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Molecular Mechanism Underlying the Synergetic Effect of Jasmonate on Abscisic Acid Signaling during Seed Germination in Arabidopsis[OPEN]

Jinjing Pan,[a,b,c,d,e] Yanru Hu,[b,d] Houping Wang,[a,b,d] Qiang Guo,[f] Yani Chen,[f] Gregg A. Howe,[f] and Diqiu Yu[a,b,d,1]

[a] State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan, School of Life Sciences, Yunnan University, Kunming 650091, China
[b] CAS Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan 650223, China
[c] College of Tobacco Science, Yunnan Agricultural University, Kunming, Yunnan 650201, China
[d] Center of Economic Botany, Core Botanical Gardens, Chinese Academy of Sciences, Menglun, Mengla, Yunnan 666303, China
[e] University of Chinese Academy of Sciences, Beijing 100049, China
[f] Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

ORCID IDs: 0000-0002-2023-8505 (J.P.); 0000-0002-1570-8752 (Y.H.); 0000-0001-7069-3509 (H.W.); 0000-0002-6619-8211 (Q.G.); 0000-0001-7200-2628 (Y.C.); 0000-0002-9218-979X (G.A.H.); 0000-0001-7507-7617 (D.Y.)

Abscisic acid (ABA) is known to suppress seed germination and post-germinative growth of Arabidopsis (*Arabidopsis thaliana*), and jasmonate (JA) enhances ABA function. However, the molecular mechanism underlying the crosstalk between the ABA and JA signaling pathways remains largely elusive. Here, we show that exogenous coronatine, a JA analog structurally similar to the active conjugate jasmonate-isoleucine, significantly enhances the delayed seed germination response to ABA. Disruption of the JA receptor CORONATINE INSENSITIVE1 or accumulation of the JA signaling repressor JASMONATE ZIM-DOMAIN (JAZ) reduced ABA signaling, while *jaz* mutants enhanced ABA responses. Mechanistic investigations revealed that several JAZ repressors of JA signaling physically interact with ABSCISIC ACID INSENSITIVE3 (ABI3), a critical transcription factor that positively modulates ABA signaling, and that JAZ proteins repress the transcription of ABI3 and ABI5. Further genetic analyses showed that JA activates ABA signaling and requires functional ABI3 and ABI5. Overexpression of *ABI3* and *ABI5* simultaneously suppressed the ABA-insensitive phenotypes of the *coi1-2* mutant and JAZ-accumulating (*JAZ-ΔJas*) plants. Together, our results reveal a previously uncharacterized signaling module in which JAZ repressors of the JA pathway regulate the ABA-responsive ABI3 and ABI5 transcription factors to integrate JA and ABA signals during seed germination and post-germinative growth.

INTRODUCTION

The processes of seed germination and subsequent seedling establishment are precisely regulated by external and internal cues, including phytohormones. Abscisic acid (ABA) is an important phytohormone that regulates numerous physiological processes in plants, including seed germination, stomatal aperture, and seedling growth, as well as plant responses to various abiotic and biotic stresses (Mauch-Mani and Mauch, 2005; Finkelstein et al., 2008; Fujita et al., 2011; Hauser et al., 2011; Nakashima and Yamaguchi-Shinozaki, 2013). For example, ABA promotes seed dormancy and suppresses seed germination. In the presence of ABA, the ABA receptors PYRABACTIN RESISTANCE (PYR)/REGULATORY COMPONENT OF ABSCISIC ACID RECEPTOR (RCAR) recognize the ABA molecule (Ma et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009). Upon binding to ABA, these receptors form a stable complex with type 2C protein phosphatases (PP2Cs), leading to the release of SNF1-related kinases 2 (SnRK2s) from PP2C-SnRK2 complexes (Cutler et al., 2010). The activated SnRK2s subsequently phosphorylate downstream transcription factors such as ABSCISIC ACID-INSENSITIVE5 (ABI5), ABI4, and ABI3 to mediate ABA responses (Kobayashi et al., 2005; Furihata et al., 2006; Fujii et al., 2007; Fujii and Zhu, 2009; Nakashima et al., 2009; Zhu et al., 2020).

The transcription factors ABI3 and ABI5 are highly induced by ABA and positively modulate ABA responses to suppress seed germination (Finkelstein, 1994; Lopez-Molina et al., 2001; Brocard et al., 2002; Finkelstein et al., 2005). Mechanistic investigations have revealed that ABI3 and/or ABI5 physically interact with several critical regulators to integrate multiple signaling pathways during seed germination. For instance, Park et al. (2011) demonstrated that ABI3 forms a protein complex with PHYTOCHROME INTERACTING FACTOR3-LIKE5 (PIL5) that activates the expression of *SOMNUS* (*SOM*) in imbibed seeds. Therefore, the *SOM* promoter integrates ABA and light signals to mediate seed germination. Previously, we showed that ABI5 physically interacts with the BRASSINOSTEROID INSENSITIVE2 (BIN2) brassinosteroid -related protein kinases and that the BIN2-ABI5

[1] Address correspondence to ydq@ynu.edu.cn.
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cascade mediates the antagonism between brassinosteroids and ABA during seed germination (Hu and Yu, 2014). Lim et al. (2013) showed that ABI3, ABI5, and DELLA proteins interact to integrate ABA and gibberellin signals to modulate the expression of a subset of high temperature–inducible genes, leading to the inhibition of seed germination.

The phytohormone jasmonate (JA) functions as a critical signaling molecule, with roles in plant growth, development, and stress responses (Pauwels et al., 2010; Hu et al., 2013; Qi et al., 2015; Du et al., 2017). It is perceived by the F-box protein CORONATINE (COR) INSENSITIVE1 (COI1), which assembles in the SCF^{COI1} protein complex that targets JASMONATE ZIM-DOMAIN (JAZ) proteins for degradation (Xu et al., 2002; Chini et al., 2007; Thines et al., 2007; Yan et al., 2009). The degradation of JAZ repressors alleviates the inhibition of downstream transcription factors, thus activating the transcriptional reprogramming of JA signaling (Chini et al., 2007; Thines et al., 2007; Pauwels et al., 2010). JAZ proteins have been shown to interact with multiple transcription factors to regulate diverse aspects of JA-regulated physiological processes, such as root growth, male fertility, anthocyanin accumulation, trichome development, senescence, and stress responses (Cheng et al., 2009; Niu et al., 2011; Fernández-Calvo et al., 2011; Kazan and Manners, 2013; Schweizer et al., 2013). In addition, JA signaling is involved in seed germination and interacts with ABA signaling. For example, methyl jasmonate (MeJA) has an inhibitory effect on the seed germination of several crops and Arabidopsis (*Arabidopsis thaliana*; Wilen et al., 1991; Krock et al., 2002; Preston et al., 2002; Norastehnia et al., 2007; Barrero et al., 2009; Dave et al., 2011, 2016). Several studies have provided evidence for crosstalk between ABA and JA signaling. For example, the genes encoding the ABA receptors PYRABACTIN RESISTANCE (PYR)/PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABSCISIC ACID RECEPTOR4 (PYL4) and PYL5 were found to be upregulated by JA, and *pyl4* and *pyl5* knockout mutants were hypersensitive to JA (Lackman et al., 2011). Conversely, *MYC2*-overexpressing plants exhibited hypersensitivity to ABA (Abe et al., 2003; Lorenzo et al., 2004). The increased inhibition of germination in a line overexpressing a mutant *MYC2* was associated with ABA hypersensitivity (Goossens et al., 2015). The results of those studies indicated that the ABA receptors PYL4/5 and the transcriptional factor MYC2 play vital roles in the crosstalk between JA and ABA. Further research showed that the E3 RING ligase KEG interacts with JAZ12 and regulates its stability (Pauwels et al., 2015). Another study provided evidence for the link between ABA and JA signaling through the direct interaction of the ABA receptor PYL6 with MYC2 (Aleman et al., 2016); the *pyl6* mutant was more sensitive to the combination of JA and ABA than to ABA alone. Dave et al. (2011) found that 12-oxo-phytodienoic acid (OPDA, the precursor of JA) inhibited the wild-type germination and acted with ABA to regulate seed germination in Arabidopsis. The abundance of ABI5 protein was increased by the inhibitory effect of OPDA and the combination of OPDA and ABA on seed germination. Ju et al. (2019) reported that JAZ proteins interact with ABI5 to modulate seed germination.

Although JA signaling has been implicated in seed germination in several species, the detailed molecular mechanism of how JA regulates these crucial physiological processes remains largely unknown. In this study, we found that exogenous application of 1 μM COR or 10 μM MeJA alone did not inhibit seed germination. However, JA significantly delayed germination in the presence of ABA, showing that JA stimulates ABA signaling to delay seed germination. We discovered that the COI1/JAZ-mediated JA signaling pathway is positively involved in ABA-delayed seed germination. Mechanistic analyses revealed that several JAZ proteins physically interact with the ABA-responsive ABI3 transcription factor and repress ABI3/ABI5-mediated transcriptional activation of downstream targets. Further genetic analyses showed that JA activates ABA signaling to delay seed germination and requires functional ABI3 and ABI5. Overexpression of *ABI3* and *ABI5* simultaneously suppressed the ABA-insensitive phenotypes of *coi1* mutants and *JAZ-ΔJas* (JAZ1, JAZ5, and JAZ8 lacking the Jas domain) plants. Our results provide a mechanistic understanding of how the integration of the JA and ABA signaling pathways is mediated by the JAZ-ABI3/ABI5 module during seed germination.

RESULTS

Exogenous JA Enhances ABA Signaling to Delay Seed Germination

To investigate the molecular mechanisms underlying JA regulation of seed germination, we first confirmed the regulatory effect of JA by evaluating the germination of the wild-type (Columbia-0 [Col-0]) Arabidopsis seeds on medium supplemented with MeJA and/or ABA. To avoid the effects of Suc and/or nitrate on seed germination, we performed germination assays using water agar medium. Consistent with a previous study by Dave et al. (2011), the Col-0 seeds displayed similar percentages of germination and expanded cotyledons on medium with or without 10 μM MeJA (Supplemental Figure 1), confirming that 10 μM MeJA alone has no inhibitory effect on germination. We then analyzed the performance of Col-0 seeds on medium containing 10 μM MeJA with or without 0.5 μM ABA. Compared with seeds treated with MeJA or ABA alone, those treated with both MeJA and ABA showed much lower percentages of germination and expanded cotyledons (Supplemental Figure 1), suggesting that MeJA enhances ABA signaling during seed germination (Staswick et al., 1992; Ellis and Turner, 2002). To verify these observations, we used COR, a JA analog that is structurally similar to the active conjugate jasmonate-isoleucine (JA-Ile). Whereas 1 μM COR did not inhibit germination and cotyledon expansion, Col-0 plants exhibited lower percentages of germination and of expanded cotyledons on both COR medium and on ABA medium (Figures 1A to 1C).

To corroborate the regulatory effect of COR on ABA signaling during seed germination, we used reverse transcription quantitative PCR (RT-qPCR) to determine the transcript levels of several well-characterized ABA-responsive genes, including *ALCOHOL DEHYDROGENASE1* (*ADH1*), *LATE EMBRYOGENESIS ABUNDANT6* (*EM6*), and *EM1* (Finkelstein and Lynch, 2000; Carles et al., 2002; Lopez-Molina et al., 2002), in Col-0 germinating seeds treated with ABA and COR simultaneously and individually. The transcript levels of these genes were higher in seeds treated with both ABA and COR than in seeds treated with ABA or COR alone

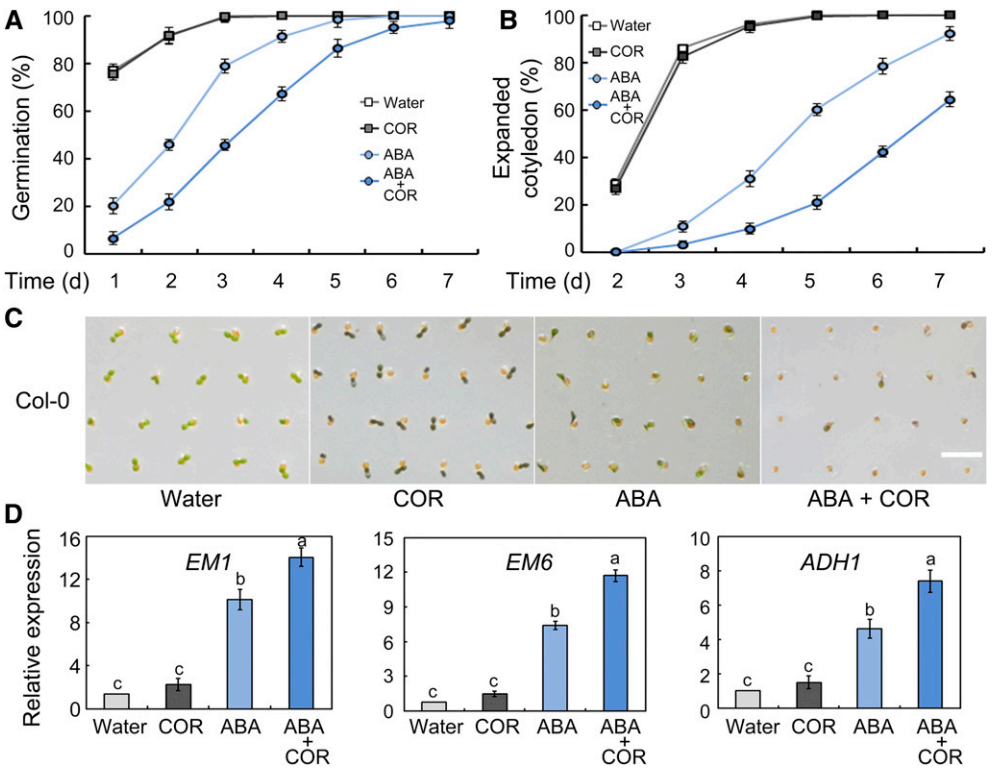

Figure 1. JA Enhances ABA Signaling during Seed Germination.

