The Plant Cell, Vol. 31: 1520–1538, July 2019, www.plantcell.org © 2019 ASPB.

The Transcription Factor INDUCER OF CBF EXPRESSION1 Interacts with ABSCISIC ACID INSENSITIVE5 and DELLA Proteins to Fine-Tune Abscisic Acid Signaling during Seed Germination in Arabidopsis

Yanru Hu,[a,1,2] Xiao Han,[a,b,1] Milian Yang,[a,b,1] Minghui Zhang,[a,b] Jinjing Pan,[a,b] and Diqiu Yu[a]

[a] CAS Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan 650223, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China

ORCID IDs: 0000-0002-1570-8752 (Y.H.); 0000-0003-0173-9889 (X.H.); 0000-0003-3511-3630 (M.Y.); 0000-0002-3721-0226 (M.Z.); 0000-0002-2023-8505 (J.P.); 0000-0001-7507-7617 (D.Y.)

ABSCISIC ACID INSENSITIVE5 (ABI5) is a crucial regulator of abscisic acid (ABA) signaling pathways involved in repressing seed germination and postgerminative growth in Arabidopsis (*Arabidopsis thaliana*). ABI5 is precisely modulated at the posttranslational level; however, the transcriptional regulatory mechanisms underlying ABI5 and its interacting transcription factors remain largely unknown. Here, we found that INDUCER OF CBF EXPRESSION1 (ICE1) physically associates with ABI5. ICE1 negatively regulates ABA responses during seed germination and directly suppresses ABA-responsive *LATE EMBRYOGENESIS ABUNDANT6* (*EM6*) and *EM1* expression. Genetic analysis demonstrated that the ABA-hypersensitive phenotype of the *ice1* mutant requires ABI5. ICE1 interferes with the transcriptional activity of ABI5 to mediate downstream regulons. Importantly, ICE1 also interacts with DELLA proteins, which stimulate ABI5 during ABA signaling. Disruption of ICE1 partially restored the ABA-hyposensitive phenotype of the *della* mutant, *gai-t6 rga-t2 rgl1-1 rgl2-1*, indicating that ICE1 functions antagonistically with DELLA in ABA signaling. Consistently, DELLA proteins repress ICE1's transcriptional function and the antagonistic effect of ICE1 on ABI5. Collectively, our study demonstrates that ICE1 antagonizes ABI5 and DELLA activity to maintain the appropriate level of ABA signaling during seed germination, providing a mechanistic understanding of how ABA signaling is fine-tuned by a transcriptional complex involving ABI5 and its interacting partners.

INTRODUCTION

Seed germination and postgerminative growth are strictly regulated by environmental and intrinsic cues. Among these, the phytohormone abscisic acid (ABA) functions as a crucial signal to promote seed maturation and dormancy but to inhibit seed germination and subsequent seedling establishment (Finkelstein et al., 2002, 2008; Gubler et al., 2005). ABA is perceived by the receptors PYRABACTIN RESISTANCE/PYRABACTIN RESISTANCE1-LIKE/REGULATORY COMPONENT OF ABSCISIC ACID RECEPTOR (Ma et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Park et al., 2009; Santiago et al., 2009). When binding to ABA, these receptors physically interact with type 2C PROTEIN PHOSPHATASE to form a stable complex, leading to the release of SUCROSE NONFERMENTIN1-RELATED KINASE2 (SnRK2s) from type 2C PROTEIN PHOSPHATASE-SnRK2 complexes (Cutler et al., 2010). The activated SnRK2s subsequently phosphorylate downstream components, such as ABSCISIC ACID-INSENSITIVE5 (ABI5) and its homologous ABSCISIC ACID-RESPONSIVE ELEMENT BINDING FACTORs, to modulate ABA responses (Kobayashi et al., 2005; Furihata et al., 2006; Fujii et al., 2007; Fujii and Zhu, 2009; Nakashima et al., 2009).

The basic leucine zipper (bZIP)–type transcription factor ABI5, which is primarily expressed in mature seeds and is strongly responsive to ABA treatment, positively regulates ABA signaling to suppress seed germination and early seedling establishment (Finkelstein, 1994; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Lopez-Molina et al., 2001, 2002; Brocard et al., 2002; Finkelstein et al., 2005). Mechanistic investigations revealed that several regulators precisely modulate the stability and/or activity of ABI5 through posttranslational protein modifications (Yu et al., 2015). For instance, the SnRK2 kinases phosphorylate and stabilize ABI5 to inhibit seed germination in the presence of ABA (Kobayashi et al., 2005; Furihata et al., 2006; Fujii et al., 2007; Fujii and Zhu, 2009; Nakashima et al., 2009). Similarly, BRASSINOSTEROID INSENSITIVE2 and SALT OVERLY SENSITIVE2-LIKE PROTEIN KINASE5 kinases also phosphorylate and activate ABI5 to enhance downstream ABA signaling (Hu and Yu, 2014; Zhou et al., 2015). By contrast, the small ubiquitin-related modifier E3 ligase SAP AND MIZ1 negately regulates ABA signaling via sumoylation of ABI5 (Miura et al., 2009). Moreover, several negative regulators of ABI5 facilitate its degradation and/or decrease ABA signaling to promote seed germination, such as KEEP ON GOING, ABI Five Binding Protein, PROTEIN PHOSPHATASE6, SENSITIVE TO ABA1, DWD HYPERSENSITIVE TO ABA1, and

[1] These authors contributed equally to this work.
[2] Address correspondence to: huyanru@xtbg.ac.cn.
The authors responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) are: Yanru Hu (huyanru@xtbg.ac.cn) and Diqiu Yu (ydq@xtbg.ac.cn).
www.plantcell.org/cgi/doi/10.1105/tpc.18.00825

DWD HYPERSENSITIVE TO ABA2 (Stone et al., 2006; Garcia et al., 2008; Lee et al., 2010; Liu and Stone, 2010; Dai et al., 2013; Lynch et al., 2017; Ji et al., 2019).

Several studies have suggested that ABI5 might also serve as a critical modulator to integrate multiple signaling pathways during seed germination and/or postgerminative growth. For example, we recently found that the BRASSINOSTEROID INSENSITIVE2 kinase physically interacts with ABI5 to mediate the antagonistic effect of brassinosteroids on ABA during seed germination (Hu and Yu, 2014). Similarly, cytokinin antagonizes ABA-mediated inhibition of cotyledon greening (Guan et al., 2014). Further analysis revealed that cytokinin signaling promotes the degradation of ABI5 via the 26S proteasome pathway. Chen et al. (2008) showed that ABI5 also integrates ABA and light signaling to modulate seed germination and postgerminative growth. ABI5 might also function as the final downstream repressor of seed germination in the counterbalance of ABA and gibberellin (GA) signaling (Piskurewicz et al., 2008). ABI5 also associates with several crucial transcription regulators, such as ABI3, ABI4, ABSCISIC ACID-RESPONSIVE ELEMENT BINDING FACTORs, MYB49, BRASSINOSTEROID INSENSITIVE1-EMSSUPPRESSOR1, and JASMONATE ZIM-DOMAIN, to regulate ABA responses (Söderman et al., 2000; Nakamura et al., 2001; Lopez-Molina et al., 2002; Yoshida et al., 2015; Ju et al., 2019; Zhang et al., 2019; Zhao et al., 2019). For example, ABI5 directly interacts with and acts downstream of ABI3 to modulate ABA signaling during seed germination (Nakamura et al., 2001; Lopez-Molina et al., 2002). However, despite the abundant evidence for the involvement of ABI5 in ABA and other signaling pathways during seed germination, details of the transcriptional regulatory mechanisms underlying ABI5 and its interacting factors remain largely unknown. Dissecting critical transcription factors that physically associate with ABI5 may shed light on the molecular basis of the tight regulation of ABA signaling during seed germination.

DELLA proteins are crucial repressors of GA signaling that share a conserved DELLA motif (Peng et al., 1997; Silverstone et al., 1998; Dill et al., 2001; Lee et al., 2002; Tyler et al., 2004; Davière and Achard, 2016). Arabidopsis (*Arabidopsis thaliana*) contains five DELLA proteins: GA-INSENSITIVE (GAI); REPRESSOR OF GA1-3 (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3 (Peng et al., 1997; Silverstone et al., 1998; Tyler et al., 2004; Davière and Achard, 2016). In the presence of GA, the GA receptors GA INSENSITIVE DWARF1 ([GID1]a, GID1b, and GID1c) and the F-box ubiquitin ligase SLEEPY1 (as an $SCF^{SLEEPY1}$ complex) recruit DELLA proteins for ubiquitination and subsequent degradation (Dill et al., 2004; Ueguchi-Tanaka et al., 2005; Nakajima et al., 2006; Harberd et al., 2009; Claeys et al., 2014). RGL2 is a critical suppressor of seed germination (Lee et al., 2002; Tyler et al., 2004; Piskurewicz et al., 2008), and GAI and RGA also repress seed germination (Cao et al., 2005; Piskurewicz et al., 2009; Chahtane et al., 2018). All five *DELLA* genes are expressed during seed germination (Tyler et al., 2004), and *GAI*, *RGA*, and *RGL2* are responsive to ABA during seed germination (Piskurewicz et al., 2008). Moreover, DELLA proteins function together with ABI5 to activate the expression of several high-temperature-responsive genes, thereby inhibiting seed germination (Lim et al., 2013).

Here, we aimed to discover further details about the molecular regulation of ABA signaling during seed germination. We used the yeast two-hybrid (Y2H) system to screen an Arabidopsis cDNA library for ABI5-interacting transcription factors and determined that the basic helix-loop-helix (bHLH) transcription factor INDUCER OF CBF EXPRESSION1 (ICE1) physically interacts with ABI5 in yeast (*Saccharomyces cerevisiae*) and in planta. ICE1 is a negative modulator of ABA signaling during seed germination and directly suppresses the expression of ABA-responsive *LATE EMBRYOGENESIS ABUNDANT6* (*EM6*) and *EM1.* Furthermore, ICE1 antagonizes the transcriptional function of ABI5 to regulate these downstream target genes. ICE1 physically associates with DELLA proteins. Disruption of ICE1 partially rescued the ABA-hyposensitive phenotype of the *gai-t6 rga-t2 rgl1-1 rgl2-1* mutant (*della*) during seed germination. Mechanistic and phenotypic investigations revealed that DELLA proteins repress the regulatory effect of ICE1. Collectively, these results indicate that ICE1 functions together with ABI5 and DELLA proteins to maintain the appropriate level of ABA signaling during seed germination, and they provide a mechanistic understanding of how ABA signaling is fine-tuned by a transcriptional complex involving ABI5 and its interacting factors.

RESULTS

ABI5 Physically Interacts with ICE1

The ABI5 transcription factor is widely known as a crucial positive regulator of ABA signaling during seed germination and early seedling growth. To obtain further details about ABI5-associated transcriptional regulation, we used the Y2H system to identify transcription factors that interact with ABI5 and potentially modulate ABA responses. The full-length ABI5 sequence with a deletion of the nine N-terminal amino acids from positions 1 to 9 was fused to the Gal4 DNA binding domain of the bait vector (BD-ABI5; Nakamura et al., 2001). Sequence analysis of putative positive colonies suggested that ICE1 interacts with ABI5 in yeast. ICE1, which contains 494 amino acid residues, is a member of the bHLH transcription factor family (Chinnusamy et al., 2003). To confirm the ABI5–ICE1 interaction, we introduced the full-length ICE1 into the Gal4 activation domain of the prey vector (AD-ICE1). The BD-ABI5 and AD-ICE1 plasmids were cotransformed into yeast to examine protein–protein interactions (Figure 1A).

