Profiling of associated proteins reveals dual functions of the phosphatase ABI1 in abscisic acid signaling

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Abstract

Clade A PP2C phosphatases are central components of the ABA-receptor coupled core signaling pathway, and are involved in multiple stress responses and developmental processes. However, the direct targets or partner proteins of A clade PP2Cs that participate in these biological processes are largely unknown. Here, we used a TurboID-based proximity labeling method to identify putative associated proteins of one A clade PP2C phosphatase, ABI1. By combining the results from affinity purification or proximity labeling of biotinylated proteins, we identified more than four hundred putative ABI1-associated proteins, including dozens of known ABI1-interacting proteins, as well as proteins involved in TOR signaling, phosphoregulation, and other biological processes. We found that RAFs, a group of protein kinases that phosphorylate and activate SnRK2s in ABA and osmotic stress signaling, are direct substrates of ABI1. A conserved serine residue located in the P-loop of the kinase domain, corresponding to Ser619 in RAF3, is a major functional ABI1 target site. ABI1-mediated dephosphorylation on this site strongly promotes the kinase activity of most B2 and B3 RAFs. Thus, ABI1 has dual functions in ABA signaling by dephosphorylating and inhibiting SnRK2 to prevent SnRK2 activation in unstressed conditions, while dephosphorylating B2 and B3 subgroup RAFs to maintain their basal kinase activity. PP2C-mediated dephosphorylation at the conserved serine residue may be a mechanism for RAF activation in both plants and animals, with potential implications for tumorigenesis in humans.

Introduction

Phosphorylation is one of the most important and universal post-translational modifications in both prokaryotes and eukaryotes. In the model plant Arabidopsis, 43,903 and 37,387 phosphosites from 8,577 and 8,008 phosphoproteins, respectively, were identified by two independent studies (Mergner et al., 2020; Wang et al., 2018b). Protein phosphorylation is determined by two opposite catalytical enzymes: the ‘writer’ protein kinases transfer a phosphoryl group from ATP (adenosine 5’-triphosphate) to proteins, and the ‘eraser’ protein phosphatases remove the phosphoryl group. The Arabidopsis genome encodes about 1,100 protein kinases and 150 protein phosphatases (Champion et al., 2004; Kerk et al., 2008). Identifying the physiological substrates or associated proteins of protein kinases/phosphatases is essential for understanding their biological functions.

As immunoprecipitated protein kinases, or even recombinant protein kinases purified from bacteria, keep their kinase activity and specificity, several high-throughput approaches have been developed to identify their substrates. For example, Popescu et al (2009) used a protein array-based method that identified 180 putative substrates of mitogen-activated protein kinase MPK6 (Popescu et al., 2009). Quantitative phosphoproteomics also reveals dozens of substrates of SnRK2 protein kinases upon applying ABA and dehydration (Umezawa et al., 2013; Wang et al., 2013). Recently, using a modified kinase assay linked phosphoproteomics (KALIP2) method, thousands of putative substrates of MPK6 and SnRK2.6 were identified under various abiotic and biotic stresses (Wang et al., 2020). Identification of putative substrates by high-throughput proteomics has led to discoveries of novel components and mechanisms regulated by the SnRK2s (Lin et al., 2020; Umezawa et al., 2013; Wang et al., 2018b).
Among the ~150 protein phosphatases encoded by the Arabidopsis genome, there are 24 tyrosine phosphatases, 76 PPM (PP2C) superfamily protein phosphatases, and 26 PPP family phosphatases. The lower number of protein phosphatases compared to kinases in the Arabidopsis genome suggests that, on average, a protein phosphatase has more targets than a protein kinase. Unlike protein kinases, the substrate specificities of protein phosphatases may be governed by associated regulatory proteins, and recombinant protein phosphatases might lose their specificity \textit{in vitro}. In addition, the target of a phosphatase needs to be phosphorylated before assaying the dephosphorylation reaction, which makes it difficult to identify phosphatase substrates by either biochemistry or mass spectrometry methods (Roy and Cyert, 2020). Several experimental approaches have been used to identify phosphatase substrates, like quantitative phosphoproteomics, protein-protein interaction mapping, and peptide-based dephosphorylation assay (Sun et al., 2008). Recently, a proximity labeling (PL) approach was used to identify the putative substrates of animal calcineurin phosphatase (Wigington et al., 2020). Compared to other PL enzymes, like APEX and Bio-ID, TurboID has considerably higher biotin transferase enzyme activity in an unharmed condition to plants (Branon et al., 2018; Yang et al., 2021). Recently, TurboID-based PL method was used to identify the associated proteins of Nucleotide-binding leucine-rich repeat (NLR), transcription factor FAMA, protein kinase BIN2, etc. in plants (Arora et al., 2020; Huang et al., 2020; Kim et al., 2019; Mair et al., 2019; Zhang et al., 2019b). However, whether TurboID-based PL methods could be used to identify the substrates or associated proteins of plant protein phosphatases needs to be tested.

The A Clade PP2Cs in the PP2C superfamily are the most well-studied protein phosphatases in the model plant Arabidopsis. The nine A Clade members – ABI1, ABI2, HAB1, HAB2, PP2CA, HAI1, HAI2, HAI3, and AHG1 – are central components in the ABA-receptor coupled core signaling pathway (Cutler et al., 2009; Ma et al., 2009; Park et al., 2009; Rubio et al., 2009; Soon et al., 2012). Under non-stressed conditions, A Clade PP2Cs dephosphorylate and directly bind to SnRK2s to inhibit their activities. Cold, drought, and other environmental stresses increase the ABA concentration by \textit{de novo} biosynthesis or release from deactivated forms like ABA-GE (Chen et al., 2020a; Lee et al., 2006). In the presence of ABA, ABA receptor RYR1/PYL/RCAR proteins bind to and inhibit PP2Cs. The SnRK2 protein kinases are then released from the PP2C-mediated inhibition and phosphorylated by RAF-like protein kinases (Katsuta et al., 2020; Lin et al., 2021; Lin et al., 2020; Soma et al., 2020; Takahashi et al., 2020). The reactivated SnRK2s phosphorylate downstream targets to initiate transcription of ABA-responsive genes or other adaptational processes. Besides regulating SnRK2s, A clade PP2Cs also interact with and dephosphorylate other protein kinases (Chen et al., 2016; Geiger et al., 2010; Hua et al., 2012; Kumar et al., 2017; Léran et al., 2015; Lynch et al., 2012; Lyzenga et al., 2013; Mitula et al., 2015; Rodrigues et al., 2013; Wang et al., 2018a; Yu et al., 2012), and other non-kinase proteins (Brandt et al., 2015; Chen et al., 2020b; Chérel et al., 2002; Ganz et al., 2021; Himmelbach et al., 2002; Léran et al., 2015; Li et al., 2012; Ludwików et al., 2014; Marczak et al., 2020; Miao et al., 2006), and directly participate in various biological processes. The numerous and dynamic roles of Clade A PP2C-interacting proteins in ABA signaling and other biological processes make A Clade PP2Cs ideal candidates for identification of associated proteins by PL.
Here, we used a TurboID-based PL system (Branon et al., 2018) to identify the associated proteins of ABI1, the most well-studied Clade A PP2C. A total of 425 ABI1-associated proteins were isolated by affinity purification of biotinylated proteins or PL from TurboID-ABI1-Myc transgenic plants. Besides dozens of known ABI1-interacting proteins, we identified hundreds of unreported ABI1-associated proteins that may be involved in ABA signaling, stress-adaptation, and Target of Rapamycin (TOR) signaling. RAF protein kinases, which are involved in SnRK2 activation after PP2C release, also associated with ABI1. ABI1-mediated RAF dephosphorylation enhances the kinase activity of most B2 and B3 RAF kinases, which may be essential for the RAF kinase activation. Our results suggest that TurboID-based PL is an efficient way to identify the associated proteins of protein phosphatases and could provide valuable information to understand the functions of protein phosphatases in various biological processes.

