Supplementary information, Data S1 Extended experimental procedures

Constructs, mutagenesis and protein purification

BRI1-CD (residues 815-1196), BAK1-CD (residues 255-615), and the corresponding kinase-dead mutants were cloned into pET-21b or pMAL-c2X vector, to generate the C-terminal 6×His-fusion or N-terminal MBP-fusion proteins. BRI1-KD (residues 863-1162) and BSK3-KD (residues 36-329) were cloned into a modified pET-Duet vector for generation of the N-terminal 6×His-fusion proteins with a precession protease cutting site. BKI1-Cter (residues 257-337) and its fragments were cloned into the pMAL-c2X vector to generate the N-terminal MBP-fusion proteins. BKI1-BIM (residues 306-325) was synthetized by Zhongke Yaguang Inc.. Mutations of BRI1-KD and BKI1-Cter were generated by an overlap PCR procedure and subjected to DNA sequencing.

All proteins, overexpressed in BL21(DE3) cells at 20 ℃, were first purified over Ni-NTA (QIAGEN) or amylase resin (NEB) columns, and then by ion-exchange chromatography (Source-15Q/15S; GE Healthcare) followed by gel filtration chromatography (Superdex-200; GE Healthcare). The SeMet-labelled BRI1-KD protein was expressed in minimal medium supplemented with 50 mg/L seleno-L-methionine.

To produce the dephosphorylated BRI1-CD and BAK1-CD, recombinant BRI1-CD or BAK1-CD was incubated with GST-PP2Cα at a 20:1 ratio for 1 h at 25 °C in the buffer containing 10 mM $MgCl_2$ and 100 mM NaCl. After incubation, GST-PP2Cα was

removed by GST-columns. Proteins were further purified by gel filtration chromatography.

All protein concentrations were determined spectrophotometrically using theoretical molar extinction coefficients at 280 nm, following the method of Gill and von Hippel (Gill and von Hippel, 1989).

Western blotting

The phosphorylation states of the proteins were assessed by western blotting using anti-phosphothreonine (anti-pThr) antibody (Cell Signaling Technology, Inc.).

To validate the phosphorylation states of the recombinant, kinase-dead, and dephosphorylated BRI1-CD and BAK1-CD, equal amount of the indicated proteins (2 μM) was resolved by SDS-PAGE and analyzed by western blotting.

Phosphorylation of the kinase-dead mutants of BRI1-CD and BAK1-CD by the recombinant BRI1-CD and BAK1-CD were analyzed by western blotting. Some 50 nM BRI1-CD or BAK1-CD was added into the standard reaction buffer containing 5 μM MBP-mBRI1-CD or MBP-mBAK1-CD at 25 ℃ for 1 h. The standard reaction buffer contains 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM ATP and 10 mM $MgCl_2$. After incubation, reactions were terminated and proteins were resolved by SDS-PAGE and analyzed by western blotting.

Autophosphorylation of the dephosphorylated BRI1-CD and BAK1-CD were also assessed by western blotting. Some 2 μM dephosphorylated BRI1-CD or BAK1-CD

protein was incubated at 25 ℃ for 1 h in the standard reaction buffer. Autophosphorylation signals were analyzed by western blotting after incubation.

To detect the transphosphorylation of the dephosphorylated BRI1-CD and BAK1-CD, dephosphorylated BRI1-CD (2 μM) and BAK1-CD (2 μM) were incubated together at 25 ℃ for 1 h in the standard reaction buffer. After incubation, the reactions were terminated and the phosphorylation signals were detected by western blotting. To assess BKI1-mediated inhibition *in vitro*, above experiments wrer conducted in the presence of 20 μM BKI1-Cter. After incubation, the transphosphorylation level was analyzed as above.

Kinase assays for BRI1

The kinase activity of BRI1 was determined using an enzyme-coupled spectrophotometric assay (Roskoski, 1983). This assay couples the production of ADP with the oxidation of NADH by pyruvate kinase and lactate dehydrogenase (LDH). Kinase assays were conducted at 25 ℃ in the kinase reaction buffer containing 100 mM MOPS, pH 7.0, 100 mM NaCl, 1 mM ATP, 10 mM $MgCl_2$, 0.2 mM NADH, 1.0 mM phosphoenolpyruvate (PEP), 20 units/mL LDH, and 15 units/mL pyruvate kinase. Reaction was initiated by the addition of the enzyme to the reaction mixture. The formation of NAD^+ can be followed by the absorbance decrease at 340 nm on a PerkinElmer Lambda 45 spectrophotometer equipped with a magnetic stirrer in the cuvette holder.

