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Arabidopsis Raf-Like Kinase Raf10 Is a Regulatory Component of Core ABA Signaling

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Abscisic acid (ABA) is a phytohormone essential for seed development and seedling growth under unfavorable environmental conditions. The signaling pathway leading to ABA response has been established, but relatively little is known about the functional regulation of the constituent signaling components. Here, we present several lines of evidence that Arabidopsis Raf-like kinase Raf10 modulates the core ABA signaling downstream of signal perception step. In particular, Raf10 phosphorylates subclass III SnRK2s (SnRK2.2, SnRK2.3, and SnRK2.6), which are key positive regulators, and our study focused on SnRK2.3 indicates that Raf10 enhances its kinase activity and may facilitate its release from negative regulators. Raf10 also phosphorylates transcription factors (ABI5, ABF2, and ABI3) critical for ABA-regulted gene expression. Furthermore, Raf10 was found to be essential for the *in vivo* functions of SnRK2s and ABI5. Collectively, our data demonstrate that Raf10 is a novel regulatory component of core ABA signaling.

Keywords: ABI5, abscisic acid, phytohormone, Raf10, signaling, SnRK2s

INTRODUCTION

Abscisic aicd (ABA) is a major plant hormone essential for plant growth and development (Finkelstein, 2013; Nambara and Marion-Poll, 2005). ABA controls seed maturation process during normal growth and establishes seed dormancy, thereby preventing embryos from precocious germination (Bentsink and Koornneef, 2008; Holdsworth et al., 2008; Kanno et al., 2010). During the postgermination growth phase, endogenous ABA level increases dramatically when plants are exposed to adverse environmental conditions such as drought, high salinity and heat, and it mediates adaptive responses to abiotic stresses (Munemasa et al., 2015; Takahashi et al., 2018b; Xiong et al., 2002). The function of ABA in stress response is essential for plant survival, and ABA-deficient mutants grow poorly even under normal growth condition. At the molecular level, ABA controls expression of numerous genes involved in seed development and adaptive stress responses (Fujita et al., 2011; Takahashi et al., 2018a; Yoshida et al., 2015b).

A large number of genetic and molecular studies have been conducted to identify ABA signaling components and to delineate ABA signaling pathway (Finkelstein, 2013). The studies revealed that the core ABA signaling pathway consists of four essential components: PYR/PYL/RCAR family of receptors, clade A type 2C protein phosphatases (PP2Cs), subclass III Snf-1 related protein kinase 2s (SnRK2s), and the ABFs/AREBs/ABI5 subfamily of bZIP factors (Cutler et al., 2010; Fujii et al., 2009; Raghavendra et al., 2010). In the absence

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of ABA, PP2Cs bind SnRK2s and dephosphorylate them to inhibit their kinase function (Umezawa et al., 2009; Vlad et al., 2009) and, thus, subsequent steps of ABA signaling. In the presence of ABA, ABA-bound receptors interact with PP2Cs to inhibit their phosphatase activity (Nishimura et al., 2010; Park et al., 2009; Santiago et al., 2012). This Receptor-ABA-PP2C ternary complex formation results in release of SnRK2s from PP2C inhibition. SnRK2s are then activated and phosphorylate the ABFs/AREBs/ABI5 subfamily of bZIP factors (Choi et al., 2000; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Uno et al., 2000) and ion channels. Following their activation by SnRK2s, the bZIP factors turn on the expression of numerous ABA-responsive genes (Fujita et al., 2013; Yoshida et al., 2010; 2015a), whose products mediate ABA response. Thus, after signal perception, SnRK2s activation and phosphorylation of ABFs/AREBs/ABI5 by SnRK2s play central positive roles in ABA signaling. To be activated, SnRK2s are first released from PP2C inhibition by ABA-bound receptors. Actual activation of SnRK2s kinase function is achieved by autophosphorylation and/or phosphorylation by unknown kinase (Boudsocq et al., 2007; Ng et al., 2011; Vlad et al., 2010; Yoshida et al., 2006a). Activated SnRK2s phosphorylae several amino acids within N-terminal and C-terminal conserved regions of ABFs/AREBs/ABI5 (Furihata et al., 2006; Kobayashi et al., 2005; Sirichandra et al., 2010; Wang et al., 2013).

Each component in the core ABA signaling pathway consists of multiple members. In Arabidopsis, for example, the ABA receptor family consists of 14 members (i.e., PYR1 and PYL1-PYL13) (Ma et al., 2009; Park et al., 2009), whereas the clade A PP2Cs include nine members (i.e., ABI1, ABI2, HAB1, HAB2, AHG1, AHG3, and HAI1-HAI3) (Schweighofer et al., 2004; Singh et al., 2016). The SnRK2 family, on the other hand, consists of 10 members, of which three subclass III SnRK2s (i.e., SnRK2.2, SnRK2.3, and SnRK2.6) play critical roles in ABA signaling (Fujii and Zhu, 2009; Fujii et al., 2007; 2011; Fujita et al., 2009; Nakashima et al., 2009). The ABI5/ABFs/AREBs subfamily of bZIP factors consist of at least nine members (Bensmihen et al., 2002; Kim, 2006; Kim et al., 2002), and, whereas ABF/AREBs (i.e., ABF1-ABF4) function in vegetative tissues, ABI5 and several other bZIP proteins function mainly in seed. Involvement of multiple members in each step of ABA signaling suggests that a large variety of combinatorial specificity is possible within the frame of the core ABA signaling network.

Raf-like kinase Raf10 and its homolg Raf11 are important regulators of ABA response, controlling seed dormancy and ABA sensitivity of seedlings (Lee et al., 2015). *Raf10* overexpression (OX) lines, for example, are highly dormant and transgenic lines with high *Raf10* expression levels do not germinate at all. By contrast, seed dormancy is significantly compromised in *raf10* knockout (KO) mutant. Raf10 also regulates ABA response of seedlings, and its OX lines are hypersensitive to ABA, whereas its KO line is insensitive to ABA. Transcriptiome analysis shows that Raf10 and Raf11 affect expression of a large number of ABA-responsive genes, including seed-specific or seed-abundant genes. Importantly, the Raf10-modulated genes include regulatory genes such as *ABI3* (Giraudat et al., 1992), *ABI5* (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000), and *SOMNUS* (Park et al., 2011), which play key roles in controlling seed development, seed dormancy, and seed germination. Despite its significant regulatory role in ABA response, the mechanism of Raf10 and Raf11 functions is unknown.

Here, we present several lines of evidence that Raf10 is a novel regulatory element in core ABA signaling network. Raf10 is classified as one of the Raf-like MAP kinase kinase kinases (MAPKKKs) based on its amino acid sequence (Ichimura et al., 2002). However, it does not function as a canonical MAPKKK. We show that Raf10 interacts with ABA signaling components such as PP2Cs, SnRK2s, ABI5/ABFs, and ABI3 and phosphorylates them. We also show, by deletion analyses and site-directed mutagenesis, that Raf10 phosphorylates the regulatory domain of SnRK2s and C-terminal portion of ABI5. Additionally, we assessed effects of Raf10 phosphorylation, and the results indicate that Raf10 positively regulates functions of SnRK2s and ABI5 *in vitro* and *in planta*. Collectively, our data support the multifaceted regulatory role of Raf10 in ABA signaling and reveal that Raf10 is a novel regulatory component of core ABA signaling modulating the signaling steps downstream of signal perception.

MATERIALS AND METHODS

Yeast two-hybrid assay

Yeast two-hybrid assays were performed as described before (Lee et al., 2009). Bait constructs were prepared in pPC62LexA, prey constructs were prepared in pYESTrp2, and L40 (*MATα, his3Δ200, trp1-901, leu2-3112, ade2, LYS2::[LexAop(x4)-HIS3], URA3::LexAop[x8]-LacZ, GAL4*) was used as host strain. In some cases, pGBKT7 and pGADT7 (Clontech Laboratories, USA) were used as bait and prey vector, respectively, and AH109 (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3:: MEL1$_{UAS}$-MEL1 $_{TATA}$-lacZ*) was employed as a host strain. X-gal overlay assay was conducted according to (Duttweiler, 1996), and liquid β-galactosidase assay was carried out according to standard procedure (Guthrie and Fink, 1991). Coding regions of relevant protein genes were amplified and, after restriction digestion, cloned into bait or prey vectors. Primer sequences with restriction sites are listed in Supplementary Table S1.

Recombinant protein preparation and *in vitro* kinase assay

Recombinant proteins were prepared according to (Choi et al., 2017; Lee et al., 2015), using a modified pMAL vector (New England Biolabs, USA) with maltose binding protein (MBP) and 6X His double tags. Coding regions of relevant proteins were amplified using primers enlisted in Supplementary Table S1 and cloned into the vector. Kinase assays were conducted according to (Choi et al., 2017; Lee et al., 2015). Site-directed mutagenesis was conducted using the Q5®Site-Directed Mutagenesis Kit (New England Biolabs) according to the supplier's instruction. Primers are listed in Supplementary Table S1.

