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GSK3-like kinases positively modulate abscisic acid signaling through phosphorylating subgroup III SnRK2s in *Arabidopsis*

Zhenying Cai[a], Jingjing Liu[a], Haijiao Wang[b], Cangjing Yang[a], Yuxiao Chen[a], Yongchi Li[a], Shanjin Pan[c], Rui Dong[a], Guiliang Tang[c], Juan de Dios Barajas-Lopez[d], Hiroaki Fujii[d], and Xuelu Wang[a,b,1]

[a]State Key Laboratory of Genetic Engineering and Institute of Plant Biology, School of Life Sciences, Fudan University, Shanghai 200433, People's Republic of China; [b]College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, People's Republic of China; [c]Department of Biological Sciences, Michigan Technological University, Houghton, MI 49931; and [d]Molecular Plant Biology Unit, Department of Biochemistry, University of Turku, FIN-20014, Turku, Finland

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***Arabidopsis* glycogen synthase kinase 3 (GSK3)-like kinases have versatile functions in plant development and in responding to abiotic stresses. Although physiological evidence suggested a potential role of GSK3-like kinases in abscisic acid (ABA) signaling, the underlying molecular mechanism was largely unknown. Here we identified members of Snf1-related kinase 2s (SnRK2s), SnRK2.2 and SnRK2.3, that can interact with and be phosphorylated by a GSK3-like kinase, brassinosteroid insensitive 2 (BIN2). *bin2-3 bil1 bil2*, a loss-of-function mutant of *BIN2* and its two closest homologs, *BIN2 like 1 (BIL1)* and *BIN2 like 2 (BIL2)*, was hyposensitive to ABA in primary root inhibition, ABA-responsive gene expression, and phosphorylating ABA Response Element Binding Factor (ABF) 2 fragment by in-gel kinase assays, whereas *bin2-1*, a gain-of-function mutation of *BIN2*, was hypersensitive to ABA, suggesting that these GSK3-like kinases function as positive regulators in ABA signaling. Furthermore, BIN2 phosphorylated SnRK2.3 on T180, and SnRK2.3^{T180A} had decreased kinase activity in both autophosphorylation and phosphorylating ABFs. Bikinin, a GSK3 kinase inhibitor, inhibited the SnRK2.3 kinase activity and its T180 phosphorylation in vivo. Our genetic analysis further demonstrated that BIN2 regulates ABA signaling downstream of the PYRABACTIN RESISTANCE1/PYR1-LIKE/REGULATORY COMPONENTS OF ABA RECEPTORS receptors and clade A protein phosphatase 2C but relies on SnRK2.2 and SnRK2.3. These findings provide significant insight into the modulation of ABA signaling by *Arabidopsis* GSK3-like kinases.**

signal transduction | phosphorylation cascades | kinase activation

Abscisic acid (ABA) is a key phytohormone in responding to various abiotic stresses and in plant development, such as embryogenesis, seed dormancy and germination, and root elongation (1–4). Since the discovery of ABA receptors, PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) (5, 6), a core ABA signaling pathway has been proposed. Without ABA, clade A protein phosphatase 2Cs (PP2Cs) inhibit the activity of subgroup III Snf1-related kinase 2s (SnRK2s) by physical interaction and dephosphorylation (7, 8), leading to inhibition of downstream transcription factors required for ABA-responsive gene expression (9–11). Perception of ABA causes conformational changes of PYR/PYL/RCAR proteins, which facilitate their binding to PP2Cs to release their inhibition on SnRK2s (7, 11). The activated SnRK2s phosphorylate transcription factors, such as ABA Response Element Binding Factors (ABFs), to regulate ABA responsive gene expression (7, 11).

The *Arabidopsis* subgroup III SnRK2 family contains three members, SnRK2.2, SnRK2.3, and SnRK2.6 (12, 13). *SnRK2.6* is specifically expressed in guard cells (12) to regulate ABA-mediated stomata movement. *SnRK2.2* and *SnRK2.3* are ubiquitously expressed and responsible for ABA-regulated seed germination and primary root elongation (13). Their triple knockout *snrk2.2 snrk2.3 snrk2.6* displays a considerable resistance to ABA, whereas single or double mutants could not, suggesting their redundant role in mediating ABA signaling (14, 15). Besides ABA, osmotic stresses also activate SnRK2s, probably through a mechanism independent of ABA biosynthesis and clade A PP2Cs (3, 16–19). However, how SnRK2s are activated is not fully understood. It is reported that several members of SnRK2s can be regulated by upstream kinases (17, 20), and autophosphorylation activity of recombinant SnRK2.2 and SnRK2.3 is only one-tenth to one-fifth of that of SnRK2.6, suggesting that some SnRK2s may be activated by yet unknown kinases in vivo (21).

Glycogen synthase kinase 3s (GSK3s) can phosphorylate a number of proteins to regulate their activity, stability, and subcellular localization in diverse systems (22, 23). In *Arabidopsis*, GSK3/Shaggy-like kinases (ASKs) contain at least 10 members and participate in many biological processes (23). BRASSINOSTEROID INSENSITIVE 2 (BIN2), a GSK3-like kinase, functions as a negative regulator in brassinosteroid (BR) signaling by phosphorylating transcription factors BRASSINAZOLE RESISTANT 1 and BRI1-EMS-SUPPRESSOR 1 (BES1) (24, 25). BIN2 also participates in many other biological processes, such as auxin signaling (26) and stomata development (27, 28). Plant GSK3-like kinases apparently play important roles in responding to many

Significance

Abscisic acid (ABA) is a key stress-responsive hormone. Subgroup III Snf1-related kinase 2s (SnRK2s) are crucial positive regulators in ABA signaling pathway, but it is still not clear how SnRK2s are activated. In addition, besides ABA, some abiotic stresses can also activate SnRK2s with unknown mechanisms. Here we provide several lines of evidence to strongly support that brassinosteroid insensitive 2, a glycogen synthase kinase 3 (GSK3)-like kinase, interacts with and phosphorylates SnRK2s on specific novel sites to activate SnRK2s, which provides significant insights into the function of GSK3-like kinases in ABA signaling and transactivation of SnRK2s. Moreover, many GSK3-like kinases are regulated at transcriptional and/or posttranslational levels by many abiotic stresses, implying the presence of direct regulation of ABA signaling by other abiotic stresses.

