*RGA-B*, >1%; *GAI-B*, >0.6%) than others (<0.2%), suggesting that PIL5 binds to specific promoter fragments in vivo. The *GAI-B* and *RGA-B* promoter fragments, which contained G-box elements, showed the highest enrichments, whereas promoter fragments farther from *GAI-B* and *RGA-B* were less enriched, suggesting that PIL5 binds to G-box elements in vivo. Notably, not all of the tested G-box elements served as binding sites for PIL5. G-box elements are present in the promoters of *GA3ox1* and *GA3ox2* and in the coding region of *GAI*, but DNA fragments containing those G-box elements were not enriched by PIL5.

To further show that PIL5 binds to the G-box elements found in the promoters of *GAI* and *RGA*, we performed in vitro binding assays with a recombinant PIL5. Our results revealed that the recombinant PIL5 protein bound well to biotin-labeled *GAI* and *RGA* promoter fragments containing G-box elements in vitro (Figures 6A to 6C). The binding to these fragments was effectively out-competed by unlabeled corresponding fragments but was not out-competed by fragments containing mutated G-box elements. Together, these results suggest that PIL5 binds directly to the promoters of *GAI* and *RGA* through G-box elements. The direct binding of PIL5 protein to only the *GAI* and *RGA* promoters further suggests that PIL5 directly regulates the expression of *GAI* and *RGA* but not that of the GA metabolic genes and *SPY*.

Since the above results suggested that PIL5 indirectly regulates GA metabolic genes, we examined whether PIL5 regulates the expression of GA metabolic genes through GAI and RGA by comparing the expression patterns of GA metabolic genes in wild-type and *gai-t6 rga-28* double mutant plants in the presence of paclobutrazol. The expression levels of *GA3ox2* and *GA2ox2* were similar in wild-type and double mutant seeds and showed similar inductions in response to red light (Figure 7). By contrast, red light irradiation induced the expression of *GA3ox1* in both wild-type and double mutant seeds, but the basal expression level of *GA3ox1* was higher in the double mutant than in red light–irradiated wild-type seeds. This observation is contradictory to the notion that the expression of *GA3ox1* is feedback-regulated by the activity in the GA response pathway. A previous report indicated that the amount of *GA3ox1* transcript is lower in *gai-t6 rga-24 ga1* triple mutant seedlings than in the *ga1* single mutant (Dill and Sun, 2001). The difference is likely due to the difference between germinating seeds and seedlings, as the *GA3ox1* mRNA level in light-grown *gai-t6 rga-28* mutant seedlings was lower than in the wild type (see Supplemental Figure 2 online). Together, these results suggest that PIL5 regulates the expression of *GA3ox2*