Unless stated otherwise, 0.5 to 1.0 μg of recombinant proteins were used as kinases or substrates. Reactions were conducted at 30°C in a buffer (25 mM Tris–HCl, pH 7.5, 10 mM

$MgCl_2$, 10 μM ATP) containing 2 μCi of [γ-^{32}P]-ATP. After the reaction, the mixtures were separated by SDS-PAGE on 10% to 12% gels, and gels were stained with Coomassie Brilliant Blue R (CBB), dried and autoradiographed.

Bimolecular fluorescence complementation assay

Bimolecular fluorescence complementation assays (BiFCs) were performed as described by (Choi et al., 2017), using the system developed by Walter et al. (2004). Briefly, constructs for transient expression were prepared in pSPYNE-35S and pSPYCE-35S complementation vectors after coding regions of relevant protein genes were amplified using primers listed in Supplementary Table S1. Each construct was transformed into *Agrobaterium* (C58C1), and pairwise combinations of constructs were then infiltrated into tobacco (*Nicotiana benthamiana*) leaves by Agroinfiltration (Voinnet et al., 2003; Witte et al., 2004). Epifluorescence signals were weak, and for better results, protoplasts were prepared from infiltrated leaves (Yoo et al., 2007) and observed under the fluorescence microscope (Olympus BX51; Olympus, Japan).

Coimmunoprecipitation assay

For coimmunoprecipitation assay, proteins were transiently expressed in tobacco leaves (*N. benthamiana*) after Agroinfiltration. Coding regions of relevant protein genes were amplified using primers in Supplementary Table S1 and cloned into 6xmyc-pBA002 or 3xHA-pBA002 (Zhou et al., 2005). Vectors were individually transformed into *Agrobacterium* C58C1, and each pair of transformed bacteria was infiltrated into tobacco leaves after co-cultivation. Leaves were flash-frozen 48 h after infiltration.

To prepare protein extracts, infiltrated tobacco leaves were ground into fine powder with liquid nitrogen and homogenized in extraction buffer containing 25 mM Tris pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100 supplemented with protease inhibitors (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail; Roche, Switzerland). Five hundred μg of pre-cleared lysates was used for Co-IP assay with 1 μg of Antibody-Coupled Dynabeads (Dynabeads Magnetic Beads; Thermo Fisher Scientific, USA) and were incubated for one hour at 4°C with gentle rotation. Beads were washed three times with washing buffer containing PBS 1×, 1% Triton® X-100, 3% BSA and boiled in SDS sample loading buffer and processed to Western blot. Anti-HA and anti-c-myc from mouse (1:3,000 dilution; Santa Cruz Biotechnology, USA) was used as first antibody. Anti-mouse (1:5,000) from GE Healthcare (USA) was used as second antibody. Protein bands were detected using SuperSignal™ West Femto substrate (Thermo Fisher Scientific) and processed with X-ray film exposure or Chemidoc (Bio-Rad, USA) imaging system.

In-gel kinase assay

In-gel kinase assay was according Wooten (2002). Protein extracts were prepared from maturing, green siliques in a buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerophosphate, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml antipain, 5 μg/ml aprotinin, 5% glycerol). Extracts (20 μg of proteins) were separated by SDS-PAGE on 10% to 12% gel embedded with 2 to 3 mg of recombinant proteins as substrates. MBP-tagged recombinant proteins were prepared as described above. After electrophoresis, gels were washed extensively to remove SDS and went through the denaturation-renaturation step. Kinase reactions were done for 2 h in the presence of 5 to 10 μCi of [γ-P^{32}]-ATP in a buffer containing 25 mM HEPES, pH 7.5, 13 mM $MgCl_2$, 2 mM EGTA, pH 7.0, 5 mM NaF, and 0.1 mM Na_3VO_4. After reactions, gels were washed, stained with CBB, dried and autoradiographed.

Transgenic analysis

Standard procedure was followed to cross *abi1-1* with *Raf10* OX lines. The *Raf10* OX line (*Raf10* OX #10) used in the cross is described elsewhere (Lee et al., 2015). After the initial cross, resulting F1 plants were self-fertilized to set F2 seeds. The F2 seeds were sown on MS media containing kanamycin (km), a selectable marker for the Raf10 OX line. The km-resistant seedlings were transferred to soil. To select *abi1-1* homozygous plants among the km-resistant F2 plants, we performed genotyping polymerase chain reaction (PCR) using CAPS primers as reported (Assmann et al., 2000); 5'-AAGATGCTGTTTCGACTATACC-3' and 5'-TTTCTCCTTAGCTATCTCCTCC-3'. Resulting PCR products were digested with *Nco*I and subject to gel electrophoresis for genotyping. After confirming homozygosity of *abi1-1*, the plants were grown to set F3 seeds. For the final phenotype analysis, F4 seeds amplified from the F3 were used after further confirmation of *abi1-1* genotype.

To generate *ABI5* OX lines, the *ABI5* coding region was amplified and cloned into pBI121 (Jefferson et al., 1987). Arabidopsis transformation was according (ref). A number of transgenic lines were generated in Col-0 or *raf10 raf11* genetic background, and, after preliminary analysis of several T3 homozygous lines, two were chosen and seeds were amplified. Final phenotype analysis was performed with the two T4 homozygous lines, and, representative results are shown.

RESULTS

Dimerization is required for Raf10 kinase function

Raf10 belongs to the B2 subgroup of Arabidopsis MAPKKKs (Ichimura et al., 2002), which consists of six members. The subgroup members possess two highly conserved regions in addition to their C-terminal kinase domains: A coiled coil region near their N-termini and a PAS/PAC domain (Moglich et al., 2009) downstream of it (Fig. 1A). We noticed that the coiled coil regions contain four heptad repeats of leucine residues (Fig. 1A), which may function as a leucine zipper dimerization motif. Moreover, in the course of our experiments to identify Raf10-interacting proteins, we found that Raf10 and its homolog Raf11 form homo and hetero dimers each other. To determine if the zipper region of Raf10 is involved in dimerization, we conducted yeast two-hybrid assay. As shown in Figure 1B (middle panel), full-length Raf10 interacted with Raf10 and Raf11 in the assay. Interactions, however, were abolished when the leucine repeat region was deleted. Similarly, full-length Raf11 could interact with Raf10 and Raf11 (Fig. 1B, right panel), but deletion of the leucine repeat

