The Arabidopsis circadian clock protein PRR5 interacts with and stimulates ABI5 to modulate abscisic acid signaling during seed germination

Milian Yang ,1,2,3 Xiao Han ,1,2 Jiajia Yang ,1,2,3 Yanjuan Jiang 1,2 and Yanru Hu 1,2,*†

1 CAS Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan 650223, China
2 Center of Economic Botany, Core Botanical Gardens, Chinese Academy of Sciences, Kunming, Yunnan 650223, China
3 University of Chinese Academy of Sciences, Beijing 100049, China

*Author for correspondence: huyanru@xtbg.ac.cn
†Senior author.

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plcell) is: Yanru Hu (huyanru@xtbg.ac.cn).

Abstract

Seed germination and postgerminative growth require the precise coordination of multiple intrinsic and environmental signals. The phytohormone abscisic acid (ABA) suppresses these processes in Arabidopsis thaliana and the circadian clock contributes to the regulation of ABA signaling. However, the molecular mechanism underlying circadian clock-mediated ABA signaling remains largely unknown. Here, we found that the core circadian clock proteins PSEUDO-RESPONSE REGULATOR5 (PRR5) and PRR7 physically associate with ABSCISIC ACID-INSENSITIVE5 (ABI5), a crucial transcription factor of ABA signaling. PRR5 and PRR7 positively modulate ABA signaling redundantly during seed germination. Disrupting PRR5 and PRR7 simultaneously rendered germinating seeds hyposensitive to ABA, whereas the overexpression of PRR5 enhanced ABA signaling to inhibit seed germination. Consistent with this, the expression of several ABA-responsive genes is upregulated by PRR proteins. Genetic analysis demonstrated that PRR5 promotes ABA signaling mainly dependently on ABI5. Further mechanistic investigation revealed that PRR5 stimulates the transcriptional function of ABI5 without affecting its stability. Collectively, our results indicate that these PRR proteins function synergistically with ABI5 to activate ABA responses during seed germination, thus providing a mechanistic understanding of how ABA signaling and the circadian clock are directly integrated through a transcriptional complex involving ABI5 and central circadian clock components.

Introduction

Seed germination and subsequent seedling establishment, two crucial developmental stages in flowering plants, require the precise coordination of multiple environmental and intrinsic signals. Among them, the phytohormone abscisic acid (ABA) is a pivotal signal that represses germination and subsequent seedling establishment, and stimulates seed maturation and dormancy in Arabidopsis thaliana (Finkelstein et al., 2002, 2008; Gubler et al., 2005; Finch-Savage and Leubner-Metzger, 2006). The presence of ABA is sensed by
the PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR family of proteins (Ma et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Park et al., 2009; Santiago et al., 2009). The recognition of ABA by these receptors leads to the repression of the co-receptor, type 2C protein phosphatases (PP2Cs), permitting the activation of a group of specific kinases termed SNF1-RELATED KINASE2 (SnRK2s; Ma et al., 2009; Park et al., 2009; Cutler et al., 2010; Zhao et al., 2020). SnRK2s subsequently phosphorylate and stabilize downstream regulators, such as the basic leucine zipper (bZIP)-type transcription factor ABSCISIC ACID-INSENSITIVE5 (ABI5) and its homologs ABSCISIC ACID-RESPONSIVE ELEMENT BINDING FACTORS, to mediate the expression of ABA-responsive genes (Kobayashi et al., 2005; Furihata et al., 2006; Fuji et al., 2007; Fuji and Zhu, 2009; Nakashima et al., 2009).

The ABI5 transcription factor, mainly expressed in dry seeds and strongly induced by ABA, plays a critical role in ABA-inhibited seed germination and postgerminative growth (Finkelstein, 1994; Finkelstein and Lynch, 2000a; Lopez-Molina and Chua, 2000; Lopez-Molina et al., 2001, 2002; Brocard et al., 2002; Finkelstein et al., 2005; Skubacz et al., 2016; Fan et al., 2019). ABI5 is tightly regulated through protein posttranslational modifications (Stone et al., 2006; Garcia et al., 2008; Miura et al., 2009; Lee et al., 2010; Liu and Stone, 2010; Albertos et al., 2015; Yu et al., 2015; Lynch et al., 2017; Ji et al., 2019). For instance, ABI5 is activated through phosphorylation by the SnRK2s and other related kinases in response to ABA but repressed by PP6 (Kobayashi et al., 2005; Furihata et al., 2006; Fuji et al., 2007; Fuji and Zhu, 2009; Nakashima et al., 2009; Dai et al., 2013; Hu and Yu, 2014; Zhou et al., 2015; Chen et al., 2021). ABI5 also acts as a key integrator between ABA and other signaling pathways during seed germination and postgerminative growth (Lim et al., 2013; Yu et al., 2015; Kim et al., 2016; Yang et al., 2016; Hu et al., 2019; Ju et al., 2019; Pan et al., 2020). For example, the kinase BRASSINOSTEROID-INSENSITIVE2 phosphorylates ABI5 to mediate the antagonism of brassinosteroids to ABA during seed germination (Hu and Yu, 2014), and cytokinin promotes degradation of ABI5 via the 26S proteasome pathway to antagonize ABA-inhibited cotyledon greening (Guan et al., 2014). Although much progress has been made in recent years, a comprehensive understanding of the transcriptional mechanisms underlying the crosstalk between ABA and other critical signals during seed germination remain elusive.

The circadian clock is an endogenous time-keeping system that provides an adaptive advantage to higher plants by synchronizing internal biological processes with external daily environmental cycles (Dunlap, 1999; Dodd et al., 2005; Pruneda-Paz and Kay, 2010; Atkins and Dodd, 2014; Hsu and Harmer, 2014; Grundy et al., 2015; Sanchez and Kay, 2016; Webb et al., 2019; Simon et al., 2020). The oscillatory mechanism of the clock is based on transcriptional–translational feedback loops that connect morning- and evening-phase circuits (Harmer, 2009; Pokhilko et al., 2012; Carré and Velfingstad, 2013; Hsu and Harmer, 2014; Greenham and McClung, 2015; Uehara et al., 2019; Nakamichi, 2020). In the feedback loop, the genes encoding MYB transcription factors CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) and LATE ELONGATED HYCOTYL (LHY) are expressed in the early morning (Wang and Tobin, 1998; Harmer, 2009), and CCA1 and LHY directly suppress the transcription of the pseudo-response regulator genes PRR9, PRR7, PRR5, and TIMING OF CAB EXPRESSION1 (TOC1, also known as PRR1; Harmer et al., 2000; Matsushika et al., 2000; Strayer et al., 2000; Alabadi et al., 2001; Farré and Liu, 2013; Adams et al., 2015). These PRR genes are expressed when LHY and CCA1 protein levels decrease and, in turn, the PRR proteins act to inhibit LHY and CCA1 transcription until the following morning (Alabadi et al., 2001; Perales and Más, 2007; Nakamichi et al., 2010, 2012; Gendron et al., 2012; Wang et al., 2010, 2013; Li et al., 2020a, 2020b).

