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BRASSINOSTEROID INSENSITIVE2 Interacts with ABSCISIC ACID INSENSITIVE5 to Mediate the Antagonism of Brassinosteroids to Abscisic Acid during Seed Germination in *Arabidopsis*[W]

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Seed germination and postgerminative growth are regulated by a delicate hormonal balance. Abscisic acid (ABA) represses *Arabidopsis thaliana* seed germination and postgerminative growth, while brassinosteroids (BRs) antagonize ABA-mediated inhibition and promote these processes. However, the molecular mechanism underlying BR-repressed ABA signaling remains largely unknown. Here, we show that the Glycogen Synthase Kinase 3-like kinase BRASSINOSTEROID INSENSITIVE2 (BIN2), a critical repressor of BR signaling, positively regulates ABA responses during seed germination and postgerminative growth. Mechanistic investigation revealed that BIN2 physically interacts with ABSCISIC ACID INSENSITIVE5 (ABI5), a bZIP transcription factor. Further genetic analysis demonstrated that the ABA-hypersensitive phenotype of *BIN2*-overexpressing plants requires ABI5. BIN2 was found to phosphorylate and stabilize ABI5 in the presence of ABA, while application of epibrassinolide (the active form of BRs) inhibited the regulation of ABI5 by BIN2. Consistently, the ABA-induced accumulation of ABI5 was affected in *BIN2*-related mutants. Moreover, mutations of the BIN2 phosphorylation sites on ABI5 made the mutant protein respond to ABA improperly. Additionally, the expression of several ABI5 regulons was positively modulated by BIN2. These results provide evidence that BIN2 phosphorylates and stabilizes ABI5 to mediate ABA response during seed germination, while BRs repress the BIN2-ABI5 cascade to antagonize ABA-mediated inhibition.

INTRODUCTION

Seed germination and postgerminative growth are critical developmental phases in the life cycle of a flowering plant. Seed germination initiates with the uptake of water by the quiescent dry seed and terminates with the elongation of the embryonic axis (Bewley and Black, 1994). In *Arabidopsis thaliana*, the visible characterization of seed germination is the penetration of the structures surrounding the embryo by the radicle (Bewley, 1997). A major physiological process during seed germination is the mobilization of the major storage reserves used for the energy supply of a seed during germination. After seed germination, subsequent postgerminative growth leads to the establishment of a seedling, which is morphologically featured by cotyledon opening, cotyledon greening, and radicle growth (Bewley and Black, 1994). Both seed germination and postgerminative growth are strictly regulated by internal and external cues, including phytohormones.

The phytohormone abscisic acid (ABA) acts as a crucial signal to regulate multiple physiological processes in plants. In *Arabidopsis*, ABA is involved in seed dormancy and germination, stomatal closure, seedling growth, and plant adaptation to various environmental challenges (Leung and Giraudat, 1998; Finkelstein et al., 2002, 2008; Hauser et al., 2011; Nakashima and Yamaguchi-Shinozaki, 2013). ABA is known to inhibit seed germination and postgerminative growth but to promote seed maturation and seed dormancy. Previous studies using genetic and molecular approaches have identified a number of components that mediate ABA response. Among these components, the bZIP-type transcription factor ABSCISIC ACID INSENSITIVE5 (ABI5) was identified through analysis of the recessive ABA-insensitive *abi5-1* mutant (Finkelstein, 1994; Finkelstein and Lynch, 2000). *ABI5*, mainly expressed in seeds and strongly induced by ABA, plays a critical role in modulating seed germination and early seedling growth (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). Further studies have revealed that the ABI5 transcription factor is tightly regulated posttranslationally. For example, the protein kinases SNF1-RELATED PROTEIN KINASE2.2 (SnRK2.2), SnRK2.3, and SnRK2.6 have been shown to phosphorylate and stabilize ABI5 protein in response to ABA (Kobayashi et al., 2005; Furihata et al., 2006; Fujii et al., 2007; Fujii and Zhu, 2009; Nakashima et al., 2009). In contrast, the phosphatase PROTEIN PHOSPHATASE6 dephosphorylates ABI5 protein (Dai et al., 2013). Moreover, several ABI5-interacting proteins have been reported to affect ABI5 degradation, including DWD HYPERSENSITIVE TO ABA1 (DWA1), DWA2, KEEP ON GOING, and the SMALL UBIQUITINRELATED MODIFIER E3 ligase SIZ1 (SAP and Miz) (Stone et al., 2006; Miura et al., 2009; Lee et al., 2010; Liu and Stone, 2010).

Brassinosteroids (BRs) are a group of plant steroid hormones that play essential roles in plant growth, development, and stress

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responses (Clouse and Sasse, 1998; Wang and He, 2004; Vert et al., 2005; Haubrick and Assmann, 2006; Kim and Wang, 2010; Gudesblat and Russinova, 2011; Zhu et al., 2013). BRs are perceived by the plasma membrane-localized receptor kinase BRASSINOSTEROID INSENSITIVE1 (BRI1) (Li and Chory, 1997; Hothorn et al., 2011; She et al., 2011). When BR levels are high, BRs bind to and stimulate BRI1; the resulting downstream signal transduction leads to inhibition of the GSK3-like kinase BRASSINOSTEROID INSENSITIVE2 (BIN2) and its homologs BIN2-LIKE1 (BIL1) and BIL2, which are pivotal repressors of BR signaling (Li et al., 2001, 2002; Li and Nam, 2002; Nam and Li, 2002; Tang et al., 2008; Kim et al., 2009; Yan et al., 2009; Kim and Wang, 2010; Guo et al., 2013). The inhibition of BIN2 leads to dephosphorylation and nuclear accumulation of BRASSINAZOLE RESISTANT1 (BZR1) family transcription factors that regulate BR-responsive gene expression (He et al., 2002; Wang et al., 2002; Yin et al., 2002; Ryu et al., 2007; Li et al., 2010; Clouse, 2011). Plants defective in BR biosynthesis or signaling display pleiotropic dwarf phenotypes, such as short leaf petioles and hypocotyls, dark-green and epinastic leaves, reduced apical dominance, and delayed flowering (Clouse et al., 1996; Li et al., 1996; Szekeres et al., 1996; Li and Chory, 1999; Krishna, 2003). Moreover, several studies have revealed that BRs play a critical antagonistic role in the inhibitory effect of ABA during seed germination (Clouse et al., 1996; Ephritikhine et al., 1999; Leubner-Metzger, 2001; Steber and McCourt, 2001; Chen et al., 2004; Zhang et al., 2009). Applying brassinolide renders wild-type plants tolerant to ABA during seed germination, whereas blocking BR biosynthesis or perception confers ABA hypersensitivity. However, the exact molecular mechanism underlying the regulatory effect of BRs on ABA remains to be elucidated.

In this study, we found that the kinase BIN2 positively modulates plant responses to ABA during seed germination. BIN2 was found to physically interact in the nucleus with the transcription factor ABI5. Further genetic analysis showed that the ABA hypersensitivity of *BIN2*-overexpressing plants requires the ABI5 protein. Moreover, BIN2 phosphorylates and stabilizes ABI5 protein in the presence of ABA, whereas epibrassinolide (eBL) application represses the regulatory effect of BIN2 on ABI5. Consistently, the ABA-induced accumulation of ABI5 was affected in *BIN2*-related mutants (*bin2-1* and *bin2-3bil1bil2*). In addition, the expression of several downstream target genes of ABI5 was positively modulated by BIN2. These findings demonstrate that BIN2 interacts with and phosphorylates ABI5 to positively regulate ABA response during seed germination, while BRs suppress the activity of BIN2 to antagonize ABA's inhibitory role.

RESULTS

BIN2 Positively Regulates ABA Response during Seed Germination

Previous studies have indicated that BRs act antagonistically with ABA to promote seed germination. Phenotypic analysis has shown that several analyzed BR biosynthesis- and perception-defective mutants are more sensitive to ABA during seed germination (Clouse et al., 1996; Ephritikhine et al., 1999; Steber and McCourt, 2001; Chen et al., 2004; Zhang et al., 2009). To negatively regulate BR signaling, the GSK3-like kinase BIN2 interacts with and phosphorylates its substrates (He et al., 2002; Yin et al., 2002). Li et al. (2001) demonstrated that root elongation of the gain-of-function mutant *bin2-1* seedlings is ABA hypersensitive. To further investigate whether BIN2 is also involved in ABA-mediated inhibition of seed germination, we evaluated germination of *bin2-1* seeds on half-strength Murashige and Skoog (MS) medium supplemented with various concentrations of ABA. Seeds of *bin2-1*/+ heterozygous plants were used in these experiments because *bin2-1* homozygous plants are completely infertile (Li et al., 2001). We found that *bin2-1*/+ progeny were hypersensitive to ABA during seed germination. As shown in Figure 1A, *bin2-1*/+ seeds had much lower germination percentages than the wild type at the tested ABA concentrations. Likewise, *bin2-1*/+ progeny displayed much lower greening in comparison with the wild type (Figures 1B and 1C). Further analysis indicated that among the progeny of *bin2-1*/+ plants incubated on half-strength MS medium containing 0.75 μM ABA for 7 d (Figure 1C), nearly all seedlings with green cotyledons were wild type (n = 80, 97.5%). These observations suggest that BIN2 may function as a positive regulator of ABA responses during seed germination and early seedling growth.