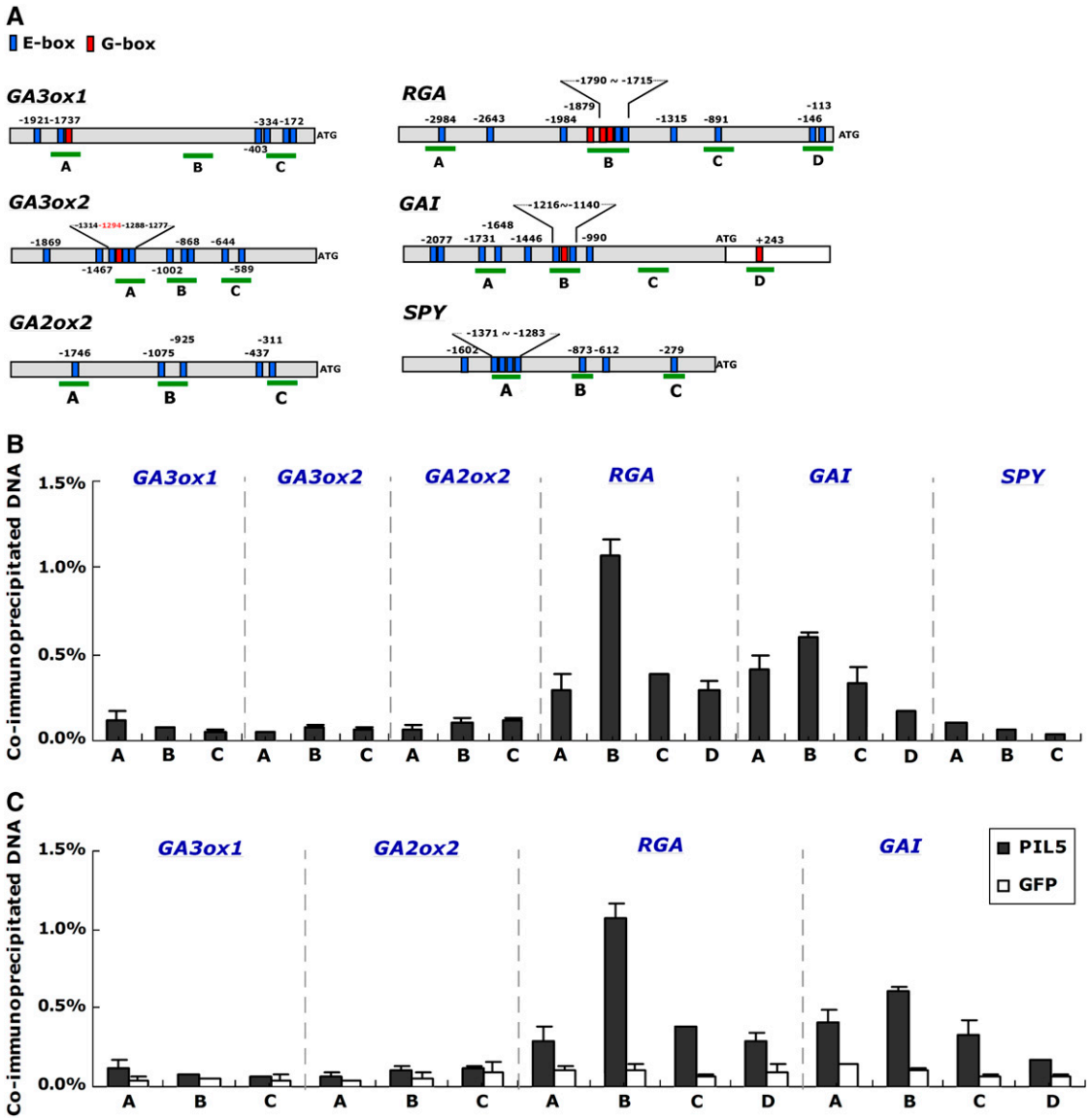

Figure 5. PIL5 Binds Directly to the Promoters of *GAI* and *RGA* in Vivo.

(A) Promoter structures of various GA metabolic and GA signaling genes. Blue boxes indicate E-box elements (CANNTG), red boxes indicate G-box elements (CACGTG), and green lines with uppercase letters indicate the various promoter fragments used for PCR.

(B) Percentage of DNA fragments coimmunoprecipitated with the anti-myc antibody relative to the input DNA.

(C) Percentage of DNA fragments coimmunoprecipitated with the anti-myc antibody relative to input DNA. PIL5, PIL5-myc transgenic seeds were used for the ChIP assay; GFP, GFP-myc transgenic seeds were used for the ChIP assay. Similar results were obtained from three independent experiments, and a typical result is presented with SD values.

and *GA2ox2* independent of GA signaling but regulates *GA3ox1* both dependently and independently of GAI and RGA in seeds.

PIL5 Regulates ABA Metabolism through Both GA-Dependent and GA-Independent Pathways

Another plant hormone that regulates seed germination, ABA, is known to establish and maintain seed dormancy; its reduction is necessary to break seed dormancy and to weaken the endosperm for the completion of seed germination (Finch-Savage and Leubner-Metzger, 2006; Muller et al., 2006). A recent study indicated that ABA levels are also regulated by phytochromes as a result of altered expression of some ABA metabolic genes (Seo et al., 2006). Since PIL5 is a phytochrome-interacting transcription factor that regulates seed germination, we investigated whether PIL5 regulates the expression of ABA metabolic genes in germinating seeds.

Expression analysis was performed under PHYB-dependent germination conditions using *ga1* mutant seeds to eliminate de

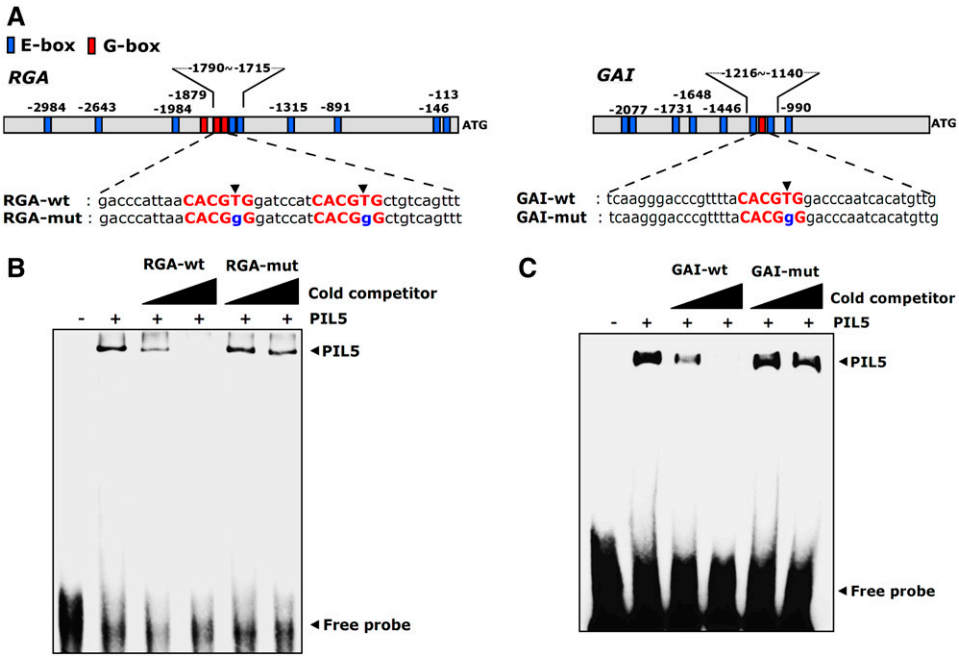

Figure 6. PIL5 Binds Directly to G-Box Elements in the Promoters of *GAI* and *RGA* in Vitro.

(A) Probe sequences corresponding to the *RGA* and *GAI* promoters. wt, probe with an intact G-box element; mut, probe with a mutated G-box element.
(B) Electrophoretic mobility shift assay of a *RGA* promoter fragment. PIL5 binds specifically to the RGA-wt probe but not to the RGA-mut probe.
(C) Electrophoretic mobility shift assay of a *GAI* promoter fragment. PIL5 binds specifically to the GAI-wt probe but not to the GAI-mut probe. Triangles indicate increasing amounts of unlabeled probes for competition in **(B)** and **(C)**.

novo GA biosynthesis in response to red light. Our results showed that among the tested ABA biosynthetic genes, *ABA1*, *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE6* (*NCED6*), and *NCED9* were repressed by red light, whereas *ABA2* was not significantly affected (Figure 8A) and *ABSCISIC ALDEHYDE OXIDASE3* (*AAO3*) was only marginally affected by red light. By contrast, the expression of an ABA catabolic gene, *CYP707A2*, was increased by red light. In the *pil5 ga1* double mutant, *ABA1*, *NCED6*, and *NCED9* were repressed irrespective of light conditions, whereas *CYP707A2* was activated further by red light. Consistent with these findings, overexpression of PIL5 increased the expression levels of *ABA1*, *NCED6*, and *NCED9* and decreased the expression of *CYP707A2* (Figure 8B). These results collectively suggest that three of the ABA biosynthetic genes are regulated by light through PIL5, whereas the ABA catabolic gene is regulated by light both dependently and independently of PIL5.

