SOS2-LIKE PROTEIN KINASE5, an SNF1-RELATED PROTEIN KINASE3-Type Protein Kinase, Is Important for Abscisic Acid Responses in Arabidopsis through Phosphorylation of ABSCISIC ACID-INSENSITIVE5[1][OPEN]

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Abscisic acid (ABA) plays an essential role in seed germination. In this study, we demonstrate that one SNF1-RELATED PROTEIN KINASE3-type protein kinase, SOS2-LIKE PROTEIN KINASE5 (PKS5), is involved in ABA signal transduction via the phosphorylation of an interacting protein, ABSCISIC ACID-INSENSITIVE5 (ABI5). We found that *pks5-3* and *pks5-4*, two previously identified PKS5 superactive kinase mutants with point mutations in the PKS5 FISL/NAF (a conserved peptide that is necessary for interaction with SOS3 or SOS3-LIKE CALCIUM BINDING PROTEINs) motif and the kinase domain, respectively, are hypersensitive to ABA during seed germination. PKS5 was found to interact with ABI5 in yeast (*Saccharomyces cerevisiae*), and this interaction was further confirmed in planta using bimolecular fluorescence complementation. Genetic studies revealed that *ABI5* is epistatic to *PKS5*. PKS5 phosphorylates a serine (Ser) residue at position 42 in ABI5 and regulates ABA-responsive gene expression. This phosphorylation was induced by ABA in vivo and transactivated ABI5. Expression of ABI5, in which Ser-42 was mutated to alanine, could not fully rescue the ABA-insensitive phenotypes of the *abi5-8* and *pks5-4abi5-8* mutants. In contrast, mutating Ser-42 to aspartate rescued the ABA insensitivity of these mutants. These data demonstrate that PKS5-mediated phosphorylation of ABI5 at Ser-42 is critical for the ABA regulation of seed germination and gene expression in Arabidopsis (*Arabidopsis thaliana*).

The phytohormone abscisic acid (ABA) plays important roles in plant growth and developmental processes (Finkelstein et al., 2002; Cutler et al., 2010). Recently, a phosphorylation cascade has been shown to play a fundamental role in early ABA signaling (Fujita et al., 2009; Cutler et al., 2010). When bound to ABA, PYRABACIN RESISTANCE1/PYRABACIN RESISTANCE1-LIKE/REGULATORY COMPONENTS OF ABSCISIC ACID RECEPTOR-type ABA receptors (hereafter referred to as PYLs) interact with and inhibit clade A PROTEIN PHOSPHATASE2C (PP2C). This leads to the release of SNF1-RELATED PROTEIN KINASE2 (SnRK2)-type protein kinases from PP2C-SnRK2 complexes, allowing SnRK2s to phosphorylate and activate downstream effectors of ABA responses, such as ABSCISIC ACID-INSENSITIVE5 (ABI5) and other ABSCISIC ACID-RESPONSIVE ELEMENT-BINDING FACTORs (ABFs; Fujita et al., 2009; Cutler et al., 2010).

The phosphorylation of ABI5 and ABFs by ABA-activated kinases is required for ABA-responsive gene expression (Lopez-Molina et al., 2001, 2003; Furihata et al., 2006; Rodrigues et al., 2013). ABI5 and ABFs

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belong to the group A subfamily of Arabidopsis (*Arabidopsis thaliana*) BASIC LEUCINE ZIPPER (bZIP) transcription factors. Group A bZIP proteins contain three conserved N-terminal (C1–C3) domains and one conserved C-terminal (C4) domain, each of which harbors putative phosphorylation sites (Furihata et al., 2006; Fujita et al., 2009; Zhou et al., 2013). The three conserved motifs in the N-terminal domains of ABI5 and ABSCISIC ACID-RESPONSIVE ELEMENT BINDING PROTEIN1 are phosphorylated in response to ABA (Lopez-Molina et al., 2002; Furihata et al., 2006). Plants expressing mutations at all of the N-terminal phosphoamino acid positions ($ABI5^{S42AS145AT201A}$) are insensitive to ABA (Wang et al., 2013c). Recently, in-gel kinase assays revealed that the Ser residues in the C2 domains of ABF1, ABF2, ABF4, and ABI5 are phosphorylated by SnRK2- and CALCIUM-DEPENDENT PROTEIN KINASE (CDPK)-type protein kinases (Furihata et al., 2006; Fujii et al., 2007; Zhu et al., 2007; Fujii and Zhu, 2009; Fujita et al., 2009). The ABA-dependent phosphorylation of the C1 motif of ABF2 (Ser-45, corresponding to Ser-42 on ABI5) has also been detected in vivo (Umezawa et al., 2013; Wang et al., 2013b). Phosphorylation at Ser-45 of ABF2 is also important for stimulating ABA-responsive gene expression (Umezawa et al., 2013), indicating that the phosphorylation of this Ser residue may also be important for the activity of other group A bZIP factors. Moreover, the biological role of Ser phosphorylation in the C1 domain has not been well determined.

SOS3-LIKE CALCIUM BINDING PROTEINs (SCaBPs), also known as CALCINEURIN B-LIKE PROTEINs (CBLs), are calcium-binding proteins involved in plant ABA signaling (Guo et al., 2002; Pandey et al., 2004, 2008; Quan et al., 2007). After the perception of elevated calcium elicited by various environmental stimuli, SCaBPs interact with and activate a group of protein kinases, SOS2-LIKE PROTEIN KINASEs (PKSs), also known as CALCINEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASEs (CIPKs; Luan et al., 2002; Gong et al., 2004). Many PKSs have been shown to be involved in ABA responses, serving as both positive or negative regulators of plant ABA signaling (Gong et al., 2002; Guo et al., 2002; Kim et al., 2003; Song et al., 2005; D'Angelo et al., 2006; Pandey et al., 2008; Qin et al., 2008; Lyzenga et al., 2013; Lumba et al., 2014). PKS3 (also known as CIPK15 or SnRK3.1; Table I) negatively regulates ABA signaling via the phosphorylation of ETHYLENE RESPONSE FACTOR7 (ERF7), which is an APETALA2/ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN-type transcription factor (Guo et al., 2002; Song et al., 2005). Another PKS, PKS26 (also known as CIPK26 or SnRK3.26; Table I), is involved in ABA signaling by interacting with the RING-type E3 ligase KEEP ON GOING and components of the ABA signaling network, such as ABI1, ABI2, and ABI5 (Lyzenga et al., 2013). Recently, PKS24 (also known as CIPK14 or SnRK3.15; Table I) and PKS5 (also known as CIPK11 or SnRK3.22; Table I) were identified to participate in ABA signaling through a systems biology approach that focused on ABA-dependent gene expression (Lumba et al., 2014). Interestingly, PKS5- and PKS24-interacting proteins are significantly enriched in transcription factors, including MYB, NAC (for NAM [NO APICAL MERISTEM], ATAF1/2 [ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR1/2], and CUC2 [CUP-SHAPED COTYLEDON2]), ERF, and bZIP transcription factors, and some of these transcription factors (i.e. ANAC18, RAP2.2, and ATHB6) are also phosphorylated by PKS5 or PKS24 in vitro (Lumba et al., 2014). These results suggest that different downstream targets and regulating mechanisms may be employed by individual PKSs in the context of negative or positive regulation of ABA signaling.

In this study, we found that *pks5-3* and *pks5-4*, two previously identified superactive PKS5 kinase mutants (Yang et al., 2010), are also involved in ABA responses during seed germination, root elongation, and gene expression. PKS5 participates in ABA responses at least partly by interacting with ABI5 and regulating ABI5 activity via the phosphorylation at Ser-42 in the C1 motif of ABI5.

RESULTS

The Expression of *PKS5* Is Induced by ABA

Previously, the expression of *PKS5* was shown to be highly induced by ABA (Fuglsang et al., 2007). To

Table I. *The SCaBP/PKS nomenclature used in this study*

Gene Name	Synonyms	References
PKS5	CIPK11, SnRK3.22, At2g30360	Fuglsang et al. (2007); Yang et al. (2010); Xie et al. (2010); Lumba et al. (2014)
SOS2	CIPK24, SnRK3.11, At5g35410	Guo et al. (2001); Quan et al. (2007); Lin et al. (2009)
PKS3	CIPK15, SnRK3.1, At5g01810	Guo et al. (2002); Song et al. (2005)
PKS26	CIPK26, SnRK3.26, At5g21326	Lyzenga et al. (2013)
PKS24	CIPK14, SnRK3.15, At5g01820	Qin et al. (2008); Lumba et al. (2014)
PKS18	CIPK20, SnRK3.6, At5g45820	Gong et al. (2002)
SOS3	CBL4, At5g24270	Guo et al. (2001); Quan et al. (2007); Lin et al. (2009)
SCaBP1	CBL2, At5g55990	Fuglsang et al. (2007); Du et al. (2011); Lin et al. (2014)
SCaBP4	CBL5, At4g01420	Guo et al. (2001); Lin et al. (2009)
SCaBP6	CBL3, At4g26570	Guo et al. (2001); Lin et al. (2009)

investigate the possible involvement of the PKS5 protein kinase in ABA responses, we determined its transcriptional level in response to ABA using microarray data from the AtGenExpress Visualization Tool (Kilian et al., 2007) and the Electronic Fluorescent Pictograph browser (Winter et al., 2007). Based on AtGenExpress Visualization Tool data, *PKS5* transcripts were up-regulated 3.1 times in seeds after treatment with 3 μM ABA for 24 h and 8.3 times in seedlings after treatment with 3 μM ABA for 3 h. Significant ABA-inducible expression of *PKS5* was also observed in seedlings after treatment with 10 μM ABA for 3 h based on Electronic Fluorescent Pictograph browser data (Supplemental Fig. S1). We also monitored the expression profile of *PKS5* in response to ABA. As shown in Figure 1A, *PKS5* expression was quickly induced in seedlings after treatment with 10 μM ABA for approximately 1 h and observed to plateau at 6 h. Expression declined steadily thereafter and was almost back to noninduced levels by 12 h.

The Kinase Activity of PKS5 Is Stimulated by ABA

To learn more about the involvement of PKS5 in ABA signaling, we measured its kinase activity in response to ABA treatment. Six tandem myc tags were fused to the N terminus of PKS5 expressed under the control of the *ACT2* promoter (An et al., 1996), and the resulting plasmid was transformed into the Arabidopsis *pks5-1* (a transfer DNA [T-DNA] insertion knockout mutant) genetic background. The 6×myc-PKS5 protein was immunoprecipitated from transgenic seedlings left untreated (0 h) or treated with 100 μM ABA for 1 h. The general kinase substrate MYELIN BASIC PROTEIN (MBP) was used to detect the transphosphorylation activity of PKS5. To our surprise, ABA treatment significantly enhanced the ability of PKS5 to phosphorylate MBP (Fig. 1, B and C). These results demonstrate that ABA also stimulates the kinase activity of PKS5 in planta.