To further determine the role of BIN2 in ABA responses during seed germination, we next analyzed the performance of the loss-of-function mutant *bin2-3*. The *bin2-3* mutant displayed germination and greening percentages in response to ABA similar to those of the wild type (Figures 1D and 1E). Yan et al. (2009) reported that BIN2 acts together with its homologs BIL1 and BIL2 to regulate BR signaling; we thus wondered whether BIN2 also functions redundantly with BIL1 and BIL2 to modulate ABA responses. To test this possibility, we examined ABA response of the triple mutant *bin2-3 bil1 bil2* during seed germination. As shown in Figures 1D to 1F, the *bin2-3 bil1 bil2* mutant had much higher germination and greening than the wild type on half-strength MS medium containing various concentrations of ABA. To further confirm our results, we used bikinin, a highly specific inhibitor of seven *Arabidopsis* GSK3-like kinases (De Rybel et al., 2009), to investigate the function of BIN2 and its homologs in ABA responses. As shown in Figures 1G and 1H, bikinin significantly increased the germination and greening of wild-type seeds and repressed the ABA hypersensitivity of *bin2-1*/+ seeds. Taken together, these observations further support the notion that BIN2 positively regulates ABA responses during seed germination and postgerminative growth.

BIN2 Physically Interacts with ABI5

To understand how BIN2 modulates ABA responses during seed germination, we used the yeast two-hybrid system to identify its potentially interacting proteins involved in ABA responses. We fused the full-length of BIN2 to the Gal4 DNA binding domain of the bait vector (BD-BIN2). After screening, two independent clones encoding the ABI5 transcription factor were identified based on prototrophy for His and Ade. To confirm the interaction, we introduced the full length of ABI5 into the Gal4 activation domain of the prey vector (AD-ABI5). The bait and prey vectors were cotransformed into yeast and the protein-protein interaction was reconstructed (Figure 2A). As BIN2 functions redundantly with its homologs BIL1 and BIL2 to mediate ABA response (Figures 1D to 1F), we thus tested whether BIL1 and BIL2 also interact with ABI5

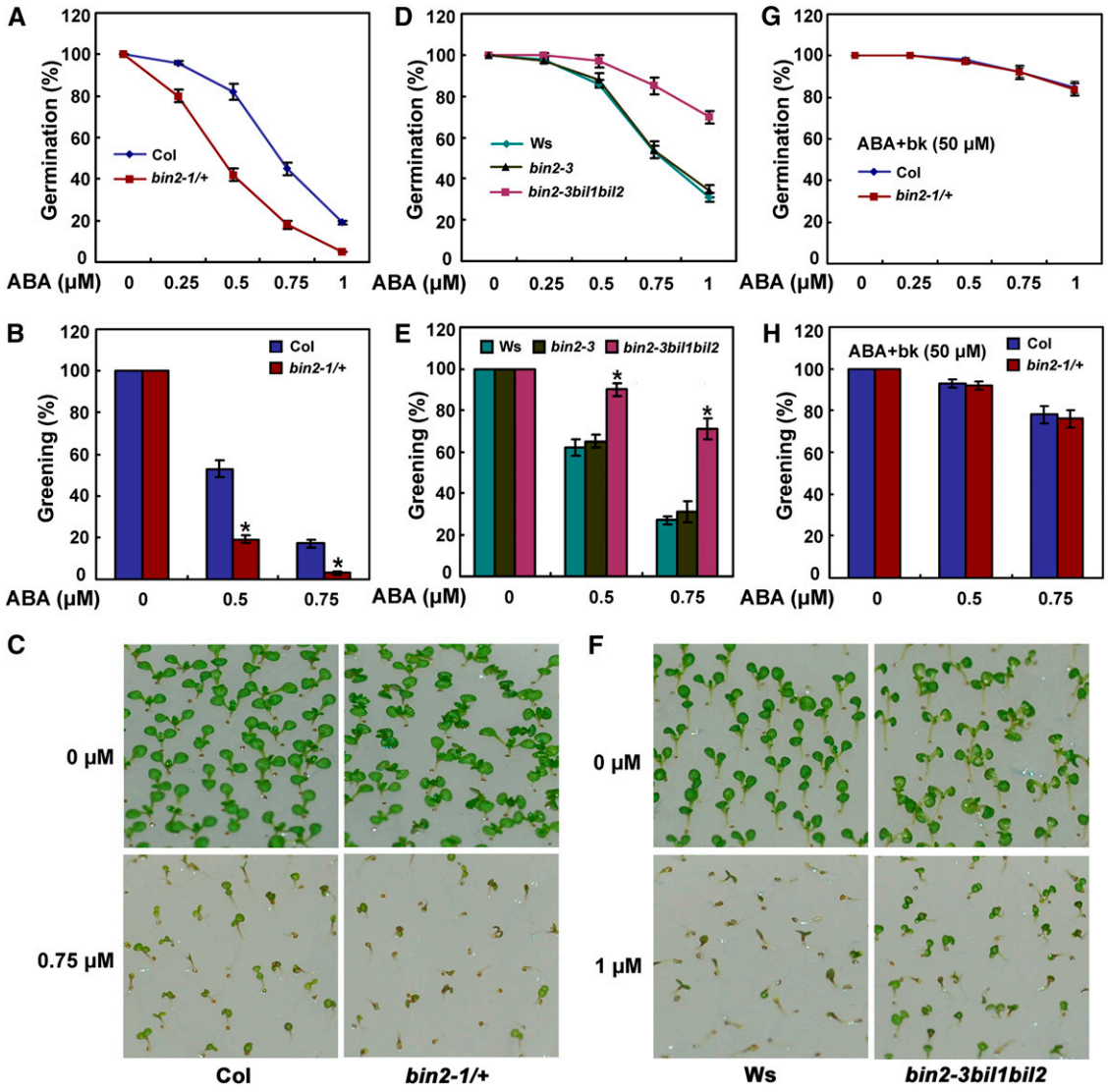

Figure 1. ABA Response of *bin2-1* and *bin2-3 bil1 bil2* during Seed Germination.

(A) Enhanced sensitivity of *bin2-1*/+ seed germination to ABA. Germination of wild-type (Col) and *bin2-1*/+ seeds was scored 4 d after stratification on half-strength MS medium supplemented with different concentrations of ABA.

(B) Enhanced sensitivity of *bin2-1*/+ cotyledon greening to ABA. Cotyledon greening was scored 5 d after stratification on half-strength MS medium containing 0.5 or 0.75 μM ABA.

(C) Seedlings of *bin2-1*/+ and wild type (Col) observed 7 d after germination on half-strength MS medium supplemented with 0 or 0.75 μM ABA.

(D) Enhanced tolerance of *bin2-3 bil1 bil2* seed germination to ABA. Germination of wild-type (Ws) and *bin2-3 bil1 bil2* seeds was scored 4 d after stratification on half-strength MS medium supplemented with different concentrations of ABA.

(E) Cotyledon greening of *bin2-3 bil1 bil2* seedlings was more tolerant to ABA. Cotyledon greening was scored 5 d after stratification on half-strength MS medium containing 0.5 or 0.75 μM ABA.

(F) Seedlings of *bin2-3 bil1 bil2* and wild type (Ws) 6 d after germination on half-strength MS medium supplemented with 0 or 1 μM ABA.

(G) Germination of wild-type (Col) and *bin2-1*/+ seeds was scored 4 d after stratification on half-strength MS medium containing 50 μM bikinin (bk) and various concentrations of ABA.

(H) Cotyledon greening of wild-type (Col) and *bin2-1*/+ seedlings was scored 5 d after stratification on half-strength MS medium containing 50 μM bikinin (bk) and 0.5 or 0.75 μM ABA. All experiments described above were performed three times, each evaluating more than 150 seeds. Values are means ± SD. Differences between the mutant and wild type are significant (*$P < 0.05$).

protein. As shown in Figure 2A, ABI5 also interacted with BIL1 and BIL2 in the yeast two-hybrid system. The BIN2-ABI5 interaction in planta was further verified by bimolecular fluorescence complementation (BiFC) and coimmunoprecipitation (CoIP) assays. For the BiFC assays, BIN2 was fused to a C-terminal yellow fluorescent protein (YFP) fragment (BIN2-cYFP), and the ABI5 protein was fused to an N-terminal YFP fragment (ABI5-nYFP). When fused BIN2-cYFP was coexpressed with ABI5-nYFP in leaves of tobacco (*Nicotiana benthamiana*), a YFP fluorescence signal—as revealed by staining with 4′,6-diamidino-2-phenylindole—was observed in transformed cell nuclei (Figure 2B). No fluorescence was detected in the negative control experiments (Figure 2B). In addition to the BiFC assays, the BIN2-ABI5 interaction was corroborated by CoIP assays using plant total proteins (Figure 2C). These results demonstrate that the BIN2 kinase physically interacts with the ABI5 transcription factor in plant cell nuclei.

