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The PP6 Phosphatase Regulates ABI5 Phosphorylation and Abscisic Acid Signaling in *Arabidopsis*[C][W]

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The basic Leucine zipper transcription factor ABSCISIC ACID INSENSITIVE5 (ABI5) is a key regulator of abscisic acid (ABA)–mediated seed germination and postgermination seedling growth. While a family of SUCROSE NONFERMENTING1-related protein kinase2s (SnRK2s) is responsible for ABA-induced phosphorylation and stabilization of ABI5, the phosphatase(s) responsible for dephosphorylating ABI5 is still unknown. Here, we demonstrate that mutations in *FyPP1* (for *Phytochrome-associated serine/threonine protein phosphatase1*) and *FyPP3*, two homologous genes encoding the catalytic subunits of Ser/Thr PROTEIN PHOSPHATASE6 (PP6), cause an ABA hypersensitive phenotype in *Arabidopsis thaliana*, including ABA-mediated inhibition of seed germination and seedling growth. Conversely, overexpression of *FyPP* causes reduced sensitivity to ABA. The ABA hypersensitive phenotype of *FyPP* loss-of-function mutants is ABI5 dependent, and the amount of phosphorylated and total ABI5 proteins inversely correlates with the levels of FyPP proteins. Moreover, FyPP proteins physically interact with ABI5 in vitro and in vivo, and the strength of the interaction depends on the ABI5 phosphorylation status. In vitro phosphorylation assays show that FyPP proteins directly dephosphorylate ABI5. Furthermore, genetic and biochemical assays show that FyPP proteins act antagonistically with SnRK2 kinases to regulate ABI5 phosphorylation and ABA responses. Thus, *Arabidopsis* PP6 phosphatase regulates ABA signaling through dephosphorylation and destabilization of ABI5.

INTRODUCTION

The phytohormone abscisic acid (ABA) is a major phytohormone that regulates plant growth and development, including seed dormancy, seed germination, seedling growth, and stomatal aperture, as well as plant responses to various abiotic and biotic stresses, such as drought, salt, and cold stresses and pathogen infection (Mauch-Mani and Mauch, 2005; Fujita et al., 2011; Hauser et al., 2011). Molecular genetic studies in *Arabidopsis thaliana* have led to the identification of a number of mutants affected in ABA signaling. Among them, the type-2C protein phosphatases ABSCISIC ACID INSENSITIVE1 (ABI1) and ABI2 were identified initially through the analysis of the dominant ABA-insensitive *abi1-1* and *abi2-1* mutants (Koornneef et al., 1982; Leung et al., 1994, 1997; Meyer et al., 1994). Subsequent characterization of loss-of-function alleles indicated that ABI1 and ABI2 negatively regulate many ABA responses, including inhibition of seed germination, seedling growth, and promotion of stomatal closure (Sheen, 1998; Gosti et al., 1999; Merlot et al., 2001). On the other hand, *abi3*, *abi4*, and *abi5* were identified as recessive mutants that show ABA insensitivity during seed germination and early seedling development (Koornneef et al., 1982; Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000).

The *ABI3*, *ABI4*, and *ABI5* genes encode B3-type, APETALA2 domain and basic Leucine zipper (bZIP)–type transcription factors (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000). ABI5 is a protein that functions as a transcriptional activator by binding to an ABA-responsive element (ABRE; Giraudat, 1995; Busk and Pagès, 1998; Hattori et al., 2002), a conserved *cis*-acting element found in the promoters of many ABA-induced genes (Yamaguchi-Shinozaki and Shinozaki, 1994). Consistent with a dominant role in seed germination and seedling development, ABI3, ABI4, and ABI5 are expressed mainly in seeds, with only low levels of expression in vegetative tissues (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000).

In addition, a number of bZIP transcription factors involved in ABA signaling have been identified through characterization of

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the ABRE binding factors (ABFs; also referred to as AREBs), including ABF1, ABF2/AREB1, ABF3, ABF4/AREB2, and ABI5 (Guiltinan et al., 1990; Yamaguchi-Shinozaki and Shinozaki, 1994). ABF2/AREB1, ABF3, and ABF4/AREB2 have been reported to play key roles in the response to drought stress (Yoshida et al., 2010). Plants overexpressing *ABF3* and *ABF4* exhibit enhanced drought tolerance and altered expression of ABA/stress-regulated genes (Kang et al., 2002). ABF2/AREB1 regulates ABRE-dependent ABA signaling that enhances drought tolerance in vegetative tissues (Fujita et al., 2005). In addition, *ABI5* is regulated by sugar and stress (Brocard et al., 2002; Arroyo et al., 2003), indicating that ABI5 also plays a role in stress response adjustment (Fujita et al., 2011).

Among these bZIP transcription factors, ABI5 plays a central role in regulating seed germination and postgermination seedling growth (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Lopez-Molina et al., 2001, 2002). ABI5 protein levels are tightly regulated. They accumulate to high levels in dry seeds or young seedlings treated with ABA; however, after germination or removal of ABA from the seedlings, ABI5 proteins are rapidly degraded (Lopez-Molina et al., 2001; Piskurewicz et al., 2008), suggesting that the levels of ABI5 protein play an important role in regulating ABA signaling. In support of this notion, several mutants affected in ABI5 degradation show a hypersensitive ABA response (Stone et al., 2006; Lee et al., 2010). A REALLY INTERESTING NEW GENE-ANKYRIN E3 ligase, KEEP ON GOING (KEG), was identified as a negative regulator of ABA signaling that is required for ABI5 degradation (Stone et al., 2006; Liu and Stone, 2010). Lee et al. (2010) reported that DWD HYPERSENSITIVE TO ABA1 (DWA1) and DWA2 are negative regulators of ABA signaling and function as the substrate receptors for a CULLIN4 E3 ligase that targets ABI5 for degradation. The ABI5-interacting protein (AFP) was reported to facilitate ABI5 degradation through the 26S proteasome–dependent pathway (Lopez-Molina et al., 2003). However, a recent study proposed that NOVEL INTERACTOR OF JAZ and its related AFP proteins act as transcriptional corepressors of TOPLESS proteins to regulate ABA responses through direct interaction with ABI5 (Pauwels et al., 2010). ABI5 abundance is also regulated by SMALL UBIQUITIN-RELATED MODIFIER modification (Miura et al., 2009). The requirement of multiple E3 ligases (for ubiquitination and sumoylation) for regulation of ABI5 levels illustrates the complexity of ABI5 regulation and its importance in regulating ABA signaling in plants.

Given the importance of ABI5 protein levels in ABA signaling, it is striking to note that ABI5 overaccumulation is not sufficient to confer postgerminative growth arrest. Transgenic plants overexpressing *ABI5* show increased ABI5 protein levels but grow normally in the absence of ABA (Lopez-Molina et al., 2001; Stone et al., 2006). Notably, additional higher molecular mass forms of ABI5 were observed in the *keg-1* mutant but not observed in *ABI5*-overexpressing plants (Stone et al., 2006). Together with the previous observation that ABA is necessary to stimulate ABI5 activity (Lopez-Molina et al., 2001), it was speculated that the severe postgerminative growth arrest of *keg-1* mutants might be caused by the accumulation of additional, presumably phosphorylated and active forms of ABI5 (Stone et al., 2006; Liu and Stone, 2010). Collectively, these data suggest that ABI5 is phosphorylated in response to ABA, and phosphorylation activates the transcription factor and enhances its stability and activity (Lopez-Molina et al., 2001; Piskurewicz et al., 2008).

Recent studies showed that three SUCROSE NON-FERMENTING1-related protein kinases, SnRK2.2 (SRK2D), OPEN STOMATA1 (OST1/SnRK2.6/SRK2E), and SnRK2.3 (SRK2I), are responsible for phosphorylation of ABI5 and ABI5-like bZIP transcription factors in response to ABA (Kobayashi et al., 2005; Fujii et al., 2007; Fujii and Zhu, 2009; Nakashima et al., 2009). The triple mutants of *snrk2.2/2.3/2.6* are much more resistant to ABA and hypersensitive to drought stress than any single or double mutants (Furihata et al., 2006; Fujii and Zhu, 2009; Nakashima et al., 2009), indicating that SnRK2.2, SnRK2.6, and SnRK2.3 are positive regulators of ABA signaling and they have overlapping functions in ABA signaling. Given the importance of SnRK2-mediated phosphorylation and activation of ABI5 in the inhibition of seed germination, dephosphorylation and subsequent degradation of ABI5 should be critical for the initiation of seed germination. However, the phosphatase(s) responsible for dephosphorylation of ABI5 has not yet been identified. In addition, it is not yet clear whether other phosphatases in addition to those already characterized function in ABA signaling.

The Ser/Thr-specific phosphoprotein phosphatases (PPPs) execute the major phosphatase activities in eukaryotes (Olsen et al., 2006). The *Arabidopsis* genome encodes 26 catalytic (C) subunits of PPPs related to type 1 (PP1), type 2A (PP2A), PP2B, and the so-called novel phosphatases, including PROTEIN PHOSPHATASE4 (PP4), PP5, PP6, and PP7 (Farkas et al., 2007). The PP2A holoenzyme consists of an enzymatically active catalytic subunit (PP2Ac), a 65-kD regulatory A subunit (PP2A A), and a variable regulatory B subunit (PP2AB) (Farkas et al., 2007). Although there are high sequence similarities among the C subunits of PP6 and PP2A phosphatases, they were classified as different phosphatases because PP6 requires Zn^{2+} for its activity, whereas PP2A does not require any cation (Kim et al., 2002; Wang et al., 2007; Dai et al., 2012a). PPPs are ubiquitous enzymes in all eukaryotes, but their regulatory functions are largely unknown in plants (Farkas et al., 2007).

In a recent study, we showed that *Arabidopsis* FyPP1 (for Phytochrome-associated serine/threonine protein phosphatase1) and FyPP3, two homologous catalytic subunits of PP6, physically interact with SAL (for SAPS-domain like protein) and PP2AA proteins (RCN1/PP2AA1, PP2AA2, and PP2AA3) to form a PP6-type heterotrimeric holoenzyme that directly regulates the phosphorylation status of PIN-FORMED (PIN) proteins (auxin efflux carriers) and subsequently affects auxin efflux and plant development (Dai et al., 2012a, 2012b). In this study, we extend these findings by showing that FyPP also plays a critical role in regulating ABA signaling in *Arabidopsis*. We show that mutants without FyPP activity accumulate higher levels of ABI5 protein and display an ABA-hypersensitive phenotype in seed germination and postgermination seedling growth. We further show that FyPP proteins directly interact with and dephosphorylate ABI5. Genetic interaction studies demonstrate an antagonistic interaction between FyPP1/3 with SnRK2 in regulating ABA responses. Our findings suggest that FyPP1 and FyPP3 function

as two negative regulators of ABA signaling through the dephosphorylation of ABI5.

RESULTS

FyPP Genes Negatively Regulate ABA Signaling

FyPP1 and FyPP3, the catalytic subunits of *Arabidopsis* PP6 phosphatases (PP6c) in *Arabidopsis*, share high sequence homology (99% amino acid identity) (Dai et al., 2012a). Publically available data (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter et al., 2007) show that *FyPP1* and *FyPP3* expression is upregulated by imbibition during seed germination, which is opposite to that of *ABI5* (see Supplemental Figure 1 online), suggesting the involvement of FyPP1 and FyPP3 in regulating seed germination. To test this, we first investigated the germination of Columbia (Col; the wild type), *fypp1* (*f1*), and *fypp3* (*f3*) seeds and seeds from self-pollinated *f1*$^{-/+}$ *f3* plants (*f1* is heterozygous and *f3* is homozygous) on germination medium (GM) plates containing various concentrations of ABA. We used seeds from self-pollinated *f1*$^{-/+}$ *f3* plants in these experiments because the *f1 f3* homozygous double mutant plants are completely infertile (Dai et al., 2012a). The results showed that both *f1* and *f3* single homozygous mutants had similar germination and greening percentages as Col at the tested ABA concentrations, while the seeds from self-pollinated *f1*$^{-/+}$ *f3* plants had much lower germination and greening percentages than Col at the tested ABA concentrations (Figures 1A to 1C; see Supplemental Figure 2 online). Genotyping analysis revealed that among the progeny of self-pollinated *f1*$^{-/+}$ *f3* plants incubated on 2.5 μM ABA plates, all geminated seeds were of the *f3* genotype (n = 20 genotyped, 100%), while the nongermination seeds (n = 95 genotyped) consisted of three genotypes: *f3* (12.6%), *f1*$^{-/+}$ *f3* (58%), and *f1 f3* (29.4%). These observations indicate that seeds of the *f1*$^{-/+}$ *f3* and *f1 f3* genotypes were hypersensitive to ABA.