To more precisely identify the ICE1 region responsible for the interaction with ABI5, we fused five truncated ICE1 variants to the Gal4 activation domain of the prey vector (Figure 1A; Hu et al., 2013) and analyzed the interactions between ABI5 and these derivatives using the Y2H system. Deletion of the 260 N-terminal residues of ICE1 ($AD\text{-}ICE1^{261–494}$) did not affect the interaction between ABI5 and ICE1; however, deletion of the 234 C-terminal residues of ICE1, including the bHLH domain ($AD\text{-}ICE1^{1–260}$), completely eliminated its interaction with ABI5 (Figure 1A). Further mapping showed that the 234 C-terminal residues of ICE1 are essential for its interaction with ABI5, as two derivatives of ICE1 with C-terminal deletions of amino acids 261 to 420 or 421 to 494 did not interact with ABI5 (Figure 1A). Similarly, to investigate which region of ABI5 is required for its interaction with ICE1, we performed directed Y2H analysis, finding that the C-terminal region 165 to 442 of ABI5 (including the bZIP domain) is responsible for the ABI5–ICE1 interaction (Figure 1B).

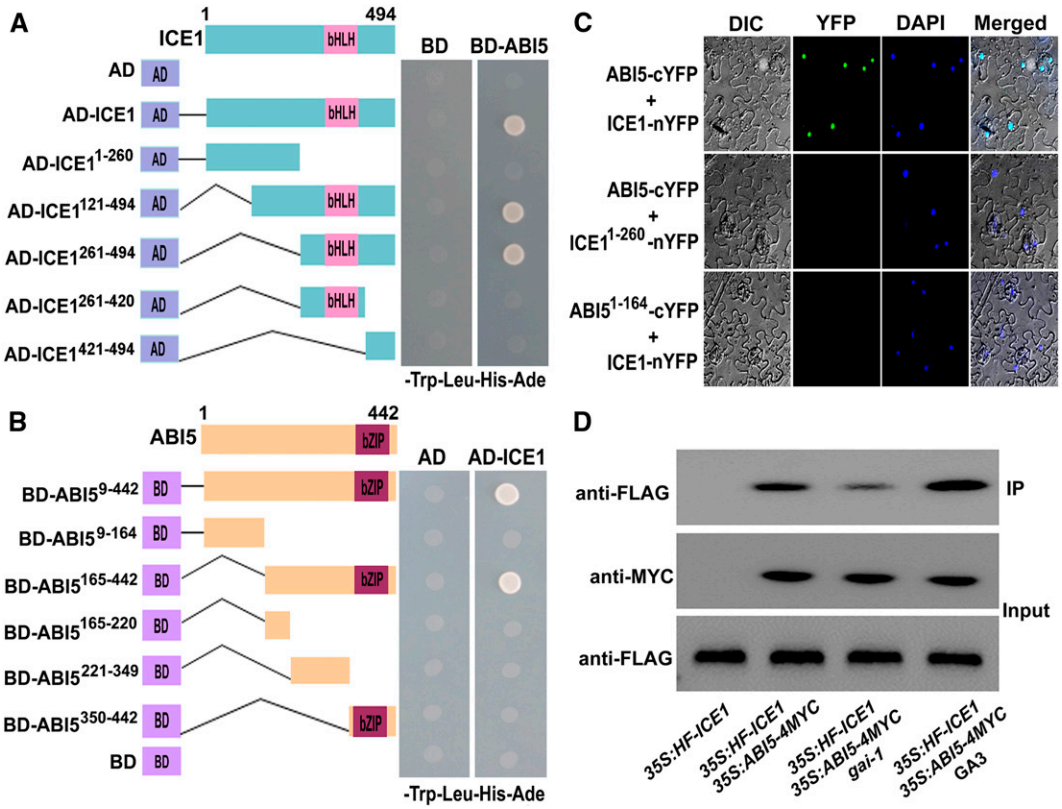

Figure 1. Physical Interaction Between ABI5 and ICE1.

(A) Mapping the ABI5-interacting domain of ICE1 using a Y2H assay. Interaction is indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade and containing 20 mM 3-aminotriazole . pGBKT7 (BD) and pGADT7 (AD) vectors were used as negative controls.

(B) Mapping the ICE1-interacting domain of ABI5 using a Y2H assay. Interaction is indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade and containing 20 mM 3-AT. BD and AD vectors were used as negative controls.

(C) BiFC analyses. Fluorescence was observed in the nuclear compartment of transformed cells, which resulted from complementation of the C-terminal region of YFP fused with ABI5 (ABI5-cYFP) with the N-terminal region of YFP fused with ICE1 (ICE1-nYFP). The experiments were performed at least four times using different batches of *N. benthamiana* plants; for each biological replicate, more than 12 plants were infiltrated and more than 600 cells were examined. DIC, differential interference contrast.

(D) CoIP analyses. Whole proteins were extracted from 0.5 μM ABA-treated (for 1 d) germinating seeds of various transgenic Arabidopsis lines with or without 1 μM GA3 treatment as indicated. The ABI5-4MYC protein was immunoprecipitated using anti-MYC M2 agarose beads, and the coIPed HF-fused ICE1 was then detected using an anti-FLAG antibody. Protein inputs for ABI5-4MYC and HF-ICE1 were also detected and shown. The experiments were repeated three times with similar results using three batches of seeds as biological replicates. IP, immunoprecipitation.

The physical interaction between ABI5 and ICE1 was further corroborated by bimolecular fluorescence complementation (BiFC) and coimmunoprecipitation (CoIP) assays in planta. For the BiFC assays, ABI5 was fused to the C-terminal yellow fluorescent protein (YFP) fragment (ABI5-cYFP) driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter, and ICE1 was ligated with the N-terminal YFP fragment to generate ICE1-nYFP. When fused ABI5-cYFP was co-infiltrated with ICE1-nYFP into wild tobacco (*Nicotiana benthamiana*) leaves, strong YFP signals were observed in the nuclear compartments of transformed cells, as revealed by staining with 4′,6-diamidino-2-phenylindole (DAPI; Figure 1C). However, no fluorescence was detected in negative controls in which ABI5-cYFP was coexpressed with ICE1$^{1–260}$-nYFP (the N-terminal domain of ICE1 fused with nYFP) or ABI5$^{1–164}$-cYFP (the N terminus of ABI5 ligated with cYFP) was coexpressed with ICE1-nYFP (Figure 1C; Supplemental Figure 1). The ABI5–ICE1 interaction was further confirmed by CoIP assays in transgenic Arabidopsis simultaneously overexpressing *ABI5* and *ICE1* (*35S:ABI5-4MYC/35S:HF-ICE1*; Figure 1D), which was constructed by crossing *35S:HF-ICE1* (containing a HA-FLAG-ICE1 construct driven by the CaMV 35S promoter; Ding et al., 2015) with previously described *35S:ABI5-4MYC* plants (containing a functional ABI5-4MYC construct; Chen et al., 2012; Hu and Yu, 2014). Taken together, these results demonstrate that ABI5 physically associates with ICE1 in plant cell nuclei, suggesting that ICE1 functions as an interacting partner of ABI5 to modulate ABA signaling.

ICE1 Negatively Modulates ABA Responses during Seed Germination and Directly Suppresses the Expression of ABA-Responsive Genes *EM6* and *EM1*

The transcription factor ICE1 plays a central role in cold-response pathways in Arabidopsis (Chinnusamy et al., 2007; Zhu, 2016; Hu

et al., 2017; Shi et al., 2018). In addition to cold-stress responses, ICE1 is also involved in several developmental processes, such as stomatal development and anther dehydration (Kanaoka et al., 2008; Denay et al., 2014; Lee et al., 2017; Wei et al., 2018). Interestingly, ICE1 was identified as a critical negative regulator of the ABA signaling pathway and seed dormancy (Liang and Yang, 2015; MacGregor et al., 2019). Phenotypic analysis showed that a T-DNA insertion loss-of-function mutant of *ICE1*, *ice1* (SALK_003155), was more sensitive to ABA than the Columbia (Col) wild type during seed germination and postgerminative growth (Liang and Yang, 2015). To confirm the role of ICE1 in ABA signaling, the authors introduced the genomic sequence of *ICE1* driven by its native promoter into the *ice1* mutant and found that these complementation plants behaved like the Col wild type in response to ABA during seed germination (Liang and Yang, 2015). Consistent with this finding, we also found that *ice1* displayed much lower germination and greening percentages than Col wild type in the presence of ABA (Supplemental Figure 2). As expected, expressing full-length ICE1 fused with green fluorescent protein (GFP) driven by its native promoter in the *ice1* mutant background complemented the mutation and produced plants (*ProICE1:ICE1-GFP/ice1*) that behaved similarly to the wild type under ABA treatment (Supplemental Figure 2). Moreover, the overexpression of *ICE1* reduced the ABA sensitivity of germinating seeds of the transgenic plants *35S:HF-ICE1* (Ding et al., 2015) and *35S:GFP-ICE1* (containing a GFP-ICE1 construct driven by the CaMV 35S promoter; Supplemental Figure 2; Chinnusamy et al., 2003). In addition, expression analysis indicated that *ICE1* is expressed in dry seeds and is responsive to ABA treatment during seed germination (Supplemental Figure 3), further supporting the notion that ICE1 is involved in ABA signaling.

To further explore the regulatory role of ICE1 in ABA signaling, we examined the expression of several well-characterized ABA-responsive genes in dry seeds and/or ABA-treated germinating seeds of *ice1* and *ICE1*-overexpressing plants (*35S:HF-ICE1* and *35S:GFP-ICE1*), including *EM6*, *EM1*, *RESPONSIVE TO ABA18* (*RAB18*), and *ALCOHOL DEHYDROGENASE1* (*ADH1*). As shown in Figure 2A, the expression levels of *EM6* and *EM1* in dry seeds were higher in *ice1* compared with the wild type (Col), whereas they were lower in *ICE1*-overexpressing plants. Moreover, *EM6*, *EM1*, *RAB18*, and *ADH1* transcript levels were higher in ABA-treated germinating *ice1* seeds than in the wild-type (Col) germinating seeds (Figure 2B). By contrast, the expression of these genes in response to ABA was reduced in germinating seeds of *ICE1*-overexpressing plants compared with the wild type (Col; Figure 2B). These results demonstrate that ICE1 negatively modulates the expression of several downstream ABA-responsive genes.

EM6 and *EM1* are direct targets of the transcription factor ABI5 (Finkelstein and Lynch, 2000; Carles et al., 2002; Lopez-Molina et al., 2002; Nakabayashi et al., 2005; Reeves et al., 2011). As ICE1 physically interacts with ABI5 and modulates the expression of *EM6* and *EM1*, we investigated whether ICE1 also directly binds to the promoter sequence of these two genes. ICE1 is a bHLH transcription factor that recognizes the MYC-recognition sequences (CANNTG, also known as E-box) in gene promoters (Chinnusamy et al., 2003; Kim et al., 2015). Sequence analysis identified several potential MYC-recognition sequences in the promoters (1.5-kb regions upstream of the translational start sites) of *EM6* and *EM1* (Figure 2C). We then conducted chromatin immunoprecipitation (ChIP) assays using *35S:HF-ICE1* plants to analyze whether these genes were directly regulated by ICE1. The ChIP assays showed that ICE1 interacted with the promoter sequences (*pEM6-1*, *pEM6-2*, *pEM1-1*, and *pEM1-2*, respectively) of *EM6* and *EM1* (Figures 2D and 2E; Supplemental Tables 1 and 2). Furthermore, binding of ICE1 to the promoters of *EM6* and *EM1* was more prominent in ABA-treated germinating seeds than in dry seeds (Figures 2D and 2E), suggesting that the enrichment of ICE1 on target promoters is responsive to ABA treatment.

