

SOMNUS, a CCCH-Type Zinc Finger Protein in *Arabidopsis*, Negatively Regulates Light-Dependent Seed Germination Downstream of PIL5^W

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Light absorbed by seed phytochromes of *Arabidopsis thaliana* modulates abscisic acid (ABA) and gibberellic acid (GA) signaling pathways at least partly via PHYTOCHROME-INTERACTING FACTOR3-LIKE5 (PIL5), a phytochrome-interacting basic helix-loop-helix transcription factor. Here, we report a new mutant, *somnus* (*som*), that germinates in darkness, independently of various light regimens. *SOM* encodes a nucleus-localized CCCH-type zinc finger protein. The *som* mutant has lower levels of ABA and elevated levels of GA due to expressional changes in ABA and GA metabolic genes. Unlike PIL5, however, *SOM* does not regulate the expression of *GA-INSENSITIVE* and *REPRESSOR OF GA1* (*RG1/RGA1*), two *DELTA* genes encoding GA negative signaling components. Our *in vivo* analysis shows that PIL5 activates the expression of *SOM* by binding directly to its promoter, suggesting that PIL5 regulates ABA and GA metabolic genes partly through *SOM*. In agreement with these results, we also observed that the reduced germination frequency of a *PIL5* overexpression line is rescued by the *som* mutation and that this rescue is accompanied by expressional changes in ABA and GA metabolic genes. Taken together, our results indicate that *SOM* is a component in the phytochrome signal transduction pathway that regulates hormone metabolic genes downstream of PIL5 during seed germination.

INTRODUCTION

The pioneering work by Borthwick et al. (1952) led to the discovery of phytochrome as a photoreceptor that promotes germination in many plant species. *Arabidopsis thaliana* has five phytochrome genes (*PHYA* to *PHYE*) that have shared but distinct roles in regulating seed germination, hypocotyl elongation, shade avoidance, flowering, and other light responses (Mathews, 2006). *PHYA* and *PHYB* are the major phytochromes for the regulation of seed germination (Reed et al., 1994). In particular, *PHYA* mediates the very low fluence response and the far-red high irradiance response of seeds (Shinomura et al., 1994, 1996; Botto et al., 1996). By contrast, *PHYB* mediates the red light low fluence response, which can be reversed by subsequent far-red light (Reed et al., 1993). Due to the presence of *PHYB* and the absence of *PHYA* in dry seeds, only the low fluence response promotes germination early in seed imbibition. In hydrated seeds, however, *PHYA* accumulates and the very low fluence response and far-red high irradiance response can regulate germination (Shinomura et al., 1994).

Phytochromes promote seed germination by converting light signals into hormonal signals. The activation of phytochromes

decreases the level of abscisic acid (ABA) and increases the level of gibberellic acid (GA) (Yamaguchi et al., 1998; Ogawa et al., 2003; Seo et al., 2006; Oh et al., 2007). The light-promoted decrease in ABA is largely due to the transcriptional repression of ABA anabolic genes (*ABA-DEFICIENT1* [*ABA1*], *NINE-CIS-EPOXYCAROTENOID DEOXYGENASE6* [*NCED6*], and *NCED9*) and the transcriptional activation of an ABA catabolic gene (*CYP707A2*) (Seo et al., 2006; Oh et al., 2007). The significance of ABA biosynthesis in seed germination can be seen in the altered germination frequencies of ABA metabolic mutants, such as *aba1*, *aba2*, *nced6*, *nced9*, and *cyp707a2* (Koornneef et al., 1982; Karsen et al., 1983; Kushiro et al., 2004; Lefebvre et al., 2006; Okamoto et al., 2006). ABA establishes and maintains seed dormancy and inhibits seed germination through various signaling components, including three positive components, *ABA-INSENSITIVE3* (*ABI3*), *ABI4*, and *ABI5* (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Soderman et al., 2000). The light-induced decrease in ABA leads to decreased activities of these factors, causing decreased expression of ABA-inducible genes and promotion of germination.

Light also regulates the level of GA by transcriptional regulation of GA anabolic genes and a GA catabolic gene (Yamaguchi, 2008). Light increases the expression of two GA anabolic genes (*GIBBERELLIN 3 β-HYDROXYLASE1* [*GA3ox1*] and *GA3ox2*) and inhibits the expression of a GA catabolic gene (*GIBBERELLIN 2-OXIDASE2* [*GA2ox2*]), and this leads to an increase in the level of bioactive GA (Ogawa et al., 2003; Oh et al., 2006). The significance of GA metabolic genes in germination can be seen in the altered germination frequencies of GA metabolic mutants, such

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as *ga requiring1* (*ga1*), *ga2*, *ga3*, *ga4*, *ga5*, and *ga2ox2* (Koorneef and Van der Veen, 1980; Talon et al., 1990; Sun et al., 1992; Sun and Kamiya, 1994; Xu et al., 1995; Williams et al., 1998; Yamauchi et al., 2007). Light-induced increase in GA leads to the degradation of DELLA proteins via SCF^{SLY1} ubiquitin E3 ligase in conjunction with GA receptors (GA INSENSITIVE DWARF 1a [GID1a], -1b, and -1c) (Itoh et al., 2003; McGinnis et al., 2003; Dill et al., 2004; Ueguchi-Tanaka et al., 2005, 2007; Griffiths et al., 2006; Iuchi et al., 2007; Jiang and Fu, 2007). DELLA proteins are nucleus-localized negative GA signaling components that inhibit various GA responses, including seed germination, stem elongation, and floral development (Peng et al., 1997, 1999; Silverstone et al., 1998; Ikeda et al., 2001; Sun and Gubler, 2004; Tyler et al., 2004). The molecular functions of DELLA proteins are not clearly defined, but a recent report shows that one DELLA protein binds directly to DNA and regulates the expression of various genes, including GA receptor genes (*GID1a* and *-1b*), GA anabolic genes (*GA2ox2* and *GA3ox1*), and *XERICO*, which encodes a ring finger ubiquitin E3 ligase that activates ABA biosynthesis (Ko et al., 2006; Zentella et al., 2007). There are five DELLA proteins in *Arabidopsis* (*GA-INSENSITIVE* [*GAI*], *RGA*, *RGA LIKE1* [*RGL1*], *RGL2*, and *RGL3*), which have shared but distinct roles in mediating different GA responses. Among them, *GAI*, *RGA*, and *RGL2* play important roles in inhibiting seed germination (Lee et al., 2002; Cao et al., 2005; Oh et al., 2007). A triple mutant of these three DELLA genes (*gai rga rgl2*) germinates irrespective of light conditions, even in the absence of GA synthesis (*ga1* mutant background) (Cao et al., 2005). This suggests that PHY-mediated light signaling promotes seed germination at least partly by increasing GA biosynthesis and the subsequent degradation of these three DELLA proteins.

In addition to ABA and GA metabolic genes, light also regulates ABA and GA signaling genes. The light activation of phytochromes inhibits the expression of two DELLA genes (*GAI* and *RGA*) in seeds (Oh et al., 2007). This indicates that PHY-mediated light signaling removes DELLA proteins by decreasing the expression of *GAI* and *RGA* at the transcriptional level and by activating the degradation of all DELLA proteins, including *RGL2*, at the protein level. PHY-mediated regulation of ABA signaling genes in seeds is less clearly understood. However, a recent microarray experiment showed that expression of *ABI3* is significantly increased in a *phyB* mutant, suggesting that light represses ABA signaling by decreasing the expression of an *ABI*-positive component (Nakabayashi et al., 2005). These results suggest that light promotes seed germination by shifting the balance of the ABA and GA pathways via transcriptional regulation of hormone metabolic and signaling genes.

In seeds, PHYTOCHROME-INTERACTING FACTOR3-LIKE5 (*PIL5*, also known as *PIF1* and *bHLH015*) plays a key role in converting light signals to alterations in the ABA and GA pathways. *PIL5* encodes a phytochrome-interacting basic helix-loop-helix (bHLH) transcription factor that is a member of bHLH subgroup 15, which includes 14 other bHLH transcription factors, including *PIF3*, *PIF4*, and *PIL6* (Toledo-Ortiz et al., 2003; Huq et al., 2004; Oh et al., 2004). Phenotypic analysis of a *pil5* mutant and *PIL5* overexpression lines shows that *PIL5* is a negative regulator of seed germination in *PHYA*- and *PHYB*-dependent seed germination (Oh et al., 2004). The negative role of *PIL5* on germination is

partly due to its regulation of GA and ABA metabolism. In particular, *PIL5* represses the expression of two GA anabolic genes (*GA3ox1* and *GA3ox2*) and activates the expression of a GA catabolic gene (*GA2ox2*), causing a decrease in the level of bioactive GA in seeds (Oh et al., 2006). By contrast, *PIL5* also activates the expression of ABA anabolic genes (*ABA1*, *NCED6*, and *NCED9*) and represses the expression of an ABA catabolic gene (*CYP707A2*), resulting in an increase in the level of ABA in seeds (Oh et al., 2007). Expression analyses show that *PIL5* also activates the expression of *GAI* and *RGA*, indicating that it regulates both GA levels and GA responsiveness (Oh et al., 2007). Consistent with this interpretation, *pil5* mutant seeds have an elevated level of GA, a decreased level of ABA, and an increased responsiveness to GA irrespective of light conditions. These expression patterns and genetic analyses indicate that phytochromes regulate the expression of these genes by inhibiting the function of *PIL5*. Inhibition of *PIL5* function by phytochromes occurs at the protein level, as shown by the phytochrome-induced rapid degradation of *PIL5* proteins in seeds and seedlings (Shen et al., 2005; Oh et al., 2006). Taken together, these previous results indicate that in *Arabidopsis* seeds, phytochromes activate the expression of hormone metabolic genes and GA signaling genes by promoting the degradation of *PIL5*.

Chromatin immunoprecipitation (ChIP) analyses indicate that *PIL5* directly regulates the expression of *GAI* and *RGA* by binding to G-box elements on their promoters (Oh et al., 2007). However, *PIL5* does not bind to the promoters of hormone metabolic genes, even though some of them contain G-box elements (Oh et al., 2007). This indicates that *PIL5* regulates the expression of ABA and GA metabolic genes indirectly. To identify components involved in PHY- and *PIL5*-mediated light signaling processes, we isolated a novel mutant called *somnus* (*som*) that germinates in complete darkness. We report here that *SOM*, which encodes a nucleus-localized CCCH-type zinc finger protein whose molecular function is unknown, regulates hormone metabolic genes downstream of *PIL5*.