The number of the BRI1 phosphorylation sites in BSK3-KD and BKI1-Cter were determined by adding 50 nM BRI1-CD into the kinase reaction buffer containing 5 μM BSK3-KD or BKI1-Cter. The profile of absorption *versus* time was recorded following the addition the enzyme. The concentrations of ADP formed in BRI1-CD-catalyzed phosphorylation reactions were determined using an extinction coefficient for NADH of 6220 M^{-1} cm^{-1} at 340 nm at pH 7.0.

To obtain the kinetic parameters for BRI1-CD-catalyzed phosphorylation of BSK3-KD, various concentrations (5 μM, 10 μM, 20 μM, 35 μM, 50 μM, 75 μM and 100 μM) of BSK3-KD were submitted to the enzyme-coupled assay in the presence of 50 nM BRI1-CD, and the relative initial rates for BRI1-CD-catalyzed phosphorylation of BSK3-KD were determined from the linear slope of progress curves obtained. The kinetic parameters were obtained by fitting the experimental data to the Michaelis-Menten equation. Similarly, the kinetic parameters for BRI1-CD-catalyzed phosphorylation of BKI1-Cter were determined by the enzyme-coupled assay using various concentrations of BKI1-Cter (0.3 μM, 1 μM, 2 μM, 3.5 μM, 5.5 μM, 7.5 μM, 10 μM and 15 μM) in the presence of 50 nM BRI1-CD.

Competitive binding assays were conducted to determine the binding affinity of BKI1-BIM for BRI1-CD. The initial rates of 50 nM BRI1-CD-catalyzed phosphorylation of BKI1-Cter were measured in the presence of various concentrations of inhibitor BKI1-BIM (0 μM, 7 μM, 14 μM, 21 μM, 28 μM and 35 μM) and substrate BKI1-Cter (2

μM, 2.5 μM, 3.5 μM, 5 μM, 10 μM, and 15 μM). The kinetic parameters were determined by fitting the experimental data to the competitive inhibition equation

$$\frac{1}{v} = \frac{\left(1+\frac{[I]}{K_i}\right)K_m}{k_{cat}[E][S]} + \frac{1}{k_{cat}[E]}$$

where v represents the initial velocity, $[I]$ represents the inhibitor (BKI1-BIM), K_m and K_i are the dissociation constants for BRI1-CD to BKI1-Cter and BKI1-BIM, respectively, and k_{cat} is the turnover number. $[E]$ is the concentration of enzyme (BRI1-CD), and $[S]$ represents the substrate (BKI1-Cter).

The effects of BKI1 interface mutants on BRI1-KD-catalyzed-BKI1-Cter phosphorylation were assessed using the enzyme-coupled assay in the presence of 50 nM BRI1-KD and 2.5 μM BKI1-Cter mutants, and the effects of BRI1 interface mutants on BRI1-KD-catalyzed BKI1-Cter/BSK3-KD phosphorylation were assessed similarly except that 5 μM BSK3-KD was used.

The role of BKI1-Cter on BRI1-CD-catalyzed BSK3-KD phosphorylation was also assessed. Reaction was initiated by adding 50 nM BRI1-CD to the kinase reaction buffer containing 5 μM BSK3-KD or BKI1-Cter in the presence of different concentrations of pBKI1-Cter. The initial velocities of BSK3-KD and BKI1-Cter phosphorylation were determined.

To quantitatively monitor the trans-activation process of the dephosphorylated BRI1-CD and BAK1-CD under different incubating conditions, phosphorylation of BSK3-KD was selected as the output to reflect the restored kinase activity of the

dephosphorylated BRI1-CD after incubation. Indicated proteins under each bar of Figure 5E were incubated together at 25 ℃ for 1 h in the standard reaction buffer. The concentrations of BRI1-CD/BAK1-CD-related proteins and BKI1-fragments used in these assays were 2 μM and 20 μM, respectively. After incubation, 50 nM variously incubated BRI1-CD or BAK1-CD were submitted to the enzyme-coupled assay in the presence of 5 μM BSK3-KD. Phosphorylation efficiency of BSK3-KD by these variously incubated enzymes were determined.

Effects of BKI1 interface mutants on the trans-activation of BRI1-CD and BAK1-CD were evaluated using the same method as mentioned above. Some 2 μM dephosphorylated BRI1-CD and BAK1-CD were first incubated in the standard reaction buffer in the presence of 20 μM BKI1 mutants as indicated in Figure 5F. After incubation, 50 nM variously incubated BRI1-CD from the reaction mixture was submitted to the enzyme-coupled assay, and the phosphorylation efficiency of 5 μM BSK3-KD were determined.