Genetic Interaction between *ABI5* and *ICE1*

Having ascertained that ICE1 physically associates with ABI5 and negatively modulates ABA responses, we then asked whether it genetically interacts with ABI5 to mediate ABA-induced repression of seed germination. To test this possibility, we generated the *abi5 ice1* double mutant by crossing *ice1* with *abi5* (*abi5-1*), a loss-of-function mutant of *ABI5* (Finkelstein, 1994; Finkelstein and Lynch, 2000). As the *abi5* mutant is in the Wassilewskija background, we backcrossed it with the Col wild type six times before crossing it with *ice1*. We examined the germination and greening percentages of *abi5*, *ice1*, and *abi5 ice1* in response to ABA. Not surprisingly, *abi5* in the Col background was also highly insensitive to ABA during seed germination and postgerminative growth (Figures 3A to 3C). Similar to *abi5* seeds, seeds of the *abi5 ice1* double mutant were also hyposensitive to ABA during seed germination and early seedling growth compared with the wild type (Figures 3A to 3C). To confirm these observations, we investigated the expression of several ABA-responsive genes in ABA-treated germinating seeds of the *abi5 ice1* double mutant, including *EM6*, *EM1*, *RAB18*, and *ADH1*. As shown in Figure 3D and Supplemental Figure 4, the expression levels of these ABA-responsive genes were reduced in *abi5 ice1* compared with those in *ice1* and the wild type (Col). These results indicate that the enhanced ABA signaling in the *ice1* mutant requires a functional ABI5 transcription factor.

ICE1 Antagonizes the Transcriptional Function of ABI5

As ICE1 interacts with ABI5 and they exert opposite regulatory effects on the expression of *EM6* and *EM1* (Figures 1to 3), we investigated whether ICE1 interferes with the transcriptional function of ABI5. To test this possibility, we used a dual-luciferase (LUC) reporter assay to examine the regulatory effect of ICE1 on the transcriptional function of ABI5 in Arabidopsis mesophyll protoplasts (Yoo et al., 2007). As *EM6* and *EM1* are direct targets of ABI5, their promoters were fused to the *LUC* gene as reporters (Figure 4A). The effector constructs contained a *GFP*, *ABI5*, or *ICE1* gene driven by the CaMV 35S promoter (Figure 4A). As expected, the expression of ABI5 dramatically stimulated the expression of *LUC* driven by the *EM6* or *EM1* promoter in response to 5 μM ABA compared with the expression of GFP alone (Figure 4B; Zhou et al., 2015; Pan et al., 2018). By contrast, the expression of ICE1 significantly repressed the expression of *LUC* driven by the *EM6* or *EM1*

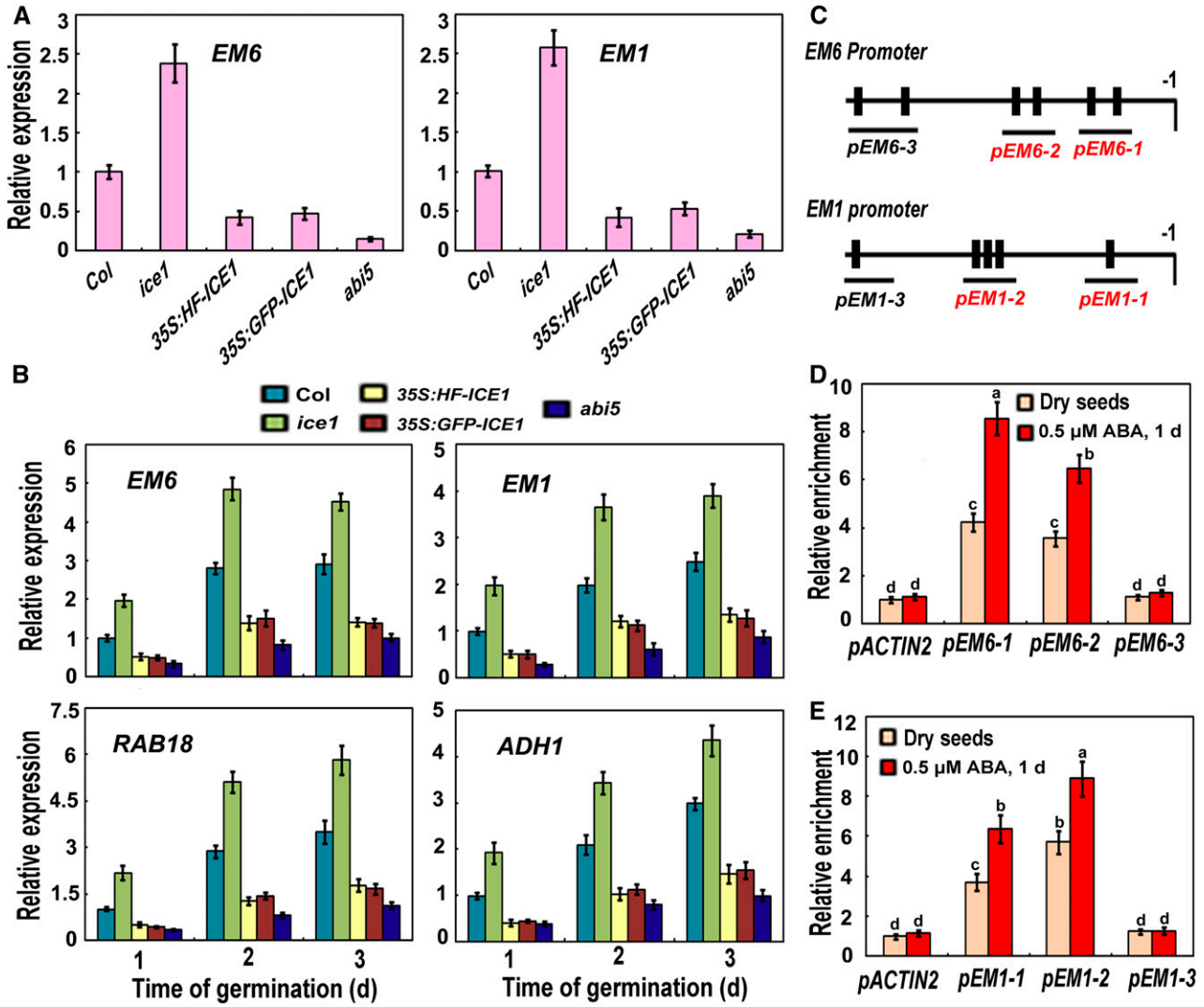

Figure 2. Expression of Several ABA-Responsive Genes in *ice1* and *ICE1*-Overexpressing Plants.

(A) Expression of *EM6* and *EM1* in dry seeds of the wild type (Col), *ice1*, *35S:HF-ICE1*, *35S:GFP-ICE1*, and *abi5*. Total RNA was extracted from three different batches of seeds as biological replicates. Each batch of seeds was pooled from more than 50 plants. Error bars show SD from three independent biological replicates. The *AT1G13320* gene was used as control.

(B) Expression of *EM6*, *EM1*, *RAB18*, and *ADH1* in ABA-treated germinating seeds of the wild type (Col), *ice1*, *35S:HF-ICE1*, *35S:GFP-ICE1*, and *abi5*. Total RNA was extracted from three different batches of seeds as biological replicates. Each batch of seeds was pooled from more than 50 plants. For each biological replicate, more than 150 seeds of the same batch were germinated on three different cultures with or without 0.5 μM ABA for the indicated times. Error bars show SD from three independent biological replicates. The *AT1G13320* gene was used as control.

(C) The promoter structure of *EM6* and *EM1* and fragments used in the ChIP assay. Black boxes on the line indicate the potential ICE1 binding sites. Lines indicate sequences detected by ChIP assays, as described in Supplemental Tables 1 and 2. In the promoter fragment names, the prefix p indicates promoter.

(D) and **(E)** ChIP-qPCR analysis of the DNA binding ratio of ICE1 to promoters of *EM6* **(D)** and *EM1* **(E)**. Three different batches of dry seeds or 0.5 μM ABA-treated (for 1 d) germinating seeds of *35S:HF-ICE1* pooled from more than 50 plants were used in ChIP using anti-HA antibody. qRT-PCR data from ChIP assay with anti-HA antibody with the *ACTIN2* promoter (*pACTIN2*) 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 ANOVA ($P < 0.05$).

promoter in response to 5 μM ABA treatment compared with the expression of GFP alone (Figure 4B). More importantly, coexpression of ICE1 with ABI5 also repressed *LUC* expression compared with the coexpression of ABI5 and GFP (Figure 4B). These findings suggest that ICE1 affects the transcriptional function of ABI5.

To further explore the antagonistic effects of ICE1 on ABI5, we investigated whether ICE1 interferes with the DNA binding activity of ABI5 in vivo. Carles et al. (2002) showed that ABI5 recognizes the *EM6* promoter region covering a G-box type *cis*-element (CACGTG), which is the same region targeted by ICE1 (indicated as *pEM6-1* in Figures 2G and 2H). As shown in

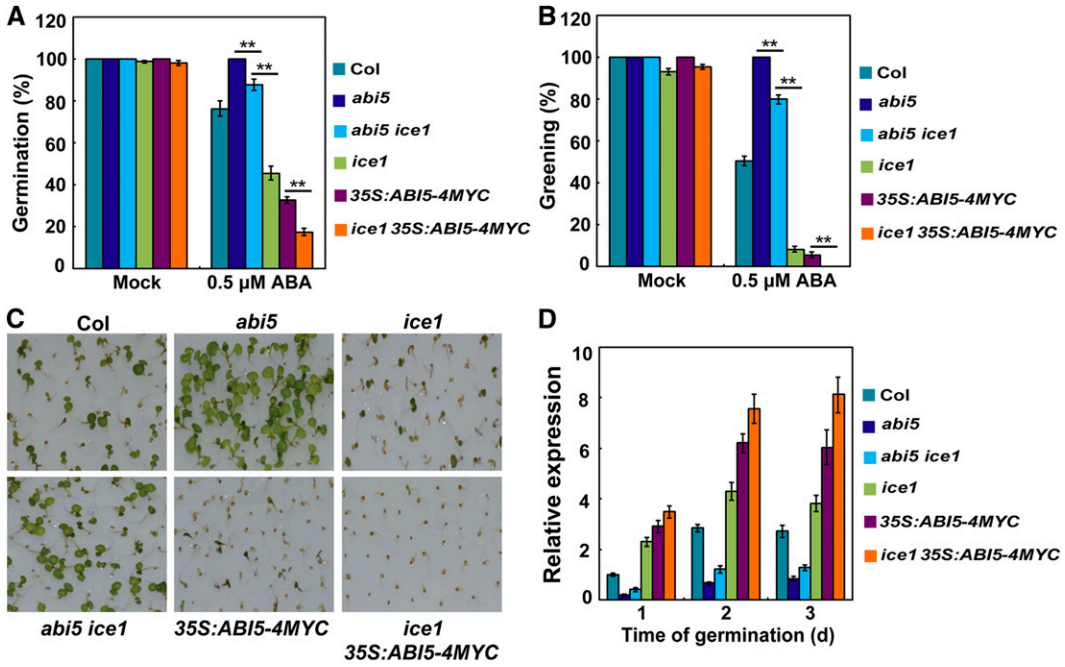

Figure 3. ABA Hypersensitivity of *ice1* Requires Functional ABI5.

(A) Germination of the *abi5 ice1* double mutant and other related mutants or transgenic plants. Seed germination was recorded 3.5 d after stratification on medium supplemented with 0.5 μM ABA.

