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Letter to the Editor

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Immunopurification and Mass Spectrometry Identifies Protein Phosphatase 2A (PP2A) and BIN2/GSK3 as Regulators of AKS Transcription Factors in *Arabidopsis*

Dear Editor,

ABA induces the phosphorylation of three basic helix-loop-helix (bHLH) transcription factors, called AKSs (ABA-responsive kinase substrates; AKS1, AKS2, and AKS3). The unphosphorylated AKSs facilitate stomatal opening through promoting the transcription of genes encoding inwardly rectifying K^+ channels (Takahashi et al., 2013). AKS1 and AKS3 are also regulators of flowering (Ito et al., 2012). However, the kinases and phosphatases that directly control the phosphorylation status of AKSs *in vivo* have not been fully characterized. Here, our proteomic analyses provide evidence supporting that AKSs are phosphorylated by GSK3 kinases and dephosphorylated by protein phosphatase 2A (PP2A).

PP2A is a ubiquitous and conserved serine/threonine phosphatase. Studies in mammals have shown that PP2A is one of the most important phosphatases for cellular regulation, with broad substrate specificity and diverse cellular functions. PP2A is a heterotrimeric complex composed of structural A, catalytic C, and regulatory B subunits. The A subunit is the scaffold required for the formation of the heterotrimeric complex, whereas the B subunit recruits specific substrates. The *Arabidopsis* genome encodes at least three A subunits, 17 B subunits, and five C subunits (Jonassen et al., 2011). Genetic studies have shown important function of PP2A in plant growth, development, and adaptation (Lillo et al., 2014), but its substrates have not been studied systematically. In addition, it is unknown whether each B subunit associates with different A subunit isoforms or non-selectively associates with all A and C subunits.

To characterize the PP2A complex, here we performed stable isotope-labeling in *Arabidopsis* (SILIA) followed by immunoprecipitation and quantitative mass spectrometry (SILIA-IP-MS) to identify proteins associated with *Arabidopsis* PP2A B′α and B′β subunits (Supplemental Figure 1). Transgenic *Arabidopsis* plants that express PP2A B′α or B′β protein fused with yellow fluorescence protein (YFP) driven by its native promoter (*B′α-YFP* and *B′β-YFP*) were grown in soil supplemented with natural nitrogen-14 fertilizer. Transgenic plants expressing YFP alone under the constitutive 35S promoter (*35S-YFP*) were grown on medium containing nitrogen-15 (^{15}N). The seeds of the ^{14}N- and ^{15}N-labeled plants were then grown on corresponding ^{14}N and ^{15}N medium for 5 days under light. Proteins were extracted from the seedlings and immunoprecipitation was performed using a polyclonal anti-YFP antibody. After the first washing step, beads of PP2A-YFP and 35S-YFP samples were mixed together and washed further. The eluted proteins were separated in SDS–PAGE gel, in-gel digested, and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The mass spectral data were analyzed using pFind and pQuant software (Supplemental Figure 2) (Liu et al., 2014; Chi et al., 2015). Our results show that both PP2A-B′α and PP2A-B′β interact with all three PP2A A subunits and five C subunits, suggesting that PP2A B′α and B′β do not distinguish the three isoforms of A subunits or the five C subunits (Supplemental Table 1 and Supplemental Figure 3). In addition, B′ε was identified in both B′α-containing complexes and in B′β-containing complexes (Supplemental Table 1 and Supplemental Figure 3).

In addition to PP2A subunits, we also identified several potential substrate proteins that interact with B′α and/or B′β (Supplemental Tables 2 and 3). These include the BZR2/BES1 transcription factor (Supplemental Figure 4), which has been shown to be phosphorylated by GSK3 kinases such as BIN2 and dephosphorylated by PP2A to activate BR-response genes (Tang et al., 2011; Kim and Wang, 2010). Interestingly, the ABA-responsive kinase substrate2 (AKS2), a bHLH transcription factor that activates the expression of potassium channel in guard cells to promote stomatal opening (Takahashi et al., 2013), was identified among the proteins associated with PP2A B′α and B′β (Figure 1–1D).