ABA-Hypersensitive Phenotype of *BIN2*-Overexpressing Plants Requires ABI5

The ABI5 transcription factor is known as a key regulator of ABA responses during seed germination and early seedling growth. Phenotypic analysis has demonstrated that the loss-of-function mutant *abi5-1* is insensitive to ABA (Finkelstein, 1994; Finkelstein and Lynch, 2000). Because BIN2 physically interacts with ABI5, we asked whether the action of BIN2 during ABA responses requires ABI5. To test this possibility, we generated *BIN2*-overexpressing transgenic plants (*Ws/35S:MYC-BIN2*) and introduced the overexpression into *abi5-1* mutant background (*abi5-1/35S:MYC-BIN2*). Similar to the gain-of-function mutant *bin2-1*/+ progeny, seeds of *Ws/35S:MYC-BIN2* were also hypersensitive to ABA during seed germination and postgerminative growth (Figure 3). However, seeds of *abi5-1/35S:MYC-BIN2* exhibited germination and greening similar to those of *abi5-1* in response to ABA (Figure 3). These observations indicate that the ABA hypersensitivity of *BIN2*-overexpressing plants requires ABI5.

BIN2 Phosphorylates and Stabilizes ABI5 in the Presence of ABA

Previous studies have shown that BIN2 physically interacts with and phosphorylates its substrates to modulate BR signaling and other physiological processes (He et al., 2002; Yin et al., 2002; Vert et al., 2008; Gudesblat et al., 2012; Kim et al., 2012;

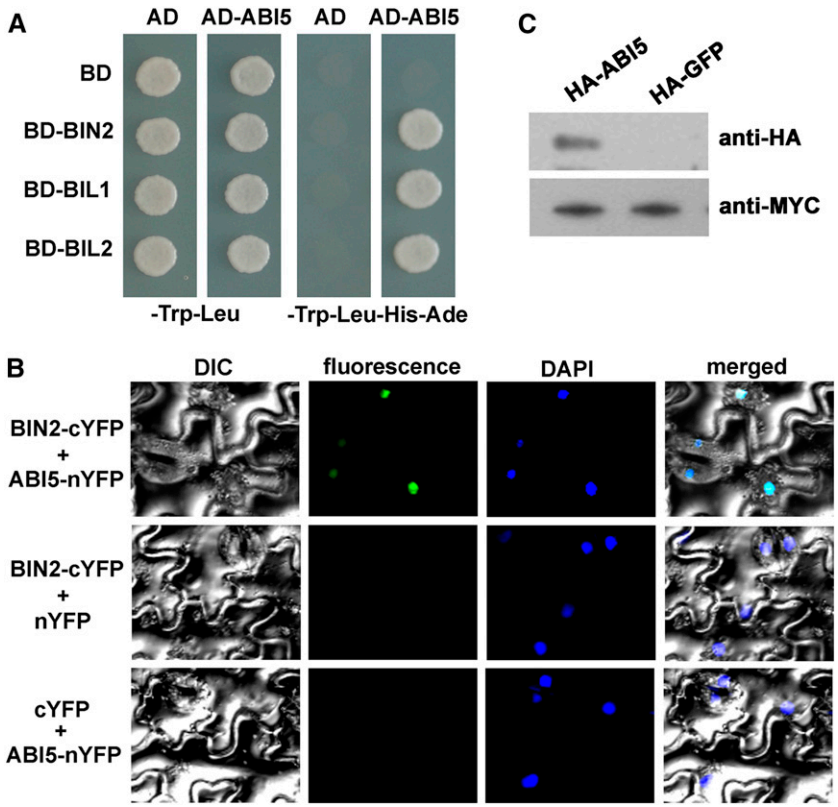

Figure 2. Physical Interaction between BIN2 and ABI5.

(A) Yeast two-hybrid assay analyses. Interaction was 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) BiFC analyses. Fluorescence was observed in the nuclear compartment of transformed cells, which resulted from complementation of the C-terminal part of YFP fused with BIN2 (BIN2-cYFP) with the N-terminal part of YFP fused with ABI5 (ABI5-nYFP).

(C) CoIP analyses. MYC-fused BIN2 was immunoprecipitated using an anti-MYC antibody, and coimmunoprecipitated HA-ABI5 was then detected using an anti-HA antibody. Protein input for MYC-BIN2 in immunoprecipitated complexes was also detected and is shown.

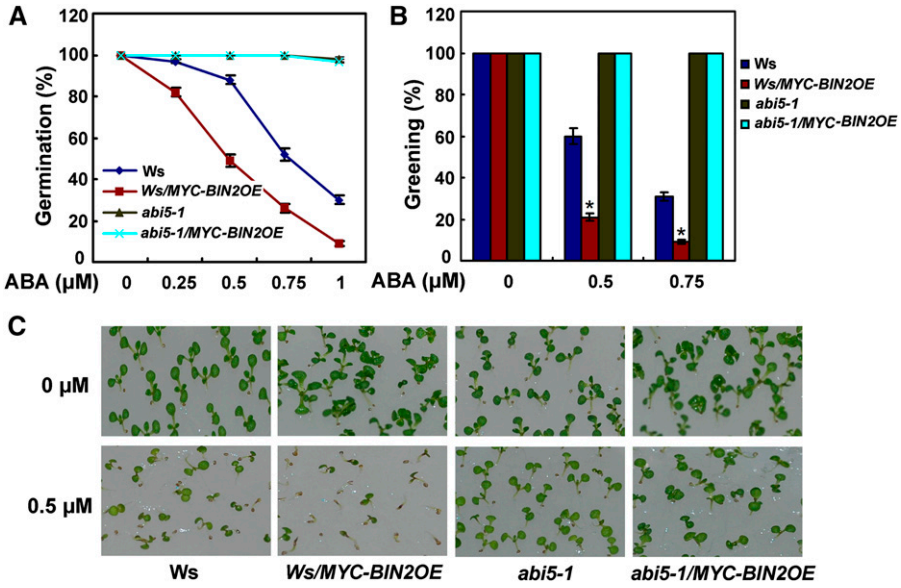

Figure 3. ABA Hypersensitivity of *BIN2*-Overexpressing Plants Requires ABI5.

(A) Germination of *BIN2*-overexpressing wild-type (*Ws/35S:MYC-BIN2*) and *abi5-1* (*abi5-1/35S:MYC-BIN2*) seeds. Germination of seeds was recorded 4 d after stratification on half-strength MS medium supplemented with different concentrations of ABA.
(B) Cotyledon greening of *Ws/35S:MYC-BIN2* and *abi5-1/35S:MYC-BIN2.* Cotyledon greening was scored 5 d after stratification on half-strength MS medium containing 0.5 or 0.75 μM ABA.
(C) Seedlings of *Ws/35S:MYC-BIN2* and *abi5-1/35S:MYC-BIN2* 6 d after germination on half-strength MS medium supplemented with 0 or 0.5 μM ABA.
Experiments described above were performed in triplicate, with each replicate evaluating more than 150 seeds. Values are means ± SD. Differences between the mutant and wild type are significant (*$P < 0.05$).

Ye et al., 2012; Khan et al., 2013; Bernardo-García et al., 2014; Cheng et al., 2014; Cho et al., 2014; Zhang et al., 2014). Because BIN2 physically interacts with ABI5, we hypothesized that BIN2 might also phosphorylate ABI5. To test this idea, we purified the bacterial-expressed 6×His-BIN2 and GST-ABI5 fused proteins and conducted in vitro kinase assays. As shown in Figure 4A, BIN2 can phosphorylate ABI5 in vitro. De Rybel et al. (2009) reported that bikinin directly binds BIN2 and inhibits its activity. To test whether BIN2 phosphorylation of ABI5 is affected by bikinin, we added bikinin to the kinase buffer and found that the phosphorylation of ABI5 by BIN2 was inhibited (Figure 4A). To confirm BIN2 phosphorylation of ABI5, we immunoprecipitated the fused protein MYC-BIN2 from *Ws/35S:MYC-BIN2* seedlings and performed kinase assays. As shown in Figure 4B, ABI5 protein was phosphorylated by immunoprecipitated MYC-BIN2 from ABA-treated seedlings. Without ABA treatment, however, the immunoprecipitated MYC-BIN2 from *Ws/35S:MYC-BIN2* seedlings did not phosphorylate ABI5 protein (Figure 4B). These results suggest that the phosphorylation of ABI5 by BIN2 from plants is ABA treatment dependent. To further verify BIN2 phosphorylation of ABI5, we performed kinase assays using total proteins extracted from ABA-treated wild-type, gain-of-function mutant *bin2-1*, and triple mutant *bin2-3 bil1 bil2* seedlings. As shown in Figure 4C, phosphorylation of ABI5 was significantly enhanced by protein extracts from *bin2-1* but was reduced by extracts from *bin2-3 bil1 bil2*. Taken together, these results indicate that BIN2 can phosphorylate ABI5 in vitro, and the phosphorylation of ABI5 by BIN2 in vivo may be dependent on ABA.

Previous studies have shown that the ABA-induced phosphorylation of ABI5 is critical for its stability (Kobayashi et al., 2005; Furihata et al., 2006; Fujii et al., 2007; Fujii and Zhu, 2009; Nakashima et al., 2009). The phosphorylation of ABI5 by BIN2 prompted us to further investigate whether BIN2 modulates ABI5 protein accumulation. To answer this question, we transiently expressed the fused protein HA-ABI5 in tobacco leaves and analyzed its accumulation using an anti-HA antibody. Interestingly, when HA-ABI5 and MYC-BIN2 were coexpressed in tobacco leaves, the accumulation of HA-ABI5 was significantly increased in response to ABA (Figure 4D). Under both ABA and eBL treatment, however, the accumulation of HA-ABI5 was reduced compared with its appearance under ABA treatment alone (Figure 4D). These observations indicate that BIN2 positively affects the ABA-induced accumulation of ABI5 protein, while BRs repress the regulation of BIN2.