(B) Cotyledon greening of the *abi5 ice1* double mutant and other related mutants or transgenic plants. Cotyledon greening was scored 5 d after stratification on medium containing 0.5 μM ABA. Experiments were performed at least three times by analyzing different batches of seeds. Each batch of seeds of the *abi5 ice1* double mutant and other related mutants or transgenic plants was pooled from more than 50 plants. For each biological replicate, we examined the seeds (more than 150) from the same batch three times as technical replicates. Values are means ± SD. ** indicates highly significant differences based on Student's *t* test ($P < 0.01$).

(C) Seedlings of the *abi5 ice1* double mutant and other related mutants or transgenic plants 6 d after germination on medium containing 0.5 μM ABA.

(D) Expression levels of *EM6* in the *abi5 ice1* double mutant and other related mutants or transgenic plants. Seeds of the *abi5 ice1* double mutant and other related mutants or transgenic plants were germinated on medium containing 0.5 μM ABA for the indicated times. Total RNA was extracted from three different batches of seeds as biological replicates. Each batch of seeds was pooled from more than 50 plants. For each biological replicate, more than 150 seeds of the same batch were geminated on three different cultures with or without 0.5 μM ABA for the indicated times. Error bars show SD from three independent biological replicates. The *AT1G13320* gene was used as control.

Figure 4C, ChIP assays revealed that the enrichment of ABI5 on the *EM6* promoter (*pEM6-1*) was reduced in transgenic plants simultaneously overexpressing *ABI5* and *ICE1* (*35S:ABI5-4MYC*/*35S:HF-ICE1*) compared with *35S:ABI5-4MYC* plants. The enrichment of ICE1 on the *EM6* promoter (*pEM6-1*) was also reduced in *35S:ABI5-4MYC*/*35S:HF-ICE1* plants compared with *35S:HF-ICE1* (Figure 4D). These results suggest that ABI5 and ICE1 reciprocally affect each other's DNA binding activity and/or competitively bind to the promoters of target genes in vivo. To further verify this observation, we generated recombinant His-ABI5 and His-ICE1 proteins in *Escherichia coli* and tested their binding activity to an oligonucleotide harboring two direct CACGTG G-box repeats (Pr in Supplemental Figure 5) using electrophoretic mobility shift assay (EMSA). As shown in Supplemental Figure 5, protein–DNA complexes with reduced migration were detected when His-ABI5 or His-ICE1 was incubated with the DNA probe. However, when ABI5 was combined with ICE1 in the binding reactions, we observed the formation of a super-shifted band with considerably lower intensity. This observation indicates that the ICE1-ABI5 heterodimer can still bind to the *cis*-element but at a dramatically reduced level, which further supports the notion that ICE1 and ABI5 reciprocally affect each other's DNA binding activity.

The antagonistic effect of ICE1 on ABI5 prompted us to ask whether the disruption of ICE1 would lead to the release of ABI5, thereby positively modulating ABA signaling during seed germination. To address this question, we crossed the *ice1* mutant with *35S:ABI5-4MYC* to generate *ice1 35S:ABI5-4MYC* plants. We analyzed the germination and greening percentages of these plants in response to ABA. Notably, the ABA-hypersensitive phenotype of *ice1 35S:ABI5-4MYC* was enhanced compared with *35S:ABI5-4MYC* (Figures 3A to 3C). Consistent with this phenotype, the expression levels of several ABA-responsive genes, such as *EM6*, *EM1*, *RAB18*, and *ADH1*, were higher in *ice1 35S:ABI5-4MYC* compared with *35S:ABI5-4MYC* (Figure 3D; Supplemental Figure 4). These results support the notion that ICE1 antagonizes the transcriptional function of ABI5 to regulate ABA signaling during seed germination.

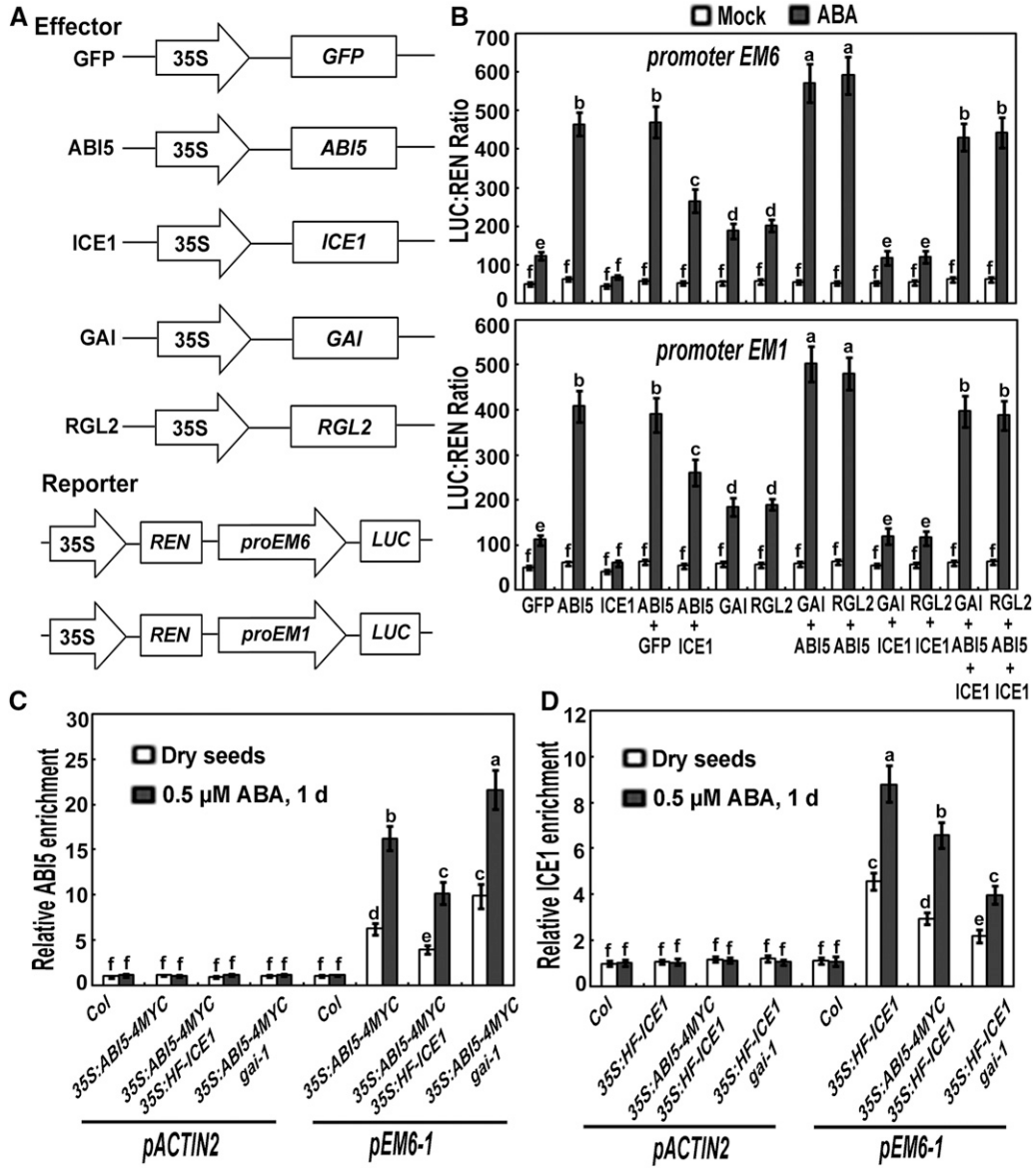

Figure 4. ICE1 Interferes With The Transcriptional Function of ABI5.

(A) Schematic of the effectors and reporters used in the transient transactivation assays.

(B) Transient dual-LUC reporter assays showing that ICE1 antagonizes ABI5 and DELLA proteins (GAI and RGL2) to modulate the expression of *EM6* or *EM1* in response to 5 μM ABA. Error bars show SD from three biological replicates using different batches of plants; each replication was from the different wild-type leaves of more than 30 plants. Bars with different letters are significantly different from each other ($P < 0.05$). Data were analyzed by ANOVA.

(C) ChIP-qPCR analysis of the DNA binding ratio of ABI5 to the promoter of *EM6* (*pEM6-1*) is repressed by ICE1 but enhanced by GAI. Three different batches of dry seeds or 0.5 μM ABA-treated (for 1 d) germinating seeds of *35S:ABI5-4MYC* and related transgenic plants pooled from more than 50 plants were used in ChIP using anti-MYC antibody. qRT-PCR data from ChIP assay with anti-MYC antibody with the *ACTIN2* promoter (*pACTIN2*) 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 ANOVA ($P < 0.05$).

(D) ChIP-qPCR analysis of the DNA binding ratio of ICE1 to the promoter of *EM6* (*pEM6-1*) is affected by ABI5 and GAI. Three different batches of dry seeds or 0.5 μM ABA-treated (for 1 d) germinating seeds of *35S:HF-ICE1* and related transgenic plants pooled from more than 50 plants were used in ChIP using anti-HA antibody. qRT-PCR data from ChIP assay with anti-HA antibody with the *ACTIN2* promoter (*pACTIN2*) 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 ANOVA ($P < 0.05$).

ICE1 Physically Associates with DELLA Proteins

To further investigate the regulatory role of ICE1 in ABA signaling, we used the Y2H system to identify its putative interacting components. The 234 C-terminal residues of ICE1 were fused to the Gal4 DNA binding domain of the bait vector to generate BD-ICE1 (Agarwal et al., 2006). After screening, two independent positive clones encoding GAI were identified based on prototrophy for His and adenine (Ade). GAI is a member of the DELLA family of proteins, which are crucial repressors of GA signaling and share the conserved DELLA motif (Peng et al., 1997; Silverstone et al., 1998; Dill et al., 2001; Lee et al., 2002; Tyler et al., 2004; Davière and Achard, 2016). To confirm the ICE1–GAI interaction and to further analyze whether ICE1 specifically interacts with GAI, we investigated the interactions of ICE1 with all five Arabidopsis DELLA proteins in the Y2H system, including GAI, RGA, RGL1, RGL2, and RGL3. As shown in Figure 5A, ICE1 strongly interacted with all five DELLA proteins in yeast. We also analyzed which region of ICE1 is essential for its interactions with DELLA proteins and found that the 234 C-terminal residues of ICE1 are required for their interactions (Figure 5B).

The ICE1–DELLA interactions were further confirmed by BiFC and CoIP assays in planta. GAI and RGL2 were selected as representatives in the BiFC and CoIP assays. For the BiFC assays, GAI and RGL2 were ligated with the C-terminal YFP fragment to generate GAI-cYFP and RGL2-cYFP. When GAI-cYFP or RGL2-cYFP was coexpressed with ICE1-nYFP in *N. benthamiana* leaves, strong YFP fluorescence was detected in the nuclei of transformed cells, as revealed by DAPI staining (Figure 5C). As negative controls, no signal was observed in experiments in which truncated GAI or RGL2 fused with cYFP ($GAI^{1–209}$-cYFP or $RGL2^{1–204}$-cYFP) was coexpressed with ICE1-nYFP or GAI-cYFP (RGL2-cYFP) was coinfiltrated with $ICE1^{1–260}$-nYFP (Figure 5C; Supplemental Figure 1C). Finally, we further verified the interactions of ICE1 with GAI and RGL2 via CoIP assays using Arabidopsis total proteins (Figures 5D and 5E). Collectively, these results indicate that ICE1 physically interacts with DELLA proteins in the nuclei of plant cells.