We next investigated the root growth of *f1 f3* single mutants and *f1 f3* double mutants on GM plates containing 0 or 1 μM ABA. The *f1 f3* double mutants have shorter roots and smaller/fused cotyledons than Col at the early seedling stage (Dai et al., 2012a; see Supplemental Figure 3 online), and we therefore chose *f1 f3* seedlings for our stress experiment based on these phenotypes. We observed that the relative root growth of the *f1 f3* mutants was much reduced compared with that of Col, whereas there were no obvious differences in the relative root growth of *f1* or *f3* single mutants and Col (see Supplemental Figure 4A online). Again, this observation indicates that the *f1 f3* double mutants, but not the *f1* or *f3* single mutants, are hypersensitive to ABA.

To confirm our observations, we investigated the ABA responses of ethanol-inducible *F3Ri/f1* lines, which were generated by introducing the *AlcA-AlcR:FyPP3RNAi* expression cassette into the *fypp1* (*f1*) single mutant background (Dai et al., 2012a). We chose two *F3Ri/f1* lines (*F3Ri/f1-3* and *F3Ri/f1-21*) for our experiments. The results showed that after ethanol induction, the seed germination and greening percentages of both *F3Ri/f1* lines were reduced compared with Col at the tested ABA concentrations, with *F3Ri/f1-3* showing a more severe phenotype than *F3Ri/f1-21* (Figures 1D and 1E). Interestingly, there was no greening of *F3Ri/f1-3* seeds after 5 d on GM plates containing ethanol but not ABA (Figure 1E). RT-PCR analysis showed that the expression of *FyPP3* was undetectable in *F3Ri/f1-3* and dramatically reduced in *F3Ri/f1-21*, while ethanol itself had no effect on *FyPP3* expression (see Supplemental Figure 5 online), indicating that the ABA hypersensitive phenotype of the *F3Ri/f1* transgenic plants after ethanol induction is specifically due to the reduced expression of the *FyPP3* gene. We also observed that the relative root growth of *F3Ri/f1-21* on GM plates with ABA was much slower than that of Col after ethanol induction (see Supplemental Figure 4B online), further confirming that the *F3Ri/f1* plants are hypersensitive to ABA.

To further investigate the function of *FyPP1* and *FyPP3* in ABA signaling, we investigated the seed germination and greening percentages of *FyPP* overexpressing (*F1OE* and *F3OE*) and *FyPP* dominant-negative (*F1DN* and *F3DN*) transgenic lines (Dai et al., 2012a). The results showed that the *F1DN* and *F3DN* seeds had much lower germination percentages after 1, 2.5, or 5 μM ABA treatment compared with Col seeds, while the *F1OE* and *F3OE* seeds had significantly higher germination percentages with 2.5 μM ABA treatment compared with Col seeds (Figure 1F). Additionally, the greening percentages of *F1DN* and *F3DN* seedlings were much lower, while the greening percentages of *F1OE* and *F3OE* seedlings were much higher than that of Col when treated with 1 μM ABA (Figure 1G). Thus, *F1DN* and *F3DN* transgenic lines showed a similar seed germination phenotype as the seeds from self-pollinated *f1*$^{-/+}$ *f3* plants and the *F3Ri/f1* transgenic lines, while the *F1OE* and *F3OE* transgenic plants displayed the opposite seed germination phenotype as seeds from self-pollinated *f1*$^{-/+}$ *f3* plants and *F3Ri/f1* transgenic lines. Taken together, these observations suggest that *Arabidopsis* FyPP1 and FyPP3 play critical roles in ABA signaling and negatively regulate ABA responses.

The ABA-Hypersensitive Phenotype of *FyPP* Loss-of-Function Mutants Is *ABI5* Dependent

Since *ABI5* is known as a key regulator of seed germination, we next tested the genetic interactions between *FyPPs* and *ABI5*. We introduced the *f1* and *f3* mutations into the *abi5-1* mutant background (Finkelstein, 1994). We generated plants that were homozygous for *f3* and *abi5* but heterozygous for *f1* (here referred to as *f1*$^{-/+}$ *f3 abi5-1*) and obtained seeds from these self-pollinated plants in our germination assays. We observed that the seeds from self-pollinated *f1*$^{-/+}$ *f3 abi5-1* plants, self-pollinated *f1*$^{-/+}$ *f3* plants, and *abi5-1* mutants, as well as the Col controls, all germinated and grew well on GM plates lacking ABA (Figure 2A; see Supplemental Figure 6A online). Seeds from self-pollinated *f1*$^{-/+}$ *f3* plants were hypersensitive to 0.5 μM ABA as expected, whereas seeds from selfed *f1*$^{-/+}$ *f3 abi5-1* plants were insensitive to 0.5 μM ABA, similar to *abi5-1* (Figure 2A; see Supplemental Figures 6B and 6C online).

We next introduced the *f1* and *f3* mutations into transgenic plants overexpressing *ABI5* (*ABI5OE* hereafter; Brocard et al., 2002). Similarly, we generated plants homozygous for *f3* and *ABI5OE* but heterozygous for *f1* (here referred to as *f1*$^{-/+}$ *f3*

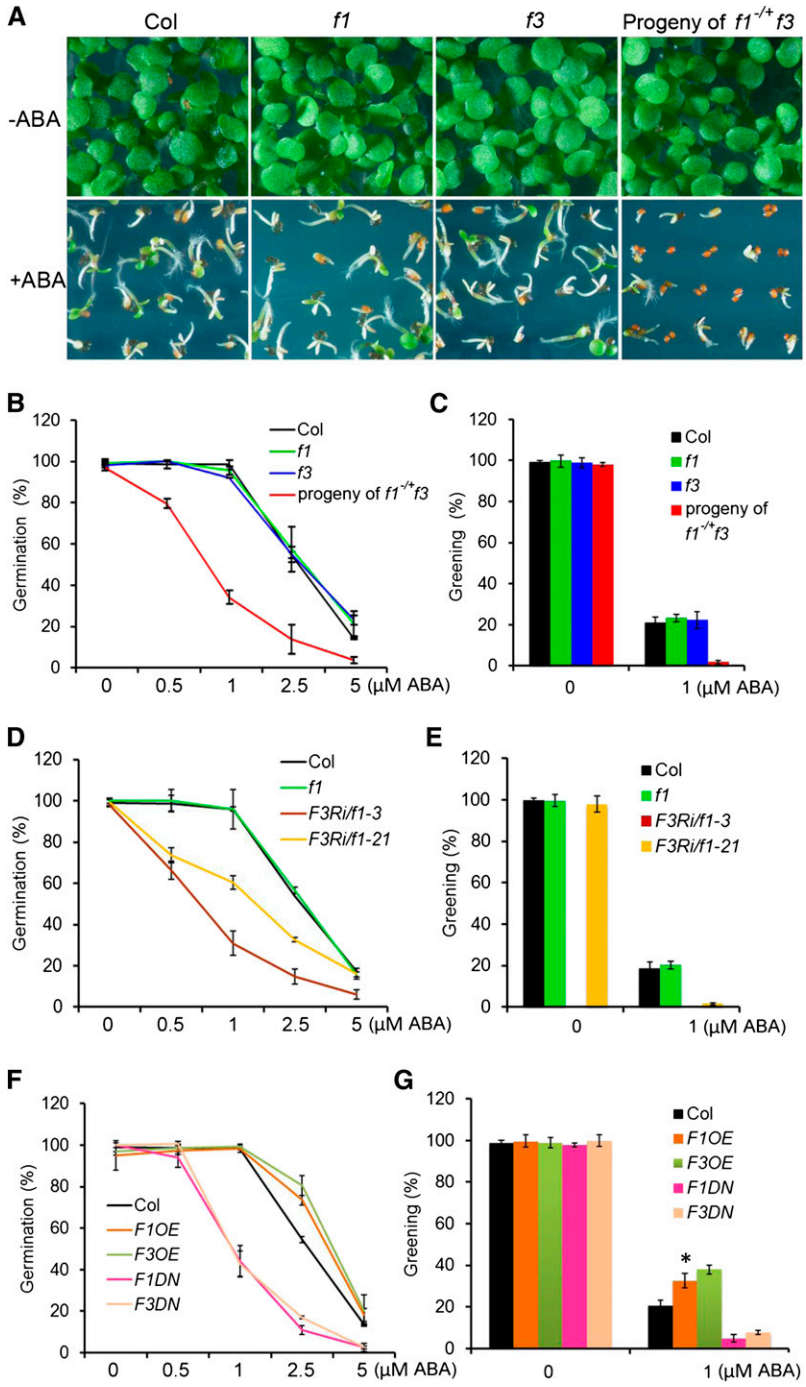

Figure 1. Phenotypic Characterization of *FyPP1* and *FyPP3* Loss- and Gain-of-Function Mutants in ABA Responses.

(A) Germination and growth of Col, *f1*, and *f3* seeds and seeds from self-pollinated *f1*$^{-/+}$*f3* plants incubated on GM plates with 0 (top panels) or 1 μM ABA (bottom panels) for 5 d. Seeds from self-pollinated *f1*$^{-/+}$*f3* plants were more sensitive to ABA than Col seeds.

(B) Germination of Col, *f1*, and *f3* seeds and seeds from self-pollinated *f1*$^{-/+}$*f3* plants incubated on GM plates with varying concentrations of ABA (0, 0.5, 1, 2.5, and 5 μM) for 5 d. Seeds from self-pollinated *f1*$^{-/+}$*f3* plants were more sensitive to ABA than Col. Germination percentages were determined from three independent experiments, with more than 100 seeds per line for each experiment. Values are means ± SD.

(C) Greening of Col, *f1*, and *f3* seeds and seeds from self-pollinated *f1*$^{-/+}$*f3* plants incubated on GM plates with 0 or 1 μM ABA for 5 d. Seeds from self-pollinated *f1*$^{-/+}$*f3* plants were more sensitive to ABA than Col seeds. Greening was determined with an average of >100 seeds from three independent experiments. Values are means ± SD.

ABI5OE) and obtained seeds from these self-pollinated plants in our experiments. Without ABA treatment, seeds from both self-pollinated *f1*$^{-/+}$ *f3* plants and *ABI5OE* plants germinated and grew well (Figure 2B). Interestingly, we observed that 24.8% of seeds (n = 250) from self-pollinated *f1*$^{-/+}$ *f3 ABI5OE* plants did not germinate after 5 d on GM plates (Figure 2B), although they eventually germinated after a longer incubation period (more than 7 d). Genotyping showed that these seeds were all *f1 f3 ABI5OE* homozygotes. After treatment with 0.5 μM ABA, the *ABI5OE* seedlings underwent growth arrest after 5 d, although their germination percentages were comparable to those of the seeds from self-pollinated *f1*$^{-/+}$*f3* plants and Col controls at this stage, while the germination percentage of the seeds from self-pollinated *f1*$^{-/+}$ *f3 ABI5OE* plants was much lower that of the *ABI5OE* seeds, and the growth of seedlings segregated from *f1*$^{-/+}$ *f3 ABI5OE* was more severely inhibited compared with the *ABI5OE* seedlings (Figure 2B). Genotyping analysis showed that after ABA treatment, all of the germinated seeds (n = 15 genotyped) from self-pollinated *f1*$^{-/+}$ *f3 ABI5OE* plants were of the *f3 ABI5* genotype, while the nongermination seeds (n = 85 genotyped) had three genotypes: *f3 ABI5OE* (20.5%), *f1*$^{-/+}$ *f3 ABI5OE* (49%), and *f1 f3 ABI5OE* (30.5%). These observations indicate that the loss of FyPP1 and FyPP3 activity enhanced the sensitivity of *ABI5OE* seeds to ABA.

We also crossed *F3OE* transgenic lines with *ABI5OE* plants. As shown in Figure 2C, without ABA treatment, Col, *F3OE*, *ABI5OE*, and *F3OE ABI5OE* double mutants germinated and grew well, while in the presence of 0.5 μM ABA, the growth of the *ABI5OE* seedlings was more severely inhibited than that of the *F3OE ABI5OE* double mutants, *F3OE* seeds, and Col wild-type controls. These results suggest that enhanced FyPP activity can largely overcome the effects of *ABI5* overexpression in response to ABA. Taken together, these data suggest that FyPP1 and FyPP3 act through the ABI5 pathway to regulate ABA responses.