RESULTS

SOM, a CCCH-Type Zinc Finger Protein, Is a Negative Regulator of PHY-Mediated Promotion of Seed Germination

Only a few light signaling components that control seed germination have been identified. Thus, we screened *Arabidopsis* T-DNA insertion lines for mutants that germinate in total darkness and isolated a mutant (*som-1*) that germinates irrespective of light conditions. In this mutant, a T-DNA was inserted in the middle of a gene (At1g03790) that had not yet been characterized (Figure 1A). We obtained two additional *som* mutant alleles (*som-2* and *som-3*) from the *Arabidopsis* Stock Center (SALK_090314 and SALK_008075, respectively) and included them in subsequent studies. The full-length *SOM* transcript was nearly undetectable by RT-PCR in three *som* mutants (*som-1*, *som-2*, and *som-3*) (Figure 1B). Unlike the full-length *SOM* transcript, an N-terminal fragment was expressed in two *som* alleles (*som-1* and *som-2*) but was nearly undetectable in the third *som* allele (*som-3*).

SOM contains a typical CCCH-type zinc finger motif (CX₈CX₅CX₃H, ZF2 in Figure 1) that is between atypical zinc

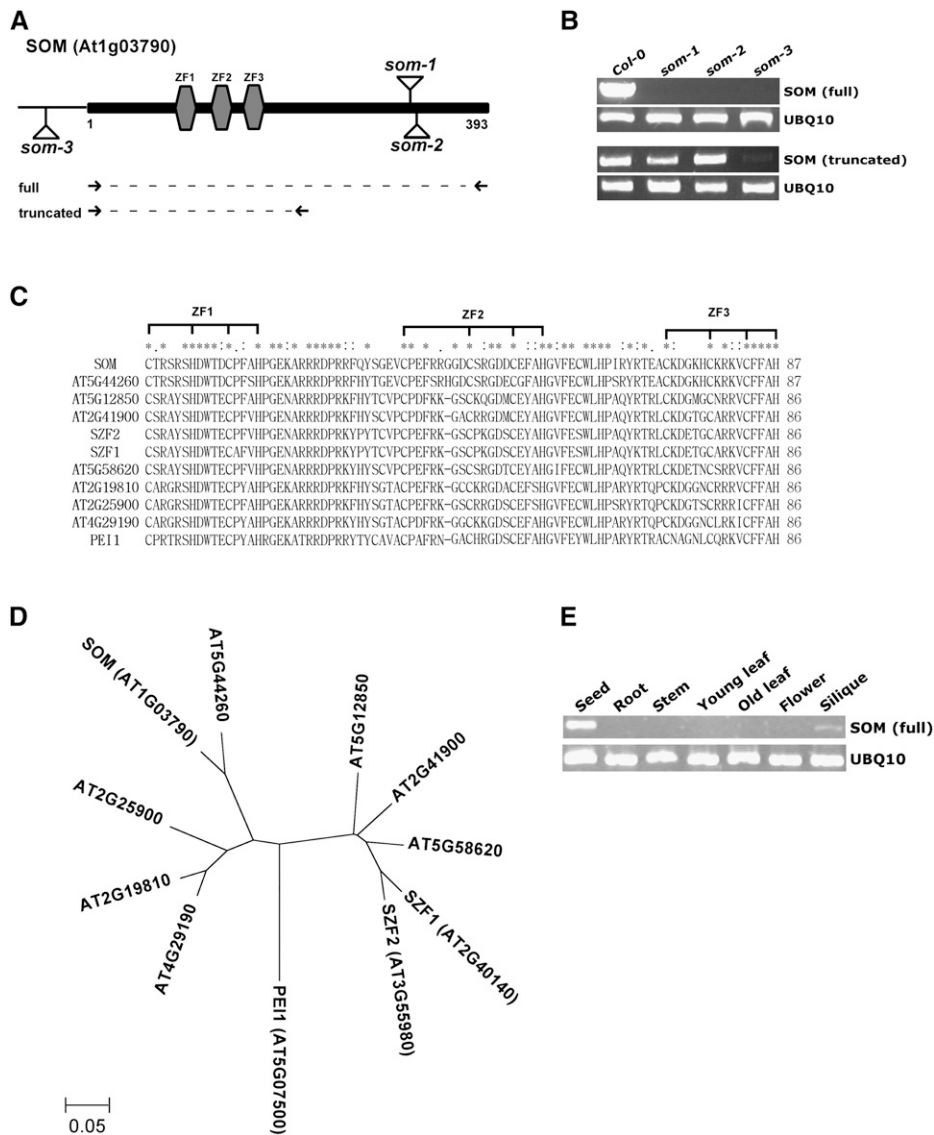


Figure 1. SOM Is a Seed-Specific Gene Encoding a Novel CCCH-Type Zinc Finger Protein.

(A) Diagram of the SOM protein, showing T-DNA insertion sites (*som-1*, *som-2*, and *som-3*) and three zinc finger motifs (gray hexagons). Numbers indicate amino acid numbers of SOM. Full indicates the position of a primer set amplifying the full-length SOM; truncated indicates the position of a primer set amplifying an N-terminal fragment of SOM. There are no introns in the SOM protein-coding sequence. Supplemental Figure 1 and Supplemental Table 1 online include detailed sequence information.

(B) Absence of expression of full-length SOM in the *som-1*, *som-2*, and *som-3* mutants, as determined by RT-PCR. The N-terminal fragment is expressed in the *som-1* and *som-2* alleles, but not in *som-3*. Expression of *UBQ10* (At4g05320) was used as an expression control.

(C) Multiple alignment of SOM and related *Arabidopsis* proteins for three zinc finger motifs (ZF1, ZF2, and ZF3). Asterisks indicate identical amino acid residues, and one or two dots indicate similar amino acid residues. Brackets indicate conserved Cys residues, characteristic of zinc finger motifs.

(D) Phylogenetic tree of SOM and related *Arabidopsis* proteins.

(E) RT-PCR analysis showing that SOM is expressed mainly in seeds. Expression of *UBQ10* was used as an expression control.

finger motifs (a $CX_5HX_4CX_3H$ motif at the N-terminal side and a $CX_5CX_4CX_3H$ motif at the C-terminal side; ZF1 and ZF3 in Figure 1). In the *Arabidopsis* genome, 11 proteins have three similar zinc finger motifs (Figures 1C and 1D), indicating that SOM belongs to a subset of the CCCH-type zinc finger family. Among these genes, At5g07500 (known as PEI1) has been characterized previously as a regulator of embryogenesis and At3g55980 and

At2g40140 (known as SZF1 and SZF2, respectively) have been characterized as regulators of the salt response (Li and Thomas, 1998; Sun et al., 2007). The molecular function of the CCCH-type zinc finger motif is not yet clearly defined. Some CCCH-type zinc finger motifs regulate RNA stability by binding to AU-rich elements, some regulate RNA processing by binding to pre-mRNA, and some regulate transcription (Hudson et al., 2004; Barreau

et al., 2005; Brown, 2005; Hall, 2005). PEI1 has been described as a DNA binding protein. Expression analysis showed that *SOM* is expressed mainly in seeds but not in adult organs and tissues (Figure 1E). Microarray data compiled in the Bio-Array Resource (<http://www.bar.utoronto.ca>) also indicate that *SOM* is a seed-specific gene (see Supplemental Figure 2 online). Taken together, the analyses indicate that *SOM* is a seed-specific protein that belongs to a subset of CCCH-type zinc finger proteins.

To test whether *SOM* is a negative regulator in PHYB-mediated promotion of germination, we analyzed the germination phenotypes of *som* mutants and *SOM* overexpression lines using a PHYB-dependent germination protocol. In this protocol, seed PHYB was inactivated by far-red light or activated by red light and seeds were then allowed to germinate in the dark for 5 d before scoring of germination. Wild-type seeds germinated when PHYB was activated by red light but not when PHYB was inactivated by far-red light (Figure 2A). As a control, *phyB* mutant seeds (*phyB-9*) did not germinate even after irradiation with red light. However, *som* mutant seeds germinated at rates of almost 100% irrespective of light conditions. Two other independent *som* alleles (*som-2* and *som-3*) also germinated irrespective of light conditions. This indicates that mutations in the *SOM* gene alter germination behavior. To determine the effect of overexpression of *SOM*, we expressed intact *SOM* (lines *SOM-OX1* and *-OX2*) and myc-tagged *SOM* (line *SOM-OX3*) under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The expression level of the *SOM* transcript was highest in the *SOM-OX1* line and lowest in the *SOM-OX3* line (see Supplemental Figure 3 online). All overexpression lines required more red light fluence for seed germination than did the wild type (Figure 2B). The germination phenotypes of both *som* mutants and *SOM* overexpression lines indicate that *SOM* is a negative regulator of seed germination.

To further investigate the relationship between *SOM* and PHYB, we generated a *phyB som* double mutant (*phyB-9 som-1*) and studied germination using the same protocol. The *phyB* mutant failed to germinate irrespective of light conditions. The *phyB som* double mutant, however, germinated well (Figure 2C), indicating that the *som* mutation is epistatic to the *phyB* mutation under these conditions. These results support the hypothesis that *SOM* is a negative regulator in the PHYB-mediated promotion of the germination process and that PHYB promotes germination at least partly by inhibiting *SOM*.

We also tested whether *SOM* is a negative regulator in the PHYA-mediated promotion of germination. For the PHYA-dependent germination protocol, we inactivated PHYB with a far-red light pulse and allowed seeds to accumulate PHYA for 2 d at 22°C in the dark. Then we irradiated these imbibed seeds with prolonged far-red light to activate PHYA and incubated seeds in the dark for an additional 5 d. With this protocol, wild-type seeds germinate when PHYA is activated by prolonged far-red light but do not germinate without the prolonged far-red light (Figure 2D). We could not determine the germination phenotypes of the *som* mutant or the *phyA som* double mutant in this PHYA-dependent germination protocol because these mutants start to germinate during the imbibition period. Therefore, we used *SOM* overexpression lines to examine the role of *SOM* under these conditions. All three overexpression lines were hyposensitive to

prolonged far-red light under this protocol (Figure 2D). Wild-type seeds germinated almost 100% even by 9.6 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ far-red light, whereas the *phyA* mutant did not germinate even by 38.4 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ far-red light. The three *SOM* overexpression lines required more far-red light than did the wild type for germination. These results indicate that *SOM* acts as a negative regulator in the PHYA-mediated promotion of germination.