Mutation of *ICE1* Partially Rescues the ABA-Hyposensitive Phenotype of a *della* Quadruple Mutant during Seed Germination

Because ICE1 directly associates with all five DELLA proteins, we wondered whether it genetically interacts with DELLA to mediate ABA responses during seed germination. All five *DELLA* genes are expressed during seed germination, and *GAI*, *RGA*, and *RGL2* are induced by ABA treatment (Tyler et al., 2004; Piskurewicz et al., 2008). Phenotypic analysis showed that seeds of the *gai-t6 rga-t2 rgl1-1 rgl2-1* quadruple mutant (*della*, in the Landsberg *erecta* [L*er*] background) displayed much higher germination and greening percentages than the L*er* wild type in the presence of ABA (Figure 6). To analyze the genetic interaction between *DELLA* and *ICE1*, we generated the *della ice1* quintuple mutant by crossing *della* with *ice1.* As the *ice1* mutant is in the Col background, we backcrossed it with the L*er* wild type six times before crossing it with *della.* As expected, the *ice1* mutant in the L*er* background was also more sensitive to ABA than the wild type during seed germination and postgerminative growth (Figure 6). Interestingly, the ABA-hyposensitive phenotype of *della* was partially rescued by the *ICE1* mutation, as the *della ice1* quintuple mutant had much lower germination and greening percentages than *della* (Figure 6). These results indicate that disruption of ICE1 partially restores the ABA-hyposensitive phenotype of the *della* mutant during seed germination.

DELLA Proteins Repress the Regulatory Effect of ICE1

DELLA proteins function by affecting the activities of transcription factors through protein–protein interactions (Davière et al., 2008; de Lucas et al., 2008; Feng et al., 2008; Hong et al., 2012; Wild et al., 2012; Yu et al., 2012; Wang et al., 2016). Having ascertained that DELLA proteins physically and genetically interact with ICE1, we asked whether DELLA affects the transcriptional function of ICE1. To test this possibility, we performed a LUC reporter assay to examine the effects of GAI and RGL2 on the transcriptional activity of ICE1 in Arabidopsis mesophyll protoplasts (Figure 4; Yoo et al., 2007). As shown in Figure 4B, coexpression of ICE1 with GAI or RGL2 increased *LUC* expression driven by the *EM6* or *EM1* promoter compared with the expression of ICE1 alone, suggesting that GAI or RGL2 interferes with the repressive effect of ICE1 on downstream target genes. Lim et al. (2013) revealed that DELLA proteins function together with ABI5 to activate the expression of several high-temperature-responsive genes, thereby inhibiting seed germination. Similarly, we found that GAI or RGL2 enhances the transcriptional activity of ABI5 to regulate ABA-inducible *EM6* and *EM1* (Figure 4B). Moreover, coexpression of ABI5 and ICE1 with GAI or RGL2 activated *LUC* expression driven by the *EM6* or *EM1* promoter compared with the coexpression of ABI5 and ICE1 (Figure 4B). These results support the hypothesis that GAI or RGL2 affects the transcriptional function of ICE1.

To further explore the regulatory effect of DELLA on ICE1, we investigated whether GAI interferes with the DNA binding activity of ICE1 in vivo. We crossed *35S:HF-ICE1* with *gai-1*, a gain-of-function mutant of *GAI* in the Col background (Wang et al., 2016), to generate *35S:HF-ICE1 gai-1.* We also generated *35S:ABI5-4MYC gai-1* by crossing *35S:ABI5-4MYC* with *gai-1.* ChIP assays revealed that the enrichment of ICE1 on the *EM6* promoter (*pEM6-1*) was reduced in *35S:HF-ICE1 gai-1* plants compared with *35S:HF-ICE1* plants (Figure 4D). By contrast, the enrichment of ABI5 on the *EM6* promoter (*pEM6-1*) was higher in *35S:ABI5-4MYC gai-1* plants compared with *35S:ABI5-4MYC* plants (Figure 4C). These results indicate that GAI represses the DNA binding activity of ICE1 but promotes that of ABI5 in vivo. To further investigate the role of DELLA in regulating ABI5 and ICE1 activity, we analyzed the ABI5–ICE1 interaction in Arabidopsis in the presence of GA3 (leading to DELLA degradation) and in the *gai-1* mutant background (hyperactive DELLA). As shown in Figure 1D, the ABI5–ICE1 interaction is strengthened by GA treatment but prevented by GAI accumulation. These results indicate that DELLA proteins affect the physical interaction between ABI5 and ICE1.

As GAI exerts opposite regulatory effects on ICE1 and ABI5, we investigated whether the ABA responses of *35S:HF-ICE1* and *35S:ABI5-4MYC* were affected by *gai-1* during seed germination. To test this possibility, we investigated the germination and

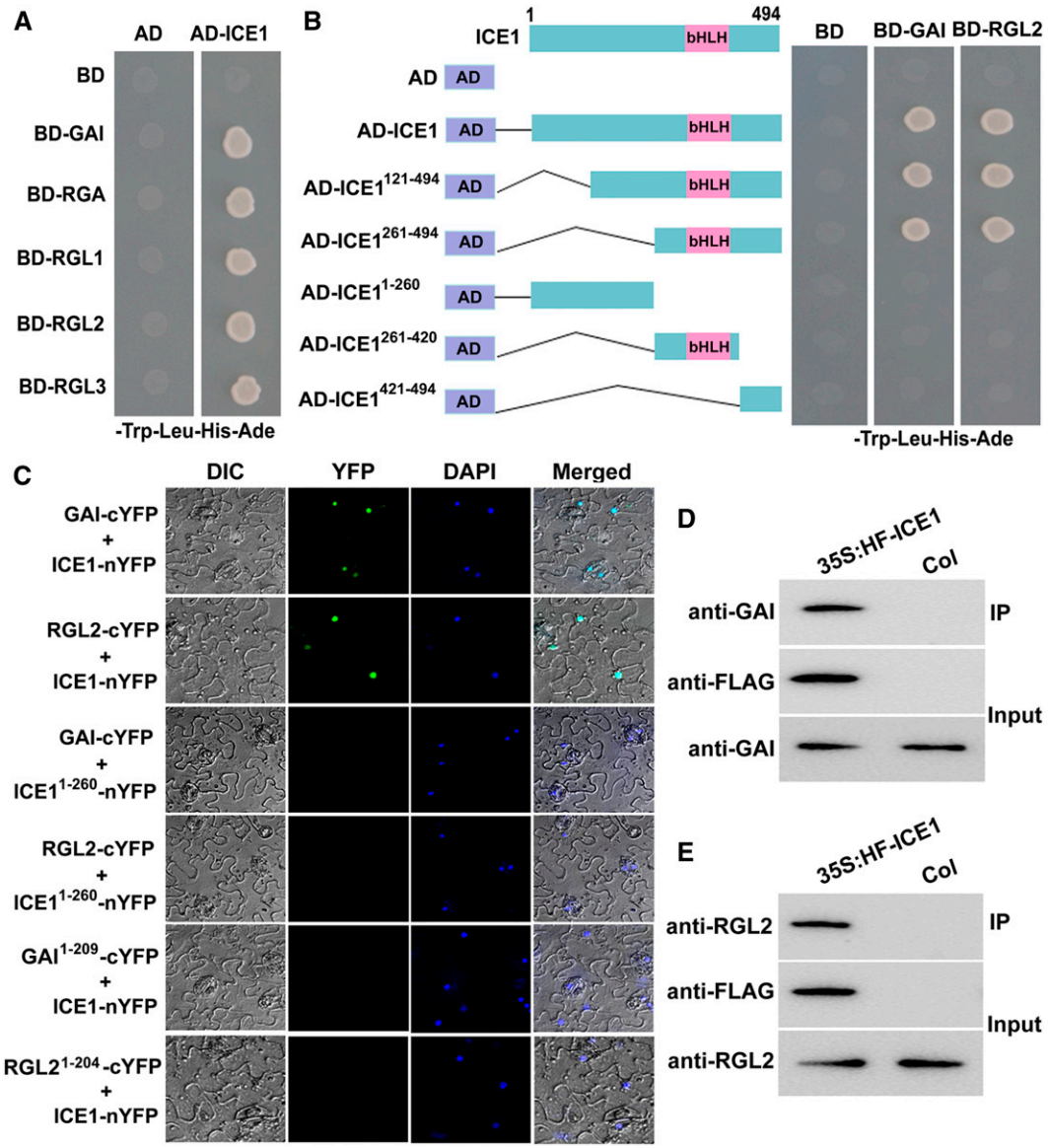

Figure 5. Physical Interactions Between ICE1 and DELLA Proteins.

(A) Y2H analyses. Interaction is indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade. Vectors pGBKT7 (BD) and pGADT7 (AD) were used as negative controls.

(B) Mapping the GAI- or RGL2-interacting domain of ICE1 using a Y2H assay. Interaction is indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade. BD and AD vectors were used as negative controls.

(C) BiFC analyses. Fluorescence was observed in the nuclear compartment of transformed cells, which resulted from complementation of the C-terminal region of YFP fused with GAI or RGL2 (GAI-cYFP or RGL2-cYFP) with the N-terminal region of YFP fused with ICE1 (ICE1-nYFP). The experiments were performed at least four times using different batches of plants; for each biological replicate, more than 12 wild tobacco plants were infiltrated and more than 600 cells were examined. DIF, differential interference contrast.

(D) and **(E)** CoIP analyses. Whole proteins were extracted from 0.5 μM ABA-treated (for 1 d) germinating seeds of the wild type or *35S:HF-ICE1* transgenic plants. The HF-fused ICE1 protein was immunoprecipitated using an anti-FLAG M2 agarose beads, and endogenous anti-GAI **(D)** or anti-RGL2 **(E)** antibody was used for blotting. Protein inputs for HF-ICE1, endogenous GAI, and RGL2 were also detected and are shown. The experiments were repeated three times with similar results using three batches of seeds as biological replicates. IP, immunoprecipitation.

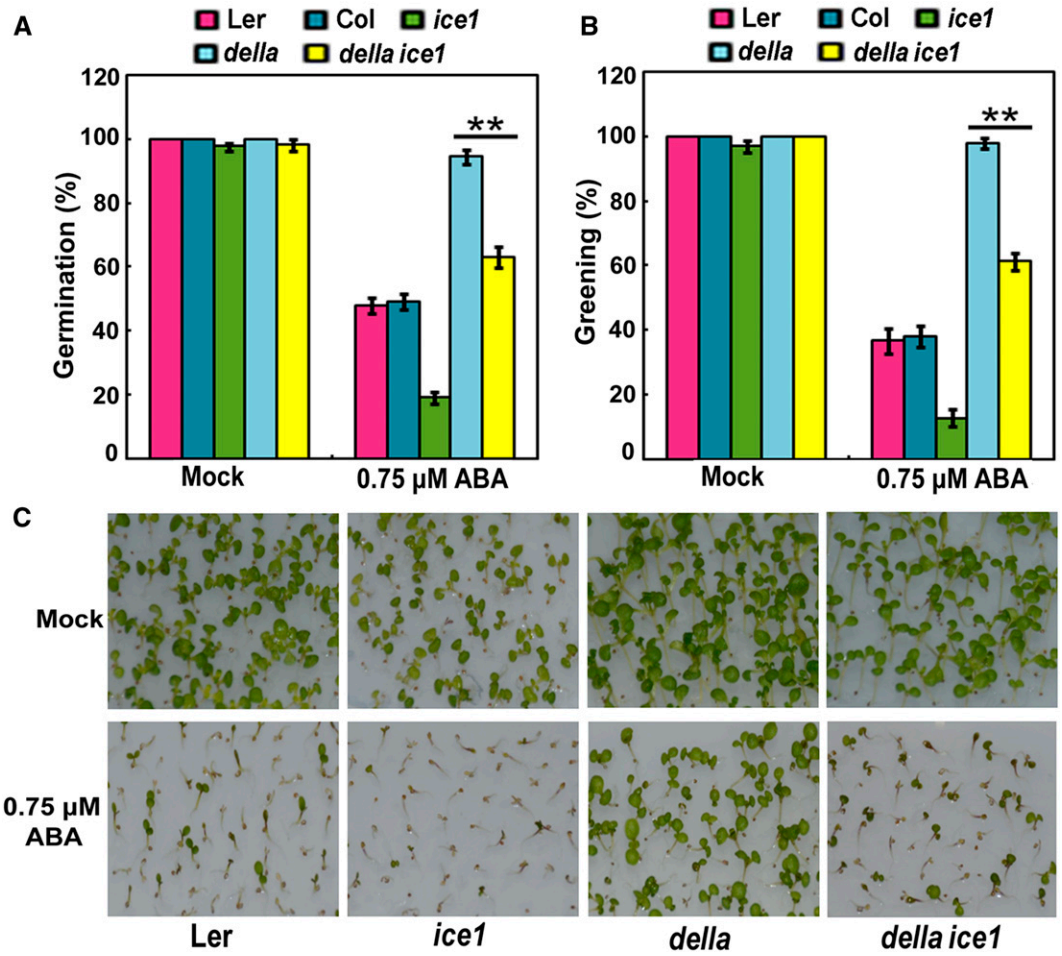

Figure 6. *ice1* Partially Rescues the ABA-Hyposensitive Phenotype of *della* Mutants.