(A) Germination of the wild-type seeds was scored 1 to 7 d after stratification on water agar medium supplemented with 1 μM COR, 0.5 μM ABA, or both 0.5 μM ABA and 1 μM COR, respectively.

(B) Percentage of the wild-type seedlings with expanded cotyledons was scored 2 to 7 d after stratification on water agar medium supplemented with 1 μM COR, 0.5 μM ABA, or both 0.5 μM ABA and 1 μM COR, respectively.

(C) Seedlings of the wild type (Col-0) observed 4.5 d after stratification on water agar medium supplemented with 1 μM COR, 0.5 μM ABA, or both 0.5 μM ABA and 1 μM COR, respectively. Average and significance were calculated over three biological replicates by analyzing seeds of different batches. Each batch of seeds for Col-0 was pooled from 50 independent plants. For every biological replicate, we examined seeds from the same batch at least three times as technical replicates. The value of each biological replicate was the average calculated over three technical replicates by analyzing more than 200 seeds. Bar = 30 mm.

(D) Expression of several ABA-responsive genes in response to ABA and COR treatment. Seeds of the wild type were germinated on water agar medium containing 1 μM COR, 0.5 μM ABA, and both 0.5 μM ABA and 1 μM COR, respectively, and then those seeds were grown on these media for 4 h in long-day conditions. Average and significance were calculated over three biological replicates. Total RNA was extracted from at least three batches of seeds as biological replicates. Each batch of seeds for the wild type was pooled from 50 independent plants. For each biological replicate, more than 250 seeds of the same batch were used for RNA extraction. *AT1G13320* gene was used as control. Data from three biological replicates were analyzed by ANOVA. Values with different letters are significantly different from each other ($P < 0.05$). Error bars show SD from three biological replicates.

(Figure 1D). Taken together, these results demonstrate that exogenous JA enhances ABA signaling to delay seed germination.

COI1/JAZ-Mediated JA Pathway Is Positively Associated with ABA Signaling during Seed Germination

Having ascertained that JA interacts with ABA synergistically to delay seed germination, we then queried whether critical components of the JA signaling pathway participate in these ABA-regulated processes. The JA receptor F-box protein COI1 is a positive modulator of JA signaling (Xie et al., 1998; Yan et al., 2009). To investigate whether COI1 is involved in ABA responses during seed germination, we analyzed the loss-of-function mutant *coi1-2.* Compared with Col-0 seeds, *coi1-2* seeds exhibited much higher percentages of germination and cotyledon greening on medium containing ABA (Figures 2A to 2D; Supplemental Figures 2A and 3). As shown in Supplemental Figure 3, Suc delayed seed germination, and nitrate enhanced this process. Next, in comparison with seeds exposed to both ABA and nitrate medium, the germination of the wild type was similar to *coi1-2* and *coi1-16* mutant, indicating that nitrate had an effect on ABA signaling during seed germination. Similarly, there were no differences in the percentages of germination and expanded cotyledon between the *coi1* mutants with Col-0 on the presence of both ABA and Suc, suggesting that Suc participated in ABA signaling to delay seed germination (Supplemental Figure 3). These results suggested that the phenotype of *coi1* mutants is conditioned by the absence of nitrate or Suc. Therefore, we used water agar medium to avoid

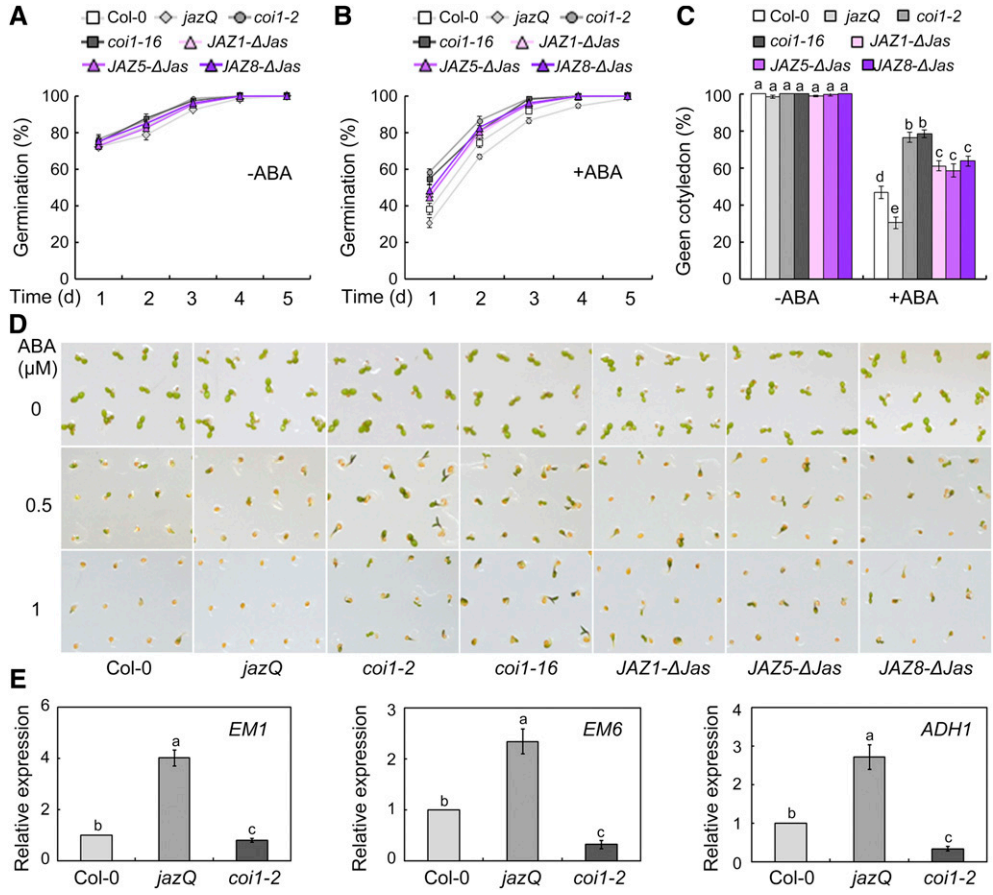

Figure 2. COI1/JAZ-Mediated JA Pathway Is Positively Associated with ABA Signaling during Seed Germination.

(A) and **(B)** Germination of the wild type (Col-0), *jazQ*, *coi1-2*, *coi1-16*, *JAZ1-ΔJas*, *JAZ5-ΔJas*, and *JAZ8-ΔJas* seeds was scored 1 to 5 d after stratification on water agar medium supplemented without ABA **(A)** or with 0.5 μM ABA **(B)**.

(C) Percentage of Col-0, *jazQ*, *coi1-2*, *coi1-16*, *JAZ1-ΔJas*, *JAZ5-ΔJas*, and *JAZ8-ΔJas* seedlings with expanded cotyledons was scored 5 d after stratification on water agar medium supplemented with 0.5 μM ABA.

(D) Seedlings of Col-0, *jazQ*, *coi1-2*, *coi1-16*, *JAZ1-ΔJas*, *JAZ5-ΔJas*, and *JAZ8-ΔJas* observed 4 d after stratification on water agar medium supplemented with 0.5 and 1 μM ABA. Average and significance were calculated over three biological replicates by analyzing seeds of different batches. Each batch of seeds for each genotype was pooled from at least 15 independent plants. For every biological replicate, we examined the seeds from the same batch at least three times as technical replicates. The value of each biological replicate was the average calculated over three technical replicates by analyzing more than 150 seeds. Data from three biological replicates were analyzed by ANOVA. Values with different letters are significantly different from each other ($P < 0.05$). Bar = 30 mm.

(E) Expression of several ABA-responsive genes in *jazQ* and *coi1-2*. Dry seeds of Col-0, *jazQ*, and *coi1-2* were collected. Average and significance were calculated over three biological replicates. Total RNA was extracted from at least three batches of seeds as biological replicates. Each batch of seeds for Col-0, *jazQ*, and *coi1-2* was pooled from at least 15 independent plants, respectively. For each biological replicate, more than 250 seeds of the same batch were used for RNA extraction. *AT1G13320* gene was used as control. Data from three biological replicates were analyzed by ANOVA. Values with different letters are significantly different from each other ($P < 0.05$). Error bars show SD from three biological replicates.

the effects of Suc and/or nitrate. In parallel experiments, compared with Col-0, in our hands and experimental conditions, *coi1-16* (another loss-of-function mutant of *COI1*) showed reduced sensitivity to ABA during seed germination and post-germinative growth (Figures 2A to 2D; Supplemental Figures 2A and 3). To further confirm that disruption of COI1 is responsible for the ABA-insensitive phenotypes of *coi1-2* and *coi1-16*, we introduced the full-length *COI1* cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter into *coi1-2* and *coi1-16* mutant plants: *coi1-2 COI1* and *coi1-16 COI1*. Expression of *COI1* in *coi1-2* and *coi1-16* fully complemented the MeJA-insensitive root elongation phenotype (Supplemental Figure 4). Moreover, *coi1-2 COI1* and *coi1-16 COI1* plants had similar responses to those of Col-0 to ABA during seed germination (Supplemental Figure 5). To avoid unspecific effects due to misexpression of *COI1* under the control of the 35S promoter, we also introduced *COI1* into the *coi1-2* and *coi1-16* backgrounds under the control of its native promoter (*coi1-2/COI1pro*:*COI1* and *coi1-16/COI1pro*:*COI1*). The *coi1-2/COI1pro*:*COI1* and *coi1-16/COI1pro*:*COI1* seeds consistently exhibited percentages of germination and cotyledon greening

similar to those of Col-0 under ABA treatment (Supplemental Figure 5). These results suggest that endogenous JA perception or signaling positively mediates ABA responses during seed germination and early seedling growth.

To further confirm the involvement of JA signaling components in ABA responses, we investigated whether genetic manipulation of JAZ proteins, which are the substrates of the SCF^{COI1} E3 ubiquitin ligase and repressors of JA signaling (Chini et al., 2007; Thines et al., 2007), affects ABA-repressed seed germination. Phenotypic analysis showed that the *jazQ* mutant (with T-DNA insertion mutations in *JAZ1/3/4/9/10* genes; Campos et al., 2016) was more sensitive than Col-0 to ABA during seed germination and seedling establishment (Figures 2A to 2D). Consistently, compared with seeds of the wild type, seeds of the *jaz* decuple mutant (*jazD*; defective in *JAZ1-7*, *-9*, *-10*, and *-13*; Guo et al., 2018) were more sensitive to ABA (Supplemental Figure 2B). To further confirm this possibility, we expressed the full-length cDNA of *JAZ1* under the control of the CaMV 35S promoter (*JAZ1-OE*). However, there were no differences in the percentages of germination and cotyledon greening between the homozygous *JAZ1-OE* plants and Col-0 plants with or without ABA. Similar results were obtained for *JAZ5-OE* and *JAZ8-OE* seeds (Supplemental Figures 6A and 6B).

Next, we generated transgenic plants overexpressing *JAZ1*, *JAZ5*, or *JAZ8* with a deleted *Jas* domain (*JAZ1-ΔJas*, *JAZ5-ΔJas*, or *JAZ8-ΔJas*). The *JAZ1-ΔJas* plants have been shown to accumulate large amounts of the JAZ1 repressor and are insensitive to JA treatment (Chini et al., 2007; Thines et al., 2007). As *JAZ1-ΔJas* homozygous plants are sterile, seeds from heterozygous plants were used in the germination assays. When incubated on medium containing 0.5 μM ABA, *JAZ1-ΔJas* heterozygous (F1 progeny from a cross with the wild type) lines displayed much higher percentages of germination and cotyledon greening than Col-0 (Figures 2A to 2D). Compared with the wild-type seeds, those of *JAZ5-ΔJas* or *JAZ8-ΔJas* were also less insensitive to ABA (Figures 2A to 2D). Among the *JAZ1-ΔJas* heterozygous seeds germinated on medium containing 0.75 μM ABA for 3.5 d, most of the seedlings with green cotyledons were *JAZ1-ΔJas* transgenic plants ($n = 80$, 91.7%). The transcript levels of several ABA-responsive genes were consistently lower in *coi1-2* than in Col-0 in response to ABA (Figure 2E) but were higher in the *jazQ* mutant than in Col-0 (Figure 2E).

Taken together, these findings provide further evidence that the endogenous JA pathway is positively involved in ABA signaling during seed germination.