Two Superactive PKS5 Mutants Are Hypersensitive to ABA

To determine if the loss or gain of function of *PKS5* leads to altered sensitivity to ABA, we examined the response of previously characterized *pks5* mutants (Fuglsang et al., 2007; Xie et al., 2010; Yang et al., 2010) to ABA during seed germination. We found that two hyperactive kinase mutants of PKS5 (Yang et al., 2010), *pks5-3* (Ser-317 replaced by Leu) and *pks5-4* (Ala-168 replaced by Val), with amino acid changes in either the FISL/NAF (a conserved 21-amino acid peptide that is necessary for interaction with SOS3 or SOS3-LIKE CALCIUM BINDING PROTEINs) motif or in the kinase activation loop (Guo et al., 2001), were hypersensitive to ABA (Fig. 2, A and B). However, sensitivity to ABA was not significantly altered in the *pks5-1* or *pks5-6* (Gly-219 replaced by Ser) mutant, both of which are *PKS5* loss-of-function mutants (Fig. 2,

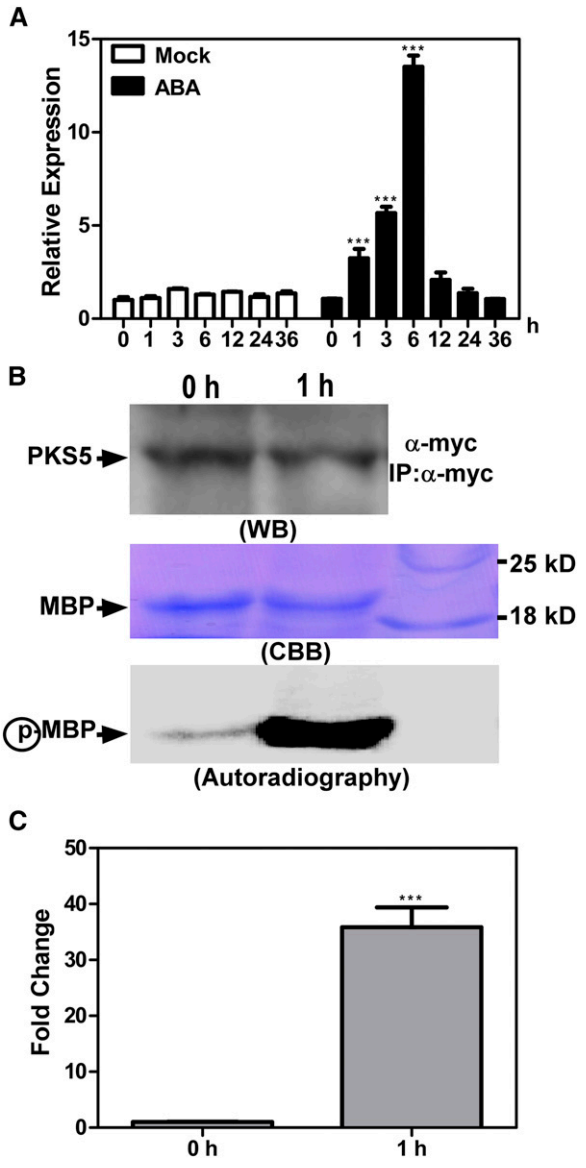

Figure 1. Expression of *PKS5* is highly induced by ABA, and ABA activates the kinase activity of PKS5. A, Expression of *PKS5* is highly induced by ABA. Seven-day-old Columbia-0 (Col-0) seedlings were left untreated or treated with 10 μM ABA, and *PKS5* expression was analyzed after 0, 1, 3, 6, 12, 24, and 36 h using quantitative reverse transcription (qRT)-PCR. B, ABA activation of PKS5 kinase activity. Two-week-old seedlings were left untreated (0 h) or treated with 100 μM ABA for 1 h. Top gel, Proteins extracted from *pks5-1* plants expressing 6myc-PKS5 were immunoprecipitated with anti-myc-conjugated agarose (5% [v/v] of immunoprecipitated products) followed by analysis with an α-myc antibody. WB, Western blot. Middle gel, Coomassie Brilliant Blue (CBB)-stained SDS-PAGE gel containing MBP as a substrate. Bottom gel, Autoradiograph showing the kinase activity of immunoprecipitated PKS5. C, Quantification of the data shown in B measured using a Typhoon 9410 phosphor imager. The relative kinase activity (fold change) was normalized and relative to the level in plants left untreated (0 h). Three independent experiments were performed. Student's *t* test was used to determine statistical significance: ***, $P < 0.001$.

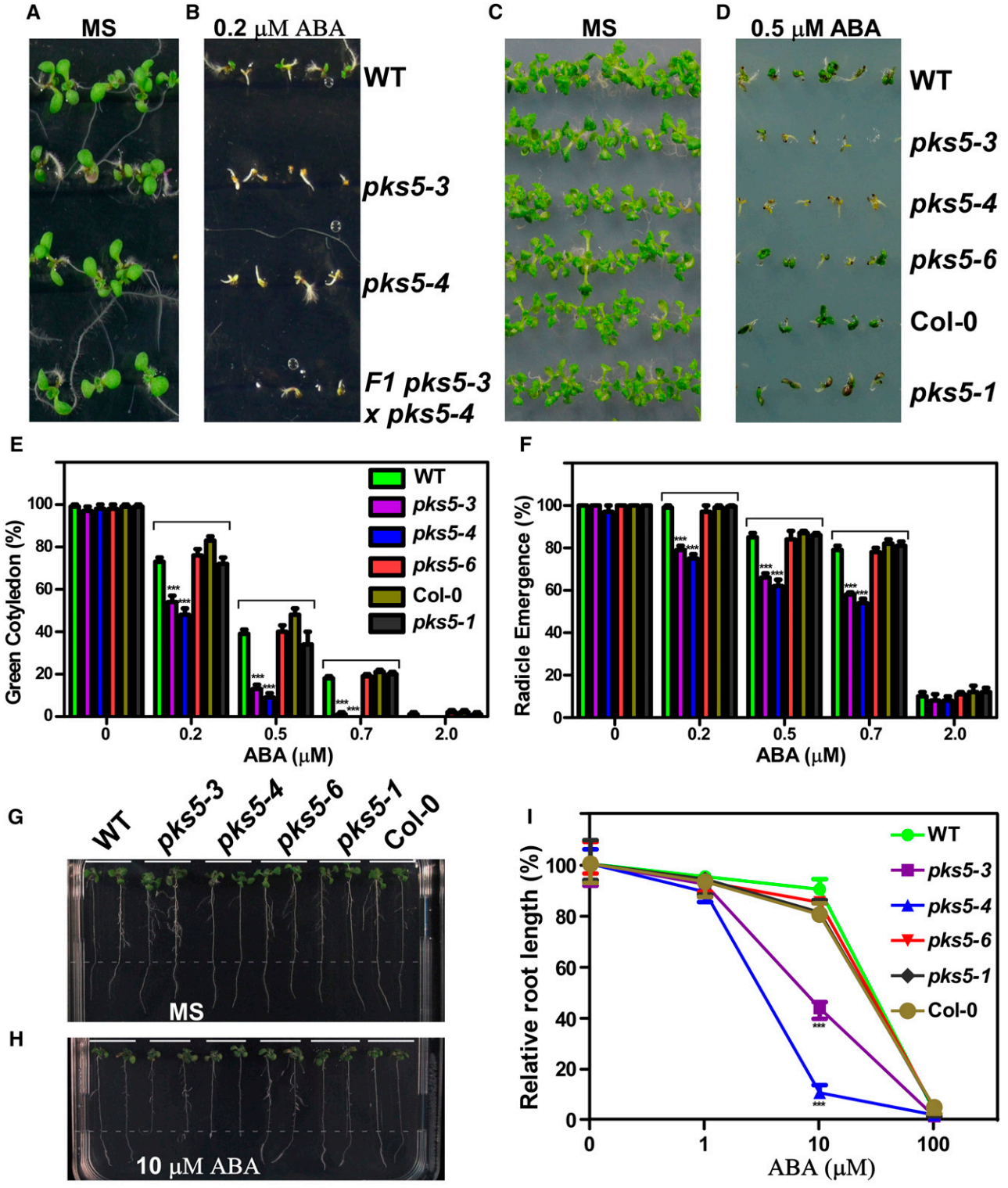

Figure 2. Responses of *pks5* mutants to ABA. A and B, The wild type (WT), *pks5-3*, *pks5-4*, and F1 progeny of *pks5-3* × *pks5-4* grown on Murashige and Skoog (MS) medium without or with 0.2 μM ABA. Photographs were taken after 7 d of growth. C and D, The wild type, *pks5-3*, *pks5-4*, *pks5-6*, Col-0, and *pks5-1* grown on MS medium without or with 0.5 μM ABA. Photographs were taken after 15 d of growth. E, Quantification of the percentage of seedlings with green cotyledons grown on MS medium containing different concentrations of ABA. The percentage of seedlings with green cotyledons was measured after 4 d (MS and 0.2 μM), 7 d (0.5 and 0.7 μM ABA), or 11 d (2 μM ABA). Each measurement included at least 100 seeds (means ± SE; $n = 3$). F, Quantification of radicle emergence for each genotype 3 d after stratification on MS medium without Suc. Each measurement

C and D). On MS medium without ABA, germination was similar in the *pks5-3* and *pks5-4* mutants and in the wild type. When germinated on medium with 0.2 μM ABA, less than 50% of *pks5-3* and *pks5-4* cotyledons were green after 4 d of incubation, while the wild type and the other *pks5* mutants had similar germination ratios with more than 70% green cotyledons (Fig. 2E). The *pks5-3* and *pks5-4* mutants were more sensitive to ABA than the wild type at all ABA concentrations tested (Fig. 2, E and F). To confirm that the ABA hypersensitivity of the *pks5-3* and *pks5-4* mutants is due to mutations in *PKS5*, we reciprocally crossed *pks5-3* and *pks5-4* and assayed the resulting F1 seeds for ABA sensitivity. Like the single mutants, the progeny of these reciprocal crosses were hypersensitive to ABA (shown for seeds from the cross where *pks5-3* was used as the female and *pks5-4* as the male; Fig. 2, A and B). These results suggest that PKS5 participates in plant responses to ABA during germination.

In addition to its inhibition of seed germination, ABA can also inhibit root elongation. To determine the ABA sensitivity of root elongation in the *pks5* mutants, 5-d-old seedlings were transferred from MS medium to medium without and with different concentrations of ABA, as described previously (Gosti et al., 1999; Fujii et al., 2007; Moes et al., 2008; Yin et al., 2009; Wang et al., 2011). Primary root length was measured 3 d later. The *pks5-3* and *pks5-4* mutants were more sensitive to ABA relative to the inhibition of root elongation when compared with the wild type (Fig. 2, G–I). No significant difference was observed in the *pks5-1* and *pks5-6* loss-of-function mutants under these conditions (Fig. 2, G–I). These data indicate that PKS5 may be a positive regulator of ABA signaling.

PKS5 Interacts with ABI5

To understand how PKS5 regulates plant responses to ABA, we analyzed PKS5-interacting factors from previously reported studies (Xie et al., 2010; Yang et al., 2010). One positive clone identified was identical to the N-terminal 257 amino acids of ABI5 (At2g36270; ABI5-N), a well-studied bZIP transcription factor involved in responses of the plant to ABA during seed germination (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001, 2002; Carles et al., 2002). To identify the region of interaction, ABI5 was divided into two segments: ABI5-N (amino acids 1–257) and ABI5-C (amino acids 258–442). A schematic diagram of these peptides is shown in Figure 3A. Both the full-length ABI5 and the ABI5-N peptide interacted with the full-length PKS5 protein, whereas the ABI5-C peptide did not (Fig. 3B). To identify the site of interaction in PKS5, PKS5-N (amino acids 1–281) and PKS5-C (amino acids 282–435), two previously reported peptides (Yang et al., 2010), were cloned and cotransformed with the ABI5 plasmids into yeast (*Saccharomyces cerevisiae*) for two-hybrid interaction analysis. PKS5-N interacted with full-length ABI5 and the ABI5-N peptide (Fig. 3C, top). The PKS5-C peptide did not interact with any portion of ABI5 (Fig. 3C, bottom), in contrast to what has been reported previously for PKS5 interaction with DnaJ HOMOLOG3 (HEAT SHOCK PROTEIN40-like; Yang et al., 2010).

To monitor the interaction of ABI5 and PKS5 in vivo, we cloned full-length ABI5 into the bimolecular fluorescence complementation (BiFC) vector pUC-SPYNE (Walter et al., 2004; Quan et al., 2007) and cloned PKS5 into the pUC-SPYCE vector. The resulting plasmids and empty vectors were introduced into Arabidopsis leaf mesophyll protoplasts. After 12 to 18 h of incubation, the yellow fluorescent protein (YFP) signal was detected only in the nuclei of PKS5-YFP^C/ABI5-YFP^N-cotransfected protoplasts (Fig. 3, D–F; Supplemental Fig. S2). A YFP signal was not detected with any single construct expressed individually or when PKS5-YFP^C and pUC-SPYNE or ABI5-YFP^N and pUC-SPYCE were expressed (Supplemental Fig. S2). We also used SOS2 (also known as CIPK24 or SnRK3.11; Table I), a well-characterized member of the PKS family, as a related kinase in the BiFC assay. No YFP signal was detected in SOS2-YFP^C/ABI5-YFP^N-cotransfected protoplasts (Supplemental Fig. S2), suggesting that the protein-protein interaction between PKS5 and ABI5 may be specific. We also used a firefly luciferase complementation assay (Chen et al., 2008; Xie et al., 2009; Wang et al., 2013a; Yuan et al., 2013) to analyze this interaction. A high level of luciferase activity was detected only when PKS5-nLUC was coexpressed with cLUC-ABI5 (Supplemental Fig. S3). Taken together, our data indicated that these two proteins interact in vivo and that the N-terminal regions of PKS5 and ABI5 are required for this interaction.