The germination phenotypes of the *som* mutant are similar to those of the previously characterized *pil5* mutant, and the phenotypes of *SOM* overexpression lines are similar to those of *PIL5* overexpression lines (Oh et al., 2004). The negative role of *SOM* in seed germination suggests that PHYA and PHYB promote germination partly by inhibiting the action of *SOM*.

Phytochromes Inhibit SOM by Repressing Its Expression in Seeds

We investigated how phytochromes inhibit *SOM* and thereby promote germination. We first examined whether phytochromes regulate the stability of the *SOM* protein. For this study, we used transgenic lines that overexpress *SOM-myc* under the control of the CaMV 35S promoter, so that *SOM* is expressed under all light conditions. We first found that the myc-tagged *SOM* was functional (Figures 2B and 2D). We then analyzed the stabilities of *SOM-myc* under different light conditions in which PHYA and PHYB were either off or on (Figure 3A). We also examined myc-tagged green fluorescent protein (GFP) and myc-tagged *PIL5* as controls. As reported previously, PHYA and PHYB activate the degradation of *PIL5* but not GFP in imbibed seeds (Oh et al., 2006). By contrast, phytochromes did not alter the protein stability of *SOM* (Figure 3A). These results indicate that activation of phytochromes does not cause the degradation of *SOM* protein.

Next, we examined whether phytochromes regulate the subcellular localization of *SOM* using transgenic lines that overexpress GFP-tagged *SOM* under the control of the CaMV 35S promoter. The GFP-tagged *SOM* is functional (Figure 3B), and we detected a fluorescent signal in the nucleus irrespective of light conditions in transgenic plants (Figure 3C). By contrast, control transgenic plants that overexpress GFP alone emit fluorescence mainly in the cytosol. In the PHYB-GFP transgenic line, dark-grown plants had a diffuse GFP signal throughout the cytosol, but light-grown plants had a few discrete fluorescent spots in the nucleus (Kircher et al., 1999; Yamaguchi et al., 1999). These results indicate that *SOM* is a nucleus-localized protein and that its localization is independent of light conditions. This suggests that phytochromes do not alter the subcellular localization of *SOM*.

Next, we examined whether phytochromes inhibit *SOM* by repressing its gene expression in seeds. In the PHYB-dependent germination protocol, the red light pulse greatly reduced the levels of the *SOM* transcript in wild-type and *phyA* mutant seeds (Figure 3D; see Supplemental Table 2 online). By contrast, the level of the *SOM* transcript remained high in *phyB* mutant seeds irrespective of light conditions. This indicates that activation of PHYB represses *SOM* transcript expression in seeds. In the PHYA-dependent germination protocol, prolonged far-red light also greatly decreased the level of the *SOM* transcript in wild-type and *phyB* mutant seeds, but not in the *phyA* mutant seeds (Figure 3E). Taken together, these results indicate that phytochromes inhibit

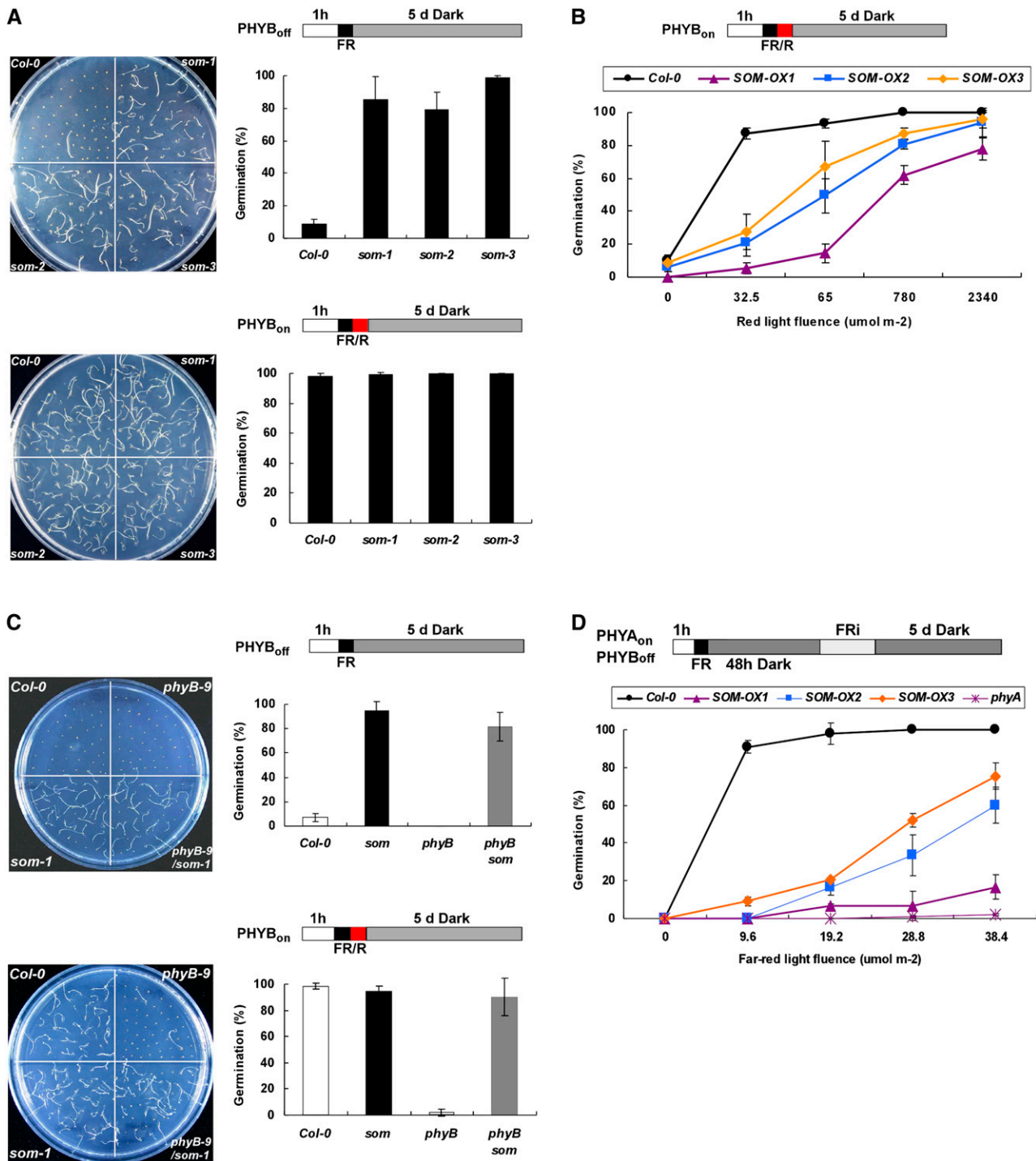


Figure 2. SOM Is a Negative Regulator in PHYA- and PHYB-Mediated Promotion of Seed Germination.

(A) Germination of *som* mutants (PHYB-dependent germination protocol). Light irradiation conditions are indicated by the diagrams above the graphs. FR, 5 min of far-red light ($3.2 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$); FR/R, 5 min of far-red light ($3.2 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and then 5 min of red light ($13 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The graphs show germination percentages of three replicates of 50 seeds each. Error bars indicate SD ($n = 3$). PHYB_{off} indicates a light scheme that inactivates PHYB by FR, and PHYB_{on} indicates a light scheme that activates PHYB by FR/R.

(B) Germination of SOM-OX transgenic lines (PHYB-dependent germination protocol). Germination percentage is shown as a function of varying red light fluences that were given immediately after 5 min of far-red light ($3.2 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The SOM-OX1 line shows the highest SOM expression levels (endogenous plus transgene), and the SOM-OX3 line shows the lowest levels (see Supplemental Figure 3 online).

SOM at least partly by repressing the expression of the *SOM* transcript in seeds.

SOM Inhibits Seed Germination by Regulating the Expression of GA and ABA Metabolic Genes

Phytochrome-mediated light signaling promotes germination partly by increasing the level of bioactive GA, decreasing the level of ABA, and increasing the responsiveness to GA (Oh et al., 2007). Since SOM regulates germination in response to light, we investigated whether SOM inhibits germination by altering the expression of hormone-related genes. Since light regulates mainly two GA anabolic genes (*GA3ox1* and *GA3ox2*) and a catabolic gene (*GA2ox2*) (Ogawa et al., 2003; Oh et al., 2006), we determined the expressions of those three metabolic genes.

First, we measured levels of GA metabolic genes in the wild type, *som* mutants, and SOM overexpressers to test whether SOM inhibits germination partly by regulating GA metabolic genes (Figure 4A). As reported previously, red light increases the expression of *GA3ox1* and *GA3ox2* in wild-type seeds (Ogawa et al., 2003). In the *som* mutant, expression of these GA anabolic genes was already high, even in far-red-light-irradiated seeds, and red light further increased their expression. By contrast, expression of *GA2ox2* was lower in the *som* mutant, and red light further decreased its expression. In contrast with the *som* mutant, the SOM overexpression line expressed lower levels of *GA3ox1* and *GA3ox2* and higher levels of *GA2ox2* in seeds treated with far-red or red light (Figure 4A). These results indicate that SOM, like PIL5, inhibits the synthesis of bioactive GA partly by repressing two GA anabolic genes and activating a GA catabolic gene. However, the expression patterns of GA metabolic genes in the *som* mutant differ from those of the *pil5* mutant. GA metabolic genes are expressed virtually independently of light conditions in the *pil5* mutant (Oh et al., 2006) but are induced by red light in the *som* mutant. These results indicate that additional factors downstream of PIL5 influence the expression of GA metabolic genes. The expression patterns of GA metabolic genes that we observed suggest that the levels of bioactive GA would be higher in *som* mutant seeds and lower in the SOM overexpression line. Thus, we measured the levels of GA₄ in different mutant seeds using the PHYB-dependent seed germination protocol (Figure 4B). The level of GA₄ was low in wild-type seeds treated with far-red light but high in seeds treated with red light, as reported previously (Oh et al., 2006). Compared with the wild type, *som* mutant seeds had a higher level of GA₄ when treated with far-red light, and red light further increased the level of GA₄. In the SOM overexpression line, the level of GA₄ was lower than in the wild type, and red light increased the level only slightly. These results are consistent with the expression patterns of GA metabolic genes.