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[1]To whom correspondence should be addressed. E-mail: xlwang@mail.hzau.edu.cn.

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abiotic stresses (23). For example, *BIL2*, a close homolog of *BIN2*, can complement yeast salt insensitive mutants (29) and mediate salt tolerance in *Arabidopsis* (30), and another GSK3-like kinase, *ASKα* (*AtSK11*) participates in regulating redox stress response through phosphorylating glucose-6-phosphate dehydrogenase in *Arabidopsis* (31). In rice, knockout of *OsGSK1*, an *AtBIN2* ortholog, showed an enhanced tolerance to cold, heat, high salt, and drought (32). Interestingly, *bin2-1*, a gain-of-function mutation of *BIN2*, was hypersensitive to ABA in primary root inhibition (33), implying its potential role in ABA signaling, but the underlying mechanism is unknown.

In this study, when we initially identified BIN2-interacting proteins with liquid chromatography tandem mass spectrometry (LC-MS/MS) using immunoprecipitated (IPed) proteins from *BIN2-FLAG* transgenic plants, we found that SnRK2.2 may interact with BIN2. We further confirmed that BIN2 physically interacts with all subgroup III SnRK2s both in vitro and in vivo and is able to phosphorylate SnRK2.2 and SnRK2.3 and enhances their kinase activity. We identified T180 as a novel phosphorylation site of SnRK2.3 by BIN2 kinase, which is important for SnRK2.3's activation. Primary root inhibition assay, ABA-responsive gene expression, and phosphorylating ABF fragment by in-gel kinase assays using *bin2-1* and *bin2-3 bil1 bil2* (34) mutants indicated that BIN2 and its homologs act as positive regulators in ABA signaling. Immuno-kinase assay and quantitative MS results indicated that bikinin inhibited the T180 phosphorylation of SnRK2.3 and its kinase activity. We generated double and multiple mutants between *BIN2*-related mutants and ABA signaling mutants, and their phenotypic analysis indicated that BIN2 enhances ABA responses through SnRK2s, but not PYR/PYL/RCARs and PP2Cs. Therefore, we propose that BIN2 phosphorylates SnRK2s to promote their activity.

Results

BIN2 Interacts with Subgroup III SnRK2s and Phosphorylates SnRK2.2 and SnRK2.3. In an early study we found that ABA inhibits primary BR signaling outputs, likely through BIN2 or its upstream components (35). To identify proteins interacting with BIN2, we carried out an LC-MS/MS experiment with IPed BIN2-FLAG complex from *35S::BIN2-FLAG* plants. Interestingly, we identified a peptide corresponding to SnRK2.2 (Fig. S1). We then tested physical interaction of BIN2 with SnRK2.2, SnRK2.3, and SnRK2.6 using a bimolecular fluorescence complementation (BiFC) assay, and we found that BIN2 interacts with all subgroup III SnRK2s in both cytoplasm and nucleus of *Nicotiana benthamiana* pavement cells (Fig. 1*A*). Pull-down assays demonstrated that GST-SnRK2s can directly interact with His-BIN2 in vitro (Fig. 1*B*). Using a semi-in vivo pull-down assay, we found that each SnRK2 protein was able to pull down BIN2-FLAG protein expressed in transgenic seedlings (Fig. 1*C*). We then conducted in vitro kinase assays to test whether BIN2 and SnRK2s can phosphorylate each other and found that BIN2 can phosphorylate both wild-type SnRK2.2 and SnRK2.3 and their kinase-dead forms, SnRK.2.2^{K52N} (K52N) and SnRK2.3^{K51N} (K51N) (Fig. 1 *D* and *E*), but not SnRK2.6 or SnRK2.6^{K50N} (K50N) (Fig. 1*F*), and the SnRK2s cannot phosphorylate either BIN2 or its kinase dead form, BIN2^{K69R} (K69R) (Fig. 1 *D*–*F*). Although SnRK2s failed to phosphorylate BIN2 in vitro, because interaction between SnRK2s and BIN2 may alter BIN2 activity, we conducted phosphorylation assays on BES1, a BIN2 substrate in the BR signaling pathway (25), by BIN2 kinase in vitro with or without SnRK2s. Additional SnRK2s did not affect BES1 phosphorylation by BIN2 kinase (Fig. S2), suggesting these SnRK2s may not directly influence BIN2 activity.