Results

Setting up of TurboID-ABI1 PL system

We hypothesized that TurboID-based PL would be an ideal system to comprehensively identify the substrates, direct protein partners, and indirect protein partners of ABI1 (Fig. 1a). We fused the coding sequence of ABI1 to either the 5’ or 3’ terminus of the coding sequence of TurboID, which is driven by a 35S promoter (Fig. 1b). A vector without ABI1 was used as a control (Fig. 1b). We first tested if the ABI1-TurboID vectors could generate functional ABI1 protein in plants, using a transient expression system in the protoplasts of Arabidopsis seedlings. In this system, the luciferase (LUC) reporter gene driven by the ABA-responsive RD29B promoter is used as an indicator of ABA response (Fujii et al., 2009). Application of ABA induced RD29B-LUC expression in the transient expression system, and this was enhanced by co-transfection of SnRK2.6 (Fig. 1c). Co-transfection of ABI1 abolished the ABA-induced RD29B-LUC expression by inhibiting the activity of SnRK2.6. Co-transfection of TurboID-ABI1-Myc, but not ABI1-TurboID-Myc, abolished the ABA-induced RD29B-LUC expression, resembling the activity of ABI1 without any tag (Fig. 1c). TurboID-ABI1-GFP showed a similar localization as ABI1-GFP in tobacco leaves (Fig. 1d). Thus, fusion of TurboID to the N-terminus of ABI1 does not affect the activity of ABI1 in plants, at least in terms of subcellular localization and ability to inhibit SnRK2.6.

We transformed the p35S-TurboID-ABI1-Myc and p35S-TurboID vectors into Col-0 wild type seedlings and generated stable expression lines. Using the TurboID-ABI1 transgenic plants, we tested the effect of different concentrations of exogenous biotin ranging from 50 to 200 µM, combined with 30, 60, 120, or 180 min of incubation time, on substrate labeling. As indicated by anti-biotin immunoblot (Fig. 1e), 180 min incubation with 100 µM exogenous biotin was sufficient for high efficiency biotin labeling in transgenic Arabidopsis. We also compared the biotinylated proteins in Col-0 wild type, TurboID-ABI1-Myc, and TurboID transgenic plant seedlings after incubation with exogenous biotin (Fig. 1f). Compared to Col-0, the TurboID-ABI1-Myc and TurboID transgenic plants had strongly enhanced and differential patterns of biotinylated proteins. To our surprise, no clear difference in biotinylated proteins was observed from the samples with or without application of ABA (Fig. 1f, left panel, two rightmost lanes).
TurboID-ABI1 PL system reveals 425 putative ABI1-associated proteins

We then used the TurboID-ABI1-Myc transgenic plants to identify ABI1-associated proteins. We incubated 2 g of TurboID-ABI1 or TurboID seedlings grown in liquid medium with 100 µM biotin for 3 h, with or without 50 µM ABA (Fig. 2a). After removing excess free biotin, we subjected the protein extract to affinity purification using magnetic streptavidin beads. The proteins coated on the beads enriched by affinity purification were then collected by on-bead trypsin digestion (Supernatant Fraction, SF). The remaining peptides on the beads were eluted (Elution Fraction, EF). Both the SF and EF were then subjected to liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). As the result, we identified 12,205 and 12,102 peptides, representing 2,430 and 2,416 unique proteins, from the SF of TurboID-ABI1-Myc samples with (ABI1-SF-ABA) or without (ABI1-SF-CK) ABA application, respectively (Fig. 2b, Supplementary Dataset 1). We also identified 9,730 peptides, representing 2,249 unique proteins, from the SF of TurboID-Myc samples with and without ABA application (TurboID-SF) (Supplementary Dataset 1). Thus, 236 proteins were exclusively present in the SF of TurboID-ABI1-Myc samples (Fig. 2b).

We also identified 752 biotinylated peptides from 384 unique proteins, and 681 biotinylated peptides from 340 unique proteins, from the EF of TurboID-ABI1-Myc sample with (ABI1-EF-ABA) or without (ABI1-EF-CK) ABA application, respectively (Fig. 2c). We identified 283 biotinylated peptides from 169 unique proteins from TurboID-Myc samples with and without ABA application (TurboID-EF) (Supplementary Dataset 2). Thus, 497 biotinylated peptides, from 224 unique proteins, were identified exclusively in EF of TurboID-ABI1-Myc samples (Fig. 2c). Combined with the 236 proteins exclusively identified from ABI1-SF (Fig. 2b), a total of 425 unique proteins were considered ABI1-associated proteins identified by TurboID-ABI1 proximity labeling (Supplementary Dataset 3). Gene ontology (GO) analysis showed that GO terms like cytoskeleton/actin filament organization, transport, response to ABA, salt or osmotic stress, growth and development, and protein phosphorylation are enriched among these ABA-associated proteins (Fig. 2d, see also Supplementary Dataset 4).

To estimate how many ABI1-associated proteins identified by this method could interact with ABI1 directly, we performed a yeast-two-hybrid (Y2H) assay between ABI1 and ten proteins randomly selected from the list of putative ABI1-associated proteins. The result showed that seven out of the ten tested proteins directly interact with ABI1, while the remaining three, MPK9, AT1G19480 and PBL16, likely do not interact directly with ABI1 (Fig. 2e).

We also performed a quantitative comparison between the ABI1-associated proteins identified from the triplicate samples with or without ABA application. 14 proteins showed increased protein abundance upon ABA application (FC >=2, p < 0.05, or only present in ABA-treated samples) and 14 proteins showed decreased protein abundance upon ABA application in the SF (FC =< –2, p < 0.05, or only present in control samples), respectively (Fig. 3a). After ABA treatment, the amount of 63 biotinylated peptides were increased in the EF (FC >=2, p < 0.05, or only present in ABA-treated samples) and 91 biotinylated peptides showed a reduction in the EF (FC =< –2, p < 0.05, or only present in control samples) (Fig. 3b).
To our surprise, we did not see clear changes in proteins, like PYLs that depend on ABA for their interaction with ABI1, or SnRK2.2 that proposed to interact with ABI1 only in the absence of ABA. Thus, TurboID-based proximity labeling may not be suitable for quantitatively profiling the dynamics of proteins associated with ABI1, perhaps because of the irreversible nature of the biotin-labeling reaction. We combined the results from ABA-treated and -untreated samples for further analysis.

