Supporting Information

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SI Materials and Methods

Identification of Protein Interacting with BIN2 by Liquid Chromatography/Tandem Mass Spectrometry. Brassinosteroid insensitive 2 (BIN2)-FLAG seedlings were ground to powder in liquid nitrogen and solubilized with 2× protein extraction buffer [100 mM Tris·HCl (pH 7.5), 300 mM NaCl, 2 mM EDTA (pH 8.0), 1% Trion X-100, 10% (vol/vol) glycerol, and protease inhibitor mixtures (AMRESCO)]. The extracts were centrifuged at 20,000 × *g* for 10 min twice, and the resulting supernatants were collected and incubated with pretreated anti-FLAG M2 agarose gel (Sigma) at 4 °C. After incubation, the agarose gel was collected by centrifugation at 800 × *g* for 2 min and washed three times with 1× protein extraction buffer. Immunoprecipitation complex was eluted by 0.1 M glycine (pH 3.5) and then digested by trypsin. The extracted peptides were concentrated and analyzed by a Finniqan LTQ mass spectrometer (Thermoquest) coupled with a surveyor HPLC system (Thermoquest).

Semi-in Vivo Pull-Down. Crude protein extract was obtained the same as in *Identification of Protein Interacting with BIN2 by Liquid Chromatography/Tandem Mass Spectrometry*, above. After centrifugation the resulting supernatants was collected and incubated with either GST or GST–Snf1-related kinase 2s (SnRK2s) preincubated GST beads for 1 h at 4 °C. Beads were washed several times and then resuspended by 1× SDS loading buffer and applied for subsequent analysis.

In Vitro Kinase Assay. For kinase assay to verify the phosphorylation modification, 0.5 μg GST-SnRK2.2, GST-SnRK2.3, and GST-SnRK2.6 or their mutated forms and 0.2 μg His-BIN2 or His-BIN2^{K69R} were used in each kinase assay. For kinase assay to verify the phosphorylation sites, 0.5 μg GST-SnRK2.3^{K51N} and double mutated forms were used in each kinase assay. For measuring the kinase activity of SnRK2.3 and its mutated form, a fragment of ABF2 (Gly73 to Gln120) was fused to GST tag (GST-ABF2$^{73\text{-}120}$) and used as a substrate of SnRK2.3. GST-ABF2$^{73\text{-}120}$ (0.5 μg) was used in the assay. For measuring the effect of BIN2 on SnRK2.3, 0.2 μg His-BIN2 or His-BIN2^{K69R} and 0.5 μg GST-ABF2$^{73\text{-}120}$ were added to the reaction. Kinase reaction buffer was composed of 25 mM Tris (pH 7.4), 12 mM $MgCl_2$, 1 mM DTT, 0.5 mM ATP, and 0.5 μL (10 μCi) [γ-^{32}P] ATP. Recombinant proteins were incubated at 30 °C for 30 min or the indicated time. The 6× SDS loading buffer was added to terminate the reaction. Samples were denatured by boiling and applied for SDS–PAGE.

In Vitro Phosphorylation Assay of BES1. In vitro phosphorylation assay was carried out according to a previous study, with some modifications (1). A half microgram of MBP-BES1, 0.2 μg GST-SnRK2s, and 0.05 μg His-BIN2 were added to kinase assay solution in the buffer containing 20 mM Tris (pH 7.4), 100 mM NaCl, 12 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP. Recombinant proteins were incubated or coincubated in kinase reaction solution at 37 °C for 30 min. The 6× SDS loading buffer was added to terminate the reaction. Samples were denatured by boiling and applied for SDS–PAGE.

Seed Germination Assay. Sterilized seeds were sown on 1/2 MS medium containing 0.4% gellan gum (Wako) without (Mock) or with 0.5 μM abscisic acid (ABA), stratified at 4 °C for 4 d, and then put into growth chamber (Percival) at 23 °C under long-day conditions (16 h light/8 h dark). The germination rates were determined at a 24-h interval by the percentage of green cotyledons. Three biological replicates ($n > 90$ for each) were conducted.

In-Gel Kinase 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. Proteins were extracted from the 10-d-old seedlings after treatment with 50 μM ABA or solvent only (Mock) for 1 h. A GST-fused ABF2 peptide (Gly73 to Gln120) was used as substrate. Other procedures were performed as described in previous research (2), with the following modification: each lane containing 100 μg proteins and 100 μCi of [**γ-^{32}P**]-ATP was used for one gel. The kinase reaction was conducted at 30 °C for 3 h. Signals were visualized with X-ray film and quantified by ImageJ.

Determination of Phosphorylation Sites of SnRK2.3 by BIN2 Kinase. GST-SnRK2.3^{K51N} was phosphorylated by His-BIN2. The phosphorylated GST-SnRK2.3^{K51N} was recovered from SDS/PAGE and subjected to in-solution alkylation/tryptic digestion followed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) as described in a previous report (3).