BIN2 and Its Homologs Play a Positive Role in Modulating ABA Signaling. Therefore, we focused our investigation on the potential roles of BIN2 in ABA signaling. We discovered that *bin2-3 bil1 bil2* was less sensitive to ABA in primary root inhibition than wild-type Ws-2 (Fig. 2 *A* and *B*). *bin2-3 bil1 bil2* was hyposensitive to ABA in seed germination (Fig. S3 *A*–*C*). The expression levels of ABA-responsive genes determined by quantitative real-time RT-PCR (qRT-PCR) were also significantly compromised in *bin2-3 bil1 bil2* (Fig. 2*C* and Fig. S3*D*). In contrast, *bin2-1* was hypersensitive to ABA in both primary root inhibition (Fig. 2 *D* and *E*) and ABA-responsive gene expression compared with wild-type Col-0 (Fig. 2*F*). Basal levels of ABA-responsive gene expression were also significantly reduced in *bin2-3 bil1 bil2* (Fig. 2*G*) and largely enhanced in *bin2-1* (Fig. 2*H*). In addition, treatment of bikinin, an inhibitor of GSK3 kinases (36), significantly inhibited ABA-responsive gene expression (Fig. 2*I*). We further conducted an in-gel kinase assay on the ABF2 fragment (Gly73 to Gln120) by their protein extracts and found that the ABA-activated bands were significantly weaker in *bin2-3 bil1 bil2* and largely stronger in *bin2-1* than that in their corresponding wild types (Fig. 2 *J* and *K* and Fig. S3 *E* and *F*). Taken together, these data suggest that BIN2 and its homologs play a positive role in modulating ABA signaling.

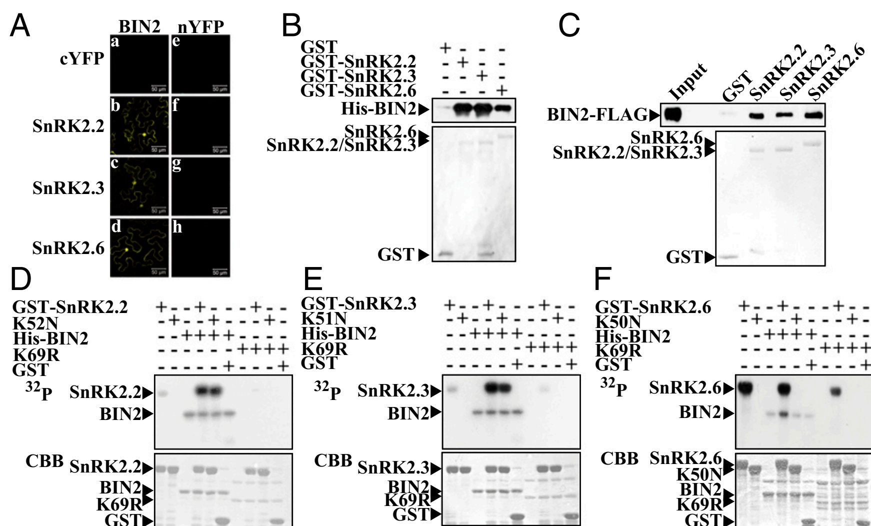

Fig. 1. BIN2 interacts with subgroup III SnRK2s and phosphorylates SnRK2.2 and SnRK2.3. (*A*) BIN2 interacts with SnRK2.2, SnRK2.3, and SnRK2.6 in BiFC assay. nYFP-BIN2 or nYFP was cotransformed into pavement cells of *N. benthamiana* with cYFP (*a* and e), SnRK2.2-cYFP (*b* and *f*), SnRK2.3-cYFP (*c* and *g*), or SnRK2.6-cYFP (*d* and *h*). (Scale bar, 50 μm.) (*B*) SnRK2.2, SnRK2.3, and SnRK2.6 interact with BIN2 in vitro. (*C*) SnRK2.2, SnRK2.3, and SnRK2.6 can pull down BIN2-FLAG in whole-protein extract from *BIN2-FLAG* transgenic plants. (*D*) BIN2 phosphorylates SnRK2.2 and SnRK2.2^{K52N} (K52N) in vitro. (*E*) BIN2 phosphorylates SnRK2.3 and SnRK2.3^{K51N} (K51N) in vitro. (*F*) BIN2 cannot phosphorylate SnRK2.6 or SnRK2.6^{K50N} (K50N) in vitro. K69R is the abbreviation for BIN2^{K69R}.