The expression patterns of the tested ABA metabolic genes suggested that the endogenous level of ABA should be lower in *pil5* mutant seeds and red light–irradiated wild-type seeds compared with far-red light–irradiated seeds. Measurement of ABA levels revealed that red light–irradiated wild-type seeds contained lower ABA levels compared with far-red light–irradiated seeds (Figure 8C). Consistent with the lower expression of ABA biosynthetic genes and the higher expression of the ABA catabolic gene in the *pil5* mutant, the ABA levels in *pil5* mutant seeds were as low as those seen in red light–irradiated wild-type seeds irrespective of light conditions. ABA levels also showed light dependence in *ga1* mutant seeds but not in *pil5 ga1* double mutant seeds, indicating that PIL5 regulates the light-dependent reduction of ABA levels in germinating seeds irrespective of de novo GA biosynthesis.

PIL5 may regulate ABA metabolic genes either directly or indirectly. Unlike the *GAI* and *RGA* promoters, the promoters of

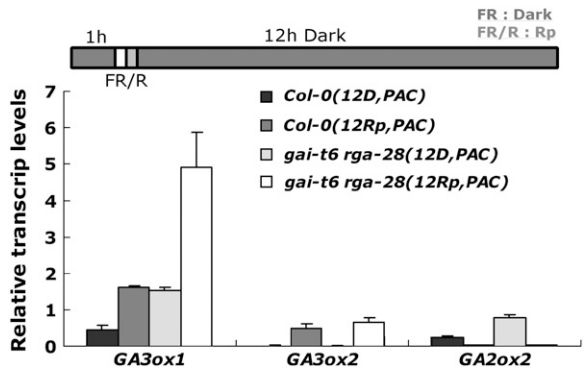

Figure 7. Light Regulates GA Metabolic Genes Both Dependently and Independently of GAI and RGA.

RT-PCR was performed, and the relative expression levels of various genes were normalized versus that of *PP2A*. Abbreviations in the diagram at top are as described for Figure 1D. PAC, paclobutrazol. Similar results were obtained from three independent experiments, and a typical result is presented with SD values.

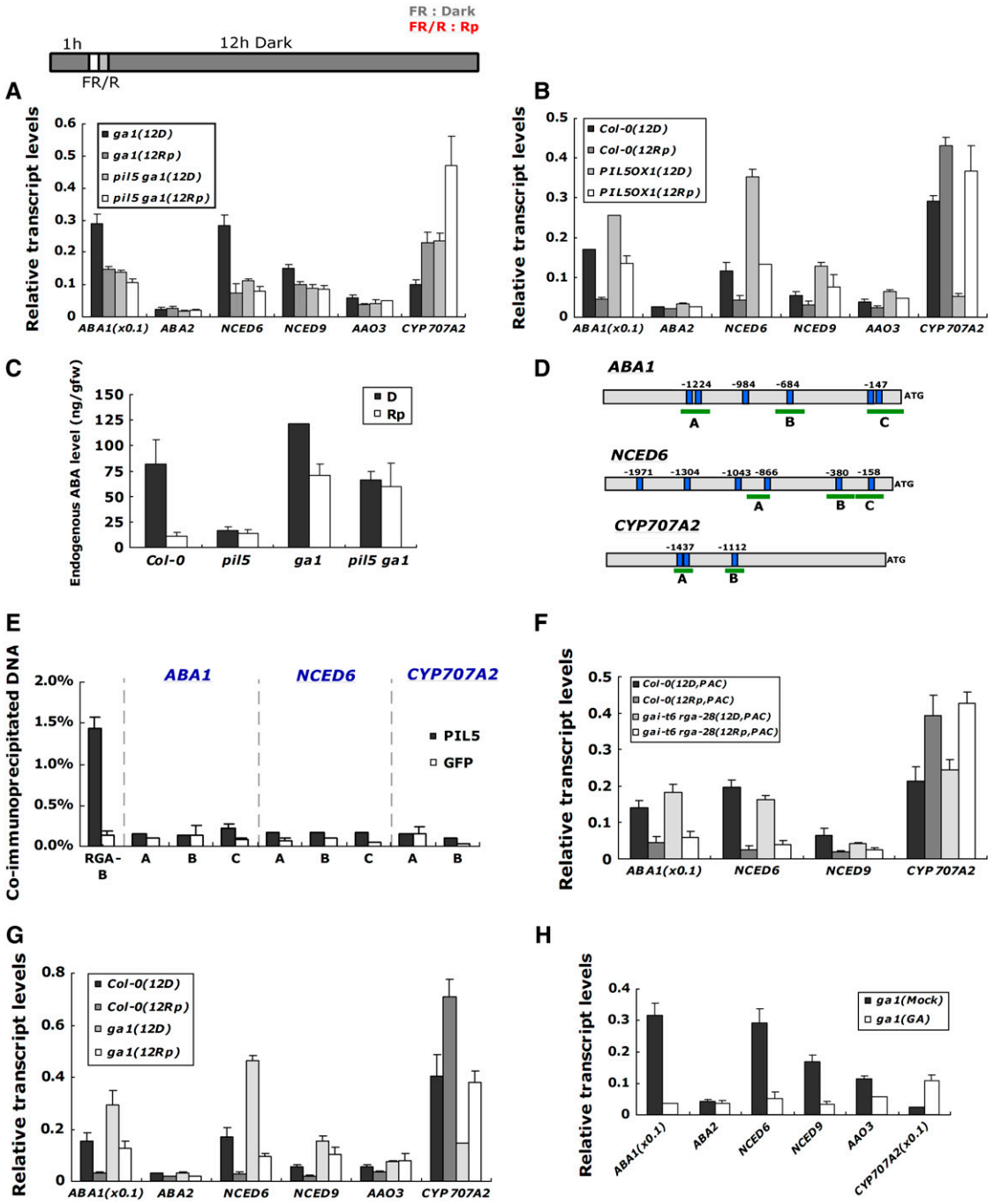

Figure 8. PIL5 Indirectly Regulates the Expression of ABA Metabolic Genes.