(A) Germination of *della ice1* and other related mutants. Seed germination was recorded 4 d after stratification on medium supplemented with 0.75 μM ABA.
(B) Cotyledon greening of *della ice1* and other related mutants. Cotyledon greening was scored 6 d after stratification on medium containing 0.75 μM ABA.
(C) Seedlings of *della ice1* and other related mutants 6 d after germination on medium with or without 0.75 μM ABA. Experiments were performed three times by analyzing different batches of seeds. Each batch of seeds of *della ice1* and other related mutants was pooled from more than 50 plants. For each biological replicate, we examined the seeds (more than 150) from the same batch three times as technical replicates. Values are means ± SD. ** indicate highly significant differences based on Student's *t* test ($P < 0.01$).

greening percentages of *35S:HF-ICE1 gai-1* and *35S:ABI5-4MYC gai-1* during seed germination in response to ABA. As shown in Figure 7, the progeny of *35S:HF-ICE1 gai-1* displayed much lower germination and greening percentages than *35S:HF-ICE1*, suggesting that the less ABA-sensitive phenotype of *35S:HF-ICE1* was compromised by *gai-1.* By contrast, the increased ABA signaling in *35S:ABI5-4MYC* was enhanced by *gai-1*, as *35S:ABI5-4MYC gai-1* seeds were much more sensitive to ABA than *35S:ABI5-4MYC* seeds during germination (Figure 7). Taken together, these observations further support the notion that GAI inhibits ICE1 but stimulates ABI5 to modulate ABA responses during seed germination.

DISCUSSION

The bZIP family protein ABI5 is widely known as a central transcription factor involved in ABA signaling, which represses seed germination and early seedling growth. Previous studies have highlighted the regulatory roles of ABI5-interacting proteins, which modify ABI5 posttranslationally (Kobayashi et al., 2005; Furihata et al., 2006; Stone et al., 2006; Fujii et al., 2007; Garcia et al., 2008; Fujii and Zhu, 2009; Miura et al., 2009; Nakashima et al., 2009; Lee et al., 2010; Liu and Stone, 2010; Dai et al., 2013; Hu and Yu, 2014; Albertos et al., 2015; Yu et al., 2015; Zhou et al., 2015). Despite recent advances in our understanding of ABI5-mediated signaling, the transcriptional regulatory mechanisms underlying ABI5 and its interacting factors have remained largely unknown. Further research was required to investigate critical transcription factors that interact with ABI5 and to elucidate the biological significance of these physical interactions. In this study, we identified the transcription factor ICE1 as an interacting partner of ABI5 (Figure 1). Phenotypic analysis showed that ICE1 negatively modulates ABA signaling during seed germination. Unlike

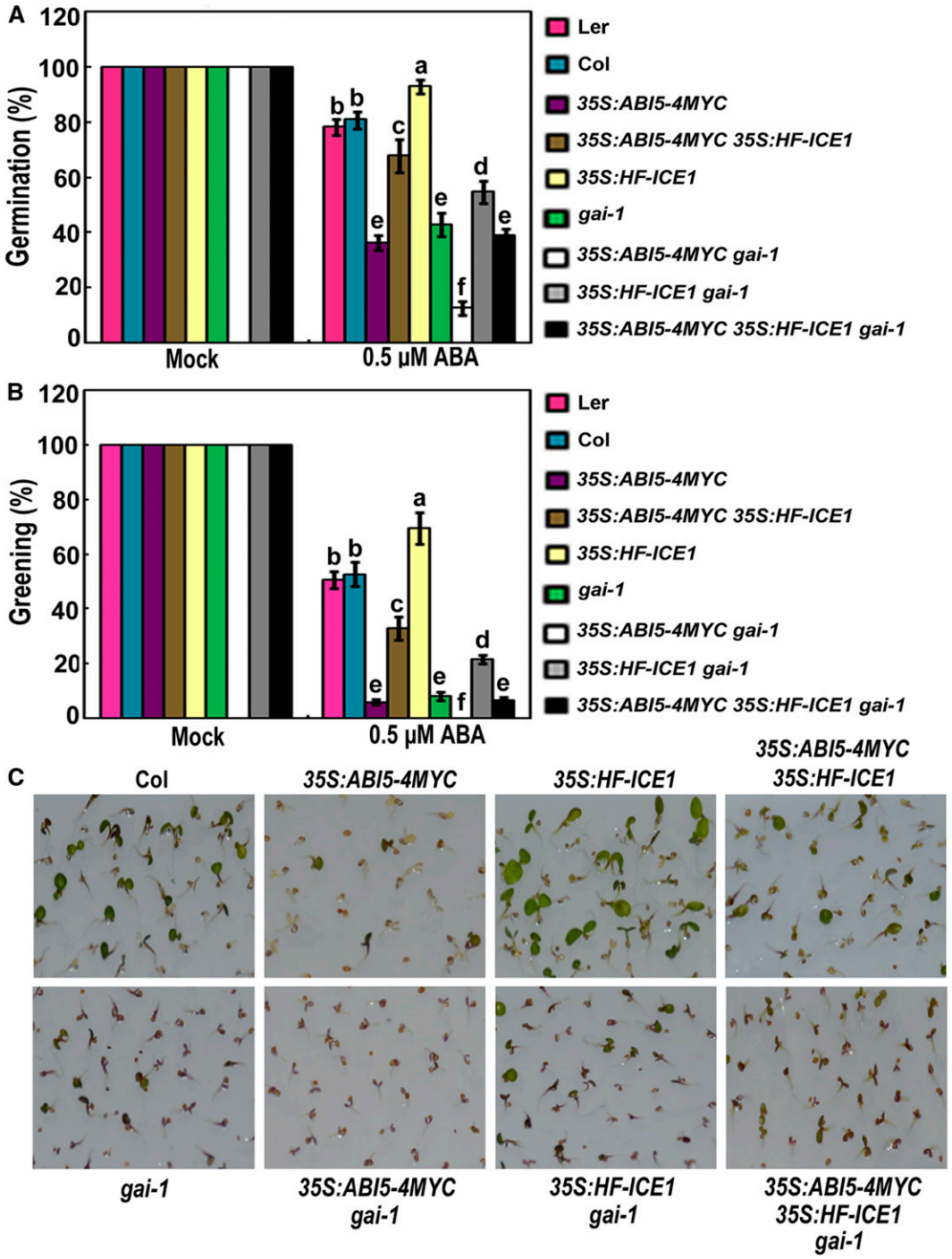

Figure 7. ABA Responses of *35S:HF-ICE1* and *35S:ABI5-4MYC* are Affected by *gai-1* during Seed Germination.

(A) Germination percentages of various genotypes in response to ABA. Seed germination was recorded 3.5 d after stratification on medium supplemented with 0.5 μM ABA.

(B) Cotyledon greening percentages of various genotypes in response to ABA. Cotyledon greening was scored 5 d after stratification on medium containing 0.5 μM ABA.

(C) Seedlings of various genotypes 6 d after germination on medium containing 0.5 μM ABA. The experiments were performed more than three times by analyzing different batches of seeds. Each batch of seeds of various plants was pooled from more than 50 plants. For each biological replicate, we examined the seeds (more than 150) from the same batch three times as technical replicates. Error bars show SD from three biological replicates using different batches of seeds, and different letters above the columns indicate significant differences based on ANOVA ($P < 0.05$).

progeny of the ABA-insensitive *abi5* mutants, seeds of the *ice1* mutant were more sensitive to ABA than the wild type during germination (Figure 2; Supplemental Figure 2). Conversely, germinating seeds overexpressing *ICE1* were less sensitive to ABA than the wild type (Figure 2; Supplemental Figure 2). Thus, ICE1 acts antagonistically with ABI5 to negatively modulate ABA responses during seed germination.

The transcription factor ICE1, a member of the bHLH transcription factor family, is a master regulator that modulates the cold response in Arabidopsis (Chinnusamy et al., 2007; Shi et al., 2018). ICE1 directly activates the expression of cold-responsive *C-REPEAT BINDING FACTOR* (*CBF*) genes, thereby positively regulating cold acclimation (Chinnusamy et al., 2003; Kim et al., 2015; Jia et al., 2016; Liu et al., 2018). The loss-of-function *ice1* mutants display significantly reduced tolerance to chilling and freezing stress with or without cold acclimation (Chinnusamy et al., 2003; Ding et al., 2015; Kim et al., 2015; Li et al., 2017a). ICE1 also plays an essential role in mediating stomatal development (Kanaoka et al., 2008; Lee et al., 2017). ICE1 and its homolog ICE2 specify three cell-state transitional steps leading to stomatal differentiation (Kanaoka et al., 2008). In this study, we found that ICE1 physically associates with ABI5 to negatively regulate ABA signaling during seed germination (Figure 1). Consistent with its ability to interact with ABI5 in vivo, ICE1 is expressed in dry seeds and is responsive to ABA in germinating seeds (Supplemental Figure 3). Further investigations indicated that ICE1 antagonizes the transcriptional function of ABI5 to modulate the expression of downstream target genes. As shown in Figure 4, the expression of *ICE1* interferes with ABI5's regulatory activity to stimulate the expression of downstream regulons. Furthermore, ICE1 and ABI5 may reciprocally affect each other's DNA binding activity and/or competitively bind the promoters of target genes in vivo (Figure 4; Supplemental Figure 5). These results support the notion that ICE1 acts as a negative interactor of ABI5 to mediate ABA responses during seed germination.

ABA treatment promotes the DNA binding activity of ABI5 (Figure 4C) that, in turn, should impede the binding of ICE1. However, we found that ABA stimulates the binding activity of ICE1 to its target promoters (Figures 2D and 2E). We speculate that other regulators might enhance the binding of ICE1 to its target promoters in response to ABA. Consistent with this possibility, Ding et al. (2015) showed that ICE1 physically interacts with several SnRK2 kinases (such as SnRK2.3 and SnRK2.6) and that SnRK2.6 phosphorylates ICE1 and enhances its binding to its target promoters. As these SnRK2 kinases play important roles in seed germination (Fujii et al., 2007; Fujii and Zhu, 2009), it is possible that SnRK2 kinases also phosphorylate ICE1 and enhance its DNA binding ability during seed germination in response to ABA treatment. Although ABI5 impedes the binding of ICE1, the final outcome of ABA regulation is enhanced ICE1 binding activity. The stimulation of ICE1 activity by ABA or other regulators might represent a balancing mechanism to antagonize ABI5 and maintain appropriate ABA signaling during seed germination. Nevertheless, the exact molecular mechanism underlying how ABA activates ICE1 binding activity remains largely unknown and deserves further investigation.