We performed yeast two-hybrid assays to determine which PP2A subunits directly interact with AKSs protein. The results show that AKS1, AKS2, and AKS3 all interact strongly with PP2A B′α, B′β, B′γ, B′κ (Figure 1E), and interact weakly with PP2A B″β and B″γ (Supplemental Figure 5A), whereas no interaction was detected between AKSs proteins and PP2A A subunits or C subunits (Supplemental Figure 5B). *In vitro* overlay assay showed that recombinant PP2A B′α and B′β bind to AKS2 protein (Figure 1F). These results indicate that AKS proteins directly interact with several PP2A regulatory isoforms, and are therefore likely dephosphorylated by PP2A.

AKS1 has been shown to activate genes, such as *KAT1*, *KAT2*, and *AKT1*, and AKS1 is inactivated by ABA-induced phosphorylation (Takahashi et al., 2013). Therefore, PP2A-mediated dephosphorylation is likely required for activation of AKSs and expression of the AKS-activated genes. Indeed, quantitative RT–PCR analysis showed that the *pp2a b′αβ* double mutant

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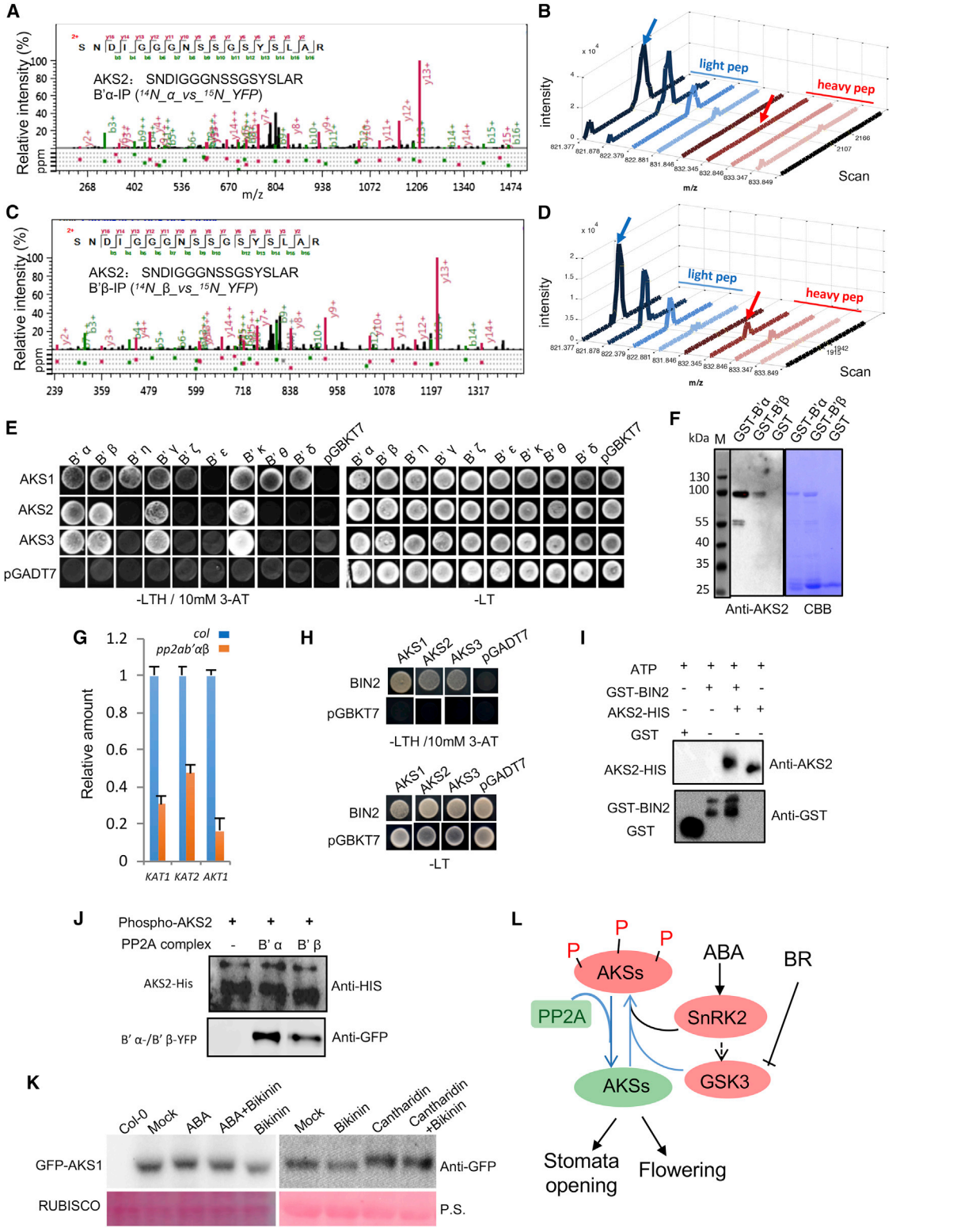