JAZ Repressors Physically Interact with ABI3

Recent studies have revealed that JAZ repressors physically interact with numerous transcription factors to modulate different aspects of JA signaling processes (Qi et al., 2011; Song et al., 2011; Zhu et al., 2011; Hu et al., 2013; Jiang et al., 2014). To test the interactions between JAZ1 and transcription factors, the full-length coding sequence (CDS) of *JAZ1* was fused to pGBK-T7 to construct a bait vector, which was transformed into the yeast (*Saccharomyces cerevisiae*) strain Y2HGOLD (Clontech). Yeast screening was performed as described previously (Hu et al., 2013), and the cDNA library was acquired from Clontech (catalog no. 630487). After screening, more than 10 independent colonies were isolated. Among these positive clones, clones encoding ABI5, TOE1, TOE2, MYC2, and NOVEL INTERACTOR OF JAZ (NINJA) were frequently sequenced. ABI3 was sequenced seven times through this screening procedure.

To further confirm the interaction between JAZs and ABI3, we generated the full-length ABI3 in the bait vector pGBKT7 (BD-ABI3), and each *JAZ* (Supplemental Data Set 1) gene was introduced into the Gal4-activation domain of the pGAD-T7 vector to act as prey (AD-JAZ). The bait and prey vectors were co-transformed into yeast. Previous studies have reported the interactional relationships of ABI4-MED18, JAZ4-WRKY57, JAZ8-WRKY57, and IAA29-WRKY57 (Supplemental Figure 7; Jiang et al., 2014; Lai et al., 2014). Physical interactions were detected between ABI3 and JAZ1, JAZ5, and JAZ8 (splicing variant of JAZ1, JAZ4, JAZ5, and JAZ8 was AT1G19180.1, AT1G48500.1, AT1G17380.1, and AT1G30135.1, respectively; Figures 3A and 3B; Supplemental Figure 7; Supplemental Data Set 1). ABI4 is a well-known transcription factor that positively modulates ABA responses during seed germination. However, no interaction was detected between ABI4 and JAZ (Figure 3B), suggesting that JAZ proteins involved in the ABA response interact with ABI3, but not ABI4.

These interactions between JAZ proteins and ABI3 were confirmed in plant cells using bimolecular florescence complementation (BiFC) and coimmunoprecipitation (Co-IP) assays. For the BiFC assays, each JAZ was fused to the N-terminal yellow fluorescent protein (YFP) fragment to generate JAZ-nYFP, while ABI3 and ABI4 were each fused to the C-terminal fragment of YFP (cYFP) to produce ABI3-cYFP and ABI4-cYFP, respectively. When ABI3-cYFP was transiently coexpressed with JAZ1-nYFP, JAZ5-nYFP, or JAZ8-nYFP in leaf cells of *Nicotiana benthamiana*, reconstituted YFP fluorescence was observed in the nucleus, as revealed by staining with 4′,6-diamidino-2-phenylindole (Figure 3C). As the negative control, ABI3-cYFP (or ABI4-cYFP) was coexpressed with JAZ4-nYFP and accumulated (or JAZ1-nYFP, JAZ5-nYFP, and JAZ8-nYFP) in *N. benthamiana* leaf cells, and no fluorescence was detected (Figure 3C; Supplemental Figure 8). For the Co-IP assays, the 3MYC-ABI3 construct was coexpressed with hemagglutinin (HA)-JAZ1, HA-JAZ5, or HA-GFP in *N. benthamiana* leaves. Cell extracts were immunopurified with an anti-MYC antibody, and the resultant immunoprecipitants were separated by SDS-PAGE and probed with an anti-HA antibody. The HA-JAZ1 and HA-JAZ5 fusion proteins were detected in cell extracts when they were coexpressed with 3MYC-ABI3 in *N. benthamiana* leaves (Figures 3D and 3E; Supplemental Figure 9). The control HA-GFP fusion protein was not detected in cell extracts when it was coexpressed with 3MYC-ABI3 (Figures 3D and 3E; Supplemental Figure 9). These results show that JAZ proteins physically interact with ABI3 in planta.

Having ascertained that JAZ proteins physically interact with ABI3, we wondered whether JAZs are involved in ABI3-mediated ABA signaling during seed germination. To test this possibility, we further analyzed the properties of JAZs in more detail. To examine *JAZ* expression in different tissues, we analyzed its transcript levels by RT-qPCR. Interestingly, relatively high transcript levels of *JAZ1*, *JAZ5*, and *JAZ8* were detected in dry seeds (Supplemental Figures 6C to 6E), indicating that JAZ proteins play roles in seed germination.

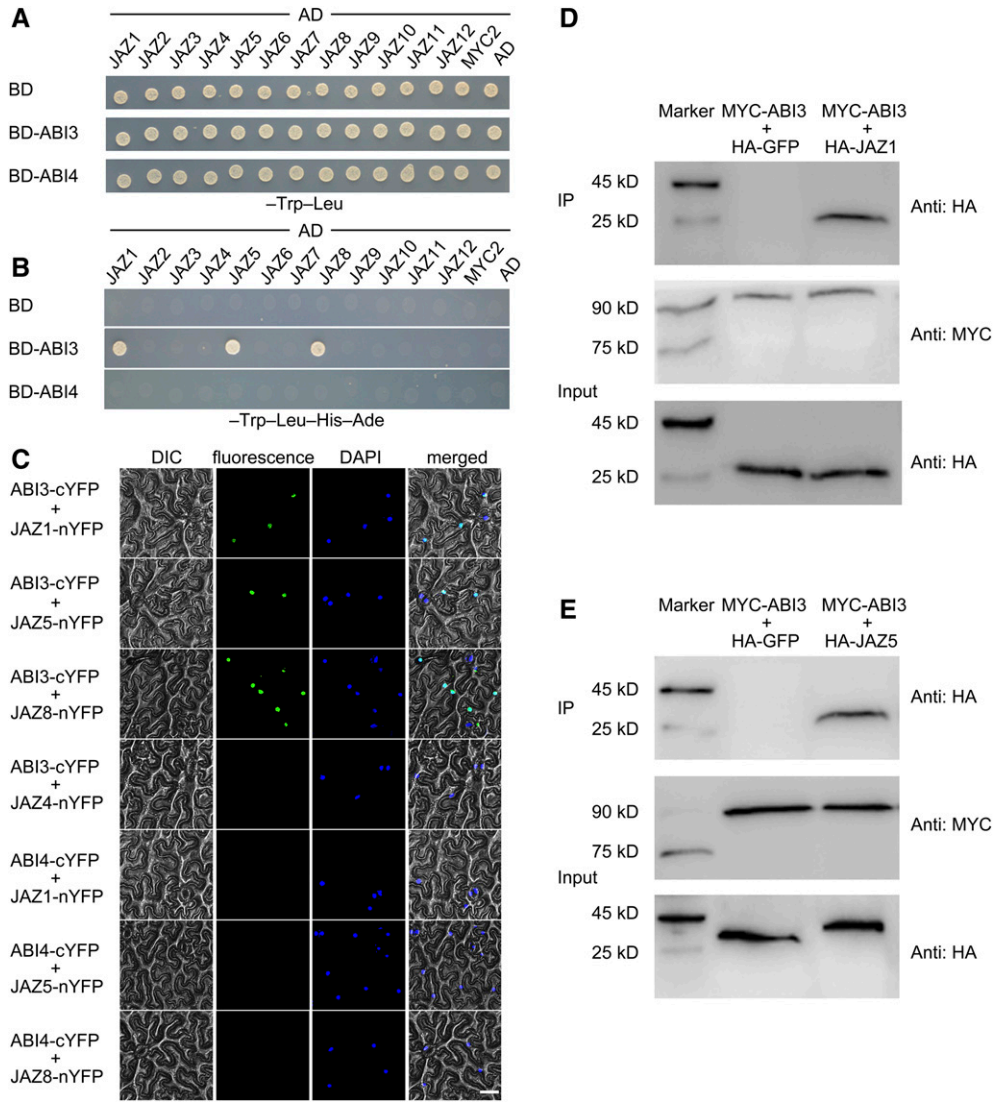

Figure 3. Physical Interactions between JAZ and ABI3.

(A) and **(B)** Y2H assay to detect interactions of ABI3 and ABI4 with JAZ proteins. Interaction was indicated by the ability of cells to grow on dropout medium lacking Leu and Trp **(A)**. Interaction was indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade and containing 20 mM 3-aminotriazole for 4 d **(B)**. pGBKT7 (BD), and pGADT7 (AD) vectors were used as the negative control.

(C) BiFC analyses. JAZ1, JAZ4, JAZ5, and JAZ8 were fused to nYFP; ABI3 and ABI4 were fused to cYFP. Fluorescence was observed in the nuclear compartment of transformed cells, which resulted from complementation of the C-terminal part of YFP fused with ABI3 (ABI3-cYFP) or ABI4-cYFP with the N-terminal part of YFP fused with JAZ (JAZ-nYFP). The localization of the nuclei was detected by 4′,6-diamidino-2-phenylindole (DAPI) staining. Bar = 15 μm. DIC, differential interference contrast.

(D) and **(E)** Co-IP analyses. Protein extracts from *N. benthamiana* leaves coexpressing MYC-tagged ABI3 with HA-fused JAZ1, JAZ5, or GFP were immunoprecipitated (IP) with HA antibody-bound agarose beads, and the immunoprecipitated proteins were analyzed by immunoblotting using anti-MYC or anti-HA antibody. The experiments were repeated three times with similar results.

The N-Terminal Fragment of ABI3 Interacts with the Middle Region of JAZ That Contains ZIM Domain

To examine which regions of ABI3 are required to interact with JAZ proteins, we performed directed yeast two-hybrid (Y2H) analyses. ABI3 was divided into the N-terminal region (BD-ABI3-N) and the C-terminal region containing the B2 and B3 domains (BD-ABI3-C; Figure 4A). Deletion of the C-terminal residues of ABI3 (BD-ABI3-C) did not affect its interactions with JAZ1 and JAZ5 (Supplemental Data Set 1); however, deletion of the N-terminal fragment of ABI3 abolished its interactions with these proteins (Figure 4A). Therefore, the N-terminal region of ABI3 is required for its interactions with JAZ1 and JAZ5.

We used the same approach to identify the domains of JAZ proteins that are required for their interactions with ABI3. JAZ1 and JAZ8 (Supplemental Data Set 1) were divided into different

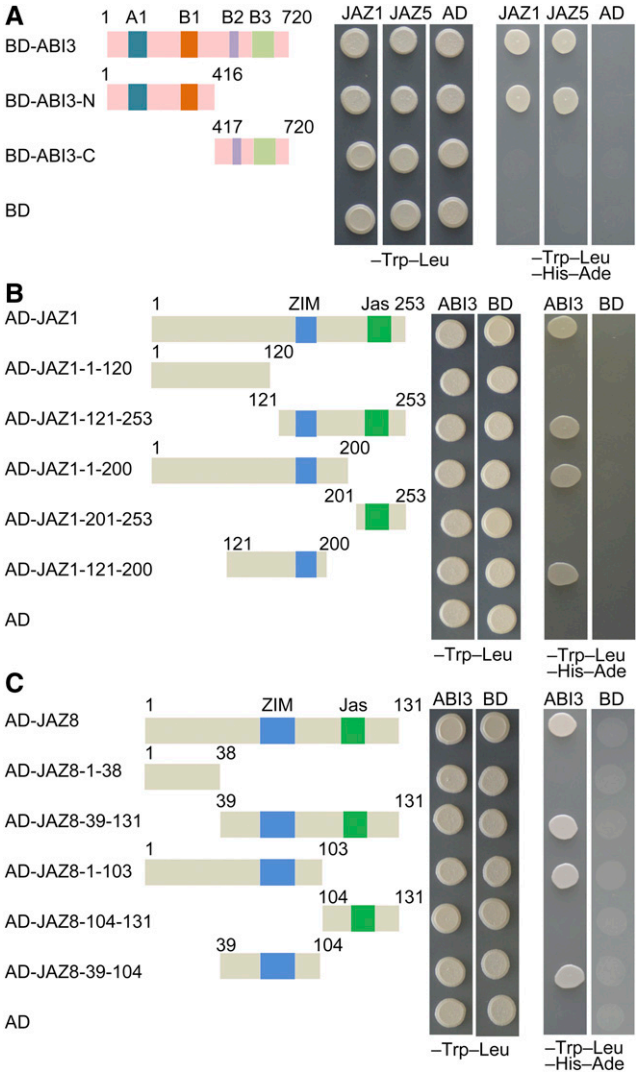

Figure 4. Y2H Assays for Identifying ABI3 and JAZ Regions Required for Their Interaction.

(A) N-Terminal fragment of ABI3 is involved in its interaction with JAZ proteins. (Left) Diagram of full-length and truncated ABI3 constructs with specific deletions. (Right) Interaction was indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade and containing 20 mM 3-aminotriazole for 4 d. The vectors pGBKT7 (BD) and pGADT7 (AD) were used as negative controls. ABI3 was divided into the N-terminal region (BD-ABI3-N, containing the A1 and B1 domains) and the C-terminal region (containing the B2 and B3 domains).