PKS5 Phosphorylates ABI5 and Activates the Expression of Downstream Genes

To investigate whether ABI5 is also a target of PKS5, ABI5 was divided into two segments, ABI5-N (amino acids 1–257) and ABI5-C (amino acids 258–442), and

Figure 2. (*Continued.*)
included at least 150 seeds (means ± SE; $n = 3$). G and H, Root growth of the wild type, *pks5-3*, *pks5-4*, *pks5-6*, Col-0, and *pks5-1* on MS medium containing 0 or 10 μM ABA. Five-day-old seedlings grown on MS medium with roots 1.5 to 2 cm long were transferred to MS medium without or with 10 μM ABA and were grown for 3 d before being photographed and analyzed. The dotted lines indicate the positions of the root tips immediately after transfer. I, Quantification of root length for seedlings treated with the indicated concentrations of ABA. More than 20 seedlings were measured in each experiment. Relative root growth represents the primary root growth of seedlings after treatment with ABA compared with the growth of seedlings without ABA treatment. More than three independent experiments were performed; one representative result is presented. Student's *t* test was used to determine statistical significance: ***, $P < 0.001$.

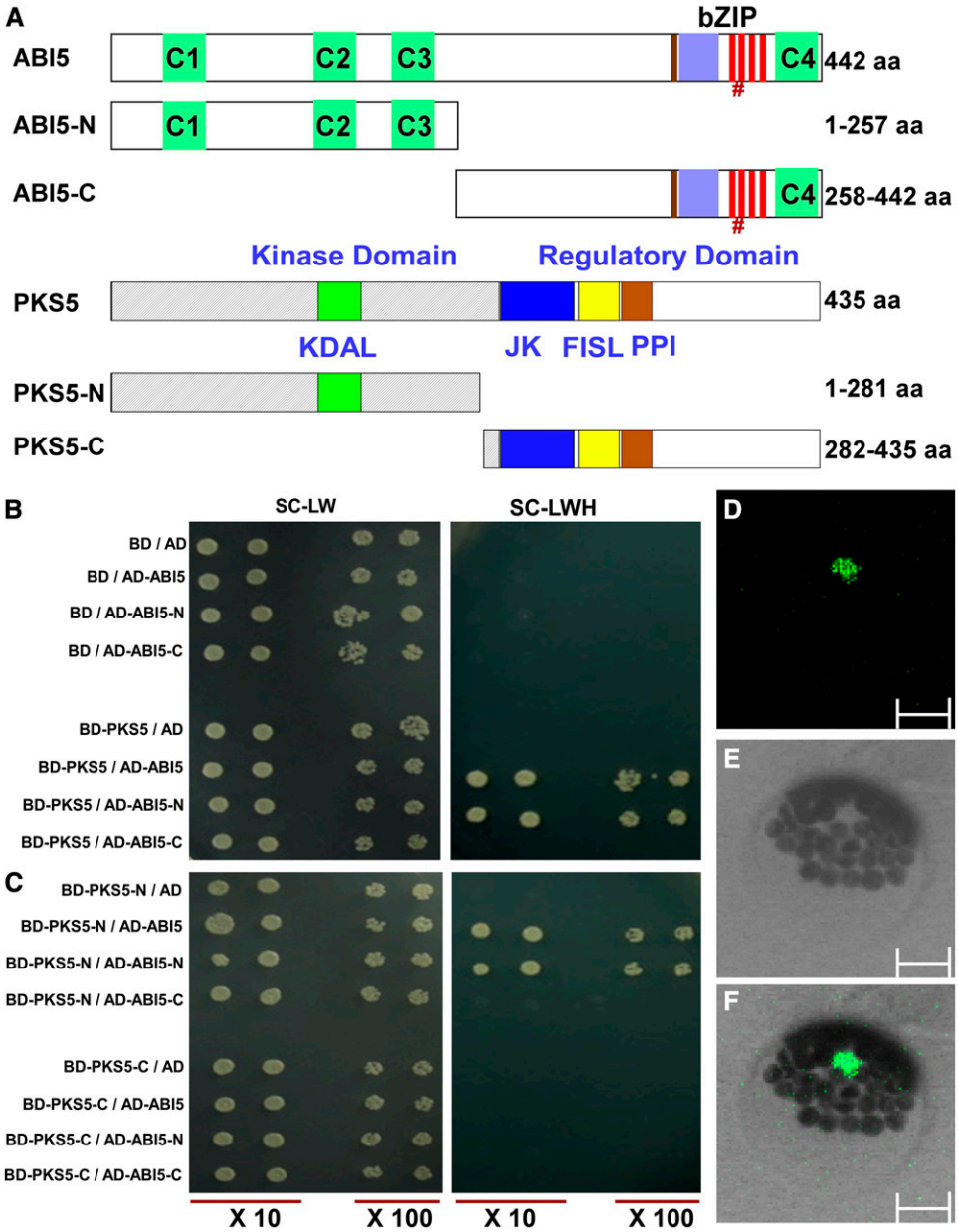

Figure 3. PKS5 interacts with ABI5. A, Schematic diagrams of the ABI5, ABI5-N, ABI5-C, PKS5, PKS5-N, and PKS5-C proteins used in the yeast two-hybrid analysis. For ABI5, ABI5-N, and ABI5-C, green boxes are conserved N- and C-terminal sequences (C1–C4); blue boxes are the basic domain; black-brown rectangles are bipartite nuclear localization signals; red rectangles are Leu residues defining the Leu zipper; and # indicates a conserved sumoylation site. For PKS5, PKS5-N, and PKS5-C, green boxes are kinase activation loops (KDAL); dark blue boxes are junction domains (JK); yellow boxes are the FISL motif (a 21-amino acid SOS3/SCaBPs-binding motif, with A, F, I, S, L, and F showing complete conservation); and brown boxes are the protein phosphatase interaction domain (PPI). aa, Amino acids. B, Yeast two-hybrid analyses of the interaction between PKS5 and ABI5, ABI5-N, or ABI5-C. AD, GAL4 DNA Activation Domain fused vector; BD, GAL4 DNA Binding Domain fused vector. C, Yeast two-hybrid assays of the interaction between PKS5-N (top) or PKS5-C (bottom) and ABI5, ABI5-N, or ABI5-C. Yeast lines expressing the indicated plasmids were grown on synthetic complete medium without Leu and Trp (SC-LW; left) and on synthetic complete medium without Leu, Trp, and His (SC-LWH; right). Yeast cells were incubated until the optical density at 600 nm reached 0.5 and then diluted 10-fold (×10) or 100-fold (×100) and used for assays. AD or AD-, GAL4 DNA Activation Domain fused vectors; BD or BD-, GAL4 DNA Binding Domain fused vectors. D to F, Interaction of PKS5 with ABI5 in vivo. Combinations of PKS5-YFP^{C} and ABI5-YFP^{N} fusion constructs were cotransformed into Arabidopsis protoplasts. The images were collected using an inverted Zeiss LSM 510 META confocal fluorescence microscope: yellow fluorescence in dark field (D); cell morphology in bright field (E); and an overlay of the bright-field and yellow fluorescence signals (F). Three independent experiments were performed. Bars = 10 μm.

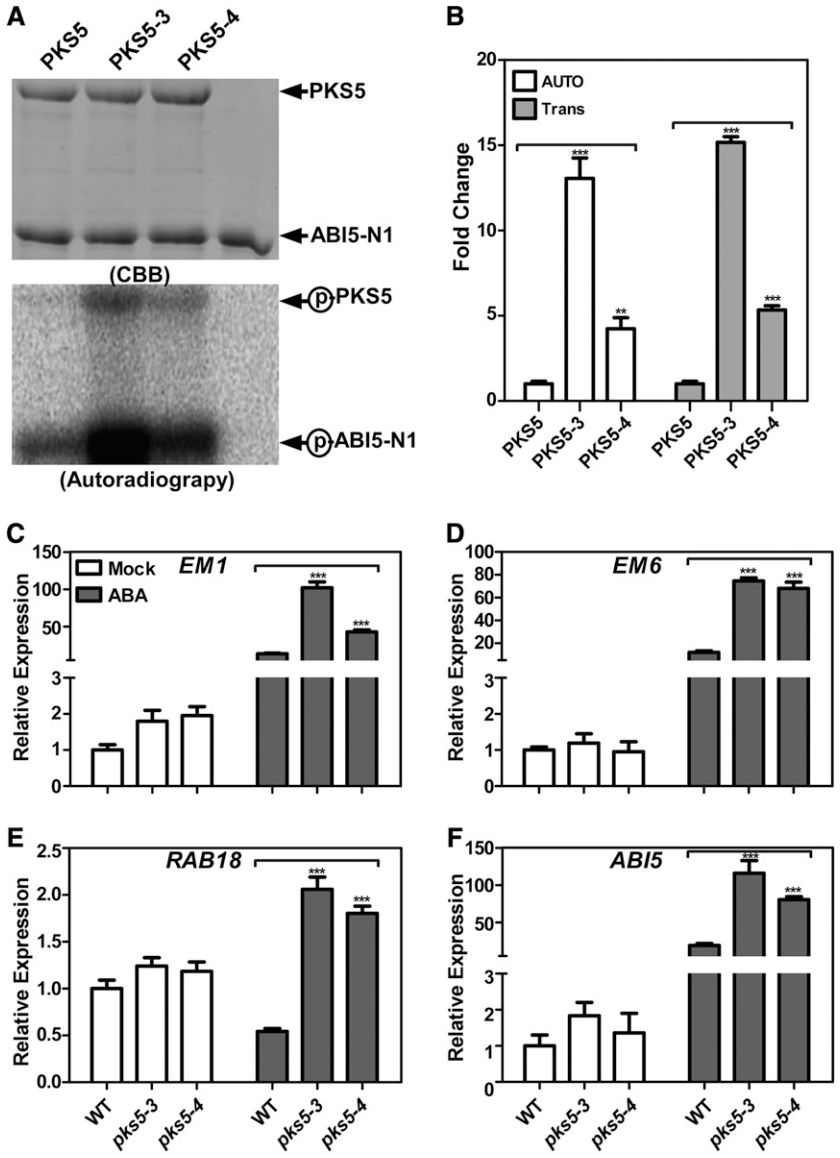

Figure 4. PKS5 phosphorylates ABI5-N1, and the expression of ABI5-regulated ABA-responsive genes is elevated in the *pks5-3* and *pks5-4* mutants in response to ABA. A, Comparison of the kinase activities of the PKS5, PKS5-3, and PKS5-4 proteins with ABI5-N1. Top gel, Coomassie Brilliant Blue (CBB)-stained SDS-PAGE gel containing the HIS-PKS5 mutant proteins and HIS-ABI5-N1. Bottom gel, Autoradiograph showing kinase activity. B, The relative autophosphorylation and transphosphorylation activities of the kinases (fold change) in A were measured with a Typhoon 9410 phosphor imager. For autophosphorylation activities (AUTO), the relative activity (fold change) was normalized to the phosphorylation level in PKS5; for transphosphorylation activities (Trans), the relative activity (fold change) was normalized to the phosphorylation level in PKS5 upon the addition of ABI5-N1. C to F, Expression of *EM1*, *EM6*, *RAB18*, and *ABI5* in wild-type (WT), *pks5-3*, and *pks5-4* plants. Wild-type and mutant seeds were sown on MS medium without or with 0.3 μM ABA and grown for 3 d. The seeds were then used for RNA extraction. Gene expression was detected by qRT-PCR. Three independent experiments were performed, and one representative result is presented. Student's *t* test was used to determine statistical significance: **, $P < 0.01$; and ***, $P < 0.001$.

fused to HIS and glutathione *S*-transferase (GST) tags, respectively. As expected, only ABI5-N was phosphorylated by PKS5 (Supplemental Fig. S4). Moreover, we found that a truncated ABI5-N fragment, ABI5-N1 (amino acids 10–257), increased both the quantity and quality of recombinant ABI5 and was also phosphorylated by PKS5 (Fig. 4A). PKS5-3 and PKS5-4 have been shown previously to be hyperactive kinases (Yang et al., 2010); therefore, we assayed their activities with ABI5-N1 as the substrate. Consistent with our previous study, recombinant PKS5-3 and PKS5-4 were more active than wild-type PKS5 in both autophosphorylation and ABI5-N1 transphosphorylation assays (Fig. 4, A and B).