Next, we examined whether SOM also inhibits germination by regulating ABA metabolic genes (Figure 4C). As reported previously, red light decreased the expression of ABA anabolic genes (*ABA1*, *NCED6*, and *NCED9*) and increased the expression of an ABA catabolic gene (*CYP707A2*) in wild-type seeds (Seo et al., 2006; Oh et al., 2007). Compared with the wild type, the *som* mutant had lower expression of ABA anabolic genes and higher expression of the ABA catabolic gene. In this mutant, red light further decreased the expression of ABA anabolic genes and increased the expression of the ABA catabolic gene. By contrast, the SOM overexpression line had higher expression of ABA anabolic genes and lower expression of the catabolic gene compared with the wild type. These results indicate that SOM, like PIL5, activates ABA biosynthesis by increasing the expression of ABA anabolic genes and repressing an ABA catabolic gene. Unlike the *pil5* mutant, however, ABA metabolic genes are still regulated by light in the *som* mutant.

The expression patterns of ABA metabolic genes suggest that ABA levels would be lower in the *som* mutant but higher in the SOM overexpression line compared with the wild type. Thus, we measured the levels of ABA in wild-type and mutant seeds (Figure 4D). The level of ABA was high in far-red-light-treated wild-type seeds but low in red-light-treated seeds, as reported previously (Oh et al., 2007). Far-red-light-irradiated *som* mutant seeds had lower levels of ABA than wild-type seeds, and red light treatment further decreased the level of ABA in this mutant. In the SOM overexpression line, seeds treated with far-red or red light had higher levels of ABA than did wild-type seeds. As with our GA studies (see above), these results are consistent with our studies of the expression patterns of ABA metabolic genes. Taken together, the results indicate that SOM regulates the levels of ABA and GA in seeds by regulating the expression of ABA and GA anabolic and catabolic genes.

SOM Does Not Regulate the Expression of *DELLA* Transcripts

PIL5 inhibits seed germination by regulating GA and ABA metabolic genes and by activating the transcription of the *DELLA* genes *GAI* and *RGA*. We investigated whether SOM also regulates the expression of these *DELLA* transcripts using the PHYB-dependent germination protocol (Figure 5A). Red light decreased the levels of *GAI* and *RGA* transcripts in wild-type seeds but had no effect on the levels of the other three *DELLA* transcripts. By contrast, the *pil5* mutant expressed constitutively low levels of *GAI* and *RGA* transcripts independent of light conditions. Both *som* mutation and SOM overexpression did not alter the expression levels of *DELLA* transcripts (Figure 5A). These results indicate that SOM does not regulate *DELLA* transcripts in seeds.

Figure 2. (continued).

(C) Germination of Columbia (Col-0), *som-1* mutant, *phyB-9* mutant, and *phyB-9 som-1* double mutant (PHYB-dependent germination protocol). Notations are as in **(A)**.

(D) Germination of SOM-OX transgenic lines (PHYA-dependent germination protocol). Seeds were imbibed for 48 h to allow the accumulation of PHYA, varying fluences of FR light (FRI) were provided to imbibed seeds, and germination was scored after 5 d in darkness. PHYA_{on} indicates a light scheme that activates PHYA by FRI. Error bars indicate SD ($n = 3$).

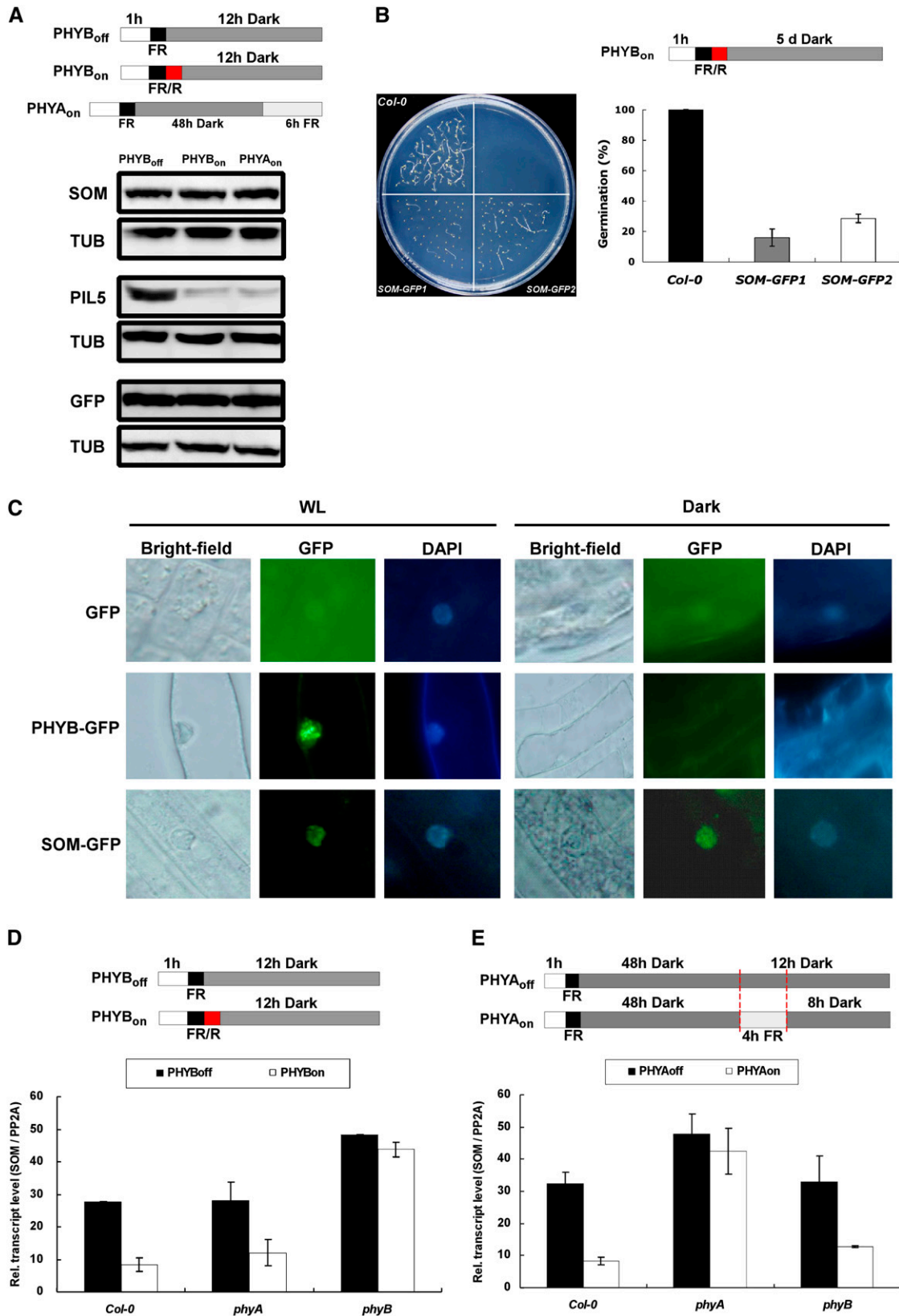


Figure 3. Phytochromes Inhibit SOM Function at the Transcriptional Level.

Next, we investigated the effect of SOM on GA responsiveness. We determined GA responsiveness by measuring the germination response of the *ga1* single mutant and the *ga1 som* double mutant in the presence of various concentrations of GA₃ (Figure 5B). GA₃ is a fungal GA that is not inactivated by GA2ox2. Thus, responsiveness to GA₃ in the absence of GA biosynthesis (*ga1* mutant background) can be used as an indicator of GA responsiveness (Oh et al., 2007). As reported previously, when we treated seeds with red light, the GA responsiveness of the *ga1* mutant increased, whereas the GA responsiveness of the *ga1 pil5* double mutant was constitutively high irrespective of light conditions (Oh et al., 2007). By contrast, the GA responsiveness of the *ga1 som* double mutant was similar to that of the *ga1* single mutant (low under far-red light and high under red light; Figure 5B). These results indicate that SOM, unlike PIL5, does not regulate the GA responsiveness of seeds.

PIL5 Regulates the Expression of SOM by Binding Its Promoter

We previously showed that PIL5 is a master regulatory transcription factor that inhibits germination partly by directly regulating the expression of two DELLA genes and partly by indirectly regulating the expression of GA and ABA metabolic genes (Oh et al., 2007). In the *som* mutant, the germination characteristics and expression patterns of the GA and ABA metabolic genes suggest that SOM may be a PIL5 downstream component that regulates GA and ABA metabolic genes during germination.

Thus, we examined the role of PHY- and PIL5-mediated light signaling in the regulation of SOM transcript accumulation in seeds. In the PHYB-dependent seed germination protocol, red light decreased the expression of SOM, but SOM expression remained high in the *phyB* mutant irrespective of light conditions (Figure 6A). This indicates that in seeds, PHYB is required for repression of the expression of SOM transcript. By contrast, the expression of SOM remained low in the *pil5* mutant irrespective of light conditions, indicating that higher expression of SOM in far-red-light-irradiated seeds requires the presence of PIL5. To further demonstrate the role of PIL5 in SOM expression, we measured the expression of SOM in the *phyB pil5* double mutant. As shown in Figure 6A, the expression level of SOM in the double

mutant was low irrespective of light conditions. This suggests that PHYB represses the expression of SOM by inhibiting PIL5. Similar to our results using the PHYB-dependent seed germination protocol, PIL5 was required for the expression of SOM transcript in the PHYA-dependent seed germination protocol (Figure 6B). Taken together, these results indicate that SOM is a downstream component of the PIL5 signaling pathway in PHYA- and PHYB-mediated light signaling during germination.

PIL5 may regulate the expression of SOM either directly (as for *GAI* and *RGA*) or indirectly (as for GA and ABA metabolic genes). Thus, we performed a ChIP assay using the *PIL5-OX* line (which expresses functional myc-tagged PIL5) and a GFP-myc overexpression line to investigate whether PIL5 regulates SOM by directly binding to its promoter. For the ChIP assay, we designed eight primer sets for various parts of the 2.9-kb SOM promoter region and a primer set for a SOM coding region. We determined the binding of PIL5 to promoter fragments by enrichment of immunoprecipitated DNA fragments over input DNA fragments. If PIL5 binds the SOM promoter, then the PIL5-bound fragment should have the highest enrichment and other fragments (which are farther from the binding site) should have decreased enrichment. In addition, the enrichment by PIL5-myc for the PIL5 binding fragment should be much higher than that by GFP-myc.