Figure 1. BIN2 Phosphorylates and PP2A Dephosphorylates AKS Transcription Factors.

(A–D) AKS2 was identified as an interacting protein of PP2A B′α and B′β using SILIA-IP-MS analysis. PP2A B′α-YFP or PP2A B′β-YFP transgenic plants were labeled with ^{14}N, and 35S-YFP plants were labeled with ^{15}N. After immunoprecipitation with anti-YFP antibody, the ^{14}N- and ^{15}N-labeled samples were mixed and analyzed by LC-MS/MS. **(A)** and **(C)** MS/MS spectra of a peptide from AKS2 protein. **(B)** and **(D)** show extracted ion chromatograms for co-eluting light and heavy isotope-labeled peptides from **(A)** and **(C)**, respectively, with the blue arrow indicating the ^{14}N mono-isotopic peak and the red arrow indicating the ^{15}N mono-isotopic peak.

(legend continued on next page)

(Tang et al., 2011) expressed these AKS-activated *KAT1*, *KAT2*, and *AKT1* genes at reduced levels compared with wild-type (Supplemental Figure 6 and Figure 1G).

To further study AKSs function, we performed mass spectrometric analysis of affinity-purified AKS1 to identify its phosphorylation sites and interacting proteins. Transgenic seedlings expressing AKS1 fused with green fluorescent protein (GFP-AKS1) were grown for 10 days and then harvested for affinity purification by using GFP-Trap, with wild-type (*col-0*) plants as a control sample. Mass spectrometric analysis identified four phosphorylated serine residues of AKS1 (Supplemental Figure 7). Further, the mass spectrometric analysis identified not only PP2A but also BIN2 kinase and several other GSK3/shaggy-like kinase family members (SK42, ATSK12, ATSK32, ATSK11) specifically in the GFP-AKS1 sample but not the wild-type control (Supplemental Table 4 and Supplemental Figure 8). Yeast two-hybrid assays confirmed that BIN2 interacts with all three AKS members (AKS1, AKS2, AKS3) (Figure 1H). *In vitro* kinase assays showed that BIN2 phosphorylated AKS2, which causes a band shift to slower mobility in the phos-tag gel (Figure 1I). Incubation of the BIN2-phosphorylated AKS2-HIS protein with PP2A that was immunoprecipitated from the *B′α-YFP* or *B′β-YFP* transgenic plants caused dephosphorylation of ASK2, as shown by shift of the ASK2 band to faster mobility in the phos-tag gel (Figure 1J). These results provide evidence showing that BIN2 can phosphorylate and PP2A containing B′α or B′β can dephosphorylate ASK2 *in vitro*.

We then tested the effects of inhibitors of BIN2/GSK3 and PP2A on the phosphorylation status of ASK1 *in vivo*. It has been reported that ABA induces AKS1 phosphorylation, and this was proposed to be mediated by SnRK2.6 kinase based on *in vitro* evidence (Takahashi et al., 2013). Consistent with the previous study, we observed an ABA-induced band shift of AKS1 that is consistent with phosphorylation of ASK1. Treatment with bikinin, a specific inhibitor of BIN2 and related GSK3 kinases (De Rybel et al., 2009), has no obvious effect on the mobility of the AKS1 band without ABA treatment, but partially reduced the AKS1 band mobility shift caused by ABA (Figure 1K), suggesting that BIN2/GSK3 activity is partially responsible for the ABA-induced AKS1 phosphorylation, while an additional kinase such as SnRK2 is likely to also contribute to the phosphorylation. Treatment with the PP2A inhibitor cantharidin (Li and Casida, 1992) caused phosphorylation of AKS1 (Figure 1K, right panel), indicating that PP2A activity is required to maintain dephosphorylation of AKS1 *in vivo*. Co-treatment with cantharidin and bikinin caused a slightly smaller shift of the AKS1 band than cantharidin treatment alone, consistent with the requirement of BIN2/GSK3 for AKS1 full phosphorylation and possible phosphorylation of AKS1 by additional kinases.