ABI5 is essential for the expression of late embryogenesis genes such as *LATE EMBRYOGENESIS ABUNDANT1* (*EM1*) and *EM6* (Finkelstein and Lynch, 2000; Carles et al., 2002; Lopez-Molina et al., 2002). To determine if the expression of *EM1*, *EM6*, *RESPONSIVE TO ABSCISIC ACID18* (*RAB18*), or *ABI5* is affected by the mutations in *PKS5*, wild-type, *pks5-3*, and *pks5-4* seeds were plated on MS medium without or with 0.3 μM ABA and treated for 3 d. The expression of *EM1*, *EM6*, and *ABI5* was dramatically induced by ABA treatment, whereas *RAB18* was only slightly induced (Fig. 4, C–F). The expression of *EM1*, *EM6*, and *ABI5* was induced by ABA much more strongly in *pks5-3* and *pks5-4* than in the wild type (Fig. 4, C, D, and F), indicating that higher PKS5 kinase activity is associated with the induction of these genes and that the phosphorylation of ABI5 by PKS5 may be involved in an ABI5 self-activation loop.

Genetic Interaction between *PKS5* and *ABI5*

To investigate whether *PKS5* genetically interacts with *ABI5*, we isolated an ABI5 T-DNA insertion line from The Arabidopsis Information Resource (SALK_013163),

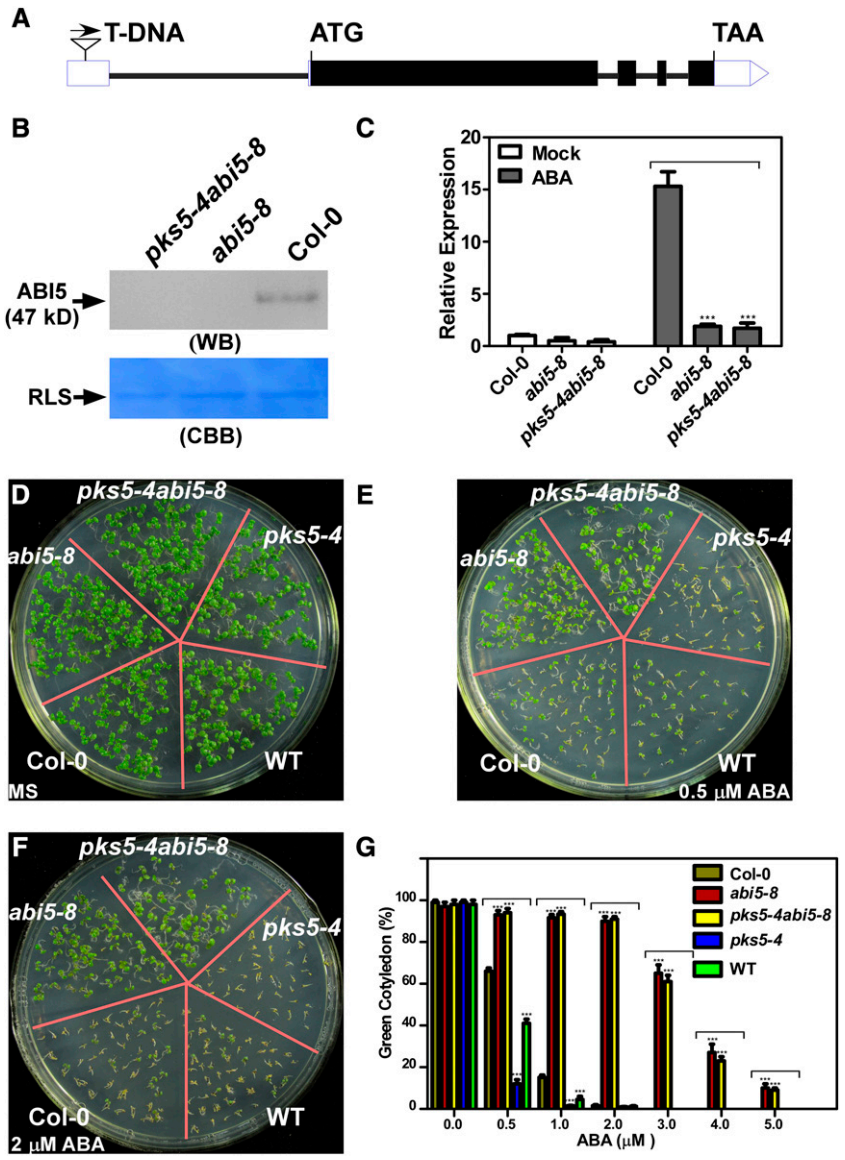

Figure 5. *ABI5* acts downstream of *PKS5* during the ABA-induced inhibition of seed germination. A, Schematic diagram of the *ABI5* gene showing the T-DNA insertion site in the *abi5-8* mutant. Open blue boxes are 5′- and 3′-untranslated regions; black boxes are exons; and lines between the boxes are introns. The T-DNA insertion is also indicated. B, Immunoblots showing ABI5 protein levels in Col-0, *abi5-8*, and *pks5-4abi5-8*. Two-day-old seedlings of Col-0, *abi5-8*, and *pks5-4abi5-8* after stratification were transferred to plates containing 5 μM ABA for 1 d. Total protein was extracted, and ABI5 levels were monitored with an α-ABI5 (Abcam) antibody. WB, Western blot. Coomassie Brilliant Blue (CBB)-stained Rubisco large subunit (RLS) served as a loading control. C, qRT-PCR showing *ABI5* transcript levels in Col-0, *abi5-8*, and *pks5-4abi5-8*. D to F, Seeds from the wild type (WT), *psk5-4*, Col-0, *abi5-8*, and *psk5-4abi5-8* were germinated on MS medium without (D) and with 0.5 μM ABA (E) or 2 μM ABA (F). Photographs were taken after 10 d of growth on MS medium without (D) and with 0.5 μM ABA (E) or after 16 d of growth on MS medium with 2 μM ABA (F). G, Quantification of the percentage of seedlings with green cotyledons on MS medium containing different concentrations of ABA. The percentage of seedlings with green cotyledons was measured after 4 d (MS), 7 d (0.5 μM ABA), or 11 d (1, 2, 3, 4, and 5 μM ABA). Each measurement included at least 150 seeds (means ± SE; $n = 3$). Three independent experiments were performed, and one representative result is presented. Student's *t* test was used to determine statistical significance: ***, $P < 0.001$.

previously designated as *abi5-8* (Zheng et al., 2012). The location of the T-DNA insertion in ABI5 is shown in Figure 5A. ABI5 protein could not be detected in *abi5-8*, and the expression of ABI5 in response to ABA was also significantly decreased in *abi5-8* (Fig. 5, B and C), indicating that it is a loss-of-function *abi5* mutant. Loss-of-function *abi5* mutants are insensitive to ABA, whereas ABI5-overexpressing transgenic plants are hypersensitive to ABA (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Lopez-Molina et al., 2001; Nambara et al., 2002). When we examined the response of *abi5-8* to ABA during seed germination, consistent and significant differences in ABA insensitivity were detected (Fig. 5, D–F), as described previously (Zheng et al., 2012). We then crossed *abi5-8* to *pks5-4* to generate *pks5-4abi5-8* double mutants. Wild type, single mutant, and double mutant seeds were sown on MS medium without or with different concentrations of ABA. On MS medium, there was no significant difference between the wild type and the mutants; more than 97% of the seeds germinated and produced seedlings with green cotyledons and true leaves. On MS medium with either 0.5 or 2 μM ABA, sensitivity to ABA was increased in *pks5-4* and decreased in *abi5-8* compared with the wild type. The *pks5-4abi5-8* double mutant displayed a pattern of insensitivity similar to that in *abi5-8* (Fig. 5, D–G; Supplemental Fig. S5A). The enhanced expression of the ABI5-regulated genes *EM1*, *EM6*, and *ABI5* observed in *pks5-4* was consistently and dramatically suppressed by *abi5-8* in the *pks5-4abi5-8* double mutant (Supplemental Fig. S5, B, C, and E). In addition to their roles in the inhibition of seed germination and the modulated expression of ABI5-regulated genes, *pks5-3* and *pks5-4* are able to regulate root elongation (Fig. 2, G–I). As a result, we also monitored seedling

sensitivity to ABA in *pks5-4abi5-8* mutants. In contrast to what was found during seed germination, *abi5-8* could not suppress the ABA sensitivity of *pks5-4* with respect to root elongation (Supplemental Fig. S6). These observations are consistent with previous studies, which reported that ABI5 is mainly involved in ABA inhibition of seed germination and ABA-regulated gene expression (Finkelstein and Lynch, 2000; Carles et al., 2002; Lopez-Molina et al., 2002, 2003). Together with the finding that ABI5 is a putative substrate of PKS5 (Fig. 4A), these data suggest that PKS5 functions in ABA signaling at least in part through its interaction with and phosphorylation of ABI5.

PKS5 Phosphorylates ABI5 Mainly at Ser-42

To identify the PKS5 phosphorylation site in ABI5, we generated three additional ABI5 N-terminal truncations: ABI5-Na (amino acids 1–132), ABI5-Nb (amino acids 131–191), and ABI5-Nc (amino acids 190–257; Fig. 6A). ABI5-Na was strongly phosphorylated by PKS5, whereas weak phosphorylation was detected in ABI5-Nb (Fig. 6B), a region shown to be phosphorylated by SnRK2.2, SnRK2.3, SnRK2.6, CDPK4, CDPK11, or SnRK1-type protein kinases (Fujii et al., 2007; Zhu et al., 2007; Zhang et al., 2008; Fujii and Zhu, 2009; Rodrigues et al., 2013). These results demonstrate that the main phosphorylation site is located in the C1 motif of the ABI5 N terminus. Several studies revealed that Ser-42 in the C1 motif and Ser-145 in the C2 motif of ABI5 are phosphorylated in vivo and in vitro (Lopez-Molina et al., 2002; Wang et al., 2013c). To determine if these sites are targets of PKS5 phosphorylation, we mutated Ser-42 and Ser-145 to Ala (to mimic non-phosphorylated status). As expected, when Ser-42 was changed to Ala (ABI5-Na^{S42A}), the mutant protein was no longer phosphorylated by PKS5 (Fig. 6B). For the Ser-145 phosphorylation, Ser-182 in the C2 motif was also mutated to Ala. While ABI5-Nb^{S182A} was still weakly phosphorylated by PKS5, ABI5-Nb^{S145A} and the ABI5-Nb$^{S145A/S182A}$ double mutant (containing both the S145A and S182A mutations) were not (Fig. 6B). These results demonstrate that Ser-42 is the principal amino acid phosphorylated by PKS5 in vitro.

PKS5 Phosphorylation of ABI5 Is Induced by ABA in Planta

To determine if ABI5 is phosphorylated by PKS5 in planta, phosphorylation site-specific antibodies were generated by immunizing rabbits with the chemically synthesized phosphopeptide C-LGRQS**pS**IYSLT-NH2 (a Ser-42 phosphospecific peptide of ABI5), and the phospho-specific antibodies (anti-phospho-Ser-42) were screened and characterized as described previously (Lin et al., 2009). To evaluate the specificity of the antibodies toward the Ser-42 phosphorylation site, the ABI5-Na and ABI5-Na^{S42A} proteins were each incubated with PKS5 in kinase buffer in the presence of ATP for 30 min. A strong cross reaction was detected only when ABI5-Na was incubated with PKS5 (Fig. 6C). Almost no signal appeared when ABI5-Na^{S42A} was incubated with PKS5, even after a significantly longer exposure. These results suggest that the antibodies specifically recognize phosphorylated Ser-42 in ABI5.