Immuno-Kinase Assay. The *pro2.3::SnRK2.3-MYC* transgenic plants were grown on 1/2 MS medium with or without (Mock) 5 μM bikinin in a growth chamber (Percival) at 23 °C under long-day conditions (16 h light/8 h dark). The 10-d-old seedlings were treated with 50 μM ABA for 1 h. Total proteins was extracted from the ABA-treated seedlings as described in *Semi-in Vivo Pull Down*, except for a final concentration of 5 mM NaF, 1 mM Na_3VO_4 in the IP buffer to inhibit the phosphatase activity. The SnRK2.3-MYC was immunoprecipitated (IPed) with anti c-myc agarose (Sigma A7470). Equal amounts of IPed SnRK2.3-MYC were used to phosphorylate the ABF peptide as described in *In Vitro Kinase Assay*.

Sample Preparation for Quantitative Mass Spectrum Analysis. Protein samples were prepared as described in *Immuno-kinase Assay*. The SnRK2.3-MYC was IPed with anti c-myc agarose (Sigma A7470) from approximated 100 mg crude lysate for one sample. An equal amount of IPed SnRK2.3-MYC was denatured by adding SDS to a final concentration of 2% (m/vol) and DTT to a final concentration of 100 mM. Each sample was loaded onto an Amicon Ultra-0.5 Centrifugal Filter with Ultracel-10 kDa membrane (Millipore), and SDS in the samples was removed by 6 M urea exchange followed by alkylation with iodoacetamide. Urea was then replaced with 100 mM NH_4HCO_3, and trypsin was added for overnight digestion at 37 °C. The peptides were finally collected from the filter by centrifugation. Phosphopeptide enrichment was performed using TiO_2 beads. In short, the peptides were reconstituted in loading buffer [70% (vol/vol) acetonitrile/2% (vol/vol) TFA, saturated by glutamic acid] and incubated with TiO_2 beads for 20 min. After incubation, the beads were washed with wash buffer A [70% (vol/vol) acetonitrile/0.5% TFA/H_2O] twice and wash buffer B [70% (vol/vol) acetonitrile/ 0.1% TFA/H_2O] twice. The phosphopeptides were then eluted from the beads with elution buffer [500 mM NH_4OH/70% (vol/vol) acetonitrile]. The eluted samples were dried for LC-MS/MS analyses.

Nanoflow LC-Electrospray Ionization-MS/MS. LC-electrospray ionization-MS/MS analysis was performed using a nanoflow EASY nLC1000 system (Thermo Fisher Scientific) coupled to an LTQ-Orbitrap

ELITE ETD mass spectrometer (Thermo Fisher Scientific). Samples were loaded and analyzed onto an EASY-Spray column (ES802, 25 cm, PepMap RSLC, C18, 2 μm, 100 Å, 75 μm i.d.). The mobile phases consisted of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in acetonitrile). After sample loading, elution of peptides was accomplished using a two-step linear gradient of 5–35% (vol/vol) B for 90 min, 35–90% (vol/vol) B for 10 min, and 90% (vol/vol) B for 5 min at a flow rate of 200 nL/min. Mass spectra were acquired in a data-dependent manner, with a full MS scan at a resolution of 240,000 using FT and the top 15 MS/MS scans in LTQ. A fragmentation threshold of 5,000, dynamic exclusion of 30 s, and an magnetic sector analyzer method with 79.98 Da loss (presenting the loss of HPO_3) were adapted for MS/MS analysis.

MS Data Analysis. Proteome Discoverer (version 1.4; Thermo Fisher Scientific) coupled with an in-house MASCOT server (version 2.3; Matrix Science) was used for peptide characterization. The raw files were searched against *Arabidopsis* database using the following parameters: trypsin was selected as the enzyme; one missed cleavage site was allowed; 10 ppm mass tolerances for MS and 0.8 Da for MS/MS fragment ions; carbamidomethylation on cysteine as fixed modification, protein *N*-acetylation, oxidation on methionine, pyroglutamate on peptide N-terminal, and phosphorylation on serine and threonine as variable modifications. The identified peptides were validated using the built-in Target Decoy PSM Validator with a false discovery rate of 0.01 and the phosphorylation sites were determined using phosphoRS 3.0.

Isolation of *abi2-3* Mutant. *abi2-3* was isolated from *bri1-301* EMS suppressor screening through germination assay on medium containing 0.75 μM ABA for insensitive seedlings. A GGC to GAC transition at nucleotide 988 (which caused a G168D substitution in amino acid) was confirmed in the *ABI2* genomic sequence through Sanger sequencing. Then we introduced *abi2-3* mutation into Col-0 background by back-crossing three times and one self-crossing to obtain homozygous *abi2-3* in Col-0.

Production of Transgenic *Arabidopsis* RNAi Lines. To create knockdown lines of SnRK2s using RNAi strategy in wild-type and *bin2-1* backgrounds, two artificial microRNAs (amiRNAs: amiR1 and amiR2) were constructed using a 2× 35S promoter and a 35S terminator in pOT2-Poly-Cis vector (4, 5). Specifically, two pairs of primers that contain the introduced amiR1 and amiR2 sequences were used for two sequential PCRs using pOT2-Poly-Cis and pOT2-amiR1 as templates sequentially, resulting in pOT2-amiR1-amiR2. The origin sequence of pOT2-amiR1-amiR2 plasmid was deleted by PCR, and PCR production containing the amiR1 and amiR2 expression cassette was introduced into a binary vector pFGC5941-PacI (4). The final construct was introduced into Col-0 and the heterozygous *bin2-1* (The *bin2-1* homozygous plants were sterile) through Agrobacterium-mediated transformation to generate the *SnRK2-RNAi*/*Col-0* and *SnRK2-RNAi*/*bin2-1*. The offspring of the heterozygous *bin2-1* includes the wild type, heterozygous *bin2-1*, and homozygous *bin2-1*, which can be distinguished by their vhypocotyl phenotype.