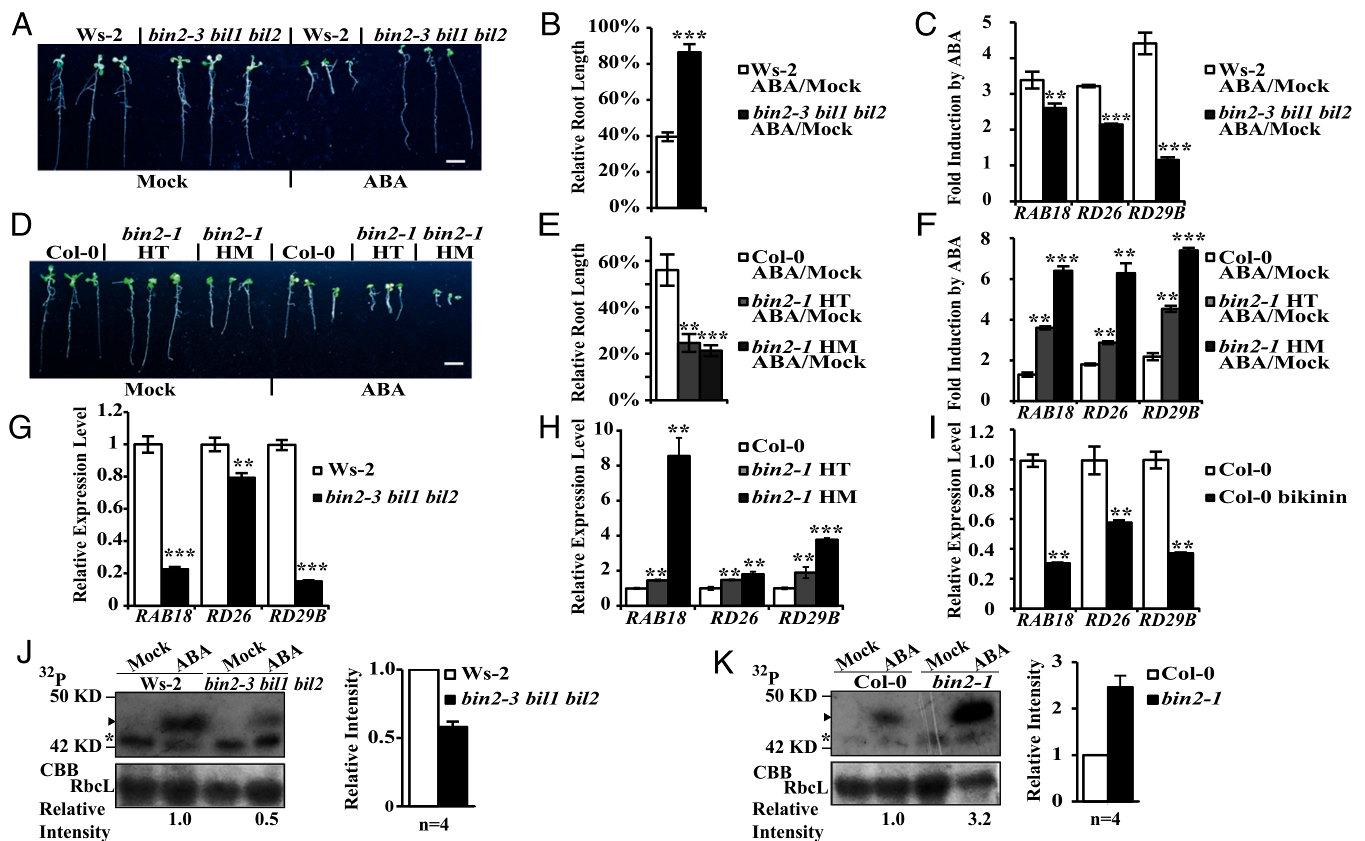

Fig. 2. BIN2 plays positive roles in modulating ABA signaling outputs. (*A*) Primary root phenotype of wild-type (Ws-2) and *bin2-3 bil1 bil2* grown on medium with or without (Mock) 10 μM ABA. (*B*) Statistic analysis of relative primary root length of Ws-2 ($n = 25$) and *bin2-3 bil1 bil2* ($n = 25$). (*C*) Induction of *RAB18, RD26*, and *RD29B* expression by ABA in Ws-2 and *bin2-3 bil1 bil2*. Seedlings were treated with 50 μM ABA or solvent only (Mock) for 1 h. (*D*) Primary root phenotype of wild-type (Col-0) and *bin2-1* grown on medium without (Mock) or with 10 μM ABA. (*E*) Statistic analysis of relative primary root length for Col-0 ($n = 20$), *bin2-1* heterozygote (HT) ($n = 35$), and *bin2-1* homozygote (HM) ($n = 20$). (*F*) Induction of *RAB18, RD26*, and *RD29B* expression by ABA in Col-0 and *bin2-1*. Seedlings were treated with 50 μM ABA or solvent only (Mock) for 1 h. (*G*) Expression levels of *RAB18, RD26*, and *RD29B* in *bin2-3 bil1 bil2* and Ws-2. Expression level of each gene in Ws-2 was normalized to "1." (*H*) Expression level of *RAB18, RD26*, and *RD29B* in *bin2-1*and Col-0. Expression level of each gene in Col-0 was normalized to "1." (*I*) Bikinin inhibits expression of *RAB18*, *RD26*, and *RD29B* in Col-0. The expression level of each gene in Col-0 grown on medium with or without (Mock) 10 μM bikinin. Expression level of each gene in Col-0 grown on medium without bikinin was normalized to "1." (*J*) Signal intensity of in-gel kinase assay on phosphorylating the ABF2 fragment after treatment with 50 μM ABA or solvent only (Mock) for 1 h. (*K*) Signal intensity of in-gel kinase assay on phosphorylating the ABF2 fragment after treatment with 50 μM ABA or solvent only (Mock) for 1 h. In *J* and *K*, the arrowheads indicate the ABA-induced bands representing the activated SnRK2s. "*" indicates unknown, none ABA-induced bands, and right graphs in *J* and *K* show relative radioactivity intensity of ABA-inducible bands (means ± SE, $n = 4$). The relative intensity of ABA-inducible bands in both Ws-2 and Col-0 after ABA treatment was normalized to "1." Coomassie brilliant blue R250 staining (CBB) of the large subunit of Rubisco (RbcL) was used as the loading control. Also see replicates 2–4 in Fig. S3 *E* and *F*. (Scale bars, 1 cm in *A* and *D*.) Primary root length of each material in mock treatment was normalized to 100% in *B* and *E*. Expression level of each gene for each material under mock treatment was normalized to "1" in *C* and *F*. Values are means ± SE for *B* and *C* and *E–I*. Student's *t* test was used to determine the significance of the indicated comparisons. Significant levels: $^{**}P < 0.01$; $^{***}P < 0.001$.