To examine ABI5 phosphorylation in vivo, we generated *35S:6myc-ABI5*, *35S:6myc-ABI5*^{S42A}, and *35S:6myc-ABI5*^{S42D} (to mimic phosphorylated status) transgenic lines. The α-myc antibody readily detected myc-labeled ABI5 in all of the transgenic plants, but no signal was observed when anti-phospho-Ser-42 antibodies were used (Fig. 6D). Previous studies have indicated that ABA stimulates ABI5 activation, which correlates with ABI5 phosphorylation (Lopez-Molina et al., 2001; Piskurewicz et al., 2008). Therefore, we reasoned that PKS5 phosphorylation of ABI5 might also be induced by ABA. To test this hypothesis, transgenic plants were left untreated or treated with 100 μM ABA for 1 h. A phospho-Ser-42 signal was detected only in protein extracts from Col-0 transgenic plants expressing *myc-ABI5* treated with ABA (Fig. 6D); no phospho-Ser-42 signal was seen in Col-0 expressing *myc-ABI5*^{S42A} or *myc-ABI5*^{S42D}. Furthermore, an enhanced phospho-Ser-42 signal was observed in *pks5-4* plants (whose activity is higher than that of PKS5) expressing *myc-ABI5* in the presence of ABA, whereas almost no phospho-Ser-42 signal was detected in *pks5-6* plants (whose activity is lower than that of PKS5) expressing *myc-ABI5* in the presence or absence of ABA (Fig. 6E). Nearly equivalent amounts of *myc-ABI5*, *myc-ABI5*^{S42A}, and *myc-ABI5*^{S42D} proteins were present in the assays, as measured by immunoblot analysis with α-myc antibodies (Fig. 6, D and E). Our data demonstrate that PKS5 specifically phosphorylates ABI5 at Ser-42 and that this phosphorylation is induced by ABA treatment.

The Ser Residue at Position 42 in ABI5 Is Important for ABA Inhibition of Seed Germination

To determine if the phosphorylation of ABI5 at Ser-42 is required for ABA to inhibit seed germination, we transformed *35S:6myc-ABI5*, *35S:6myc-ABI5*^{S42A}, and *35S:6myc-ABI5*^{S42D} into the *abi5-8* and *pks5-4abi5-8* mutants. Homozygous T3 plants (derived from different T0 transformants) of *abi5-8* or *pks5-4abi5-8* containing each construct were tested for cotyledon greening and radicle emergence in the absence and presence of different concentrations of ABA. In transgenic lines expressing *ABI5*^{S42D}, the ABA-insensitive phenotypes of *abi5-8* and *pks5-4abi5-8* were completely suppressed (Fig. 7, A–F; Supplemental Fig. S7, A–F). These results suggest that mimicking the phosphorylation status of Ser-42 (S42D) also made ABI5 functional in Arabidopsis.

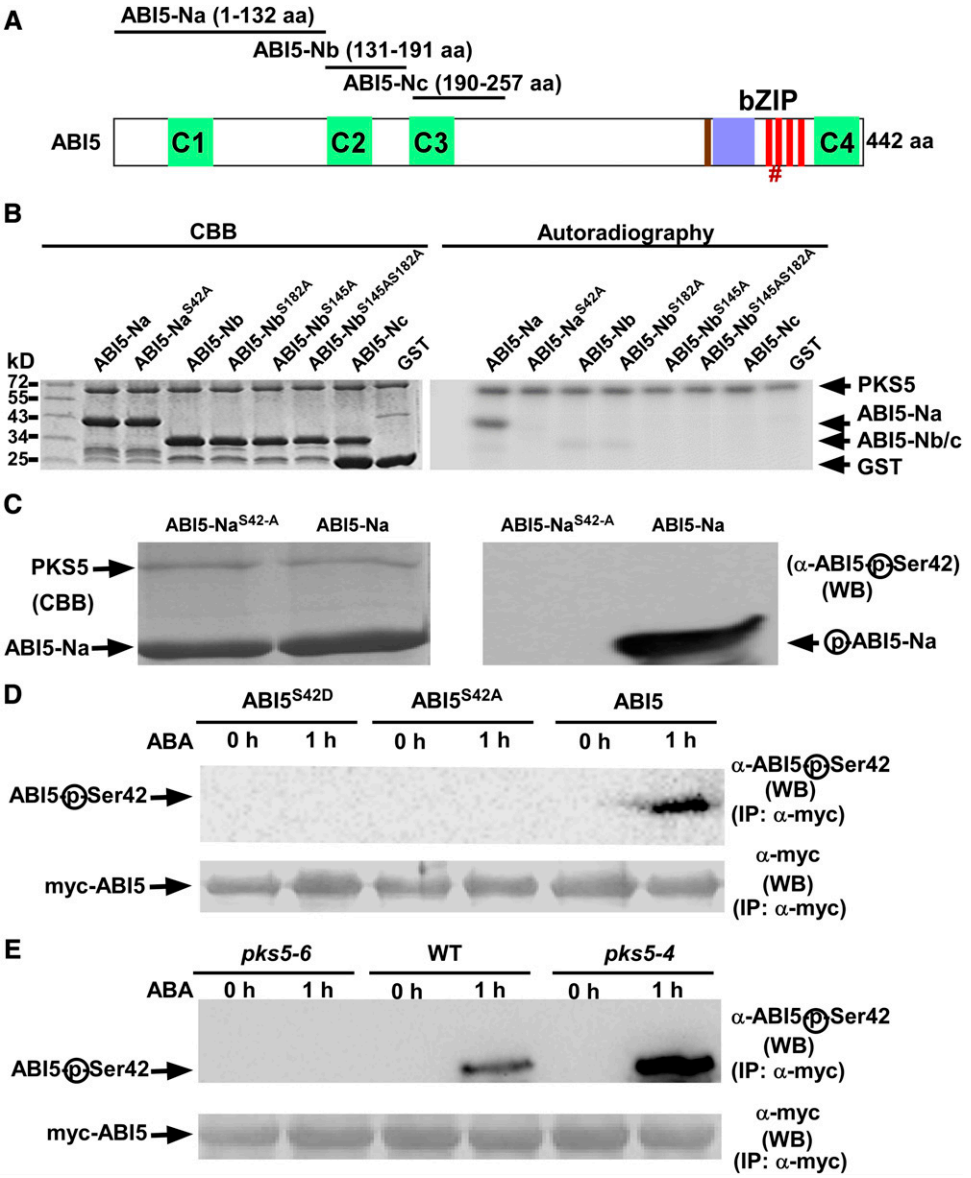

Figure 6. PKS5 phosphorylates Ser at position 42 in ABI5. A, Model of N-terminally truncated ABI5 fragments. Green boxes are conserved N- and C-terminal sequences (C1–C4); blue boxes are the basic domain; black-brown rectangles are bipartite nuclear localization signals; red rectangles are Leu residues defining the Leu zipper; and # indicates a conserved sumoylation site. aa, Amino acids. B, PKS5 phosphorylates ABI5 at Ser-42. Left gel, SDS-PAGE with Coomassie Brilliant Blue (CBB)-stained PKS5 and ABI5 proteins. Right gel, Autoradiograph showing PKS5 autophosphorylation and ABI5 phosphorylation. C, Detection of in vitro ABI5 phosphorylation at Ser-42 with an α-ABI5-pSer-42 antibody. Left, Coomassie Brilliant Blue-stained PKS5 and ABI5-Na proteins; and right, immunoblot (WB [Western blot]) showing ABI5 Ser-42 phosphorylation. D, α-ABI5-pSer-42 antibody detection of ABI5 but not $ABI5^{S42A}$ or $ABI5^{S42D}$ in transgenic Col-0 plants after exogenous ABA treatment. Proteins extracted from transgenic plants expressing myc-ABI5, myc-$ABI5^{S42A}$, and myc-$ABI5^{S42D}$ were analyzed with an α-ABI5-pSer-42 antibody (top) or an α-myc antibody (bottom). IP, Immunoprecipitation. E, Detection of enhanced ABI5 Ser-42 phosphorylation in *pks5-4* but not in *pks5-6* mutant plants. Proteins extracted from wild-type, *pks5-4*, or *pks5-6* plants expressing myc-ABI5 were analyzed with an α-ABI5-pSer-42 antibody (top) or an α-myc antibody (bottom). For treatment, 2-week-old seedlings were left untreated (0 h) or treated with 100 μM ABA for 1 h. Three independent experiments were performed, and one representative result is presented.

In contrast, no transgenic line was found among T3 seeds from more than 50 independent T0 transformants of *abi5-8* and *pks5-4abi5-8* expressing *35S:6myc-*$ABI5^{S42A}$ in which the ABA-insensitive phenotype was fully suppressed (Fig. 7, A–F; Supplemental Fig. S7, A–F). Immunoblot analysis showed that the expression of ABI5 was similar in the *35S:6myc-ABI5*, *35S:6myc-*$ABI5^{S42A}$, and *35S:6myc-*$ABI5^{S42D}$ transgenic