1. Yin Y, et al. (2002) BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. *Cell* 109(2):181–191.
2. Fujii H, Verslues PE, Zhu JK (2007) Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in *Arabidopsis*. *Plant Cell* 19(2):485–494.
3. Thingholm TE, Jørgensen TJ, Jensen ON, Larsen MR (2006) Highly selective enrichment of phosphorylated peptides using titanium dioxide. *Nat Protoc* 1(4):1929–1935.
4. Ji L, et al. (2011) ARGONAUTE10 and ARGONAUTE1 regulate the termination of floral stem cells through two microRNAs in Arabidopsis. *PLoS Genet* 7(3):e1001358.
5. Tang G, et al. (2012) Construction of short tandem target mimic (STTM) to block the functions of plant and animal microRNAs. *Methods* 58(2):118–125.

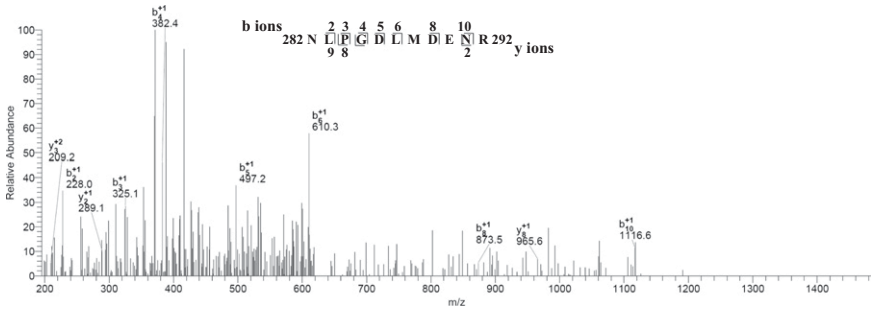

Fig. S1. Identification of SnRK2.2 as an interactor of BIN2 by LC-MS/MS.

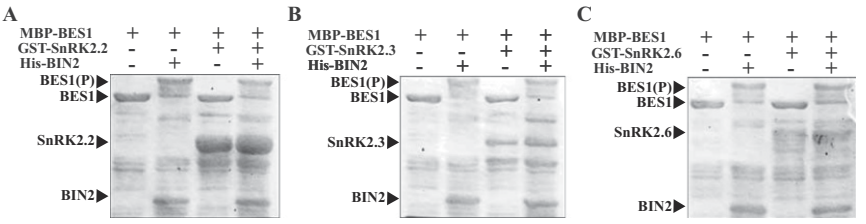

Fig. S2. Subgroup III SnRK2s do not affect BIN2's phosphorylation on BES1 in vitro. (*A*) SnRK2.2 does not affect BIN2's phosphorylation on BES1 in vitro. (*B*) SnRK2.3 does not affect BIN2's phosphorylation on BES1 in vitro. (*C*) SnRK2.6 does not affect BIN2's phosphorylation on BES1 in vitro. BES1(P), phosphorylated form of BES1; BES1, unphosphorylated form of BES1.