We recently showed that VQ18 and VQ26, two proteins of the VQ family, also physically interact with the ABI5 transcription factor (Pan et al., 2018). VQ18 and VQ26 interfere with the transcriptional activity of ABI5 and function as negative interactors of ABI5 to repress ABA signaling during seed germination. Likewise, Chen et al. (2012) revealed that the Arabidopsis Mediator subunit MEDIATOR25 physically associates with ABI5 and negatively regulates ABI5-dependent ABA responses. Mechanistic analysis further indicated that MEDIATOR25 affects the protein abundance of ABI5 and even the recruitment of ABI5 to the promoters of its targets. By contrast, Kim et al. (2016) demonstrated that the PHYTOCHROME INTERACTING FACTOR1 (PIF1) transcription factor is a positive interactor of ABI5 during ABA signaling. The distinct regulatory effects of these interacting factors on ABI5 may provide specific mechanisms to fine-tune the expression of ABI5 regulons upon exposure to ABA or particular stress conditions. It is also possible that these dual (positive or negative) influences on ABI5 might represent adaptive mechanisms that maintain appropriate ABA signaling levels, thereby establishing efficient stress tolerance while minimizing the detrimental effects of ABA on germination and postgerminative seedling growth. Nevertheless, the exact mechanisms underlying how ABI5 is affected by its interacting transcriptional modulators remain largely unknown. Further study is required to elucidate the potential regulatory relationships between ABI5 and its specific partners in mediating ABA signaling during seed germination.

While we found that the *abi5 ice1* double mutant mimicked the phenotype of *abi5*, the responses of *abi5* and *abi5 ice1* were also significantly different (Figure 3), suggesting that ICE1 also functions with other components to mediate ABA signaling during seed germination. Zhao et al. (2013) found that two MYC2 proteins from banana (*Musa acuminata*) interact with ICE1 to regulate the expression of cold-responsive genes. Interestingly, an earlier study reported that Arabidopsis MYC2 functions as a transcriptional activator in ABA signaling during seed germination (Abe et al., 2003). It is possible that ICE1 also associates with MYC2 to modulate ABA responses. Consistent with this speculation, our protein interaction analysis showed that ICE1 physically associates with MYC2 in yeast (Supplemental Figure 6). The transcription factors ABI3 and ABI4 are strongly responsive to ABA and positively regulate ABA signaling to repress seed germination (Finkelstein, 1994; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Suzuki et al., 2001; Lopez-Molina et al., 2002; Finkelstein et al., 2005). No interaction between ICE1 and ABI3 or ABI4 was observed in yeast (Supplemental Figure 6), suggesting that ICE1 does not directly associate with these transcription factors through protein–protein interactions to modulate ABA responses. However, Liang and Yang (2015) showed that the ABA-induced expression of *ABI3* and *ABI4* was significantly higher in the *ice1* mutant compared with the wild type, suggesting that ICE1 might repress the expression of *ABI3* and *ABI4* in response to ABA. Moreover, MacGregor et al. (2019) demonstrated that ICE1 directly binds to and suppresses the expression of *ABI3* to modulate seed dormancy. It is thus possible that ICE1 modulates ABA signaling partially through inhibiting the expression of *ABI3* and *ABI4* directly or indirectly during seed germination. Further investigations of the associations between ICE1 and other crucial ABA-related regulators will enhance our understanding of ICE1-mediated ABA signaling during seed germination.

In this study, we found that the ICE1 transcription factor physically interacts with DELLA proteins (Figure 5), which are crucial repressors of GA signaling that play positive roles in ABA signaling during seed germination (Peng et al., 1997; Silverstone et al., 1998; Lee et al., 2002; Tyler et al., 2004; Piskurewicz et al., 2008, 2009). All five *DELLA* genes are expressed during seed germination (Tyler et al., 2004), and *GAI*, *RGA*, and *RGL2* are responsive to ABA treatment during seed germination (Piskurewicz et al., 2008). Among these factors, RGL2 is recognized as a major regulator that promotes the ABA responses that suppress seed germination (Lee et al., 2002; Tyler et al., 2004; Piskurewicz et al., 2008). In addition to RGL2, GAI and RGA also repress seed germination (Cao et al., 2005; Piskurewicz et al., 2008, 2009; Chahtane et al., 2018). Seeds of the loss-of-function *della* quadruple mutant are less sensitive to ABA and show higher germination and greening percentages compared with the wild type (Figure 6). Our genetic analysis demonstrated that the disruption of ICE1 partially rescued the ABA-hyposensitive phenotype of the *della* mutant (Figure 6), suggesting that DELLA proteins positively modulate ABA signaling to inhibit germination partially through the ICE1 transcription factor. Further analysis demonstrated that DELLA proteins repress the transcriptional function of ICE1 and affect the physical interaction between ICE1 and ABI5 (Figures 1D; Figure 4). Consistent with these results, a gain-of-function mutation in *GAI* (*gai-1*) repressed the less sensitive phenotype of *ICE1*-overexpressing plants (*35S*:*HF-ICE1*; Figure 7). These observations collectively suggest that DELLA proteins function antagonistically with ICE1 to regulate ABA signaling during seed germination.

Lim et al. (2013) demonstrated that DELLA proteins interact with ABI5 and ABI3 to activate the expression of a subset of high-temperature-inducible genes, thus inhibiting seed germination. Further biochemical analysis revealed that DELLA proteins function together with ABI5 and ABI3 to bind to the promoter sequences of their downstream targets. Similarly, we also found that DELLA proteins (GAI and RGL2) promote the transcriptional activity of ABI5 to modulate ABA-inducible *EM6* and *EM1* (Figure 4). Importantly, the DELLA–ABI5 interaction might also affect the antagonistic influence of ICE1 on ABI5 (Figure 4). Consistent with the notion that DELLA has opposite regulatory effects on ABI5 and ICE1, a dominant mutation in *GAI* (*gai-1*) enhanced the ABA-hypersensitive phenotype of *ABI5*-overexpresing *35S*:*ABI5-4MYC* plants but repressed the ABA-hyposensitive phenotype of *ICE1*-overexpressing *35S*:*HF-ICE1* plants (Figure 7). Based on the regulatory relationships among DELLA, ICE1, and ABI5, we speculate that ICE1 maintains appropriate DELLA- and ABI5-activated ABA signaling during seed germination (Figure 8; Supplemental Figure 7). According to this model, in the presence of high concentrations of ABA, the ABA-induced DELLA proteins stimulate ABI5 to positively modulate ABA responses while repressing the regulatory effect of ICE1, thereby establishing appropriate ABA signaling to suppress seed germination and postgerminative growth. In response to low concentrations of ABA or in the presence of GA, the accumulation of DELLA proteins is reduced, leading to the release of ICE1 from the DELLA-ICE1 complex; ICE1 inhibits the expression of ABA-responsive genes to attenuate ABA signaling, thus promoting seed germination and subsequent seedling establishment.

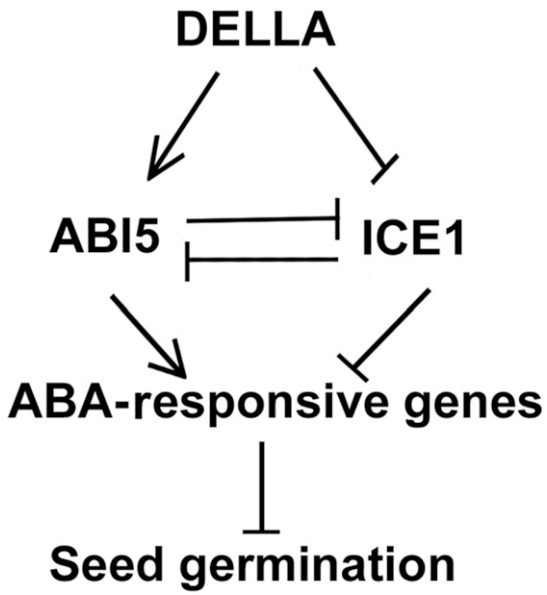

Figure 8. A Simplified Model for the Roles of DELLA, ABI5, and ICE1 Interactions in Modulating ABA Signaling During Seed Germination.

In response to high concentrations of ABA, the ABA-induced DELLA proteins stimulate ABI5 to positively modulate ABA responses while repressing the regulatory effect of ICE1, thereby establishing appropriate ABA signaling to suppress seed germination and postgerminative growth. In response to low concentrations of ABA or the presence of GA, the accumulation of DELLA proteins is reduced, leading to the release of ICE1 from the DELLA-ICE1 complex; ICE1 inhibits the expression of ABA-responsive genes, such as *EM6* and *EM1*, to attenuate ABA signaling, thus promoting seed germination and subsequent seedling establishment.

DELLA proteins are crucial regulators of the GA and ABA signaling pathways that modulate physiological processes through interacting with multiple transcription factors. In addition to ABI5 and ICE1, several transcriptional regulators such as PIF1, GLABRA1, GLABRA3, MYC2, BRASSINAZOLE-RESISTANT1, ETHYLENE INSENSITIVE3, AUXIN RESPONSE FACTOR6, CONSTANS, WRKY13, and bHLH48 are targets of DELLA that modulate diverse processes (Davière et al., 2008, 2014; de Lucas et al., 2008; Feng et al., 2008; An et al., 2012; Bai et al., 2012; Hong et al., 2012; Wild et al., 2012; Yu et al., 2012; Oh et al., 2014; Qi et al., 2014; Li et al., 2016, 2017b; Wang et al., 2016; Chen et al., 2017; Zhang et al., 2018). Among these DELLA-interacting transcription factors, MYC2 positively regulates ABA signaling and inhibits seed germination (Abe et al., 2003). PIF1 also acts as a transcriptional activator of ABA responses during seed germination (Oh et al., 2006, 2009). By contrast, BRASSINAZOLE-RESISTANT1 negatively mediates ABA signaling to promote seed germination (Yang et al., 2016). Given that DELLA proteins physically associate with these ABA-related transcription factors, it is possible that DELLA proteins function through these transcription factors to mediate ABA signaling during seed germination. Moreover, Liu et al. (2016) revealed that RGL2 associates with NUCLEAR FACTOR-Y, subunit C factors to modulate ABA-mediated seed germination. These observations, together with the current findings, imply that

DELLA proteins modulate ABA signaling to inhibit seed germination and postgerminative growth via multiple layers of regulation. Further research is required to elucidate the regulatory relationships among these DELLA-interacting ABA-related transcriptional regulators, which may shed light on the molecular basis of the DELLA-mediated ABA signaling network.

METHODS

Materials and Plant Growth Conditions

The plant hormone ABA was purchased from Sigma-Aldrich. Taq DNA polymerases were purchased from Takara Biotechnology, and other common chemicals were obtained from Shanghai Sangon. Anti-MYC, anti-FLAG, and anti-HA antibodies were purchased from Sigma-Aldrich. The wild-type and mutant Arabidopsis (*Arabidopsis thaliana*) plants used in this study were in the Col-0 or L*er* genetic background. The *ice1* (SALK_003155; Kanaoka et al., 2008), *abi5* (*abi5-1*; Finkelstein, 1994; Finkelstein and Lynch, 2000), and *della* (Wang et al., 2016) mutants were described previously. Seeds of *35S*:*HF-ICE1* and *ProICE1*:*ICE1-GFP*/*ice1* were kindly provided by Shuhua Yang (China Agricultural University). The *35S*:*GFP-ICE1* (CS68099) line was obtained from the Arabidopsis Resource Center at Ohio State University (http://abrc.osu.edu). The *ProICE1*:*β-glucuronidase* (*GUS*) line (Wei et al., 2018) was obtained from Juan Lin (Fudan University). The *35S*:*ABI5-4MYC* line was obtained from Chuanyou Li (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). The Arabidopsis plants were grown in a growth chamber at 22°C under a 14-h-light (100 μE m^{-2} s^{-1}, white fluorescent bulbs, full wavelength of light), 10-h-dark photoperiod.