BIN2 Phosphorylates SnRK2.3 on T180 to Promote Its Kinase Activity. To investigate the biochemical mechanisms by which BIN2 phosphorylates SnRK2s and enhances ABA signaling, we used a mass spectrum approach and identified a number of potential phosphorylation sites of SnRK2.3^{K51N} (K51N) by BIN2 kinase in vitro, including S172, S176, T177, and T180 (Fig. S4 *A–C*). We then mutated each site to Ala in combination with K51N mutation, and they were designated as K51N S172A, K51N S176A, K51N T177A, and K51N T180A, respectively. We also mutated S168, which is located in a typical GSK3 recognition motif S/TxxxS/T, to Ala (K51N S168A). In vitro phosphorylation assays with BIN2 kinase indicated that BIN2 can still strongly phosphorylate K51N S168A, K51N S172A, and K51N T177A but phosphorylates K51N S176A with a reduced activity and almost no phosphorylation on K51N T180A (Fig. 3*A*). The decreased phosphorylation of K51N T180A was not due to a reduced interaction between BIN2 and K51N T180A (Fig. S5*A*), suggesting that T180 of SnRK2.3 may be a key phosphorylation site by BIN2 kinase. Furthermore, SnRK2.3^{T180A} (T180A) had extremely low autophosphorylation activity and decreased ability to phosphorylate ABF2 fragment, a widely used substrate of SnRK2s (7), demonstrating that T180 of SnRK2.3 is crucial for its activation (Fig. 3*B*). However, mutation of T180 to Asp (D) and Glu (E) failed to mimic a constitutively active SnRK2.3 in vitro (Fig. S5*B*). To investigate T180 function in vivo, we compared the responses of Col-0, *SnRK2.3-OX* (line 3), and *SnRK2.3^{T180A}-OX* (line 7) to ABA by measuring expression levels of *RAB18*, *RD26*, and *RD29B*. *SnRK2.3-OX* showed hypersensitivity to ABA, whereas *SnRK2.3^{T180A}-OX* had similar sensitivity to ABA compared with Col-0, implying that T180 is a key residue for transmitting ABA signaling in vivo (Fig. 3*C*). To test whether phosphorylation on T180 of SnRK2.3 by BIN2 can enhance its kinase activity, we then measured the activity of SnRK2.3 with additional BIN2 or BIN2^{K69R} (kinase dead form) in vitro. We found that BIN2 greatly enhanced phosphorylation of SnRK2.3 and ABF2 fragment compared with BIN2^{K69R} (Fig. 3*D*), although SnRK2.3

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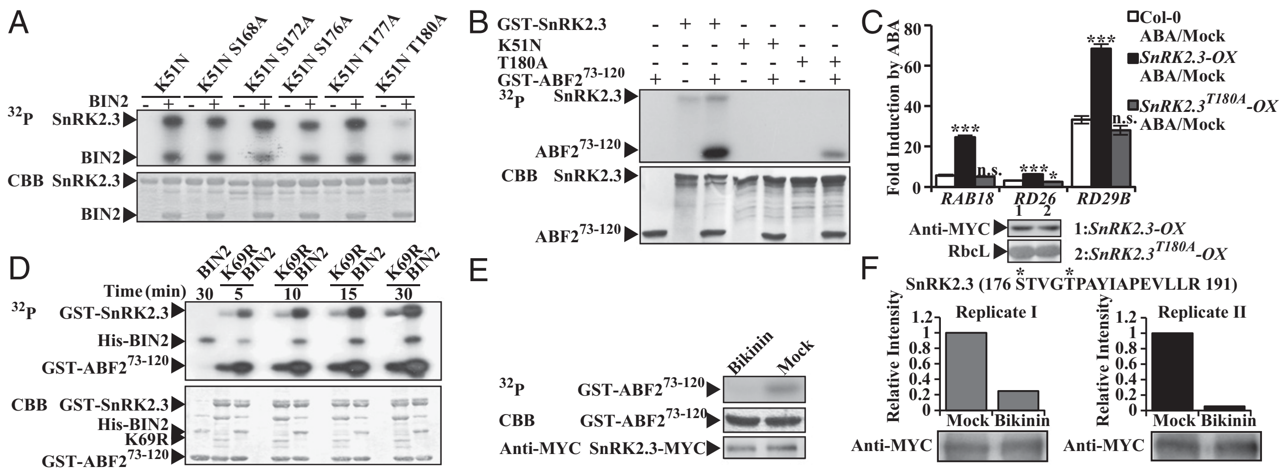

Fig. 3. BIN2 phosphorylates SnRK2.3 and enhances its kinase activity. (*A*) In vitro kinase assays of various mutant forms of SnRK2.3 with BIN2 kinase. Potentially phosphorylated Ser or Thr residues of SnRK2.3 were mutated to Ala, and each mutation was combined with a kinase dead mutation K51N. $SnRK2.3^{K51N\ S168A}$ is abbreviated for K51N S168A, and the same rule also applied for other mutant forms. (*B*) In vitro phosphorylation assays of SnRK2.3 and $SnRK2.3^{T180A}$ on the ABF2 fragment. (*C*) Relative expression levels of *RAB18*, *RD26,* and *RD29B* in Col-0, *SnRK2.3-OX*, and *SnRK2.3^{T180A}-OX* after treatment with 50 μM ABA or solvent only (Mock) for 1 h. Expression level of each gene for each material under mock treatment was normalized to "1." Values are means ± SE. Student's *t* test was used to determine the significance of the indicated comparisons. Significant levels: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; and n.s. (not significant, $P > 0.05$). (*D*) In vitro phosphorylation assays of SnRK2.3 on ABF2 fragment in the presence of BIN2 or $BIN2^{K69R}$ (K69R). (*E*) Immunokinase assays of SnRK2.3-MYC on ABF2 fragment. The *SnRK2.3-MYC-OX* plants were grown on 1/2 MS without (Mock) or with 5 μM bikinin for 10 d and treated with 50 μM ABA for 1 h. The IPed SnRK2.3-MYC was used to phosphorylate ABF2 fragment. (*F*) Quantitative mass spectrum analysis on phosphorylation of the IPed SnRK2.3-MYC. SnRK2.3-MYC was IPed from seedling as described in Fig. 3*E* and digested by trypsin. Phospho-peptides were enriched with TiO_2 beads. The sequence of phospho-peptide for quantification is shown. Phosphorylation sites are marked with "*." Signal intensity of the target phospho-peptide from the mock treatment was normalized to "1." Two independent experiments were conducted.

similarly interacted with both BIN2 and $BIN2^{K69R}$ (Fig. S5*C*), supporting that BIN2 phosphorylates SnRK2.3 at T180 to enhance its activation.