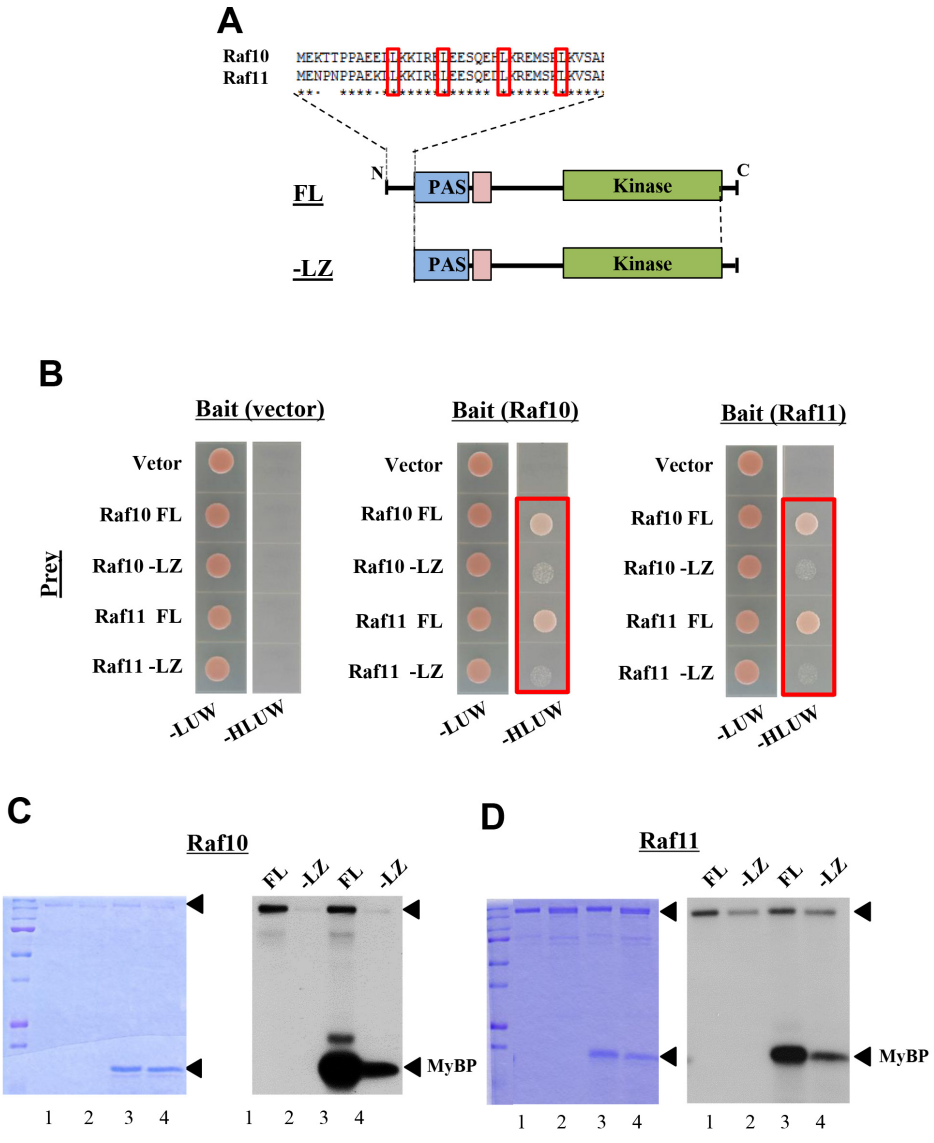

Fig. 1. Dimerization of Raf10 and Raf11. (A) Schematic diagram of Raf10 and Raf11 domain structure. Amino acid sequences of N-terminal portions are shown in the upper part. Leucine repeats are boxed. PAS, PAC domain (pink box), and Kinase domain are indicated. (B) Yeast two-hybrid assay to determine dimerization. Interactions between full-length and N-terminal deletion constructs were examined. Transformed yeast was grown on medium lacking histidine (-HLUW) supplemented with 3 mM (Raf10) or 1 mM (Raf11) 3-amino-1,2,4-triazole (3-AT). FL, full-length. -LZ, constructs lacking leucine repeats. (C and D) Kinase activity of Raf10 and Raf11 lacking the N-terminal leucine repeat regions. Kinase assays were conducted using the full-length (lanes 1 and 3) or N-terminal deleted recombinant proteins (lanes 2 and 4). Left panels, Coomassie blue-stained gel. Right panels, autoradiography showing ^{32}P-γ-ATP incorporation. MyBP, myelin basic protein.

region abolished its interaction with Raf10 and Raf11. Thus, the leucine repeat regions were necessary for the homo and hetero dimerization of Raf10 and Raf11.

We next investigated if dimerization affects Raf10 kinase activity. Raf10 recombinant proteins, full-length or truncated version lacking the leucine repeat region, were prepared, and *in vitro* kinase assays were conducted. The result (Fig. 1C) showed that autophosphorylation activity of the truncated Raf10 was significantly lower than that of the full-length Raf10. Similarly, reduced phosphorylation of myelin basic protein (MyBP) was observed with the truncated Raf10. Decrease in the auto- and MyBP-phosphorylation was also observed with Raf11 (Fig. 1D), indicating that dimerization is required for full kinase activity of Raf10 and Raf11.

Raf10 interacts with core ABA signaling components

To investigate Raf10 substrates, we examined if Raf10 could interact with ABA signaling components. We first performed yeast two-hybrid assays to examine possible interactions of Raf10 with core ABA signaling components (i.e., ABA receptors, PP2Cs, SnRK2s, and ABI5/ABFs) and ABI3 (Giraudat et al., 1992), the master regulator of seed development and seed dormancy (Bentsink and Koornneef, 2008; Holdsworth et al., 2008). *Raf10* OX and KO phenotypes are strong, and we hypothesized that it may directly control the activity of these ABA signaling components. We did not observe interactions between Raf10 and receptors, PYR1, PYL1, or PYL4 (Supplementary Fig. S1A). By contrast, PP2Cs including ABI1, ABI2, HAB1, AHG1, and AHG3 interacted with Raf10 in yeast (Figs. 2A and 2B). Among the five PP2Cs, ABI1 and AHG3 exhibited stronger interactions than other PP2Cs. Raf10 also interacted with SnRK2.2, SnRK2.3, and SnRK2.6 (Fig. 2C), which are key positive regulators of ABA response (Fujii and Zhu, 2009; Fujita et al., 2009; Nakashima et al., 2009). Similarly, Raf10 interacted with ABI5 and ABI3 (Fig. 2D and Supplementary Fig. S1B). Together, our yeast two-hybrid assay

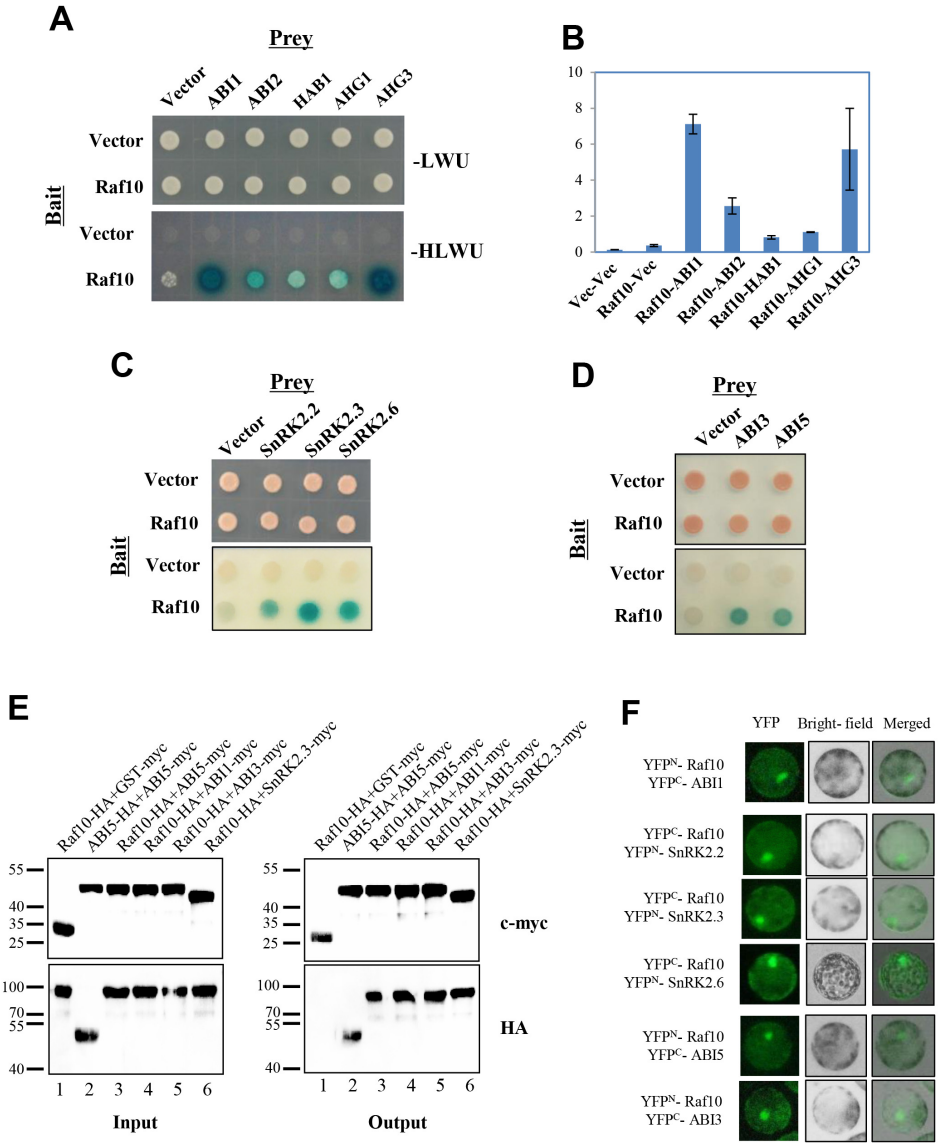

Fig. 2. Interaction of Raf10 with ABA signaling components. (A) Interactions between full-length Raf10 and PP2Cs (ABI1, ABI2, HAB1, AHG1, and AHG3) were determined by two-hybrid assay. Full-length *Raf10* was used as bait, and *PP2Cs* were used as prey. Yeast transformants were grown on SC-HWU, and *LacZ* reporter activity was detected by X-gal overlay assay. Top panel shows growth control on SC-LWU medium. (B) Liquid β-galactosidase assay. *LacZ* reporter activity was determined by liquid assay using O-nitrophenyl-β -D-galactopyrano-side (ONPG) as a substrate. The numbers indicate Miller units. (C) Interactions between full-length Raf10 and SnRK2s (SnRK2.2, SnRK2.3, and SnRK2.6) were examined by two-hybrid assay. Full-length *Raf10* was employed as bait, and *SnRK2s* were used as prey. Yeast transformatnts were grown on nonselective SC-LW medium (top panel), and *LacZ* reporter activity was determined by X-gal overlay assay (bottom panel). (D) Interactions between full-length Raf10 and ABI3 or ABI5 were examined by two-hybrid assay. Full-length *Raf10* was used as bait, and *ABI3* and *ABI5* were used as prey. Yeast transformants were grown on SC-LW medium, and *LacZ* reporter activity was determined by X-gal overlay assay. (E) Interactions between full-length Raf10 and selective ABA signaling components (ABI1, SnRK2.3, ABI3, and ABI5) were determined by coimmunoprecipitation assay. Protein extracts, prepared from tobacco (*N. benthamiana*) leaves infiltrated with 3HA-pBA and 6myc-pBA constructs, were used in the pull-down with 1 μg of c-myc antibody bound to Dynabeads. Proteins in the input and pulldown samples were detected using Western blot with either c-myc or HA antibody. The numbers in the left of each autoradiogram indicate the position and size of size markers (kDa). (F) Interactions between Raf10 and ABA signaling components (ABI1, SnRK2.2, SnRK2.3, SnRK2.6, ABI3, and ABI5) were determined by BiFC. Protoplasts prepared from tobacco (*N. benthamiana*) leaves infiltrated with pSPYNE-35S and pSPYCE-35S construct pairs were observed under fluorescent microscope. See also Supplementary Figures S1 and S2.

results indicate that Raf10 interacts with major ABA signaling components.