Our results indicate that PIL5 binds to the promoter of SOM in vivo (Figure 6C). When we immunoprecipitated cross-linked GFP-myc with anti-myc antibody, all fragments showed very low enrichments. We consider this as nonspecific background enrichment. In contrast with GFP-myc, the ChIP of PIL5-myc showed high enrichment of an *RGA* promoter fragment (*RGA-B*), which was previously shown to be a binding site of PIL5 in vivo (Oh et al., 2007). In the same ChIP sample, some of the SOM promoter fragments also showed high enrichment. Among promoter fragments, a SOM-A fragment centered at -2220 showed the lowest enrichment and is comparable with the enrichment level of GFP-myc. The enrichment by PIL5-myc peaked at two positions, a SOM-D fragment centered at -1538 and a SOM-H fragment centered at -342. The enrichment of these two fragments was comparable to that of *RGA-B*. Compared with these two fragments, the enrichment of other fragments gradually decreased with distance from the SOM-D and SOM-H fragments. Taken together, our ChIP analyses indicate that PIL5 binds to two different parts of the SOM promoter.

Figure 3. (continued).

Notations for light irradiation schemes are the same as those in Figure 2.

(A) No change in SOM protein stability under different light conditions. Proteins were extracted at the end of dark incubation from transgenic lines expressing myc-tagged SOM, myc-tagged PIL5, and myc-tagged GFP. Tubulin (TUB) was detected by anti-tubulin antibody as a loading control. Light conditions are indicated in the diagrams at top. Seeds were harvested for the protein extraction at the end of dark incubation (PHYB_{off} and PHYB_{on}) or far-red irradiation (PHYA_{on}).

(B) Germination of transgenic lines (*SOM-GFP1* and *SOM-GFP2*) expressing the SOM-GFP fusion protein. Notations are the same as in Figure 2A.

(C) Subcellular localization of SOM-GFP, PHYB-GFP, and GFP determined using transgenic lines expressing these proteins. 4',6-Diamidino-2-phenylindole (DAPI) was used to stain the nucleus. WL, white light.

(D) Relative transcript levels of SOM in Col-0, *phyA-211* mutant, and *phyB-9* mutant seeds (PHYB-dependent germination protocol). Light conditions are indicated by the diagrams at top. Seeds were harvested for the RNA extraction at the end of dark incubation. The SOM transcript levels were normalized by the *PP2A* transcript level. Error bars indicate SD of three real-time PCR experiments of one biological sample.

(E) Relative transcript levels of SOM in Col-0, *phyA-211* mutant, and *phyB-9* mutant seeds (PHYA-dependent germination protocol). Light conditions are indicated by the diagrams at top. Seeds were harvested for RNA extraction at the end of dark incubation. SOM transcript levels were normalized by the *PP2A* transcript level. Error bars indicate SD of three real-time PCR experiments.

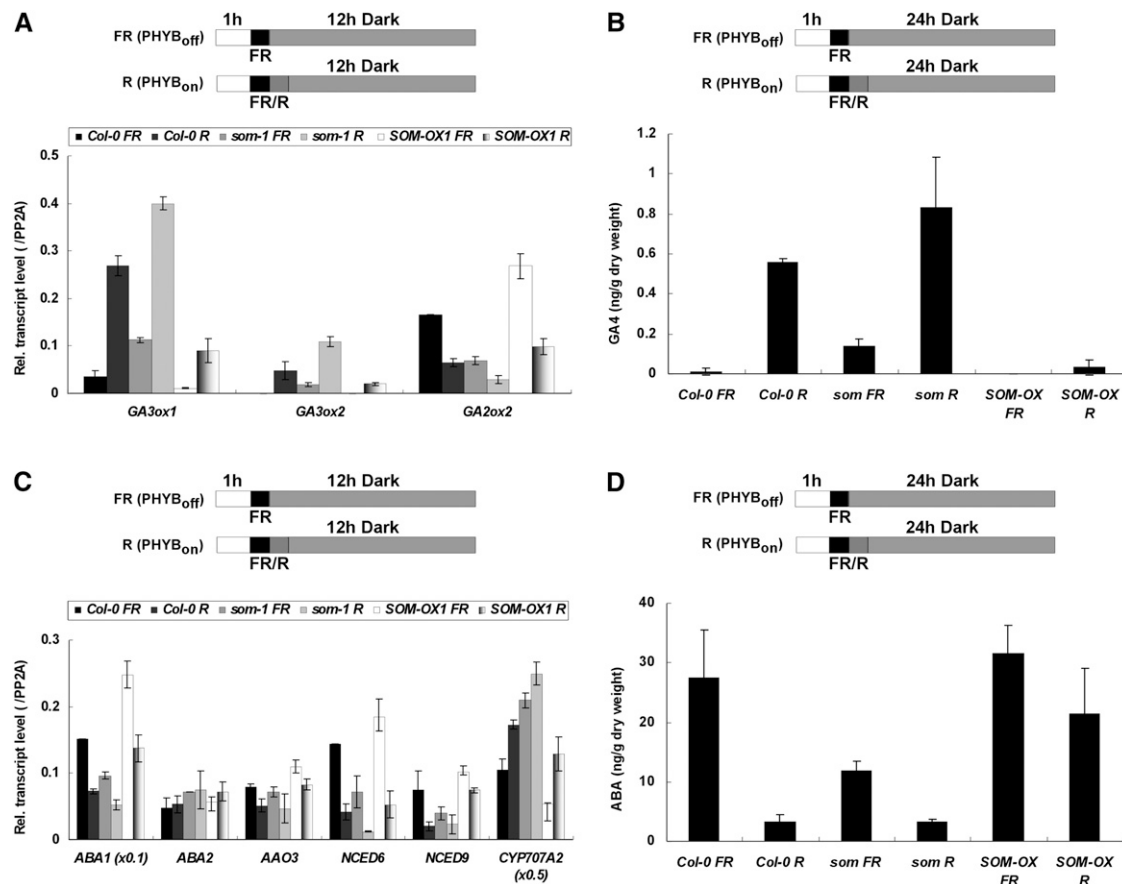


Figure 4. SOM Regulates Both GA and ABA Metabolic Genes.

Notations for light irradiation schemes are the same as those in Figure 2.

(A) Relative transcript levels of GA metabolic genes (*GA3ox1*, *GA3ox2*, and *GA2ox2*) in Col-0, *som-1* mutant, and *SOM-OX1* seeds (PHYB-dependent germination protocol). Light conditions are indicated by the diagrams at top. Error bars indicate SD of three PCR experiments.

(B) Bioactive GA levels in Col-0, *som-1* mutant, and *SOM-OX1* seeds under different light conditions. Seeds were harvested for quantification at the end of dark incubation. Error bars indicate SD of three biological samples.

(C) Relative transcript levels of ABA metabolic genes (*ABA1*, *ABA2*, *AAO3*, *NCED6*, *NCED9*, and *CYP707A2*) in Col-0, *som-1* mutant, and *SOM-OX1* seeds (PHYB-dependent germination protocol). Error bars indicate SD of three PCR experiments.

(D) ABA levels in Col-0, *som-1*, and *SOM-OX1* seeds. Error bars indicate SD of three biological samples.

Previous analyses demonstrated that PIL5 binds to G-box elements in the promoters of *GAI* and *RGA* (Oh et al., 2007). Sequence analysis of the *SOM* promoter shows that it also contains a G-box element. Curiously, however, the position of the G-box element (−2223) does not correspond to in vivo PIL5 binding promoter fragments (SOM-D and SOM-H) (Figure 6C). SOM-D and SOM-H contain E-box elements, which have been shown to serve as binding sites for many bHLH transcription factors (Ellenberger et al., 1994). We used an electrophoretic mobility shift assay (EMSA) to investigate whether PIL5 binds to E-box elements (Figure 6D). We made a double-stranded and labeled probe (38mer) containing the sequence of the part of the SOM-H fragment that contained E-box elements and studied its binding to the PIL5 protein using an EMSA. The binding was further competed out by the nonlabeled probe but was not competed out by the same probe with mutated E-box elements. These findings indicate that PIL5 can also bind to some E-box

elements that are present in the SOM-H promoter fragments and suggest that PIL5 regulates the expression of the *SOM* transcript by binding to E-box elements in its promoter.

PIL5 Regulates ABA and GA Metabolic Genes Partly through SOM

The activation of *SOM* expression by PIL5 via binding to the *SOM* promoter suggests that PIL5 may inhibit seed germination partly through SOM. To investigate this possibility, we established two *PIL5-OX som* mutant lines by crossing *PIL5-OX* and the *som* mutant. When we examined the germination response (Figure 7A), we found that *PIL5-OX* seeds did not germinate well in response to a short red light pulse, whereas almost all wild-type seeds germinated following this stimulus. Using the same protocol, the *PIL5-OX som* seeds germinated well. The higher germination frequency of *PIL5-OX som* lines was not due to the suppression

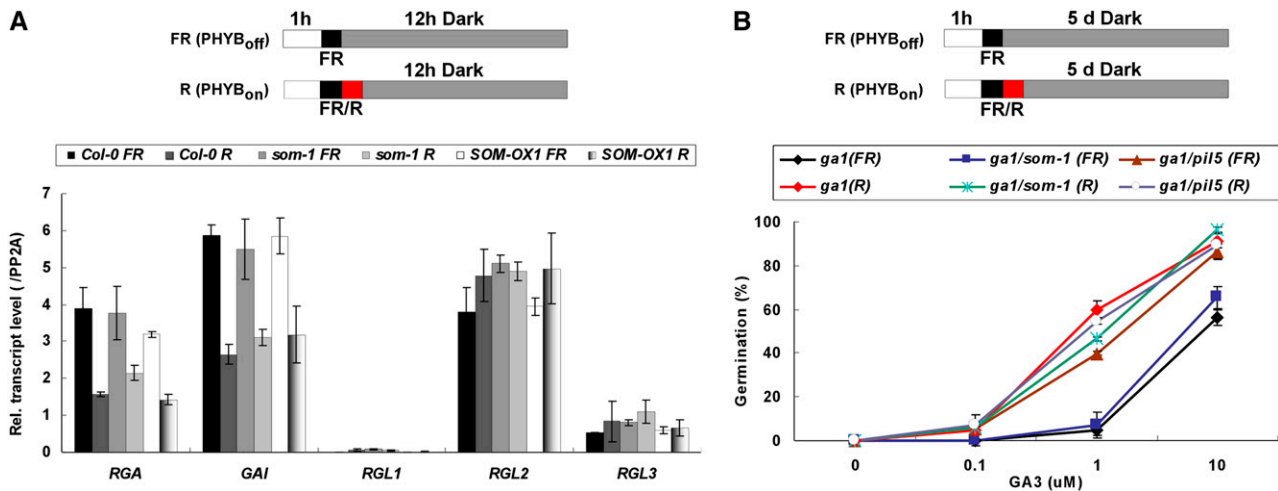


Figure 5. SOM Does Not Regulate the Expression of *GAI* and *RGA*.