To determine the effect of GSK3s on SnRK2.3 activation in vivo, we IPed SnRK2.3-MYC from transgenic seedlings grown on 1/2 MS medium with or without (Mock) 5 μM bikinin after 50 μM ABA treatment for 1 h, and we found that bikinin significantly inhibited the activity of SnRK2.3-MYC on phosphorylating ABF fragment (Fig. 3*E*), and bikinin did not directly inhibit SnRK2.3 in vitro (Fig. S5*D*). We quantified the T180 phosphorylation level of the IPed SnRK2.3-MYC from seedlings after ABA treatment. We found that T180s were significantly less phosphorylated in seedlings grown on bikinin than the mock (Fig. 3*F* and Fig. S5 *E* and *F*).

***BIN2*-Promoted ABA Signaling Is Dependent on *SnRK2s*.** To investigate whether BIN2-enhanced ABA signaling is mediated through SnRK2.2 and SnRK2.3 or other components of ABA signaling, we conducted genetic analysis by generating a set of double or multiple mutants of *BIN2*-related mutants with several known ABA signaling mutants. We first generated a multiple mutant of *bin2-1* with ABA receptor quadruple mutant *pyr1 pyl1 pyl2 pyl4.* Because *pyl2* is linked with *erecta* locus (5), we crossed *bin2-1* heterozygote with *er105* to obtain Col-0:*er* and *bin2-1:er* plants, which were used as controls. We found that *pyr1 pyl1 pyl2 pyl4* quadruple mutant was insensitive to ABA in inhibiting primary root elongation, but *bin2-1 pyr1 pyl1 pyl2 pyl4* showed an enhanced sensitivity to ABA, which was similar to the *bin2-1:er* single mutant (Fig. 4 *A* and *B*), and induction of *RAB18*, *RD26*, and *RD29B* expression by ABA in *bin2-1 pyr1 pyl1 pyl2 pyl4* was much higher than that in *pyr1 pyl1 pyl2 pyl4* (Fig. 4*C*). The intensity of ABA-induced bands was also largely higher in *bin2-1 pyr1 pyl1 pyl2 pyl4* than in *pyr1 pyl1 pyl2 pyl4* by an in-gel kinase assay (Fig. S6 *A* and *B*), implying that BIN2 can modulate ABA signaling downstream of ABA receptors. We obtained an *abi2^{G168D}* mutant in Col-0 background through a *bri1-301* suppressor screening in seed germination, which was designated as *abi2-3*. A double mutant of *bin2-1* and *abi2-3* also partially suppressed the insensitive phenotype of *abi2-3* to ABA in primary root inhibition (Fig. 4 *D* and *E*). The induction of *RAB18*, *RD26*, and *RD29B* expression by ABA was higher in *abi2-3 bin2-1* than in *abi2-3* (Fig. 4*F*).

To test whether BIN2-modulated ABA responses are mediated by *SnRK2s*, we created knockdown lines of *SnRK2s* using an RNAi approach in wild-type and *bin2-1*, designated as *SnRK2-RNAi/Col-0* and *SnRK2-RNAi/bin2-1*, respectively. In both RNAi lines, the expression level of *SnRK2.2* was reduced to 1% of wild-type, and the *SnRK2.3* expression level was reduced by 40–60% of wild-type, but the expression level of *SnRK2.6* was only slightly reduced (Fig. S6*C*). In primary root inhibition assays, *SnRK2-RNAi/bin2-1* and *SnRK2-RNAi/Col-0* showed a similar sensitivity to ABA (Fig. 4 *G* and *H*), and the induction of *RAB18*, *RD26*, and *RD29B* expression by ABA in *SnRK2s RNAi/bin2-1* was much lower than in *bin2-1* (Fig. 4*I*), suggesting that BIN2 enhances ABA signaling outputs through these SnRK2s.

Discussion

This study provides several lines of evidence to strongly support that *Arabidopsis* GSK3-like kinases play positive roles in modulating ABA signaling outputs. First, *bin2-3 bil1 bil2* was less sensitive to ABA in primary root inhibition, ABA-induced gene expression, and phosphorylating ABF fragment. Second, gain-of-function mutation of *BIN2*, *bin2-1* was hypersensitive to ABA in both assays. Third, a GSK3 kinase inhibitor, bikinin, strongly inhibited ABA signaling outputs. Fourth, BIN2 can directly interact with SnRK2s and activate their kinase activity. Fifth, bikinin inhibited T180 phosphorylation of SnRK2.3 and its kinase activity in vivo. Finally, genetic analysis also indicated that BIN2 promoted ABA signaling through SnRK2s. Phosphorylation of SnRK2s by GSK3-like kinases is apparently important for their full activation. It has been speculated that activation of SnRK2.2 and SnRK2.3 may require other components (3, 17–19).