**Many putative ABI1-associated proteins are components of ABA signaling**

The list of putative ABI1 interactors included several known components of ABA receptor-coupled core signaling ([Supplementary Table I](#)): ABA receptor proteins (PYL4, PYL6, PYL7, and PYL8), ABA-activated SnRK2.2, and Clade A PP2C phosphatase ABI2. Two B3 subgroup RAF protein kinases, RAF3 and RAF5/SIS8, were also present in the list of ABA-associated proteins. Other components of ABA signaling included CIPK21 and PIR1. PIR1 is a Ring finger family E3 ubiquitin ligase that mediates the degradation of PP2CA/AHG3 (Baek et al., 2019). The pir1 mutant has increased PP2CA protein accumulation and reduced ABA sensitivity in seed germination and root growth (Baek et al., 2019). Though PIR1 does not directly interact with ABI1 in a yeast-two-hybrid assay (Baek et al., 2019), identification of PIR1 as an ABI1-associated protein suggests a potential role for PIR1 in ABI1 degradation, in addition to its role in PP2CA degradation. Four transcription factors (KUA1/MYBH, SKIP, VOZ2, AKS2), a chromatin-remodeling factor (CHR5), and two RNA binding proteins (AtRGGA and CCR2), were in the list of ABI-associated proteins, suggesting a role for ABI1 in regulation of ABA-responsive transcription. Putative interactors HSP70-1, BTF3L, and RAFK1A are also known to be involved in ABA-regulated gene expression. ABI1 may also be involved in actin filament reorganization and stomatal movement by interacting with components of the WAVE/SCAR complex ([Supplementary Table I](#)). Taken together, these results indicate that a major subset of ABI1-associated proteins is related to ABA-mediated biological processes.

We also compared the ABI1-associated proteins with our previous study of ABA-responsive phosphoproteomics (Wang et al., 2020). The phosphorylation of 117 (27.5%) out of 425 ABI1-associated proteins was significantly induced by ABA treatment (p < 0.05, Fig. 3c, [Supplementary Dataset 5](#)). Among these 117 proteins, at least 35 proteins were not identified as putative substrates of SnRK2.6 or CKL2 in our previous result (Fig. 3c, see also [Supplementary Dataset 5](#)). Interestingly, CPK21, a known ABI1 substrate, is present in this list ([Supplementary Dataset 5](#)). CPK21 and MPK8 also interacted with ABI1 in our Y2H assay (see Fig. 2e). Thus, some proteins in the list of ABI1-associated proteins could be direct substrates of ABI1.

**ABI1-associated proteins overlap with the TOR phosphonetwork**

TOR kinase is an evolutionarily conserved regulator of growth in eukaryotes. Arabidopsis TOR complex consists of TOR kinase, the regulatory components RAPTOR1A, RAPTOR1B, LST8-1, LST8-2. We noticed that 14 out of 83 TOR-dependent phosphoproteins were present in our list of ABI1-associated proteins.
In addition, 12 out of 79 LST8-1-interacting proteins and 8 out of 98 RAPTOR1B-interacting proteins were present in our list of ABI1-associated proteins (Van Leene et al., 2019), which suggested a tight connection between the TOR signal network and ABI1-mediated processes (Fig. 3d, Supplementary Dataset 6). A basic helix-loop-helix (bHLH) transcription factor AKS1 was identified as an ABI1-associated protein, and co-immunoprecipitates with LST8-1 (Van Leene et al., 2019). AKS1 is a substrate of ABA-activated SnRK2s and controls the expression of KAT1 and stomatal aperture (Takahashi et al., 2013). The PP2C regulatory subunit TAP46, a component of the TOR signaling pathway (Ahn et al., 2011), was also present in the list of ABI1-associated proteins (Supplementary Dataset 3). TAP46 directly binds to and stabilizes ABI5, thereby positively regulating ABA signaling (Hu et al., 2014). Two subunits of the autophagy-initiating kinase complex, ATG101 and ATG1b, were identified as putative ABI1-associated proteins (Supplementary Dataset 3). ATG101 is associated with Raptor1B and LST8-1, and ATG1b is a putative TOR substrate. In total, 30 TOR substrates or TORC-associated proteins may associate with ABI1 (Supplementary Dataset 6), suggesting a close relationship between ABI1 and TOR signaling.

**Putative ABI1-interactors include protein kinases and phosphatases**

We identified 27 protein kinases and 7 protein phosphatases as ABI1-associated proteins (Table I). Besides two B2 subgroup RAFs involved in ABA-induced SnRK2 activation, four B4 subgroup RAF kinases, RAF16, RAF20, RAF24, and RAF42, were identified (Table I). B4 subgroup RAFs directly phosphorylate and mediate ABA-independent SnRK2 activation upon osmotic stress (Katsuta et al., 2020; Lin et al., 2020; Soma et al., 2020). The high-order mutant of B4 subgroup RAF kinases, Ok130-null, is hypersensitive to osmotic stress (Lin et al., 2020). One putative MAP4 kinase SIK1, two MAPKKKs, one MAPKK, and three MAP kinases, are also present as ABI1-associated proteins (Table I). ABI2, two tyrosine-specific phosphatases RLPH2 and PAS2, and three PP2A components were identified as ABI1-associated proteins, as was an inhibitor of TOPP1, AT-I2 (Table I). ABI1 may work with these phosphatases in some biological processes.

**ABI1 promotes RAF3 activity by dephosphorylating a phosphoserine in the P-loop**

Encouraged by the association between ABI1 and RAF protein kinases, we further validated the interactions between ABI1 and RAFs by Y2H assay (Supplementary Fig. 1). Ten out of fifteen tested RAFs – RAF2, RAF3, RAF5, RAF6 from the B3 subgroup; RAF7, RAF9, RAF10, RAF11, RAF12 from the B2 subgroup; and RAF18, RAF20 from the B4 subgroup – showed clear interactions with ABI1 (Supplementary Fig. 1). RAF1, RAF16, RAF24, and RAF40 appeared not to interact with ABI1 (Supplementary Fig. 1). For unknown reasons, we were not able to obtain positive colonies for the combination of ABI1 with RAF4, RAF8, RAF35, or RAF42. Nonetheless, supporting our TurboID result (Table I), the Y2H assays show that RAFs physically interact with ABI1.
B2, B3, and B4 RAF kinases are quickly activated by osmotic stress and are essential for SnRK2 activation upon ABA and osmotic stresses (Katsuta et al., 2020; Lin et al., 2021; Lin et al., 2020; Soma et al., 2020; Takahashi et al., 2020). We used various biochemistry methods to elucidate how ABI1 regulates RAF activation. *In vitro* phosphorylation assay showed that recombinant kinase domains (KDs) of representative RAFs, RAF3-KD, RAF10-KD and RAF24-KD, did not phosphorylate either ABI1 or PP2CA (Supplementary Fig. 2a). Interestingly, incubating with ABI1 significantly enhanced the kinase activities of recombinant RAF2-KD, RAF3-KD, RAF4-KD, and RAF5-KD from B3 RAF subgroup in the context of autophosphorylation activity (Fig. 4a, lanes 1-8). Incubation with ABI1 also induced the kinase activities of RAF10-KD and RAF11-KD (Fig. 4a, lanes 13-16), and reduced the activities of RAF7-KD and RAF12-KD (Fig. 4a, lanes 11, 12 and 17, 18), the RAFs from the B2 RAF subgroup. As expected, ABI1 inhibited SnRK2.6 activity (Fig. 4a, lanes 9 and 10). ABI1 inhibited the kinase activity of RAF24 and RAF40 from the B4 subgroup (Supplementary Fig. 2b).