The circadian clock integrates multiple internal and external signals to modulate plant growth, development, and physiology, such as photomorphogenesis, flowering, leaf senescence, and stress responses (Dunlap, 1999; Yamamoto et al., 2003; Dodd et al., 2005; Nakamichi et al., 2005, 2007, 2009; Fukushima et al., 2009; Pruneda-Paz and Kay, 2010; Liu et al., 2013; Atkins and Dodd, 2014; Hsu and Harmer, 2014; Sanchez and Kay, 2016; Frank et al., 2018; Kim et al., 2020; Li et al., 2020a; Simon et al., 2020). Moreover, a close relationship between circadian clock and ABA biosynthesis or signaling has been reported in Arabidopsis. For instance, the circadian clock is involved in the production of ABA, thereby conferring a competitive advantage to the plant against drought, heat, salinity, and osmotic stresses (Burschka et al., 1983; Nováková et al., 2005; Lee et al., 2006; Fukushima et al., 2009; Nakamichi et al., 2009; McAdam et al., 2011; Grundy et al., 2015; Adams et al., 2018). Several key genes that encode ABA biosynthetic enzymes, such as NINE-CIS-EPOXYCAROTENOID DIOXYGENASE3 and ABA DEFICIENT2, exhibit circadian rhythmicity (Covington et al., 2008; Fukushima et al., 2009; Penfield and Hall, 2009; Seung et al., 2012; Adams et al., 2018), and many ABA signaling components and downstream-responsive genes are rhythmically expressed (Covington et al., 2008; Michael et al., 2008; Mizuno and Yamashino, 2008; Penfield and Hall, 2009; Seung et al., 2012; Liu et al., 2013). LHY and CCA1 transcription factors have been shown to bind the promoter sequences of several genes critical for ABA biosynthesis and signaling (Adams et al., 2018). PRR5, PRR7, and PRR9 are also involved in ABA biosynthesis and signaling, and the content of ABA increases in prr5 prr7 prr9 triple mutant seedlings (18-day-old plants; Fukushima et al., 2009; Liu et al., 2013; Footitt et al., 2017). Moreover, multiple circadian clock proteins (e.g. CCA1, LHY, and TOC1) play important roles in seed dormancy and integrate environmental signaling controlling dormancy release in Arabidopsis (Penfield and Hall, 2009). Nevertheless, the exact molecular
mechanisms underlying the circadian regulation of ABA responses during seed germination are still not fully understood.

In this study, we aimed to discover transcriptional regulation details of circadian clock-mediated ABA signaling during seed germination. We used the yeast two-hybrid system to identify potential ABI5-interacting partners involved in the circadian clock, and found that PRR5 and PRR7 physically associate with ABI5 in yeast (Saccharomyces cerevisiae) and in planta. Phenotypic analysis showed that PRR5, PRR7 as well as PRR9 positively regulate ABA signaling redundantly during seed germination. The prr5 prr7 double mutant and prr5 prr7 prr9 triple mutant are hypersensitive to ABA during seed germination. Conversely, overexpressing PRR5 causes germinating seeds to become ABA-hypersensitive. Further genetic analysis demonstrated that the ABA hypersensitivity of PRR5-overexpressing plants requires functional ABI5 protein. Consistently, the mechanistic investigations revealed that PRR5 stimulates the transcriptional function of ABI5 to modulate downstream target genes. Together, our findings indicate that these PRR proteins act synergistically with ABI5 to positively regulate the ABA responses during seed germination and provide a mechanistic understanding of the crosstalk between the circadian clock and ABA signaling.

Results

ABI5 physically interacts with PRR5 and PRR7

The ABI5 transcription factor is a critical modulator of ABA signaling, which represses seed germination and early seedling growth. Importantly, ABI5 also may function as a crucial interaction node to integrate ABA signaling and other pathways. To further investigate the molecular mechanisms underlying the circadian regulation of ABA signaling during seed germination, we performed yeast two-hybrid analysis to identify possible physical interactions between ABI5 and core components of the circadian clock, including CCA1, LHY, PRR9, PRR7, PRR5, PRR3, and TOC1. The full-length ABI5 was fused to the Gal4 activation domain (AD) of the prey vector (AD-ABI5) and the full-length of the clock proteins were ligated with the Gal4 DNA-binding domain (BD) of the bait vector (BD-CCA1, BD-LHY, BD-PRR, and BD-TOC1). As shown in Figure 1A, ABI5 physically associated with PRR5 and PRR7 in the yeast two-hybrid system, and no interaction was detected between ABI5 and CCA1, LHY, PRR9, PRR3, or TOC1 (Figure 1A; Supplemental Figure S1). Parallel experiments showed that ABI3 and ABI4, two other key transcription factors involved in ABA signaling (Giraudat et al., 1992; Finkelstein, 1994; Finkelstein et al., 1998; Söderman et al., 2000), did not interact with PRR5 and PRR7 in yeast (Figure 1A), supporting the specificity of the interactions of ABI5 with PRR5 and PRR7.

To corroborate that ABI5 interacts with PRR5 and PRR7 in plant cells, we used the bimolecular fluorescence complementation (BiFC) assay. The full-length coding sequence (CDS) of ABI5 was ligated with the sequence encoding the C-terminal yellow fluorescent protein (YFP) fragment driven by the Cauliflower mosaic virus (CaMV) 35S promoter to generate ABI5-cYFP, whereas the full-length PRR5, PRR7, and PRR9 were fused with the sequence encoding the N-terminal YFP fragment to produce PRR5-nYFP, PRR7-nYFP, and PRR9-nYFP. When ABI5-cYFP was coexpressed transiently with PRR5-nYFP or PRR7-nYFP in leaf cells of wild tobacco (Nicotiana benthamiana), strong YFP fluorescence was detected in the nucleus of the transformed cells, as revealed by staining with 4′,6-diamidino-2-phenylindole (DAPI; Figure 1B). No YFP signal was observed in the negative control assays in which ABI5-cYFP was coexpressed with PRR9-nYFP and ABI51–164-cYFP (the sequence encoding the N-terminal amino acid residues 1–164 of ABI5 fused to cYFP) was coexpressed with PRR5-nYFP or PRR7-nYFP (Figure 1B). Moreover, as shown in Figure 1C, a commouniprecipitation (CoIP) assay provided further evidence of the association between ABI5 and PRR5 in transgenic Arabidopsis simultaneously overexpressing ABI5 and PRR5 (35S:ABI5-4MYC/35S:2FLAG-PRR5), which was constructed by introducing a PRR5 overexpression construct (35S:2FLAG-PRR5) into previously described 35S:ABI5-4MYC plants containing a functional ABI5-4MYC construct driven by the CaMV 35S promoter; Chen et al., 2012; Hu et al., 2019). Collectively, these results demonstrate that ABI5 physically interacts with PRR5 and PRR7, implying that PRR5 and PRR7 may function as two interacting partners of ABI5 to mediate ABA responses during seed germination.