(B) and **(C)** ZIM domain of JAZ1 **(B)** or JAZ8 **(C)** is responsible for their interactions with ABI3. (Left) Diagram of full-length and truncated JAZ constructs with specific deletions. (Right) Interaction was indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade and containing 20 mM 3-aminotriazole for 4 d. The vectors BD and AD were used as negative controls.

truncated versions and then independently fused with the Gal4-activation domain to generate prey vectors (Figures 4B and 4C). The potential interactions between these derivatives and ABI3 were then assayed using the yeast two-hybrid system. Deletion of the C-terminal 133 residues of JAZ1 (AD-JAZ1-1-120) eliminated its interaction with ABI3, while deleting its N-terminal fragment (AD-JAZ1-121-253) had no effect (Figure 4B). Further mapping revealed that the middle region of JAZ1 containing the ZIM domain was required for the JAZ1-ABI3 interaction. Likewise, the middle region of JAZ8 containing the ZIM domain was essential for the JAZ8-ABI3 interaction (Figure 4C).

JA Activation of ABA Signaling Requires Functional ABI3 and ABI5

The ABI3 and ABI5 transcription factors are critical regulators of ABA signaling during seed germination and early seedling growth. Phenotypic analyses have indicated that the loss-of-function mutants *abi3* and *abi5* are insensitive to ABA (Koornneef et al., 1984; Giraudat et al., 1992; Finkelstein, 1994; Finkelstein and Lynch, 2000). Given that several JAZ repressors of the JA signaling pathway physically associate with ABI3 and ABI5 (Figure 3; Ju et al., 2019), we hypothesized that JA stimulates ABA responses during seed germination and that this requires ABI3 and/or ABI5. To test this possibility, we determined the percentages of germination and expanded cotyledons of *abi3* (*abi3-1* mutant backcrossed six times with the Col-0 wild type) and *abi5* (*abi5-1* mutant backcrossed six times with the Col-0 wild type) seeds on media containing ABA and/or COR. Consistent with previous studies (Koornneef et al., 1984; Giraudat et al., 1992; Finkelstein, 1994), *abi3* and *abi5* seeds germinated and grew faster than Col-0 seeds on ABA-containing medium, and the germination percentages of *abi3-8* and *abi5-7* were higher than that of Col-0 (Figure 5). Furthermore, compared with Landsberg *erecta* (L*er*) and Wassilewskija (Ws), the *abi3-1* and *abi5-1* single mutants exhibited much higher percentages of germination and expanded cotyledons on media containing ABA, COR, or both ABA and COR (Figure 5). Because ABI5 physically interacts with ABI3 (Nakamura et al., 2001), we generated an *abi5 abi3* double mutant (backcrossed *abi5* crossed with backcrossed *abi3*) and analyzed its response to ABA and COR. Compared with *abi3*, *abi5*, and Col-0, a higher percentage of the double mutants had expanded cotyledons in the presence of both ABA and COR (Figure 5). These observations indicate that JA activates ABA signaling to delay seed germination and that this requires functional ABI3 and ABI5.

The ABI3 and ABI5 transcription factors are recognized as key regulators of seed dormancy in Arabidopsis (Bentsink and Koornneef, 2008). Because JAZ proteins physically interact with ABI3, we questioned whether COI1 and JAZ are also involved in modulating seed dormancy. To test this possibility, we examined the fresh mature siliques of *coi1-2* and *JAZ1-ΔJas* plants after 5 d on water-saturated filter paper at 22°C without stratification. The seed dormancy level of *coi1-2* and *JAZ1-ΔJas* was similar to that of the wild type (Supplemental Figure 10). However, seed dormancy was significantly reduced in the *abi3* mutant, consistent with previous studies (Ooms et al., 1993; Finkelstein, 1994; Liu et al., 2013). These results suggest that the COI1/JAZ-mediated JA pathway does not regulate seed dormancy in Arabidopsis

JAZ Proteins Repress the Transcriptional Activation Roles of ABI3 and ABI5

Because JAZ proteins directly interact with ABI3 (Figure 3) and ABI5 (Ju et al., 2019), we investigated whether JAZ proteins

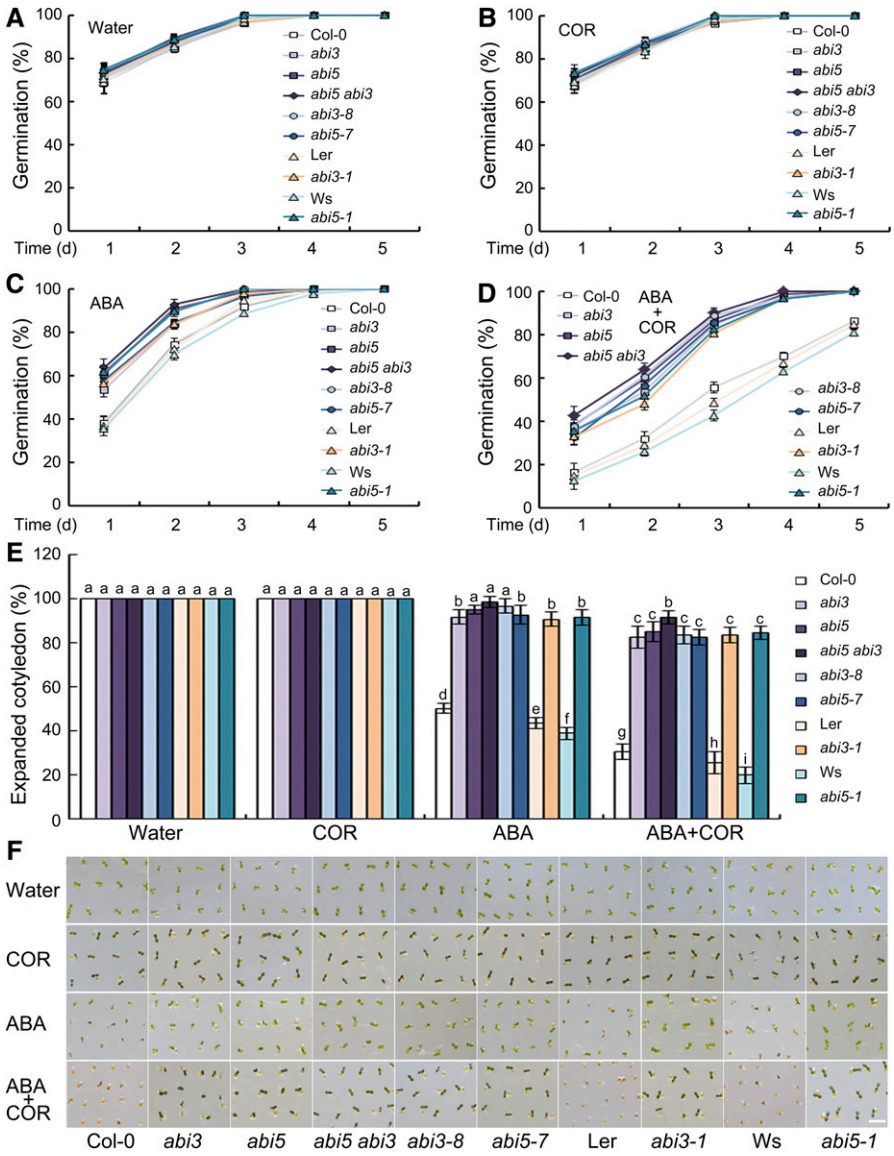

Figure 5. JA Promotes ABA Signaling during Seed Germination Requiring ABI3 and ABI5.

(A) to **(D)** Germination of Col-0, *abi3*, *abi5*, *abi5 abi3*, *abi3-8*, *abi5-7*, Ler, *abi3-1*, Ws, and *abi5-1* seeds was scored 1 to 5 d after stratification on water agar medium **(A)** supplemented with 1 μM COR **(B)**, 0.5 μM ABA **(C)**, or both 0.5 μM ABA and 1 μM COR **(D)**, respectively.

(E) Percentage of Col-0, *abi3*, *abi5*, *abi5 abi3*, *abi3-8*, *abi5-7*, Ler, *abi3-1*, Ws, and *abi5-1* seedlings with expanded cotyledons was scored 5 d after stratification on water agar medium supplemented with 1 μM COR, 0.5 μM ABA, or both 0.5 μM ABA and 1 μM COR, respectively.

(F) Seedlings of Col-0, *abi3*, *abi5*, *abi5 abi3*, *abi3-8*, *abi5-7*, Ler, *abi3-1*, Ws, and *abi5-1* observed 4 d after stratification on water agar medium supplemented with 1 μM COR, 0.5 μM ABA, or both 0.5 μM ABA and 1 μM COR, respectively. Average and significance were calculated over three biological replicates by analyzing seeds of different batches. Each batch of seeds for each genotype was pooled from at least 15 independent plants. For every biological replicate, we examined the seeds from the same batch at least three times as technical replicates. The value of each biological replicate was the average calculated over three technical replicates by analyzing more than 150 seeds. Data from three biological replicates were analyzed by ANOVA. Values with different letters are significantly different from each other ($P < 0.05$). Error bars show SD from three biological replicates. Bar = 30 mm.

interfere with the transcriptional activation roles of ABI3 and/or ABI5. To this end, we used dual-luciferase (LUC) reporter assays to examine the effects of JAZ1, JAZ5, or JAZ8 on the transcriptional functions of ABI3 or ABI5 in Arabidopsis mesophyll protoplasts (Yoo et al., 2007). Because *EM1*, *EM6*, and *ABI5* are downstream targets of ABI5 and ABI3 (Lopez-Molina and Chua, 2000; Nakamura et al., 2001; Carles et al., 2002; Lopez-Molina et al., 2002), their promoters were fused to the *LUC* gene to generate reporters (Figure 6A). The effector constructs contained *ABI5*, *ABI3*, *JAZ*, or *GFP* driven by the CaMV 35S promoter (Figure 6A). *ABI3* expression activated *LUC* expression driven by the *EM6* or *ABI5* promoter with ABA treatment, compared with the

expression of the *GFP* control (Figures 6B and 6C). Moreover, compared with expression of *ABI3* alone, coexpression of *JAZ1* or *JAZ5* with *ABI3* repressed the *LUC* expression level (Figures 6B and 6C). Similarly, *ABI5* expression dramatically activated *LUC* expression driven by the *EM6* or *EM1* promoter in the presence of ABA in comparison with expression of the *GFP* control (Figures 6D and 6E). However, compared with expression of *ABI5* alone, coexpression of *ABI5* with *JAZ8* suppressed *LUC* expression (Figures 6D and 6E). These results demonstrate that JAZ proteins repress the transcriptional activation roles of ABI3 and ABI5, thereby preventing activation of their downstream targets.

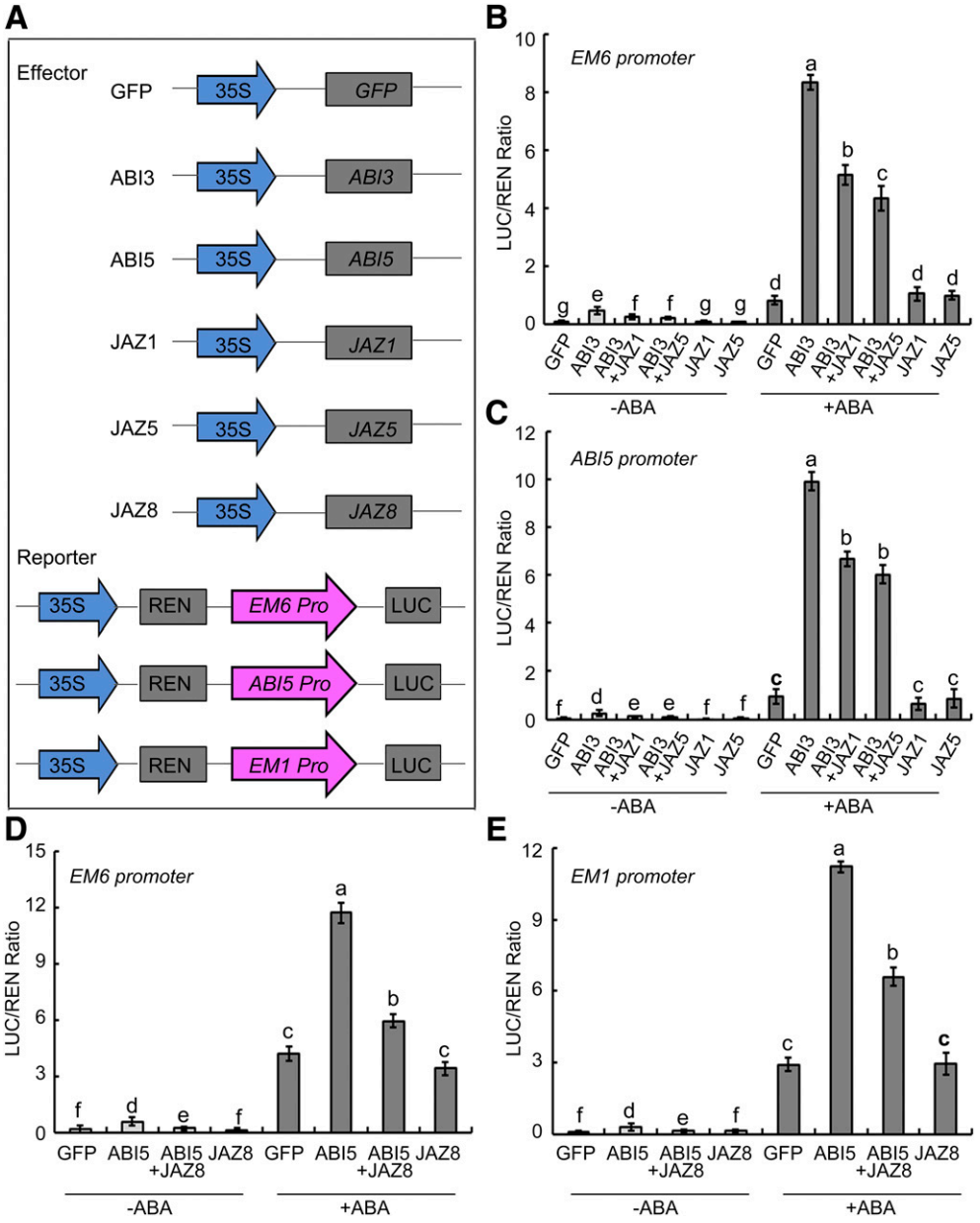

Figure 6. JAZ Proteins Repress the Transcriptional Activities of ABI3 and ABI5.