Notations for light irradiation schemes are the same as those in Figure 2.

(A) Relative transcript levels of GA signaling genes (*RGA*, *GAI*, *RGL1*, *RGL2*, and *RGL3*) in Col-0, *som-1* mutant, and *SOM-OX1* seeds (PHYB-dependent germination protocol). Error bars indicate SD of three PCR experiments.

(B) GA responsiveness of the *ga1* mutant, the *ga1 som-1* double mutant, and the *ga1 pil5* double mutant (PHYB-dependent germination protocol). GA responsiveness is determined by the germination percentage in response to varying GA₃ concentrations. Error bars indicate SD ($n = 3$).

of the *PIL5* transgene, as shown by similar amounts of *PIL5* protein in these lines (Figure 7B). The *som* mutation also did not affect the degradation of *PIL5* by light in seeds (Figure 7C). *PIL5* protein was degraded similarly both in the *PIL5-OX* and the *PIL5-OX som* lines when seeds were irradiated with red light or prolonged far-red light. Taken together, these results indicate that *PIL5* requires *SOM* for the inhibition of germination.

Since *PIL5* regulates the expression of *SOM* and *SOM* regulates the expression of hormone metabolic genes (Figure 4), *PIL5* might regulate the expression of hormone metabolic genes via *SOM*. We analyzed the expression patterns of hormone metabolic genes in the *PIL5-OX som* double mutant line (Figures 7D and 7E). As reported previously, *PIL5* repressed the expression of two GA anabolic genes (*GA3ox1* and *GA3ox2*) and activated the expression of a GA catabolic gene (*GA2ox2*). In addition, *PIL5* activated the expression of ABA anabolic genes (*ABA1*, *NCED6*, and *NCED9*) and repressed an ABA catabolic gene (*CYP707A2*). The expression levels of ABA and GA metabolic genes in the *PIL5-OX som* double mutant were intermediate between those of *PIL5-OX* and the *som* mutant (Figures 7D and 7E). In contrast with hormone metabolic genes, the expression of *GAI* and *RGA* in the *PIL5-OX som* double mutant was similar to that of *PIL5-OX* (Figure 7F). Taken together, these results indicate that *PIL5* regulates the expression of ABA and GA metabolic genes partly via *SOM*. However, the intermediate expression levels of metabolic genes in the *PIL5-OX som* double mutant suggest that there are additional *PIL5* downstream components that also regulate ABA and GA metabolic genes.

DISCUSSION

We report here that *SOM*, a novel seed-specific CCCH-type zinc finger protein, negatively regulates seed germination by activat-

ing ABA biosynthesis and inhibiting GA biosynthesis. To determine the role of *SOM* in *PHY*-mediated promotion of germination, we investigated the functional relationship between *SOM* and *PIL5*, which has been characterized previously. ChIP analysis shows that *PIL5* activates the expression of *SOM* by binding directly to its promoter, suggesting that *SOM* has a role downstream of *PIL5* in the phytochrome-mediated transduction process. Our analysis of the *PIL5-OX som* double mutant also shows that *PIL5* regulates hormone metabolic genes partly via *SOM*. Taken together, these results suggest that light regulates ABA and GA metabolic genes via *PIL5* and then via *SOM* in seeds (Figure 7G and its legend for the detailed description). The molecular function of the CCCH-type zinc finger motif that is present in *SOM* is currently unknown. Further studies are needed to determine whether *SOM* is a transcription factor that regulates hormone metabolic genes, an RNA binding protein, or a protein with some other function.

PHY- and PIL5-Mediated Light Signaling Regulates ABA and GA Metabolic Genes Partly via SOM

During seed germination, phytochromes mediate the inhibition of ABA biosynthesis and the activation of GA biosynthesis via transcriptional regulation of ABA and GA metabolic genes (Ogawa et al., 2003; Oh et al., 2006, 2007; Seo et al., 2006). Our previous germination studies have shown that *PIL5*, an important negative regulator of germination, participates in the conversion of a phytochrome-mediated light signal into ABA and GA signals (Penfield et al., 2005; Oh et al., 2006, 2007). Following light absorption, phytochromes move into the nucleus (Kircher et al., 1999, 2002; Yamaguchi et al., 1999), interact with *PIL5* (Huq et al., 2004; Oh et al., 2004), and activate the degradation of *PIL5* (Shen et al., 2005; Oh et al., 2006). Since *PIL5* activates ABA

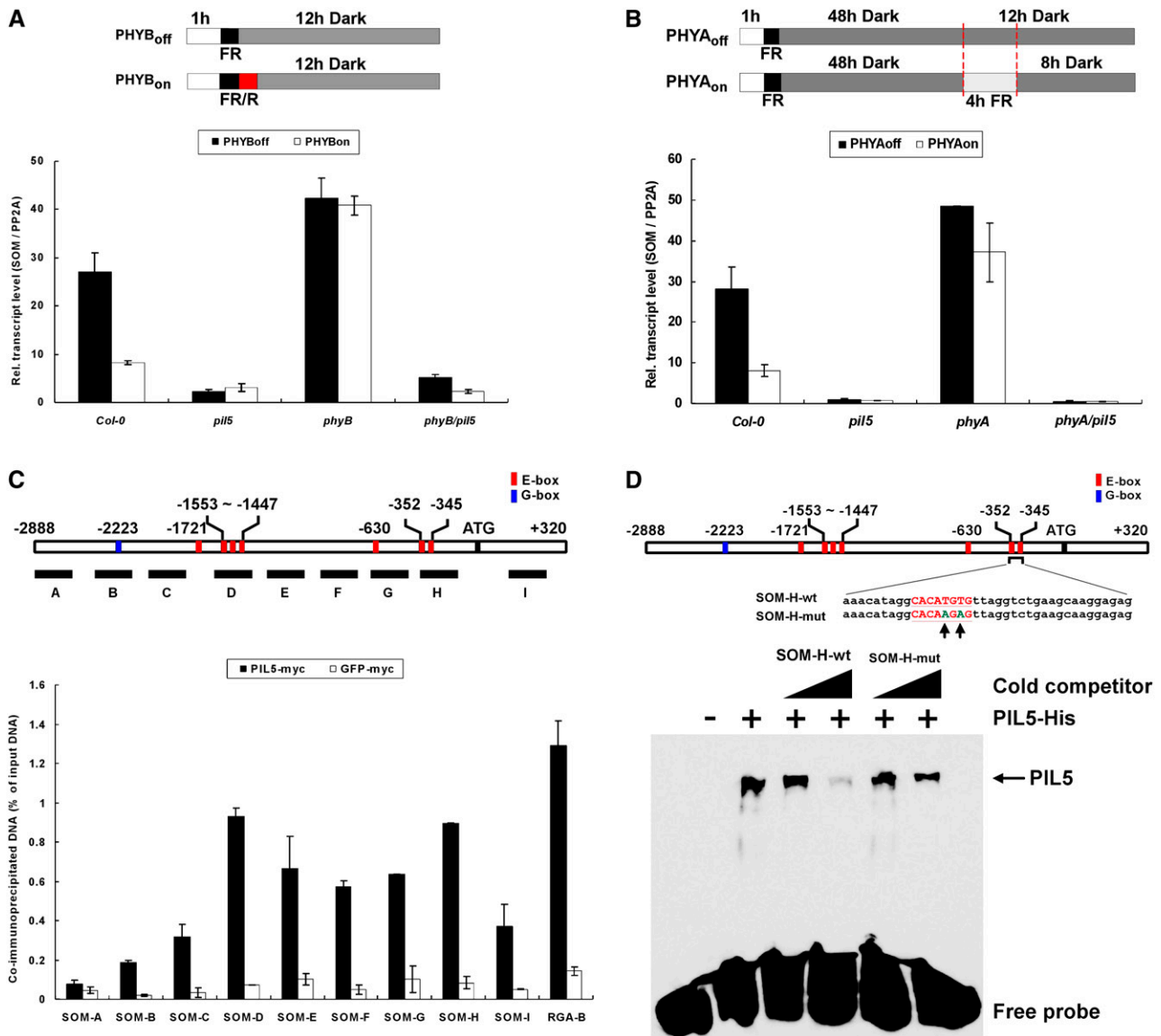


Figure 6. PIL5 Regulates the in Vivo Expression of SOM by Binding the SOM Promoter.

Notations for light irradiation schemes are the same as those in Figure 2.

(A) SOM transcript levels in *Col-0*, *pil5* mutant, *phyB-9* mutant, and *phyB-9 pil5* double mutant (PHYB-dependent germination protocol).

(B) SOM transcript levels in *Col-0*, *pil5* mutant, *phyA211* mutant, and *phyA211 pil5* double mutant (PHYA-dependent germination protocol).

(C) PIL5 ChIP analysis of the SOM promoter. The diagram at top depicts the promoter sequence of the SOM gene. The red box represents an E-box element (CANNTG) and the blue box represents a G-box element (CACGTG). Underlying thick lines marked by uppercase letters indicate various promoter fragments used for PCR. The primer sequences used for ChIP-PCR are shown in Supplemental Table 1 online. The graph indicates the percentage of DNA fragments that coimmunoprecipitated with PIL5-myc (closed bars) or GFP-myc (open bars) relative to the input DNAs. The RGA-B fragment, identified as a PIL5 binding site in an earlier report (Oh et al., 2007), was used as a positive control. Shown are a typical result and SD from three PCR experiments. Similar results were obtained from three independent experiments.

(D) In vitro binding analysis of PIL5 and E-box elements in the SOM-H fragment of the SOM promoter. The diagram at top depicts the probe sequence corresponding to the SOM-H fragment of the SOM promoter. Mutated bases in the SOM-H-mut probe are represented by green letters and indicated by arrows. SOM-H-wt, probe with intact E-box elements; SOM-H-mut, probe with mutated E-box elements. Triangles indicate increasing amounts of unlabeled probes for competition. Error bars indicate SD from three PCR experiments.