The bZIP domain of ABI5 and the C-terminal fragment of PRR5 are responsible for the interaction

To identify the region of ABI5 essential for the interaction with PRR5, we fused five truncated ABI5 variants to the Gal4 AD of the prey vector (Figure 2A) and examined the interaction between these variants and PRR5 by yeast two-hybrid analysis. As shown in Figure 2A, deletion of the N-terminal amino acid residues 1–164 of ABI5 (AD-ABI5165–442) did not affect the ABI5–PRR5 interaction, whereas deletion of the 278 C-terminal residues of ABI5 that harbor the bZIP domain (AD-ABI51–164) completely abolished the ABI5–PRR5 interaction (Figure 2A). This result shows that the C-terminal region of ABI5 was required for its interaction with PRR5. Further mapping revealed that the 93 amino acids spanning the C-terminal bZIP domain were specifically involved in the ABI5–PRR5 interaction, because an ABI5 variant in which the N-terminal amino acids 1–349 were deleted (AD-ABI5350–442) could still physically associate with PRR5 (Figure 2A).

Similarly, to determine the PRR5 region critical for the interaction with ABI5, we truncated the sequences of PRR5 to obtain variants with the N-terminal PR domain, the C-terminal fragment, or the CCT domain (Figure 2B; Kiba et al., 2007). We fused the truncated PRR5 sequences to the Gal4 DNA-BD of the pGBK7 vector as baits and performed directed yeast two-hybrid analysis. As shown in Figure 2B, the N-terminal PR domain (BD-PRR51–180) and the CCT
domain (BD-PRR5 502–558) did not interact with ABI5, whereas the C-terminal fragment (BD-PRR5 172–558) strongly interacted with ABI5. These results demonstrate that the entire C-terminal region of PRR5 is crucial in forming the ABI5–PRR5 interaction.

The prr5 prr7 double and prr5 prr7 prr9 triple mutants are hyposensitive to ABA during seed germination

Previous studies showed that PRR proteins are core clock components in Arabidopsis that regulate multiple physiological processes, such as photomorphogenesis, flowering, and stress responses (Yamamoto et al., 2003; Nakamichi et al., 2005, 2007, 2009; Farrow and Liu, 2013; Liu et al., 2013; Li et al., 2020a; Yuan et al., 2021). Because PRR5 and PRR7 physically interact with the ABI5 transcription factor, we queried whether they are involved in ABI5-mediated ABA signaling during seed germination. To test this possibility, we first analyzed the expression of PRR5, PRR7, as well as PRR9 in ABA-treated wild-type seeds. As shown in Figure 3, the expression of PRR5, PRR7, and PRR9 was rhythmic and responsive to ABA during the early stage of germination (Figure 3). Similarly, we detected the expression of ABI5 in wild-type germinating seeds, and found that the transcript levels of ABI5 also displayed a diel pattern in response to ABA (Figure 3). Then, we evaluated the germination of the loss-of-function prr5 (prr5-1 and prr5-2) and prr7 (prr7-1 and prr7-2) single mutants on half-strength Murashige and Skoog (MS) supplemented with different concentrations of ABA. As shown in Supplemental Figure S2, A and B, seeds of prr5 and prr7 single mutants displayed germination and greening percentages in response to ABA which were similar to those of wild-type seeds. To avoid the effects of sucrose and/or nitrate on seed germination (Garcia-Rubio et al., 1997; Finkelstein and Lynch, 2000b; Dekkers et al., 2004; Alboresi et al., 2005; Dave et al., 2011), we also analyzed the phenotypes of prr5 and prr7 single mutants on water agar medium and found that these mutants behaved like the wild-type upon ABA treatment during seed germination (Supplemental Figure S2, C and D). This finding shows that disruption of PRR5 or PRR7 alone had little effect on ABA responses during seed germination and subsequent seedling establishment.

Because PRR5 and PRR7 play partially overlapping roles in the circadian clock, we hypothesized that they may mediate ABA signaling redundantly during seed germination. To test this speculation, we genetically crossed prr5-1 with prr7-2 to generate a prr5 prr7 double mutant and evaluated its performance in half-strength MS medium containing different concentrations of ABA. As shown in Figure 4, A–C, the progeny of the prr5 prr7 double mutant were hyposensitive to ABA during seed germination and showed much higher germination and greening than the wild-type. PRR9 is a close homolog of PRR5 and PRR7 in regulating the circadian clock and various other physiological processes (Nakamichi et al., 2005, 2007, 2009; Farrow and Liu, 2013). To test whether
The germinating seeds of the well-characterized ABA-responsive genes in ABA-treated response to ABA, we examined the expression of several LATE EMBRYOGENESIS ABUNDANT 6 including wild-type during seed germination (Supplemental Figure S3). The seeds of the RAB18, and RD29B were more hyposensitive to ABA than the seeds of the PRR5, PRR7, and PRR9 mutants (Figure 4, A–C). These results show that PRR5, PRR7, and PRR9 may positively modulate ABA responses during seed germination.

Overexpression of PRR5 confers germinating seeds being ABA-hypersensitive
To further analyze the role of PRR5 in ABA signaling during seed germination and postgerminative growth, we generated transgenic plants overexpressing PRR5 (35S:2FLAG-PRR5) under the control of the CaMV 35S promoter. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis showed that some of overexpressing lines had elevated levels of PRR5 transcripts under normal growth condition (Supplemental Figure S4). We selected the homozygous 35S:2FLAG-PRR5-9 and 35S:2FLAG-PRR5-10 transgenic plants for further analysis (Supplemental Figure S4). Consistent with previous studies (Sato et al., 2002; Murakami et al., 2004), the F4 progeny of these transgenic plants exhibited an early flowering phenotype compared with the wild-type. We investigated the performances of 35S:2FLAG-PRR5-9 and 35S:2FLAG-PRR5-10 on half-strength MS medium with various concentrations of ABA during seed germination. As shown in Figure 6A, the progeny of 35S:2FLAG-PRR5-9 and 35S:2FLAG-PRR5-10 had much lower germination percentages than the wild-type at the ABA concentration tested. Moreover, the seeds of 35S:2FLAG-PRR5-9 and 35S:2FLAG-PRR5-10 showed significantly less greening than the seeds of the wild-type (Figure 6, B and C). Likewise, on water agar media containing ABA, 35S:2FLAG-PRR5-9 and 35S:2FLAG-PRR5-10 were also more sensitive to ABA than the wild-type during seed germination (Supplemental Figure S5). Thus, the overexpression of PRR5 enhances ABA responses during seed germination, which further supports the notion that PRR5 positively mediates ABA signaling to repress seed germination and early seedling growth in Arabidopsis.