BRs and BIN2 Modulate the Degradation of Native ABI5 Protein

To verify the regulatory effect of BRs on ABI5 stability, we further analyzed whether the ABA-induced accumulation of native ABI5 protein is modulated by BRs in *Arabidopsis*. Wild-type seeds were germinated and grown on half-strength MS medium containing 0.5 μM ABA with or without 1 μM eBL. Consistent with a previous study (Lopez-Molina et al., 2001), ABI5 protein was strongly induced by ABA in germinating wild-type seeds (Figure 5A; Supplemental Figure 1). Interestingly, the ABA-induced ABI5 protein levels were significantly reduced in germinating seeds

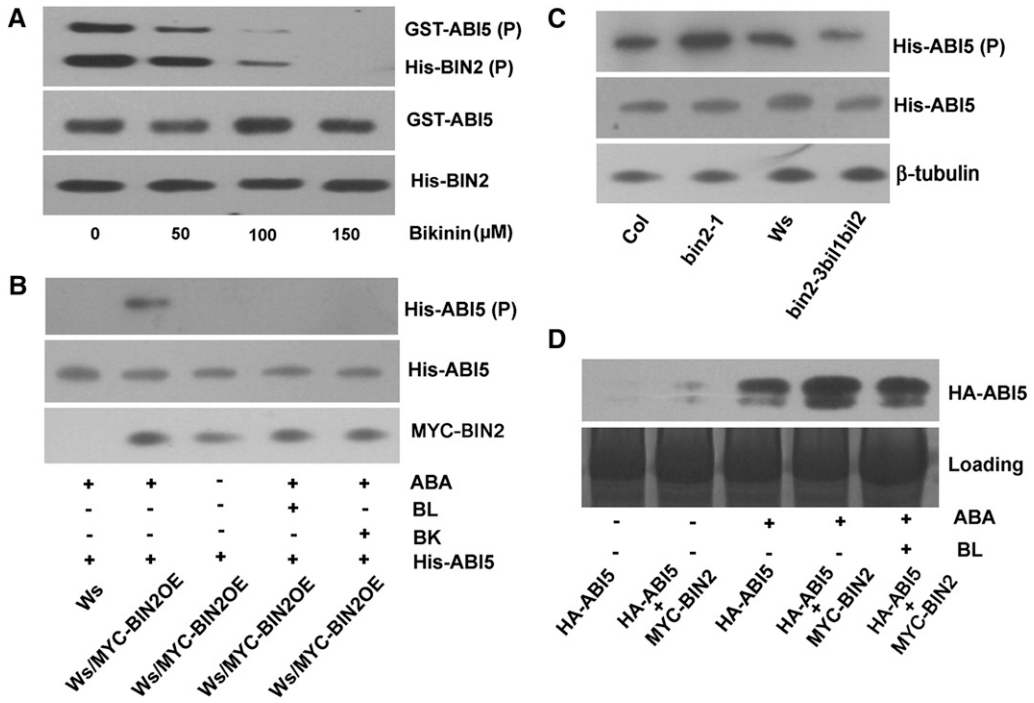

Figure 4. BIN2 Phosphorylation Stabilizes ABI5 in Response to ABA.

(A) Bacterial-expressed BIN2 phosphorylates ABI5 in vitro. Kinase assays were performed with purified 6×His-BIN2 and GST-ABI5 fused proteins from *Escherichia coli* strain BL21 (DE3) with or without the BIN2-specific inhibitor bikinin. The addition of 6×His-BIN2 and GST-ABI5 to kinase buffer were detected by protein gel blotting with an anti-His or anti-ABI5 antibodies.

(B) Phosphorylation of ABI5 by BIN2 immunoprecipitated from plants. MYC-BIN2 fused protein was immunoprecipitated from *BIN2*-overexpressing wild-type seedlings (*Ws/35S:MYC-BIN2*) subjected to ABA (50 μM), ABA (50 μM), and eBL (50 μM), or ABA (50 μM) and bikinin (BK, 100 μM) treatment. Five-day-old half-strength MS medium-grown seedlings were treated for 3 h before protein extraction. Immunoblotting for MYC-BIN2 was performed with total proteins prior to immunoprecipitation (bottom panels). The 6×His-ABI5 fused protein was also detected and is shown (middle panels).

(C) Phosphorylation of ABI5 by protein extracts from ABA-treated seedlings of wild type (Col and Ws), *bin2-1*, and *bin2-3 bil1 bil2.* Five-day-old half-strength MS medium-grown seedlings were treated with ABA (50 μM) for 3 h before protein extraction. Immunoblotting for the β-tubulin (bottom panels) was performed as a control. The 6×His-ABI5 fused protein was also detected and is shown (middle panels).

(D) Regulatory effect of BIN2 on transiently expressed ABI5 protein accumulation. MYC-BIN2 protein was coexpressed with HA-ABI5 in tobacco leaves, and HA-ABI5 was detected using an anti-HA antibody. The transformed tobacco leaves were treated with ABA (150 μM) or ABA (150 μM) plus eBL (50 μM) for 8 h before protein extraction. These experiments described above were repeated three times with similar results.

treated with eBL (Figure 5A; Supplemental Figures 1 and 2). Notably, the eBL-induced degradation of ABI5 protein was attenuated by the 26S proteasome inhibitor MG132 (Figure 5B), indicating that the 26S proteasome pathway is required for the BRs-mediated reduction of ABI5. Having ascertained that BRs induce the degradation of native ABI5, we then asked whether BIN2 is also involved in the regulation of native ABI5 degradation. To test this, we measured the ABA-induced accumulation levels of native ABI5 protein in germinating seeds of *bin2-1* and *bin2-3 bil1 bil2*. Compared with those in germinating wild-type seeds, the ABA-induced accumulation levels of ABI5 were enhanced in germinating *bin2-1* seeds but reduced in germinating *bin2-3 bil1 bil2* seeds (Figure 5C; Supplemental Figure 3), suggesting that BIN2 positively modulates the ABA-induced accumulation of native ABI5 protein.

Expression of Several ABI5 Regulons Is Reduced in ABA-Treated Germinating Seeds of *bin2-3 bil1 bil2*

To further determine the regulatory effect of BIN2 on ABI5, we examined the expression of several downstream target genes of ABI5 in ABA-treated germinating seeds of *bin2-1* and *bin2-3 bil1 bil2*, including the drought-induced genes *RD29A*, *RD29B*, *ALCOHOL DEHYDROGENASE1* (*ADH1*), *RESPONSIVE TO ABA18* (*RAB18*), *LATE EMBRYOGENESIS ABUNDANT6* (*EM6*), and *EM1.* As shown in Figure 6, the expression levels of these ABA-responsive genes were greater in germinating *bin2-1* seeds than those in germinating wild-type seeds (Figure 6). In contrast, transcripts of these genes were reduced in germinating seeds of *bin2-3 bil1 bil2* compared with those in the wild type (Figure 6). These results indicate that BIN2 positively regulates the expression of several downstream target genes of ABI5 in ABA-treated germinating seeds.

BIN2 Phosphorylation Sites Are Required for ABI5 Activity

Using mass spectrometry, Lopez-Molina et al. (2002) showed that ABI5 proteins were phosphorylated at several sites, including Thr-35, Ser-36, Ser-41, Ser-42, Ser-138, Ser-139, Ser-145, and Thr-201. Among those phosphoamino sites, Ser-42, Ser-145, and Thr-201 may be phosphorylated by SnRK2 protein kinases

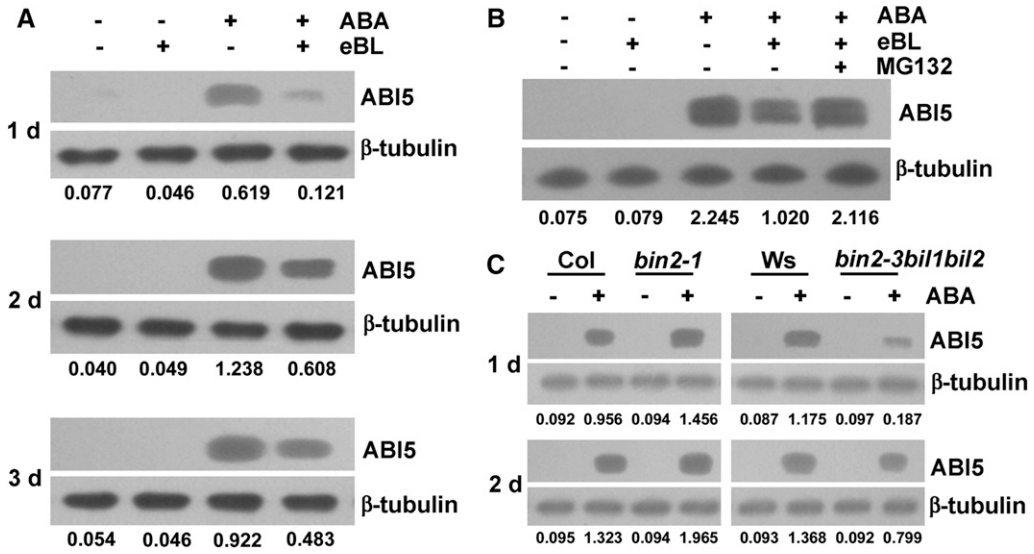

Figure 5. BRs Induce the Degradation of Native ABI5 Protein.