FyPP1 and FyPP3 Directly Interact with ABI5

We next performed a series of experiments to test whether ABI5 could serve as a substrate of FyPP proteins. Yeast two-hybrid (Y2H) assays showed that both FyPP1 and FyPP3 specifically interacted with ABI5, but not with other key regulators of ABA signaling, including the ABA receptors (Pyr and Pyl1), the PP2C phosphatases (ABI1 and ABI2), the SnRK2 kinase (SnRK2.6/OST1), and the downstream transcription factors ABI3 and ABI4 (see Supplemental Figures 7 and 8 online). Domain deletion analysis showed that the N-terminal region of FyPP1 protein mediated the interaction between FyPP1 and ABI5 in yeast cells (Figures 3A and 3B; see Supplemental Figure 8 online). Moreover, luciferase complementation imaging (LCI) assays (Chen et al., 2008) showed that both cLUC-FyPP1 and cLUC-FyPP3 interacted with nLUC fusions of ABI5 and group I ABI5-like proteins (more similar to ABI5 protein, such as AREB3 and EEL), but not nLUC fusions of group II ABI5-like proteins (less similar to ABI5 protein, such as ABF2) (Figure 3C; see Supplemental Figure 9 online). Furthermore, coimmunoprecipitation (Co-IP) assays showed that Myc-ABI5 coimmunoprecipitated with FyPP1-HA and FyPP3-3HA, but not with green fluorescent protein (GFP)-HA in tobacco (*Nicotiana tabacum*) leaves (Figure 3D), further confirming the interaction between FyPPs and ABI5 in vivo.

To identify the subcellular localization of the interaction between FyPPs and ABI5, we performed bimolecular fluorescence complementation (BiFC) in onion (*Allium cepa*) epidermal cells. We observed strong yellow fluorescent protein (YFP) signals in the nuclei of onion cells bombarded with ABI5-YFP^N (YFP N-terminal region) and FyPP3-YFP^C (YFP C-terminal region) plasmids, but no YFP signal was detected in onion cells bombarded with the YFP^N and YFP^C control plasmids (Figure 3E). In *Arabidopsis* plants, the FyPP1 and FyPP3 proteins were detected in the nucleus, the plasma membranes, and the cytosol (see Supplemental Figure 10 online). These observations indicate that the interaction between ABI5 and FyPP3 occurs in the nucleus in planta. Taken together, these data indicate that there is a direct interaction between FyPPs and ABI5.

FyPP Directly Dephosphorylates ABI5 in Vitro

Since FyPP1 and FyPP3 directly interacted with ABI5, we next tested whether FyPP1 and FyPP3 could directly dephosphorylate ABI5. A previous study reported that the Ser119-Gln190 ABI5 fragment (ABI5b) is phosphorylated by the SnRK2.2/2.3/2.6 kinases (Nakashima et al., 2009). We therefore performed in vitro phosphorylation experiments using glutathione *S*-transferase (GST)–tagged ABI5b purified from *Escherichia coli* cells as the substrate and protein extracts prepared from Col, *f1 f3*, *F1DN*,

Figure 1. (continued).

(D) Germination of Col, *f1*, and *F3Ri/f1* seeds incubated on GM plates with varying concentrations of ABA (0, 0.5, 1, 2.5, and 5 μM) for 5 d. All GM plates contained 0.1% ethanol. Ethanol-induced *F3Ri/f1* seeds were more sensitive to ABA than Col or *f1* seeds. Germination percentage was determined from three independent experiments, with more than 150 seeds per line for each experiment. Values are means ± SD.

(E) Greening of Col, *f1*, and *F3Ri/f1* seeds incubated on GM plates (plus 0.1% ethanol) with 0 or 1 μM ABA for 5 d. Ethanol-induced *F3Ri/f1* seeds were more sensitive to ABA than Col seeds or *f1* seeds. Greening was determined with an average of >150 seeds from three independent experiments. Values are means ± SD.

(F) Germination of Col, *F1DN*, *F3DN*, *F1OE*, and *F3OE* seeds incubated on GM plates with varying concentrations of ABA (0, 0.5, 1, 2.5, and 5 μM) for 5 d. *F1DN* and *F3DN* seeds were more sensitive to ABA, while *F1OE* and *F3OE* seeds were less sensitive to ABA than Col. Germination percentages were determined from three independent experiments, with more than 100 seeds per line for each experiment. Values are means ± SD.

(G) Greening of Col, *F1OE*, *F3OE*, *F1DN*, and *F3DN* seeds incubated on GM plates with 0 or 1 μM ABA for 5 d. *F1DN* and *F3DN* seeds were more sensitive to ABA, while F1OE and F3OE were less sensitive to ABA than Col seeds. Greening was determined with an average of >100 seeds from three independent experiments. Values are means ± SD. Asterisks indicate the levels of statistical significance as determined by Student's *t* test: *P < 0.01 versus Col.

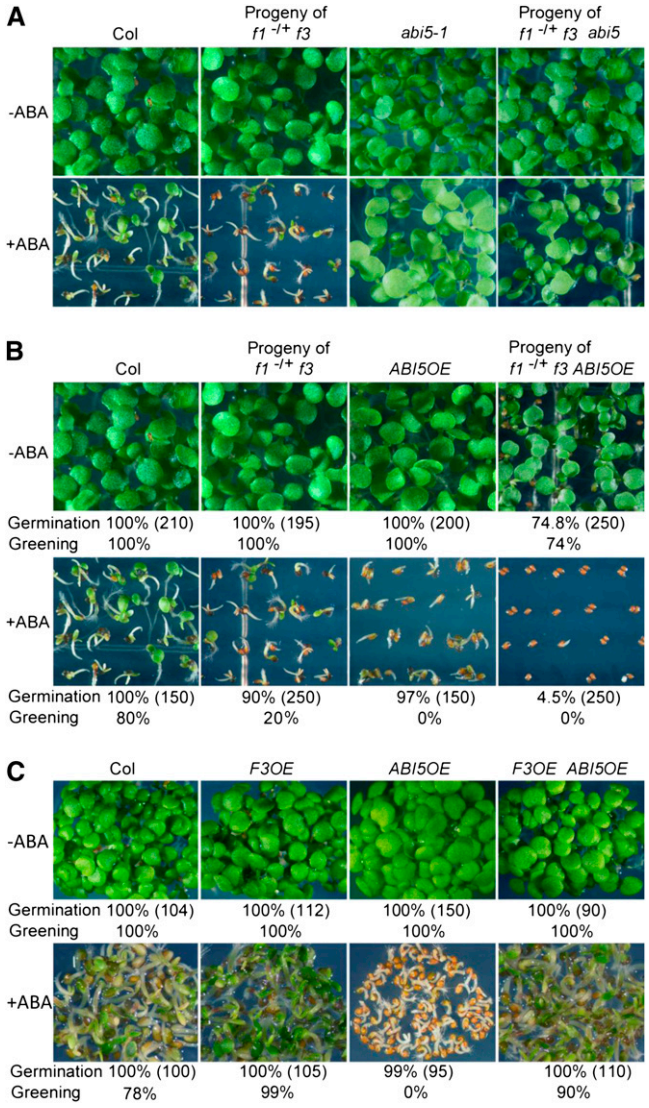

Figure 2. Genetic Interaction between *ABI5* and *FyPPs*.

(A) Germination and growth of seeds of Col, *abi5*, and self-pollinated *f1*$^{-/+}$ *f3* and *f1*$^{-/+}$ *f3 abi5* plants incubated on GM plates with 0 or 0.5 μM ABA for 5 d in white light. Seeds from self-pollinated *f1*$^{-/+}$*f3* plants showed increased sensitivity to ABA, while *abi5-1* seeds were insensitive to ABA compared with Col controls. Seeds from self-pollinated *f1*$^{-/+}$ *f3 abi5-1* plants were insensitive to ABA, similar to *abi5-1* seeds.

(B) Loss of FyPP1 and FyPP3 activity enhances the ABA-related phenotypes of *ABI5OE* seeds. Top panel shows seedlings grown on GM plates without ABA. The ungerminated seeds (25.2%) from self-pollinated *f1*$^{-/+}$ *f3 ABI5OE* plants were further phenotyped and genotyped and confirmed to be *f1 f3 ABI5OE* homozygotes. Bottom panel shows the seedlings grown on GM plates containing 0.5 μM ABA. The germination and greening percentages of each line are shown at the bottom of each panel, and the number of seeds used for the calculation is shown in parentheses.

(C) Growth of Col, *F3OE*, *ABI5OE*, and *F3OE ABI5OE* seeds on GM plates with 0 or 0.5 μM ABA. Overexpression of *FyPP3* rescued the ABA hypersensitivity phenotype of *ABI5OE* seeds. The germination and greening percentages of each line are shown at the bottom of each panel, and the number of seeds used for the calculation is shown in parentheses.

F3DN, *F1OE*, and *F3OE* seedlings treated with ABA or with control solvent as the kinase sources. Equal amounts of protein extracts and substrates were coincubated in these assays. No phosphorylated GST-ABI5b was detected in any sample without ABA treatment (Figure 4A), indicating that phosphorylation of ABI5 is ABA dependent, which is consistent with previous reports (Fujii et al., 2007; Nakashima et al., 2009). However, after ABA treatment, the amounts of phosphorylated GST-ABI5b were higher in samples incubated with protein extracts from *f1 f3*, *F1DN*, and *F3DN* transgenic seedlings, while the amounts of phosphorylated GST-ABI5b were slightly lower in samples incubated with protein extracts from *F1OE* and *F3OE* seedlings, compared with Col (Figure 4A). These data suggested that there is a correlation between FyPP1/3 activity and phosphorylation of GST-ABI5b.

To confirm this observation, we performed in vitro phosphorylation/dephosphorylation assays. We incubated equal amounts of GST-ABI5b proteins and protein extracts from ABA-treated Col or *f1 f3* seedlings. We added various amounts of purified wild-type PP6 phosphatase (GST-FyPP3) or PP6 null mutant (GST-FyPP3^{D81N}; Dai et al., 2012a) to the samples mixed with protein extracts from *f1 f3* seedlings and GST-ABI5b proteins. The results showed that the addition of exogenous GST-FyPP3, but not GST-FyPP3D81N, reduced the amounts of phosphorylated GST-ABI5b, and the more GST-FyPP3 protein added, the less phosphorylated GST-ABI5b detected (Figure 4B). Additionally, when we used mutant GST-ABI5b (ABI5b^{S145A} or ABI5bm) as the substrate, we did not observe a phosphorylation band by the *f1 f3* mutant extracts (Figure 4B). These data together suggest that FyPP1/3 proteins play a critical role in regulating the phosphorylation status of ABI5.

It was reported that the Ser/Thr sites in the conserved (Leu)xArgxxSer/Thr motif of ABI5 are the targets of SnRK2 kinases, including SnRK2.2, 2.3, and 2.6 (Furihata et al., 2006; Nakashima et al., 2009). To test whether these Ser/Thr sites mediate the interaction between ABI5 and FyPPs, we conducted site-directed mutagenesis and substituted these Ser/Thr sites with either Ala or Asp residues, thus generating the dephosphorylation mimic mutant (ABI5A4) or phosphorylation mimic mutant (ABI5D4) forms of ABI5 (Figure 4C). Y2H assays showed a stronger interaction between FyPP1 (F1NT) and ABI5D4 than the interaction between FyPP1 (F1NT) and wild-type ABI5 (Figure 4D; see Supplemental Figure 8 online). This observation suggests that phosphorylation of these Ser/Thr sites in the conserved (Leu)xArgxxSer/Thr motifs plays an important role in mediating the interaction between ABI5 and FyPP.