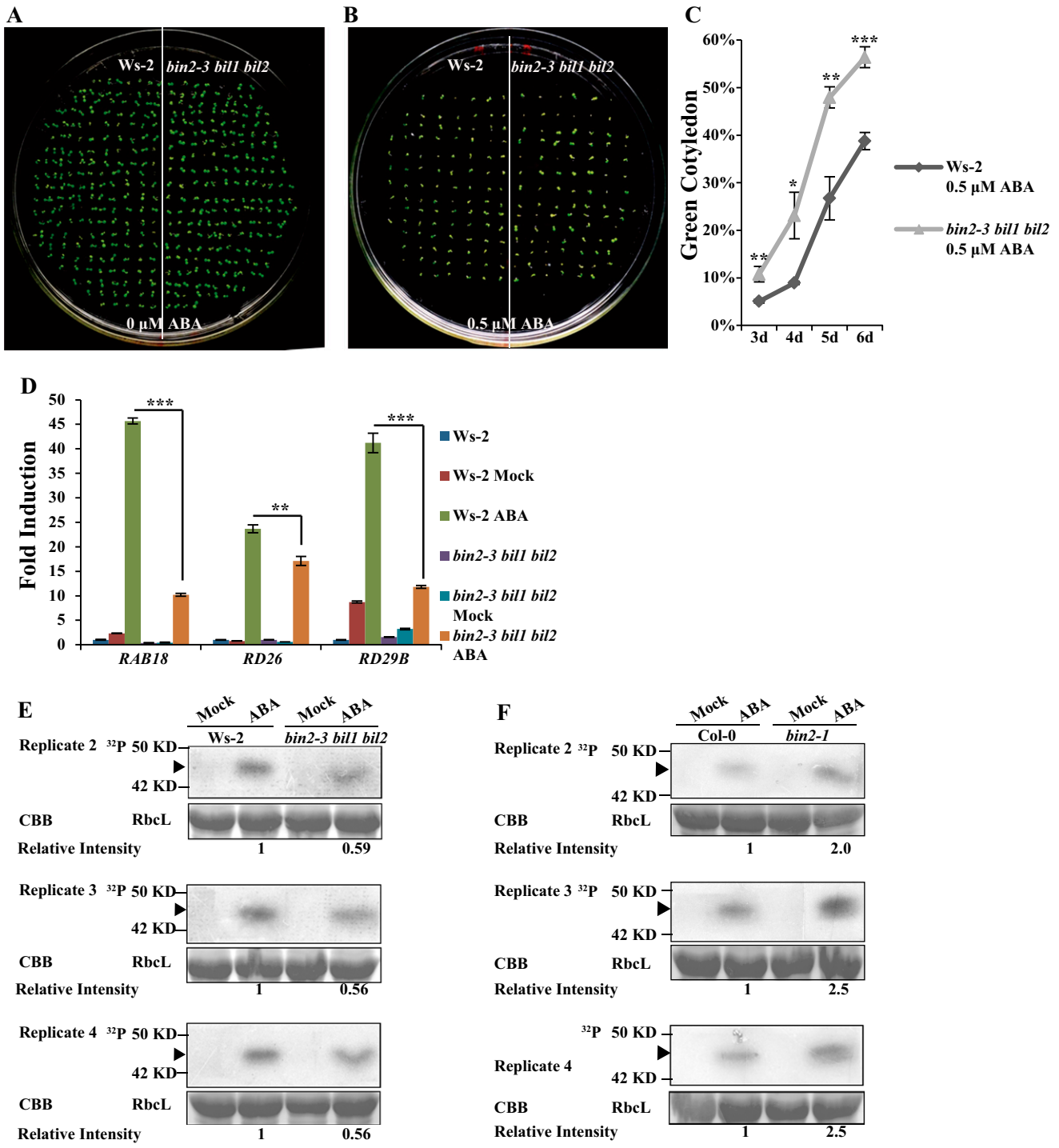

Fig. S3. BIN2 and its homologs function as positive regulators in ABA signaling. (*A*) Photography of 6-d-old Ws-2 and *bin2-3 bil1 bil2* grown on medium without ABA (Mock). (*B*) Photography of 6-d-old Ws-2 and *bin2-3 bil1 bil2* grown on medium with 0.5 μM ABA. (*C*) Statistical analysis of germination rate (by the percentage of green cotyledons) with Ws-2 and *bin2-3 bil1 bil2* grown on medium with 0.5 μM ABA. Three repetitions were used for each material ($n > 95$ for each material in one repetition). Ws-2 and *bin2-3 bil1 bil2* had equal germination rates (~99%) on medium without ABA. (*D*) Expression level of *RAB18*, *RD26*, and *RD29B* in Ws-2 and *bin2-3 bil1 bil2* with 50 μM ABA or solvent only (Mock) treatment for 3 h. Expression level of each gene in Ws-2 with no treatment was normalized to "1." (*E*) The signal intensity of in-gel kinase assay with the GST-ABF2$^{73\text{-}120}$ as substrate in Ws-2 and *bin2-3 bil1 bil2* for biological replicates 2–4 and quantification of these biological replicates. The relative radioactivity intensity of ABA-inducible bands of Ws-2 after ABA treatment was normalized to "1." (*F*) The signal intensity of in-gel kinase assay with the GST-ABF2$^{73\text{-}120}$ as substrate in Col-0 and *bin2-1* for biological replicates 2–4 and quantification of these biological replicates. The relative radioactivity intensity of ABA-inducible bands of Col-0 after ABA treatment was normalized to "1." For *E* and *F*, seedlings were either treated with 50 μM ABA or solvent only (Mock) for 1 h. The arrowhead indicates ABA-induced bands representing the activation status of SnRK2s. Coomassie brilliant blue R250 stain (CBB) of the large unit of Rubisco (RbcL) was used as the loading control. Significant levels for *C* and *D*: $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