We then examined the ABA- and osmotic stress-induced RAF-SnRK2 activation in wild type and PP2C mutants using an in-gel kinase assay, which involves separating plant proteins in SDS-polyacrylamide gels containing histone as a kinase substrate and renaturation of the proteins with DTT after removal of SDS from the gels before detecting protein bands with kinase activities by incubation with [γ-32P]ATP. ABA-induced SnRK2 activation, consistent with previous results (Fujii et al., 2009), was increased in \textit{abi1abi2hab1pp2ca}, a mutant lacking four A clade PP2Cs, (Fig. 4b and 4c). Interestingly, mannitol-induced RAF activation was strongly impaired in the \textit{abi1abi2hab1pp2ca} mutant (Fig. 4b and 4c). Combining the *in vitro* and *in vivo* data together, A Clade PP2Cs may have a positive role in RAF activation, which differs from their role in inhibiting SnRK2s in ABA signaling.

To determine the ABI1 target site in RAF-KD, we performed stable-isotope dimethyl labeling-based quantitative proteomics to compare the phosphosites in recombinant RAF-KD, with or without ABI1 incubation (Fig. 5a). The RAF3-KD, whose activity showed the most dramatical induction after ABI1-incubation, was chosen for further analysis. Phosphorylation of five serine/threonine residues in RAF3 – Ser619, Thr727, Thr835, Thr875, and Ser878 – was strongly decreased after incubating with ABI1 (Fold change < 0.1), while the phosphorylation of other phosphosites was relatively unaffected (Fig. 5b, Supplementary Dataset 7). Site-directed mutagenesis of these putative ABI1 target sites revealed that the phosphorylation of Ser619, within the glycine-rich phosphate-binding (P)-loop and corresponding to Ser467 in human b-Raf, regulates RAF3 activity (Fig. 5c and d). The phosphomimicking Ser to Gln (S to D) mutation at Ser619 impaired RAF3-KD kinase activity, while phosphomimicking mutation of other sites did not impair RAF3-KD activity. Consistent with this observation, RAF3^{S619D} and RAF3^{S619E}, showed reduced ability to rescue ABA-induced \textit{RD29B-LUC} expression in the protoplasts of \textit{OK100-nonu}, when compared to wild type RAF3 (Fig. 5e). Compared to Ser619, mutations on either Tyr620 or Thr727 only slightly affect the activity of RAF3 in the transient expression assay, in the context of ability to rescue ABA-induced \textit{RD29B-LUC} expression in the protoplasts of \textit{OK100-nonu} (Supplementary Fig. 2c). Thus, Ser619 in RAF3 is the direct target site of ABI1, and phosphorylation of this site represses RAF3 activity.
Discussion

Identifying the associated partners and substrates is one of most efficient ways to study the biological function of a certain protein phosphatase. In this study, using the well-studied protein phosphatase ABI1 as a model, we tested TurboID-based PL in identification of phosphatase-associated proteins in Arabidopsis. We combined the two fractions from affinity purification of biotinylated proteins and PL, and identified more than four hundred proteins associated with TurboID-ABI1. Several known substrates, regulators, or partners of ABI1 were present in the list of ABI-associated proteins. The overlap between ABI1-associated proteins and proteins with increased phosphorylation upon ABA treatment revealed that some proteins in this list might be direct targets of ABI1. Upon stress conditions, ABA-receptor complex represses ABI1 activity and might result in the increased phosphorylation of some ABI1 targets, like SnRK2s or CPK21. Hundreds of additional ABI1-associated proteins suggested broader roles of ABI1 in ABA signaling, stress responses, or TOR-mediated regulation of growth and development. The identification of these ABI1-associated proteins provides a valuable resource to understand the functions of ABI1 in various biological processes. Thus, the PL-based approach is an efficient way to identify the putative targets or functional partners of protein phosphatases in plants.

In our previous study, we reported that RAFs keep a basal level of activation even under unstressed conditions, which ensures rapid phosphorylation of SnRK2s upon their release from PP2C-mediated inhibition (Lin et al., 2021). After phosphorylation and activation by RAFs, the activated SnRK2s then intermolecularly transphosphorylate other inactivated SnRK2s to quickly amplifying the activation (Lin et al., 2021). In this study, we revealed that ABI1 directly dephosphorylates RAFs to enhance RAF activity. ABI1-mediated RAF dephosphorylation might be a mechanism to keep the basal level kinase activity of RAFs. Meanwhile, PP2C dephosphorylates and inhibits SnRK2 to prevent SnRK2 activation and stress signaling in the absence of ABA. Therefore, we propose a dual role for ABI1 – it ensures that the B2/B3 RAF-SnRK2.2/2.3/2.6 cascade can quickly respond to ABA or osmotic stresses but prevents the leaky activation of SnRK2 under non-stressed conditions (Supplementary Fig. 3).

RAFs are conserved kinases in both animals and plants. The functional ABI1 target site in RAF3, Ser619, corresponds to Ser467 in human b-Raf (Fig. 5d). In human b-Raf, Ser467 and nearby Ser465 are inhibitory phosphosites repressing b-Raf activity and tumorigenesis, likely by stabilizing the inactive “DFG-out” conformation of RAF (Hey and Pritchard, 2013; Holderfield et al., 2013). However, how the phosphorylation of Ser465/Ser467 is erased in humans is unknown. We propose that PP2C-mediated dephosphorylation could be a conserved mechanism for RAF activation in both plants and animals. Although humans have a canonical RAF-MAPK cascade, instead of the noncanonical RAF-SnRK2 cascade in plants, discovery of PP2C in RAF-dephosphorylation provides a valuable clue for RAF regulation and cancer therapy in human cells.

We noticed that, in addition to enhancing B3 RAF activity, ABI1 dephosphorylates B4 RAFs and abolishes their kinase activity. Osmotic stress may activate B4 RAF kinases through either phosphorylation or phosphorylation-independent manners, which could abolish ABI1-mediated inhibition. It is also notable
that Tyr620, next to the ABI1 target site Ser619, also undergoes phosphorylation and may contribute to the RAF3 regulation in plants (Supplementary Fig. 2c). This residue has been substituted with Cys or Phe in most B2 and B4 RAFs, which could be the reason for differential effects of ABI1 dephosphorylation on activity of different RAF kinases. As serine/threonine phosphatases, ABI1 and other A Clade PP2Cs likely do not dephosphorylate Tyr620 in plants, and the tyrosine phosphatase dephosphorylating Tyr620 in RAF3 needs to be identified in the future.