FyPP Regulates ABI5 Protein Stability

Phosphorylation plays a critical role in regulating protein stability (Hardtke et al., 2000; Karin and Ben-Neriah, 2000; Deshaies and Ferrell, 2001; Joo et al., 2008). We next tested whether FyPP1 and FyPP3 may also be involved in regulating ABI5 protein stability. We germinated Col, *F1DN*, *F3DN*, *F1OE*, and *F3OE* seeds and seeds from self-pollinated *f1*$^{-/+}$*f3* plants on GM plates containing 0 or 1 μM ABA. After varying incubation time (0, 1, 2, 4, and 6 d) under continuous white light, we extracted their total RNAs and proteins. We used the anti-ABI5–specific

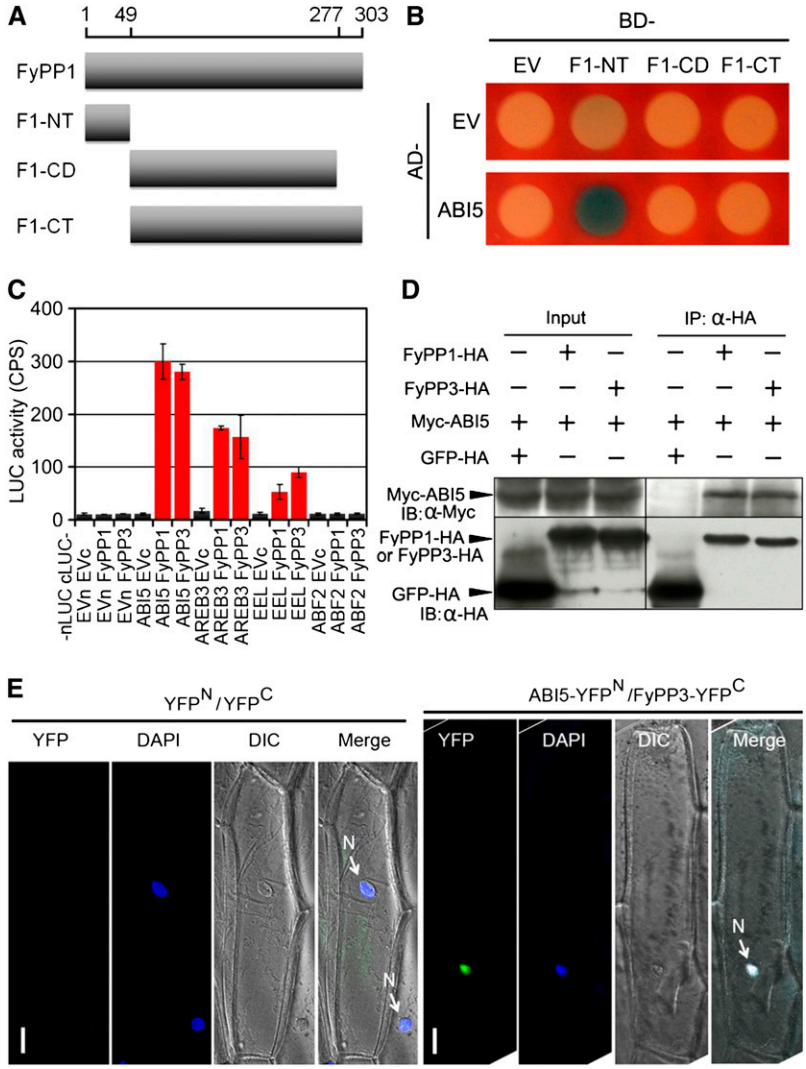

Figure 3. Protein–Protein Interactions between FyPPs and ABI5.

(A) Diagram of the FyPP1 protein constructs used in Y2H assays. F1-NT, FyPP1 N-terminal region (amino acids 1 to 49); F1-CD, FyPP1 catalytic domain (amino acids 50 to 277); F1-CT, FyPP1 C-terminal region (amino acids 50 to 303).

(B) ABI5 protein interacted with the F1-NT domain of FyPP1 protein in yeast cells. AD, B42 activation domain; BD, LexA DNA binding domain; EV, empty vector control.

(C) LCI assays showing that when fused with nLUC, ABI5 and group I ABI5-like proteins (AREB3 and EEL), but not group II protein (ABF2), interacted with both cLUC-FyPP1 and cLUC-FyPP3 in plant cells. Values are means ± SD, $n = 3$. EVc, cLUC empty vector; EVn, nLUC empty vector.

(D) Co-IP of ABI5 and FyPP1 or FyPP3 in plant cells. α-HA affinity matrix was used for immunoprecipitation (IP); α-HA and α-Myc antibodies were used for immunoblotting (IB). Input, total protein before immunoprecipitation.

(E) BiFC assays showing that AB15 and FyPP3 interacted in the nucleus. ABI5-YFP^N and FyPP3-YFP^C fusion proteins were expressed in onion epidermal cells through cobombardment. No YFP signal was observed in onion cells cobombarded with the YFP^N (YFP protein N-terminal) and YFP^C (YFP protein C-terminal) control plasmids. The nuclei were stained by DAPI (4',6-diamidino-2-phenylindole, blue). Bars = 50 μM.

antibodies in the immunoblot analysis. Stone et al. (2006) detected 52.5-, 51-, and 50-kD ABI5 bands with the 52.5- and 50-kD bands predominating in immunoblot assays using this α-ABI5 antibody, whereas we detected two bands in this study and in the previous report (Figures 5A and 5B; Lee et al., 2010). The results showed that in the presence of ABA, ABI5 proteins were hyperaccumulated in the *F1DN* and *F3DN* seeds and seeds from self-pollinated *f1*$^{-/+}$ *f3* plants, but reduced in the *F1OE* and *F3OE* seeds, compared with Col (Figure 5A). Consistent with previous reports, ABI5 proteins were quickly degraded in germinated seeds due to diminished ABA levels (Figure 5A; Piskurewicz et al., 2008). Quantitative RT-PCR analysis showed that *ABI5* expression was highly induced in all lines in response to ABA compared with the Col controls (see Supplemental Figure 11A online). These results suggest that the differential accumulation of ABI5 proteins in these plants is most

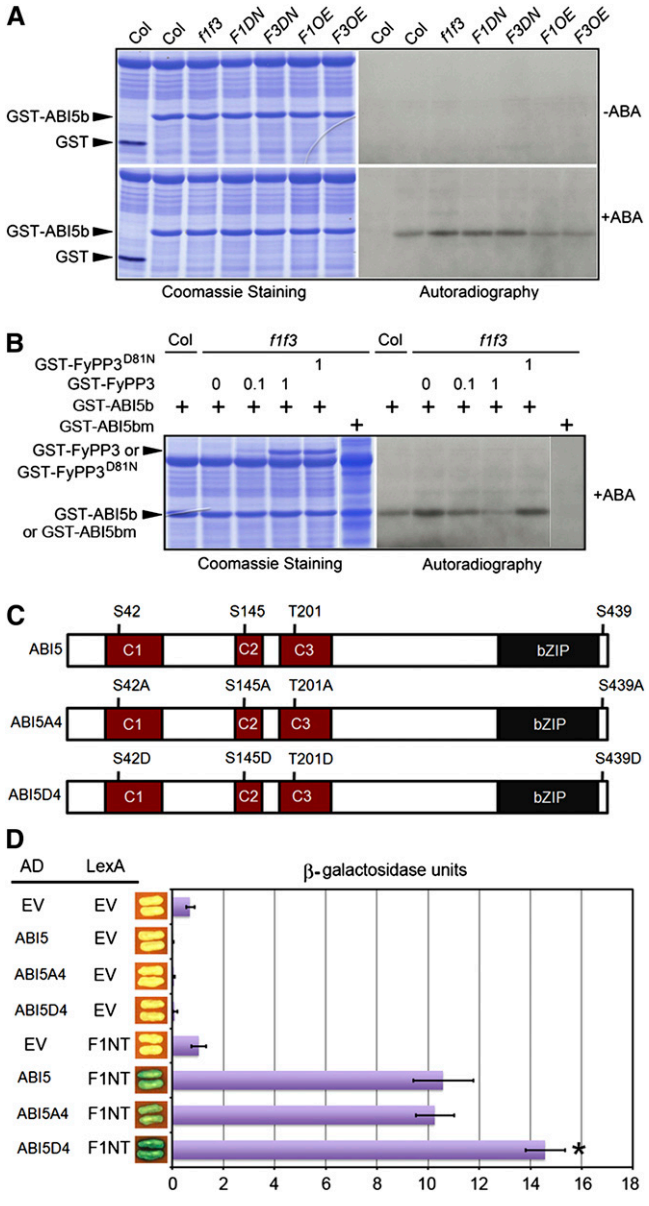

Figure 4. FyPP Directly Dephosphorylates ABI5.

(A) In vitro kinase assay of GST-ABI5b (Ser119-Gln190). In the absence of ABA, there was no detectable phosphorylated GST-ABI5b when treated with the plant extracts derived from Col, *f1 f3*, *F1DN*, *F3DN*, *F1OE*, and *F3OE* seedlings. After treatment with ABA, there were increased amounts of phosphorylated GST-ABI5b when incubated with the plant extracts derived from *f1 f3*, *F1DN*, and *F3DN* seedlings, in contrast with the reduced abundance of phosphorylated GST-ABI5b when incubated with the plant extracts derived from *F1OE* and *F3OE* seedlings.

(B) In vitro dephosphorylation of GST-ABI5b by GST-FyPP3. GST-FyPP3 reversed the ABA-dependent dephosphorylation of GST-ABI5b treated with the plant extracts derived from f1 f3 seedlings. Increasing amounts of GST-FyPP3 decreased the amount of phosphorylated GST-ABI5b, while the inactive phosphatase (GST-FyPP3D81N) had no effect on the phosphorylation status of GST-ABI5b. A mutant form of ABI5 (GST-ABI5bS145A) was used as a negative control in the experiment. The amounts of GST-FyPP3 and GST-FyPP3D^{81N} proteins used in the assay are indicated by the numbers (0, 0.1, and 1 μg).

(C) Schematic representation of the domain structure of the dephosphorylation mimic mutant ABI5A4 and the phosphorylation mimic mutant ABI5D4 used for the Y2H assays. The labeled Ser (S) and Thr (T) residues were mutated to Ala (A) or Asp (D), respectively.

(D) Y2H assays between FyPP1 N-terminal region (F1NT) and various ABI5 mutants shown in **(A)**. The phosphorylation mimic mutant ABI5D4 showed enhanced interaction with F1NT. Values are means ± SD; $n = 3$. Asterisks indicate the levels of statistical significance as determined by Student's t test: *P < 0.02 versus F1NT–ABI5 interaction. EV, empty vector control.

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likely due to posttranscriptional regulation. Consistent with this, seeds from self-pollinated *f1*$^{-/+}$ *f3* plants and *F1DN* and *F3DN* mutants had lower germination rates after ABA treatment, whereas *F1OE* and *F3OE* seeds had higher germination rates, compared with Col (see Supplemental Figures 2 and 12 online).

It was reported that ABI5 is highly induced and stabilized by ABA but is degraded rapidly after the removal of ABA (Lopez-Molina et al., 2001). To investigate how FyPPs function in this process, we treated Col, *f1*, *F3Ri/f1-21*, *F1DN*, *F3DN*, *F1OE*, and *F3OE* seeds with 5 μM ABA for 3 d and then removed the ABA and grew the seeds on GM plates. We harvested samples after various incubation time points (0, 8, 12, 16, and 20 h) for protein and RNA extraction. Immunoblot analysis showed that after the removal of ABA, ABI5 was more abundant in the *FyPP* loss-of-function mutants, such as *F1DN*, *F3DN*, and *F3Ri/f1-21* seeds, after ethanol induction, but this protein was more rapidly degraded in the *F1OE* and *F3OE* seeds compared with Col (Figure 5B). Gene expression assays showed that *ABI5* mRNA was inhibited to comparable levels in all seeds after removal of ABA (see Supplemental Figures 11B to 11E online). Together, these observations support the notion that FyPPs are essential for post-transcriptional regulation of ABI5 in response to ABA removal.

We next investigated the effect of ABA on the expression of ABI5-regulated ABA-responsive genes in the *FyPP* loss-of-function mutants. Two RD genes, *RD29A* and *RD29B*, have ABREs in their promoters and are transactivated by ABI5 (Nakashima et al., 2006). Finkelstein et al. (2005) also showed dramatically reduced ABA inducibility of *RD29B* in *abi5* seedlings. Our quantitative RT-PCR assays showed that the expression of both *RD29A* and *RD29B* was upregulated in Col, *f1 f3*, *F1DN*, and *F3DN* mutants after ABA treatment, with a hyperinduction of *RD29A* and *RD29B* in the *FyPP* loss-of-function mutants (Figure 5C). These observations suggest that the ABA hypersensitivity of *FyPP* loss-of-function mutants was due to the hyperaccumulation of ABI5 proteins, resulting in upregulation of downstream ABA-responsive gene expression.

FyPP1 and FyPP3 Function Antagonistically with SnRK2 in Mediating ABA Signaling

'Because FyPP/PP6 phosphatases and SnRK2 kinases show antagonistic effects on ABA signaling, we were interested in understanding the genetic interactions between SnRK2 and FyPPs.