Measurement of Germination and Greening Percentages

The germination and greening percentages of the wild type and mutant seeds were performed as described previously (Hu and Yu, 2014). Seeds were stratified for 4 d at 4°C. 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 in a seedling. To measure the ABA sensitivity of germination and greening, seeds were plated on ABA-containing Murashige and Skoog medium with 2% (w/v) Suc. Three independent experiments were conducted, and similar results were obtained.

Y2H Assays

Multiple truncated forms of ABI5 were fused to pGBKT7 to generate the bait vectors (BD-ABI5) and transformed into yeast strain Y2HGold (Clontech; Chen et al., 2012). The cDNA library was obtained from Clontech (catalog no. 630487). Yeast screening was performed as described previously (Hu et al., 2013). To confirm the ABI5–ICE1 interaction, the full-length coding sequence of ICE1 was cloned into the prey vector pGADT7 (AD-ICE1). To further identify the partners of ICE1 involved in ABA signaling, the C-terminal portion (amino acids 261 to 494) of ICE1 was fused to pGBKT7 to generate the bait vector (BD-ICE1; Agarwal et al., 2006). To confirm the DELLA–ICE1 interactions, truncated DELLA proteins with deleted activation domains were fused to pGBKT7 to generate the bait vectors (BD-GAI, BD-RGA, BD-RGL1, BD-RGL2, and BD-RGL3); these bait vectors were cotransformed with AD-ICE1 into yeast strain Y2HGold. The primers used for cloning are listed in Supplemental Table 3.

BiFC Assays

The cDNA sequences of the N-terminal 173-amino acid–enhanced YFP (nYFP) and C-terminal 64-amino acid YFP (cYFP) fragments were PCR amplified and individually cloned into tagging pFGC5941 plasmids behind the FLAG or MYC tag sequences to generate pFGC-nYFP and pFGC-cYFP, respectively (Kim et al., 2008). Full-length or the 164 N-terminal residues of ABI5 were inserted into pFGC-cYFP to generate a C-terminal in-frame fusion with cYFP (ABI5-cYFP or $ABI5^{1-164}$-cYFP). Similarly, full-length or truncated RGL2 or GAI was fused with cYFP to generate RGL2-cYFP, $RGL2^{1-204}$-cYFP, GAI-cYFP, or GAI^{1-209}-cYFP. Full-length or the 260 N-terminal residues of ICE1 were inserted into pFGC-nYFP to generate an N-terminal in-frame fusion with nYFP (ICE1-nYFP or $ICE1^{1-260}$-nYFP). The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101, and infiltration of wild tobacco (*Nicotiana benthamiana*) leaves was performed as described previously (Hu et al., 2013). The experiments were performed at least four times using different batches of plants; for each biological replicate, more than 12 wild tobacco plants were infiltrated and more than 600 cells were examined. Infected leaves were analyzed 48 h after infiltration. YFP and DAPI fluorescence were detected under a confocal laser-scanning microscope (Olympus).

CoIP Assays

To analyze the ABI5–ICE1 interaction, whole proteins were extracted from 0.5 μM ABA-treated (for 1 d) germinating seeds of *35S*:*HF-ICE1* or *35S*:*ABI5-4MYC*/*35S*:*HF-ICE1*. CoIP assays were performed using whole protein extracts as described previously (Hu et al., 2013). Briefly, the ABI5-4MYC protein was immunoprecipitated using anti-MYC M2 agarose beads, and coimmunoprecipitated HA-FLAG (HF)–fused ICE1 was detected using an anti-FLAG antibody (catalog no. F7425, Sigma-Aldrich; 1:12,000). To analyze the DELLA–ICE1 interaction, whole proteins were extracted from 0.5 μM ABA-treated (for 1 d) germinating seeds of the wild type or *35S*:*HF-ICE1* transgenic plants. The HF-fused ICE1 protein was immunoprecipitated using anti-FLAG M2 agarose beads, and endogenous anti-GAI (catalog no. AS111631, Agrisera; 1:10,000) or anti-RGL2 antibody (catalog no. AS111803, Agrisera; 1:10,000) was used for blotting.

RNA Extraction and RT-qPCR

Total RNA was extracted using the TRIzol reagent (Invitrogen), and RT-qPCR was performed as described by Hu et al. (2013). Briefly, first-strand cDNA was synthesized from 1.5 μg of DNase-treated RNA in a 20-μL reaction volume using *Moloney murine leukemia virus* reverse transcriptase (Fermentas) with oligo(dT)18 primer. RT-qPCR was performed using 2× SYBR Green I master mix on a LightCycler 480 real-time PCR machine (Roche), according to the manufacturer's instructions. At least three biological replicates for each sample were used for RT-qPCR analysis, and at least two technical replicates were analyzed for each biological replicate. The *At1g13320* gene, which encodes a subunit of Ser/Thr protein phosphatase 2A and is stably expressed in seed samples during seed germination (Czechowski et al., 2005), was used as a control. The gene-specific primers used for transcript analysis are listed in Supplemental Table 4.

GUS Staining

The putative promoter of *ICE1* (2589 bp) was amplified from genomic DNA and fused with the *β-glucuronidase* (*GUS*) reporter gene to generate a *ProICE1*:*GUS* construct (Chinnusamy et al., 2003). The *ProICE1*:*GUS* transgenic seeds were germinated on Murashige and Skoog medium with or without 0.5 μM ABA. The histochemical detection of GUS activity was performed with X-gluc as the substrate. Transgenic plants were subjected to GUS staining as described by Chen et al. (2010).

ChIP Analyses

The ChIP assay was performed essentially as described previously (Mukhopadhyay et al., 2008; Jiang et al., 2014). Briefly, dry seeds or the ABA-treated germinating wild type, *35S*:*HF-ICE1*, *35S*:*ABI5-4MYC*, *35S*:*ABI5-4MYC 35S*:*HF-ICE1*, *35S*:*ABI5-4MYC gai-1*, or *35S*:*HF-ICE1 gai-1* seeds were crosslinked in 1% formaldehyde and their chromatin was isolated. The anti-HA antibody or anti-MYC antibody was used to immunoprecipitate the protein–DNA complex, and the precipitated DNA was purified using a PCR purification kit (Qiagen) for RT-qPCR analysis. To quantitatively assess ICE1–DNA (target promoters) or ABI5–DNA binding, RT-qPCR analysis was performed as described previously (Mukhopadhyay et al., 2008) with the *ACTIN2* 3′ untranslated region sequence as an endogenous control. The relative quantity is presented as the DNA binding ratio. The results presented were obtained from at least three independent experiments.

Transient Transactivation Assay

Full-length *ABI5*, *ICE1*, *RGL2*, *GAI*, and *GFP* sequences were amplified and cloned into the pGreenII 62-SK vector as effectors. The putative promoter sequences of *EM6* (1273 bp) and *EM1* (2000 bp) were amplified by PCR and cloned into the pGreenII 0800-LUC vector as reporters (Hellens et al., 2005). Combinations of plasmids were transformed into Arabidopsis leaf mesophyll protoplasts according to the Sheen laboratory protocol (Sheen, 2001). Transfected cells were cultured for 10 to 16 h with or without 5 μM ABA, and relative LUC activity was analyzed using a Dual-LUC Reporter Assay system (Promega). This measured the activities of firefly LUC and the internal control *Renilla reniformis* LUC. The primers and restriction enzymes/sites used for cloning are listed in Supplemental Table 3.

Electrophoretic Mobility Shift Assay

The full-length coding sequence of ABI5 and ICE1 were subcloned into the expression vector pET-28a and pET-32a, respectively (Hu and Yu, 2014; Ding et al., 2015) and transformed into *Escherichia coli*. Expression of the recombinant proteins was induced by treatment with isopropyl β-D-1-thiogalactopyranoside. The expressed proteins were purified according to the manual provided by Novagen. The EMSA was performed using a Light Shift Chemiluminescent EMSA Kit (Pierce; http://www.piercenet.com) according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed by analysis of variance (ANOVA). The results are shown in Supplemental Table 5.

Accession Numbers

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: *ABI5*, AT2G36270; *ABI4*, AT2G40220; *ABI3*, AT3G24650; *ICE1*, AT3G26744; *GAI*, At1g14920; *RGA*, At2g01570; *RGL1*, At1g66350; *RGL2*, At3g03450; *RGL3*, At5g17490; *MYC2*, AT1G32640; *EM1*, AT3G51810; *EM6*, AT2G40170; *ADH1*, AT1G77120; *RAB18*, AT1G43890; and *ACTIN2*, AT3G18780.

Supplemental Data

Supplemental Figure 1. Expression of truncated versions of ABI5 or ICE1 protein in *N. benthamiana* leaves.

Supplemental Figure 2. Phenotypic analysis of *ice1* and other related transgenic plants in response to ABA.

Supplemental Figure 3. Expression of *ICE1* in response to ABA during seed germination.

Supplemental Figure 4. Expression levels of several ABA-responsive genes in *abi5 ice1* and other related plants.

Supplemental Figure 5. ABI5 and ICE1 reciprocally affect each other's DNA binding activity in vitro.

Supplemental Figure 6. Yeast two-hybrid assay analysis of the interactions between ICE1 and several ABA-related transcription factors.

Supplemental Figure 7. A simplified model for the roles of interacting partners of ABI5, ICE1, and DELLA in mediating ABA signaling during seed germination.

Supplemental Table 1. Information for detecting the ICE1 binding promoter sequences of *EM6*.

Supplemental Table 2. Information for detecting the ICE1 binding promoter sequences of *EM1*.

Supplemental Table 3. Primers used for cloning.

Supplemental Table 4. Primers used for qRT-PCR analysis.

Supplemental Table 5. ANOVA tables.

ACKNOWLEDGMENTS

We thank Shuhua Yang (China Agricultural University), Chuanyou Li (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences), Xingliang Hou (South China Botanical Garden, Chinese Academy of Sciences), Dapeng Zhang (Tsinghua University), Xiaolan Chen (Yunnan University), Zhixiang Chen (Purdue University), Ming Luo (South China Botanical Garden, Chinese Academy of Sciences), and Juan Lin (Fudan University) for sharing research materials. We thank the Arabidopsis Resource Center at Ohio State University for the *35S*:*GFP-ICE1* (CS68099) seeds. We also thank the Central Laboratory of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences for technical support. This work was supported by the Yunnan Applied Basic Research Projects (grants 2017FA005 and 2016FB026 to Y.H.), the Natural Science Foundation of China (grants 31670281 and 31870258 to Y.H.), the Youth Innovation Promotion Association of the of Chinese Academy of Sciences (to Y.H.), and Candidates of the Young and Middle Aged Academic Leaders of Yunnan Province (grant 2017HB067 to Y.H.).

AUTHOR CONTRIBUTIONS

Y.H. and D.Y. designed this study; Y.H., X.H., M.Y., M.Z., and J.P. performed experiments; Y.H., X.H., and M.Y. interpreted data and wrote the article. All authors read and approved the final article.

Received October 29, 2018; revised April 19, 2019; accepted May 6, 2019; published May 13, 2019.

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