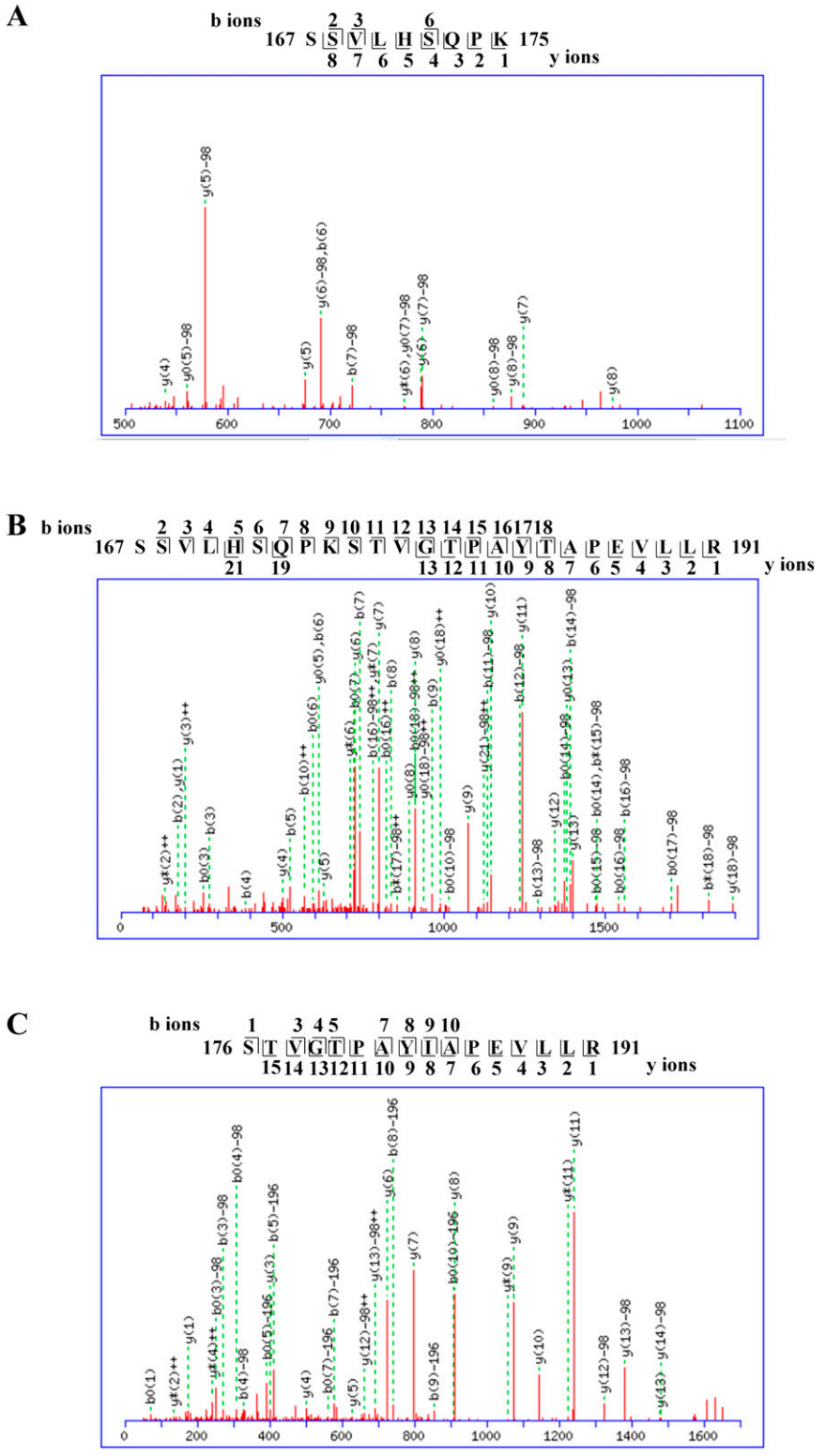

Fig. S4. Identification of BIN2 phosphorylation sites on SnRK2.3 by LC-MS/MS. (*A*) S172 is a potential phosphorylation site of SnRK2.3. (*B*) S176 is a potential phosphorylation site of SnRK2.3. (*C*) T177 and T180 are potential phosphorylation sites of SnRK2.3.