(A) Relative expression levels of ABA metabolic genes in *ga1* and *pil5 ga1* seeds were normalized versus that of *PP2A*. Abbreviations in the diagram at top are as described for Figure 1D.

(B) Relative expression levels of ABA metabolic genes in Col-0 and *PIL5OX1* seeds. The light treatment scheme and notations are as described for **(A)**.

(C) Endogenous ABA levels in Col-0, *pil5*, *ga1*, and *pil5 ga1* seeds.

(D) Promoter structures of the *ABA1*, *NCED6*, and *CYP707A2* genes. Blue boxes indicate E-box elements (CANNTG), and green lines with uppercase letters indicate the various promoter fragments used for PCR.

(E) Percentage of DNA fragments coimmunoprecipitated with the anti-myc antibody relative to input DNA. PIL5, PIL5-myc transgenic seeds were used for the ChIP assay; GFP, GFP-myc transgenic seeds were used for the ChIP assay.

(F) Relative expression levels of ABA metabolic genes in Col-0 and *gai-t6 rga-28* seeds. The light treatment scheme and notations are as described for **(A)**. PAC, paclobutrazol.

the examined ABA metabolic genes do not contain G-box elements that could serve as binding sites for PIL5 (Figure 8D). Consistent with the absence of G-box elements, ChIP analysis showed that the relative amounts of precipitated promoter fragments belonging to *ABA1*, *NCED6*, and *CYP707A2* were low (<0.2%) (Figure 8E). This indicates that PIL5 does not bind directly to the promoters of these genes and that PIL5 indirectly regulates ABA metabolism.

Since PIL5 indirectly regulates ABA metabolism in the absence of de novo GA biosynthesis (Figure 8A), we examined whether PIL5 regulates ABA metabolic genes through *GAI* and *RGA* by determining the expression of ABA metabolic genes in the *gai-t6 rga28* double mutant in the presence of paclobutrazol. Our results revealed that the ABA metabolic genes were expressed similarly between wild-type and double mutant seeds (Figure 8F). The light-dependent expression patterns of ABA biosynthetic genes were also similar between these seeds, suggesting that PIL5 regulates ABA metabolic genes independently of *GAI* and *RGA* in this GA-independent pathway.

Curiously, the overall ABA level was lower in the Col-0 background compared with the *ga1* mutant background (Figure 8C). This observation raised the possibility that GA might also regulate ABA metabolism. To investigate whether the higher ABA level in the *ga1* mutant was reflected in the expression patterns of ABA metabolic genes, we compared expression patterns between wild-type and *ga1* mutant seeds. Our results revealed that the ABA biosynthetic genes *ABA1*, *NCED6*, and *NCED9* were more highly expressed in the *ga1* mutant than in wild-type seeds, whereas the ABA catabolic gene, *CYP707A2*, was expressed at a lower level in the *ga1* mutant versus wild-type seeds (Figure 8G). These results suggest that ABA is synthesized more and degraded less in the *ga1* mutant compared with the wild type, explaining the higher level of ABA in the *ga1* mutant. The alterations in the expression levels of these genes in response to red light, however, were more robust in the wild type than in the *ga1* mutant, consistent with the higher fold change in ABA level in wild-type seeds versus *ga1* mutant seeds (Figure 8C).

To further examine whether GA antagonizes ABA biosynthesis, we tested whether the application of exogenous GA altered the expression of ABA metabolic genes in the *ga1* mutant. Our results revealed that exogenous GA treatment transcriptionally repressed all of the tested ABA biosynthetic genes except *ABA2* and transcriptionally activated *CYP707A2*, indicating that ABA metabolism is also regulated by a GA-dependent pathway (Figure 8H). Since GAI and RGA do not regulate ABA metabolism (Figure 8F), other DELLA proteins are likely to regulate ABA metabolism in the GA-dependent pathway. Since PIL5 regulates bioactive GA levels (Oh et al., 2006), our results further suggest that PIL5 regulates ABA metabolism not only through GA-independent pathways but also through GA-dependent pathways.

Decreasing ABA Level Is Necessary for Endosperm Rupture

To investigate the physiological significance of the reductions in ABA levels seen following red light irradiation of wild-type seeds or in the *pil5* mutation during seed germination, we determined the germination frequencies of Col-0 and *pil5* seeds in the presence or absence of ABA. In the absence of ABA, wild-type seeds germinated only after red light irradiation, while the *pil5* mutant germinated irrespective of light conditions (Figure 9A). In the presence of ABA, both Col-0 and *pil5* seeds failed to germinate even after red light irradiation (Figure 9A). However, careful observation revealed that although radicles did not protrude from the endosperm layer of these seeds, the testa was ruptured (Figure 9B). These results support previous observations that ABA inhibits endosperm rupture during seed germination (Muller et al., 2006). However, the lack of inhibition of testa rupture by exogenously provided ABA could be due to its inability to penetrate into the embryo. To investigate this possibility, we examined the testa rupture of the *cyp707a2* mutant in the presence of both ABA and GA in the dark. Since the *cyp707a2* mutant accumulates ABA inside its seed, the exogenous supply of ABA increases both endogenous and exogenous ABA levels. Like that of wild-type seeds, the testa of *cyp707a2* mutant seeds was ruptured but the endosperm was not ruptured. These results further support the role of ABA in inhibiting endosperm rupture during seed germination and suggest that PIL5 regulates both testa rupture and endosperm rupture through GA and ABA, respectively, in germinating seeds.