Genetic interaction between ABIS and PRR5
Having ascertained that PRR5 interacts with ABIS and positively modulates ABA responses, we asked whether the action of PRR5 in mediating ABA signaling required functional ABIS. To test this possibility, we generated abi5 35S:2FLAG-PRR5 plants by genetically crossing 35S:2FLAG-PRR5-10 with abi5 (abi5-1), which is a loss-of-function mutant of ABIS in the Wassilewskija background (Finkelstein, 1994; Finkelstein and Lynch, 2000a) and was introduced into the Columbia (Col) background through backcrossing it with the Col wild-type six times (Hu et al., 2019). Similar to abi5 seeds, progeny of abi5 35S:2FLAG-PRR5 was also hyposensitive to ABA during seed germination, with much higher percentages of germination and greening compared with those of the wild-type and 35S:2FLAG-PRR5-10 plants (Figure 7, A–C). These results show that the ABA hypersensitivity of 35S:2FLAG-PRR5-10 requires a functional ABIS
transcription factor. However, the responses of abi5 35S:2FLAG-PRR5 after exposure to ABA were different from those of the abi5 mutant (Figure 7, A–C). To further elucidate the genetic relationship between ABI5 and PRR5 in ABA signaling, we crossed abi5 with the prr5 prr7 double mutant to produce a prr5 prr7 abi5 triple mutant, and investigated its phenotype in the presence of ABA during seed germination. As shown in Figure 8, A–C, the prr5 prr7 abi5 triple mutant had higher germination and greening percentages than prr5 prr7 and abi5 on half-strength MS medium containing 1.5-μM ABA, implying that PRR5 and PRR7 may associate with other proteins besides ABI5 to mediate ABA signaling.

PRR5 stimulates the transcriptional function of ABI5

Recent studies have revealed that several interacting partners of ABI5 exert their regulatory effects mainly by stimulating or repressing the transcriptional function of ABI5 (Lim et al., 2013; Kim et al., 2016; Hu et al., 2019; Ji et al., 2019; Ju et al., 2019; Zhang et al., 2019; Zhao et al., 2019; Pan et al., 2020). Because PRR5 physically and genetically interacts with ABI5, we examined whether it also affects the ability of ABI5 to activate downstream targets. To test this, we initially investigated the possible regulatory effect of PRR5 on the transcriptional function of ABI5 in wild-type Arabidopsis mesophyll protoplasts using a dual-luciferase (LUC) reporter approach (Yoo et al., 2007). The effectors contained an ABI5, PRR5, PRR7, or GFP (green fluorescent protein) gene under the control of the CaMV 35S promoter (Figure 9A). Because EM6 and EM1 are direct downstream targets of ABI5 (Finkelstein and Lynch, 2000a; Lopez-Molina and Chua, 2000; Nakamura et al., 2001; Carles et al., 2002; Reeves et al., 2011), we fused their promoters with the LUC gene to produce reporter constructs (Figure 9A). Consistent with previous studies (Zhou et al., 2015; Pan et al., 2018; Hu et al., 2019), expression of ABI5 significantly increased the expression level of LUC driven by the EM6 or EM1 promoters in the presence of 5-μM ABA compared with the expression of GFP alone (Figure 9B). More importantly, the coexpression of PRR5 with ABI5 further enhanced the LUC expression level when compared with the coexpression of GFP and ABI5 (Figure 9B). Similar results were found when PRR7 was coexpressed with ABI5 in these assays (Figure 9B). These results suggest that PRR5 and PRR7 may stimulate the transcriptional function of ABI5 to modulate downstream EM6 or EM1 under ABA treatment.

To verify that the transcriptional function of ABI5 is enhanced by PRR proteins, we compared the ability of ABI5 to activate downstream targets in mesophyll protoplasts of the wild-type and the prr5 prr7 prr9 triple mutant. As shown in Figure 9C, LUC expression driven by the EM6 promoter in response to ABA was reduced in prr5 prr7 prr9 protoplasts compared with its expression in wild-type protoplasts.
Similar results were found when the EM1 promoter was used in these assays (Figure 9C). These findings further support the notion that PRR5 and PRR7 stimulate ABI5’s transcriptional function to modulate downstream genes. Considering that PRR5 and PRR7 interact with ABI5 and enhance its transcriptional function to activate EM6 and EM1, we queried whether PRR proteins directly mediate the expression of EM6 and EM1 through binding their promoters. The evidence based on the yeast one-hybrid analysis showed that PRR5 and PRR7 did not recognize the promoter sequences of EM6 and EM1 (Supplemental Figures S6 and S7). However, the possibility that PRR proteins are recruited to EM6 and EM1 promoters in vivo through interacting with other crucial transcription factors (e.g. ABI5) cannot be ruled out. Previous studies revealed that ABI5 recognizes the EM6 and EM1 promoter regions (such as pEM6-1 and pEM1-1 shown in Supplemental Table S1) covering a G-box-type cis-element (CACGTG; Carles et al., 2002; Chen et al., 2012). Chromatin immunoprecipitation (ChIP) was performed in ABA-treated germinating seeds of 35S:2FLAG-PRR5-10 and abi5 35S:2FLAG-PRR5 plants upon ABA treatment. The results showed that PRR5 was enriched at the promoter regions of EM6 and EM1 (pEM6-1 and pEM1-1) targeted by ABI5 in 35S:2FLAG-PRR5-10 plants (Figure 10, A and B). However, the enrichment of PRR5 on pEM6-1 and pEM1-1 was significantly decreased in abi5 35S:2FLAG-PRR5 compared with 35S:2FLAG-PRR5-10 (Figure 10, A and B). These findings imply that PRR5 associates with the promoters of EM6 and EM1 mainly through ABI5 in vivo.

Hayama et al. (2017) revealed that PRR proteins modulate the stability of their interacting CONSTANS (CO) transcription factor, which promoted us to analyze whether PRR proteins also affects the accumulation of ABI5. The results showed that ABA-induced accumulation of ABI5 was similar in prr5 prr7 prr9 and wild-type plants (Figure 9D), suggesting that PRR proteins did not regulate the stability of ABI5. As PRR proteins exert stimulative effect on ABI5, we investigated whether the ABA responses of 35S:ABI5-4MYC were enhanced by the overexpression of PRR5 during seed germination. To test this possibility, we compared the germination and greening percentages of 35S:ABI5-4MYC and 35S:ABI5-4MYC/35S:2FLAG-PRR5-10 plants during seed germination in response to ABA. As shown in Figure 10, C–E, the progeny of 35S:ABI5-4MYC/35S:2FLAG-PRR5-10 displayed much lower germination and greening percentages than 35S:ABI5-4MYC, suggesting that the increased ABA signaling

Figure 4 ABA responses of prr5 prr7, prr5 prr9, and prr5 prr7 prr9 mutants during seed germination. A, Germination of the WT, prr5 prr7, prr5 prr9, and prr5 prr7 prr9 mutants. Seed germination was recorded 2 days after stratification on half-strength MS medium supplemented with different concentrations of ABA. B, Cotyledon greening of the WT, prr5 prr7, prr5 prr9, and prr5 prr7 prr9 mutants. Cotyledon greening was scored 4.5 days after stratification on half-strength MS medium supplemented with different concentrations of ABA. Experiments were performed seven times by analyzing different batches of seeds. Each batch of seeds of WT, prr5 prr7, prr5 prr9, and prr5 prr7 prr9 mutants was pooled from more than 60 independent plants. For each biological replicate, more than 120 seeds were examined. Values are means ± sd. C, Seedlings of WT, prr5 prr7, prr5 prr9, and prr5 prr7 prr9 mutants 4.5 days after germination on half-strength MS medium containing 0.5-μM ABA.
in 35S:ABI5-4MYC was enhanced by PRR5 overexpression. The phenotypic observation further supports our proposal that PRR5 stimulates ABI5 to modulate ABA signaling during seed germination.