To confirm interactions between Raf10 and ABA signaling components, we conducted coimmunoprecipitation assay. Raf10 and selective ABA signaling components (i.e., ABI1, ABI3, ABI5, and SnRK2.3), tagged with HA and myc, respectively, were transiently expressed in tobacco leaves after Agroinfiltration, and proteins were immunoprecipitated with myc antibody. Immnonoprecipitates were then analyzed by Western blot analysis. Figure 2E (lanes 3-6) shows that ABI5, ABI1, ABI3, and SnRK2.3 coprecipitated with Raf10. In the control assay (lanes 1 and 2), ABI5, which forms homodimers, coprecipitated with ABI5, but Raf10 and GST did not, as expected.

To confirm further interactions of Raf10 with ABA signaling components, we performed BiFCs (Walter et al., 2004). *Raf10* and ABA signaling components (*ABI1*, three *SnRK2s*, *ABI5*, and *ABI3*) were individually cloned into pSPYNE-35S and pSPYCE-35S, respectively, and pairwise combinations of Raf10 and ABA signaling components were transiently expressed in tobacco cells (*N. benthamiana*) after Agroinfiltration (Voinnet et al., 2003; Witte et al., 2004). Figure 2F and Supplementary Figure S2 show that fluorescent signals were detected with the Raf10-ABI1 and Raf10-SnRK2s (i.e., Raf10-SnRK2.2, Raf10-SnRK2.3, and Raf10-SnRK2.6) pairs. Similarly, positive signals were observed with ABI5-Raf10 and ABI3-Raf10 pairs. Collectively, our results indicate that Raf10 interacts with major ABA signaling components, PP2Cs, SnRK2s, ABI5, and ABI3.

Raf10 phosphorylates ABA signaling components

Above interaction studies suggest that Raf10 may phosphorylate ABA signaling components, or, in the case of ABI1 and other PP2Cs, Raf10 may be dephosphorylated by them. To test the possibilities, we conducted *in vitro* kinase assays. First, recombinant proteins of full-length SnRK2.2 (amino acids 1-362) or its partial fragments containing the kinase domain (amino acid 1-290) or C-terminal part (amino acids 291-362) were prepared, and their phosphorylation by recombinant Raf10 was examined. Figure 3A (lane 2) shows

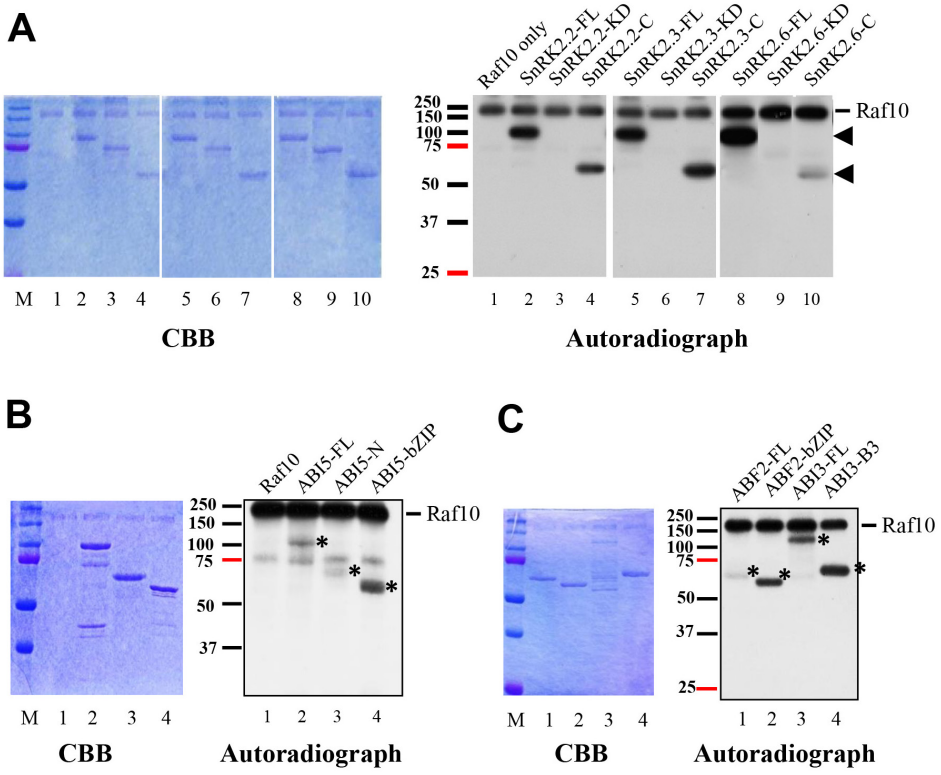

Fig. 3. Phosphorylation of SnRK2s. (A) Phosphorylation of SnRK2s (SnRK2.2, SnRK2.3, and SnRK2.6) by Raf10. Approximately 1 μg of full-length Raf10 and SnRK2s were used in the *in vitro* kinase assay. FL, full-length; KD, kinase domain; C, C-terminal domain. Left panel, CBB-stained gel. Right panel, autoradiogram. (B) Phosphorylation of ABI5 by Raf10. ABI5 recombinant proteins. FL, full-length protein (amino acid 1-442); N, N-terminal fragment (amino acid 1-350); bZIP, bZIP domain-containing C-terminal fragment (amino acids 341-442). Left panel, CBB-stained gel. Right panel, autoradiogram. (C) Phosphorylation of ABF2 and ABI3 by Raf10. The same amount of recombinant ABF2 proteins were used in lanes 1 and 2, respectively. FL, full-length (amino acids 1-416); bZIP, C-terminal bZIP-containing fragment (amino acids 321-416). In lanes 3 and 4, recombinant ABI3 proteins were used as substrates. FL, full-length (amino acids 1-720); B3, B3 domain (amino acids 561-720). Left panel, CBB-stained gel. Right panel, autoradiogram. The position of size makers is presented on the left of autoradiograms. The arrowheads and the asterisks indicate relevant phosphorylation band position. See also Supplementary Figures S3 and S4.

that full-length SnRK2.2 was phosphorylated by Raf10. The kinase domain of SnRK2.2, on the other hand, was not phosphorylated (lane 3), but the C-terminal part of SnRK2.2 was phosphorylated (lane 4). Similar phosphorylation pattern, i.e., phosphorylation of the full-length protein (lane 5) and its C-terminal part (lane 7) but not the kinase domain (lane 6), was observed with SnRK2.3. The full-length SnRK2.6 (lane 8) and its C-terminal portion (lane 10) were also phosphorylated by Raf10, although C-terminal phosphorylation was relatively weak. In our assay condition, SnRK2.2 and SnRK2.3 autophosphorylation was not detectable, and, although SnRK2.6 autophosphorylation was detectable, degree of phosphorylation by Raf10 was much stronger than autophosphorylation (Supplementary Fig. S3). Thus, our *in vitro* kinase assay results show that Raf10 phosphorylates C-terminal portions of SnRK2.2, SnRK2.3 and SnRK2.6.