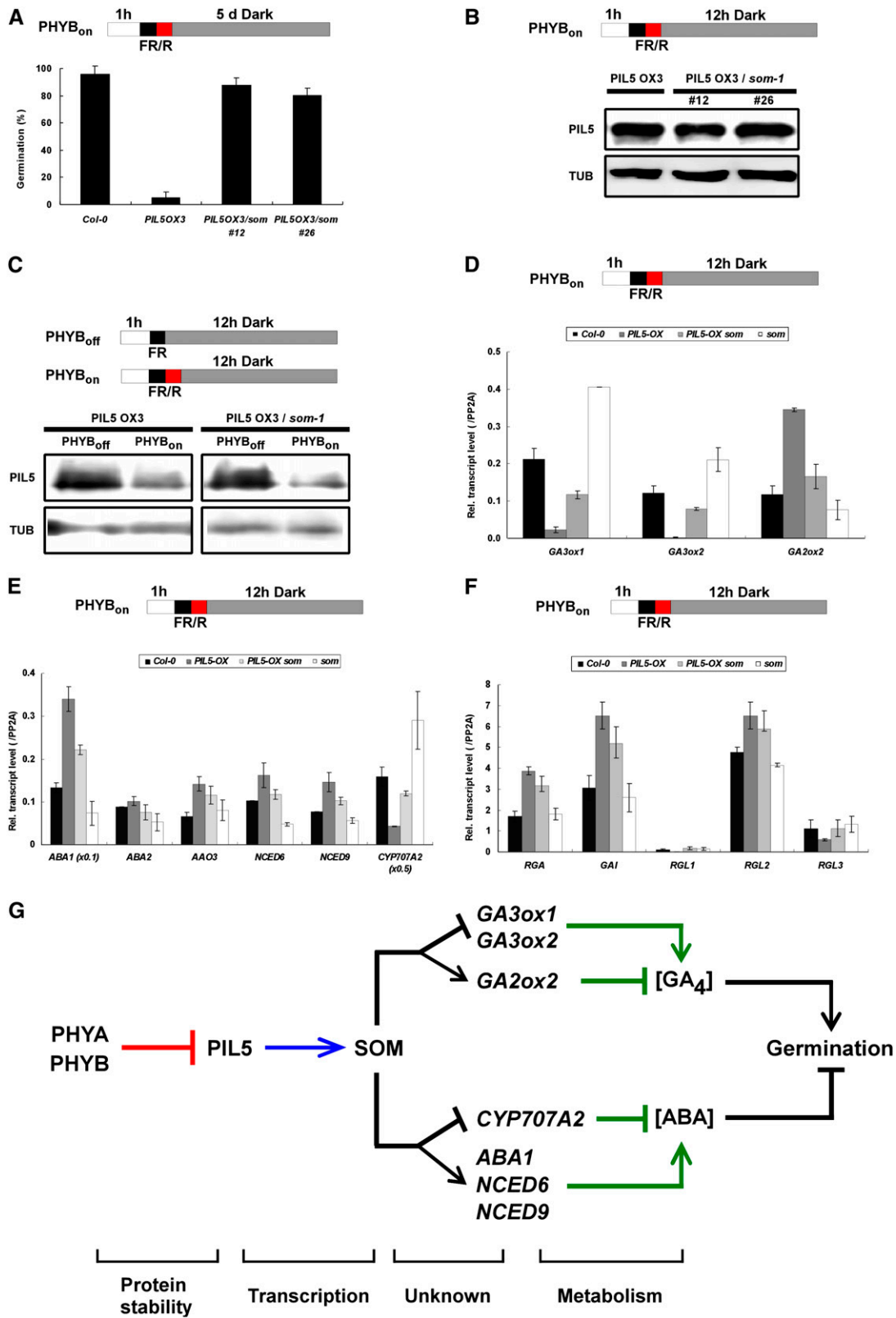


Figure 7. PIL5 Regulates ABA and GA Metabolic Genes Partly through SOM.

biosynthesis, represses GA biosynthesis, and increases the expression of two DELLA genes (*GAI* and *RGA*), the degradation of PIL5 by phytochromes decreases the level of ABA, increases the level of GA, and decreases GA negative signaling components, resulting in germination (Oh et al., 2006, 2007). ChIP analyses showed that PIL5 regulates the expression of *GAI* and *RGA* by binding directly to their promoters, but it regulates hormone metabolic genes indirectly (Oh et al., 2007). This suggested the presence of other signaling components that link PIL5 to the expression of hormone metabolic genes.

The results of this study show that SOM is one of the light signaling components that regulate the expression of ABA and GA metabolic genes. Similar to the *pil5* mutant, the *som* mutant germinates irrespective of light conditions. The expression analyses show that SOM activates ABA biosynthesis by activating three ABA anabolic genes (*ABA1*, *NCED6*, and *NCED9*) and represses an ABA catabolic gene (*CYP707A2*), resulting in the increased ABA level. Our studies also show that SOM inhibits GA biosynthesis by repressing two GA anabolic genes (*GA3ox1* and *GA3ox2*) and activating a GA catabolic gene (*GA2ox2*), resulting in the decreased GA level. In contrast with PIL5, however, SOM does not regulate the expression of *GAI* and *RGA*, so there is no change in the GA responsiveness of *som* mutants. The SOM regulation of hormone metabolic genes, but not DELLA genes, indicates that SOM regulates a subset of genes that are regulated by PIL5.

In imbibed seeds, light absorption by phytochromes alters the level of SOM transcripts via PIL5. Red light represses the expression of *SOM* in a PHYB-dependent manner, as supported by results showing that red light-dependent repression of *SOM* occurs in the wild type, whereas red light-independent constitutively high expression of *SOM* occurs in the *phyB* mutant. The repression of *SOM* by PHYB requires PIL5, as supported by results showing light-independent constitutively low expression of *SOM* in the *pil5* mutant and the *phyB pil5* double mutant. Prolonged far-red light also represses the expression of *SOM* in a PHYA-dependent manner. The light-dependent repression of *SOM* by prolonged far-red light is greatly decreased in the *phyA* mutant. The prolonged far-red light dependence of *SOM* expression is completely absent in the *pil5* mutant and in the *phyA pil5*

double mutant. Taken together, these results suggest that PHYA and PHYB repress the expression of *SOM* via PIL5 in imbibed seeds.

The persistent light-dependent expression of ABA and GA metabolic genes in the *som* mutant suggests that PIL5 regulates these metabolic genes not just via *SOM* but also via other signaling components. The *som* mutant has altered expression levels of ABA and GA metabolic genes, but light also regulates these levels in this mutant. These expression patterns are paralleled by the concentrations of ABA and GA that we measured in imbibed seeds. ABA levels in the *som* mutant are already lower than in the wild type, even before red light treatment, but they are decreased even more by red light in the *som* mutant. Similarly, GA levels are already higher in the *som* mutant before red light treatment than in the wild type, but they are further increased by red light. This response differs from that of the *pil5* mutant, which exhibits light-independent expression levels of hormone metabolic genes and light-independent hormone levels. Therefore, if PIL5 regulates these metabolic genes solely through *SOM*, the expression levels and hormone levels should also be light-independent in the *som* mutant. Taken together, our results suggest that, in addition to *SOM*, other components regulate the expression of ABA and GA metabolic genes downstream of PIL5.

PIL5 Regulates the Expression of SOM by Binding Its Promoter

The transmission of PHY-mediated light signals to *SOM* requires transcriptional and posttranslational regulation. Phytochromes transmit perceived light signals to PIL5 by interacting with PIL5 and activating its degradation, a posttranslational process (Shen et al., 2005; Oh et al., 2006). Other researches have shown that phytochromes also mediate the degradation of other negative components, such as PIF3, PIF4, and PIL6 (Bauer et al., 2004; Park et al., 2004; Shen et al., 2007; Lorrain et al., 2008). This suggests that a major mode of PHY-mediated light signaling involves activation of the degradation of specific proteins (Huq, 2006; Bae and Choi, 2008). Signal transmission from PIL5 to *SOM* occurs at the transcriptional level. ChIP analyses show that PIL5 binds to the promoter of *SOM* in vivo, indicating that PIL5 directly targets

Figure 7. (continued).

Notations for light irradiation schemes are the same as those in Figure 2.

(A) Germination of Col-0, *PIL5-OX*, and *PIL5-OX som* mutant seeds (PHYB-dependent germination protocol). Error bars indicate SD ($n = 3$).

(B) Similar levels of PIL5 protein in the seeds of *PIL5-OX* and two *PIL5-OX som* lines (lines 12 and 26).

(C) No change in PIL5 protein stability in the *PIL5-OX som* double mutant (line 12) seeds (PHYB-dependent germination protocol).

(D) Relative transcript levels of GA metabolic genes (*GA3ox1*, *GA3ox2*, and *GA2ox2*) in Col-0, *PIL5-OX*, *PIL5-OX som*, and *som* mutant seeds (PHYB-dependent germination protocol). Error bars indicate SD from three PCR experiments.

(E) Relative transcript levels of ABA metabolic genes (*ABA1*, *ABA2*, *AAO3*, *NCED6*, *NCED9*, and *CYP707A2*) in Col-0, *PIL5-OX*, *PIL5-OX som*, and *som* mutant seeds (PHYB-dependent germination protocol). Error bars indicate SD from three PCR experiments.

(F) Relative transcript levels of GA signaling genes (*RGA*, *GAI*, *RGL1*, *RGL2*, and *RGL3*) in Col-0, *PIL5-OX*, *PIL5-OX som*, and *som* mutant seeds (PHYB-dependent germination protocol). Error bars indicate SD from three PCR experiments.

(G) Proposed role of *SOM* in seed germination. Both PHYA and PHYB inhibit PIL5 by degrading the PIL5 protein, whereas PIL5 activates the expression of *SOM* by directly binding its promoter. *SOM* decreases GA_4 levels by repressing GA anabolic genes and activating a GA catabolic gene. For ABA, *SOM* increases the level of ABA by activating ABA anabolic genes and repressing an ABA catabolic gene. Decreased GA_4 cannot promote seed germination, whereas the increased ABA inhibits seed germination. Other unknown factors that also regulate ABA and GA metabolic genes downstream of PIL5 and DELLA proteins, including two PIL5 target DELLA proteins (*GAI* and *RGA*) that inhibit seed germination downstream of PIL5, are not indicated in the diagram. The mode of action for each step is indicated at bottom.

SOM as well as *GAI* and *RGA*. Curiously, the PIL5 binding sites in the *SOM* promoter do not correspond to a G-box element (CACGTG), an element to which PIL5 is known to bind (Huq et al., 2004; Oh et al., 2007). Instead, the binding sites are E-box elements (CANNTG), known binding elements of many bHLH transcription factors, indicating that PIL5 binds not only to the G-box element but also to some E-box elements. Irrespective of binding sequence elements, the binding of PIL5 to its target promoters activates the expression of target genes, including *SOM*, *GAI*, and *RGA*, and these regulate ABA and GA metabolic genes and GA responsiveness. In seeds, light activation of phytochromes decreases the level of the PIL5 protein and, consequently, the levels of PIL5 downstream target genes. PIL5-mediated regulation of some downstream target genes, such as *SOM*, alters the levels of ABA and GA inside the imbibed seeds. PIL5-mediated regulation of other downstream target genes, such as *GAI* and *RGA*, regulates hormone responsiveness.