(A) Seeds of wild type (Col) were germinated on half-strength MS medium containing 0.5 μM ABA with or without 1 μM eBL for the indicated times. The accumulation of native ABI5 protein was detected by immunoblotting with an anti-ABI5 antibody. Immunoblotting was performed with the β-tubulin antibody as a loading control. The relative levels of ABI5 were normalized to β-tubulin.

(B) BR-induced degradation of native ABI5 protein is via the 26S proteasome pathway. Seeds of the wild type (Col) were germinated on half-strength MS medium for 24 h, transferred to medium containing various combinations of 0.5 μM ABA, 1 μM eBL, and 50 μM MG132, and cultured for an additional 48 h. The accumulation of native ABI5 protein was detected by immunoblotting as described in **(A)**. Immunoblotting was performed with the β-tubulin antibody as a loading control. The relative levels of ABI5 were normalized to β-tubulin.

(C) Involvement of BIN2 in degradation of native ABI5 protein. Seeds of the wild type (Col and Ws), *bin2-1*, and *bin2-3 bil1 bil2* were germinated on half-strength MS medium containing 0.5 μM ABA for the indicated times. The accumulation of native ABI5 protein was detected by immunoblotting as described in (A). Immunoblotting was performed with the β-tubulin antibody as a loading control. The relative levels of ABI5 were normalized to β-tubulin. The experiments described above were repeated three times with similar results.

(Kobayashi et al., 2005; Furihata et al., 2006; Fujii et al., 2007; Fujii and Zhu, 2009; Nakashima et al., 2009). Interestingly, Ser-41 may be a potential phosphorylation site of the kinase BIN2; Ser-41 is located in the region containing one typical conserved recognition site (S/TxxxS/T, S/T corresponds to Ser or Thr and x denotes any other residue) of GSK3 kinases (Woodgett, 2001). To analyze whether BIN2 phosphorylates ABI5 on Ser-41, we mutated Ser-41 to Ala (GST-ABI5 S41A) and performed kinase assays. As shown in Figure 7A, BIN2 phosphorylates GST-ABI5 S41A with reduced activity, suggesting that Ser-41 of ABI5 is a site of phosphorylation by BIN2. As BIN2 still phosphorylated the mutant protein GST-ABI5 S41A (Figure 7A), other unconserved sites on ABI5 may also be phosphorylated by BIN2. Recently, Gudesblat et al. (2012) showed that SPEECHLESS (SPCH) was phosphorylated by BIN2 at several unconserved sites (e.g., Ser-38, Ser-43, and Ser-171), which are close to the typical recognition sites. To test whether BIN2 also phosphorylates the unconserved sites (Thr-35 and Ser-36) close to the typical recognition site of Ser-41 on ABI5, we further mutated those two amino acids to Ala, resulting in the mutant combination GST-ABI5 S41A T35A S36A. As shown in Figure 7A, the phosphorylation level of GST-ABI5 S41A T35A S36A by BIN2 in vitro was further reduced compared with that of GST-ABI5 S41A, indicating that Thr-35 and Ser-36 are also BIN2 target sites. Moreover, besides Ser-41, several potential BIN2 phosphorylation sites in ABI5 (SNGRS, TGDPS, SPVSS, and SAARS) with canonical S/TxxxS/T sequences were found. To test whether those sites are also BIN2 phosphorylation sites, we mutated those amino acids to Ala and the mutant proteins were designated as GST-ABI5 S176A S180A, GST-ABI5 T255A S259A, GST-ABI5 S314A S318A, and GST-ABI5 S368A S372A, respectively. As shown in Figure 7A, the phosphorylation level of GST-ABI5 S368A S372A by BIN2 was slightly reduced compared with that of wild-type ABI5. We then mutated Ser-368 and Ser-372 sites to Ala in combination with S41A T35A S36A mutation and designated the mutant protein as GST-ABI5 S41A T35A S36A S368A S372A. Interestingly, in vitro kinase assays showed that BIN2 almost didn't phosphorylate GST-ABI5 S41A T35A S36A S368A S372A (Figure 7A). The decreased phosphorylation of GST-ABI5 S41A T35A S36A S368A S372A by BIN2 was not due to a reduced interaction between BIN2 and the mutant form of ABI5 (Supplemental Figure 4), indicating that Ser-368 and Ser-372 are also BIN2 phosphorylation sites.

To functionally analyze the involvement of BIN2 phosphorylation sites in ABI5 activity, we fused the mutant protein ABI5 S41A T35A S36A with HA tag, designated as the HA-ABI5 mutant, and introduced the mutant protein into the *abi5-1* background under its native promoter (*abi5-1/HA-ABI5 mutant*). As shown in Figure 7B, the mutated form of ABI5 failed to fully rescue the ABA-tolerant phenotype of *abi5-1* during seed germination. Consistently, the ABA-induced accumulation of the mutant form of ABI5 was

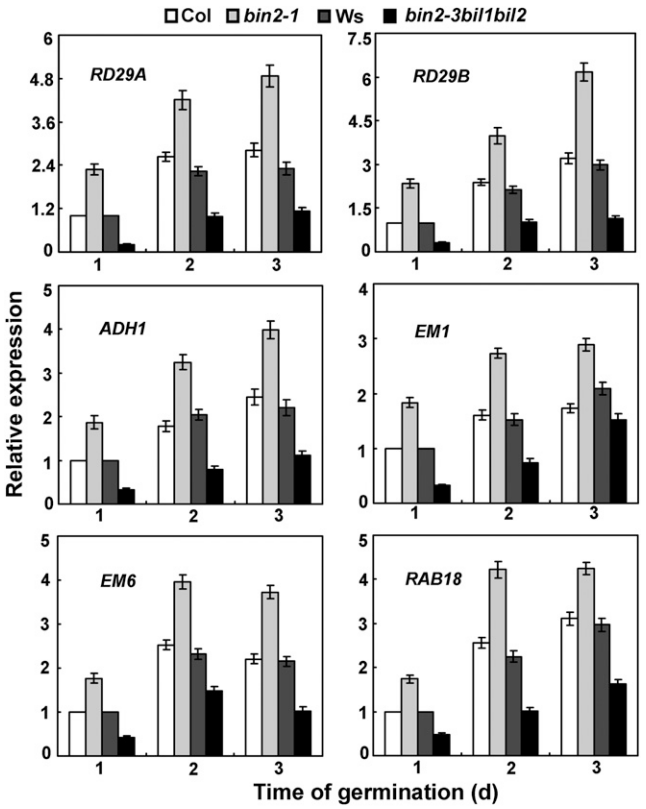

Figure 6. Expression of ABI5 Regulons in *bin2-1* and *bin2-3 bil1 bil2*.

Seeds of wild types (Col and Ws), *bin2-1*, and *bin2-3 bil1 bil2* were germinated on medium containing 0.5 μM ABA for indicated times. Total RNAs were extracted from those germinating seeds, and RT-qPCR analysis was performed. Error bars show SD from three independent RNA extractions.

reduced compared with that of wild-type form (Figure 7C). Moreover, the expression levels of *RD29B* were lower in the *abi5-1/HA-ABI5* mutant than those in *abi5-1/HA-ABI5* (Figure 7D). These results indicate that those BIN2 phosphorylation sites are essential for ABI5 function in response to ABA during seed germination.

BRs Partially Repress ABA Hypersensitivity of *ABI5*-Overexpressing Plants

Previous study showed that overexpression of *ABI5* renders transgenic plants hypersensitive to ABA (Lopez-Molina et al., 2001). As BRs are involved in the degradation of ABI5 protein, we queried whether BRs affect ABA hypersensitivity of *ABI5*-overexpressing plants. To test this possibility, we initially evaluated the performance of wild type and *ABI5*-overexpressing plants (*35S:ABI5-4MYC*; Chen et al., 2012) in half-strength MS medium containing 0.5 μM ABA with or without 1 μM eBL. Consistent with a previous study (Zhang et al., 2009), the ABA sensitivity of wild-type plants was partially reversed by applying eBL (Figures 8A and 8B). Interestingly, application of eBL also partially repressed the ABA-hypersensitive phenotype of *ABI5*-overexpressing plants (Figures 8A and 8B). To confirm this observation, we further analyzed the ABA-induced accumulation of ABI5-4MYC fused protein in response to eBL using anti-MYC antibody. As shown in Figure 8C, under both ABA and eBL treatment, the accumulation of ABI5-4MYC fused protein was reduced compared with its appearance under ABA treatment alone. Consistent with these findings, the ABA-induced expression levels of *RD29B* and *RAB18* were reduced under both ABA and eBL treatment compared with those under ABA treatment alone (Figure 8D).