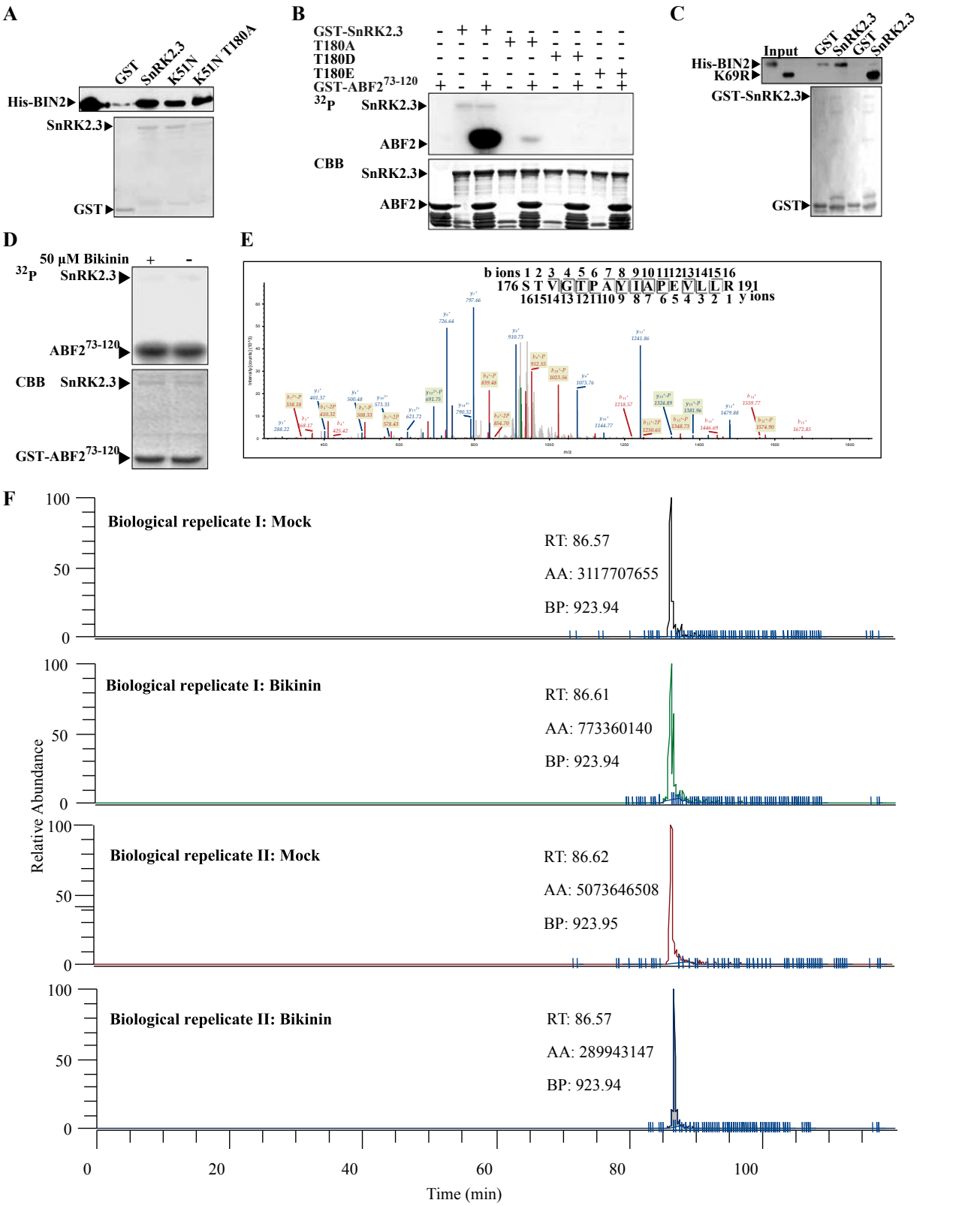

Fig. S5. BIN2 interacts and phosphorylates SnRK2.3 on T180. (*A*) In vitro pull-down of His-BIN2 with GST-SnRK2.3 or mutant form of GST-SnRK2.3. SnRK2.3$^{K51N\ T180A}$ is abbreviated for K51N T180A; the same rule applied for other mutant forms. (*B*) In vitro phosphorylation assays of the wild-type and mutant forms of SnRK2.3 on ABF2 peptide. (*C*) In vitro pull-down of GST-SnRK2.3 with His-BIN2 or His-BIN2^{K69R} (K69R). (*D*) In vitro phosphorylation of SnRK2.3 on the ABF2 fragment with or without 50 μM bikinin. (*E*) Single-scan MS/MS spectrum and manual validation identifying phospho-peptide of SnRK2.3 (176–191). (*F*) Relative abundance of the SnRK2.3 phospho-peptide STVGTPAYIAPEVLLR (176–191) that eluted at the time shown in the chromatogram. The peak area (AA) is a measure of the abundance of the phospho-peptide. RT, retention time; BP, base peak *m/z*. Two independent biological replicates were conducted.