**Discussion**

The circadian clock is an endogenous biological oscillator that modulates a wide range of physiological processes in plants, such as photomorphogenesis, flowering, and stress responses (Yamamoto et al., 2003; Fukushija et al., 2009; Pruneda-Paz and Kay, 2010; Liu et al., 2013; Atkins and Dodd, 2014; Hsu and Harmer, 2014; Sanchez and Kay, 2016; Frank et al., 2018; Kim et al., 2020; Li et al., 2020a; Simon et al., 2020). The circadian clock also plays crucial roles in the control of ABA biosynthesis and downstream responses (Novaková et al., 2005; Lee et al., 2006; Covington et al., 2008; Mizuno and Yamashino, 2008; Fukushija et al., 2009; Nakamichi et al., 2009; Penfield and Hall, 2009; McAdam et al., 2011; Seung et al., 2012; Grundy et al., 2015; Adams et al., 2018). However, the detailed mechanisms underlying how ABA signaling is circadian regulated remain elusive. An in-depth understanding of the regulatory effects of central circadian clock components on ABA signaling may help reveal the molecular basis of circadian-mediated ABA signaling. The bZIP-type ABI5 transcription factor is a master regulator of ABA signaling that represses seed germination and early seedling growth (Finkelstein, 1994; Finkelstein and Lynch, 2000a; Lopez-Molina and Chua, 2000; Lopez-Molina et al., 2001, 2002; Brocard et al., 2002; Finkelstein et al., 2005; Skubacz et al., 2016). ABI5 also functions as a critical node to integrate multiple signaling pathways during seed germination and/or postgerminative growth (Lim et al., 2013; Yu et al., 2015; Kim et al., 2016; Hu et al., 2019; Ju et al., 2019; Pan et al., 2020). Despite recent advances, the direct involvement of ABI5 in circadian-modulated ABA responses and the underlying molecular mechanisms are largely unknown.

In this study, we showed that ABI5 physically interacts with PRR5 and PRR7 (Figure 1, A–C), two core proteins of the circadian clock (Yamamoto et al., 2003; Nakamichi et al., 2005, 2010; Farré and Liu, 2013). The interaction between ABI5 and PRR5 or PRR7 was specific because ABI5 did not associate with close homologs of PRR5 and PRR7, such as PRR9 and TOC1 (Figure 1; Supplemental Figure S1). In addition, no interaction was detected between PRR5 or PRR7 and other critical modulators of ABA signaling, such as ABI3 and ABI4 (Figure 1A). Further analysis showed that the bZIP domain of ABI5 and the C-terminal region of PRR5 are essential for the interaction (Figure 2, A and B). In line with the PRR5–ABI5 and PRR7–ABI5 physical interactions, the phenotypic analysis showed that PRR5 and PRR7 positively modulate ABA responses during seed germination. Similar to seeds of the abi5 mutant, progeny of the prr5 prr7

![Figure 5](https://example.com/figure5.png)

**Figure 5** Expression levels of several ABA-responsive Genes in prr5 prr7 prr9. RT-qPCR analysis of the ABA-induced expression of EM6, EM1, RAB18, and RD29B in the WT and prr5 prr7 prr9. Total RNA was extracted from three different batches of germinating seeds (2 days, harvested from ZT0 to ZT36) of WT and prr5 prr7 prr9 with 0.5-μM ABA treatment grown under 16-h-light/8-h-dark for indicated times. Time is expressed as hours from dawn (ZT0). The PP2A (AT1G13320) gene was used as control. Error bars show sd from three independent biological replicates. Values are means ± sd.
double mutant and prr5 prr7 prr9 triple mutant were hypo-
sensitive to ABA treatment, with much higher percentages 
of germination and greening than the seeds of the wild-type 
(Figure 4, A–4; Footitt et al., 2017). Consistent with this re-
sult, PRR5 and PRR7 are positively involved in the expression 
of several downstream ABA-responsive genes, including 
EM6, EM1, RAB18, and RD29B (Figure 5). Conversely, the 
overexpression of PRR5 confers germinating seeds with more 
sensitivity to ABA compared with the wild-type (Figure 6, 
A–C). On the basis of these results, we concluded that PRR5 
and PRR7 interact with ABI5 to activate ABA signaling dur-
ning seed germination and subsequent seedling establishment 
in Arabidopsis.

In addition to PRR5, PRR7, and PRR9 proteins, multiple 
key components of the circadian clock are essential for 
modulating ABA signaling and/or seed dormancy (Penfield 
and Hall, 2009; Footitt et al., 2011, 2017; Finch-Savage and 
Footitt, 2017; Adams et al., 2018). For instance, LHY and 
CCA1 recognize the promoter regions of several genes 
critical for ABA biosynthesis and downstream responses 
(Adams et al., 2018). Phenotypic analysis showed that the 
 germination of lhy mutant was impaired in the presence of 
ABA, whereas LHY overexpression led to increased seed 
germination (Adams et al., 2018). Moreover, disruption of 
the clock proteins LHY, CCA1, and GIAGIAGA (GI) resulted 
in germination defects in response to low temperature, 
alternating temperatures, and dry after-ripening (Penfield and 
Hall, 2009). Further investigations revealed that the tran-
script levels of central clock genes, such as LHY, CCA1, GI, 
TOC1, PRR7, and PRR9, do not oscillate in dry seeds 
(Penfield and Hall, 2009; Footitt et al., 2017). Those studies 
collectively showed that clock genes do not function in a 
circadian context in dry seeds and have crucial roles in the 
suppression of germination (Penfield and Hall, 2009; Footitt 
et al., 2011, 2017; Finch-Savage and Footitt, 2017; Figures 4 
and 6). Interestingly, the expression of several clock genes 
displays rhythmic patterns during seed imbibition and the 
clock is restarted (Zhong et al., 1998; Penfield and Hall, 2009; 
Footitt et al., 2017). Consistently, we also found that PRR5, 
PRR7, and PRR9, similar to ABI5, are rhythmically expressed 
and responsive to ABA during seed germination (Figure 3). 
The expression phase of ABI5 overlaps with those of PRR5 
and PRR7, consistent with their ability to interact with 
plants when expressed normally (Figures 1 and 3).
Interestingly, all analyzed ABA-responsive genes are expressed in the same phase as ABI5 (Figures 3 and 5). We speculated that protein levels for PRR5/7 and ABI5 may follow a similar pattern as their transcript accumulation, but this depends on possible post-transcriptional regulation. Moreover, PRR7 transcription maintains high levels in the cold winter months and tracks seed dormancy in the deeply dormant winter annual ecotype Cape Verde Island (Footitt et al., 2013, 2014, 2017). It is possible that PRR7, as well as its close homologs PRR5 and PRR9 functions in the winter months to enhance ABA signaling and suppress seed germination.