We next investigated if Raf10 could phosphorylate ABI5 and one of the ABFs/AREBs family member ABF2, which also interacts with Raf10 (Supplementary Fig. S1B). Figure 3B shows that full-length ABI5 was phosphorylated by Raf10 (lane 2). The assay using its partial fragments containing its N-terminal (amino acids 1-350) (lane 3) or C-terminal

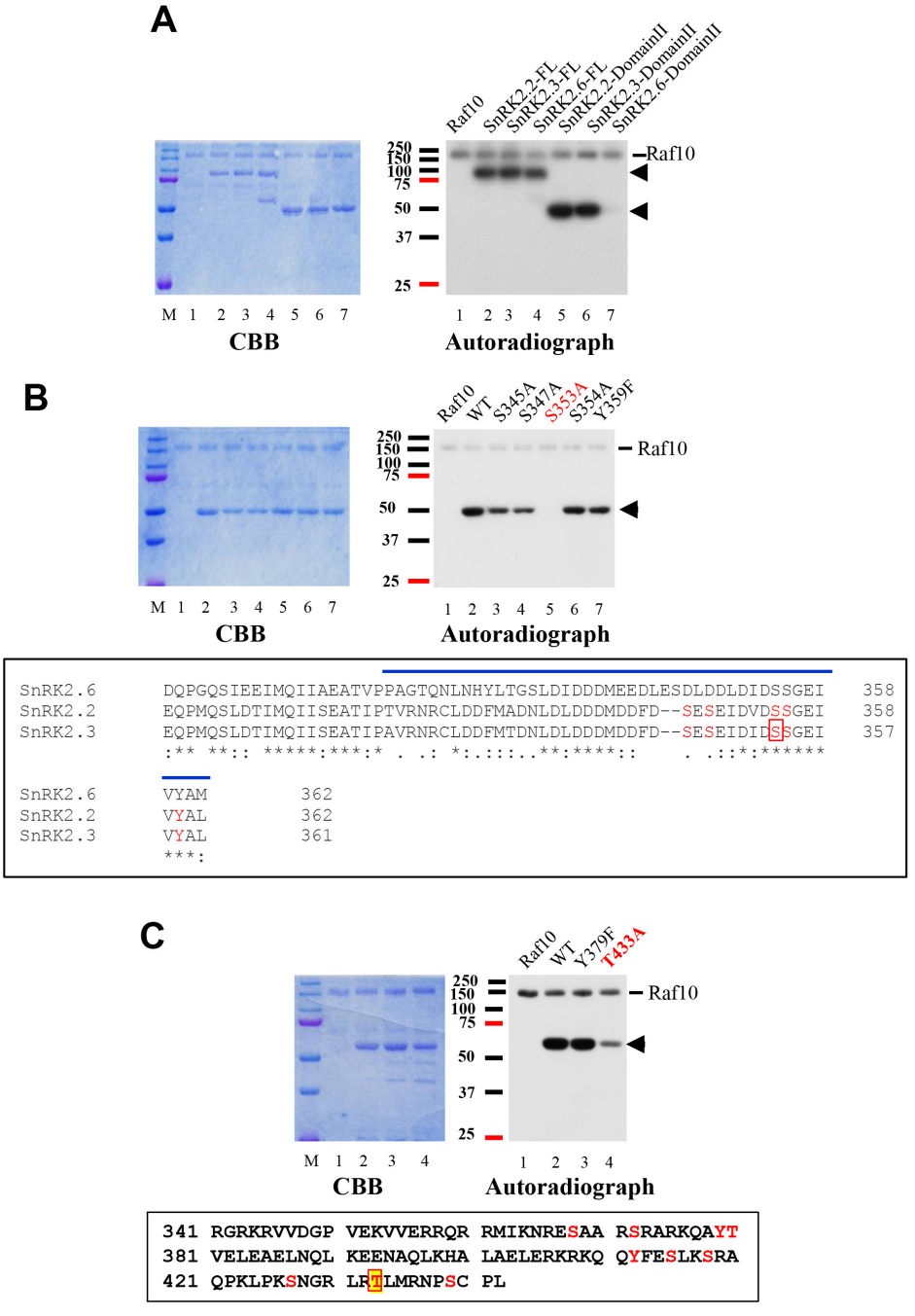

Fig. 4. Site-directed mutagenesis to determine phosphorylation sites. (A) Phosphorylation of SnRK2 Domain II (ABA box) by Raf10. Assays were performed as in Figure 3, using full-length (FL) or Domain II constructs. Arrowheads indicate substrate band position. Left panel, CBB-stained gel. Right panel, autoradiogram. (B) Site-directed mutagenesis of SnRK2.3 Domain II (ABA box). Wild-type and mutant recombinant proteins were used as substrates in the assay. The bottom panel shows amino acid sequences of the C-terminal regions of snRK2s. The bar indicates Domain II (ABA box). Potential phosphorylation sites in SnRK2.2 and SnRK2.3 are indicated in red fonts, and S353 is boxed. Left panel, CBB-stained gel. Right panel, autoradiogram. (C) Site-directed mutagenesis of ABI5 C-terminal region. Phosphorylation of two mutant proteins is shown. The bottom panel shows the amino acid sequence of the C-terminal region. Potential phosphorylation sites examined in our study are indicated by red fonts. Left panel, CBB-stained gel. Right panel, autoradiogram. Size markers are shown on the left of autoradiograms in (A-C). See also Supplementary Figure S5.

bZIP-containing region (amino acids 341-442) (lane 4) indicated that, although both fragments were phosphorylated, its C-terminal portion was much more strongly phosphorylated. A similar phosphorylation pattern was observed with ABF2 (Fig. 3C), i.e., weak phosphorylation of its N-terminal portion (amino acids 1-330) (lane 1) and stronger phosphorylation of its bZIP region (amino acids 321-416) (lane 2). We also observed phosphorylation of full-length ABI3 (amino acids 1-720) (Fig. 3C, lane 3) and its B3 DNA-binding domain (amino acids 561-720) (lane 4) by Raf10. However, phosphorylation of PP2Cs (i.e., ABI1 or AHG3) by Raf10 was not detected (Supplementary Fig. S4, see also Discussion). In summary, our results indicate that Raf10 phosphorylates SnRK2s, ABI5, ABF2, and ABI3 *in vitro*.

Raf10 phosphorylates S353 of SnRK2.3 and T433 of ABI5

C-terminal portions of SnRK2s consist of Domain I and Domain II (Belin et al., 2006; Yoshida et al., 2006a). Domain I (i.e., SnRK2 box) is responsible for the ABA-independent activation of SnRK2s, whereas Domain II, also known as ABA box, mediates interactions between SnRK2s and PP2Cs. Because Raf10 phosphorylates the C-terminal portion of SnRK2s, we asked if Raf10 could phosphorylate the ABA box by carrying out *in vitro* kinase assays. Figure 4A shows that Raf10 indeed phosphorylated the ABA box of SnRK2.2 (lane 5) and SnRK2.3 (lane 6). In the same assay, SnRK2.6 ABA box was also phosphorylated, but degree of phosphorylation was much weaker (lane 7) compared with those of SnRK2.2 and SnRK2.3.

We next set out to identify phosphorylation sites within the ABA box. We chose SnRK2.3 for the study. As shown in Figure 4B (bottom panel), the ABA box of SnRK2.3 contain five putative phosphorylation sites. The amino acids were changed to alanine by site-directed mutagenesis, and we conducted *in vitro* kinase assay using the mutant proteins as substrates for Raf10. Figure 4B (top panel) shows that S353A substitution among five substitutions abolished ^{32}P incorporation completely (lane 5), indicating that S353 of SnRK2.3 is likely the target phosphorylation site of Raf10.

We performed similar assays to determine phosphorylation sites of ABI5 C-terminal region containing its bZIP domain. There are 10 putative phosphorylation sites within the region (Fig. 4C, bottom panel), and we conducted site-directed mutagenesis of the 10 amino acid residues. Kinase assays using the mutant proteins (Fig. 4C and Supplementary Figs. S5A-S5D) revealed that T433A substitution reduced ^{32}P incorporation significantly (Fig. 4C, lane 4), albeit not completely. The result indicates that T433 of ABI5 bZIP region may be one of the Raf10 phosphorylation sites although it may not be the only phosphorylation site. Phosphorylation of ABI5 T433 by Raf10 was confirmed by mass spectrometry (i.e., LC-MS/MS) (Supplementary Fig. S5E).

Raf10 may modulate the ABI1-SnRK2.3 interaction and the SnRK2.3 activity

As mentioned above, ABA box is the interaction domain between SnRK2s and PP2Cs. Because Raf10 phosphorylates S353 of SnRK2.3 in the ABA box, we reasoned that phosphorylation of the amino acid may affect ABI1-SnRK2.3 interaction. To test the hypothesis, we prepared two substitution mutants, S353A and S353D, of SnRK2.3, and examined their interactions with ABI1 by yeast two-hybrid assay. As shown in Figure 5A, wild-type SnRK2.3 interacted with ABI1. S353A mutant also interacted with ABI1, indicating that the mutation did not affect SnRK2.3-ABI1 interaction significantly. However, SnRK2.3-ABI1 interaction was much weaker with the S353D mutant, which may be considered a phosphomimic. Weaker interaction between ABI1 and the SnRK2.3 S353D mutant was also observed in coimmunoprecipitation assay (Fig. 5B), further supporting the conclusion that ABI1-SnRK2.3 interaction was compromised by the mutation. The results suggest the possibility that phosphorylation of SnRK2.3 S353 by Raf10 may negatively affect SnRK2.3-ABI1 interaction.