DISCUSSION

BIN2 Is a Critical Node for BR-ABA Antagonism during Seed Germination

Previous studies have highlighted the close relationship between BRs and ABA during seed germination and postgerminative growth, demonstrating that BRs act antagonistically with ABA to promote these processes (Clouse et al., 1996; Ephritikhine et al., 1999; Leubner-Metzger, 2001; Steber and McCourt, 2001; Chen et al., 2004; Zhang et al., 2009). However, the exact molecular mechanism underlying the antagonistic effect of BRs on ABA remains unclear. In this study, we further characterized the regulatory role of the BIN2 kinase, a critical repressor of BR signaling, in mediating ABA response during seed germination and subsequent postgerminative growth. We found evidence that BIN2 positively modulates ABA response (Figure 1) and serves as an integration node for BR and ABA signals to regulate seed germination (Figure 2). BIN2 phosphorylates and stabilizes ABI5 in response to ABA, whereas BRs inhibit the activity of BIN2 to affect ABA response (Figures 4 and 5). Further analysis demonstrated that the action of BIN2 in ABA responses requires ABI5, as disruption of ABI5 represses the hypersensitive response of *BIN2*-overexpressing plants to ABA (Figure 3). Moreover, BIN2 was found to positively regulate the expression of several downstream target genes of ABI5 in response to ABA (Figure 6; Supplemental Figure 5). Our results thus reveal a pathway in which the BR-associated BIN2 kinase directly regulates the ABA-responsive ABI5 transcription factor to integrate BR and ABA signals during seed germination and postgerminative growth.

Recently, Cai et al. (2014) demonstrated that BIN2 physically interacts with subgroup III SnRK2 kinases and positively regulates ABA signaling to inhibit root elongation. Biochemical analysis in their study revealed that BIN2 phosphorylates SnRK2.2 and SnRK2.3. BIN2 phosphorylation is crucial for SnRK2.3 kinase activity, as mutation of the BIN2 phosphorylation site (Thr-180) on SnRK2.3 greatly blocked the activation of SnRK2.3. Further genetic analysis showed that BIN2-enhanced ABA signaling is dependent on SnRK2s, suggesting that SnRK2s are required for the role of BIN2 to mediate ABA response. However, our research showed that BIN2 physically interacts with and phosphorylates/stabilizes ABI5 (Figures 4, 5, and 7), indicating that BIN2 can directly promote ABA signaling through the downstream component of SnRK2s. These differences may result from the facts that SnRK2s and BIN2 phosphorylate ABI5 at different sites (Figure 7; Furihata et al., 2006), and stabilization/promotion of ABI5 by BIN2 phosphorylation is dependent on the phosphorylation of ABI5 by SnRK2s. Moreover, it's possible that there may be other crucial positive modulators that are regulated by SnRK2s but independent of BIN2. Consistent with our

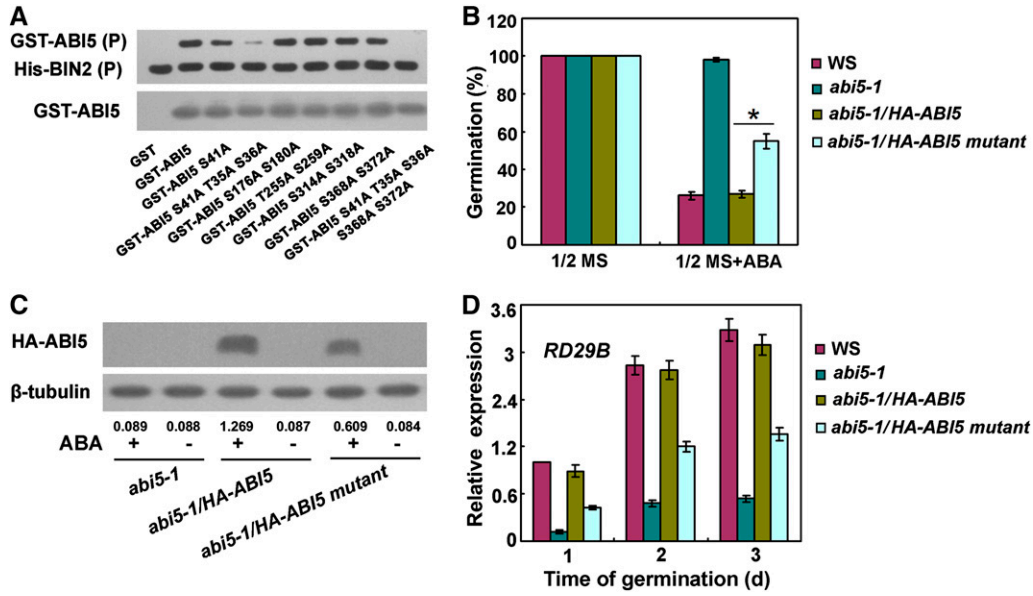

Figure 7. BIN2 Phosphorylation Sites Are Critical for ABI5 Function.

(A) Ser-41, Thr-35, Ser-36, Ser-368, and Ser-372 on ABI5 are BIN2 phosphorylation sites. Kinase assays were performed with purified 6×His-BIN2 and wild-type or mutant forms of GST-ABI5 from *E. coli* strain BL21 (DE3). The addition of GST-ABI5 to kinase buffer was detected by protein gel blotting with an anti-ABI5 antibody and is shown.

(B) Functional analysis of the involvement of Ser-41, Thr-35, and Ser-36 in ABI5 activity during seed germination. Germination of seeds was scored 4 d after stratification on half-strength MS medium with or without 0.75 μM ABA. Experiments were performed in triplicate, with each replicate evaluating more than 150 seeds. Values are means ± SD. Difference between *abi5-1/HA-ABI5* and *abi5-1/HA-ABI5* mutant is significant (*$P < 0.05$). WS, the wild type.

(C) ABA-induced accumulation of mutant form of ABI5 was reduced compared with that of wild-type form. Seeds were germinated on half-strength MS medium with or without 0.5 μM ABA for 24 h. The accumulation of HA-ABI5 fused protein was detected by immunoblotting with an anti-HA antibody. Immunoblotting was performed with the β-tubulin antibody as a loading control. The relative levels of ABI5 were normalized to β-tubulin.

(D) Expression levels of *RD29B* were lower in *abi5-1/HA-ABI5* mutant than those in *abi5-1/HA-ABI5*. Seeds were germinated on medium containing 0.5 μM ABA for indicated times. Total RNAs were extracted from those germinating seeds, and RT-qPCR analysis was performed. Error bars show SD from three independent RNA extractions. Experiments described above were repeated three times with similar results.

result (Supplemental Figure 5), Cai et al. (2014) found that BIN2 positively regulated the induced expression levels of several ABA-responsive genes in germinated seedlings (10-d-old seedlings). Interestingly, our yeast-two hybrid assays showed that BIN2 and its homologs also interact with ABSCISIC ACID RESPONSIVE ELEMENT BINDING FACTOR1 (ABF1) and/or ABF3 (Supplemental Figure 6), two critical regulators of ABA signaling in germinated seedlings. We thus speculate that BIN2 and its homologs may also directly phosphorylate and activate those factors to modulate ABA signaling.

The transcription factor BZR1, a positive regulator of the BR signaling pathway, was previously identified as a direct target of the BIN2 kinase (He et al., 2002). In the absence of BRs, BIN2 phosphorylates and destabilizes BZR1 protein (He et al., 2002). Recently, Tsugama et al. (2013) showed that overexpression of the *BZR1* gene alleviated the inhibitory effect of ABA in transgenic plants, suggesting that BZR1 negatively mediates ABA response. Consequently, BIN2 appears to positively modulate ABA responses partially by repressing the BZR1-mediated signaling pathway. As the ABA-hypersensitive response of *BIN2*-overexpressing plants is ABI5 dependent (Figure 3), there might be a regulatory relationship between BZR1- and ABI5-mediated ABA responses. However, using the yeast two-hybrid assay, we did not find any physical interaction between BZR1 and ABI5 (Supplemental Figure 7). Interestingly, Ryu et al. (2014) recently reported that BRI1-EMS-SUPPRESSOR1 (BES1), a homolog of BZR1, forms a transcriptional repressor complex with TOPLESS and HISTONE DEACETYLASE19 to regulate the expression of ABI3 and ABI5, thereby suppressing ABA signaling during early seedling development. These findings support the notion that BIN2 mediates ABA signaling partially through regulating downstream transcription factors. Furthermore, all of these studies collectively revealed that BIN2 modulates ABA signaling at multiple layers (Supplemental Figure 8), which may finely balance the interaction between BR and ABA signals to establish appropriate stress tolerance but minimize detrimental effects on plant growth and development.