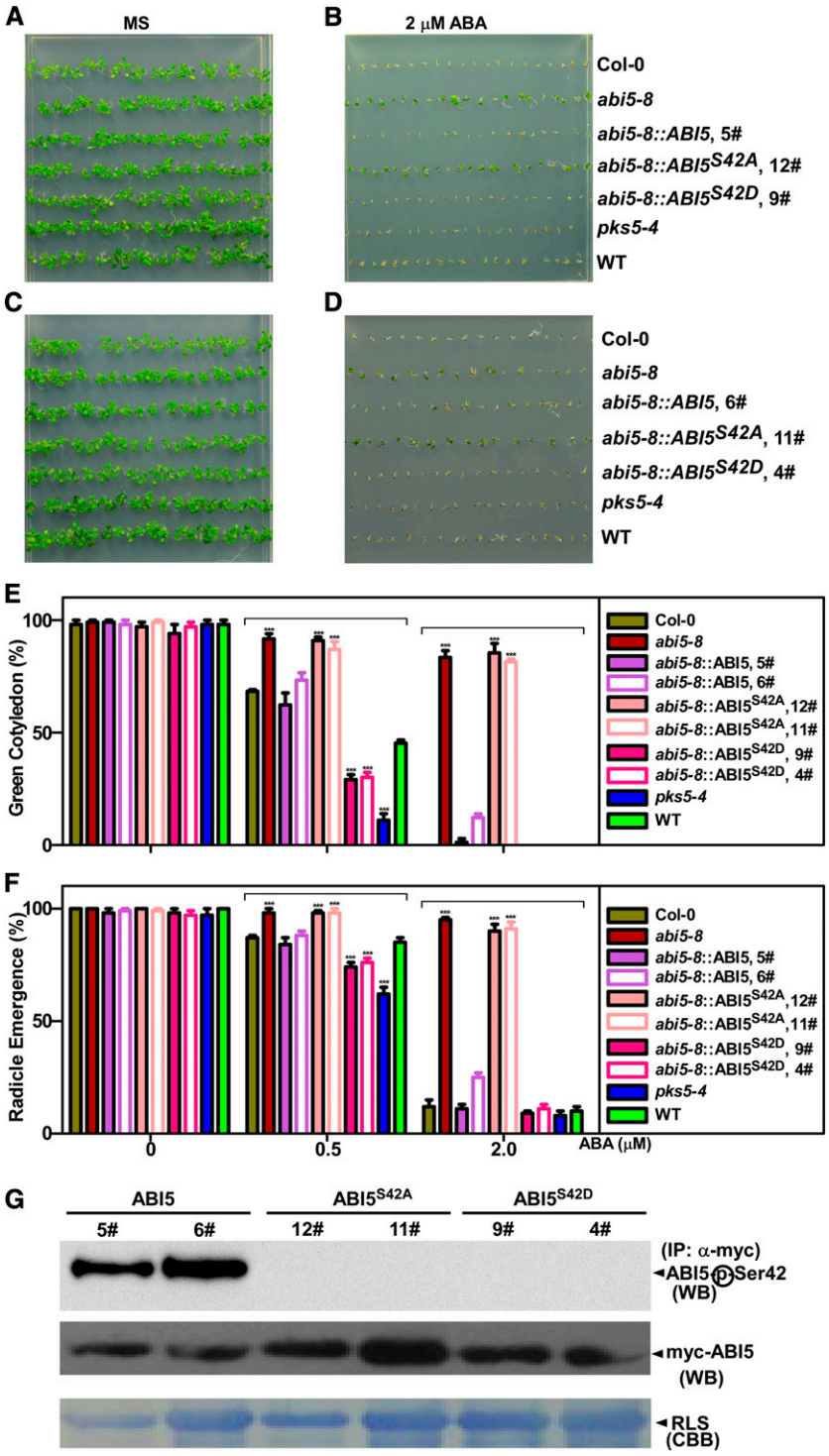

Figure 7. The Ser residue at position 42 is important for ABI5 function in the ABA inhibition of seed germination in the *abi5-8* mutant. A to D, ABA sensitivity of *abi5-8* mutants expressing myc-ABI5 (*abi5-8*::*ABI5*), myc-ABI5^{S42A} (*abi5-8*::*ABI5*^{S42A}), or myc-ABI5^{S42D} (*abi5-8*::*ABI5*^{S42D}). Photographs were taken after 11 d of growth on MS medium (A and C) or 16 d with 2 μM ABA (B and D). E, Quantification of the percentage of seedlings with green cotyledons from plants grown on MS medium containing different concentrations of ABA. The percentage of seedlings with green cotyledons was measured after 4 d (MS), 7 d (0.5 μM ABA), or 11 d (2 μM ABA). Each measurement included at least 100 seeds (means ± SE; $n = 3$). F, Quantification of radicle emergence at 3 d after stratification on MS medium without Suc. Each measurement included at least 120 seeds (means ± SE; $n = 3$). G, Immunoblots of ABI5 protein levels or ABI5 Ser-42 phosphorylation status in the *abi5-8* mutant expressing myc-ABI5, myc-ABI5^{S42A}, or myc-ABI5^{S42D}. To determine the phosphorylation status of ABI5 Ser-42, 2-week-old seedlings were pretreated with 100 μM ABA for 1 h before immunoblot analysis. IP, Immunoprecipitation; WB, western blot. Coomassie Brilliant Blue (CBB)-stained Rubisco large subunit (RLS) served as a loading control. More than five independent experiments were performed, and two representative results are presented for *abi5-8* expressing myc-ABI5 (5# and 6#), myc-ABI5^{S42A} (11# and 12#), and myc-ABI5^{S42D} (4# and 9#). Student's *t* test was used to determine statistical significance: ***, $P < 0.001$. WT, Wild type.

lines and that a phospho-Ser-42 signal for ABI5 was only detected in plants expressing *35S:6myc-ABI5* after treatment with 100 μM ABA for 1 h (Fig. 7G; Supplemental Fig. S7G), demonstrating that the differences in complementation were not due to differing levels of transgene expression. These results demonstrate that the Ser residue at position 42 in ABI5 is important for ABI5 function in Arabidopsis, most likely due to the phosphorylation of this site by PKS5 in response to ABA.

The Ser Residue at Position 42 in ABI5 Is Important for the Transactivation Activity of ABI5

The phosphorylation of ABI5 is essential to activate ABA-responsive gene expression (Lopez-Molina et al.,

2001, 2002; Piskurewicz et al., 2008). Recently, we have developed hexamers of an ABSCISIC ACID RESPONSE ELEMENT (ABRE)-driven *HIS* reporter gene system to determine the transactivation activity of ABI5 (Zhou et al., 2013). To determine if the phosphorylation of Ser-42 in ABI5 also affects the ability of ABI5 to activate ABA-responsive gene expression, yeast cells expressing pRS315-6×ABRE-HIS were transformed with an empty vector or with the pPC86-ABI5, pPC86-ABI5^{S42A}, or pPC86-ABI5^{S42D} construct (Fig. 8A). Consistent with previous reports, the expression of wild-type ABI5-induced 6×ABRE-driven *HIS* expression when compared with the vector control (Fig. 8B). The expression of ABI5^{S42A} failed to promote 6×ABRE-driven *HIS* expression above what was induced in the wild-type ABI5 control, while the phosphorylation-mimetic ABI5^{S42D} exhibited an increase in 6×ABRE-driven *HIS* expression compared with the wild-type ABI5 control (Fig. 8B).

We also investigated the biological importance of Ser-42 phosphorylation using a transactivation assay in Arabidopsis protoplasts. We transiently expressed the constructs in Arabidopsis mesophyll protoplasts and monitored transcriptional activity via *LUCIFERASE* (*LUC*) expression driven by the *EM6* promoter (Fig. 8C), a well-defined ABI5-responsive promoter (Carles et al., 2002; Tezuka et al., 2013). As was observed in our yeast one-hybrid assays, the expression of wild-type ABI5 induced more than a 3-fold increase in LUC activity relative to the vector control (Fig. 8D). In contrast, the expression of ABI5^{S42A} failed to promote LUC activity above what was induced in the vector control (Fig. 8D). The phosphorylation-mimetic ABI5^{S42D} exhibited an increase in relative LUC activity compared with activity in wild-type ABI5 (Fig. 8D). Interestingly, an apparently enhanced LUC activity was observed in *pks5-4* (whose activity is higher than that of PKS5) protoplasts, while a slightly reduced LUC activity was observed in *pks5-6* (whose activity is lower than PKS5) protoplasts when ABI5 was overexpressed in the presence of ABA (Fig. 8, E and F). As was observed in Col-0, this enhanced or decreased activity was also abolished when ABI5^{S42A} or ABI5^{S42D} was overexpressed, respectively (Fig. 8, E and F). Together with the yeast one-hybrid assay results, our data demonstrate that the Ser residue at position 42 in ABI5 is important for ABI5 transactivation and that this is most likely due to the phosphorylation of this site by PKS5 in response to ABA.

DISCUSSION

PKS5 Is a Positive Regulator of Plant ABA Signaling

A number of PKS kinases have been shown to be involved in ABA responses (Gong et al., 2002; Guo et al., 2002; Kim et al., 2003; Song et al., 2005; D'Angelo et al., 2006; Pandey et al., 2008; Qin et al., 2008; Lyzenga et al., 2013; Lumba et al., 2014); however, their roles and underlying mechanisms differ. For example, in contrast to our results for PKS5, loss-of-function mutations in *PKS3* and *PKS24* resulted in mutant plants that were hypersensitive to ABA, suggesting that these kinases may be negative regulators of ABA responses (Guo et al., 2002; Song et al., 2005; Qin et al., 2008; Lumba et al., 2014). Like PKS5 (Fig. 2), gain-of-function mutations in PKS18 (PKS18^{T169D}, a constitutively active form of PKS18, also known as CIPK20 or SnRK3.6; Table I) and PKS26 (overexpression) result in transgenic plants that were hypersensitivity to ABA, indicating that PKS18 and PKS26 also function as positive regulators of plant ABA signaling (Gong et al., 2002; Lyzenga et al., 2013). PKS26 also localizes in the cytoplasm and nucleus and phosphorylates the same bZIP transcription factor as PKS5 (Fig. 6; Yang et al., 2010; Lyzenga et al., 2013), suggesting that they may function redundantly in the same pathway. Determining the responses of multiple mutants to ABA and demonstration of the underlying mechanisms in the future will help us fully understand the case, including a role for PKS5 and other PKSs in the positive regulation of ABA signaling.

PKS5 has been proposed previously to be a negative regulator of ABA signaling (Lumba et al., 2014), which is in contrast to our observations. To try to understand this discrepancy, we analyzed the rate of radicle emergence, the percentage of green cotyledons, and root elongation in our *pks5* mutants. In all cases, consistent and significant differences in ABA sensitivity were observed in *pks5-3* and *pks5-4* (Fig. 2). Therefore, differences between these two studies in terms of *pks5* mutant alleles might be due to allele-specific phenotypes, differences in materials and methods, and variation in analyses of germination.

In addition to playing a role in the positive regulation of ABA inhibition of seed germination (Fig. 2) and in ABA-regulated gene expression (Fig. 4), PKS5 also has a key role in positively modulating ABA inhibition of root elongation (Fig. 2). Different from what was observed during seed germination (Fig. 5), the hypersensitivity of *pks5-4* to ABA with respect to root elongation could not be suppressed by *abi5-8* (Supplemental Fig. S6). Besides ABI5, a number of transcription factors have been identified to interact with PKS5, such as MYB, NAC, and ERF (Lumba et al., 2014). Interactions with these proteins may be involved in the positive regulation of PKS5-mediated ABA signaling with respect to root elongation (Fig. 9).

Biological Significance of the ABA-Dependent Phosphorylation of ABI5 at Ser-42 by PKS5

ABA-dependent polyphosphorylation plays a key role in fine-tuning ABI5 like bZIP transcription factors (Lopez-Molina et al., 2002, 2003; Fujita et al., 2005; Furihata et al., 2006; Umezawa et al., 2013; Wang et al., 2013b). Unlike the C2 motif (Fujii et al., 2007; Zhu et al., 2007; Zhang et al., 2008; Fujii and Zhu, 2009; Nakashima et al., 2009; Rodrigues et al., 2013), the kinases responsible for phosphorylating the C1 and C3 motifs have remained elusive. Here, we report that the Ser site in the C1 motif of ABI5 (Ser-42) is phosphorylated by PKS5 (Fig. 6). In contrast to