Previous studies revealed that PRR5, PRR7, and PRR9 play pivotal roles in multiple clock-associated physiological processes (Yamamoto et al., 2003; Nakamichi et al., 2005, 2007, 2010; Farré and Liu, 2013; Li et al., 2019; Yuan et al., 2021). For instance, PRR5, PRR7, and PRR9 act as transcriptional repressors in the circadian clock and interact with TOPELESS/TOPELESS-RELATED (TPL/TPR) and HISTONE DEACETYLASE6 (HDA6) to restrict the expression of the core clock genes CCA1 and LHY (Nakamichi et al., 2010; Farré and Liu, 2013; Wang et al., 2013; Liu et al., 2016).

These three PRR proteins also directly suppress cold-induced expression of CREPEAT BINDING FACTOR/DRE BINDING FACTOR1 (CBF/DREB1) genes and negatively modulate freezing tolerance (Nakamichi et al., 2009). Conversely, PRR5, PRR7, and PRR9 stabilize CO to enhance the expression of FLOWERING LOCUS T (FT) and promote flowering (Nakamichi et al., 2007; Hayama et al., 2017). PRR9 directly activates transcription of ORESARA1 (ORE1) and positively regulates leaf senescence (Kim et al., 2018). Our data show that PRR5 and PRR7 stimulate the transcriptional function of ABI5 to upregulate ABA-induced expression of EM6 and EM1 (Figure 9, B and C). PRR5 also associates with the EM6 and EM1 promoters mainly through ABI5 (Figure 10, A and B). Further phenotypic analysis found that the overexpression of ABI5 and PRR5 simultaneously confers plants much more sensitive to ABA during seed germination compared with the overexpression of ABI5 alone (Figure 10, C–E). These results collectively demonstrate that PRR5 is a positive modulator of ABI5-mediated signaling during seed germination. Given that the bZIP domain required for dimerization and DNA binding of ABI5 is involved in the interaction with PRR5 (Figure 2A), it is perhaps surprising that addition of
PRR5 enhances rather than inhibits ABI5 function. As PRR5 is recruited to EM6 and EM1 promoters in vivo through interacting with ABI5 (Figure 10, A and B), it is possible that the PRR5–ABI5 complex may function similarly as the dimers of ABI5 and have increased binding activity on promoters of target genes (e.g. EM6 and EM1). In addition, PRR5 may compete with some repressors of ABI5 to bind the bZIP domain and interfere with the regulatory effects of those repressors. Nevertheless, the detailed biochemical mechanisms underlying how these PRR proteins synergize with ABI5 to modulate downstream genes deserve further investigation. Because these PRR proteins could have dual regulatory effects (negative or positive) on their targets and/or interacting partners, they may help to establish an appropriate balance among different development- or stress-signaling pathways so that growth and stress tolerance are optimized for the prevailing conditions.

Genetic analysis found that the progeny of abi5 35S:2FLAG-PRR5, similar to the abi5 seeds, was also hyposensitive to ABA treatment compared with those of the wild-type (Figure 7, A–C). This result demonstrates that the increased ABA signaling in PRR5-overexpressing plants requires ABI5. However, the possibility that PRR5 and PRR7 associate with other proteins to modulate ABA responses during seed germination cannot be ruled out. As shown in Figure 7, A–C, although the abi5 35S:2FLAG-PRR5 plants mimicked the phenotype of abi5, the performances of abi5 35S:2FLAG-PRR5 and abi5 were significantly different. Consistent with this notion, the prr5 prr7 abi5 triple mutant exhibited higher germination and greening percentages than abi5 and prr5 prr7 in the presence of ABA (Figure 8, A–C). ABI3 and ABI4 also are crucial transcriptional regulators of ABA signaling that are involved in repressing seed germination (Giraudat et al., 1992; Finkelstein, 1994; Finkelstein et al., 1998; Suzuki et al., 2001). However, no physical interaction between PRR5 or PRR7 and ABI3 or ABI4 was detected in yeast (Figure 1A). EM6 and EM1 are direct downstream target genes of ABI5 (Carles et al., 2002). The yeast one-hybrid screening found that PRR5 and PRR7 did not bind the promoter sequence of EM6 and EM1 (Supplemental Figures S6 and S7). These observations imply that PRR5 and PRR7 may not directly interact with ABI3, ABI4, EM6, and EM1 in ABA signaling. Nevertheless, ChIP assays showed

Figure 8 ABA responses of prr5 prr7, abi5, and prr5 prr7 abi5 mutants during seed germination. A, Germination of the WT, prr5 prr7, abi5, and prr5 prr7 abi5 mutants. Seed germination was recorded 3 days after stratification on half-strength MS medium supplemented with 1.5-µM ABA. B, Cotyledon greening of the WT, prr5 prr7, abi5, and prr5 prr7 abi5 mutants. Cotyledon greening was scored 6 days after stratification on half-strength MS medium supplemented with 1.5-µM ABA. Experiments were performed five times by analyzing different batches of seeds. Each batch of seeds of the WT, prr5 prr7, abi5, and prr5 prr7 abi5 mutants was pooled from more than 60 independent plants. For each biological replicate, more than 120 seeds were examined. Values are means ± so. Bars with different letters are significantly different from each other (P < 0.05). Data were analyzed by analysis of variance (ANOVA). C, Seedlings of the WT, prr5 prr7, abi5, and prr5 prr7 abi5 6 days after germination on half-strength MS medium containing 1.5-µM ABA.
that PRR5 may associate indirectly with the promoters of EM6 and EM1 through ABI5 (Figure 10, A and B). Further elucidation of potential associations of PRR5 and PRR7 with other key regulators of ABA responses will further enhance our understanding of PRR5- and PRR7-mediated ABA signaling networks.
ABA hypersensitivity of ABI5-overexpressing plants is enhanced by PRR5 overexpression during seed germination. Figure 10 A, B, ChIP-qPCR analysis of the relative enrichment of PRR5 on the promoter regions of EM6 (pEM6-1) and EM1 (pEM1-1). Three different batches of 0.5-μM ABA-treated (for 2.5 days) germinating seeds of PRR5-overexpressing WT (35S:2FLAG-PRR5-10) and abi5 (abi5 35S:2FLAG-PRR5) pooled from more than 60 independent plants were used in ChIP using anti-FLAG antibody. qPCR data from the ChIP assay with anti-FLAG antibody with the PP2A (AT1G13320) promoter (pPP2A) as a negative control. Error bars show SD from three biological replicates using different batches of seeds, and different letters above the columns indicate significant differences based on analysis of variance (ANOVA; *P < 0.05*). C, Germination of ABI5-overexpressing WT (35S:ABI5-4MYC) and other related transgenic plants in response to ABA. Seed germination was recorded 2 days after stratification on half-strength MS medium supplemented with 0.5-μM ABA. D, Cotyledon greening of 35S:ABI5-4MYC and other related transgenic plants in response to ABA. Cotyledon greening was scored 7 days after stratification on half-strength MS medium supplemented with 0.5-μM ABA. Experiments were performed five times by analyzing different batches of seeds. Each batch of seeds of various genotypes was pooled from more than 60 independent plants. For each biological replicate, more than 120 seeds were examined. Values are means ± SD. Bars with different letters are significantly different from each other (*P < 0.05*). Data were analyzed by ANOVA. E, Seedlings of 35S:ABI5-4MYC and other related transgenic plants 7 days after germination on half-strength MS medium containing 0.5-μM ABA.
Our phenotypic investigation showed that seeds of the prr5 prr7 prr9 triple mutant had much higher germination and greening percentages than seeds of prr5 prr7 and prr5 prr9 double mutants in response to ABA (Figure 4, A–C). This observation suggests that PRR9 may act together with PRR5 and PRR7 to positively modulate ABA responses during seed germination. However, unlike PRR5 and PRR7, PRR9 did not interact with ABI5 to form a protein complex (Figure 1, A and B), implying that PRR9 is not involved directly in ABI5-mediated ABA signaling through a PRR9–ABI5 interaction during seed germination. It is possible that PRR9 may function with PRR5 and PRR7 to modulate ABA responses via other modulators in ABA signaling. To better understand the molecular mechanism of the core circadian clock proteins PRR5/7/9-regulated ABA signaling in Arabidopsis, we constructed the simplified model involving PRR5/7/9 and ABI5 shown in Figure 11. When the concentration of ABA is elevated, ABA induces the expression of ABI5 as well as PRR5/7/9 during early stage of seed germination. PRR5 and PRR7 physically interact with ABI5 and stimulate its transcriptional function to enhance ABA signaling and maintain proper seed germination and postgerminative growth. In addition, PRR5, PRR7, and PRR9 may modulate ABA responses through other components of ABA signaling and negatively involve in ABA biosynthesis. Taken together with the fact that the transcript levels of ABI5 with the fact that the transcript levels of ABI5 may be specific adaptive mechanisms to integrate diverse signals and establish appropriate ABA signaling levels, thereby ensuring efficient stress tolerance while minimizing the detrimental effect of ABA on germination and early seedling growth.