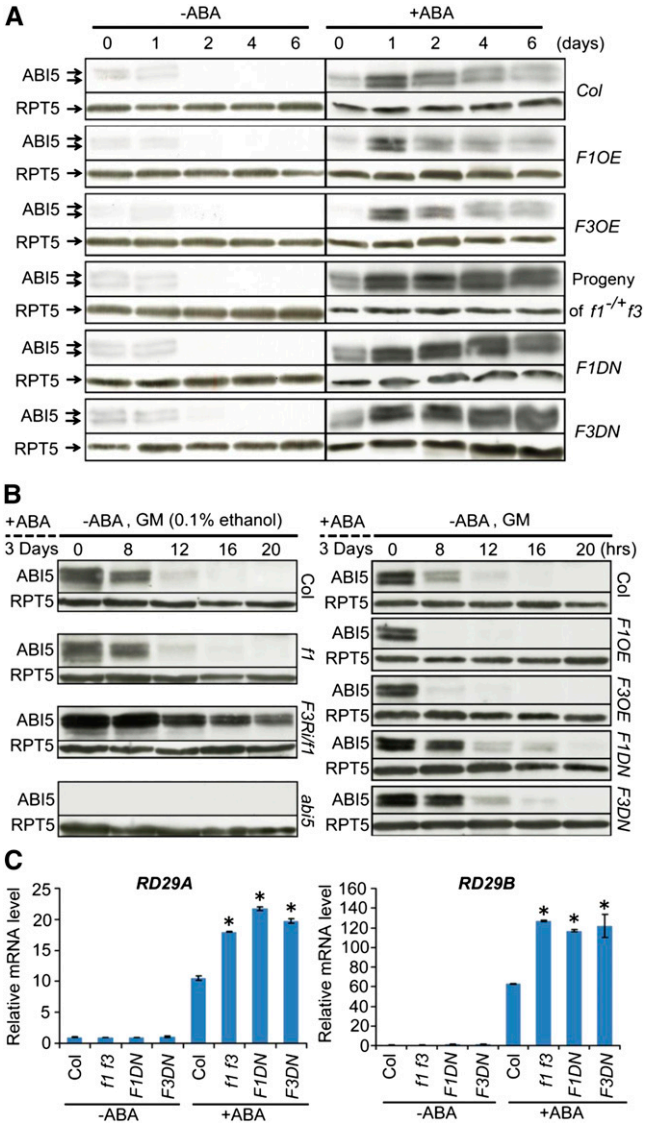

Figure 5. Role of FyPP1 and FyPP3 in the Regulation of ABI5 Protein Stability.

(A) Immunoblot assays showing that more ABI5 protein accumulated in seeds of self-pollinated *f1*$^{-/+}$ *f3*, *F1DN*, and *F3DN* lines but less accumulated in *F1OE* and *F3OE* seeds compared with Col seeds treated with ABA (1 μM) for various durations (0, 1, 2, 4, and 6 d). A total of 100 μg protein was loaded for each lane. All immunoblot assays were performed side-by-side under identical conditions. Arrows indicate the ABI5 protein bands. RPT5 was used as a loading control.

(B) Immunoblot assays showing that after ABA removal, ABI5 protein was more stable in *F3Ri/f1*, *F1DN*, and *F3DN* lines but more rapidly degraded in *F1OE* and *F3OE* seeds compared with Col seeds. The seeds were treated with ABA (5 μM) in white light for 3 d and then harvested at different time points after removal of the ABA. The medium used for each line is indicated on the top of each panel. *abi5-1* mutant seeds were used as a negative control in the experiment. A total of 100 μg protein was loaded for each lane. All immunoblot assays were performed side-by-side under identical conditions. RPT5 was used as a loading control.

(C) Quantitative RT-PCR assay showing that the expression of *RD29A* and *RD29B* was hyperinduced in *f1 f3*, *F1DN*, and *F3DN* seedlings treated with ABA compared with ABA-treated Col. Five-day-old seedlings were incubated in 1× Murashige and Skoog liquid medium with ABA (100 μM) or control solvent (DMSO) for 1.5 h before harvest. Values are means ± SD; $n = 3$. Asterisks indicate the levels of statistical significance as determined by Student's *t* test: *P < 0.01 versus Col.

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OST1 (SnRK2.6) plays a critical role in mediating various ABA responses, and its loss-of-function mutant (*ost1* or *snrk2.6*) displays a hypersensitive phenotype in response to drought stress and resistance to ABA during seed germination (Li et al., 2000; Mustilli et al., 2002; Yoshida et al., 2006; Zheng et al., 2010). Additionally, the single mutants of *snrk2.2* and *snrk2.3* do not display obvious ABA-related phenotypes, whereas *ost1* is more resistant to ABA during seed germination than the wild type (Fujii et al., 2007; Zheng et al., 2010). We therefore introduced the *ost1* mutation into *f1*$^{-/+}$ *f3* and *F3DN* mutant backgrounds. We generated plants homozygous for *f3* and *ost1* but heterozygous for *f1* (here referred to as *f1*$^{-/+}$ *f3 ost1*) and obtained seeds from these self-pollinated plants for our germination/growth assays. Our results showed that seeds from self-pollinated *f1*$^{-/+}$ *f3* plants and *F3DN* mutants were sensitive to ABA as expected, while seeds from self-pollinated *f1*$^{-/+}$ *f3 ost1* plants or the *F3DN ost1* double mutants were more tolerant to ABA than their *f1*$^{-/+}$ *f3* or *F3DN* parental lines, respectively (Figures 6A and 6B). On the other hand, compared with the *ost1* mutants, seeds from self-pollinated *f1*$^{-/+}$ *f3 ost1* plants and the *F3DN ost1* double mutants were still sensitive to ABA (Figures 6A to 6C). Consistent with this observation, the ABI5 protein levels in ABA-treated seeds of self-pollinated *f1*$^{-/+}$ *f3 ost1* plants and *F3DN ost1* double mutants were reduced compared with ABA-treated *f1*$^{-/+}$ *f3* or *F3DN* parental lines, respectively (Figure 6D). These results suggest that *ost1* attenuates the ABA hypersensitivity of seeds from self-pollinated *f1*$^{-/+}$*f3* and *F3DN* and that FyPPs function antagonistically with OST1/SnRK2 kinases in mediating ABA signaling.

ABA Promotes the Degradation of FyPP Proteins in Seedlings

Since *FyPP* loss-of-function mutants are hypersensitive to ABA, we were interested in understanding how ABA regulates FyPP1 and FyPP3 protein stability. We treated 4-d-old *F1OE* and *F3OE* seedlings with the protein synthesis inhibitor cycloheximide for 15 h and then added ABA or the control solvent DMSO. We harvested the samples after different incubation time (0, 1, 2, 5, 8, and 24 h) and performed immunoblot assays. The results showed that the YFP-FyPP1 protein levels in *F1OE* seedlings and the YFP-FyPP3 protein levels in *F3OE* seedlings decreased after treatment with ABA, but not after the addition of DMSO (Figures 7A to 7D). These observations suggest that ABA promotes degradation of FyPP proteins.

DISCUSSION

In this study, we characterized the function of *FyPP1* and *FyPP3*, two homologous genes encoding the catalytic subunits of PP6 in ABA signaling. We showed that the *FyPP* dominant-negative and *f1 f3* double mutants are ABA hypersensitive.

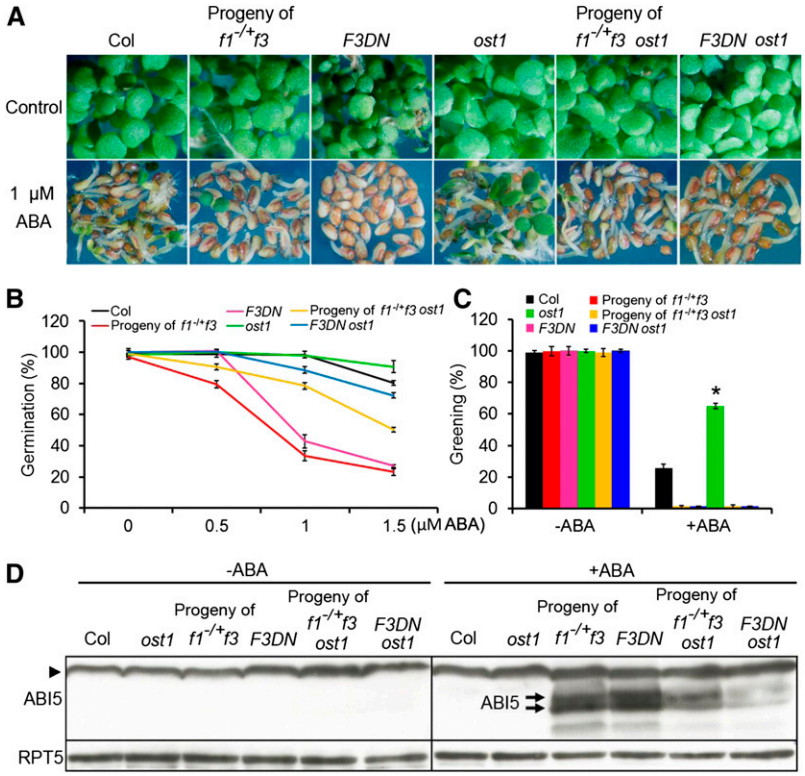

Figure 6. Antagonistic Relationship between *OST1* (*SnRK2.6*) and *FyPPs*.

(A) Germination and growth of Col, *ost1*, *F3DN*, and *ost1 F3DN* seeds and seeds from self-pollinated *f1*$^{-/+}$ *f3* and *f1*$^{-/+}$ *f3 ost1* plants incubated on GM plates with or without ABA. Seeds were incubated under white light for 5 d.

(B) Germination of Col, *ost1*, *F3DN*, and *ost1 F3DN* seeds and seeds from self-pollinated *f1*$^{-/+}$ *f3* and *f1*$^{-/+}$ *f3 ost1* plants. Seeds were grown under white light for 5 d on GM plates with the indicated concentrations of ABA. Germination was determined with an average of >100 seeds from three independent experiments. Values are means ± SD.

(C) Greening of Col, *ost1*, *F3DN*, and *ost1 F3DN* seeds and seeds from self-pollinated *f1*$^{-/+}$ *f3* and *f1*$^{-/+}$ *f3 ost1* plants incubated on GM plates with (1 μM) or without ABA treatment for 5 d. Greening was determined with an average of >100 seeds from three independent experiments. Values are means ± SD. Asterisks indicate the levels of statistical significance as determined by Student's *t* test: *P < 0.01 versus Col.

(D) Immunoblot analysis of ABI5 accumulation in kinase- and phosphatase-deficient mutants. In the absence of ABA, there was no detectable ABI5 protein in Col, *ost1*, *f1*$^{-/+}$ *f3*, *F3DN*, *f1*$^{-/+}$ *f3 ost1*, and *F3DN ost1* seedlings. After ABA treatment (1 μM, 8 d), ABI5 (indicated by the arrows) hyperaccumulated in seeds of self-pollinated *f1*$^{-/+}$ *f3* or *F3DN* lines, in contrast with the reduced accumulation of ABI5 in seeds of self-pollinated *f1*$^{-/+}$ *f3 ost1* plants and *F3DN ost1* lines. There was no detectable ABI5 protein in Col and *ost1* seedlings at this stage. The arrowhead indicates the nonspecific band recognized by the ABI5 antibody. Arrows indicate the ABI5 bands. RPT5 was used as a loading control. A total of 100 μg protein was loaded for each lane.

FyPP1 and FyPP3 protein levels are downregulated by ABA. We also showed that FyPP1 and FyPP3 interact with ABI5 in yeast and in planta and directly dephosphorylate ABI5 in vitro. Furthermore, we demonstrated that the ABA-hypersensitive phenotype of the *f1 f3* mutants is ABI5 dependent, and FyPPs function antagonistically with SnRK2 in regulating ABI5 phosphorylation and ABA signaling.

FyPP1 and FyPP3 Function as Negative Regulators of ABA Signaling

In this study, we collected several lines of evidence supporting the hypothesis that FyPP1 and FyPP3 play a negative role in regulating ABA signaling. First, the *f1 f3* double mutants, *FyPP* dominant-negative mutants, and ethanol-induced *F3Ri/f1* lines all showed a hypersensitive response to ABA during seed germination and root growth. The ABA-hypersensitive phenotype of these *FyPP* loss-of-function mutants closely resembles other loss-of-function mutants of negative regulators of ABA signaling, such as ABI1, ABI2, PP2Ac2, KEG, DWA1, and DWA2 (Sheen, 1998; Gosti et al., 1999; Merlot et al., 2001; Stone et al., 2006; Pernas et al., 2007; Lee et al., 2010; Liu and Stone, 2010). Second, two representative ABA-responsive genes, *RD29A* and *RD29B*, are hyperinduced in the *f1 f3* double mutants and *FyPP* dominant-negative mutants after ABA treatment. Third, the *F1OE* and *F3OE* lines showed increased tolerance to ABA during germination, which is opposite to the ABA response of *FyPP* loss-of-function mutants. Notably, the levels of FyPP1 and FyPP3 proteins are downregulated by ABA, suggesting a possible feedback loop between ABA signaling and FyPP stability/activity.