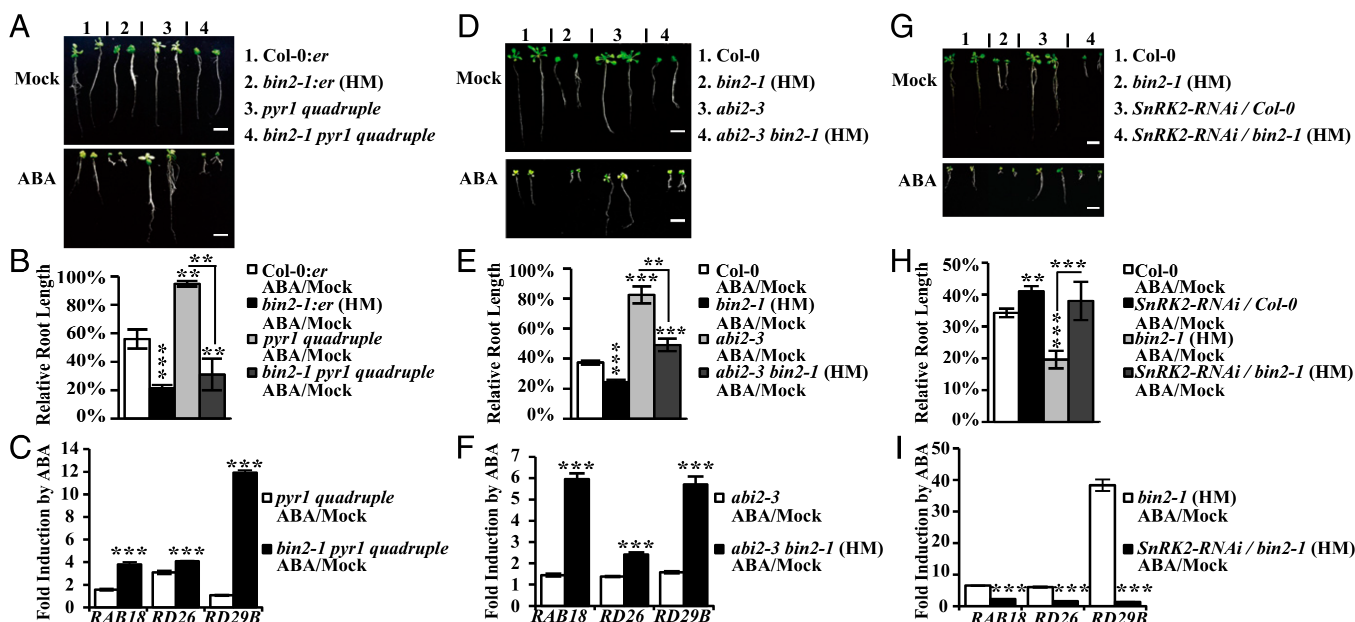

Fig. 4. BIN2 promotes ABA signaling outputs through subgroup III SnRK2s. (*A*) Root phenotype of Col-0:*er*, *bin2-1:er*, *pyr1 pyl1 pyl2 pyl4,* and *bin2-1 pyr1 pyl1 pyl2 pyl4* grown on medium with or without (Mock) 10 μM ABA. (*B*) Relative primary root length in Col-0:*er* ($n = 25$), *bin2-1:er* ($n = 15$), *pyr1 pyl1 pyl2 pyl4* ($n = 25$), and *bin2-1 pyr1 pyl1 pyl2 pyl4* ($n = 20$). (*C*) Induction of *RAB18, RD26,* and *RD29B* expression by ABA in *pyr1 pyl1 pyl2 pyl4* and *bin2-1 pyr1 pyl1 pyl2 pyl4*. Seedlings were treated with 50 μM ABA or solvent only (Mock) for 1 h. (*D*) Root phenotype of Col-0, *abi2-3*, *bin2-1,* and *abi2-3 bin2-1*. (*E*) Relative primary root length in Col-0 ($n = 25$), *bin2-1* ($n = 15$), *abi2-3* ($n = 25$), and *abi2-3 bin2-1* ($n = 20$). (*F*) Induction of *RAB18, RD26,* and *RD29B* expression by ABA in *abi2-3* and *abi2-3 bin2-1*. Seedlings were treated with 50 μM ABA or solvent only (Mock) for 1 h. (*G*) Primary root phenotype of Col-0, *bin2-1*, *SnRK2-RNAi/Col-0,* and *SnRK2-RNAi/bin2-1*. (*H*) Relative primary root length in Col-0 ($n = 20$), *SnRK2-RNAi/Col-0* ($n = 20$), *bin2-1* (HM) ($n = 20$), and *SnRK2-RNAi/bin2-1* (HM) ($n = 20$). (*I*) Induction of *RAB18*, *RD26*, and *RD29B* expression by ABA in *bin2-1* and *SnRK2-RNAi/bin2-1*. Seedlings were treated with 50 μM ABA or solvent only (Mock) for 1 h. (Scale bars, 1 cm in *A*, *D*, and *G*). Primary root length of each material in mock treatment was normalized to 100%. Expression level of each gene for each material under mock treatment was normalized to "1" in *C*, *F*, and *I*. Values are means ± SE in *B*, *C*, *E*, *F*, *H*, and *I*. Student's *t* test was used to evaluate the significance of indicated comparisons. Significant levels: $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

GSK3-like kinases acting as positive modulators to promote SnRK2.2 and SnRK2.3 activity is providing a direct example.

Arabidopsis GSK3-like kinases may play important roles in integrating environmental cues with ABA signaling. It was reported that both ABA and NaCl can induce expression of *BIL2*, and plants overexpressing *BIL2* exhibited an elevated tolerance to salt stress (29, 30). Furthermore, high salinity can activate ASKα, and its overexpression also enhanced seedlings' tolerance to salt (31), and most *Arabidopsis* GSK3-like kinases are up-regulated by NaCl and osmotic stress (37). In addition, low humidity, NaCl, and sorbitol can rapidly activate SnRK2s, which is independent of Clade A PP2C and ABA biosynthesis (16, 17). Therefore, many abiotic stresses may activate SnRK2s by regulating certain GSK3-like kinases at transcriptional or posttranslational levels.