In summary, our quantitative proteomic study demonstrates that PP2A B′α and B′β subunits interact with all the isoforms of PP2A A and C subunits, with little specificity to different isoforms of the other subunits. We also identified AKS transcription factors as substrates of PP2A, and demonstrated essential roles of PP2A in the dephosphorylation of ASK1 and activation of the expression of its target genes involved in stomata opening. Proteomic analysis of AKS1-associated proteins not only confirmed its interaction with PP2A, but also revealed its direct interaction with BIN2 and several other GSK3 kinases. Our study thus demonstrates that the phosphorylation status of the AKS transcription factors is controlled not only by the SnRK2 kinase but also the GSK3 kinases and PP2A phosphatase in *Arabidopsis* (Figure 1J). As AKSs are known regulators of stomata opening and flowering, our study implicates PP2A and GSK3 kinases in regulating stomata opening and flowering, possibly downstream of the brassinosteroid and ABA pathways.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

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(E) Yeast two-hybrid assays confirmed the direct interactions between AKSs (AKS1, AKS2, AKS3) and PP2A B′ regulatory subunits.
(F) Gel blot overlay assay of the direct binding between B′α/B′β and AKS2. Recombinant GST-PP2A B′α, GST-PP2A B′β, and GST were separated on SDS–PAGE gels, blotted on nitrocellulose membrane, and probed with MBP-AKS2-HIS followed by anti-AKS2 antibody. Right: a replicate gel stained with Coomassie brilliant blue (CBB).
(G) Transcript abundance of *KAT1*, *KAT2*, *AKT1* in seedlings of *col-0* and *pp2ab′ αβ* double mutant were determined by real-time quantitative RT–PCR. Transcript abundance of each gene was normalized to the amount of *TUB8* ($n = 3$ individual experiments).
(H) Yeast two-hybrid assays show direct interactions between AKSs and BIN2/GSK3 proteins.
(I) The AKS2-HIS, GST-BIN2, and GST proteins were incubated as indicated with ATP in kinase buffer for 3 h, then separated in phos-tag-PAGE gel and immunoblotted using anti-AKS2 and anti-GST antibodies. Phosphorylation is indicated by band mobility shift.
(J) PP2A dephosphorylates the BIN2-phosphorylated AKS2-HIS. AKS2-HIS was phosphorylated by BIN2 *in vitro*, then incubated with immunoprecipitated PP2A (using PP2A B′α-YFP and B′β-YFP) for 3 h in the presence of 30 μm bikinin. The proteins were separated by phos-tag-PAGE gels, and immunoblotted using anti-HIS antibody to detect phosphorylated and unphosphorylated AKS2-HIS and using anti-YFP antibody to detect B′α-YFP and B′β-YFP in another blot.
(K) Six-day-old light-grown seedlings of 35S:GFP-AKS1 transgenic plants were treated with mock solution, 50 μm ABA, 50 μm ABA plus 50 μm bikinin, 50 μm bikinin, 50 μm cantharidin, 50 μm cantharidin plus 50 μm bikinin for 1 h. GFP-AKS1 phosphorylation was analyzed by immunoblotting using anti-GFP antibody. Col-0 plant was used as a negative control. The blot was stained with Ponceau S (P.S; bottom).
(L) A diagram of the model that GSK3 kinases and PP2A phosphatase regulate the phosphorylation and dephosphorylation of AKS proteins to control the stomatal opening behavior and flowering time in *Arabidopsis*. Black lines show previously reported mechanisms, and blue lines show mechanisms uncovered in this study.

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