Another possible effect of SnRK2 phosphorylation by Raf10 is modulation of SnRK2 kinase function. The subclass III SnRK2s (SnRK2.2, SnRK2.3, and SnRK2.6) have autophosphorylation activity that results in their autoactivation (Ng et al., 2011). Autoactivation capability, however, varies significantly among the three kinases. SnRK2.6 autoactivation is highly efficient, but autoactivation of SnRK2.2 and SnRK2.3 is much less efficient, and it has been suggested that they may be activated by unknown kinases. To test if Raf10 would affect SnRK2.3 activity, we conducted *in vitro* kinase assay, using the N-terminal part of ABI5 (amino acids 1-250) as a substrate. The N-terminal portions of ABI5 and ABFs are major targets of phosphorylation by SnRK2s (Fujii et al., 2007; Nakashima et al., 2009), and we examined ABI5 N-terminal phosphorylation by SnRK2.3. Figure 5C shows that wild-type SnRK2.3 phosphorylated the substrate (lane 2). Phosphorylated band intensity was much weaker when the SnRK2.3 S353A mutant was used (lane 4). In contrast, band intensity was much stronger when the phosphomimic S353D mutant form of SnRK2.3 was used. The result, i.e., enhanced kinase activity of the phosphomimic mutant form of SnRK2.3 indicates that Raf10 phosphorylation of SnRK2.3 S353 is likely to enhance SnRK2.3 kinase activity to phosphorylate ABI5 N-terminal portion.

Raf10 affects SnRK2 activity and ABI5 phosphorylation *in vivo*

To investigate if Raf10 affects SnRK2 activity and ABI5 phosphorylation *in vivo*, we conducted an in-gel kinase assay. We used protein extracts prepared from immature seeds because Raf10 functions mainly in seeds and during early seedling growth and, as a substrate, ABI5 C-terminal fragment containing its bZIP domain. Our results so far indicated that Raf10 phosphorylated SnRK2s and the ABI5 C-terminal fragment, and we reasoned that the phosphorylation events would lead to changes in the phosphorylation pattern of the ABI5 C-terminal fragment. Raf10 functions as a dimer, as shown above (Fig. 1). Thus, it would not be possible to detect Raf10 activity in the assay, because our protocol involves two denaturation steps (See Methods). We indeed did not see a kinase activity corresponding to Raf10, i.e., a band at 86 kDa position (Fig. 6A). However, we detected other kinase activities. In the wild-type Arabidopsis plants, a kinase activity at approximately 42 to 45 kDa position was detected

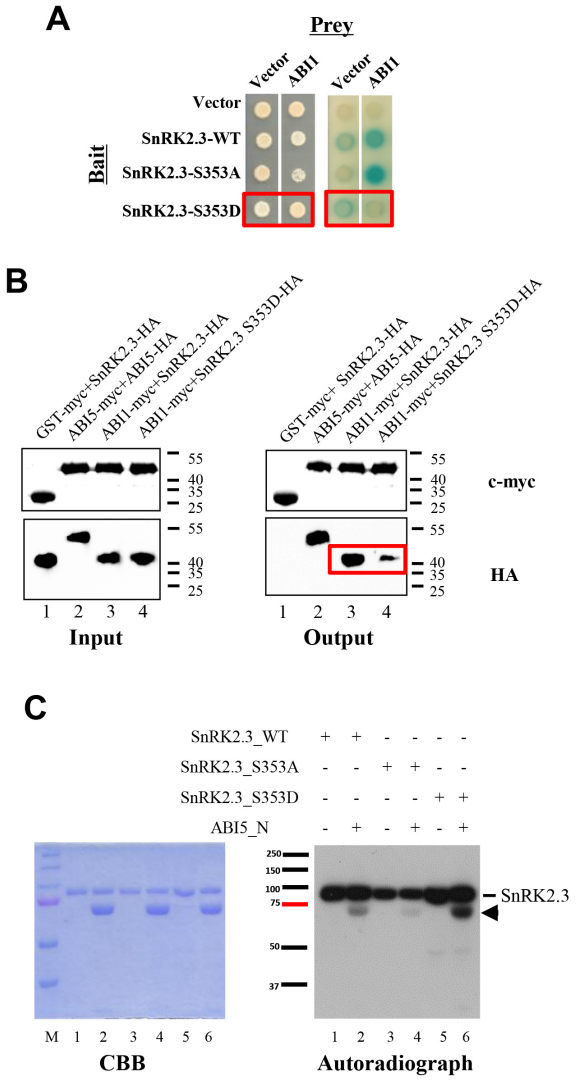

Fig. 5. Effect of SnRK2.3 phosphorylation by Raf10. (A) Yeast two-hybrid assay to determine S353 phosphorylation effect on ABI1-SnRK2.3 interaction. Interaction between the SnRK2.3 phosphomimic (S353D) mutant and ABI1 are indicated by the red box. (B) Coimmunoprecipitation assay to determine S353 phosphorylation effect on ABI1-SnRK2.3 interaction. HA- or myc-tagged proteins were transiently expressed in tobacco (*N. benthamiana*) leaves, and coimmunoprecipitation was conducted using tobacco leaf extracts. Pull-down was conducted using 500 μg of extracts and 1 μg of myc antibody. Proteins in the input and pull-down samples were analyzed by Western blot analysis. Size markers are shown beside the blots. The bands corresponding to ABI1 are highlighted by red box. (C) Kinase assay to examine S353 phosphorylation effect on SnRK2.3 kinase activity. Kinase activity of wild-type and the phosphomimic (S353D) mutant of SnRK2.3 was determined using an ABI5 N-terminal fragment (amino acids 1-250) as substrates. The arrowhead shows substrate band position. Exposure time for autoradiograph was at least 10 times longer than that in Figures 3 and 4. See also Supplementary Figure S6.

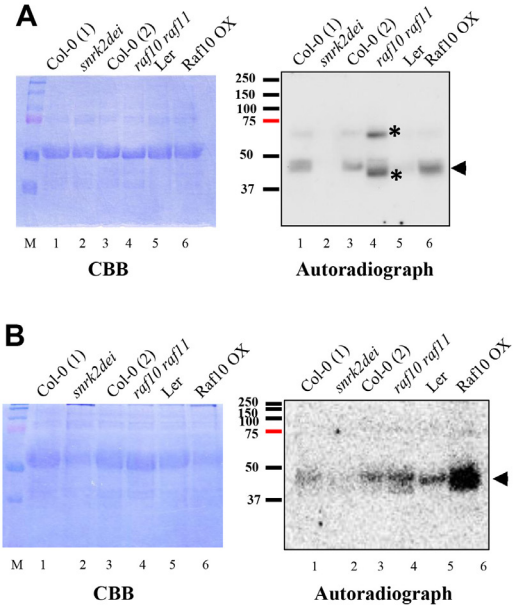

Fig. 6. In-gel kinase assay to determine Raf10 effect on ABI5 phosphorylation. (A) In-gel kinase assay was conducted using seed extracts (20 μg each) prepared from immature, green siliques and MBP-ABI5 C-terminal fragment (2 mg) embedded in the gel as a substrate. The band corresponding to SnRK2s is indicated by an arrowhead, and unknown kinase bands are indicated by asterisks. (B) In-gel kinase assay using an MBP-ABI5 N-terminal fragment as a substrate. Seed extracts (20 μg) and 3 mg of recombinant proteins were used. The arrowhead indicates the band corresponding to SnRK2s. Left panels, CBB-stained gel. Right panels, autoradiograph.

(lanes 1 and 3). The kinase activity was not detected at all in the SnRK2 triple mutant, *snrk2dei*, suggesting that it is likely to be SnRK2s. The kinase activity was almost completely diminished in the *raf10 raf11* double KO mutant. By contrast, its intensity increased greatly in the Raf10 OX line. Raf10-dependence of the kinase activity suggests that Raf10 positively regulates its activity. In the assay, two unknown kinases at ~40 kDa and ~65 kDa position, respectively, were detected whose activity was enhanced in the *raf10 raf11* double KO mutant. The result implies that there may be unknown kinases which phosphorylate the ABI5 C-terminal region and whose activities are regulated negatively by Raf10.

Similar in-gel kinase assay was performed with the N-terminal portion of ABI5 (amino acids 1-250) as a substrate (Fig. 6B). The protein fragment contains major SnRK2 phosphorylation sites within its three conserved regions (i.e., C1-C3) (Furihata et al., 2006). As in the case of ABI5 C-terminal fragment, a kinase activity with molecular mass of 42 to 45 kDa was detected, which was not detected in the *snrk2dei* triple mutant. Unlike the case of C-terminal fragment, we did not observe significant change in its intensity in the *raf10 raf11* mutant. However, its activity increased greatly in the Raf10 OX line, suggesting that Raf10 is sufficient to activate the kinase activity.

Raf10 overexpression suppresses the hypermorphic *abi1-1* phenotype

Our results suggested that Raf10 may affect ABA response by modulating signaling steps downstream of PP2Cs, e.g., by modulating ABI1-SnRK2 interactions or SnRK2s activity. To test the hypothesis, we crossed *abi1-1* with a *Raf10* OX line and determined ABA sensitivity of the progeny. *abi1-1* is a hypermorphic ABA-insensitive mutant which constitutively bind SnRK2s to inhibit their activity. *abi1-1* plants thus are ABA-insensitive even in the presence of ABA. If Raf10 functions downstream of PP2Cs, by disrupting ABI1-SnRK2 interactions or by directly promoting SnRK2 activity, the progeny of the cross would exhibit ABA-hypersensitive, *Raf10* OX phenotype. Figure 7A shows that the double mutant progeny of the cross exhibited enhanced seed dormancy as the *Raf10* OX line did. Also, the progeny exhibited ABA hypersensitivity during early seedling growth (Fig. 7B). Thus, the ABA insensitive phenotype of *abi1-1* was fully suppressed by *Raf10* overexpression. The result is consistent with the hypothesis that Raf10 enhances ABA sensitivity by activating signaling steps downstream of PP2Cs, probably by disrupting PP2C-SnRK2 interactions or by promoting SnRK2 activity.