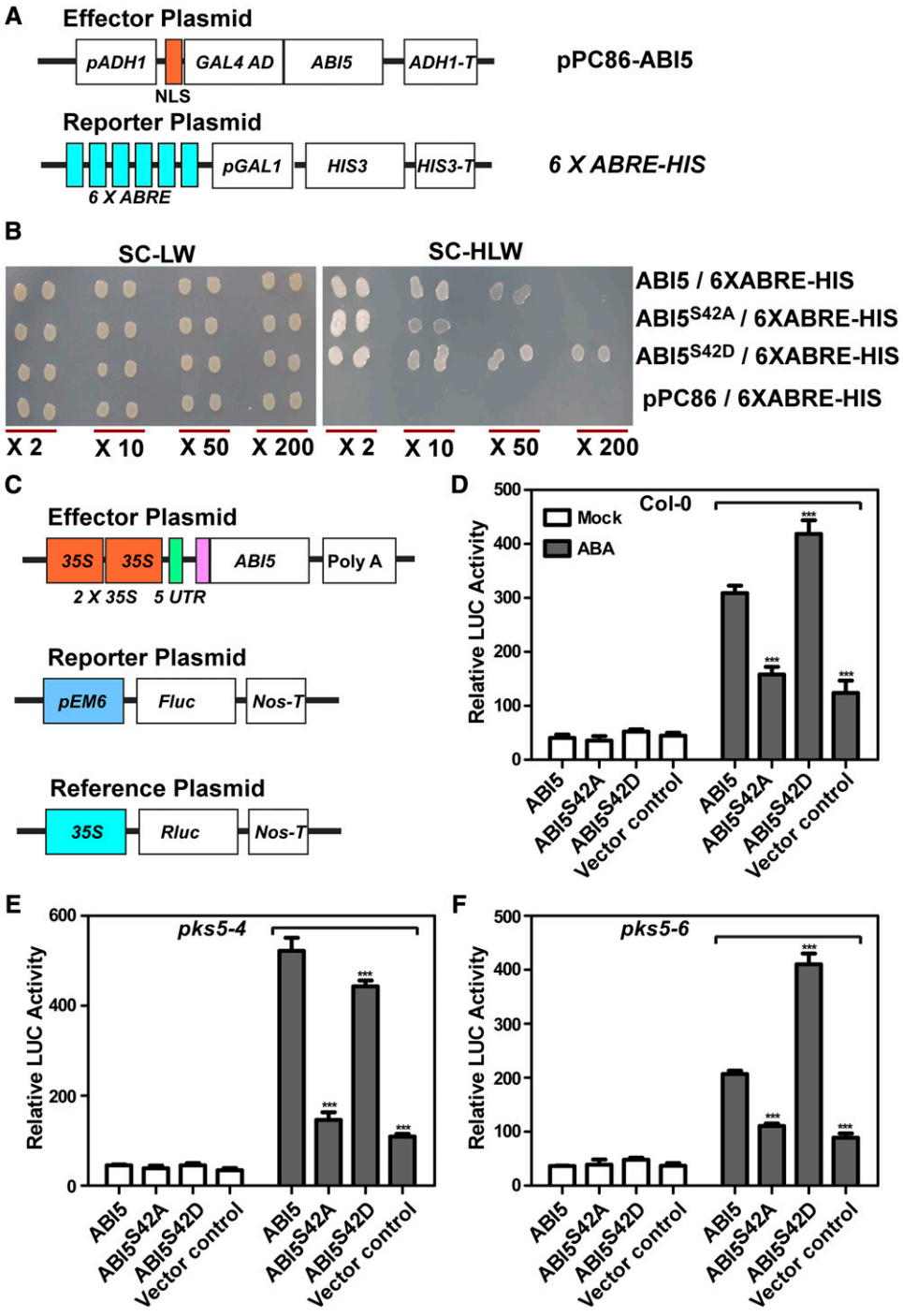

Figure 8. The Ser residue at position 42 in ABI5 is important for its transactivating activity. A, Schematic diagram of the effector and reporter constructs used in the yeast one-hybrid assay. *pADH1*, *ALCOHOL DEHYDROGENASE1* promoter; NLS, nuclear localization signal; *GAL4 AD*, *GAL4* activation domain; *ADH1-T*, *ADH1* terminator; *6×ABRE*, a hexamer of *ABRE*; *pGAL1*, minipromoter of *GAL1*; *HIS3-T*, *HIS3* terminator. B, Yeast one-hybrid analysis of ABI5 proteins. yWAM2 yeast lines expressing the indicated plasmids were grown on synthetic complete medium without Leu and Trp (SC-LW; left) and on synthetic complete medium without Leu, Trp, and His (SC-HLW; right). Yeast cells were incubated until the optical density at 600 nm reached 0.5 and then diluted 2-fold (×2), 10-fold (×10), 50-fold (×50), or 200-fold (×200) and used for assays. C, Schematic diagram of the effector, reporter, and reference constructs used in cotransfection experiments. For the effector construct, *2×35S* is a tandem repeat of the *Cauliflower mosaic virus* (CaMV) 35S promoter; 5 UTR is a 5′-untranslated region derived from *Tobacco etch virus*; the purple box is a hexamer of the myc tag; and Poly A is the poly(A) signal derived from CaMV. For the reporter construct, *pEM6* contains 1.3 kb of the *EM6* promoter; Fluc is the firefly (*Photinus pyralis*) *LUC* gene; and Nos-T is the nopaline synthase

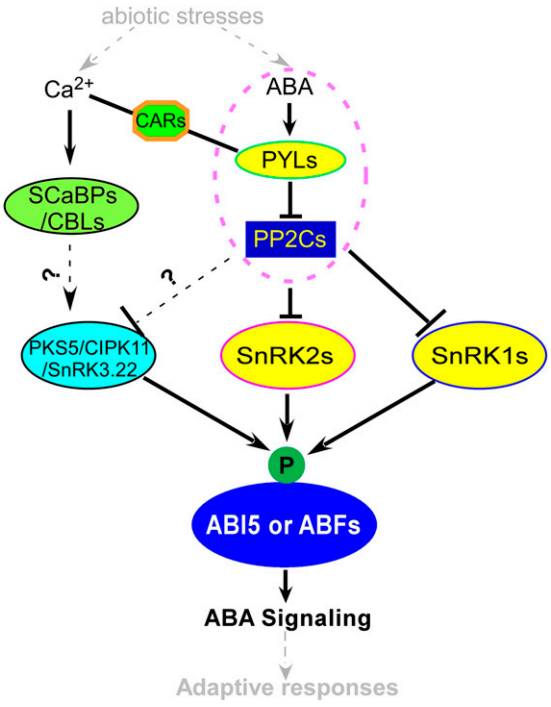

Figure 9. Working model of PKS5-mediated ABA signaling processes relative to the core ABA signaling pathway. A simplified model for PKS5/CIPK11/SnRK3.22-mediated ABA signaling relative to SnRK2- and SnRK1-mediated ABA signaling processes is shown. When challenged by abiotic stresses (i.e. high salt or drought), endogenous ABA or Ca^{2+} significantly increases, which is a requirement for the establishment of adaptive responses in plants. When bound to ABA, PYL receptors inhibit clade A PP2Cs. The inhibition of PP2Cs in turn leads to SnRK2 or SnRK1 activation. Activated SnRK2s or SnRK1s mediate ABA signaling through the phosphorylation of downstream effectors (ABI5 or ABFs). Once activated by unknown upstream proteins, PKS5 is able to positively modulate ABA signaling at least partly via the phosphorylation of ABI5 at Ser-42. The complex of Ca^{2+}-C2-DOMAIN ABSCISIC ACID-RELATED PROTEIN (CARs)-PYLs may facilitate the ABA signaling in plants. Arrows depict activation, and intersecting lines indicate inhibition. Abiotic stresses and adaptive responses are shown in semitransparent format. The core ABA signaling pathway is circled with pink dotted lines. The activation of PKS5 by ABA likely undergoes cross talk at other levels (dotted lines). For a detailed description, see "Discussion."

SnRK2-, CDPK-, and SnRK1-type kinases (Fujii et al., 2007; Zhu et al., 2007; Zhang et al., 2008; Fujii and Zhu, 2009; Nakashima et al., 2009; Rodrigues et al., 2013), PKS5 mainly phosphorylated ABI5 in the C1 motif in vitro, with only very weak phosphorylation observed in the C2 motif (Fig. 6A). In agreement with previous studies (Lopez-Molina et al., 2002; Fujii et al., 2009; Wang et al., 2013b), our transactivation assay data suggest that the phosphorylation of ABI5 at Ser-42 also enhances its activity (Fig. 8). Moreover, our transgenic work revealed that the identified phosphorylation site at Ser-42 has significance for the function of ABI5 (Fig. 7; Supplemental Fig. S7). It is currently not clear why transgenic lines expressing $ABI5^{S42D}$ in *pks5-4abi5-8* showed sensitivity to 0.5 μM ABA similar to that of *pks5-4*, while the expression of ABI5 in *pks5-4abi5-8* showed sensitivity to 0.5 μM ABA similar to that of Col-0 or the wild type (Supplemental Fig. S7E). This phenomenon is different from what is observed with transgenic lines expressing $ABI5^{S42D}$ or ABI5 in *abi5-8* (Fig. 7E) and might be attributed to the ecotype of the transgenic plants (*abi5-8* is in the Col-0 ecotype, while *pks5-4abi5-8* is in the Col-0 *er105* background); alternatively, the phosphorylation of Ser-42 may impact the phosphorylation of other sites.

Possible ABA-Dependent Activation Mechanisms of PKS5

As is the case for the regulation of plasma membrane H^+-ATPase and plant responses to salt at alkaline pH (Yang et al., 2010), the superactive effect of PKS5-3 and PKS5-4 on the kinase activity of PKS5 in whole plants can only be observed in the presence of environmental stimuli (salt or salt at alkaline pH treatment in the former study and exogenous ABA treatment for our assay; Figs. 2, 6E, and 9). PKS5 interacts with SCaBP1 (also known as CBL2; Table I; Fuglsang et al., 2007) and phosphorylates a Ser residue (Ser-216) in the PFPF (a conserved 23-amino acid peptide that is necessary for phosphorylation of SCaBP proteins by their interacting PKSs) motif of SCaBP1 (Du et al., 2011); however, SCaBP1 cannot activate PKS5 in vitro (Lin et al., 2014). In addition to SCaBP1, PKS5 also interacts with other SCaBPs (i.e. SCaBP4/CBL5, SCaBP6/CBL3, and SOS3/CBL4; Table I) and can phosphorylate many of these SCaBPs (Guo et al., 2001; Xie et al., 2009; Du et al., 2011; Lin et al., 2014). Therefore, it is likely that other SCaBP calcium sensors that perceive the cytosolic calcium signature may participate in activating PKS5 in planta (Fig. 9).

A protein phosphatase interaction motif is conserved in Arabidopsis PKS proteins, including PKS5, and is essential for many PKS kinases (i.e. SOS2, PKS3, PKS5, and PKS26) to interact with PP2Cs, such as ABI1, ABI2, or ABSCISIC ACID-HYPERSENSITIVE GERMINATION3 (AHG3; Guo et al., 2002; Ohta et al., 2003; Gong et al., 2004; Lyzenga et al., 2013; Lumba et al., 2014). PKS5 is able to interact with AHG3, which is a well-known clade

Figure 8. (*Continued.*)
terminator. For the reference construct, 35S is the CaMV 35S promoter; Rluc is the *LUC* gene from *Renilla reniformis*, also known as sea pansy; and Nos-T is the nopaline synthase terminator. D to F, Transactivation activity of the *EM6* promoter based on ABI5 expression. Transactivation experiments were performed using protoplasts prepared from Col-0 (D), *pks5-4* (E), or *pks5-6* (F) leaves. Transfected cells were cultured for 16 h without or with 5 μM ABA, and relative LUC activity was assayed according to the Dual-Luciferase Reporter Assay Protocol provided by Promega. The empty vector control was also included as a negative control. The values shown are average Fluc activities normalized to Rluc activities. Error bars indicate SE. Three independent experiments were performed. Student's *t* test was used to determine statistical significance: ***, $P < 0.001$.

A PP2C involved in ABA signaling (Cutler et al., 2010; Umezawa et al., 2010; Lumba et al., 2014). Recently, calcium-dependent lipid binding to the C2 domain of CARs has been demonstrated to facilitate ABA signaling by affecting the subcellular localization of the ABA-PYLs-PP2C complex (Rodriguez et al., 2014). Inhibition of PP2C is a requirement for SnRK1- and SnRK2-type protein kianse activation by ABA (Cutler et al., 2010; Umezawa et al., 2010; Rodrigues et al., 2013). Thus, it will also be important to elucidate how PKS5 is regulated by AHG3-like PP2C protein complexes in the presence or absence of ABA (Fig. 9).

All of these findings indicate that PKS5 may be activated by abiotic stress- or ABA-induced cytosolic calcium elevation through SCaBPs or other calcium-binding proteins. ABA may also activate PKS5 through the inhibition of AHG3 and other clade A PP2C protein complexes. Moreover, posttranslational modifications, such as the phosphorylation of PKS5 by unknown upstream regulators (i.e. kinase), may regulate the kinase activity of PKS5 in response to ABA or abiotic stresses in planta (Fig. 9).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Plants were grown in controlled-environment growth chambers under a 16-h-light (22°C)/8-h-dark (20°C) cycle. The following Arabidopsis (*Arabidopsis thaliana*) strains were used in this study: Col-0 and the wild type (Col-0 *erecta105*), which was used for generating TARGETING INDUCED LOCAL LESIONS IN GENOMES (TILLING) mutants (Till et al., 2003). A T-DNA insertion mutant of *abi5* (SALK_013163; designated as *abi5-8*) was obtained from the Arabidopsis Biological Resource Center and identified by T-DNA insertion-based PCR as described previously (Zheng et al., 2012). The identification and manipulation of the *pks5-3* (CS90635), *pks5-4* (CS91114), *pks5-6* (CS91337), and *pks5-1* (SALK_108074) mutants were as described previously (Yang et al., 2010). The *pks5-4abi5-8* double mutant was obtained by crossing *abi5-8* to *pks5-4* and confirmed by T-DNA insertion-based PCR and gene sequencing.

To determine the responses of seeds to the ABA inhibition of germination and root elongation, seeds harvested at the same time were used for cotyledon greening, radicle emergence, and root elongation assays as described (Fujii et al., 2007; Fujii and Zhu, 2009; Yin et al., 2009; Wang et al., 2011; Zheng et al., 2012; Lumba et al., 2014). Briefly, wild-type and mutant seeds were sterilized in a solution containing 20% (v/v) sodium hypochlorite and 0.1% (v/v) Triton X-100 for 10 min, washed five times with sterilized water, and sown on MS medium (Phytotech) with 0.3% (w/v) Phytagel (Sigma-Aldrich) with different concentrations of ABA (Sigma-Aldrich). The plates were incubated in growth chambers at 4°C for 4 d followed by incubation at 23°C under continual illumination. To quantify the percentage of seedlings with green cotyledons, seeds were sown on MS medium containing different concentrations of ABA and analyzed on the indicated days after stratification as described (Fujii et al., 2007; Zheng et al., 2012; Lumba et al., 2014). For radicle emergence assays, seeds were sown on MS medium without Suc and analyzed 3 d after stratification as described previously (Fujii et al., 2007). For root elongation assays, 5-d-old seedlings grown on MS medium with roots 1.5 to 2 cm long were transferred to MS medium containing 0, 1, 10, or 100 μM ABA and were grown for 3 d before being photographed and analyzed as described (Gosti et al., 1999; Moes et al., 2008; Yin et al., 2009; Wang et al., 2011). For ABA treatments, 2-week-old seedlings grown on MS medium were left untreated or treated with 100 μM ABA for 1 h as described (Fujii and Zhu, 2009; Umezawa et al., 2013; Wang et al., 2013b).