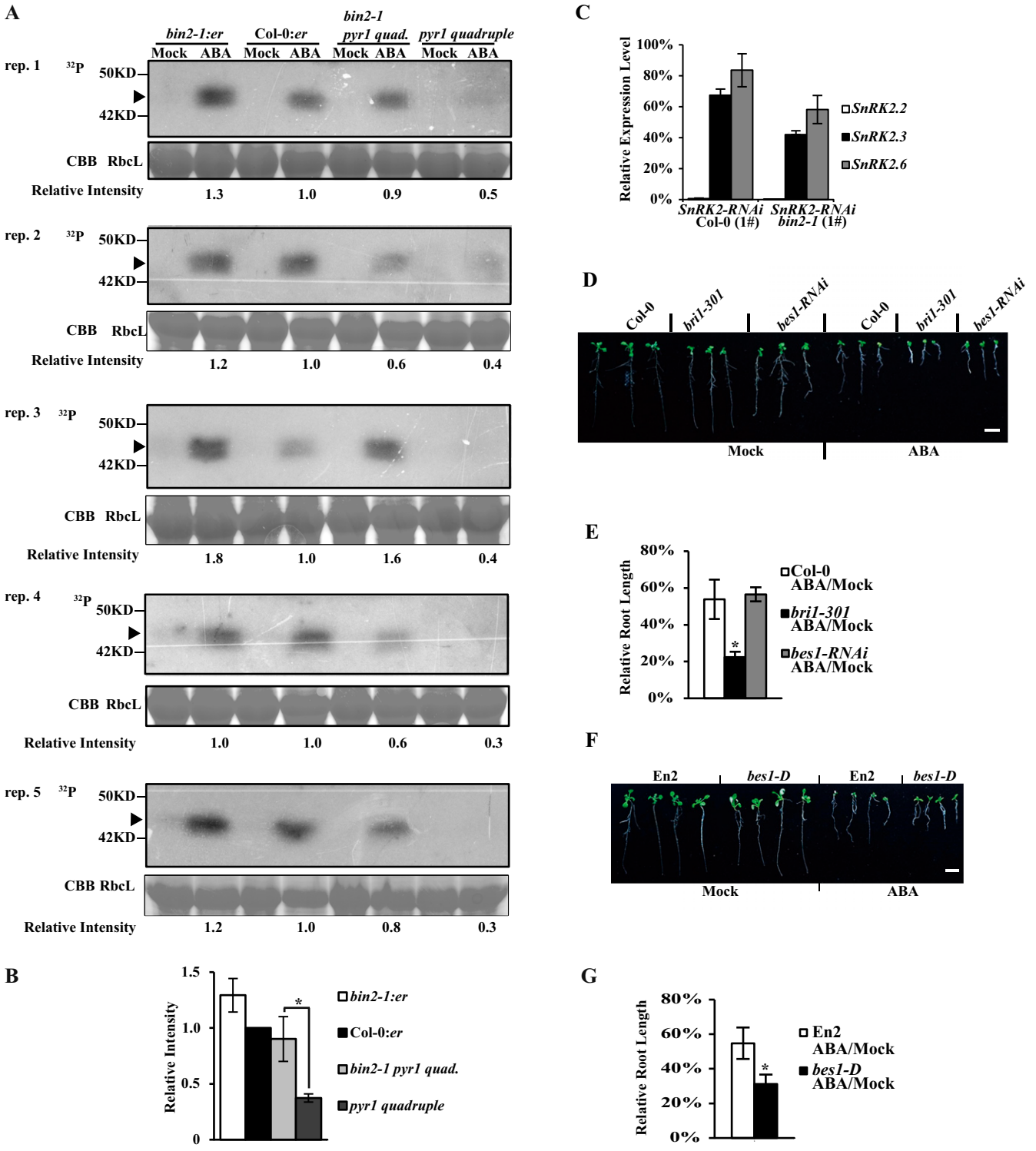

Fig. S6. BIN2 promotes ABA signaling through the subgroup III SnRK2s. (*A*) The signal intensity of in-gel kinase assay with the GST-ABF2$^{73\text{-}120}$ as substrate in Col-0:*er*, *bin2-1:er*, *bin2-1 pyr1 quadruple* (*bin2-1 pyr1 quad.*), and *pyr1 quadruple*. Seedlings were either treated with 50 μM ABA or solvent only (Mock) for 1 h. The arrowhead indicates ABA-induced bands representing the activation status of SnRK2s. Coomassie brilliant blue R250 staining (CBB) of the large unit of Rubisco (RbcL) was used as the loading control. Results of five biological replicates were shown. (*B*) Relative intensity of the ABA-inducible band. Data are means ± SE ($n = 5$). The relative radioactivity intensity of ABA-inducible bands of Col-0:*er* after ABA treatment was normalized to "1." (*C*) Expression level of subgroup III SnRK2s in SnRK2 RNAi line. (*D*) *bri1-301* is hypersensitive to ABA in primary root inhibition. (*E*) Statistic analysis of primary root length of Col-0 ($n = 20$), *bri1-301* ($n = 20$), and *bes1-RNAi* ($n = 20$). Primary root length of each material in mock treatment was normalized to 100%. Values are means ± SE. (*F*) *bes1-D* is hypersensitive to ABA in primary root inhibition compared with wild-type En2. (*G*) Statistic analysis of primary root length of En2 ($n = 20$) and *bes1-D* ($n = 20$). Primary root length of each material in mock treatment was normalized to 100%. Values are means ± SE. Student's *t* test was used to determine the significance of the indicated comparisons (*B*, *E*, and *G*). Significant levels: $^*P < 0.05$. (Scale bar, 1 in *D* and *F*.)

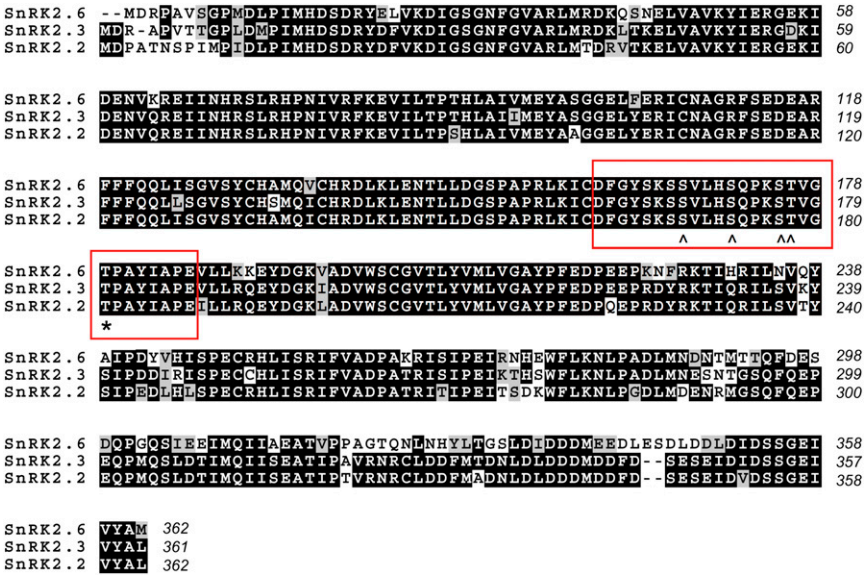

Fig. S7. Amino acid sequence alignment of subgroup III SnRK2s. The activation loops (A loop) among subgroup III SnRK2s are shown in red rectangles. Residues corresponding to S168, S172, S176, and T177 of SnRK2.3 are marked by "^." Residues corresponding to T180 of SnRK2.3 are marked with "*."