Our finding also supports a close relationship between ABA and TOR signal networks (Wang et al., 2018b). Our previous work, as well as several other studies, showed that mutants of TORC components have enhanced ABA sensitivities. Under unstressed conditions, TOR phosphorylates ABA receptors to block stress response and promote growth/development. On the other hand, SnRK2s physically interact with and strongly phosphorylate RAPTOR1B, a regulatory component of TORC. The SnRK2-mediated phosphorylation promotes the disassociation of TORC, to inhibit growth and development under stress conditions. By this phosphorylation-loop, the PYL-PP2C-SnRK2 module and TOR complex reciprocally balance stress response and growth to couple to the ever-changing environment. A recent study suggested that PP2CA, an ABI1 homolog, co-immunoprecipitates with SnRK1 and may also contribute to the balance of stress response and growth (Belda-Palazon et al., 2020). However, SnRK1 protein kinases were not present in our list of ABI1-associated proteins. It is worth noting that PL and Co-IP identify the protein complex, including proteins either physically interacting or not directly interacting with the bait protein (Fig. 1a). For example, at least two ABI1-associated proteins, PBL16 and AT4G10480, do not physically interact with ABI1, as indicated by Y2H assay (Fig. 2e). In addition, the tissue-specific and development stage-dependent expression, or even protein stability during the sample preparation, may affect the identification of protein complexes. That might be the reason why some known ABI1-interacting proteins were missing from our list of ABI1-associated proteins.

Materials And Methods

Plant materials and growth condition

Nicotiana benthamiana seeds were grown on soil for 3-4 weeks in the green house. TurboID-MYC and TurboID-ABI1-MYC was overexpressed in Arabidopsis Col-0 ecotype. Arabidopsis seedlings were sowed on half Murashige-Skoog medium supplemented with 0.7% agar powder, incubated in the dark for 3 days at 4°C, and transferred to a growth chamber for germination at 22°C under long-day conditions (16 h of light).

Immunoblot analysis

10-day-old Arabidopsis seedlings with or without the indicated treatment were ground to fine powders in liquid nitrogen, dissolved in 2 × SDS buffer, and boiled for 10 min. After centrifugation, supernatants were separated by 10% SDS-PAGE. After transfer to PVDF membranes (Bio-Rad), proteins were analyzed by immunoblot with antibodies against c-Myc (Abmart, M20002S) or Biotin (Abcam, ab53494).
**Yeast-two-hybrid assay**

To detect protein interactions between ABI1 and ABI1-associated proteins, *pGBKT7-ABI1* was co-transformed with *pGADT7* plasmids with coding sequences of randomly selected proteins from the list of ABI1-associated proteins into AH109 cells. The positive clones were selected on synthetic complete medium lacking Leu and Trp and transferred to selective SD medium lacking Leu, Trp, His, and in the presence of 3-Amino-1,2,4-Triazol (3-AT). To determine the intensity of protein interaction, saturated yeast cultures were diluted and spotted onto selection medium. Photographs were taken after 4 days incubation at 30 °C.

**Confocal microscopy.**

The plasmids *p35S::ABI1-GFP, p35S::ABI1-TurboID-GFP* and *p35S::TurboID-ABI1-GFP* were individually transformed into *Agrobacterium* GV3101 cells, and injected into *Nicotiana benthamiana* leaves. 48-72 hours after infiltration, the transformed tobacco leaves were examined by confocal microscopy on a Leica SP8 laser scanning confocal microscope at 488 nm laser excitation and 500 to 550 nm emission for GFP.

**Protoplast isolation and transactivation assay**

4-week-old Arabidopsis seedlings grown under a short photoperiod (8 hours light /16 hours dark) were used for protoplast isolation. The young leaves were treated with enzyme solution containing cellulase R10 (Yakult Pharmaceutical Industry) and macerozyme R10 (Yakult Pharmaceutical Industry) in the dark for 3 hours. The enzyme/protoplast solution was diluted with equal volume of W5 solution (2 mM MES, pH 5.7, 154 mM NaCl, 125 Mm CaCl$_2$, and 5 mM KCl) to stop the enzymatic hydrolysis. The protoplasts were pelleted at 500 g for 2 min. Protoplasts were resuspended in W5 solution and kept for 30 min on ice in the dark. Before transfection, the protoplasts were resuspended in MMG solution (4 mM MES, pH 5.7, 0.4 Mannitol, and 15 mM MgCl$_2$). Protoplasts were gently and thoroughly mixed with the plasmid mixture and PEG solution (40% w/v PEG-4000, 0.2 M mannitol, and 100 mM CaCl$_2$), and incubated for 5 min. The transfection was stopped by adding W5 solution and the protoplasts were washed with 1 mL W5 solution. After transfection, protoplasts were incubated for 5 hours under light in incubation solution (0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7), with or without 5 µM ABA. The *RD29B* promoter fused to the LUC coding sequence was used as an ABA-responsive reporter (7 µg of plasmid per transfection). *ZmUBQ::GUS* was included in each sample as control (2 µg per transfection). *ABF2-HA, SnRK2.6-Flag, ABI1-GFP, TurboID-ABI1-GFP, ABI1-TurboID-GFP* were used at 3 µg per transfection.

**Affinity purification of biotinylated proteins**

*TurboID-ABI1-MYC* and *TurboID-MYC* seedlings were treated with the indicated concentration of biotin for the indicated time, with or without 50 µM ABA treatment. Two grams of the plant tissue were ground with liquid nitrogen. The tissue powder was resuspended in 3 mL IP buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.1% SDS, 0.5% sodium deoxycholate, 10 mM NaF, 1 x Protease...
Inhibitor Cocktail (Roche, 45868700)]. After vortex mixing, the tubes were centrifuged at 12,000 g for 10 min. For desalting, the supernatant was run through the Spin Desalting Column (Thermo Fisher Scientific) to remove the excess biotin in lysates.

After desalting, protein amount was quantified using the BCA Protein Assay Kit (Thermo Fisher Scientific). Fourteen mg of proteins were incubated with 150 µL streptavidin beads (Thermo Fisher Scientific) on a rotator overnight at 4°C to enrich the biotinylated proteins. After washing 3 min with washing buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM NaF, 1 mM EDTA) three times, the streptavidin beads were subjected to on-bead digestion.

**On-bead digestion of biotinylated proteins**

The streptavidin beads were suspended in digestion buffer (12 mM sodium deoxycholate and 12 mM sodium lauroyl sarcosinate in 100 mM Tris-HCl, pH 8.5) and then were 4-fold diluted with 50 mM TEAB buffer). Protein was then digested with 2.5 µg Lys-C (Wako, Japan) for 4 h at 37°C, and 1 µg trypsin (Promega, V5113) for an additional 4 h at 37°C. After digestion, the digestion buffer and beads were separated by centrifugation. The detergents in the supernatants were removed by acidifying the solution using 10% TFA and then centrifuged at 16,000 g for 20 min. After desalting using a SEP-PAK C18 cartridge, the supernatants (named SF in the text) were subjected to LC-MS/MS analysis.

The biotinylated peptides coated on the beads were eluted by elution buffer (80% ACN, 0.2% TFA, 0.1% FA) three times and dried in a vacuum concentrator (Thermo Fisher Scientific). The elution fraction (named EF in the text) was resuspended in 0.1% formic acid (FA), desalted using C18 ZipTips (Millipore, Burlington, MA), and analyzed by LC-MS/MS.