To address the Raf10 effect on the function of ABI5 *in vivo*, we generated transgenic plants overexpressing ABI5 in the *raf10 raf11* mutant background and determined their ABA sensitivity during the germination/cotyledon greening stage. ABI5 is most effective in inhibiting growth at this stage of plant development (Lopez-Molina et al., 2001). We hypothesized that Raf10 would be required for full ABI5 function if Raf10 promotes the function of SnRK2s or directly modulates ABI5 function. Figure 7C shows that growth of *ABI5* OX line seedlings in the wild-type background (Col-0) were

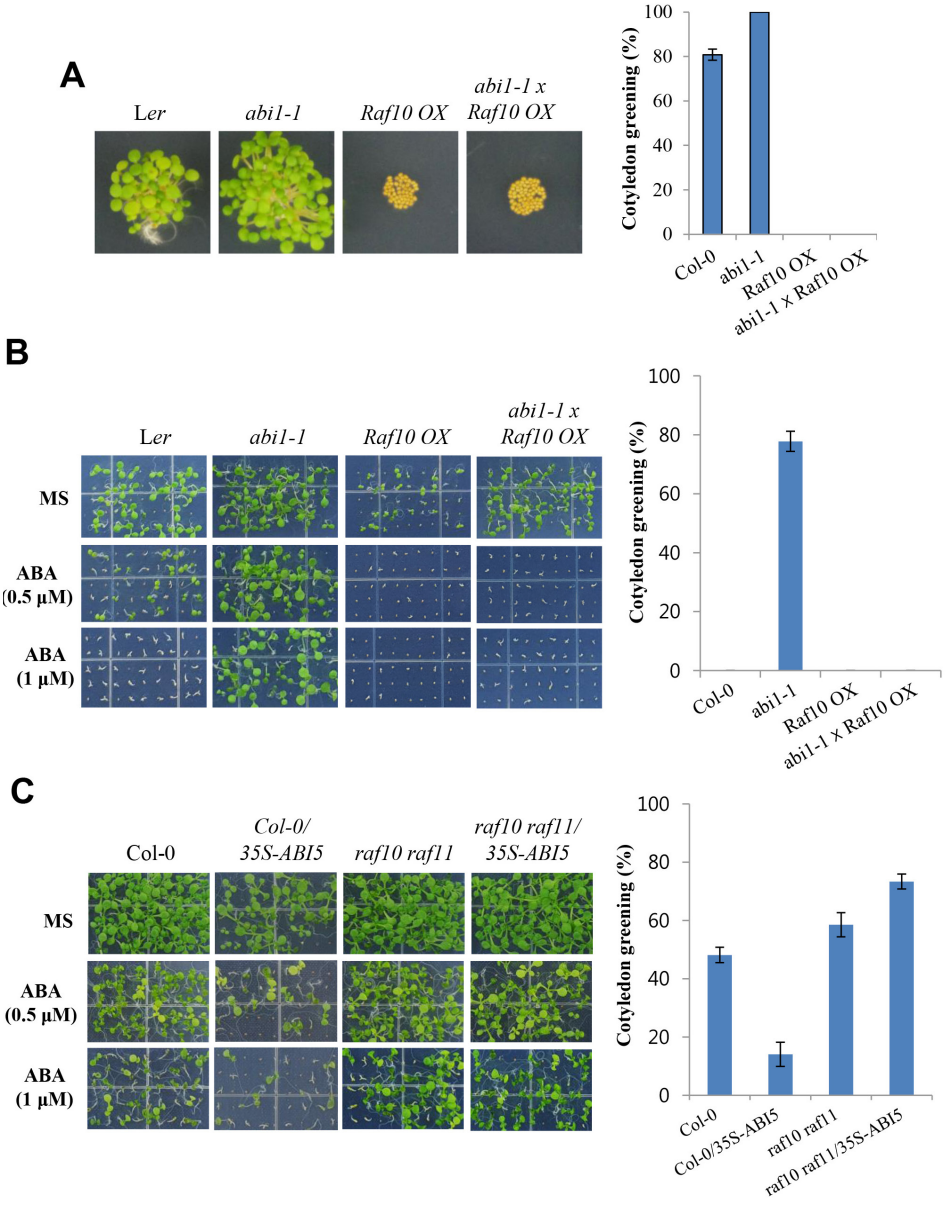

Fig. 7. Raf10 suppresses *abi1-1* phenotypes and is required for ABI5 function. (A) Dormancy of the double mutant progeny from the *abi1-1* × *Raf10* OX line cross. Freshly harvested seeds were plated without stratification and grown for 5 days before pictures were taken. Cotyledon greening efficiency was determined 7 days after the plating. (B) ABA sensitivity of the double mutant progeny from the *abi1-1* × *Raf10* OX line cross was determined during seed germination/cotyledon greening stage. Mature, dry seeds were plated and grown for 5 days before pictures were taken, and the cotyledon greening efficiency at 1 µM ABA was determined. (C) ABA sensitivity of *ABI5* OX lines was determined during germination/cotyledon greening stage. Seeds were plated and grown for 7 days, and cotyledon greening efficiency was determined.

ABA-hypersensitive. By contrast, *ABI5* OX line seedlings in the *raf10 raf11* mutant background were ABA-insensitive, as the parental *raf10 raf11* mutant plants were. Expression levels of *ABI5* in Col-0 and the *raf10 raf11* background were similar, and we interpret the result to indicate the Raf10 dependence of ABI5 function, i.e., Raf10 was required for normal function of ABI5.

DISCUSSION

We presented several lines of evidence that Raf10 is a novel regulatory component of core ABA signaling. Raf10 interacted with major ABA signaling components and phosphorylated positive regulators functioning downstream of the signal perception step. Consistent with the positive regulatory role of Raf10 in ABA response (Lee et al., 2015), phosphorylation by Raf10 resulted in enahnced SnRK2.3 activity, and Raf10 was required for the *in vivo* functions of SnRK2s and ABI5.

Our results show that Raf10 functions as a dimer and the N-terminal, lecine repeat region is dimerization motif (Fig. 1). In our two-hybrid screen to identify Raf10-interacting proteins, we found that Raf10 also interacts with Raf11 and Raf12 in addition to Raf10. Thus, Raf10 forms homodimer as well as heterodimers with other B2 subgroup members. At present, we do not know if other B2 subgroup members form heterodimers with Raf10 and play roles in ABA response. However, considering that Raf11 play similar role to Raf10 (Lee et al., 2015), it is possible that dimerization between B2 subgroup members provides an additional layer of functional complexity among the group members.

Raf10 is classified as a Raf-like MAPKKK (Ichimura et al., 2002). However, it does not phosphorylate any MAPKKs (data not shown). Rather, our interaction studies show that Raf10 interacts with three of the four core ABA signaling components (Fig. 2 and Supplementary Fig. S1): PP2Cs, SnRK2s, and ABI5/ABF/AREBs. Raf10 also interacts with ABI3, key regulator of seed development and seed dromancy (Bentsink and Koornneef, 2008; Holdsworth et al., 2008). Broad interaction capability with multiple ABA signaling components implies that Raf10 would play a general or multifaceted role in ABA response, consistent with strong ABA-associated phenotypes observed in *Raf10* OX and KO lines (Lee et al., 2015).

Raf10 interacted with all five PP2Cs (ABI1, ABI2, HAB1, AHG1, and AHG3) we tested in our interaction assay (Figs. 2A and 2B). Especially, the Raf10-ABI1 and Raf10-AHG3 interactions were stonger than other Raf10-PP2C interactions, implying that Raf10 may function via ABI1 and AHG3. It is noteworthy in this regard that Raf10 interacts strongly with AHG3, which is the most active PP2Cs in seed and functions mainly during seed germination as Raf10 does (Kuhn et al., 2006; Yoshida et al., 2006b). Given that Raf10 is a kinase whereas PP2Cs are phosphtases, there are two possible outcomes of the Raf10-PP2C interactions: Raf10 phosphorylates PP2Cs, or PP2Cs dephosphorylate Raf10. Our *in vitro* kinase assay result (Supplementary Fig. S4) suggests that the latter would be more likely. Both autophosphorylation and the substrate (myelin basic protein) band intensity decreased in a PP2C concentration-dependent manner in the assay employing Raf10 as a kinase and ABI1 and AHG3 as substrates. Neither ABI1 or AHG3 was phosphorylated by Raf10. Our current working hypothesis is that Raf10 is negatively regulated by PP2Cs.