DISCUSSION

Previously, we showed that PIL5 inhibits seed germination by repressing two GA synthetic genes (*GA3ox1* and *GA3ox2*) and activating a GA catabolic gene (*GA2ox2*) (Oh et al., 2006). However, the light-dependent change of germination frequency in *ga1* mutant seeds incubated on media containing GA_3, which is not degradable by GA2ox2, showed that light increases the GA responsiveness of these seeds independently of those GA metabolic genes. In the *pil5 ga1* double mutant, the GA responsiveness was high irrespective of light conditions, suggesting that PIL5 inhibits the light-dependent enhancement of GA responsiveness. Since altered GA responsiveness was observed even in the *ga1* mutant, our results indicate that PIL5 must affect other components of GA signaling.

The change of responsiveness to exogenously supplied GA could be due to changes in any of the GA signaling components, which include SPY and SHORT INTERNODES (Jacobsen and Olszewski, 1993; Fridborg et al., 2001) and the DELLA proteins, which play a central role in GA responsiveness. Bioactive GAs bind to their soluble receptor, GIBBERELLIN-INSENSITIE DWARF1,

Figure 8. (continued).

(G) Relative expression levels of ABA metabolic genes in Col-0 and *ga1* seeds. The light treatment scheme and notations are as described for **(A)**.
(H) Relative expression levels of ABA metabolic genes in *ga1* seeds irradiated with a far-red light pulse and then incubated in the dark for 12 h on mock medium or medium containing 100 μM GA_{4+7}. Similar results were obtained from three independent experiments, and a typical result is presented with SD values.

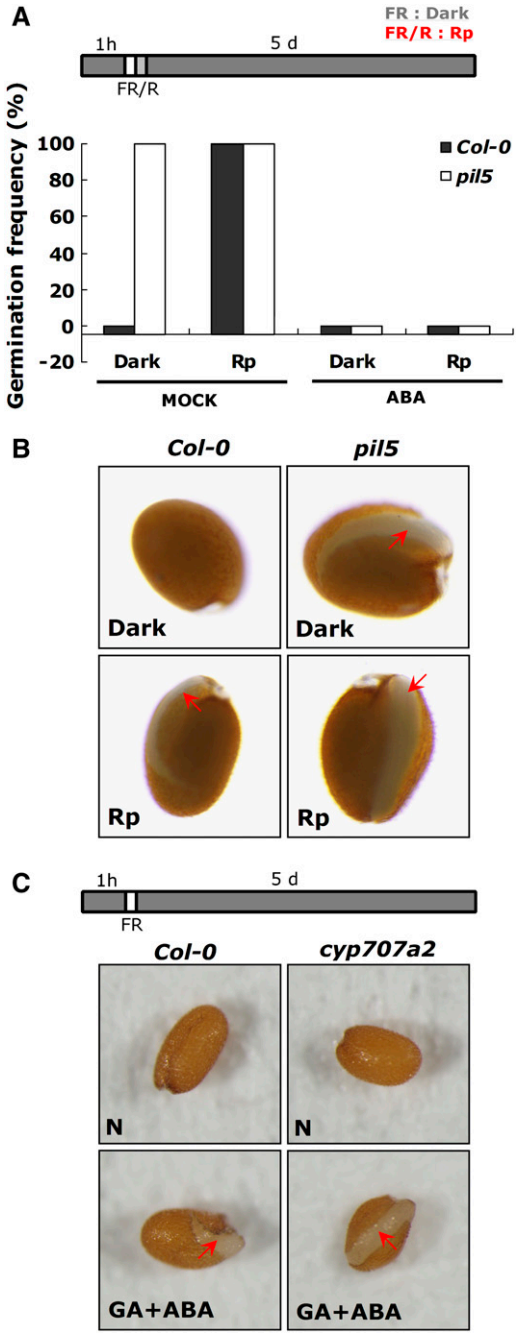

Figure 9. Decreased ABA Levels Are Necessary for Endosperm Rupture.

(A) Germination frequencies of Col-0 and *pil5* seeds incubated on mock medium or 3 μM ABA-containing medium under PHYB-dependent germination conditions. Abbreviations in the diagram at top are as given for Figure 1A.

(B) Representative images of Col-0 and *pil5* seeds incubated on 3 μM ABA-containing medium. Dark, seeds were subjected to a far-red light pulse and then incubated in the dark; Rp, seeds were subjected to a red light pulse followed by a far-red light pulse and then incubated in the dark. Red arrows indicate ruptured testa.

(C) Representative images of Col-0 and *cyp707a2* seeds incubated either on agar plates (N) or on agar plates containing 100 μM GA_{4+7} plus 10 μM ABA-containing medium (GA+ABA). The diagram at top depicts the light treatment scheme for the experiments. Red arrows indicate ruptured testa.

and promote the ubiquitination of DELLA proteins by the $SCF^{SLY1,SNE}$ E3 ligase complex (McGinnis et al., 2003; Dill et al., 2004; Strader et al., 2004; Ueguchi-Tanaka et al., 2005). The ubiquitinated DELLA proteins are subsequently degraded by the 26S proteasome, and the resulting decrease in DELLA protein levels initiates a transcriptional cascade leading to expressional changes in a subset of genes encoding cell wall–modifying enzymes, hydrolytic enzymes, etc. (Cao et al., 2006).