Crystallization, data collection and structure determination

The native (150 μM) or SeMet-labelled (200 μM) BRI1-KD protein was mixed with BKI1-BIM peptide in 1:2 molar ratio. The BRI1-KD-BIM complexes were incubated on ice for 1 h, in the presence of 1 mM AMP-PNP and 2 mM $MgCl_2$, and then subjected to crystallization trials. Crystals of both native and SeMet BRI1-KD-BIM complexes were grown using hanging drops vapor-diffusion method by mixing the protein sample with

equal volume of reservoir solution containing 0.1 M HEPES, pH 7.5, 20% PEG 3350 and 0.2 M Li Citrate. Crystals were cryo-protected in reservoir solutions supplemented with 20% ethylene glycol and flash-frozen under a cold nitrogen stream at 100 K. After crystal screening, good data sets of BRI1-KD-BIM were collected at beamline BL17U of the Shanghai Synchrotron Radiation Facility (SSRF) (λ = 0.9793 Å for the native crystal and 0.9789 Å for the SeMet crystal) at 100 K. The diffraction data sets were processed with the HKL2000 package (Otwinowski and Minor, 1997). The native BRI1-KD-BIM complex structure was solved by molecular replacement using Phaser (McCoy et al., 2007), with Pto kinase domain (PDB ID: 2QKW) as the search model. Standard refinements were performed with the programs PHENIX (Adams et al., 2002) and Coot (Emsley and Cowtan, 2004). The SeMet BRI1-KD-BIM structure was determined by molecular-replacement using the native structure as search model, and the selenium anomalous signals were used to validate the atomic model of native BRI1-KD. The data processing and refinement statistics of the native and SeMet BRI1-KD-BIM complexes were summarized in Table 1. Structure of BRI1-KD in isolation was rebuilt with the diffraction data (4OAC.cif) downloaded from PDB, and refinement statistics were summarized in Table S2. All structural representations in this paper were prepared with PyMOL (http://www.pymol.org).

Agrobacterium-mediated generation of transgenic plants

For wildtype and mutated BKI1 overexpression in *Arabidopsis*, the PCR fragments of full length BKI1s were inserted into pCAMBIA-1302 binary vector with a CaMV 35S promoter at 5' side and a *GFP* coding sequence at 3' side. The mutations were obtained via overlap PCR method. *Agrobacterium tumefaciens* strain GV3101 were transformed with these binary plasmids. The bacteria was resuspended to OD_{600} ~1.0 in the transition buffer (Sucrose 50 g, MS 2.2 g, Silwet-77 200 μL, and 6-BA 10 μL for 1 liter, pH 5.8~6.0). Floral dip method was used to generate transgenic lines in the Columbia (Col-0) background. Seeds were screened on hygromycin-containing 1/2 MS plate with 0.4% phytagar (GIBCO), and then transferred to soil. T_2 seedlings were used for phenotype analysis and other experiments. Plants were grown at 23 ℃ under long-day conditions (16 h light / 8 h dark cycle).

Protein extraction from plants for immunoblot analysis

Plant leaves were ground to fine powder in liquid nitrogen. Total protein was extracted with 2×extraction buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1 mM PMSF, 5 mM DTT, 0.5% SDS, and 10 mM β-mercaptoethanol), separated with a 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane. The membrane was blocked, incubated with anti-GFP antibodies, and goat anti-rabbit HRP-conjugated secondary antibodies. Signals were visualized with X-ray film.

Confocal imaging

Confocal microscopy images of various versions of BKI1-GFP in 4-day-old T_2 transgenic seedlings was taken with an inverted Leica SP8 microscope using 488 nm exciting laser and HyD scanner for observation. For exogenous BR treatment, the root tips were first observed without and then treated with 1 μM epi-brassinolide (100 mM stock in DMSO) for 10 min on slides.

Isothermal titration calorimetry

To assess the binding affinity between BKI1-Cter and BRI1-CD, we titrated BKI1-Cter into BRI1-CD using ITC carried out at 25℃ on a VP-ITC MicroCalorimeter (GE Healthcare). All proteins were prepared in a buffer containing 25 mM HEPES (pH 7.4) and 150 mM NaCl. The sample cell was loaded with 1.43 mL of BRI1-CD (100 μM), and titration was carried out using a 284-μL syringe filled with BKI1-Cter (1 mM). Each titration experiment consisted of 25 consecutive injections of 10-μL volume and 20-s duration each, with a 5-min interval between injections. To correct for the heat effects of dilution and mixing, control experiments were performed in which BKI1-Cter was injected into an identical solution but without BRI1-CD. To determine the binding affinity between pBKI1-Cter and BRI1-CD, The BRI1-CD (100 μM) was similarly titrated with pBKI1-Cter (1 mM). The calorimetric data were analyzed with a one-site binding model assuming a binding stoichiometry of 1:1 using ORIGIN software supplied with the instrument.

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