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

(B) and **(C)** JAZ1 and JAZ5 repress ABI3 to activate *EM6* **(B)** and *ABI5* **(C)** in response to 5 μM ABA. Error bars show SD from three biological replicates; each biological replication was from different wild-type leaves. Data were analyzed by ANOVA. Values with different letters are significantly different from each other ($P < 0.05$). REN, *Renilla* LUC.

(D) and **(E)** JAZ8 represses ABI5 to activate *EM6* and *EM1* in response to 5 μM ABA. Error bars show SD from three biological replicates; each biological replication was from different wild-type leaves. Data were analyzed by ANOVA. Values with different letters are significantly different from each other ($P < 0.05$). REN, *Renilla* LUC.

Overexpression of *ABI3* and *ABI5* Simultaneously Suppresses ABA-Insensitive Phenotypes of *coi1-2* and *JAZ8-ΔJas*

Having ascertained that JAZ proteins suppress the transcriptional roles of ABI3 and ABI5 (Figure 6), we queried whether overexpression of *ABI3* and/or *ABI5* could overcome JAZ repression and rescue the ABA-insensitive phenotypes of the *coi1* mutants in which JAZ proteins are highly accumulated (Chini et al., 2007; Thines et al., 2007). To test this hypothesis, we generated transgenic plants overexpressing *ABI3* (*ABI3-OE*). The RT-qPCR analyses showed that the *ABI3-OE* lines constitutively expressed *ABI3* at high levels (Supplemental Figure 11A). Next, we overexpressed *ABI3-OE-5* in the *coi1-2* and *coi1-16* backgrounds to produce *coi1-2 ABI3-OE* and *coi1-16 ABI3-OE* plants. Compared with Col-0, the *coi1-2 ABI3-OE* and *coi1-16 ABI3-OE* plants displayed higher percentages of germination and expanded cotyledons (Figure 7; Supplemental Figures 12A and 12B). However, *coi1-2 ABI3-OE* and *coi1-16 ABI3-OE* seeds were more sensitive to ABA and COR during germination than were *coi1-2* and *coi1-16* seeds. Because ABI5 and ABI3 positively modulate ABA signaling during seed germination, we simultaneously overexpressed *ABI3* and *ABI5* by overexpressing *ABI5* (the validated overexpression line *ABI5-OE-8*; Supplemental Figure 11B) in the *coi1-2 ABI3-OE* and *coi1-16 ABI3-OE* backgrounds (*coi1-2 ABI3-OE ABI5-OE* and *coi1-16 ABI3-OE ABI5-OE*). Compared with *coi1-2* and *coi1-16*, the *coi1-2 ABI3-OE ABI5-OE* and *coi1-16 ABI3-OE ABI5-OE* lines exhibited significantly lower percentages of germination and expanded cotyledons when grown on medium supplemented with both ABA and COR (Figure 7; Supplemental Figures 12A and 12B). Thus, simultaneous overexpression of *ABI3* and *ABI5* suppressed the ABA-insensitive phenotypes of the *coi1-2* and *coi1-16* mutants.

To further understand the regulatory relationship between JAZ repressors and ABI3 or ABI5, we determined whether overexpression of *ABI3* and/or *ABI5* could rescue the ABA-insensitive phenotypes of *JAZ-ΔJas* transgenic plants in which JAZ proteins are also highly accumulated (Figure 2; Chini et al., 2007; Thines et al., 2007). To test this hypothesis, we generated *JAZ8-ΔJas* plants and then crossed them with *ABI3-OE-5* to create *JAZ8-ΔJas ABI3-OE* plants. The percentages of germination and expanded cotyledons were lower in *JAZ8-ΔJas ABI3-OE* lines than in *JAZ8-ΔJas* lines in response to both ABA and COR (Figure 8). Moreover, overexpression of *ABI5* (*ABI5-OE-8*) in the *JAZ8-ΔJas ABI3-OE* background rendered the transgenic lines much more sensitive to ABA and COR during seed germination (Figure 8). Similar data were also obtained when *ABI3* and *ABI5* were expressed in the *JAZ5-ΔJas* background (Supplemental Figures 12C and 12D). Thus, simultaneous overexpression of *ABI3* and *ABI5* suppressed the ABA-insensitive phenotypes of *JAZ-ΔJas* plants. These findings support the idea that ABI3/ABI5 and JAZ proteins modulate ABA and JA signaling during seed germination.

Because ABI3 interacts with JAZ, the *jazQ* and *jazD* mutants were sensitive to ABA during seed germination, and overexpression of *ABI3* and *ABI5* suppressed the ABA-insensitivity of *coi1-2* and *coi1-16*. We speculated that signaling mediated by the receptor COI is required to induce ABA responses. Therefore, we performed RNA-sequencing experiments to profile the transcriptomes of *coi1-2*, *coi1-16*, and Col-0 with or without ABA treatments. Total RNAs were isolated from seeds treated with or without ABA. We sequenced each sample on the Illumina HiSeq X Ten platform. Sequenced reads were trimmed to remove adaptor sequences, and low-complexity or low-quality sequences were removed using Trimmomatic (0.36) with the following parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW: 4:15 MINLEN:50. Clean reads were mapped to the genome of TAIR10.1_NCBI_year_2018 using Hisat2 (2.2.1.0) with default parameters. The read counts of each gene were obtained using htseq-count (0.9.1).

Under normal conditions (no ABA treatment), compared with the wild type, *coi1-2* and *coi1-16* had 84 upregulated genes and 160 downregulated genes (Figure 9A). We classified the genes coregulated by COI1 under normal conditions and found that they encode proteins with various biological functions, including seed oil body biogenesis, lipid storage, and response to ABA (Figure 9B). Under ABA treatment, 106 genes were upregulated and 210 genes were downregulated in both *coi1-2* and *coi1-16*, compared with the wild type (Figure 9C).

To identify the genes responsible for the ABA-insensitive phenotypes of the *coi1-2* and *coi1-16* mutants, we examined the overlap between coregulated genes in the two mutants and those regulated by ABA signaling in the wild type. Among all the coregulated genes in *coi1-2* and *coi1-16*, 16 were responsive to ABA (Figure 9D; Supplemental Data Set 2). We then compared the expression levels of these coregulated and ABA-responsive genes under normal and ABA treatment conditions. A heatmap analysis indicated that the expression levels of most coregulated genes were lower in *coi1-2* and *coi1-16* than in the wild type under both normal and ABA treatment conditions (Figure 9E).

We used RT-qPCR to confirm the transcript levels of selected genes from the set of coregulated and ABA-responsive genes, including *CRU1*, *CRU3*, *LEA*, and *RAB18*. The analyses revealed that those genes' transcript levels were lower in *coi1-2* and *coi1-16* than in the wild type (Figure 9F).

Ju et al. (2019) found that ABA treatments could promote JA biosynthesis. Consistent with this, qRT-PCR confirmed that several JA biosynthesis genes (*ACX1*, *AOC3*, *KAT5*, and *OPR3*) were upregulated by ABA treatment in the wild type (Supplemental Figures 13E to 13H). The transcription of ABA biosynthesis genes (*ABA1*, *ABA2*, *ABA3*, and *NCED3*) also was upregulated by exogenous ABA (Supplemental Figures 13A to 13D). However, under ABA treatment, the ABA induction of these ABA biosynthetic genes was reduced in *coi1-2* and *coi1-16* compared with the wild type (Supplemental Figures 13I to 13L). Similarly, the transcript levels of these JA biosynthetic genes were decreased in the *coi1-2* and *coi1-16* mutants (Supplemental Figures 13M to 13P). These results indicate that ABA treatment promotes JA and ABA biosynthesis, while ABA-induced JA and ABA biosyntheses are reduced in the *coi1-2* and *coi1-16* mutants and increased in the *jazQ* mutant (Supplemental Figure 13).

DISCUSSION

The phytohormone JA is ubiquitous in the plant kingdom and regulates multiple physiological aspects of plant development, growth, and stress responses. Several studies have highlighted the involvement of JA in seed germination and have suggested

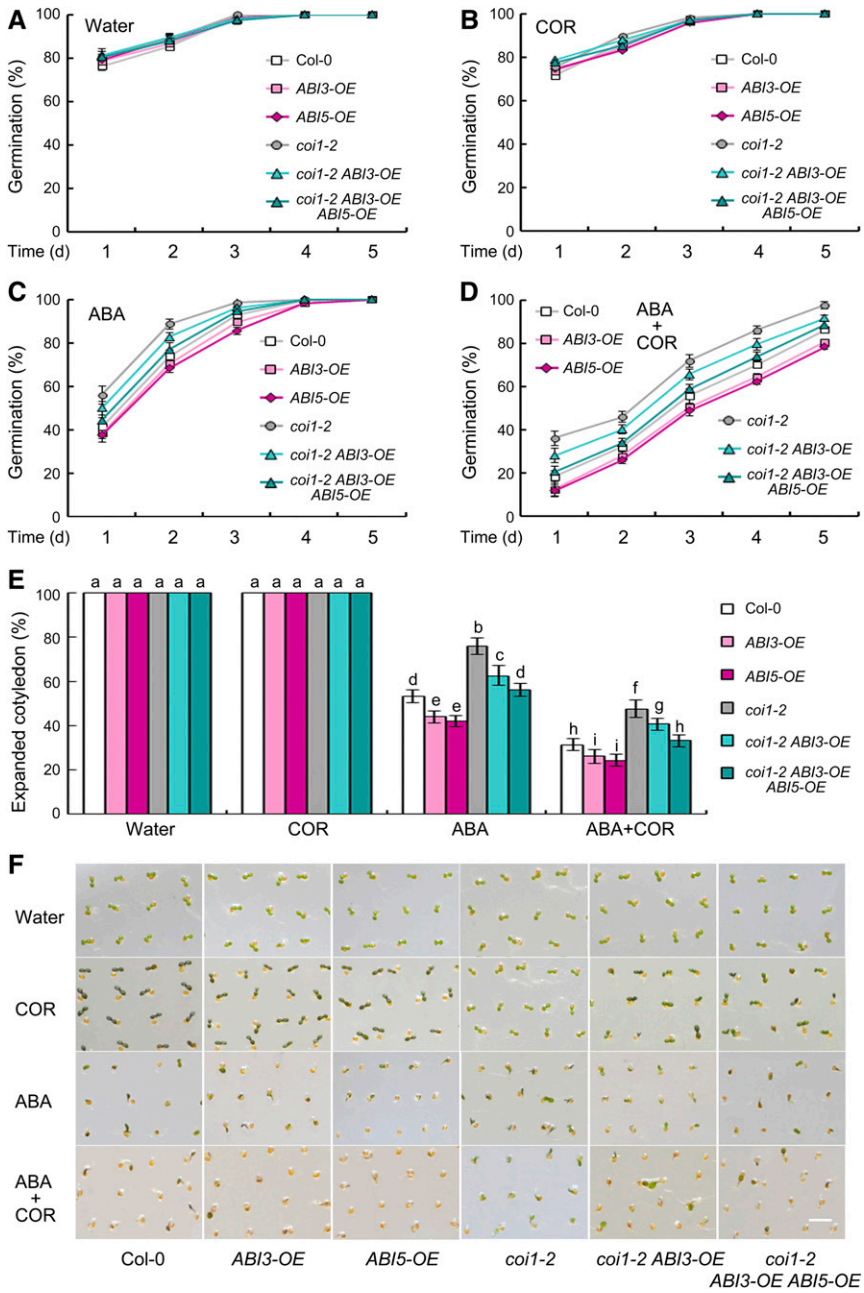

Figure 7. Overexpression of *ABI3* and *ABI5* Simultaneously Suppresses the ABA-Insensitive Phenotypes of *coi1-2* Mutant.