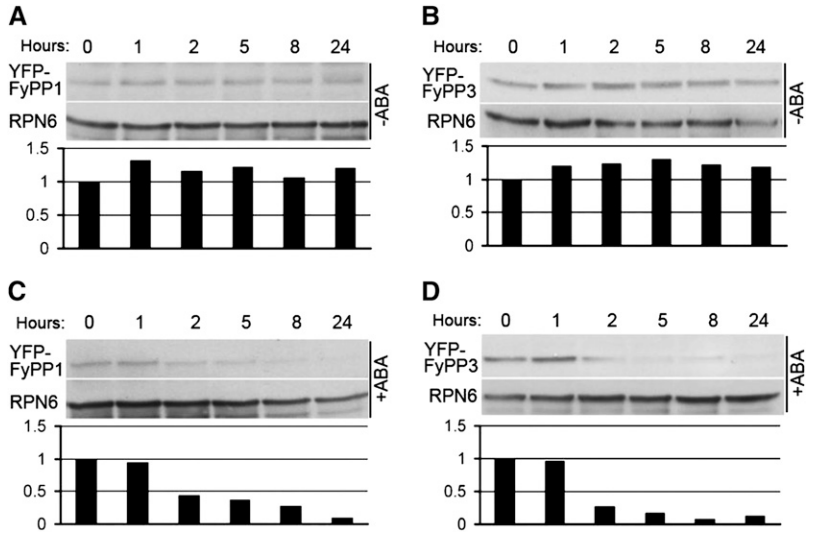

Figure 7. Degradation of FyPP1 and FyPP3 Protein upon ABA Treatment.

(A) YFP-FyPP1 protein levels in seedlings treated with control solvent (DMSO) for different durations (0, 1, 2, 5, 8, and 24 h).
(B) YFP-FyPP3 protein levels in seedlings treated with control solvent (DMSO) for different durations (0, 1, 2, 5, 8, and 24 h).
(C) YFP-FyPP1 protein levels in seedlings treated with ABA for different durations (0, 1, 2, 5, 8, and 24 h).
(D) YFP-FyPP3 protein levels in seedlings treated with ABA for different durations (0, 1, 2, 5, 8, and 24 h).
The relative amounts of YFP-FyPP proteins are shown below the immunoblot assays; 100 μg total protein was loaded for each lane.

The PP2A and PP2A-like PPPs, such as PP4 and PP6, are major Ser/Thr phosphatases in most eukaryotic cells. The in vivo activities of these PPPs are regulated by a set of regulatory subunits. In a previous study, we showed that FyPP1 or FyPP3 physically interacts with SAL (SAP domain proteins) and PP2AA proteins (RCN1 or PP2AA1, PP2AA2, and PP2AA3) to form a PP6-type heterotrimeric holoenzyme complex that directly interacts with and dephosphorylates a subset of PIN proteins to regulate polar auxin transport and plant development. We demonstrated that *FyPPs*, *SAL*, and *PP2A As* have comparable roles in regulating auxin transport and plant development. In addition, there is a synergistic interaction between FyPP1 (or 3) (catalytic subunit), RCN1 (PP2AA1), and SAL1 in the regulation of plant development (Dai et al., 2012a). Intriguingly, RCN1 was previously reported as a positive regulator of ABA signaling, as *rcn1* mutants show reduced ABA sensitivity in seed germination and ABA-induced gene expression (Kwak et al., 2002). The ABA-insensitive phenotype of *rcn1* is similar to that of *FyPP* overexpression lines but opposite to that of the *FyPP* loss-of-function mutants. Notably, another recent study reported that overexpression of *PP2Ac2* causes an ABA-insensitive phenotype, while the *pp2ac2* loss-of-function mutant shows an ABA-hypersensitive phenotype (Pernas et al., 2007), suggesting that PP2Ac also plays a negative regulatory role in ABA signaling. As we previously demonstrated, RCN1 and FyPPs can assemble into functional PP6 heterotrimeric holoenzyme complexes (Dai et al., 2012a). It is not completely clear now why the loss-of-function mutants of PP2AA (such as RCN1) and FyPP1/FyPP3 display opposite ABA phenotypes. At least two possible scenarios can be envisaged. (1) PP2AA proteins (PP2AA1 or RCN1, PP2AA2, and PP2AA3) are promiscuous and can function as the regulatory subunits for both PP2A and PP6 phosphatase holoenzymes (Dai et al., 2012a), and the loss-of-function phenotype of RCN1 might be due to the combined effects on PP2A and PP6 activities, rather than simply reflecting the effect on PP6 alone. (2) It is possible that the A regulatory subunits (such as RCN1) may exert different regulatory effects (activation or inhibition) on the phosphatase activity toward different substrates or different developmental tissues or signaling pathways. For example, it has been shown that α4 is a common regulatory subunit of PP2A, PP4, and PP6 in mammals. Binding of α4 to PP2Ac inhibits its activity on phosphorylated 4E-BP1 (Nanahoshi et al., 1998), while addition of α4 to PP2Ac promotes PP2A activity when using myelin basic protein as the substrate (Murata et al., 1997). This variation in α4 effects on PP2Ac activity also occurs in different cell lines. For example, overexpression of α4 in COS-1 cells results in increased activities of cellular PP2A, whereas in COS7 cells, α4 overexpression has differential effects on the phosphorylation of endogenous phosphoproteins (Prickett and Brautigan, 2006; Nien et a., 2007). In addition, when using myelin basic protein as the substrate, the activity of PP6 bound to α4 is severely reduced in contrast with the increased activity of PP2A bound to α4, suggesting that a single regulatory subunit can recognize two kinetically identical catalytic subunits to induce different allosteric effects that alter enzyme activity (Prickett and Brautigan, 2006). Characterization of other regulatory subunits of PP2A and PP6 (such as the SAL proteins) and their genetic interactions with the catalytic subunits will help clarify these issues.

FyPP1 and FyPP3 Regulate ABI5 Phosphorylation and Stability

Phosphorylation and degradation are two important posttranslational modifications of proteins. The posttranslational nature of both processes ensures a rapid response to exogenous and endogenous

cues without going through the more time-consuming transcriptional regulation. Such a mechanism is widely used in plants in response to many stresses and environmental signals. In previously characterized examples, phosphorylation of a substrate in response to a specific signal mostly serves as a recognition tag for an E3-ubiquitin ligase, which facilitates degradation of the marked protein (Hardtke et al., 2000; Karin and Ben-Neriah, 2000; Deshaies and Ferrell, 2001). For example, a group of basic helix-loop-helix transcription factors called phytochrome-interacting factors (PIFs) promote etiolation and repress photomorphogenesis in darkness. Photoactivation of phytochrome induces intranuclear phosphorylation of the PIFs and their subsequent proteasome-mediated degradation. These data indicate that phytochrome-induced phosphorylation of target proteins may represent the primary intermolecular signaling action of the activated photoreceptor. The resultant proteolysis of the PIFs triggers the transition from skotomorphogenic to photomorphogenic development (deetiolation), upon initial exposure of seedlings to light, by directly altering the expression of PIF target genes (J. Li et al., 2011). There are also cases in which phosphorylation protects the protein from proteasome-mediated degradation while dephosphorylation promotes protein degradation. For example, phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6 leads to the accumulation of 1-aminocyclopropane-1-carboxylic acid synthase proteins and the induction of ethylene synthesis (Joo et al., 2008). Similarly, it was reported that PP2A dephosphorylates BRI1, leading to decreased BRI1 abundance and decreased brassinosteroid signaling (Wu et al., 2011).

ABI5 is known to play a key role in regulating seed germination and seedling growth. In dry seeds, high levels of ABA promote SnRK2-dependent phosphorylation and stabilization of ABI5 proteins (Fujii et al., 2007; Nakashima et al., 2009). However, during seed germination, ABI5 proteins are rapidly degraded due to the diminished ABA content (Piskurewicz et al., 2008). Furthermore, the ABA-induced inhibition of seed germination and the severity of ABA-triggered postgermination growth arrest are correlated with increased ABI5 protein levels (Lopez-Molina et al., 2001). These studies indicate that phosphorylation stabilizes ABI5 and that the abundance of ABI5 protein plays an important role in regulating seed germination and postgermination growth. As phosphorylated ABI5 proteins are stable and functional, dephosphorylation of ABI5 should be an essential prerequisite for the degradation of ABI5 and the initiation of seed germination. Our results provide substantial evidence that FyPP1 and FyPP3 plays a key role in promoting dephosphorylation and subsequent degradation of ABI5, allowing seed germination and postgermination growth to occur. First, our genetic interaction studies showed that the ABA hypersensitivity of *f1 f3* double mutants during seed germination was dependent on *ABI5* function, and overexpression of *FyPP3* in the *ABI5OE* background largely rescued the ABA-related phenotypes of *ABI5OE* seeds (Figure 2). Second, we showed that after ABA treatment, the *f1 f3*, *F1DN*, and *F3DN* mutants accumulated higher levels of ABI5 protein (Figure 5A). This observation suggests that the activity of FyPPs is necessary for maintaining proper ABI5 levels in response to ABA. Third, we showed that after removal of ABA, the ABI5 protein is more stable in *FyPP* loss-of-function mutants, but it is more quickly degraded in the *FyPP* overexpression lines (Figure 5B), further suggesting that FyPP activities play a critical role in regulating ABI5 stability and ABA signaling. Fourth, both FyPP1 and FyPP3 interacted with ABI5 in yeast and plant cells, and these interactions happened in the nucleus (Figure 3; see Supplemental Figure 10 online). Furthermore, phosphorylated ABI5 interacted better than nonphosphorylated ABI5 with FyPP1 protein in yeast cells, indicating the substrate specificity of FyPP1 and FyPP3 toward ABI5 (Figures 4C and 4D). Finally, the plant extracts from *f1 f3*, *F1DN*, and *F3DN* exhibited enhanced phosphorylation activity on ABI5 proteins in an ABA-dependent manner, and the exogenous wild-type FyPP3 proteins, but not the phosphatase null mutant FyPP3^{D81N}, reversed the phosphorylation activity of *f1 f3* plant extracts on ABI5 proteins (Figure 4). Together, these data support the notion that FyPP1 and FyPP3 act to dephosphorylate ABI5, thus promoting degradation of ABI5 and abrogating the inhibitory effect of ABA on seed germination and postgermination growth.

Reversible phosphorylation of proteins mediated by kinases and phosphatases plays essential roles in regulating plant growth and development, including signaling by various

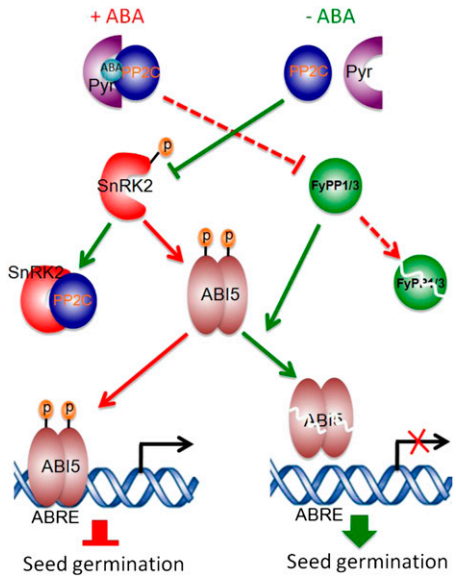

Figure 8. A Model Showing the Antagonistic Interaction between SnRK2 Kinases and FyPP/PP6 Phosphatases in ABA Signaling.