**LC-MS/MS Analysis**

The peptides were dissolved in 4 µL of 0.2% FA and injected into an Easy-nLC 1200 (Thermo Fisher Scientific). Peptides were separated on a 15 cm in-house packed column (360 µm OD × 75 µm ID) containing C18 resin (2.2 µm, 100 Å, Michrom Bioresources). The mobile phase buffer consisted of 0.1% FA in ultra-pure water (Buffer A) with an eluting buffer of 0.1% FA in 80% ACN (Buffer B) run over a linear 93 min gradient of 5-40% buffer B at flow rate of 300 nL/min. The Easy-nLC 1200 was coupled online with an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was operated in the data-dependent mode in which a full-scan MS (from m/z 350–2,000 with the resolution of 120,000) was followed by top 10 higher-energy collision dissociation (HCD) MS/MS scans of the most abundant ions with dynamic exclusion for 45 s.

The dried dimethyl-labeled peptides were resuspended in 0.1% (v/v) FA, and then analyzed by Q Exactive HFX mass spectrometer (Thermo, San Jose, CA). The peptides were separated by Easynano1200 LC (Thermo, San Jose, CA), equipped with 150 mm in-house C18 analytical column. The peptide mixture was separated on the analytical column with the following conditions at 300 nL/min, 90 min run with a liquid gradient from 5-90% of solvent B (80% acetonitrile/0.1% FA). The parameters for a full MS survey scan were set as a resolution of 60,000 at 400 m/z over the m/z range of 300-1800, Automatic gain controls
(AGC) target of 3E6, maximum ion injection time (IT) of 50 ms. The top 20 multiply-charged parent ions were selected by data dependent MS/MS mode, fragmented by the higher-energy collision dissociation (HCD) with the normalized collision energy of 29% at the m/z scan range of 200-2000. For the MS/MS detection, the resolution was set at 15,000, AGC target value was 1E5, and the maximum IT was 100 ms. Dynamic exclusion was enabled for 30 s.

**Analysis of mass spectrometry data**

The raw files were searched directly against the Arabidopsis thaliana database (TAIR10 with 35,386 entries) with no redundant entries using Proteome Discoverer (version 2.4). Peptide precursor mass tolerance was set at 20 ppm, and MS/MS tolerance was set at 0.05 Da. Three missed cleavage sites of trypsin were allowed. Search criteria included a static carbamidomethylation of cysteines (+57.0214 Da) and variable modifications of oxidation (+15.9949 Da) on methionine residues and biotin (+226.078 Da) on lysine residues. The false discovery rates of proteins, peptides and biotinylated peptides were set at 1% FDR.

The raw files dimethyl-labeled MS were searched directly against ABI1 protein using Maxquant (version1.6.6.60). The following parameters were used: carbamidomethylation of Cys was set as a fixed modification, phosphorylation of STY, oxidation of M and acetylation of protein N terminal were set as variable modifications, and a maximum of two missed cleavages was allowed. The false discovery rate for peptide, protein, and site identification was set to 1% FDR. For quantitative analysis of proteins and biotinylated peptides, the data was normalized by total intensity.

**In-gel kinase assay**

In-gel kinase assay was performed as previous described (Lin et al., 2020). In brief, 20 µg extract of total proteins were electrophoresed on 10% SDS/PAGE embedded with histone in the separating gel. The gel was then washed three times at room temperature for 30 min each with washing buffer. The kinase was renatured and incubated at 4°C overnight with three changes of renaturing buffer. The gel was further incubated at room temperature in reaction buffer with 200 nM ATP plus 50 µCi of \( {\gamma}^{32}P \)ATP for 90 min. The reaction was stopped by transferring the gel into 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate. The gel was then washed for 5 h with five changes. Radioactivity was detected with a Personal Molecular Imager (Bio-Rad). The radioactivity from RAF bands was calculated by ImageJ.

**Protein purification and in vitro kinase assay**

For *in vitro* kinase assays, full length coding sequence of ABI1 and PP2CA and kinase domains of RAFs were cloned into either *pGEX-4T-1*, *pET28a*, or *pET-SUMO* vectors and transformed into BL21 or ArcticExpression cells. The recombinant proteins were expressed and purified as standard protocol. For the PP2C phosphorylation assay, recombinant kinase domains of RAFs were incubated with boiled ABI1 and PP2CA in reaction buffer (25 mM Tris HCl, pH 7.4, 12 mM MgCl\(_2\), 2 mM DTT), with 1 µM ATP plus 1 µCi of \( {\gamma}^{32}P \) ATP for 30 min at 25°C. For kinase activity assay of RAFs, 2 µg recombinant kinase domains of RAFs were pre-incubated with 0.5 µg ABI1 for 30 min in the reaction buffer (25 mM Tris HCl, pH 7.4, 12 mM MgCl\(_2\), 2 mM DTT, 1 mM ATP) at 25°C.
pH 7.4, 12 mM MgCl$_2$, 2 mM DTT). After incubation, 1 µM ATP plus 1 µCi of [$\gamma$-$^{32}$P] ATP were added to the reaction and incubated for additional 30 min. The reactions were stopped by boiling in SDS sample buffer and proteins were separated by 10% SDS-PAGE.

**Dephosphorylation assay and dimethyl-labeled quantification**

For dephosphorylation assay, 100 µg RAF3-KD coated on Glutathione Sepharose (Cytiva) was incubated with or without 50 µg HIS-SUMO-ABI1 for 60 min at 25°C in reaction buffer (25 mM Tris HCl, pH 7.4, 12 mM MgCl$_2$, 0.5 mM DTT). After removing HIS-SUMO-ABI1 by centrifugation, the RAF3-KD was digested with 2 µg trypsin (Sigma-Aldrich, St. Louis, MO) overnight. The tryptic peptides were acidified by 10% TFA and then centrifuged at 16,000 g for 20 min, and then desalted using a SEP-PAK C18 cartridge (Waters, Milford, MA). Peptide amount was determined using the BCA assay (Thermo Fisher Scientific, Waltham, MA). 20 µg tryptic peptides from RAF3-KD, incubated with or without ABI1, were dissolved in 100 µL of 100 mM TEAB, mixed with 4 µL of 4% $^{13}$CD$_2$O or $^{12}$CH$_2$O, respectively, and added to 4 µL of freshly prepared 0.6 M sodium cyanoborohydride. The mixture was agitated for 60 min at room temperature and the reaction was stopped by adding 16 µL of 1% NH$_4$OH on ice and agitating the mixture for 1 min. The dimethyl-labeled peptides were then mixed with 20 µL of 10% FA, and the two differently labeled peptides were combined and desalted by SEP-PAK C18 cartridge (Waters, Milford, MA).

Phosphopeptide enrichment was performed according to the reported IMAC StageTip protocol with some modifications. The in-house-constructed IMAC tip was made by capping the end with a 20 µm polypropylene frits disk (Agilent). The tip was packed with 5 mg of Ni-NTA silica resin by centrifugation at 200 g for 1 min. Ni$^{2+}$ ions were removed by 100 µL of 100 mM EDTA solution. The tip was then activated with 100 µL of 100 mM FeCl$_3$ and equilibrated with 100 µL of loading buffer (6% (v/v) acetic acid at pH 3.0) prior to sample loading. The dimethyl-labeled peptides (200 µg) were reconstituted in 100 µL of loading buffer and loaded onto the IMAC tip. After successive washes with 200 µL of washing buffer (4% (v/v) TFA, 25% acetonitrile (ACN)) and 100 µL of loading buffer, the bound phosphopeptides were eluted with 150 µL of 200 mM NH$_4$H$_2$PO$_4$. The eluted phosphopeptides were loaded into a C18 StageTip and separated into eight fractions using high-pH reverse phase fractionation. The phosphopeptides were dried using a SpeedVac.