(A) to **(D)** Germination of Col-0, *ABI3-OE*, *ABI5-OE*, *coi1-2*, *coi1-2 ABI3-OE*, and *coi1-2 ABI3-OE ABI5-OE* seeds was scored 1 to 5 d after stratification on water agar medium **(A)** supplemented with 1 μM COR **(B)**, 0.5 μM ABA **(C)**, or both 0.5 μM ABA and 1 μM COR **(D)**, respectively.
(E) Percentage of Col-0, *ABI3-OE*, *ABI5-OE*, *coi1-2*, *coi1-2 ABI3-OE*, and *coi1-2 ABI3-OE ABI5-OE* seedlings with expanded cotyledons was scored 5 d after stratification on water agar medium supplemented with 1 μM COR, 0.5 μM ABA, or both 0.5 μM ABA and 1 μM COR, respectively.
(F) Seedlings of Col-0, *ABI3-OE*, *ABI5-OE*, *coi1-2*, *coi1-2 ABI3-OE*, and *coi1-2 ABI3-OE ABI5-OE* observed 4 d after stratification on water agar medium supplemented with 1 μM COR, 0.5 μM ABA, or both 0.5 μM ABA and 1 μM COR, respectively. Average and significance were calculated over three biological replicates by analyzing seeds of different batches. Each batch of seeds for each genotype was pooled from at least 18 independent plants. For every biological replicate, we examined the seeds from the same batch at least three times as technical replicates. The value of each biological replicate was the average calculated over three technical replicates by analyzing more than 150 seeds. Data from three biological replicates were analyzed by ANOVA. Values with different letters are significantly different from each other ($P < 0.05$). Error bars show SD from three biological replicates. Bar = 30 mm.

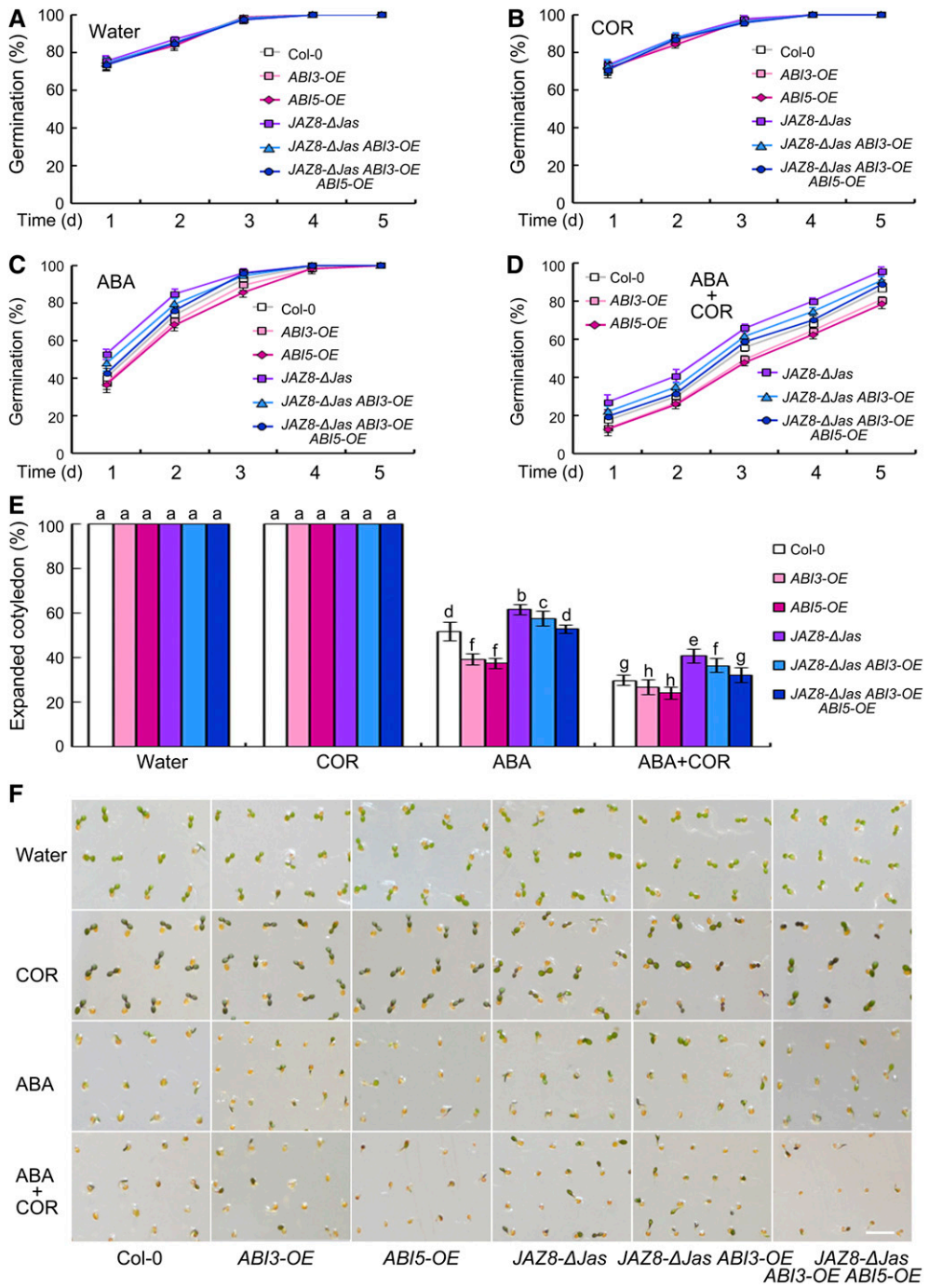

Figure 8. Overexpression of *ABI3* and *ABI5* Simultaneously Suppresses the ABA-Insensitive Phenotypes of *JAZ8-ΔJas*.

(A) to **(D)** Germination of Col-0, *ABI3-OE*, *ABI5-OE*, *JAZ8-ΔJas*, *JAZ8-ΔJas ABI3-OE*, and *JAZ8-ΔJas ABI3-OE ABI5-OE* seeds was scored 1 to 5 d after stratification on water agar medium **(A)** supplemented with 1 μM COR **(B)**, 0.5 μM ABA **(C)**, or both 0.5 μM ABA and 1 μM COR **(D)**, respectively.
(E) Percentage of Col-0, *ABI3-OE*, *ABI5-OE*, *JAZ8-ΔJas*, *JAZ8-ΔJas ABI3-OE*, and *JAZ8-ΔJas ABI3-OE ABI5-OE* seedlings with expanded cotyledons was scored 5 d after stratification on water agar medium supplemented with 1 μM COR, 0.5 μM ABA, or both 0.5 μM ABA and 1 μM COR, respectively.
(F) Seedlings of Col-0, *ABI3-OE*, *ABI5-OE*, *JAZ8-ΔJas*, *JAZ8-ΔJas ABI3-OE*, and *JAZ8-ΔJas ABI3-OE ABI5-OE* observed 4 d after stratification on water agar medium supplemented with 1 μM COR, 0.5 μM ABA, or both 0.5 μM ABA and 1 μM COR, respectively. Average and significance were calculated over three biological replicates by analyzing seeds of different batches. Each batch of seeds for each genotype was pooled from at least 18 independent plants. For every biological replicate, we examined the seeds from the same batch at least three times as technical replicates. The value of each biological replicate was the average calculated over three technical replicates by analyzing more than 150 seeds. Data from three biological replicates were analyzed by ANOVA. Values with different letters are significantly different from each other ($P < 0.05$). Error bars show SD from three biological replicates. Bar = 30 mm.

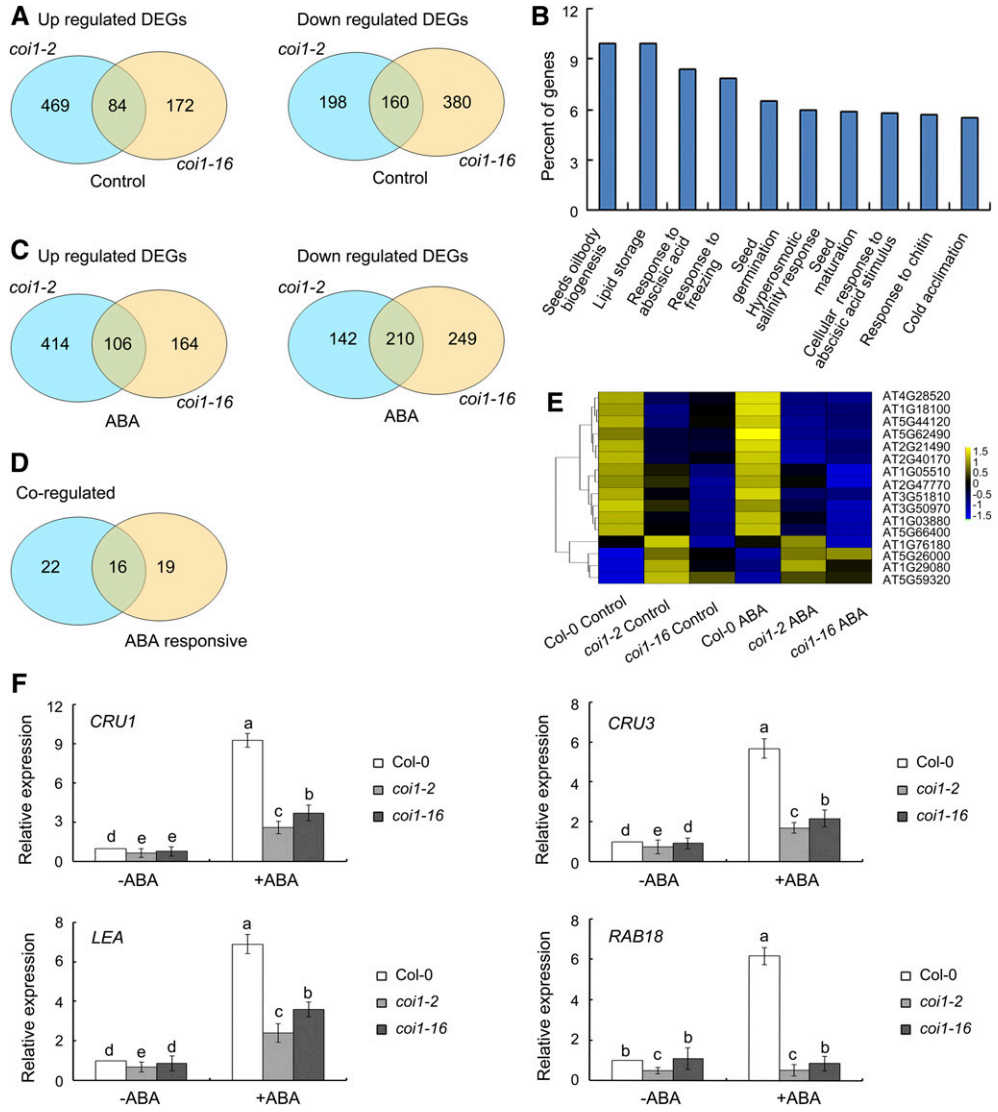

Figure 9. Transcriptomic Analysis of Regulatory Gene Expression Profiles by COI1 in Response to ABA.

(A) Venn diagram shows the number of overlapped up- and downregulated DEGs in *coi1-2* and *coi1-16* mutant compared with the wild type without ABA treatments.

(B) Gene Ontology analysis of *coi1-2* and *coi1-16* coregulated genes.

(C) Venn diagram shows the overlapped number of up- and downregulated DEGs in *coi1-2* and *coi1-16* mutant compared with the wild type under ABA treatment.

(D) Venn diagram shows the overlapped number of co–up- and downregulated genes in *coi1-2* and *coi1-16* mutant, which are ABA responsive genes in the wild type.

(E) Heatmap shows that ABA responsive genes coregulated by *coi1-2* and *coi1-16* mutant.

(F) RT-qPCR analysis of expression level of several selected ABA responsive genes coregulated by ABA and COI1. Seeds of wild type, *coi1-2*, and *coi1-16* were germinated on medium containing 0.5 μM ABA treated with 4 h. Average and significance were calculated over three biological replicates. Total RNA was extracted from at least three batches of seeds as biological replicates. Each batch of seeds for each genotype was pooled from at least 20 independent plants. For each biological replicate, more than 250 seeds of the same batch were used for RNA extraction. *AT1G13320* gene was used as control. Three biological replicates data were analyzed by ANOVA. Values with different letters are significant different from each other ($P < 0.05$).

that it may interact with ABA to mediate these processes (Wilen et al., 1991; Krock et al., 2002; Preston et al., 2002; Norastehnia et al., 2007; Barrero et al., 2009; Dave et al., 2011). However, the detailed molecular mechanisms underlying JA regulation of seed germination and its crosstalk with ABA remain elusive. In this study, we further investigated the regulatory role of JA in modulating ABA responses during seed germination and subsequent post-germinative growth. Consistent with previous studies, our

results demonstrate that exogenous JA (COR) has a positive role in activating ABA responses to delay seed germination. Col-0 seeds displayed low percentages of germination and of expanded cotyledons in the presence of both ABA and JA (Figure 1; Supplemental Figure 1), while lines with blocked endogenous JA perception or signaling were less sensitive to ABA during seed germination (Figure 2; Supplemental Figure 2). Based on these findings, we conclude that the JA signal activates ABA responses to delay seed germination in Arabidopsis.