The DELLA proteins serve as integrators of various hormonal and environmental signals (Achard et al., 2003, 2006; Fu and Harberd, 2003) and are modulated through the direct and/or indirect regulation of their stability. Since phytochromes are known to regulate the degradation of various light-signaling components, including PIL5, PIF3, ELONGATED HYPOCOTYL5, and LONG AFTER FAR-RED LIGHT1 (Osterlund et al., 2000; Seo et al., 2003; Bauer et al., 2004; Park et al., 2004; Shen et al., 2005; Oh et al., 2006), it is plausible that phytochromes could enhance GA responsiveness in seeds by directly promoting the degradation of DELLA proteins. Our analysis of seeds constitutively expressing DELLA proteins (GAI or RGA) showed that the activation of phytochromes by light destabilizes GAI and RGA in seeds (Figure 2A). However, de novo GA biosynthesis was required for the light-induced destabilization of GAI or RGA (Figure 2B). Since the enhancement of GA responsiveness by red light was observed even in the *ga1* mutant (Figure 1A), the destabilization of GAI and RGA was not responsible for the light-induced GA responsiveness observed under GA-deficient conditions. However, since we have not tested the stabilities of the other three DELLA proteins under different light conditions, we cannot exclude the possibility that the stabilities of other DELLA proteins are regulated by phytochromes even in the absence of GA biosynthesis.

The expression levels of *DELLA* genes are known to differ at various developmental stages, suggesting that these GA-negative signaling components may also be transcriptionally regulated (Tyler et al., 2004). Since phytochromes are known to initiate the transcriptional cascades that alter the expression levels of 10 to 30% of the *Arabidopsis* transcriptome (Ma et al., 2001), we hypothesized that *DELLA* gene expression in seeds could be affected by phytochromes. Consistent with this notion, gene expression analysis revealed that the expression levels of two *DELLA* genes, *GAI* and *RGA*, were reduced by red light, whereas the other DELLA genes were unaffected (Figure 3A). In *pil5 ga1* double mutant seeds, the expression levels of *GAI* and *RGA* were low irrespective of light conditions, suggesting that PIL5 mediates the light-dependent expression of *GAI* and *RGA* in seeds. Two major lines of evidence indicate that the light-induced repression of *GAI* and *RGA* is functionally relevant for the light-dependent regulation of seed germination. First, GAI and RGA negatively regulate seed germination, as inferred from

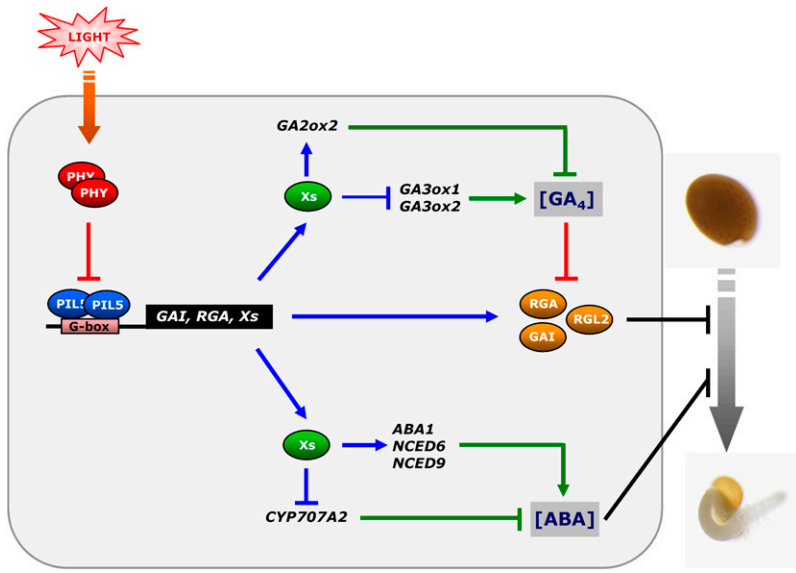

Figure 10. Proposed Molecular Events Leading to Seed Germination in *Arabidopsis*.

In the dark, PIL5 activates the expression of various genes, including *GAI*, *RGA*, and other unknown factors (*X*s), by binding directly to their promoters through G-box elements, resulting in increased protein levels of GAI, RGA and X factors. The X factors repress GA biosynthetic and ABA catabolic genes and activate GA catabolic and ABA biosynthetic genes, resulting in decreased GA levels and increased ABA levels. The decrease in GA stabilizes DELLA proteins, leading to increased DELLA protein levels and suppression of GA responses, including testa rupture and subsequent germination. Upon light irradiation, activated phytochromes induce PIL5 degradation, leading to decreased levels of GAI and RGA proteins and ABA. The level of RGL2, although not transcriptionally regulated by PIL5 directly, is also decreased due to the increased bioactive GA. As a result, various physiological processes are initiated, including the mobilization of storage molecules and the hydrolysis of cell walls, and the seeds eventually germinate. Red lines, events occurring at the protein level; blue lines, events occurring at the transcriptional level; green lines, events occurring via enzymatic activities. The potential regulatory circuit between GA and ABA is not shown here.

the light-independent germination of the *ga1 gai rga rgl2* quadruple loss-of-function mutant (Cao et al., 2005) and further supported by our data showing that the *gai-t6 rga-28* double loss-of-function mutant is hypersensitive to red light for germination (Figure 4A). Second, PIL5 transcriptionally activates *GAI* and *RGA*, as functionally manifested in the higher germination frequency of the *PIL5OX rga-28* double mutant compared with the *PILOX* single mutant and the higher germination frequency of the *pil5 gai-1* double mutant compared with the *gai-1* single dominant gain-of-function mutant (Figures 4C and 4F). Since PIL5 protein is rapidly degraded in seeds in response to light, our data collectively suggest that phytochromes induce GA responses in seeds partly by repressing the transcription of *GAI* and *RGA* via the degradation of PIL5.