Raf10 could phosphorylate ABI5, ABF2, and ABI3 (Figs. 3B and 3C). At present, we do not know the outcomes of these phosphorylation events. The C-terminal portions of ABI5 and ABF2 contain the DNA-binding, bZIP regions. The B3 domain of ABI3 is also DNA-binding domain. Thus, one of the possible outcomes of Raf10 phosphorylation of the transcription factors is modulation of their DNA-binding property. Indeed, the T433E phosphomimic mutant of ABI5 exhibited higher DNA-binding activity in yeast (Supplementary Fig. S6A) (Kim et al., 1997). Additionally, the ABI5 mutant interacted more efficiently with SnRK2s in yeast and plant cells (Supplementary Figs. S6B and S6C). These observations suggest that Raf10 phosphrylation may positively affect ABI5 function by enhancing its DNA-binding activity or its interaction with SnRK2s.

Among the 10 SnRK2s, the three subclass III SnRK2s (i.e., SnRK2.2, SnRK2.3, and SnRK2.6) play pivotal roles in ABA response (Fujii and Zhu, 2009; Fujita et al., 2009; Nakashima et al., 2009). Virtually all aspects of ABA response are impaired in the *snrk2dei* triple mutant. The kinases phosphorylate and activate, among others, the ABI5/ABF/AREB subfaminly of bZIP proteins, which turn on numerous ABA/stress-responsive genes (Yoshida et al., 2015a). Our data indicate that Raf10 could phosphorylate these subclass III SnRK2s and that phosphorylation sites are within their ABA boxes (Figs. 3 and 4). The ABA box is important for PP2C-SnRK2 interactions and ABA-dependent SnRK2s activity (Belin et al., 2006; Soon et al., 2012; Yoshida et al., 2006a), and our study focused on SnRK2.3 shows that Raf10 may modulate both aspects of SnRK2.3 function. The phoshomimic, S353D muatnt of SnRK2.3 interacted with ABI1 less efficiently than the wild-type SnRK2.3 (Figs. 5A and 5B), implying that Raf10 would promote the SnRK2.3 function by facilitating its release from ABI1 inhibition. Raf10 may also directly enhance SnRK2.3 kinase activity. In the kinase assay using the ABI5 N-terminal fragment as a substrate (Fig. 5C), the phosphomimic mutant exhibited several-fold higher activity than wild-type SnRK2.3. Although SnRK2s are activated by autophosphorylation, the autoactivation efficiency of SnRK2.2 and SnRK2.3 is markedly low, and activation by unknown kinase has been suggested (Boudsocq et al., 2007; Ng et al., 2011). ABA boxe are outside activation loops, and their phosphorylation may not be directly associated with their autoactivation. Nonetheless, the ABA box is essential for SnRK2s activity (Belin et al., 2006; Yoshida et al., 2006a), and our result indicates that Raf10 may enhance SnRK2.3 activity by phosphorylating its ABA box. It is noteworthy in this regard that Raf10 phosphorylation of SnRK2.2 and SnRK2.3, whose autoactivation is inefficient, was much more stronger than that of SnRK2.6 (Figs. 3A and 4A), whose autoactivation is very efficient.

The significance of Raf10 in modulation of SnRK2s function is further corroborated by our in-gel kinase assay results. In the assay using ABI5 C-terminal region as a substrate (Fig. 6A), we detected a kinase activity. The size of the kinase, approximately 42 to 45 kDa, is same as those of SnRK2s, and it

was not detected at all in the *snrk2dei* triple mutant. Based on these observations, we reason that the kinase activity corresponds to SnRK2s. The kinase activity was abolished almost completely in the *raf10 raf11* double KO mutant, but it increased signifcantly in the *Raf10* OX line. Thus, Raf10 was essential and sufficient for the SnRK2s activity to phosphorylate the ABI5 C-terminal region. We obtained similar result, i.e., Raf10-dependence of SnRK2 activity, in the in-gel kinase assay using an ABI5 N-terminal fragment, which contains major SnRK2 phosphorylation stes (Furihata et al., 2006) (Fig. 6B). In the assay, we detected a kinase, which was the same size as the kinase detected in the ABI5 C-terminal assay and diminished in the *snrk2dei* mutant. The kinase activity was not changed noticeably in the *raf10 raf11* double KO mutant, unlike in the case of the C-terminal fragment. However, remarkable increase was observed in the *Raf10* OX line. The result is consistent with our *in vitro* kinase assay result (Fig. 5C) and suggests that, although not essential, Raf10 is sufficient to activate their function to phosphorylate ABI5 N-terminal region. Together, results of our in-gel kinase assays demonstrate that Raf10 promotes activity of SnRK2s to phosphorylate ABI5.

In the *abi1-1* mutant, SnRK2s are inhibited by PP2Cs even in the presence of ABA, resulting in constitutive ABA-insensitivity regardless of ABA presence (Park et al., 2009). Our analysis of the double mutant progeny of the cross between *abi1-1* and *Raf10* OX line shows that the mutant progeny was ABA-hypersensitive as the *Raf10* OX line. Thus, *Raf10* OX fully suppressed the *abi1-1* phenotype, suggesting that Raf10 activated SnRK2s functions or downstream signaling steps. In principle, several mechanisms are conceivable for suppression of *abi1-1* phenotype. One possibility is that Raf10 blocks ABI1 function, i.e., its binding to SnRK2s, either by reducing ABI1 protein level or inhibiting its function. We did not observe major changes in ABI1 protein level in *Raf10* OX or KO lines (data not shown), but our data (Fig. 5) indicate that ABI1-SnRK2.3 interaction and, possibly, other PP2C-SnRK2 interactions, may be inhibited by Raf10 phosphorylation of SnRK2.3 S353. This will make release of SnRK2s from PP2C inhibition more feasible. Another possible mechanism for ABA hypersensitivity would be direct enhancement of SnRK2s kinase activity resulting from phosphorylation by Raf10. This is in line with our data (Figs. 5C and 6) discussed above. Considering that Raf10 interacts with ABI5 and ABFs/AREBs, it is also probable that Raf10 would modulate the function of ABI5 and related bZIP factors positively, thereby increasing ABA sensitivity. In this regard, it is noteworthy that Raf10 is required for full ABI5 function *in planta* (Fig. 7C). The role of Raf10 may be indirect, i.e., via promotion of the SnRK2s activity to phosphorylate ABI5, but it is also possible that Raf10 directly activates its function. As yet, we do not know which mechanism would be most likely. However, all three mechanisms are consistent with our data, suggesting that Raf10 function may be multifaceted.

Previously, several kinases have been reported to modulate the functions of SnRK2s. A Raf-like kinase from the moss *Physcomitrella patens*, ARK, has been demonstrated to be an activator of SnRK2s (Saruhashi et al., 2015). Arabidopsis homologs of ARK belong to the B3 subgroup, and, unlike Raf10, ARK phosphorylates a residue in the activation loop of the moss SnRK2B. In Arabidopsis, BIN2, a GSK3-like kinase, which is a negative regulator of brassinosteroid signaling, phosphorylates T180 of SnRK2.3 to enhance its activity (Cai et al., 2014). Casein kinase 2, on the otherhand, phoshphorylates the ABA box of SnRK2.6 and promotes its degradation (Vilela et al., 2015), and HT1 (high leaf temperature 1) phosphorylates SnRK2.6 to inhibit its SLAC1 ion channel phosphorylation (Tian et al., 2015). Raf10 is different from the kinases in its substrate specificity and in its mode of action.

In conclusion, our work demonstrates that Raf10 is a novel regulatory components in core ABA signaling. Our current working model of Raf10 function is depicted in Supplementary Figure S7. Raf10 modulates primarily SnRK2s functions either by directly activating their kinase activity or by promoting their release from PP2C inhibition. It may also regulates the activity of ABI5 and related bZIP factors. The model is based on our observation that SnRK2s activity is highly dependent on Raf10 (Fig. 6). Enhanced kinase activity of SnRK2.3 phosphomimic mutant (Fig. 5C), the weaker interaction of the mutant with ABI1 (Figs. 5A and 5B), and the Raf10-dependence of ABI5 function (Fig. 7C) support our model. Although Raf10 modulates the subclass III SnRK2s, weaker phosphorylation of SnRK2.6 (Figs. 3A and 4A) suggests that SnRK2.2 and SnRK2.3 would be affected more by Raf10 than SnRK2.6. An important question to be addressed in the future is how Raf10 function is regulated. *Raf10* expression is constitutive, but it functions mainly in seed and during early seedling development stage (Lee et al., 2015). Presently, we do not know how this tissue-specificity is achieved. Given that ABI1 and AHG3 can dephosphorylate Raf10 (Supplementary Fig. S4), Raf10 activity may be inhibited by ABI1, whereas ABA activates its function by inhibiting ABI1.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

Disclosure

The authors have no potential conflicts of interest to disclose.

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