Intriguingly, we found that mutation of *COI1* leads to decreased ABA signaling during seed germination. This finding is in disagreement with the results of two previous studies (Ellis and Turner, 2002; Fernández-Arbaizar et al., 2012). In those studies, the authors analyzed one of the same *coi1* alleles that we used, *coi1-16*, and found that it was hypersensitive to ABA in comparison with Col-0. To verify the role of COI1 in mediating the ABA response, we also investigated another *coi1* allele, *coi1-2*, in the presence of ABA. Like our *coi1-16* mutant, *coi1-2* seeds also exhibited much higher percentages of germination and greening cotyledons than Col-0 seeds on medium containing ABA (Figure 2; Supplemental Figure 2A). Consistent with this phenotype, the induced expression levels of several well-characterized ABA-responsive genes were lower in *coi1-2* than in the wild type (Figure 2E). More importantly, to further corroborate that mutation of *COI1* is responsible for the ABA-insensitive phenotypes of *coi1-2* and *coi1-16*, we expressed the full-length *COI1* gene driven by its native promoter or the CaMV 35S promoter in the *coi1-2* and *coi1-16* mutant backgrounds. As expected, expression of full-length *COI1* in the *coi1-2* or *coi1-16* background resulted in the mutant plants responding similarly to Col-0 under ABA treatment (Supplemental Figure 5). Moreover, on medium containing ABA, the germination and greening percentages were much higher in heterozygous *JAZ-ΔJas* plants, which accumulate higher levels of JAZ proteins, than in Col-0 (Figures 2 and 8), similar to the effects seen for the *coi1* mutants. By contrast, the germination rates were lower in the *jazQ* and *jazD* mutants than in the wild type under ABA treatment. Therefore, our results show that JA synergistically regulates ABA signaling during seed germination.

The *coi1-16* phenotype and germination rates of seeds may be dependent on our experimental conditions because many external factors, including light, humidity, temperature, and availability of nitrogenous compounds, are known to affect ABA responses during seed germination. For example, the light intensity under long-light conditions is one factor that differs among various laboratories. The germination media also differ among different studies. Some studies conducted germination assays on water agar medium to avoid the effects of nitrate and Suc (this study; Dave et al., 2011), whereas different media were used in other studies (Ellis and Turner, 2002; Fernández-Arbaizar et al., 2012). Differences in these factors may account for the inconsistency of the germination rates and the *coi1-16* phenotype in response to ABA among different studies. In our hands and experimental conditions, the ABA sensitivity of *coi1* mutants was changed under conditions where exogenous nitrate or Suc was present (Supplemental Figure 3). The data suggested that *coi1* mutants may have different ABA sensitivity according to the imbibition conditions. Furthermore, our germination data were not identical on half-strength Murashige and Skoog and water agar media, but seeds displayed a similar percentage of expanded cotyledons in both media.

Recently, several studies have provided evidence of crosstalk between JA and ABA signaling. The results of those studies supported that JA interacts with ABA signaling to regulate physiological process (Lackman et al., 2011; Pauwels et al., 2015). Consistent with the results of Dave et al. (2011), our results show that COR acts along with ABA to regulate seed germination in Arabidopsis. Despite recent advances in research on the link between JA and ABA signaling, the exact mechanisms underlying the crosstalk between JA signaling and other developmental processes and signaling pathways remain to be elucidated. Characterization of the physical interactions between JAZ proteins and the critical components of other signaling pathways may shed light on the molecular basis of the regulation of the JA signal transduction network.

In this study, we found that JAZ proteins physically associate with an important transcription factor in ABA signaling, ABI3, and further demonstrated that those JAZ proteins repress the transcriptional activation functions of ABI3 and ABI5 (Figures 3 and 6). In our analyses, these JAZ repressors specifically interacted with ABI3 and ABI5, but they did not form complexes with ABI4, an APETALA2 (AP2) family transcription factor that positively modulates ABA signaling in yeast (Figure 3; Ju et al., 2019). Further analyses revealed that the ZIM domain of JAZ is required for interactions between JAZ and ABI3 (Figure 4). Deletion of the C-terminal 133 residues of JAZ that contain the ZIM domain eliminated the interaction between JAZ with ABI3, while deletion of the N-terminal region (121 to 253 residues) did not affect this interaction. Interestingly, this finding differs from that of several previous studies, which showed that the Jas domain is essential for most of the interactions between JAZ repressors and downstream transcription factors (Pauwels and Goossens, 2011). Zhai et al. (2015) found that the deletion of a 53–amino acid residue N-terminal region (including the ZIM domain) of JAZ1 eliminated its interaction with TOE1, indicating that this region is required for the JAZ-TOE1 interaction. Other studies also reported that the region containing the ZIM domain is vital for interactions with other proteins (Cheng et al., 2011; Song et al., 2011; Jiang et al., 2014). Importantly, the ZIM domain of most JAZ proteins can recruit TPL or TPR proteins indirectly through the ethylene-response factor amphiphilic repression (EAR) motif–containing NINJA adaptor protein to repress JA responses (Shyu et al., 2012; Thatcher et al., 2016; Howe et al., 2018). In addition, sequence variations in the hypervariable region of the degron affect JAZ stability and JA-regulated physiological responses. JAZ8-mediated repression depends on an EAR motif at the JAZ8 N terminus, which binds the corepressor TOPLESS and represses its transcriptional activation function (Shyu et al., 2012). Therefore, different domains of JAZ proteins interact with different proteins. In future studies, the identification of JAZ-associated transcription factors and further mapping of specific JAZ domains or residues required for these interactions may enhance our understanding of JAZ-regulated targets.

The transcription factors ABI3 and ABI5, which are mainly expressed in seeds and are strongly induced by ABA, play critical roles in modulating ABA responses to suppress seed germination and early seedling growth (Finkelstein, 1994; Finkelstein and

Lynch, 2000; Lopez-Molina and Chua, 2000; Lopez-Molina et al., 2001, 2002; Suzuki et al., 2001; Brocard et al., 2002; Finkelstein et al., 2005; Bedi et al., 2016). Previous studies have shown that the loss-of-function mutants *abi3* and *abi5* are insensitive to ABA treatment (Koornneef et al., 1984; Giraudat et al., 1992; Finkelstein, 1994; Finkelstein and Lynch, 2000). Interestingly, our phenotypic analyses showed that *abi3*, *abi5*, and an *abi5 abi3* double mutant also exhibited higher percentages of expanded cotyledons than Col-0 on medium containing ABA and COR (Figure 5). These findings demonstrate that JA stimulates ABA signaling to delay seed germination and post-germinative growth and that this requires functional ABI3 and ABI5. We found that overexpression of *ABI3* and *ABI5* simultaneously suppresses the ABA-insensitive phenotypes of *coi1-2* and *JAZ8-ΔJas* mutants (Figures 7 and 8). This finding further supports the hypothesis that ABI3/ABI5 and JAZ proteins modulate ABA and JA signaling during seed germination. Together, the results of our biochemical and genetic analyses reveal a previously unknown signaling module in which JAZ repressors in the JA pathway directly modulate ABA-responsive ABI3 and ABI5 transcription factors to integrate JA and ABA signals during seed germination and post-germinative growth.

In a recent study by Pan et al. (2018), two VQ (containing the conserved FxxxVQxxTG motif) proteins, VQ18 and VQ26, were shown to physically associate with ABI5 and interfere with its transcriptional role. As with the JAZ proteins in this study, VQ18 and VQ26 do not mediate seed dormancy but, rather, they negatively modulate ABA signaling during seed germination and early seedling establishment. However, Liu et al. (2013) found that two transcription factors of auxin signaling, AUXIN RESPONSE FACTOR10 (ARF10) and ARF16, positively regulate *ABI3* expression and promote both seed dormancy and ABA signaling during seed germination. Furthermore, Vaistij et al. (2013) revealed that MOTHER-OF-FT-ANDTFL1 (MFT), a member of the phosphatidyl ethanolamine binding protein (PEBP) family, regulates *ABI5* expression to stimulate seed dormancy but represses ABA responses during seed germination. Together, the results of these studies show that the components that regulate or interact with ABI3 and/or ABI5 may have distinct roles in regulating seed dormancy or ABA signaling. Further research is required to dissect the crucial regulators of ABI3/ABI5 and elucidate the exact mechanisms underlying ABI3/ABI5-mediated seed dormancy and regulation of the ABA signaling network.

JA is a hormone induced by pathogen attack, wounding, and some abiotic stresses (Kazan, 2015; Howe et al., 2018), and ABA also is induced by several abiotic stresses. It is easy to imagine that when seeds are exposed to soil, environmental factors induce the accumulation of JA and ABA. In plants, ABA and JA are two important hormones that regulate diverse aspects of plant developmental and stress responses. Some observations have revealed clues about the crosstalk between the ABA and JA signaling pathways. For instance, ABA induces the expression of *PLIP2* and *PLIP3*, which may participate in JA biosynthesis (Wang et al., 2018). Another study showed that ABA does indeed promote JA biosynthesis (Ju et al., 2019). Consistent with these studies, we found that ABA treatments enhanced JA and ABA biosynthesis. The accumulated JA may then cooperate with ABA to delay seed germination for avoiding a bad environment. Our results reveal some of the mechanisms underlying the crosstalk between ABA and JA: JAZ proteins physically interact with the transcription factors ABI3/ABI5 and repress their activities to delay seed germination. As shown in Figure 10, we propose the following working model: under unfavorable growth conditions, JAZ proteins are degraded and then ABI3 and ABI5 are released from JAZ-mediated repression to activate ABA signaling; simultaneously, the transcription factors ABI3 and ABI5 are activated by ABA, thereby delaying the process of seed germination.

METHODS

Plant Materials and Growth Conditions

Most of the Arabidopsis (*Arabidopsis thaliana*) lines used in this study were in the Col-0 background. Additional Arabidopsis accessions used were Ws and Ler. The *abi3-1* (Jiang and Yu, 2009), *abi3-8* (Lin et al., 2020), *abi5-1* (Finkelstein, 1994; Finkelstein and Lynch, 2000), and *abi5-7* (Zhou et al., 2015) mutants have been described previously. Seeds of *abi3* and *abi5* were obtained from Dapeng Zhang (Tsinghua University), seeds overexpressing *ABI5* (Chen et al., 2012; Hu and Yu, 2014) were obtained from Chuanyou Li (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences), and *coi1-2* was kindly provided by Zhixiang Chen (Purdue University) and Daoxin Xie (Tsinghua University). The *coi1-16* mutant was obtained from the ABRC.

The seeds were surface sterilized in 20% (v/v) bleach for 15 min before being sown on water agar medium supplemented with 0.7% (w/v) agar and maintained at 4°C for 3 d. After 7 d, the seedlings were transferred to soil. The plants were grown in chambers under long-day conditions, consisting of 14 h of light (120 μE m^{-2} s^{-1} at 22°C, white fluorescent bulbs, full wavelength of light) and 10 h of dark (0 μE m^{-2} s^{-1} at 19°C), or short-day conditions, consisting of 8 h of light (120 μE m^{-2} s^{-1} at 22°C) and 16 h of dark (0 μE m^{-2} s^{-1} at 19°C). *Nicotiana benthamiana* plants were grown in a controlled environment cabinet under long-day conditions (120 μE m^{-2} s^{-1} at 22°C) for BiFC and Co-IP assays.

Chemicals and Enzymes

The plant hormones ABA, MeJA, and COR and the 26S proteasome inhibitor MG132 were purchased from Sigma-Aldrich. The Taq DNA polymerase was purchased from Takara Biotechnology. Other common chemicals were purchased from Shanghai Sangon Biotechnology.

Generation of Transgenic Arabidopsis Lines

To generate *JAZ-* and *ABI3-OE* transgenic lines, the full-length cDNA of *ABI3* was cloned into the binary vector pOCA30 (Pan et al., 2018) in the sense orientation behind the CaMV 35S promoter (Hu et al., 2013). The *JAZ1*, *JAZ5*, or *JAZ8* cDNAs had the *Jas* domain deleted (Thines et al., 2007), and each was independently cloned into the binary vector pOCA30 in the sense orientation behind the CaMV 35S promoter. The heterozygous *JAZ-ΔJas* plants were the F1 progeny of *JAZ-ΔJas* crossed with the wild type, and the seeds of this cross were used in a germination assay. The primers and restriction enzyme sites used to amplify sequences and generate vectors are listed in Supplemental Table 1.

The *coi1-2 ABI3-OE* (or *coi1-16 ABI3-OE*) and *JAZ8-ΔJas ABI3-OE* (*JAZ5-ΔJas ABI3-OE*) plants were generated by crossing an appropriate transgenic plant with *ABI3-OE-5*. The results of those crosses were respectively crossed with *ABI5-OE-8* plants to produce *coi1-2 ABI3-OE ABI5-OE* (or *coi1-16 ABI3-OE ABI5-OE*) and *JAZ8-ΔJas ABI3-OE ABI5-OE* (*JAZ5-ΔJas ABI3-OE ABI5-OE*) plants.