In addition to the transcriptional repression of *GAI* and *RGA*, the degradation of GAI, RGA, and RGL2 by bioactive GA is also necessary for germination in response to light. The *ga1 gai rga* triple loss-of-function mutant failed to germinate, indicating that the *gai rga* double loss-of-function mutant still required GA biosynthesis for germination. However, the *ga1 gai rga rgl2* quadruple loss-of-function mutant showed light-independent germination, indicating that the newly synthesized bioactive GA is likely to activate the degradation of RGL2 in this context (Tyler et al., 2004). Our data showed that the *gai-t6 rga-28* double mutant is hypersensitive to red light but still requires red light (Figure 4A). Since our expression analysis indicated that transcription of the other DELLA genes was not regulated by light (Figure 3A), these results suggest that light promotes seed germination by both lowering DELLA protein levels and transcriptionally repressing *GAI* and *RGA*. In the *pil5* mutant, *GAI* and *RGA* are transcriptionally repressed and at the same time bioactive GA level is increased (Oh et al., 2006); thus, the mutant germinates irrespective of light conditions. Together, our results collectively indicate that phytochromes promote seed germination by activating the degradation of PIL5, which results in transcriptional repression of *GAI* and *RGA*, and also activating de novo GA synthesis, leading to the degradation of RGL2 and other DELLA proteins.

Our ChIP analyses indicated that PIL5 binds to the promoters of *GAI* and *RGA* in vivo but not to the other tested promoters. In the *GAI* and *RGA* promoters, PIL5 bound strongly to promoter fragments containing G-box elements but not to those containing E-box elements. In vitro binding assays further confirmed that PIL5 directly and specifically bound G-box elements from the *GAI* and *RGA* promoters. Since PIL5 has transcriptional activation activity, its direct binding to the promoters of *GAI* and *RGA* but not those of GA and ABA metabolic genes suggests that PIL5 directly regulates the transcription of *GAI* and *RGA* but indirectly regulates GA and ABA metabolic genes through yet unknown factors. Notably, we found that only some G-box elements served as PIL5 binding sites in vivo. Among the known PIL5-regulated genes, the promoters of *GA3ox1* and *GA3ox2* also

contain G-box elements, but fragments containing these sequences did not show PIL5 binding activity in vivo. This indicates that a G-box element alone is not sufficient to specify PIL5 binding in vivo, suggesting that flanking sequences in addition to the core G-box element might provide additional specificity. Alternatively, PIL5 might bind to G-box elements in the presence or absence of specific neighboring factors in the same promoter. The future identification of additional direct target promoters will be necessary to clarify the nature of PIL5 binding in vivo.

GA and ABA Antagonistically Regulate Each Other

Our data show that GA decreases ABA levels by repressing the expression of ABA biosynthetic genes and activating the expression of an ABA catabolic gene. Conversely, a previous report showed that ABA decreases GA levels by repressing GA biosynthetic genes in seeds (Seo et al., 2006). Thus, it appears as though ABA and GA antagonistically regulate each other by regulating transcription of the corresponding metabolic genes. The balance between ABA and GA in this antagonistic regulatory circuit could be easily shifted toward either side by a stimulus, such as an environmental factor. If such a signal led to a slight reduction in the ABA level, then the level of GA would increase due to decreased ABA-mediated repression. The increased GA level would then further decrease the ABA level, leading to additional increases in the GA level, and so on. Therefore, although the initial signal would trigger only a small change in the ABA level, the final output would be a large increase in GA and a large decrease in ABA. The opposite would be true in response to a signal triggering a small decrease in GA. Such an ABA–GA antagonistic regulatory circuit could explain the close coupling between dormancy breakdown and germination in seeds. Similarly, it might also explain why it has proven difficult for researchers to delineate whether a given factor regulates seed dormancy by affecting ABA metabolism or regulates germination by affecting GA metabolism, because both hormones would be affected simultaneously regardless of the direct target hormone. Further analysis will be required to confirm the presence of this ABA–GA antagonistic regulatory circuit in seeds.

Regulation of Seed Germination by PIL5-Mediated Light Signaling

Collectively, our results indicate that PIL5 regulates the expression levels of GA- and ABA-related genes either directly or indirectly. Based on our findings, we propose the following model to describe putative molecular events initiated by phytochromes in seeds (Figure 10). In darkness, PIL5 binds to the promoters of *GAI* and *RGA* and activates their expression. Simultaneously, PIL5 also activates the expression of more unknown factors, *X*s, which initiate the transcriptional cascades needed for the repression of GA biosynthetic and ABA catabolic genes and the activation of GA catabolic and ABA biosynthetic genes. Due to the subsequent decreases in bioactive GA levels and increases in DELLA protein levels, GA responses (e.g., testa rupture) are suppressed and seeds do not germinate. When phytochromes are activated by light, PIL5 protein is rapidly degraded. The degradation of PIL5 shuts down the transcription of *GAI*, *RGA*, and the *X*s, leading to increases in bioactive GA and decreases in both ABA levels and DELLA protein levels. These changes trigger various processes associated with germination, including mobilization of storage molecules, hydrolysis of cell walls, and rupture of the testa and the endosperm. Finally, a radicle emerges through the ruptured endosperm and testa, completing seed germination.