In the presence of ABA (+ABA), ABA mediates the formation of an ABA receptor-ABA-PP2C phosphatase complex (such as Pyr-ABA-PP2C complex). This causes the autophosphorylation and activation of the downstream SnRK2 kinases, including SnRK2.2, SnRK2.3, and SnRK2.6. Meanwhile, ABA promotes the degradation of the PP6 catalytic subunits, including FyPP1 and FyPP3. The active SnRK2 kinases then phosphorylate and activate the downstream transcription factors, such as ABI5, which further activate the expression of ABRE-containing genes and repress seed germination. By contrast, in the absence of ABA (-ABA), PP2C phosphatases persist, allowing them to inhibit SnRK2 kinase activity by forming PP2C-SnRK2 protein complexes and dephosphorylating SnRK2 kinases. Simultaneously, FyPP/PP6 perceives this signal and dephosphorylates ABI5, triggering its degradation and altering the expression of ABRE-containing genes, therefore promoting seed germination. The red lines indicate events happening in the presence of ABA. The green lines indicate events happening in the absence of ABA. The dashed lines indicate events happening through unknown mechanisms. p, phosphate.

phytohormones, such as auxin, ethylene, and brassinosteroids (Michniewicz et al., 2007; Kamiyoshihara et al., 2010; Tang et al., 2011), by modulating the stability and activity of key intermediates in various signaling pathways. The virtual elimination of ABA responses in the *snrk2.2/2.3/2.6* triple mutant (Fujii et al., 2007; Nakashima et al., 2009) and the hypersensitivity of the *f1 f3*, *F3Ri/f1*, *F1DN*, and *F3DN* mutants to ABA (Figure 1) suggest that the SnRK2 kinases and FyPP/PP6 phosphatases might function antagonistically in ABA signaling. It is known that phosphorylation of ABI5 is necessary for ABI5 function because overexpression of ABI5 alone is not sufficient to suppress seed germination (Lopez-Molina et al., 2001). Our data show that loss of *FyPP* function is sufficient to trigger ABI5-dependent inhibition of seed germination (Figure 2B), which is very similar to the previous report that overexpression of a *SnRK2* kinase (PKABA1) in the *ABI5-OE* background is sufficient to activate ABI5 protein and suppress seed germination (Piskurewicz et al., 2008). Phosphorylation of ABI5 by SnRK2 kinase is ABA dependent (Fujii et al., 2007; Nakashima et al., 2009), while the *FyPP* loss-of-function mutants, such as *f1 f3*, *F1DN*, and *F3DN*, showed enhanced ABA-dependent phosphorylation of ABI5 proteins (Figure 4). These observations suggest that the loss of FyPP activity and the overproduction of SnRK2 activity produced similar effects on ABI5 phosphorylation and function. Additionally, the genetic interactions between FyPPs and SnRK2.6 kinase indicate that *snrk2.6* mutants attenuated the ABA hypersensitivity phenotypes of the *FyPP* loss-of-function mutants (Figure 6), again suggesting an antagonistic role of FyPP and SnRK2.6 kinase in mediating ABA signaling. Taken together, these data provide strong evidence for the following model: SnRK2 kinase-mediated phosphorylation acts to promote the stability and function of ABI5 upon the perception of ABA, while FyPP/PP6-mediated dephosphorylation promotes the degradation and inactivation of ABI5 after the removal of ABA (Figure 8). Thus, SnRK2- and FyPP/PP6-mediated phosphorylation and dephosphorylation of ABI5 function as a molecular switch that regulates ABA signaling.

It is intriguing to note that previous studies showed that a subfamily of Mg^{2+}-dependent Ser/Thr protein phosphatases (PP2Cs), including ABI1, ABI2, and HAB1, physically interact with SnRK2 kinases, including SnRK2.2, SnRK2.3, and SnRK6/OST1, and dephosphorylate a Ser residue in the kinase activation loop in the absence of ABA (Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009; Soon et al., 2012). When ABA is present, ABA associates with the PYR/PYL/RCAR family of ABA receptors to promote the binding of these receptors to the catalytic site of PP2Cs. This inhibits their phosphatase activity and subsequently activates SnRK2 kinases through autophosphorylation (Ma et al., 2009; Park et al., 2009; Soon et al., 2012). The active SnRK2s relay the ABA signal to downstream effectors, such as ABI5/bZIP transcriptional factors (Cutler et al., 2010; Hubbard et al., 2010). Protein–protein interaction assays between ABI1 and the key transcriptional regulators involved in ABA signaling, such as ABI3, ABI4, and ABI5, failed to detect binding to PP2C (Nakamura, et al., 2001). Thus, the ABA signaling pathway appears to involve at least two distinct families of phosphatases. The main role of PP2Cs is most likely to act as a hub in mediating early ABA signaling events, modulating the phosphorylation and activity of SnRK2 kinases, whereas FyPP/PP6 specifically dephosphorylates ABI5 (and possibly other ABI5-like bZIP transcription factors) and promotes its degradation, causing subsequent changes in gene expression and ABA responses (Figure 8). Interestingly, our LCI assays failed to detect protein–protein interaction between FyPP proteins and group II ABI5-like bZIP transcription factors involved in the drought response, such as ABF2, ABF3, and ABF4 (Figure 3C). Despite this observation, we cannot exclude the possibility that FyPP1/3 may also regulate these bZIP transcription factors through the help of additional regulatory factors, as the interaction between phosphatase and substrate in vivo is usually dynamic and sometimes needs the help of other regulators (Shi, 2009). It will be interesting to investigate how the phosphorylation and activity of these ABI5-like bZIP transcription factors are regulated in future studies.

FyPP1 and FyPP3 Act in the Nexus of Multiple Signaling Pathways

It was previously reported that *Arabidopsis* FyPP1 and FyPP3 play an essential role in regulating flowering time. Recombinant FyPP efficiently dephosphorylates oat (*Avena sativa*) phytochrome A in a spectral form–dependent manner, and overexpression of *FyPP* caused delayed flowering while reducing the expression of *FyPP*-accelerated flowering in *Arabidopsis* (Kim et al., 2002). In a recent study, we showed that FyPP1 or FyPP3 interacts with the SAL proteins and the A subunits of PP2A (PP2AAs) to form PP6 heterotrimeric holoenzymes that act antagonistically with the PID/AGC3 kinases in the regulation of PIN-FORMED (PIN) protein phosphorylation, subsequently regulating directional auxin transport and root development (Dai et al., 2012a). H. Li et al. (2011) also reported that FyPP1 and PID mediated the switch of PIN1 polar localization in the regulation of pavement cell development in *Arabidopsis* leaves. In this work, we have shown that FyPP1 and FyPP3 act as negative regulators in ABA signaling. They function antagonistically with SnRK2 kinases to modulate the reversible phosphorylation of ABI5 and subsequently regulate seed germination and postgermination development. Thus, it appears that coupled with various kinases, the FyPP/PP6 phosphatases play a multifaceted role and may function in the nexus of multiple signaling pathways to regulate diverse plant developmental processes by mediating the reversible phosphorylation of key developmental regulators. Further studies of these phosphatases will undoubtedly provide more molecular and biochemical insights into the roles and functional modes of PPP phosphatases in the regulation of plant development and responses to environmental stimuli.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana lines *fypp1* (*f1*), *fypp3* (*f3*), *f1*$^{-/+}$ *f3*, *f1 f3*, *F3Ri/f1*, *F1OE*, *F3OE*, *F1DN*, and *F3DN* lines were described previously (Dai et al., 2012a). The *ost1* (or *snrk2.6*, SALK_008068; Zheng et al., 2010) mutant is in the Col background and was obtained from the SALK Institute. *abi5-1* mutants and *ABI5OE* transgenic lines were reported previously (Finkelstein, 1994; Brocard et al., 2002). The *F3Ri/f1* lines were generated by introducing an ethanol-inducible expression cassette *AlcA-AlcR*:

FyPP3RNAi into *fypp1* backgrounds as described previously (Dai et al., 2012a). More *F3Ri/f1* lines were screened in this study based on the expression of *FyPP3* after ethanol induction. Two types of *F3Ri/f1* lines were identified by RT-PCR: *FyPP3* undetectable (e.g., *F3Ri/f1*-3) and *FyPP3* weakly expressed (e.g., *F3Ri/f1*-21). *F3Ri/f1*-3 and *F3Ri/f1*-21 were then used in this study. Because the *f1 f3* double mutants have shorter roots and smaller/fused cotyledons (see Supplemental Figure 3 online; Dai et al., 2012a), we were able to choose *f1 f3* plants for ABA treatment. Seeds were sterilized as described (Dai et al., 2012a). *Arabidopsis* seedlings were grown as previously described (Lee et al., 2010). Specifically, for the assays shown in Supplemental Figure 4 online, seeds were germinated and grown vertically on GM (Lee et al., 2010) plates (with 0.1% ethanol for Supplemental Figure 4B online) for 3 d under continuous white light at 22°C in a growth chamber after stratification for 4 d in darkness. The seedlings were transferred onto fresh GM plates (with 0.1% ethanol for Supplemental Figure 4B online) with 0 or 1 μM ABA. The positions of the primary root tips were marked. The seedlings were then grown vertically for four more days in the same growth chamber, and the additional root growth was measured with a ruler. The percentage of relative root elongation was calculated with three replicates based on control plants grown on unsupplemented media. To minimize the effect of harvesting time on seed germination, all seed batches compared in this study were harvested on the same day from plants grown in the same growth chamber with identical environmental conditions.

Because *f1 f3* homozygous plants are completely sterile (Dai et al., 2012a), seeds from self-pollinated *f1*$^{-/+}$ *f3* plants (*f1* is heterozygous, and *f3* is homozygous), *f1*$^{-/+}$ *f3 abi5-1* (homozygous for *f3* and *abi5* but heterozygous for *f1*), *f1*$^{-/+}$ *f3 ABI5OE* (homozygous for *f3* and *ABI5OE* but heterozygous for *f1*), and *f1*$^{-/+}$ *f3 ost1* (homozygous for *f3* and *ost1* but heterozygous for *f1*) were used for the seed germination assays (for Figures 1, 2, and 6; see Supplemental Figures 2 and 6 online), immunoblot assays (for Figures 5 and 6), and quantitative RT-PCR assay (for Supplemental Figure 11 online). The progeny produced from these selfed plants were of mixed genotypes, including the following: a mixture of *f3*, *f1*$^{-/+}$ *f3*, and *f1 f3*, with a theoretical segregation ratio of 1:2:1 for self-pollinated *f1*$^{-/+}$ *f3* plants; a mixture of *f3 abi5-1*, *f1*$^{-/+}$ *f3 abi5-1*, and *f1 f3 abi5-1* genotypes, with a theoretical segregation ratio of 1:2:1 for self-pollinated *f1*$^{-/+}$ *f3 abi5-1* plants; a mixture of *f3 ABI5OE*, *f1*$^{-/+}$ *f3 ABI5OE*, and *f1 f3 ABI5OE* genotypes, with a theoretical segregation ratio of 1:2:1 for self-pollinated *f1*$^{-/+}$ *f3 ABI5OE* plants; and a mixture of *f3 ost1*, *f1*$^{-/+}$ *f3 ost1*, and *f1 f3 ost1* genotypes, with a theoretical segregation ratio of 1:2:1 for self-pollinated *f1*$^{-/+}$ *f3 ost1* plants.

Generation of Constructs

For Y2H assays, full-length coding sequences (CDSs) of *Pyr*, *Pyl1*, *ABI1*, *ABI2*, *ABI3*, *ABI4*, *ABI5*, and *OST1* were amplified by RT-PCR with primers Pyr-F/R, Pyl1-F/R, ABI1-F/R, ABI2-F/R, ABI3-F/R, ABI4-F/R, ABI5-F1/R1, and OST1-F/R, respectively; with *Eco*RI or *Xho*I sites at the end of F or R primers, respectively, for cloning *Pyr*, *ABI2*, *ABI5*, and *OST1* fragments; *Eco*RI or *Sal*I sites at the end of F or R primers, respectively, for cloning *Pyl1*; *Mfe*I or *Xho*I sites at the end of F or R primers, respectively, for cloning *ABI1*; *Mfe*I or *Sal*I sites at the end of F or R primers, respectively, for cloning *ABI3* and *ABI4*. *Eco*RI- and *Xho*I-digested *Pyr*, *ABI2*, *ABI5*, and *OST1* fragments were then inserted into pEG and pJG vectors digested with the same enzymes to generate pEG-*Pyr*, pEG-*ABI2*, pEG-*ABI5*, pEG-*OST1*, pJG-*Pyr*, pJG-*ABI2*, pJG-*ABI5*, and pJG-*OST1* plasmids. The *Pyl1* fragment digested with *Eco*RI and *Sal*I was inserted into pEG and pJG vectors digested with the same enzymes to generate pEG-*Pyl1* and pJG-*Pyl1* plasmids, respectively. The *ABI1* fragment digested with *Mfe*I and *Xho*I was inserted into the pEG and pJG vectors digested with the same enzymes to generate pEG-*ABI1* and pJG-*ABI1* plasmids, respectively. *ABI3* and *ABI4* fragments digested with *Mfe*I and *Sal*I were inserted into pEG and pJG vectors digested with the same enzymes to generate pEG-*ABI3* and pEG-*ABI4*, and pJG-*ABI3* and pJG-*ABI4* plasmids, respectively. The *ABI5A4* and *ABI5D4* mutant genes were generated using the same strategy used to clone *FyPP1*^{D81N} (Dai et al., 2012a). *ABI5A4* and *ABI5D4* fragments digested with *Eco*RI and *Xho*I were inserted into pEG and pJG vectors digested with the same enzymes to generate pEG-*ABI5A4* and pEG-*ABI5D4*, and pJG-*ABI5A4* and pJG-*ABI5D4* plasmids, respectively. pEG-*FyPP1*, pEG-*F1NT*, pEG-*F1CD*, pEG-*F1CT*, pEG-*FyPP3*, pJG-*FyPP1*, pJG-*F1NT*, pJG-*F1CD*, pJG-*F1CT*, and pJG-*FyPP3* plasmids were generated as described (Dai et al., 2012a).