Although BR and ABA have antagonistic effects on seed germination (35, 38), the effect of BRs on ABA signaling outputs is apparently complicated. It was known that BR-deficient mutant *de-etiolated-2 mutant (det2)* (38) and BR-perception mutant *bri1-301* (35) were hypersensitive to ABA in seed germination. BR-deficient mutants *constitutive photomorphogenesis and dwarfism* (*cpd*) and *det2* (39) and BR-perception mutant *bri1* were hypersensitive to ABA in primary root inhibition (40) (Fig. S6 *D* and *E*). A knockout mutant of *Brassinosteroid signaling kinase 5* (*BSK5*), a positive regulator in BR signaling upstream of BIN2, was also hypersensitive to ABA in seed germination and primary root inhibition (41). However, *bes1-D* and *bzr1-D*, dominant mutants with enhanced BR signaling outputs and acting downstream of BIN2, were also hypersensitive to ABA in primary root inhibition (39) (Fig. S6 *F* and *G*). Therefore, BR signaling may not only play a role in inhibiting BIN2 activity to inhibit ABA signaling but also promote ABA signaling through downstream transcription factors.

We identified T180 as a novel transphosphorylation site of SnRK2.3 by BIN2, which was not among these well-known autophosphorylation sites of SnRK2s (21). Addition of bikinin to 1/2 MS medium inhibited the T180 phosphorylation in vivo (Fig. 3*F*). The subgroup III SnRK2s share high similarity in amino acid sequence with an identical activation loop (Fig. S7). It is likely that BIN2 can phosphorylate the conserved T181 of SnRK2.2 to enhance its activity. Interestingly, in vivo phosphorylation of SnRK2.3 on T180 and SnRK2.2 on T181 had been found in a previous study after ABA treatment (42). In addition, the fact that SnRK2.6^{K50N} cannot be phosphorylated by BIN2 indicated that BIN2 may not activate SnRK2.6 in vitro. Because SnRK2.6 may also be activated through a PP2C-independent pathway (16, 17), it could be regulated by other GSK3-like kinases or other proteins in vivo. On the basis of these current and previous studies, we propose that certain GSK3-like kinases may be induced or activated by various abiotic stresses, including NaCl, drought, and wounding at transcription or protein levels (30, 31, 37). The activated GSK3-like kinases promote SnRK2.2 and SnRK2.3 by phosphorylating T181 and T180, probably also S177 and S176, respectively, to enhance ABA responses (Fig. S8). In addition, ABA early signaling may also regulate GSK3-like kinases, such as induction of *BIL2* expression, to form a positive feedback loop to enhance ABA signaling. Further studies are needed to fully understand the mechanisms of how abiotic stresses, such as NaCl, activate GSK3-like kinases.

Materials and Methods

Plant Materials and Growth Condition. *Arabidopsis* materials, including various mutants, transgenic plants, and their corresponding wild types used in

this study, are summarized in Table S1. Plants were grown in a growth room at 23 °C under long-day conditions (16 h light/8 h dark). For primary root inhibition assay and RNA samples for marker gene expression, seedlings were grown on 1/2 MS medium in a growth chamber (Percival) at 23 °C under long-day conditions (16 h light/8 h dark). *SI Materials and Methods* describes in detail *abi2-3* and the construction of *SnRK2 RNAi* lines (Table S2).

Primary Root Inhibition Assay. Seeds were sterilized and sown on 1/2 MS medium containing 0.4% gellan gum and 1% (m/vol) sucrose. After stratification at 4 °C for 4 d, plates were put into a growth chamber under long-day conditions (16 h light/8 h dark) at 23 °C. After 4 d, seedlings were transferred to 1/2 MS medium containing 1% (m/vol) sucrose with or without 10 μM ABA under long-day conditions at 23 °C. Seedlings were allowed to grow for another 10 d and then collected for phenotypic analysis.

Semi-in Vivo Pull-Down. Details in *SI Materials and Methods*.

Gene Expression Analysis by Quantitative Real-Time PCR. Seeds were sterilized and then sown to 1/2 MS medium containing 0.4% gellan gum, with or without 10 μM bikinin (Calbiochem). The growth condition was the same as that in *Primary Root Inhibition Assay*. Ten-day-old seedlings were collected for indicated treatments and then were ground to fine powder in liquid nitrogen. Total RNA was extracted using the Tiangen RNApre Plant Kit. Procedures described previously (43) were used for cDNA synthesis and qRT-PCR. Primers for qRT-PCR are listed in Table S3. PCRs were performed with an Eppendorf iCycler.

Kinase Assay with the IPed SnRK2.3-MYC. Details in *SI Materials and Methods*.

In-Gel Kinase Assay. Details in *SI Materials and Methods*.

In Vitro Kinase Assay. Details in *SI Materials and Methods*.

Transient Transformation and BiFC Assay. *Agrobacterium* strain *LBA4404* was transformed with *SnRK2-cYFP*, *nYFP-BIN2*, or control vectors and then infiltrated into young leaves of *N. benthamiana*. Plants were put in the dark for 1 d and then allowed to grow under long-day conditions (16 h light/8 h dark). Fluorescence signals in pavement cells were visualized by confocal microscopy (Zeiss) after 24–36 h.

Quantitative Mass Spectrum Analysis on the in Vivo Phosphorylation of SnRK2.3. Details in *SI Materials and Methods*.

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