**Data Availability**

The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD029281 (Username: reviewer_pxd029281@ebi.ac.uk; Password: vwCXMRmL).

**Declarations**

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Author contributions

PW designed the research; CZ, YW, ZL, RL, BJ, GQ, and CCH performed the research; CZ, RL, and PW analyzed the data; CZ, RL, CCH, CPS, and PW wrote the paper.

Competing Interests

The authors declare no competing interests.

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**Table**

Table I. Protein kinases and phosphatases present in our list of ABI1-associated proteins
| AGI ID   | Name             | Annotated Function                                                                                                                                                                                                 | Reference                                                                                           |
|----------|------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|
| AT3G50500| SnRK2.2          | An ABA-activated SnRK2, phosphorylates substrates to transduce ABA signaling                                                                                                                                   | (Lin et al., 2020; Soma et al., 2020)                                                              |
| AT5G11850| RAF3             | Phosphorylates and activates ABA-dependent SnRK2s                                                                                                                                                               |                                                                                                      |
| AT1G73660| RAF5/SIS8        | Phosphorylates and activates ABA-independent SnRK2s. The osmotic stress-induced ABA-independent SnRK2 activation is abolished in high-order mutant OK130-null and the OK130-null mutant is hypersensitive to osmotic stress. | (Katsuta et al., 2020; Lin et al., 2020; Takahashi et al., 2020)                                      |
| AT1G04700| RAF16            |                                                                                                                                                                                                                 |                                                                                                      |
| AT1G79570| RAF20            |                                                                                                                                                                                                                 |                                                                                                      |
| AT2G35050| RAF24            | Phosphorylates and activates ABA-independent SnRK2s. The osmotic stress-induced ABA-independent SnRK2 activation is abolished in high-order mutant OK130-null and the OK130-null mutant is hypersensitive to osmotic stress. | (Katsuta et al., 2020; Lin et al., 2020; Takahashi et al., 2020)                                      |
| AT3G46920| RAF42            |                                                                                                                                                                                                                 |                                                                                                      |
| AT4G04720| CPK21            | Inhibited by ABI1, phosphorylates SLAC1 to activate the guard cell anion channel                                                                                                                                  | (Geiger et al., 2011)                                                                                   |
| AT2G23070| CKA4             | Participates protein phosphorylation in the chloroplast stroma; cka4 mutant shows a reduced sensitivity to ABA                                                                                                 | (Wang et al., 2014)                                                                                   |
| AT3G18040| MPK9             | Expresses preferentially in guard cells and is involved in ROS-mediated ABA signaling                                                                                                                             | (Jammes et al., 2009)                                                                                   |
| AT1G18150| MPK8             | Involves in ROS-dependent wounding response, regulates GA Sensitivity                                                                                                                                           | (Takahashi et al., 2011; Zhang et al., 2019a)                                                          |
| AT2G42880| MPK20            |                                                                                                                                                                                                                 |                                                                                                      |
| AT3G13530| MKKK7            | Negatively regulates flagellin-triggered signaling and basal immunity                                                                                                                                          | (Mithoe et al., 2016)                                                                                   |
| AT1G63700| YDA, MKKK4       | A component of the stomatal development regulatory pathway                                                                                                                                                      | (Lukowitz et al., 2004)                                                                                 |
| AT5G50000| CBC2/RAF33       | Regulate stomatal aperture by integrating signals from blue light and CO₂                                                                                                                                       | (Hiyama et al., 2017)                                                                                   |
| AT5G61350| ERU/CAP1         | A close homology of FERONIA, regulates root hair tip growth                                                                                                                                                      | (Bai et al., 2014; Haruta et al., 2014)                                                               |
| AT4G28490| HAESA            | The receptor of IDA, controls floral organ abscission                                                                                                                                                           | (Jinn et al., 2000)                                                                                   |
| AT5G56460| PBL16            |                                                                                                                                                                                                                 |                                                                                                      |
| AT3G24550| PERK1            |                                                                                                                                                                                                                 |                                                                                                      |
| AT1G69220| SIK1             | An MAP4K, positively regulates immunity by                                                                                                                                                                      | (Zhang et al., 2019a)                                                                                 |
stabilizing BIK1 to promote the extracellular ROS burst (Iwabuchi et al., 2018)

| Accession | Gene Symbol | Description                                                                                      | Reference               |
|-----------|-------------|--------------------------------------------------------------------------------------------------|-------------------------|
| AT1G73450 | DYRK-2B     | Interacts with ANGUSTIFOLIA and regulates the actin filaments                                    | (Iwabuchi et al., 2019) |
| AT3G45780 | PHOT1       | Blue-light photoreceptor, whose mutants are defective in blue-light response                     |                         |
| AT3G53930 | ATG1b       | Subunit of ATG1 kinase complex that regulates autophagy                                           | (Huang et al., 2019)    |
| AT2G40730 | CTEXP       | Protein kinase superfamily protein with ARM repeat domain-containing protein                     |                         |
| AT3G15220 |             | Protein kinase superfamily protein                                                                |                         |
| AT4G10730 |             | Protein kinase superfamily protein                                                                |                         |
| AT5G35960 |             | Protein kinase superfamily protein                                                                |                         |
| AT5G58950 |             | Protein kinase superfamily protein                                                                |                         |
| AT2G27960 | CKS1        | A catalytic subunit of cyclin-dependent kinase 1. Physically interacts with CDKs and plays an essential, but not entirely resolved, role in the regulation of the cell cycle | (De Veylder et al., 2001) |

**Phosphatases**

| Accession | Gene Symbol | Description                                                                                      | Reference               |
|-----------|-------------|--------------------------------------------------------------------------------------------------|-------------------------|
| AT5G57050 | ABI2        | A Clade A PP2C, dephosphorylates and inhibits SnRK2, inhibited by PYLs                            | (Merlot et al., 2001)  |
| AT5G10480 | PAS2        | Involves in cell division and differentiation, interacts with phosphorylated form CDKA;1        | (Eckardt, 2006)         |
| AT5G53000 | TAP46       | A PP2A-associated protein, tap46 knockdown mutants are less sensitive to ABA                     | (Hu et al., 2014)       |
| AT4G15410 | PUX5        | PP2A 55 kDa regulatory subunit B prime gamma                                                     |                         |
| AT1G17720 | ATB BETA    | PP2A regulatory subunit PR55                                                                       |                         |
| AT3G09970 | RLPH2       | Phospho-tyrosine-specific phospho-protein phosphatase                                             | (Uhrig et al., 2016)    |
| AT5G52200 | AT-I2       | Inhibitor of PP1 phosphatase, negatively regulated ABA signaling                                  | (Hou et al., 2016)      |