ABA-Activated BIN2 Phosphorylation Is Essential for ABI5 Function

Protein interaction assays demonstrated that BIN2 physically interacts with ABI5 in yeast and plant cells (Figure 2), and further analysis showed that bacterial-expressed BIN2 can phosphorylate ABI5 in vitro (Figures 4A and 7A). Although the BIN2-ABI5 physical interaction and the phosphorylation of ABI5 by bacterial-expressed BIN2 in vitro are independent of ABA, the phosphorylation of ABI5

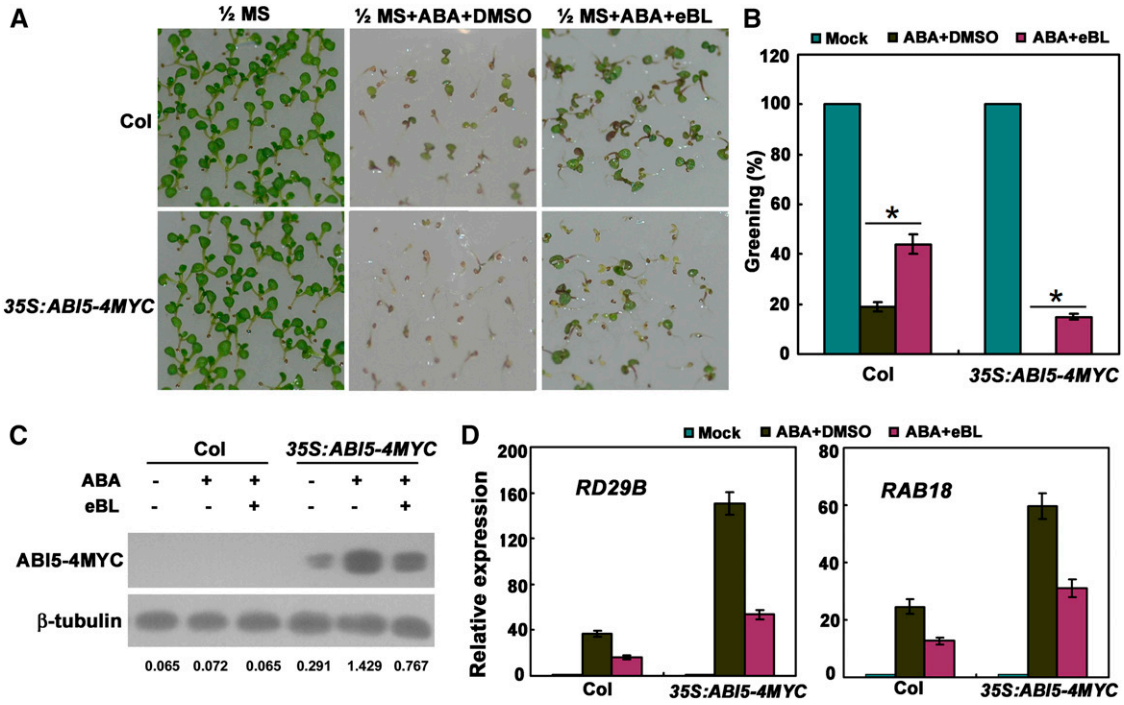

Figure 8. BRs Partially Repress ABA-Hypersensitive Phenotype of *ABI5*-Overexpressing Plants.

(A) Seedlings of wild-type (Col) and *ABI5*-overexpressing plants (*35S:ABI5-4MYC*) 7 d after germination on half-strength MS medium supplemented with 0.75 μM ABA with or without 1 μM eBL. Experiments were repeated three times with similar results.

(B) Cotyledon greening of the wild type (Col) and *35S:ABI5-4MYC* 5 d after stratification on half-strength MS medium containing 0.75 μM ABA with or without 1 μM eBL. Values are means ± SD. Differences between eBL-treated seedlings and eBL-nontreated seedlings are significant (*$P < 0.05$).

(C) Regulatory effect of BRs on the accumulation of overexpressed ABI5-4MYC fused protein. Seven-day-old wild-type (Col) and *35S:ABI5-4MYC* seedlings were treated with 100 μM ABA with or without 50 μM eBL for 6 h before protein extraction. The accumulation of ABI5-4MYC fused protein was detected by immunoblotting with an anti-MYC antibody. Immunoblotting was performed with the β-tubulin antibody as a loading control. The relative levels of ABI5 were normalized to β-tubulin. Experiments were repeated three times with similar results.

(D) ABA-induced expression of *RD29B* and *RAB18* in *35S:ABI5-4MYC* was repressed by BRs. Five-day-old seedlings of the wild type (Col) and *35S:ABI5-4MYC* were treated with 100 μM ABA with or without 50 μM eBL for 4 h before RNA extraction. Error bars show SD from three independent RNA extractions.

by plant-expressed BIN2 is ABA dependent (Figure 4B). These observations suggest that there may be other factors interfering with plant-expressed BIN2 to phosphorylate ABI5 in the absence of ABA and that high ABA levels may remove these factors to stimulate BIN2 activity and phosphorylate ABI5. Similarly, Zhang et al. (2009) found that ABA induces the phosphorylation of BES1, whereas once the BIN2 activity was inhibited, ABA failed to induce BES1 phosphorylation, further indicating that ABA can activate the activity of BIN2 kinase. Nevertheless, the mechanisms underlying how ABA stimulates BIN2 kinase to phosphorylate its substrates remain elusive, and further research is required to fill in the gaps in our knowledge of this process.

Phosphorylation/dephosphorylation and degradation are critical posttranslational modifications of the ABI5 protein. Previous studies have revealed that high ABA levels promote AB15 phosphorylation and stabilization (Fujii et al., 2007; Nakashima et al., 2009) and that the kinases SnRK2.2, SnRK2.3, and SnRK2.6 are responsible for this ABA-responsive phosphorylation (Kobayashi et al., 2005; Fujii et al., 2007; Fujii and Zhu, 2009; Nakashima et al., 2009). In this study, we demonstrated that the GSK3-like kinase BIN2 also phosphorylates and stabilizes the ABI5 protein in response to ABA (Figures 4, 5, and 7). When coexpressed with BIN2, ABI5 protein levels significantly increased after ABA treatment (Figure 4D). The ABA-induced accumulation of ABI5 was enhanced in germinating *bin2-1* seeds but reduced in germinating *bin2-3 bil1 bil2* seeds (Figure 5C) compared with those in germinating wild-type seeds. More importantly, mutations of several BIN2 phosphorylation sites on ABI5 made the mutant protein fail to properly respond to ABA (Figure 7). The mutated form of ABI5 protein accumulated abnormally and failed to fully rescue the phenotype of *abi5-1* during seed germination (Figure 7). These results provide evidence that direct phosphorylation of ABI5 by BIN2 is crucial for the regulation of ABA responses by ABI5 during seed germination.

Based on the ABA responses of triple mutants *bin2-3 bil1 bil2* and *snrk2.2 snrk2.3 snrk2.6* (Figure 1; Fujii and Zhu, 2009), we speculate that SnRK2 kinase-mediated AB15 phosphorylation may play a dominant role in the regulation of ABI5 during seed germination. Nevertheless, BIN2 is also involved in ABA-induced phosphorylation and stability of ABI5 (Figures 4, 5, 7, and 8) and

is important for maintaining a delicate balance of hormonal signaling during seed germination (Supplemental Figure 8). In the presence of BRs, BR signaling suppresses the activity of BIN2 to phosphorylate the ABA-responsive transcription factor ABI5, thereby antagonizing the inhibitory role of ABA and promoting seed germination. In the absence of BRs, however, ABA stimulates BIN2 activity to phosphorylate and stabilize ABI5, thereby repressing seed germination.

In addition to ABA and BR signaling pathways, a previous study indicated that gibberellin signaling is also involved in the degradation of ABI5 during seed germination (Piskurewicz et al., 2008). Guan et al. (2014) recently demonstrated that cytokinin signaling antagonizes ABA-mediated inhibition of cotyledon greening in *Arabidopsis*. Further analysis in their study revealed that cytokinin signaling also promotes the degradation of ABI5 via the 26S proteasome pathway (Guan et al., 2014). Moreover, ABI5 also integrates light and ABA signaling during seed germination and early seedling development (Chen et al., 2008). Collectively, these previous results and our findings suggest that ABI5 serves as a critical modulator to integrate multiple signaling pathways during seed germination and/or postgerminative growth. Further investigating the crosstalk between these signaling pathways may enhance our understanding of the regulatory mechanisms of ABI5-repressed seed germination and/or postgerminative growth.

Dual Roles of BIN2 Phosphorylation in Regulating Its Substrates

Functioning as a crucial regulator of BR signaling, BIN2 was previously reported to physically interact with and phosphorylate its substrates to modulate BR signaling and other physiological programs (He et al., 2002; Yin et al., 2002; Vert et al., 2008; Gudesblat et al., 2012; Kim et al., 2012; Ye et al., 2012; Khan et al., 2013; Bernardo-García et al., 2014; Cheng et al., 2014; Cho et al., 2014; Zhang et al., 2014). Accumulating evidence has demonstrated that BIN2 plays dual (positive or negative) roles in regulating the stability/activity of its various substrates (Supplemental Figure 9). For example, Yin et al. (2002) showed that BIN2 phosphorylates and destabilizes BES1 to negatively regulate BR signaling. In the absence of BRs, BIN2 phosphorylation promotes the degradation and prevents the nuclear accumulation of BES1. Similarly, He et al. (2002) found that BIN2 directly phosphorylates BZR1 and negatively regulates its protein accumulation in vivo. Recently, BIN2 was found to regulate stomatal development by inhibiting a mitogen-activated protein kinase (MAPK) pathway (Gudesblat et al., 2012; Kim et al., 2012; Khan et al., 2013). BIN2 phosphorylates YDA (a MAPK kinase kinase), MKK4, and MKK5 (two MAPK kinases) and inhibits their kinase activities (Kim et al., 2012; Khan et al., 2013). In addition, BIN2 also negatively modulates the stability of SPCH (Gudesblat et al., 2012), a transcription factor acting downstream of the MAPK cascade. Recently, Cheng et al. (2014) found that BIN2 phosphorylates ENHANCER OF GLABRA3 (EGL3) and TRANSPARENT TESTA GLABRA1 (TTG1) and inhibits the activity of the WEREWOLF-EGL3-TTG1 transcription complex, thereby regulating root epidermal cell patterning. BIN2 phosphorylation was also shown to negatively affect the DNA binding activity of its substrate, such as AUXIN RESPONSE FACTOR2 (ARF2) (Vert et al., 2008). Taken together, BIN2 phosphorylation is negatively involved in modulating the stability or activity of those substrates to mediate various physiological processes.