**Materials and methods**

**Materials and plant growth conditions**

Taq DNA polymerases were obtained from Takara Biotechnology (Dalian, China), and other common chemicals were purchased from Shanghai Sangon (Shanghai, China). The phytohormone ABA was purchased from Sigma-Aldrich. The wild-type and mutant A. thaliana plants used in this study were in the Columbia (Col-0) genetic background. The prr5-1 (SALK_006280), prr5-2 (SALK_135000C), prr7-1 (SALK_091569C), and prr7-2 (SALK_030430C) mutants were obtained from the Arabidopsis Resource Center at Ohio State University (http://abrc.osu.edu). The prr5 prr7 double mutant was generated by genetically crossing prr5-1 with prr7-2 using standard techniques. Seeds of prr5-1 prr9-1 (prr5 prr9) and prr5-1 prr7-2 prr9-1 (prr5 prr7 prr9) were provided by Prof. Lei Wang (Institute of Botany, Chinese Academy of Sciences). The transgenic line 35S:ABIS-4MYC (Chen et al., 2012) was provided by Prof. Chuanyou Li (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). To generate 35S:2FLAG-PRR5 transgenic plants, the full-length cDNAs of PRR5 behind the 2FLAG tag sequences were cloned into the binary vector pOCA30 in the sense orientation behind the CaMV 35S promoter (Hu et al., 2013). The Arabidopsis plants were grown in an artificial growth chamber at 22°C under a 16-h-light (100-μE m⁻² s⁻¹, white fluorescent bulbs, full-spectrum light), 8-h-dark photoperiod.

**Figure 11** Simplified model for the interactions of ABI5 and PRR proteins in modulating ABA signaling during seed germination. When the concentration of ABA is elevated, ABA induces the expression of ABI5 as well as PRR5, PRR7, and PRR9 during seed germination. PRR5 and PRR7 physically interact with ABI5 and stimulate its transcriptional function to enhance ABA signaling and maintain proper seed germination and postgerminative growth. In addition, PRR5, PRR7, and PRR9 are negatively involved in ABA biosynthesis.
Yeast two-hybrid assays
The full-length CDS of **CCA1**, **LHY**, **PRR9**, **PRR7**, **PRR5**, **PRR3**, and **TOC1** were fused to pGCKT7 (Clontech) to generate bait vectors (BD-CCA1, BD-LHY, BD-PRR, and BD-TOC1) that contain the Gal4 DNA-BD. Full-length CDS of **ABI5**, **ABI4**, and **ABI3** were inserted into pGADT7 (Clontech) to produce prey vectors (AD-ABI) with the Gal4 AD. To identify specific regions critical for the interactions, multiple truncated PRR5 sequences were fused to pGBK7 and truncated ABI5 sequences were ligated with pGADT7. Yeast two-hybrid assays were performed as described previously (Hu et al., 2013). The bait and prey vectors were cotransformed into the yeast strain AH109 and physical interactions were indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade for 4 days after plating. The primers used for cloning are listed in Supplemental Data Set S1.

BiFC assays
The cDNA sequences encoding the C-terminal 64-amino acid enhanced YFP (cYFP) fragments and N-terminal 173-amino acid YFP (nYFP) were PCR amplified and individually inserted into tagging pFCCS941 plasmids to produce pFGC-cYFP and pFGC-nYFP, respectively (Kim et al., 2008). Full-length cDNA or the sequences encoding the 164 N-terminal residues of ABI5 were cloned into pFGC-cYFP to produce a C-terminal in-frame fusion with cYFP (ABI5-cYFP or ABI51–164-cYFP). Full-length PRR5, PRR7, and PRR9 were inserted into pFGC-nYFP to generate an N-terminal in-frame fusion with nYFP (PRR5-nYFP, PRR7-nYFP, and PRR9-nYFP). The resulting plasmids were transformed into Agrobacterium tumefaciens strain GV3101, and infiltration of wild tobacco (N. benthamiana) leaves was performed at zeitgeber time 12 as described previously (Hu et al., 2019). Infected leaves with YFP and DAPI fluorescence were detected 40–52 h after infiltration under a confocal laser-scanning microscope (Olympus, Tokyo, Japan). The experiments were performed at least four times using different batches of wild tobacco plants; for each biological replicate, more than 12 tobacco plants were infiltrated and more than 600 cells were analyzed. The primers used for cloning are listed in Supplemental Data Set S1.

ColP assays
To confirm the ABI5–PRR5 interaction, whole proteins were extracted from samples harvested at ZT12 of 0.5-μM ABA-treated (for 2.5 days) germinating seeds of transgenic Arabidopsis simultaneously overexpressing ABI5 and PRR5 (35S:ABI5-4MYC/35S:2FLAG-PRR5), which was constructed by introducing PRR5 overexpression (35S:2FLAG-PRR5) into previously described 35S:ABI5-4MYC plants (Chen et al., 2012; Hu et al., 2019). Total proteins were prepared from Arabidopsis plants with an extraction buffer containing 50-mM Tris–HCl (pH 7.4), 1-mM EDTA, 150-mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 1-mM PMSF, and 1x Roche Protease Inhibitor Cocktail. Immunoprecipitation experiments were performed with protein A/G Plus-agarose beads following the manufacturer’s protocol. In brief, cell lysates were precleared with the protein A/G Plus-agarose beads and incubated with the anti-MYC antibody (catalog no. A7470, Sigma-Aldrich; 1:250) and the protein A/G Plus-agarose beads at 4°C overnight in the extraction buffer. The beads were washed twice extensively with the extraction buffer and the co-immunoprecipitated protein was then detected by immunoblotting using an anti-FLAG antibody (catalog no. F7425, Sigma-Aldrich; 1:10,000).