For LCI assays, the full-length CDSs of *ABI5*, AREB3, EEL, and ABF2 were amplified by PCR with the primer pairs ABI5-F1/R1, AREB3-F/R, EEL-F/R, and ABF2-F/R with *Kpn*I or *Xho*I sites at the end of F or R primers for *ABI5* and *Kpn*I or *Sal*I sites at the end of F or R primers for *AREB3*, *EEL*, and *ABF2*, respectively. *ABI5* was digested with *Kpn*I and *Xho*I and *AREB3*, *EEL*, and *ABF2* were digested with *Kpn*I and *Sal*I and then inserted into the pCAMBIA1300-*cLUC* and *-nLUC* vectors (Chen et al., 2008) to generate pCAMBIA-*ABI5-nLUC*, pCAMBIA-*AREB3-nLUC*, pCAMBIA-*EEL-nLUC*, pCAMBIA-*ABF2-nLUC*, pCAMBIA-*cLUC-ABI5*, pCAMBIA-*cLUC-AREB3*, pCAMBIA-*cLUC-EEL*, and pCAMBIA-*cLUC-ABF2*. pCAMBIA-*FyPP1-nLUC*, pCAMBIA-*FyPP3-nLUC*, pCAMBIA-*cLUC-FyPP1*, and pCAMBIA-*cLUC-FyPP3* plasmids were generated as described previously (Dai et al., 2012a).

For BiFC assays, the CDS of *ABI5* was amplified by PCR with the primer pair ABI5-F3/R3 with *Sal*I and *Bam*HI sites at the end of F and R primers, respectively. *ABI5* digested with *Sal*I and *Bam*HI was then cloned into the pY2N vector containing the C-terminal (156 to 239 amino acids) domain of the YFP fluorescent protein (YFP^{C}) to generate the pY2N-*ABI5* plasmid. The Y1C-FyPP3 plasmid was generated as described (Dai et al., 2012a).

For Co-IP assays, *ABI5* was amplified with ABI5-F2/R2 with *Bam*HI and *Spe*I at the end of F and R primers, respectively. *ABI5* digested with *Bam*HI and *Spe*I was then inserted into the pCAMBIA-*Myc* vector (from Fang Chen, Yale University) digested with the same enzymes to generate pCAMBIA-*Myc-ABI5*. pCAMBIA-*FyPP1-3HA* and pCAMBIA-*FyPP3-3HA* were generated as described (Dai et al., 2012a).

For in vitro protein expression, ABI5b fragment (ABI5 Ser119-Gln190; Nakashima et al., 2009) was amplified by PCR with the primer pair ABI5b-F/R, with *Eco*RI and *Xho*I sites at the end of F and R primers, respectively. The *ABI5bm* (*ABI5b*^{S145A}) mutant fragment was generated using the same strategy used to clone *FyPP1*^{D81N} (Dai et al., 2012a). *ABI5b* and *ABI5bm* digested with *Eco*RI and *Xho*I was inserted into the pGEX-4T-1 vector digested with the same enzymes to generate pGEX-*ABI5b* and PGEX-*ABI5bm*. pGEX-FyPP3 and pGEX-FyPP3^{D81N} plasmids were generated as described (Dai et al., 2012a).

All genes/fragments were confirmed by sequencing. The primers used in PCR cloning are shown in Supplemental Table 1 online.

Germination Assays

The germination of *Arabidopsis* seeds was described previously (Piskurewicz et al., 2008). Seed germination was determined based on the appearance of an embryonic axis (i.e., radicle) protrusion, as observed under a microscope. Seedling greening was determined based on the appearance of green cotyledons in a seedling.

Y2H Assays

Y2H assays were conducted as described previously (Yang et al., 2005; Dai et al., 2012a).

LCI Assays

The LCI assays were performed as described (Chen et al., 2008; Dai et al., 2012a).

BiFC Assays

The BiFC assays were described previously (Bracha-Drori et al., 2004; Shen et al., 2009; Dai et al., 2012a).

Co-IP Assays

Various plasmids were transformed into *Agrobacterium tumefaciens* strain GV2260 as previously described (Dai et al., 2012a). Various bacterial strains were coinfiltrated into young tobacco (*Nicotiana tabacum*) leaves as previously described (Chen et al., 2008) and grown for 3 d. Protein extraction and co-IP were performed as described (Dai et al., 2012a). Immunoprecipitation products were separated by electrophoresis using 10% acrylamide gels, and the target proteins were detected by protein gel blots using α-HA or α-Myc antibodies (Roche) at a dilution of 1:1000 in 5% milk.

Protein Isolation and Immunoblot Analysis

Plant proteins were isolated with extraction buffer containing 50 mM Tris-Cl, pH 7.5, 6 mM NaCl, 1 mM $MgCl_2$, 1 mM PMSF, 1× protease inhibitor cocktail (Sigma-Aldrich), and 1% Nonidet P-40. For Figure 7, 4-d-old *F1OE* and *F3OE* seedlings were treated with the protein synthesis inhibitor cycloheximide for 15 h, and then ABA (0.2 mM) or control solvent (DMSO) was added. Samples were then harvested after different incubation times. Yeast proteins were extracted using the Y-PER Yeast Protein Extraction Reagent (Thermo Scientific) according to the manufacturer's instructions. To determine the protein concentration, various amounts of BSA protein were added to 1× Bradford protein assay buffer (Bio-Rad) and incubated at room temperature for 5 min before reading the A_{600} value. A standard curve of A_{600} versus concentration of BSA was then generated. The total protein concentrations were determined by extrapolating the A_{600} values of the sample proteins in 1× Bradford solution against the BSA standard curve. The extracts were mixed with 2× SDS sample buffer, boiled for 5 min, and then separated on 10% SDS protein gels. The membrane transfer and immunoblot assays were performed as described previously (Lee et al., 2010). For Figure 7, α-GFP antibodies (Invitrogen) were used at a dilution of 1:1000, and α-RPN6 (Chen et al., 2006) antibodies were used at a 1:2000 dilution. For Figure 5, α-ABI5 antibodies were used at a 1:1000 dilution. α-RPT5 antibodies (Kwok et al., 1999) were used at a 1:1500 dilution. For Supplemental Figure 8 online, α-LexA (Abcam) and α-B42 (Sigma-Aldrich) antibodies were used at a 1:2000 dilution.

In Vitro Phosphorylation Assays

GST and recombinant GST-FyPP3, GST-FyPP3^{D81N}, and GST-ABI5b proteins were expressed in *Escherichia coli* strain BL21 and purified as described previously (Park et al., 2008). Six-day-old seedlings were treated with 100 μM ABA or control solvent DMSO for 30 min. Samples were harvested into liquid nitrogen. Total proteins were extracted with 1× kinase/phosphatase buffer (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, and 1 mM Zn^{2+}), plus 1× protease inhibitor and 1 mM PMSF. In vitro kinase assays with plant extracts were performed essentially as described previously (Michniewicz et al., 2007; Dai et al., 2012a) with a few modifications. For Figures 4A and 4B, 2 μg of GST, GST-ABI5b, or GST-ABI5bm (ABI5b^{145A}) proteins and 25 μg of plant seedling extracts were mixed in 1× kinase/phosphatase buffer, 1× protease inhibitor, 1 mM PMSF, and 1× ATP solution (100 μM ATP and 1 μCi [γ-^{32}P]ATP) in a total volume of 50 μL. Various amounts of exogenous GST-FyPP3 or GST-FyPP3^{D81N} fusion proteins were added to the reactions shown in Figure 4B. The samples were incubated at 30°C for 30 min, and the reactions were stopped by adding 5× loading buffer and boiling for 5 min. The products were separated by electrophoresis using 12% acrylamide gels. The gels were stained, dried, and then visualized by exposure to x-ray films.

RNA Isolation and RT-PCR/Real-Time PCR (Quantitative PCR) Analysis

Samples were harvested in liquid nitrogen. Total RNAs were isolated using RNeasy plant mini kits (Qiagen). One microgram of total RNA of each sample was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's introductions. RT-PCR was performed as described (Lee et al., 2010). For real-time PCR, 50 ng of cDNAs was used for each reaction using the SYBR Green kit according to the manufacturer's introductions in an Applied Biosystems real-time PCR machine. Expression levels were normalized to that of an actin gene. All quantitative PCR experiments were independently performed in triplicate, and representative results were shown. The primers for RT-PCR and quantitative PCR are shown in Supplemental Table 1 online.

Confocal Observations

For Supplemental Figure 10 online, seedlings were grown to 4 d old and the roots were then harvested for confocal observation. GFP fluorescence was observed with a Carl Zeiss LSM510 confocal microscope.

Accession Numbers

Sequence data from this study can be found in the Arabidopsis Genome Initiative database under the following accession numbers: *At1g50370* (*FyPP1*), *At3g19980* (*FyPP3*), *At4g17870* (*Pyr*), *At5g46790* (*PyI1*), *At4g26080* (*ABI1*), *At5g57050* (*ABI2*), *At3g24650* (*ABI3*), *At2g40220* (*ABI4*), *At2g36270* (*ABI5*), *At4g33950* (*OST1*), *At2g41070* (*EEL*), *At3g56850* (*AREB3*), *At3g44460* (*DPBF2*), *At1g49720* (ABF1) *At1g45249* (*ABF2*), *At4g34000* (*ABF3*), *At3g19290* (*ABF4*), *At5g52310* (*RD29A*), and *At5g52300* (*RD29B*).

Supplemental Data

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

Supplemental Figure 1. Expression of *FyPP1*, *FyPP3*, and *ABI5* during Seed Germination.

Supplemental Figure 2. Germination Rates of Col, *f1*, and *f3* Seeds and Seeds from Self-Pollinated *f1*$^{-/+}$ *f3* Plants Treated with or without ABA at Various Concentrations.

Supplemental Figure 3. Phenotypes of *f1 f3* Homozygotes at the Seedling Stage.

Supplemental Figure 4. Relative Root Growth of *f1 f3* and Ethanol-Induced *F3Ri/f1* Mutants after ABA Treatment.

Supplemental Figure 5. Expression of *FyPP3* in *F3Ri/f1* Lines.

Supplemental Figure 6. Germination Phenotypes of Col and *abi5-1* Seeds and Seeds from Self-Pollinated *f1*$^{-/+}$ *f3* and *f1*$^{-/+}$ *f3 abi5*-1 Plants.

Supplemental Figure 7. Yeast Two-Hybrid Assays between FyPP1/3 and Several Key Regulators in ABA Signaling.

Supplemental Figure 8. Expression of Fusion Proteins in the Yeast Cells for Yeast Two-Hybrid Assays in This Work.

Supplemental Figure 9. Sequence Alignment of the bZIP Transcription Factors.

Supplemental Figure 10. Subcellular Localization of YFP-FyPP1 and YFP-FyPP3 in *Arabidopsis* Roots.

Supplemental Figure 11. Expression Analysis of *ABI5* mRNA.

Supplemental Figure 12. Germination rates of Col, *F1OE*, *F3OE*, *F1DN*, and *F3DN* Seeds Treated with or without ABA.

Supplemental Table 1. List of the Primer Sequences Used in This Study.

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AUTHOR CONTRIBUTIONS

M.D., X.W.D., and H.W. designed the research. M.D. cloned the constructs, generated the transgenic plants, established the mutants and crosses, and performed in vitro phosphorylation assay, protein–protein interaction assays, and expression analysis. F.C. performed BiFC assays. Q.X., T.M., K.M., J.-H.L., C.D.N., L.G., W.T., and J.W. carried out genotyping and some expression analysis. M.D. and H.W. wrote the article.

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