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. For germination assays, seeds were dried at 22°C in white paper bags for at least 1 month. Transgenic plants overexpressing myc-tagged RGA and GAI were generated by cloning the full-length *RGA* and *GAI* cDNAs into the pCAMBIA-HTM vector and transforming the vectors into ecotype Col-0. The *pil5-1* mutant (Salk_072677), *ga1* mutant (Salk_109115), *rga-28* mutant (Salk_089146), and *cyp707a2-1* mutant (Salk_072410) were obtained from the Salk Institute (Alonso et al., 2003). The *gai-t6* mutant was a kind gift from N.P. Harberd at the John Innes Centre. The *gai-1* and *gai-t6* mutants were backcrossed to Col-0 six times, and a resulting homozygous line was used in this study. All other plants used in this study were of the Col-0 background.

Germination Assay

Germination assays were performed as described previously (Oh et al., 2006). For the PHYB-dependent germination assay, triplicate sets of 60 seeds for each mutant were surface-sterilized and plated in the presence or absence of various hormones (GA_3, dimethyl-GA_4, and ABA). For irradiations, seeds were exposed to far-red light (3.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or red light (13 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 5 min unless specified otherwise. The seeds were then incubated in the dark for 5 d, and germination frequency was measured. Seeds with protruded radicles were considered germinated seeds. For the PHYA-dependent germination assay, sterilized and plated seeds were allowed to imbibe for 1 h, irradiated with far-red light for 5 min, and then incubated in the dark for 48 h to allow the accumulation of PHYA. After this dark incubation, the seeds were irradiated with far-red light for 12 h and incubated in the dark for an additional 4 d, and germination frequency was measured. GA_3 and ABA were purchased from Duchefa, and 2,2-dimethyl-GA_4 was synthesized using a previously reported method (Beale et al., 1984) with modifications (see Supplemental Methods online).

Gene Expression Analysis and Protein Analysis

For quantitative RT-PCR, seeds were irradiated with far-red light or red light and incubated in the dark for 12 h, and total RNA was extracted using the Spectrum plant total RNA kit (Sigma-Aldrich) according to the manufacturer's protocol. Total RNA was used to synthesize cDNA, the fragments of interest were amplified by real-time PCR using specific primers (see Supplemental Table 1 online), and the resulting expression levels were normalized versus that of *PP2A*, as described previously (Czechowski et al., 2005).

For protein analysis, seeds were irradiated as described above and incubated on medium containing 100 μM paclobutrazol with or without 100 μM GA_{4+7} (Duchefa) as described previously (Oh et al., 2006). Total proteins were extracted from imbibed seeds by denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, and 8 M urea, pH 8.0), and myc-tagged protein levels were determined by protein gel blot using an anti-myc antibody, as described previously (Oh et al., 2006). RGA protein levels

were examined by immunoblot analysis using anti-RGA antibodies from rat (DUR18), as described previously (McGinnis et al., 2003). RPT5 protein level was determined using anti-RPT5 antibody (Biomol).

Analysis of Endogenous ABA

ABA was extracted with 80% methanol containing 1% acetic acid, and [$^2H_6$]ABA (Icon Isotopes) was added to the extract as an internal standard. ABA-containing fractions were purified as described previously (Priest et al., 2006) and then subjected to liquid chromatography-selected reaction monitoring using a system consisting of a quadrupole/time-of-flight tandem mass spectrometer (Q-Tof Premier; Waters) and an Acquity Ultra Performance liquid chromatograph (Waters) equipped with a reverse-phase column (Acquity UPLC BEH-C18; Waters).

ChIP Assay

For the ChIP assay, *PILOX3* seeds were irradiated with far-red light, incubated in the dark for 6 h, and then cross-linked in 1% formaldehyde solution under a vacuum for 1 h. The seeds were then ground to powder in liquid nitrogen, and chromatin complexes were isolated and sonicated as described previously (Tai et al., 2005). The sonicated chromatin complexes were precipitated with a monoclonal anti-myc antibody as described previously (Gendrel et al., 2005). The cross-linking was then reversed, and the amount of each precipitated DNA fragment was determined by real-time PCR using specific primers (see Supplemental Table 1 online).

Electrophoretic Mobility Shift Assay

His-tagged PIL5 protein was expressed in *Escherichia coli* and purified with nickel-nitrilotriacetic acid agarose, and electrophoretic mobility shift assay was performed using biotin-labeled probes and the Lightshift Chemiluminescent EMSA kit (Pierce). The sequences of the biotin-labeled and cold probes used are depicted in Figure 6A. The PIL5 proteins and biotin-labeled probe were incubated together in binding buffer [10 mM Tris, 150 mM KCl, 1 mM DTT, 0.05% Nonidet P-40, and 10 ng/μL polydeoxy (inosinate-cytidylate), pH 7.5] for 20 min at room temperature, the mixture was resolved by 5% native polyacrylamide gel electrophoresis, and the labeled probe was detected according to the instructions provided with the electrophoretic mobility shift assay kit.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: *GA1* (At4g02780); *GA3ox1* (At1g15550); *GA3ox2* (At1g80340); *GA2ox2* (At1g30040); *GAI* (At1g14920); *RGA* (At2g01570); *RGL1* (At1g66350); *RGL2* (At3g03450); *RGL3* (At5g17490); *SLY1* (At4g24210); *SPY* (At3g11540); *CP1* (At4g36880); *EXP1* (At1g69530); *EXP3* (At2g37640); *PIL5* (At2g20180); *ABA1* (At5g67030); *ABA2* (At1g52340); *NCED6* (At3g24220); *NCED9* (At1g78390); *AAO3* (At2g23740); and *CYP707A2* (At2g29090).

Supplemental Data

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

Supplemental Figure 1. Germination Frequency of Various Mutants in the Presence of 2,2-Dimethyl-GA_4.

Supplemental Figure 2. Relative Expression Level of GA3ox1 in Light-Grown Seedlings.

Supplemental Table 1. Primer Sequences.

Supplemental Methods. Synthesis of 2,2-Dimethyl-GA_4.

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