**Figures**
Figure 1

Setting up of TurboID-ABI1 proximity labeling system. a. Overview of the experimental setup. b. Constructions used in the transient assay experiments. c. The expression of RD29B-LUC reporter gene in the protoplasts cotransfected with different ABI1 constructions. Error bars, SD (n = 3 individual transfections). Two-tailed paired t-tests, **p < 0.01. d. Localization of ABI1-GFP and TurboID-ABI1-GFP in N. benthamiana. e. Biotin transferase activity of TurboID-ABI1-Myc transgenic plants incubated with different concentrations of biotin. Anti-biotin (upper panel) and anti-Myc (bottom panel) immunoblots show the biotin transferase activity and abundance of TurboID-ABI1-Myc, respectively. f. The biotinylated proteins in wild type, TurboID-Myc, and TurboID-ABI1-Myc transgenic plants without or with ABA treatment. Anti-biotin (left panel) and anti-Myc (right panel) immunoblots show the biotinylated proteins and abundance of TurboID-Myc and TurboID-ABI1-Myc proteins. Images shown are representative of at least two independent experiments.
Figure 2

TurboID-ABI1 PL system reveals 425 putative ABI1-associated proteins. a. The workflow for identifying ABI1-associated proteins TurboID-based PL system. b. The proteins identified by biotin affinity purification from TurboID-ABI1-Myc seedling without (ABI1-SF-CK) or with (ABI1-SF-ABA) ABA treatment. The proteins identified by biotin affinity purification from TurboID-Myc transgenic plants were used as background (TurboID-SF). c. The biotinylated peptides (upper) and proteins (bottom) on the beads after
trypsin digestion, from samples of TurboID-ABI1-Myc seedling without (ABI1-EF-CK) or with (ABI1-EF-ABA) ABA treatment. The samples from TurboID-Myc transgenic plants were used as background (TurboID-EF).
d. Enriched GO terms of ABI1-associated proteins. e. Yeast-two-hybrid assays validating the interaction between ABI1 and randomly selected ABI1-associated proteins.

Figure 3
Quantitative and overlapping analysis of ABI1-associated proteins. a. A label-free quantitative (LFQ) MS analysis of the proteins identified by biotin affinity purification from the TurboID-ABI1-Myc seedling without (ABI1-SF-CK) or with (ABI1-SF-ABA) ABA treatment. The red or green spots indicate the proteins shown significant increased (fold change > 2, p < 0.05) or decreased (fold change < 0.5, p < 0.05) amount in ABI1-SF-ABA, compared to ABI1-SF-CK, respectively. The proteins in Table I or Supplementary Table I are highlighted as blue. b. A label-free quantitative MS analysis of the biotinylated peptides identified from the TurboID-ABI1-Myc seedling without (ABI1-EF-CK) or with (ABI1-EF-ABA) ABA treatment. The red or green spots indicate the biotinylated peptides shown significant increased (fold change > 2, p < 0.05) or decreased (fold change < 0.5, p < 0.05) amount in ABI1-EF-ABA, compared to ABI1-EF-CK, respectively. The peptides from proteins in Table I or II are highlighted as blue. c. Identification of 35 putative ABI1 targets by overlapping ABI1-associated proteins with ABA-responsive phosphoproteomics, and putative targets of SnRK2.6 and CKL2. The list of ABA-responsive phosphoproteomics and putative targets of SnRK2.6 and CKL2 were obtained from Wang et al., 2020. d. Overlap of ABI1-associated proteins, putative TOR-target proteins, and LST8-1 and RAPTOR1B interactors. The list of putative TOR-target proteins and LST8-1 and RAPTOR1B interactors were obtained from Van Leene et al., 2019.
### Figure 4

ABI1 physically interacts with and positively regulates RAF activity. 

**a.** ABI1 regulates kinase activity of recombinant RAF-KDs. The recombinant RAF-KDs were incubated with ABI1 purified from E. coli in the presence of [γ-32p]ATP. Autoradiograph (upper) and Coomassie staining (lower) show phosphorylation and loading, respectively, of purified RAF-KDs and PP2Cs. Recombinant SnRK2.6 was used as a control. Images shown are representative of at least two independent experiments. 

|         | B3 subgroup |         | B2 subgroup |
|---------|-------------|---------|-------------|
|         | RAF2-KD     | RAF3-KD | RAFLK-KD    |
| ABI1 kDa| +           | +       | +           |
| lane 1  | 100         | 70      | 100         |
| lane 2  | 90          | 60      | 90          |
| lane 3  | 80          | 50      | 80          |
| lane 4  | 70          | 60      | 70          |
| lane 5  | 60          | 50      | 60          |
| lane 6  | 50          | 40      | 50          |
| lane 7  | 40          | 30      | 40          |
| lane 8  | 30          | 20      | 30          |

**b.** In-gel kinase assay result

**c.** Relative intensity

ABI1 positively regulates kinase activity of recombinant RAF-KDs. The recombinant RAF-KDs were incubated with ABI1 purified from E. coli in the presence of [γ-32p]ATP. Autoradiograph (upper) and Coomassie staining (lower) show phosphorylation and loading, respectively, of purified RAF-KDs and PP2Cs. Recombinant SnRK2.6 was used as a control. Images shown are representative of at least two independent experiments.
showing the ABA- and mannitol-triggered RAF-SnRK2 activation in wild type and abi1abi2hab1pp2ca mutant. Images shown are representative of three independent experiments. c. The relative radioactivity of the RAFs in control and mannitol-treated wild type and abi1abi2hab1pp2ca samples. Radioactivity levels of the OK bands were normalized using wild type after mannitol treatment. Error bars, SD (n = 3 independent biological replicates). Two-tailed paired t-tests, ***p < 0.001.

Figure 5

ABI1 promotes RAF3 activity by dephosphorylating a phosphoserine in the P-loop. a. Overview of dimethyl-labeling-based quantitative proteomics for phosphosite identification. b. Five serine/threonine residues showed abolished phosphorylation after incubation with ABI1. An unaffected phosphosite, Ser725, was used as control. Data shown are representative of at least two independent experiments. c. RAF3 activity measured for glutamine substitution mutants. The kinase activity of wild type and mutated recombinant RAF3-KD in the presence of $\gamma$-32P]ATP. Autoradiograph (upper) and Coomassie staining (lower) show phosphorylation and loading of purified RAF3-KD, respectively. Images shown are representative of at least two independent experiments. d. Sequence alignment showing the conserved phosphosites (highlighted) in the P-loop of Arabidopsis B2, B3 RAFs, PpARK/PpCTR1 from Physcomitrella patens, and human b-RAF. Arrows indicate phosphosites identified in human b-Raf. e. Activation of the RD29B-LUC reporter gene by wild type and the mutated RAF3 in the protoplasts of OK100-nonu. Error bars, SD (n = 6 individual transfections). Two-tailed paired t-tests, **p < 0.01, ***p < 0.001.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- nrreportingsummaryABI1.pdf
- SIABI1TurbolDNov16.pdf
- SupplementaryDataset1APproteins.xlsx
- SupplementaryDataset2BioinPeptides.xlsx
- SupplementaryDataset3ABI1associatedproteins.xlsx
- SupplementaryDataset4GO.xlsx
- SupplementaryDataset5Overlap.xlsx
- SupplementaryDataset6TORsignaling.xlsx
- SupplementaryDataset7RAF3targetsites.xlsx
- SupplementaryDataset8Primers.xlsx