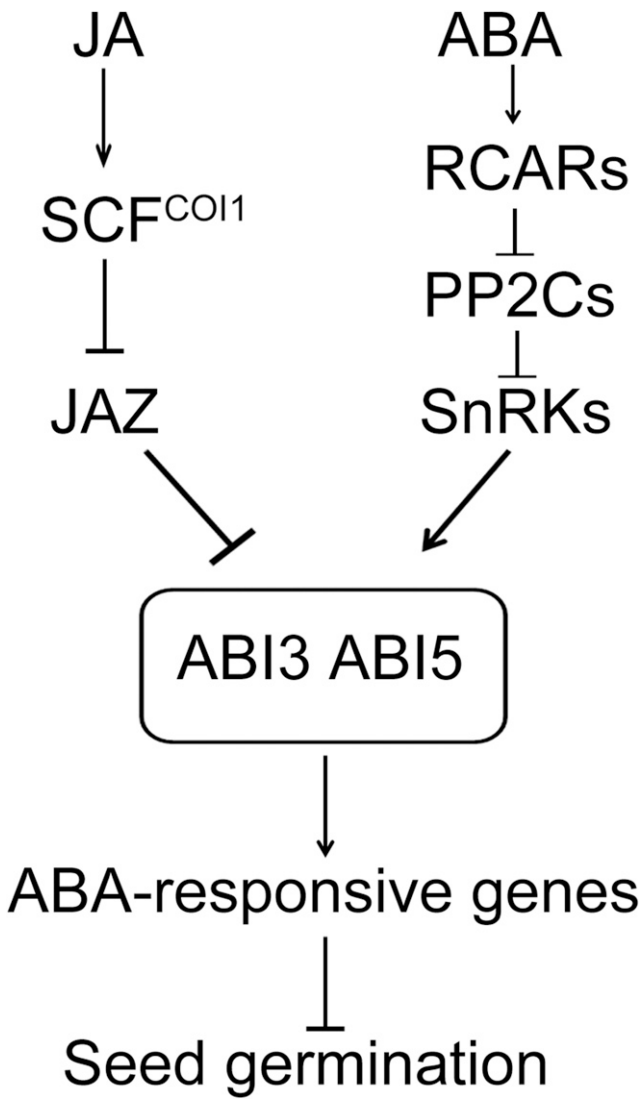

Figure 10. Proposed Working Model Describing the Interaction of JA and ABA Signaling in Mediating Seed Germination.

Under normal growth conditions, the JAZ proteins interact directly with ABI3/ABI5 and repress the transcriptional activities of ABI3/ABI5, leading to repression of the ABA pathway. Under unfavorable growth conditions, JA biosynthesis is increased, which leads to the degradation of JAZ proteins, and then ABI3 and ABI5 are released from JAZ-mediated inhibition to activate ABA signaling; simultaneously, the accumulation of ABA activates the transcription factors ABI3 and ABI5, thereby delaying the process of seed germination.

Because the *abi5-1* mutant was in the Ws background, we backcrossed it with the Col-0 wild type six times before use. Similarly, the *abi3-1* mutant (in the Ler background) was backcrossed with the Col-0 wild type six times before use. The *abi5 abi3* double mutant was generated by crossing the backcrossed *abi5* mutant with the backcrossed *abi3* mutant.

Seed Germination and Dormancy Assays

Plants of each genotype were grown side by side under the same conditions. Seeds of each genotype were harvested from independent plants and stored at the same time, and they were pooled before germination assays. We scored germination as radicle emergence from the seed coat and endosperm, and we also recorded cotyledon opening and cotyledon expansion (Dave et al., 2011; Piskurewicz and Lopez-Molina, 2016). For the stratification treatment, seeds were stratified at 4°C in the dark for 3 d.

To examine the phenotypes of germinated materials, every batch of seeds was grown on water agar medium supplemented with or without ABA and/or COR for the indicated periods in long-day conditions (14 h of light and 10 h of dark). Every batch of dry mature seeds for each genotype was pooled from at least 15 independent plants (each figure represents data from seeds that matured and were harvested at the same time). Average values were calculated from three biological replicates and compared using statistical tests to determine the significance of differences. For every biological replicate, we tested the seeds from the same batch at least three times as technical replicates.

For seed dormancy analysis, for each genotype, we examined fresh mature siliques on plants grown under the same conditions and maturing at the same time after 5 d of growth on water-saturated filter paper at 22°C without stratification (Liu et al., 2013).

Expression Analyses

Seeds were placed on media containing hormones as indicated, and total RNA was extracted from seeds after the indicated periods. Total RNA was extracted using TRIzol reagent (Invitrogen) for real-time RT-PCR analysis. Superscript II (Invitrogen) was used according to the manufacturer's instructions, and 1 μg of DNase-treated RNA was reverse transcribed in a 20-μL reaction volume. Each qRT-PCR was conducted using 1 μL of cDNA and the SYBR Premix Ex Taq kit (Takara Biotechnology) on a LightCycler 480 real-time PCR instrument (Roche). At least three independent biological samples for each replicate were analyzed. *AT1G13320* was used as an internal control. The independent biological data were subjected to ANOVA (Supplemental Data Set 3). The $2^{-\Delta\Delta Ct}$ method was used for relative quantification of gene transcript levels (Livak and Schmittgen, 2001). The RT-qPCR primers used in these analyses are listed in Supplemental Table 2.

RNA Sequencing and Data Analysis

The Col-0, *coi1-2*, and *coi1-16* seeds were grown on media with or without 1 μM ABA for 24 h and then the materials were collected. Three biological replicates were prepared for each sample, and RNA was extracted from a mixed sample of seeds. RNA was extracted using TRIzol reagent using the ethanol precipitation protocol and then sequenced (Oebiotech). Clean reads were mapped to the Arabidopsis genome (TAIR10; www.arabidopsis.org) after screening and trimming. Cufflinks software was used to determine expression values (Tarazona et al., 2011). Genes with estimated absolute fold changes ≥1 were identified as reliable differentially expressed genes (DEGs). DEGs were analyzed using DESeq (Anders and Huber, 2010). Multiple testing was corrected via false discovery rate estimation and q-values below 0.05 were considered to indicate differential expression. Subsequently, Gene Ontology enrichment analysis was performed using TopGO (Alexa et al., 2006). The RT-qPCR primers used in the analysis shown in Figure 9F are listed in Supplemental Table 2.

Y2H Screening and Confirmation

The full-length CDS of *ABI3* was fused to pGBKT7 to construct the bait vector, which was transformed into the yeast strain Y2HGOLD (Clontech). Yeast screening was performed as described previously (Hu et al., 2013). Each of the 12 JAZs (JAZ1, JAZ2, JAZ3, JAZ4, JAZ5, JAZ6, JAZ7, JAZ8, JAZ9, JAZ10, JAZ11, and JAZ12) was introduced into the prey vector pGADT7. These plasmids were cotransfected into yeast strain AH109. The transfected yeast cells were plated on SD/–Leu/–Trp medium and SD/–Ade/–His/–Leu/–Trp medium and cultured at 28°C for 4 d. The primers and restriction enzyme sites used to amplify sequences and generate vectors are listed in Supplemental Table 3.

BiFC Assays

The full-length CDSs of *JAZ1*, *JAZ4*, *JAZ5*, and *JAZ8* were fused in frame to the YFP C terminus of the C-YFP to form JAZ1-cYFP, JAZ4-cYFP, JAZ5-cYFP, and JAZ8-cYFP, respectively. The *ABI3* and *ABI4* full-length CDSs were cloned in frame to the YFP N terminus, respectively. Each of the cloning plasmids was introduced into *Agrobacterium tumefaciens* strain EHA105 and then infiltrated into *N. benthamiana* leaves as described previously (Wang et al., 2016). At 48 h after infiltration, infected tissues were analyzed under a confocal laser-scanning microscope (Leica). The primers and restriction enzyme sites are listed in Supplemental Table 3.

Co-IP Assays

The Co-IP assays were performed as described previously (Pan et al., 2018). Briefly, the full-length CDSs of *JAZ1* and *JAZ5* were separately cloned into a tagging vector with HA-tag in the sense orientation downstream of the CaMV 35S promoter. ABI3 was fused to a 3×MYC-tag to form the ABI3-3MYC fusion protein (Pan et al., 2018). The fusion proteins were transiently coexpressed in *N. benthamiana* leaves. All of the infected leaves were treated with 50 μM MG132 and 5 μM ABA after being infiltrated for 40 h. The leaves were collected after 48 h and homogenized in extraction buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1% [w/v] Triton X-100, 0.2% [w/v] Nonidet P-40, 0.6 mM phenylmethylsulfonyl fluoride, and 25 μM MG132 with 1× Roche protease inhibitor cocktail). Next, MYC-fused ABI3 was immunoprecipitated using an anti-MYC mouse antibody (1:5000; catalog no. A7470, Sigma-Aldrich; Pan et al., 2018), and the coimmunoprecipitated proteins were detected using an anti-HA rabbit antibody (1:10,000; catalog no. H9658, Sigma-Aldrich). The primers and restriction enzyme sites are listed in Supplemental Table 1. The antibodies used for this study included anti-mouse MYC (1:5000; Sigma-Aldrich; Pan et al., 2018) and anti-rabbit HA (1:10,000; Sigma-Aldrich).

Transient Transactivation Assay

To detect the transactivation activity of ABI3 and ABI5 proteins, the promoter regions of *ABI5* (~2326 bp), *EM6* (~1273 bp), and *EM1* (~2000 bp) were amplified by PCR and independently cloned into the pGreenII 0800-LUC vector as reporter plasmids (Hellens et al., 2005). JAZ1, JAZ5, or JAZ8 was amplified and independently cloned into the pGreenII 62-SK vector as effector plasmids (Hellens et al., 2005). Combinations of plasmids were introduced into mesophilic protoplasts from Arabidopsis according to the protocol of Sheen (2001). Transfected cells were cultured for 10 to 16 h with or without 5 μM ABA and then relative LUC activity was determined using the Dual-Luciferase Reporter Assay Protocol machine (Promega), which measures the activities of firefly LUC and the internal control *Renilla* LUC. The primers and restriction enzyme sites are listed in Supplemental Table 4.

Statistical Analysis

ANOVA was performed using SPSS software. A value of $P < 0.05$ was considered to be statistically significant. The results of statistical analyses are shown in Supplemental Data Set 3.

Accession Numbers

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: *ABA1* (AT5G67030); *ABA2* (AT1G52340); *ABA3* (AT1G16540); *ABI3* (AT3G24650); *ABI4* (AT2G40220); *ABI5* (AT2G36270); *ACX1* (AT4G16760); *ADH1* (AT1G77120); *AOC3* (AT3G25780); *AT1G13320 COI1* (AT2G39940); *CRU1* (AT5G44120); *CRU3* (AT4G28520); *EM1* (AT3G51810); *EM6* (AT2G40170); *KAT5* (AT5G48880); *LEA* (AT2G21490); IAA29 (AT4G32280); *JAZ1* (AT1G19180); *JAZ4* (AT1G48500); *JAZ5* (AT1G17380); *JAZ8* (AT1G30135); *MED18* (AT2G22370); *MYC2* (AT1G32640); *NCED3* (AT3G14440); *OPR3* (AT2G06050); *RAB18* (AT1G43890); *WRKY57* (AT1G69310).

Supplemental Data

Supplemental Figure 1. JA enhances ABA signaling during seed germination. Supports Figure 1.

Supplemental Figure 2. The ABA-sensitivity of *coi1-2*, *coi1-16*, and *jazD* seeds. Supports Figure 2.

Supplemental Figure 3. The phenotype of wild type (Col-0), *coi1-2*, *coi1-16*, and *abi3-8* under different conditions. Supports Figure 2.

Supplemental Figure 4. The MeJA-sensitivity of *coi1-2 COI1, coi1-2/COI1pro:COI1*, *coi1-16 COI1*, and *coi1-16/COI1pro:COI1* seedlings. Supports Figure 2.

Supplemental Figure 5. The ABA-sensitivity of *coi1-2 COI1, coi1-2/COI1pro:COI1*, *coi1-16 COI1*, and *coi1-16/COI1pro:COI1* seeds. Supports Figure 2.

Supplemental Figure 6. The phenotype of plants overexpressing the full-length JAZ and expression patterns of *JAZ*. Supports Figure 2 and 3.

Supplemental Figure 7. Y2H assays for the interaction of JAZ and ABI3, ABI4, WRKY57. Supports Figure 3.

Supplemental Figure 8. Expression of ABI3, ABI4, JAZ1, JAZ4, JAZ5, or JAZ8 protein in *N. benthamiana* leaves. Supports Figure 3.

Supplemental Figure 9. Expression of ABI3, JAZ1, JAZ5, or GFP protein in *N. benthamiana* leaves. Supports Figure 3.

Supplemental Figure 10. The phenotype of seed dormancy in Col-0, *ABI3-OE, abi3*, *coi1-2* and *JAZ1-ΔJas*. Supports Figure 5.

Supplemental Figure 11. RT-qPCR analysis of *ABI3* or *ABI5* expression in overexpression lines. Supports Figure 7 and 8.

Supplemental Figure 12. Overexpressing ABI3 and ABI5 simultaneously rescued the ABA-insensitive phenotypes of *coi1-16* and *JAZ5-ΔJas*. Supports Figure 7 and 8.

Supplemental Figure 13. RT-qPCR analysis of several JA and ABA biogenesis genes in Col-0, *coi1-2* and *coi1-16*. Supports Figure 9 and 10.

Supplemental Table 1. Primers used for transgenic plants construction and CoIP assay.

Supplemental Table 2. Primers used for qRT-PCR.

Supplemental Table 3. Primers used for yeast-two hybrid assays and BiFC assay.

Supplemental Table 4. Primers used for transient expression assay.

Supplemental Data Set 1. Sequence of JAZ' splicing variant. Supports Figure 3 and 4.

Supplemental Data Set 2. Data of transcriptomic analysis for COI1 regulated genes. Supports Figure 9.

Supplemental Data Set 3. Statistical analysis of ANOVA results for the data shown in figures.

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AUTHOR CONTRIBUTIONS

J.P., G.A.H., and D.Y. designed the experiments; J.P., H.W., Q.G., and Y.C. performed the experiments; J.P., G.A.H., and D.Y. analyzed the data; J.P., and Y.H. wrote the article. All authors read and approved the final article.

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