In contrast, recent studies have shown that several proteins are positively regulated by BIN2. In this study, we provide evidence that BIN2 phosphorylates and stabilizes ABI5 protein in response to ABA (Figures 4, 5, and 7). Likewise, BIN2 phosphorylation stabilizes HOMEODOMAIN-LEUCINE ZIPPER PROTEIN1 and MYELOBLASTOSIS FAMILY TRANSCRIPTION FACTOR LIKE-2, which were reported to mediate the BR signaling pathway (Ye et al., 2012; Zhang et al., 2014). Moreover, BIN2 phosphorylation was demonstrated to positively affect the activity of its interacting kinase. Cai et al. (2014) recently showed that BIN2 phosphorylation enhances the kinase activity of SnRK2.3 to positively modulate ABA signaling. In addition, Cho et al. (2014) found that BIN2 phosphorylation of ARF7 and ARF19 attenuates their interaction with AUXIN/INDOLE-3-ACETIC ACID repressor proteins, subsequently activating these ARF proteins and potentiating auxin signaling output to regulate lateral root development.

METHODS

Materials and Plant Growth Conditions

Plant hormones, bikinin, and the 26S proteasome inhibitor MG132 were purchased from Sigma-Aldrich. Taq DNA polymerases were purchased from Takara Biotechnology, and other common chemicals were obtained from Shanghai Sangon. The anti-MYC, anti-HA, anti-His, and antitubulin antibodies were purchased from Sigma-Aldrich, and the anti-ABI5 antibody was kindly provided by Jianru Zuo (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). Seeds of *Arabidopsis thaliana bin2-1* and *bri1-5* were kindly provided by Zhiyong Wang (Stanford University), and seeds of *bin2-3* and *bin2-3 bil1 bil2* were obtained from Jianming Li (Shanghai Center for Plant Stress Biology and Institute of Plant Physiology and Ecology, Chinese Academy of Sciences). Seeds of *35S:ABI5-4MYC* were obtained from Chuanyou Li (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). *bin2-1* and *35S:ABI5-4MYC* are in the *Arabidopsis* Columbia-0 (Col) background, while *bin2-3*, *bin2-3 bil1 bil2*, *bri1-5*, and *abi5-1* are in the Wassilewskija (Ws) background. *Arabidopsis* plants were grown in an artificial growth chamber at 22°C under a 14-h-light/10-h-dark photoperiod.

Determination of Germination and Greening

The germination of wild-type and mutant seeds was performed as described previously (Xiong et al., 2001). Germination was determined based on the appearance of an 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. Three independent experiments were conducted and similar results were obtained.

Yeast Two-Hybrid Screening and Confirmation

The full-length of BIN2, BIL1, or BIL2 was fused to the bait vector pGBKT7 and then transformed into the yeast strain Y2HGold (Clontech). The cDNA library was obtained from Clontech (catalog number 630487). Yeast screening was performed as described previously (Hu et al., 2013a). To confirm the interaction, full-length encoding sequences of ABI5, ABF1, ABF2, ABF3, ABF4, and AREB3 were cloned into the prey vector pGADT7. Primers used for generating various clones in this study are listed in Supplemental Table 1.

BiFC Assays

cDNA sequences of the N-terminal, 173-amino acid, enhanced YFP (nYFP) and C-terminal, 64-amino acid (cYFP) fragments were PCR amplified and cloned into pFGC5941 to generate pFGC-nYFP and pFGC-cYFP, respectively (Kim et al., 2008). Full-length BIN2-encoding sequences were inserted into pFGC-cYFP to generate a C-terminal in-frame fusion with cYFP, while ABI5-encoding sequences were introduced into pFGC-nYFP to form an N-terminal in-frame fusion with nYFP. The resulting plasmids were introduced into *Agrobacterium tumefaciens* (strain GV3101), with infiltration of tobacco (*Nicotiana benthamiana*) performed as described previously (Hu et al., 2013a). Infected leaves were analyzed 48 h after infiltration. YFP and 4′,6-diamidino-2-phenylindole fluorescence were observed under a confocal laser scanning microscope (Olympus).

CoIP Assays

Full-length coding sequences of BIN2 and ABI5 were individually cloned into tagging plasmids behind MYC or HA tag sequences in the sense orientation behind the cauliflower mosaic virus 35S promoter (Hu et al., 2013a). MYC-fused BIN2 and HA-fused ABI5 were then transiently coexpressed in *N. benthamiana.* Infected leaves were sectioned 48 h after infiltration. CoIP assays were performed using leaf protein extracts as described previously (Hu et al., 2013a). Briefly, MYC-fused BIN2 were immunoprecipitated using an anti-MYC antibody (1:250), and the coimmunoprecipitated protein was then detected using an anti-HA antibody (Sigma-Aldrich) (1:10,000).

In Vitro Kinase Assays

In vitro kinase assays were performed as described previously (Yin et al., 2002). The 6×His-BIN2 and GST-ABI5 fused proteins were purified from *Escherichia coli* strain BL21 (DE3). For in vitro kinase assays, the purified 6×His-BIN2 (2 μg) was incubated with GST-ABI5 (2 μg) in 20 μL of kinase buffer (20 mM Tris, pH 7.5, 100 mM NaCl, and 12 mM $MgCl_2$) containing 100 μM ATP and 10 μCi of [γ-^{32}P]ATP with or without bikinin. When using immunoprecipitated MYC-BIN2 fused protein in vitro kinase assays, total proteins were extracted from seedlings with buffer (10 mM Tris, pH 7.5, 0.5% Nonidet P-40, 2 mM EDTA, 150 mM NaCl, 1 mM PMSF, and 1× protease inhibitor). The MYC-fused BIN2 kinase was immunoprecipitated from total proteins, and the 6×His-ABI5 fusion protein was expressed in *E. coli* strain BL21 (DE3). When using plant protein extracts (20 μg) for in vitro kinase assays, the total proteins were extracted from seedlings with 1× kinase buffer containing 1 mM DTT, 1 mM PMSF, and 1× protease inhibitor. After incubation at 30°C for 30 min, the reactions were stopped by the addition of 6× loading buffer followed by boiling for 5 min. Proteins were separated by electrophoresis using 12% acrylamide gels, and phosphorylation was detected by exposing the dried gels to x-ray films.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted using the Trizol reagent (Invitrogen). Quantitative real-time PCR (RT-qPCR) was performed as described by Hu et al. (2013b). Briefly, first-strand cDNA was synthesized from 1.5 μg DNase-treated RNA in a 20-μL reaction volume using M-MuLV reverse transcriptase (Fermentas) with oligo (dT)18 primer. RT-qPCR was performed using 2× SYBR Green I master mix on a Roche LightCycler 480 real-time PCR machine, according to the manufacturer's instructions. At least three biological replicates for each sample were used for RT-qPCR analysis and at least two technical replicates were analyzed for each biological replicate. The *ACTIN2* gene was used as a control. Gene-specific primers used to detect transcripts are listed in Supplemental Table 2.

Accession Numbers

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: *BIN2*, AT4G18710; *BIL1*, AT2G30980; *BIL2*, AT1G06390; *ABI5*, AT2G36270; *ABF1*, AT1G49720; *ABF2*, AT1G45249; *ABF3*, AT4G34000; *ABF4*, AT3G19290; *AREB3*, AT3G56850; *SnRK2.2*, AT3G50500; *SnRK2.3*, AT5G66880; *SnRK2.6*, AT4G33950; *BRI1*, AT4G39400; *BZR1*, AT1G75080; *RD29A*, AT5G52310; *RD29B*, AT5G52300; *ADH1*, AT1G77120; *EM1*, AT3G51810; *EM6*, AT2G40170; and *ACTIN2*, AT3G18780.

Supplemental Data

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

Supplemental Figure 1. Germination Rates of Samples Corresponding to Those Used for Protein Extracts in Figure 5A.

Supplemental Figure 2. Immunoprecipitated ABI5 Treated with Calf Alkaline Phosphatase and Detected by Protein Gel Blotting.

Supplemental Figure 3. Germination Rates of Samples Corresponding to Those Used for Protein Extracts in Figure 5C.

Supplemental Figure 4. Yeast Two-Hybrid Assay Analysis of the Interactions between BIN2 and the Mutant Forms of ABI5.

Supplemental Figure 5. ABA-Induced Expression of ABI5 Regulons in Five-Day-Old Seedlings of *bin2-1* and *bin2-3 bil1 bil2*.

Supplemental Figure 6. Yeast Two-Hybrid Assay Analysis of the Interactions between BIN2 and the Homologs of ABI5.

Supplemental Figure 7. Yeast Two-Hybrid Assay Analysis of the Interaction between ABI5 and BZR1.

Supplemental Figure 8. A Simplified Model for the Antagonistic Effect of BRs on ABA during Seed Germination.

Supplemental Figure 9. A Simplified Model for the Regulation of Various Substrates by BIN2 Kinase.

Supplemental Table 1. Primers Used for Generating Various Clones.

Supplemental Table 2. Primers Used for RT-qPCR.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Y.H. designed and performed experiments, interpreted data, and wrote the article. D.Y. designed experiments, interpreted data, and edited the article. Both authors read and approved the final article.

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BRASSINOSTEROID INSENSITIVE2 Interacts with ABSCISIC ACID INSENSITIVE5 to Mediate the Antagonism of Brassinosteroids to Abscisic Acid during Seed Germination in *Arabidopsis*

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