The Molecular Function of SOM Is Unknown

We have not yet determined the exact molecular functions of *SOM*. Our analyses show that *SOM* is located in the nucleus and has three zinc finger motifs, including one tristetraprolin (TTP)-type zinc finger motif (CX₈CX₅CX₃H) and two atypical zinc finger motifs (CX₅HX₄CX₃H and CX₅CX₄CX₃H). Other plant proteins have similar zinc finger motifs. These include PE11 from *Arabidopsis*, which regulates embryogenesis (Li and Thomas, 1998), SZF1 and SZF2 from *Arabidopsis*, which regulate salt responses (Sun et al., 2007), and DOS from rice (*Oryza sativa*), which regulates leaf senescence (Kong et al., 2006). Another *Arabidopsis* zinc finger protein, FRIGIDA-ESSENTIAL1 (FES1), has a single zinc finger motif (CX₇CX₅CX₃H) and has been shown to delay flowering in the presence of FRIGIDA by activating the expression of *FLOWERING LOCUS C* (Schmitz et al., 2005). The molecular functions of PE11, DOS, and FES1 are also unknown.

Zinc finger proteins with tandem TTP-type zinc finger motifs regulate various developmental processes mainly by binding to RNA molecules and modulating their stabilities or processing (Blackshear, 2002). In the mouse, TTP regulates inflammation by binding to AU-rich elements that are in the 3' untranslated region of tumor necrosis factor- α mRNA and destabilizing this mRNA (Taylor et al., 1996; Carballo et al., 1998; Lai et al., 2000). In *Arabidopsis*, HUA1 regulates floral organ development by binding to *AGAMOUS* pre-mRNA and facilitating its processing (Li et al., 2001). Taken together, the molecular functions of tandem TTP-type zinc finger proteins suggest that *SOM* may also act as an RNA binding protein. However, since TTP-type zinc finger proteins have other molecular functions, this requires experimental verification.

The Expression of SOM during Seed Maturation

Beyond its role in PHY- and PIL5-mediated light signaling, it will be interesting to determine the role of *SOM* in other seed germination conditions. The *SOM* promoter contains both a PIL5 binding G-box element and a putative RY element (CATGCAT/A). Since the RY motif is known to be a binding site for ABI3 (Finkelstein et al., 2002), the expression of *SOM* could be regulated by ABI3.

According to the microarray data compiled in the Bio-Array Resource, expression levels of *ABI3* increase dramatically during seed maturation and decrease during imbibition under white light (see Supplemental Figure 2 online). The increased expression of *ABI3* during seed maturation likely plays a role in seed dormancy (Finkelstein et al., 2008). Curiously, the expression level of *SOM* also increases dramatically during seed maturation. Unlike *SOM*, however, the expression level of *PIL5* remains low during seed maturation. Taken together, these data are consistent with a role for ABI3 and/or FUS3 in regulating *SOM* expression during seed maturation.

The Bio-Array Resource database also indicates, however, that the expression level of *SOM* at 24 h after imbibition under white light is not significantly different between the wild type and the *abi3* mutant (see Supplemental Figure 2 online). Thus, the similar expression patterns of *SOM* and *ABI3* transcripts during seed maturation could also be caused by shared regulatory factors. Alternatively, ABI3 may regulate *SOM* redundantly with other factors such as FUSCA3, such that a single mutation fails to dramatically affect *SOM* expression. Further studies are necessary to establish the functional relationships between *SOM*, ABI3, and FUS3 during seed maturation.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana plants were grown in a room with a 16-h-light/8-h-dark cycle at 22 to 24°C. Following seed harvesting, seeds were dried at 22°C in white paper bags for at least 1 month prior to germination assays. The first *som* mutant line (*som-1*) was identified by screening T-DNA insertion pools made in the laboratory. The second and the third *som* mutants (*som-2* and *som-3*) were obtained from the Arabidopsis Stock Center (Salk_090314 and SALK_008075) (Alonso, 2003). T-DNA insertion sites were determined by amplifying the flanking regions and sequencing them (see Supplemental Figure 1 online). For generation of the *SOM-OX1* and *SOM-OX2* transgenic lines, full-length cDNA of *SOM* (At1g03790) was amplified with a primer set (5'-AACTCCTAGGATGGATGTCGTTTGTACGGAACATCAAA-3' and 5'-ATCAGGATCCTTCAAGTCAAGAGATCATTGACCCATC-3'), cloned into a binary vector (pBI121), and transformed into *Arabidopsis*, and homozygous lines were selected. Transgenic lines expressing myc-tagged *SOM* (*SOM-OX3*) and GFP-tagged *SOM* (*SOM-GFP*) under the control of the CaMV 35S promoter were generated with a primer set (5'-AACTCCTAGGATGGATGTCGTTTGTACGGAACATCAAA-3' and 5'-ATCAGGATCCTTCAAGAGATCATTGACCCATCCTAG-3'). Subcellular localization of *SOM-GFP* was determined by fluorescence microscopy (BX51; Olympus). All plants used in this study (the wild type, *phyA-211*, *phyB-9*, *ga1* [Salk_109115], *som-1*, *som-2*, *som-3*, and *SOM-OXs*) were in the Col-0 background.

Germination Assay

Germination assays were performed as described previously (Oh et al., 2007). For the PHYB-dependent germination protocol, triplicate sets of ~50 seeds for each mutant were surface-sterilized and plated on aqueous agar medium (0.6% phytoagar, pH 5.7) under white light. Plates were left on a clean bench before light irradiation. One hour after the beginning of seed sterilization, seeds were irradiated with 5 min of far-red light (3.2 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) or red light (13 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), unless specified otherwise, and incubated in the dark for 5 d, and then germination (protrusion of the radicle) was assayed. Growth chambers with 740-nm LEDs (far-red light)

or 670-nm LEDs (red light) were used for the light treatment (Vision). For the PHYA-dependent germination assay, sterilized and plated seeds were irradiated by far-red light for 5 min and then incubated in the dark for 48 h to allow the accumulation of PHYA. After the dark incubation, seeds were irradiated with various fluence levels of far-red light and incubated in the dark for 5 d for germination scoring. Germination was scored after a 4-d dark incubation period. To determine the effect of GA₃, triplicate sets of ~50 seeds were placed on agar plates containing variable concentrations of GA₃.

Gene Expression Analysis, Protein Analysis, and Sequence Analysis

For real-time PCR, total RNA was extracted from seeds that were incubated in the dark for 12 h after far-red or red light irradiation. We used the Spectrum Plant Total RNA kit (Sigma-Aldrich) according to the manufacturer's protocol. The total RNA was used to synthesize cDNA. The relative transcript level of each gene was determined by real-time PCR using specific primers (see Supplemental Table 1 online) by comparison with the level of PP2A (see Supplemental Table 2 online), SYBR Green Supermix (Bio-Rad), and the iCycler IQ PCR machine (Bio-Rad), as described previously (Oh et al., 2007). Expression patterns were confirmed using at least three different biological samples, and representative data are presented in the figures.

To determine the effect of light on the stability of SOM protein, dark-incubated imbibed seeds were treated with either red or far-red light. Light treatment schemes were identical to those for the seed germination assay except that seeds were harvested at 12 h after red light irradiation or immediately after 6 h of far-red light irradiation. Total protein was extracted from ~50 μ L of seeds, and myc-tagged protein levels were determined by protein gel blotting using an anti-myc antibody, as described previously (Oh et al., 2006).

Amino acid sequences of SOM and its homologs were retrieved from the database and aligned by Clustal in the MEGA4 program (www.megasoftware.net) using a defaulting setting. A bootstrap phylogenetic tree was constructed using the neighbor-joining method embedded in the MEGA4 program.

Quantification of Endogenous ABA and GA Levels

GA measurements were performed on a liquid chromatography–tandem mass spectrometry 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), as described previously (Varbanova et al., 2007). ²H-labeled GAs (purchased from Lewis Mander, Australian National University) were used as internal standards. Endogenous ABA levels were determined by liquid chromatography–tandem mass spectrometry using [²H₆]ABA (Icon Services) as an internal standard, as reported previously (Saika et al., 2007).

ChIP Assay and EMSA

For the ChIP assay, *PIL5-OX3* seeds were incubated in the dark for 6 h after far-red light irradiation. The ChIP assay was performed as described previously (Tai et al., 2005; Oh et al., 2007). After reverse cross-linking, the amount of each precipitated DNA fragment was determined by real-time PCR using specific primers (see Supplemental Table 1 online).

For the EMSA, recombinant His-tagged PIL5 protein was purified using a nickel-nitrilotriacetic acid agarose column. The ability of PIL5 to bind to biotin-labeled oligonucleotide probes (Forward, 5'-AAACATAGGCAC-ATGTGTTAGGTCTGAAGCAAGGAGAG-3'; Reverse, 5'-CTCTCCTTGC-TTCAGACCTAACACATGTGCTATGTTT-3') was determined with the Lightshift Chemiluminescent EMSA kit (Pierce). Briefly, PIL5 protein and biotin-labeled probes were incubated in the binding buffer (10 mM Tris and 10 ng/ μ L polydeoxy [inosinate-cytidylate], pH 7.5) for 20 min at room

temperature, and the binding mixture was loaded onto a 5% native polyacrylamide gel. Detection of biotin-labeled probe was performed according to the manufacturer's instructions.

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), *PIL5* (At2g20180), *ABA1* (At5g67030), *ABA2* (At1g52340), *NCED6* (At3g24220), *NCED9* (At1g78390), *AAO3* (At2g23740), *CYP707A2* (At2g29090), *SOM* (At1g03790), *UBQ10* (At4g05320), and *PP2A* (At1g13320).

Supplemental Data

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

Supplemental Figure 1. T-DNA Insertion Sites of *som-1*, *som-2*, and *som-3*.

Supplemental Figure 2. Expression Profile of *SOM* during Seed Maturation.

Supplemental Figure 3. Expression Levels of *SOM* in Three *SOM*-OXs.

Supplemental Table 1. Primer Sequences.

Supplemental Table 2. Relative Expression Levels of Various Genes Used for Drawing Figures.

Supplemental Data Set 1. Text File Corresponding to the Alignment in Figures 1C and 1D.

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