Plasmid Construction

The full-length coding sequence of *PKS5* was cloned into the pET28a vector between the *Bam*HI and *Sal*I sites. *PKS5-3* and *PKS5-4* were also cloned into pET28a at these sites. All of the N-terminally truncated ABI5 proteins, ABI5-N1 (amino acids 10–257), ABI5-Na (amino acids 1–132), ABI5-Nb (amino acids 131–191), and ABI5-Nc (amino acids 190–257), were cloned into the pGEX-6p-1 vector between *Bam*HI and *Sal*I to generate GST-tagged proteins. ABI5-N1 was also cloned into pET28a between the *Bam*HI and *Sal*I sites. ABI5-C (amino acids 258–442) was cloned into pGEX-6p-1. To produce myc-tagged PKS5, full-length *PKS5* was digested from the pET28a-PKS5 construct using *Bam*HI and *Sal*I and subcloned into the p1307-6myc binary vector (Zhou et al., 2013). The full-length *ABI5* coding sequence was also cloned into the p1307-6myc vector between the *Bam*HI and *Sal*I sites.

To introduce the S42A, S145A, S182A, and T201A mutations into ABI5, we performed two-round-PCR-based nucleotide replacement as described previously (Quan et al., 2007; Xie et al., 2010). The primers used to construct the plasmids are listed in Supplemental Table S1. All plasmids were confirmed by sequencing to avoid cloning errors.

Immunoprecipitation and Kinase Assays

Total proteins were extracted from *pks5-1* plants expressing 6myc-PKS5 by homogenizing in 2× cold immunoprecipitation buffer containing 20 mM Tris-Cl at pH 7.5, 1% (v/v) Nonidet P-40, 4 mM EDTA, 300 mM NaCl, 2× protease inhibitor cocktail (Roche), and 2× phosphatase inhibitor (Roche). Debris was removed by centrifugation at 4°C and 13,000 rpm for 20 min. Immunoprecipitation was performed by incubating the supernatant with anti-myc-conjugated agarose (Abmart) at 4°C for 12 h. Precipitated proteins were then washed extensively with immunoprecipitation buffer prior to the kinase assay.

GST- and His-tagged recombinant fusion protein purification and kinase activity assays were performed as described previously (Lin et al., 2009; Yang et al., 2010).

Yeast Two-Hybrid Assays

PKS5, *PKS5-N* (amino acids 1–281), and *PKS5-C* (amino acids 282–435) were cloned into the pGBKT7 vector between the *Bam*HI and *Sal*I sites, and *ABI5*, *ABI5-N* (amino acids 1–257), and *ABI5-C* (amino acids 258–442) were cloned into the pGADT7 vector between the *Bam*HI and *Sal*I sites. The primers used to construct the plasmids are listed in Supplemental Table S1. Combinations of the indicated plasmids were transformed into the AH109 yeast (*Saccharomyces cerevisiae*) strain using the lithium acetate/single-stranded carrier DNA/polyethylene glycol (PEG) method (Xie et al., 2010; Yang et al., 2010). The transformed yeast cells were selected on synthetic complete medium lacking Leu and Trp. Interactions were determined by measuring the growth of serial dilutions of transformed yeast cells on synthetic complete medium lacking Leu, Trp, and His for 2 to 3 d (Lin et al., 2009; Xie et al., 2010; Yang et al., 2010).

BiFC

To detect the interaction between PKS5 and ABI5 in vivo, full-length PKS5 and ABI5 were digested from pET28a-PKS5 and p1307-myc-ABI5 using *Bam*HI and *Sal*I and were subcloned in frame into the pUC-SPYCE and pUC-SPYNE vectors (Walter et al., 2004; Quan et al., 2007) to obtain pUC-SPYCE-PKS5 and pUC-SPYNE-ABI5. For transient expression, these plasmids were isolated and purified using the Plasmid Maxiprep Kit (Vigorous Biotechnology) and then introduced into Arabidopsis leaf mesophyll protoplasts according to the PEG-Ca^{2+} protocol, as described previously (Quan et al., 2007; Xie et al., 2010; Wang et al., 2013a; Yuan et al., 2013). Transfected protoplasts were incubated for 12 to 16 h at 23°C under continuous light; subsequently, YFP fluorescence was assayed using a Zeiss LSM510 Meta confocal microscope with excitation at 513 nm.

qRT-PCR Analysis

Total RNA was extracted from 3-d-old seedlings growing on 0.3% (w/v) MS medium without or with 0.3 μM ABA using the RNAiso Plus reagent (TaKaRa). A total of 5 μg of treated RNA was used for reverse transcription with the PrimeScript II First Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. qRT-PCR was performed using a CFX 96 real-time PCR machine (Bio-Rad) and the SYBR Premix Ex Taq Kit (TaKaRa) to monitor double-stranded DNA products as reported previously (Wang et al., 2013a; Yuan et al., 2013). The relative expression of *EM1*, *EM6*, *RAB18*, and *ABI5* was normalized to the expression of *ACTIN2* and expressed relative to that of mock-treated seedlings as described previously (Yang et al., 2010). The primers used for qRT-PCR are listed in Supplemental Table S2.

Preparation of Anti-Phospho-Ser-42 ABI5 Polyclonal Antibodies

Two 11-amino acid peptides (corresponding to amino acids 37–47 of ABI5) with N-terminal Cys residues, C-LGRQS**pS**IYSLT-NH_2 (phosphorylated form) and C-LGRQS**S**IYSLT-NH_2 (nonphosphorylated form), were synthesized by Abmart (www.ab-mart.com.cn) and used to immunize rabbits to generate anti-phospho-Ser-42 site-specific antibodies. Anti-phospho-Ser-42 site-specific antibodies were screened and purified as described previously (Lin et al., 2009).

ABI5^{S42} Phosphorylation in Planta

To introduce the S42A and S42D substitutions into ABI5, we performed two-round-PCR-based nucleotide replacement as described previously (Quan et al., 2007; Xie et al., 2010). *ABI5*^{S42A} and *ABI5*^{S42D} were cloned into the p1307-myc binary vector downstream of the myc tag. The resulting constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into Arabidopsis Col-0, *abi5-8*, wild type, *pks5-4*, *pks5-4abi5-8*, or *pks5-6*. Two-week-old seedlings from independent T3 homozygous lines were left untreated or treated with 100 μM ABA for 1 h. Plant protein was extracted using 2× cold immunoprecipitation buffer. Myc-tagged ABI5 proteins were immunoprecipitated by incubating the supernatant with α-myc-conjugated agarose (Abmart) at 4°C for 12 h. The resulting samples were then analyzed by SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Millipore). The blots were probed with primary α-ABI5-phospho-Ser-42 or α-myc (Abmart) antibodies, and the signals from horseradish peroxidase-conjugated anti-mouse antiserum were detected on film using the enhanced chemiluminescence substrate (GE Healthcare).

Yeast One-Hybrid Assays

The pPC86-ABI5 effector plasmid and the pRS315-6×ABRE-HIS reporter plasmid were as described previously (Zhou et al., 2013). To obtain pPC86-ABI5^{S42A} and pPC86-ABI5^{S42D}, full-length coding sequence fragments were amplified from p1307-myc-ABI5^{S42A} and p1307-myc-ABI5^{S42D} using gene-specific primers. The yWAM2 yeast strain was used for yeast one-hybrid assays. The transactivation activities of the ABI5 proteins were determined by measuring the growth of serial dilutions of transformed yeast cells on a synthetic complete medium lacking Leu, Trp, and His for 2 to 3 d.

Transactivation Activity of ABI5 Proteins in Vivo

To detect the transactivation activity of the ABI5 proteins in planta, the promoter region of *EM6* (approximately 1.3 kb) was amplified by PCR and cloned between the *Hin*dIII and *Bam*HI sites of the pUC19-Fluc-NosT reporter plasmid. Plants expressing the p1307-myc-ABI5, p1307-myc-ABI5^{S42A}, and p1307-myc-ABI5^{S42D} constructs were used as effector plasmids. The reference plasmid was obtained from Promega. Combinations of plasmids were isolated and purified via the Plasmid Maxiprep Kit (Vigorous Biotechnology) and introduced into Arabidopsis leaf mesophyll protoplasts according to the PEG-Ca^{2+} protocol provided by the Sheen laboratory (Sheen, 2001). Transfected cells were cultured for 12 to 16 h without or with 5 μM ABA, and relative LUC activity was assayed according to the Dual-Luciferase Reporter Assay Protocol provided by Promega. The Tecan Infinite M200 microplate reader (Tecan Austria) was used to measure the relative LUC activity according to the protocol of the Magellan Standard version 7.1 software provided by the manufacturer as described previously (Wang et al., 2013a; Yuan et al., 2013).

For protoplast transfection, leaf strips (0.5–1 mm) were cut from the middle part of rosette leaves and submerged in a cellulase/macerozyme solution (1.5% [w/v] cellulase R10 [Yakult Honsha], 0.4% [w/v] macerozyme R10 [Yakult Honsha], 0.4 M mannitol, 20 mM KCl, 20 mM MES, pH 5.7, 10 mM $CaCl_2$, 5 mM mercaptoethanol, and 0.1% [w/v] bovine serum albumin). The enzyme/protoplast solution was filtered through a 75-μm nylon mesh, washed twice with W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES, pH 5.7), and resuspended in MMg solution (0.4 M mannitol, 15 mM $MgCl_2$, and 4 mM MES, pH 5.7) at 1 to 2 × 10^5 cells mL^{-1} immediately prior to transfection. For transfection, the plasmid DNA mixture (less than 20 μL) was added to 200 μL of protoplasts in a microfuge tube and mixed well with 220 μL of PEG-Ca^{2+} solution (4 g of PEG 4000, 3 mL of water, 2.5 mL of 0.8 M mannitol, and 1 mL of 1 M $CaCl_2$). After 15 min, the protoplasts were washed twice with W5 solution, resuspended at approximately 2 to 4 × 10^4 cells mL^{-1} in W5 solution with 0 or 5 μm ABA, and incubated in a growth chamber for 12 to 16 h.

The *EM6* promoter fused with LUC (*pEM6*-Fluc-NosT) was used as an ABI5-regulated ABA-responsive reporter gene (7 μg of plasmid per transfection). 35S-Rluc-NosT was included in each sample as an internal control (2 μg per transfection). Effector plasmid of ABI5 (p1307-myc-ABI5, p1307-myc-ABI5^{S42A}, and p1307-myc-ABI5^{S42D}) was used at 3 μg per transfection.

Sequence data from this article can be found in The Arabidopsis Information Resource database under the following accession numbers: At2g30360 (*PKS5*), At2g36270 (*ABI5*), At3g51810 (*EM1*), At2g40170 (*EM6*), and At5g66400 (*RAB18*).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Inducible expression of *PKS5* by ABA obtained from the Electronic Fluorescent Pictograph browser (Winter et al., 2007; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

Supplemental Figure S2. BiFC demonstrates that PKS5 and ABI5 interact in vivo.

Supplemental Figure S3. A firefly luciferase complementation also demonstrates that PKS5 and ABI5 interact in vivo.

Supplemental Figure S4. PKS5 phosphorylates the ABI5 N terminus.

Supplemental Figure S5. Quantification of radicle emergence and expression of ABI5-regulated ABA-responsive genes in each genotype.

Supplemental Figure S6. ABA inhibition of root elongation of the *pks5-4abi5-8* double mutant.

Supplemental Figure S7. The Ser residue at position 42 is important for ABI5 function in ABA inhibition of seed germination in the *pks5-4abi5-8* double mutant.

Supplemental Table S1. DNA primer pairs used for construct generation.

Supplemental Table S2. DNA primer pairs used for mutant identification and qRT-PCR.

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