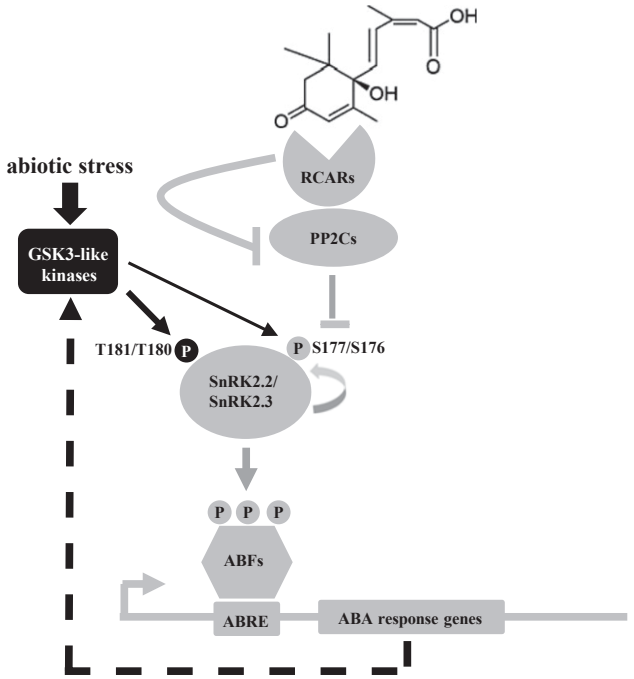

Fig. S8. A proposed model. GSK3-like kinases activate SnRK2s mainly through phosphorylation on T181 of SnRK2.2 and T180 of SnRK2.3 to enhance ABA signaling. Some abiotic stresses, such as low humidity and high salt, can activate *Arabidopsis* GSK3 kinases at transcriptional and/or protein levels. ABA may also induce the expression of some members of GSK3-likes kinases, which acts as a positive feedback loop to accelerate ABA signaling.

Table S1. Plant materials used in this research

Plant material information
Col-0
bin2-1(bin2^{E263K},Col-0 background)
*abi2-3(*abi2^{G168D},Col-0 background*)*
abi2-3 bin2-1(Col-0 background)
SnRK2-RNAi (Col-0 background)
SnRK2-RNAi/bin2-1
35S::BIN2-FLAG (Col-0 background)
pSnRK2.3::SnRK2.3-MYC (Col-0 background)
pSnRK2.3::SnRK2.3^{T180A}*-MYC* (Col-0 background)
bri1-301(Col-0 background)
bes1-RNAi (Col-0 background)
Ws-2 (Wassilewskija 2)
bin2-3 bil1 bil2 (Ws-2 background)
Col-0:*er*
pyr1 pyl1 pyl2 pyl4
bin2-1:er
pyr1 pyl1 pyl2 pyl4 bin2-1
En2 (Enkheim-2)
bes1-D (En2 background)

Table S2. Primers used for construction of *SnRK2* RNAi lines

Primer name	Primer sequence (5′–3′)
M0SL-SnRK2.236-3-SwaI-PR	gccATTTAAATagccgaattggAAGATTGATGAAAATGTTCAAatccgagcccgatggtgagactTTT
M0SL-SnRK2.236-3-SwaI-PF	gccATTTAAATgcagggattggAAGATTGATAAAAATGTTCATatcggatcctcgaggtgtaaaaaaactcg
M4SL-SnRK2.236-3-PmeI-PR	gccGTTTAAACACCCCATTAGCAACATTCACAACCAAAATCAAATCCGTGGCACCAGCTCACAGTcat
M4SL-SnRK2.236-3-PmeI-PF	gccGTTTAAACccagcgttagcAACATTCACGACCAAAATCATatcggttgcccaagctgaataaaaaaa

Table S3. Primers used for quantitative RT-PCR

Primer name	Primer sequence (5′–3′)
RAB18-qRT-S	TTGTAACGCAGTCGCATT
RAB18-qRT-R	GATGCTCATTACACACTCATG
RD29B-qRT-S	AAGAACGTCGTTGCCTCA
RD29B-qRT-R	GCCCGTAAGCAGTAACAG
RD26-qRT-S	AGGTCTTAATCCAATTCCAG
RD26-qRT-R	GAGTTCTGCTGCCGATTC
SnRK2.2-RT-S	ATTGCCTTTGTCTTTCTC
SnRK2.2-RT-A	ATCACATCAGATTAAGTCG
SnRK2.3-RT-S	AGTGTTCTGATCCTCTGGTT
SnRK2.3-RT-A	ATTTGCTTTTGTGTGACATTGG
SnRK2.6-RT-S	GGATATCAATTCCTGAAATAAGG
SnRK2.6-RT-A	TCATCAAACTGAGTGGT