Determination of germination and greening
The germination and greening of the wild-type and mutant seeds were determined as described previously (Hu et al., 2019). Briefly, seeds were first hydrated at ZT0, sown on medium with or without supplementation of ABA, and cold stratified at 4°C/dark for 4 days. Then, they were transferred at ZT0 to an artificial growth chamber at 22°C under 16-h light and 8-h-dark conditions for germination. Germination was determined based on the appearance of the embryonic axis (i.e. radicle protrusion) as observed under a microscope. Seedling greening was determined based on the appearance of green cotyledons on seedlings. To analyze the ABA sensitivity of germination and greening, seeds were plated on water agar (0.6%) medium or half-strength MS medium supplemented with ABA. More than three independent experiments were performed, and similar results were obtained.

RNA extraction and RT-qPCR
Total RNA was extracted from germinating seeds (2 days, harvested from ZT0 to ZT36) of the wild-type and/or *prr5 prr7 prr9* with or without 0.5-μM ABA treatment using the Trizol reagent (Invitrogen) and RT-qPCR was performed as described previously (Han et al., 2020). Briefly, 1.0-μg DNase-treated RNA was reverse-transcribed in a 20-μL reaction volume with oligo (dT)18 primer using Moloney murine leukemia virus reverse transcriptase (Fermentas). Then, 1.0-μL cDNA was used for RT-qPCR with the SYBR Premix Ex Taq kit (Takara) on a Roche LightCycler 480 real-time PCR machine, according to the manufacturer's instructions. At least three biological replicates for each sample were used for RT-qPCR analysis. The *At1g13320* gene, which encodes a subunit of Ser/Thr PP2A and is stably expressed in seed samples during germination (Czechowski et al., 2005), was used as the control. The gene-specific primers used for the RT-qPCR are listed in Supplemental Data Set S1.

Transient transactivation assays
Full-length **ABI5**, **PRR5**, **PRR7**, and **GFP** sequences were PCR amplified and cloned into the pGreenII 62-SK vector as effectors (Hellens et al., 2005). The putative promoter sequences of **EM1** (2,000 bp) and **EM6** (1,273 bp) were amplified and fused to the pGreenII 0800-LUC vector as reporters (Hellens et al., 2005). Combinations of plasmids were transformed into the wild-type or *prr5 prr7 prr9* mutant Arabidopsis leaf mesophyll protoplasts according to the Sheen laboratory protocol (Sheen, 2001). Transfected cells
were cultured for 10–16 h with or without 5-μM ABA treatment, and the relative LUC activity was analyzed using a Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA), which measured the activities of firefly LUC and the internal control Renillareniformis LUC (REN). The primers used for cloning are listed in Supplemental Data Set S1.

**Yeast one-hybrid assays**
The yeast one-hybrid assays were performed using the Matchmaker Yeast One-Hybrid System Kit (Clontech) according to the manufacturer's instructions. Full-length CDS of PRR5 and PRR7 were inserted into pGADT7 to produce AD-PRR constructs. The putative promoter fragments of EM1 and EM6 were cloned into the pAbAi vector to generate pAbAi-pEM1 and pAbAi-pEM6, which were linearized by BstB1 and then transformed into the Y1HGold yeast strain. The transformed cells were grown in the SD/-Ura plate for 3 days. AD-PRR5 and AD-PRR7 were then transformed into the strain harboring pAbAi-pEM1 or pAbAi-pEM6 and selected on the SD/-Leu plate. Cotransformed cells were cultured on an SD/-Leu plate containing aureobasidin A (AbA, 200 μg·L⁻¹) for 3 days, and positive clones were spotted in several yeast concentrations from dilution of 10⁻⁶ (OD₆₀₀ = 1.0) to 10⁻⁴. The primers used for cloning are listed in Supplemental Data Set S1.

**ChIP assays**
The ChIP assay was performed essentially as described previously (Mukhopadhyay et al., 2008; Jiang et al., 2014). Briefly, germinating seeds (with or without 0.5-μM ABA) were cross-linked in 1% formaldehyde and their chromatin isolated. The anti-FLAG antibody was used to immunoprecipitate the protein–DNA complex, and the precipitated DNA was purified using a PCR purification kit (Qiagen) for qPCR analysis. To quantitatively determine the PRR5–DNA (target promoter) binding, qPCR analysis was performed according to the procedure described previously (Mukhopadhyay et al., 2008) with the promoter sequence of PP2A (At1g13320) gene as an endogenous control. The relative quantity value was calculated by the 2⁻¹(ΔΔCt) method (Mukhopadhyay et al., 2008) and presented as the DNA binding ratio. The qPCR data from ChIPAssay with anti-FLAG antibody with the PP2A (At1g13320) promoter as a negative control. The results shown were obtained from three biological replicates using different batches of seeds. The primers used for ChIP assays are listed in Supplemental Data Set S1.

**Statistical analysis**
Statistical analysis was performed by analysis of variance. The results are shown in Supplemental Table S2.

**Accession numbers**
The genes discussed in this article can be found in the Arabidopsis Genome Initiative database as follows: ABI5, AT2G36270; ABI4, AT2G40220; ABI3, AT3G24650; PRR5, AT5G24470; PRR7, AT5G02810; PRR9, AT2G46790; PRR3, AT5G60100; TOC1, AT5G61380; LHY, AT1G01060; CCA1, AT2G46830; EM1, AT3G51810; EM6, AT2G40170; RAB18, AT1G43890; and RD29B, AT5G52300.

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

- **Supplemental Figure S1.** Yeast two-hybrid assay analysis of the interactions of ABI5 with PRR5, PRR3, TOC1, LHY, and CCA1 proteins.
- **Supplemental Figure S2.** ABA responses of prr5 and prr7 single mutants during seed germination.
- **Supplemental Figure S3.** ABA responses of prr5 prr7, prr5 prr9, and prr5 prr7 prr9 mutants during seed germination on water agar medium.
- **Supplemental Figure S4.** RT-qPCR analysis of PRR5 expression in overexpression lines.
- **Supplemental Figure S5.** ABA responses of PRRS-overexpressing plants during seed germination on water agar medium.
- **Supplemental Figure S6.** Yeast one-hybrid assay on binding of PRR5 and PRR7 to the promoter region of EM6.
- **Supplemental Figure S7.** Yeast one-hybrid assay on binding of PRR5 and PRR7 to the promoter region of EM1.
- **Supplemental Table S1.** Information for ABI5-binding promoter sequences of EM6 and EM1 (pEM6-1 and pEM1-1).
- **Supplemental Table S2.** Analysis of variance (ANOVA) tables.
- **Supplemental Data Set S1.** Primers used for cloning, RT-qPCR, and ChIP analysis.

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