Plant Communications, Volume *3*

Supplemental information

Structural analysis of receptor-like kinase SOBIR1 reveals mechanisms that regulate its phosphorylation-dependent activation

Xue Wei, Yulu Wang, Su Zhang, Tianyi Gu, Gabryel Steinmetz, Haiyan Yu, Guoguang Guo, Xin Liu, Shilong Fan, Fengzhong Wang, Yangnan Gu, and Fengjiao Xin

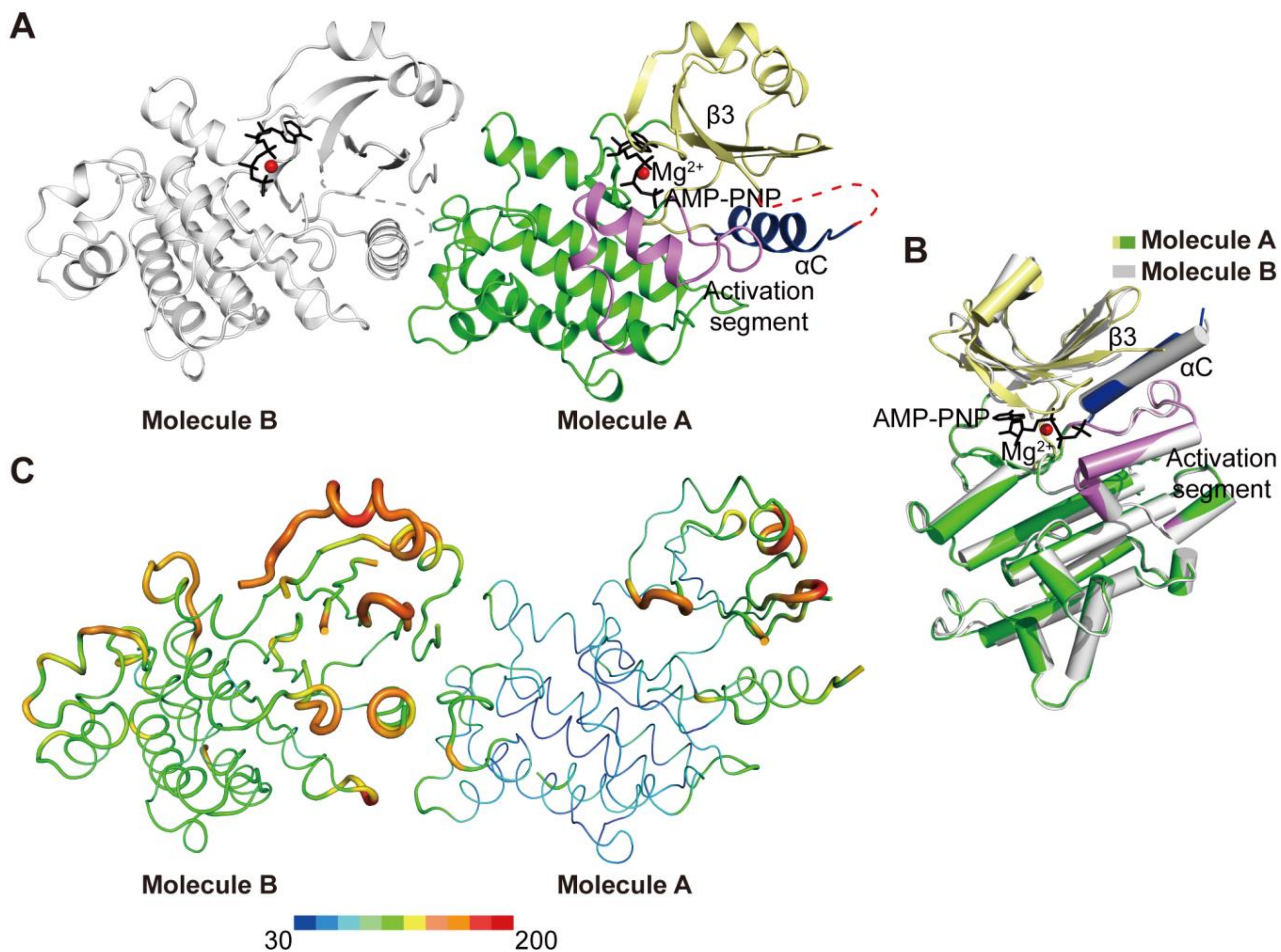

Figure S1. Overall structure of SOBIR1-KD^{D489A}. Related to Figure 2 and Table 1

(A) Schematic representation of two molecules within one asymmetric unit of SOBIR1-KD^{D489A}. Molecule A (right) uses the same color scheme as that in the schematic diagram in Figure 1A, whereas molecule B (left) is presented in gray. The bound AMP-PNPs are presented as black sticks, whereas the Mg^{2+} ions are indicated by red spheres. The disordered β3-αC loops are presented as red (molecule A) and gray (molecule B) dashed lines.

(**B)** Molecules A and B superimposed in one asymmetric unit.

(**C)** Comparison between molecule A (right) and molecule B (left), which are colored according to the temperature factors for Cα atoms.

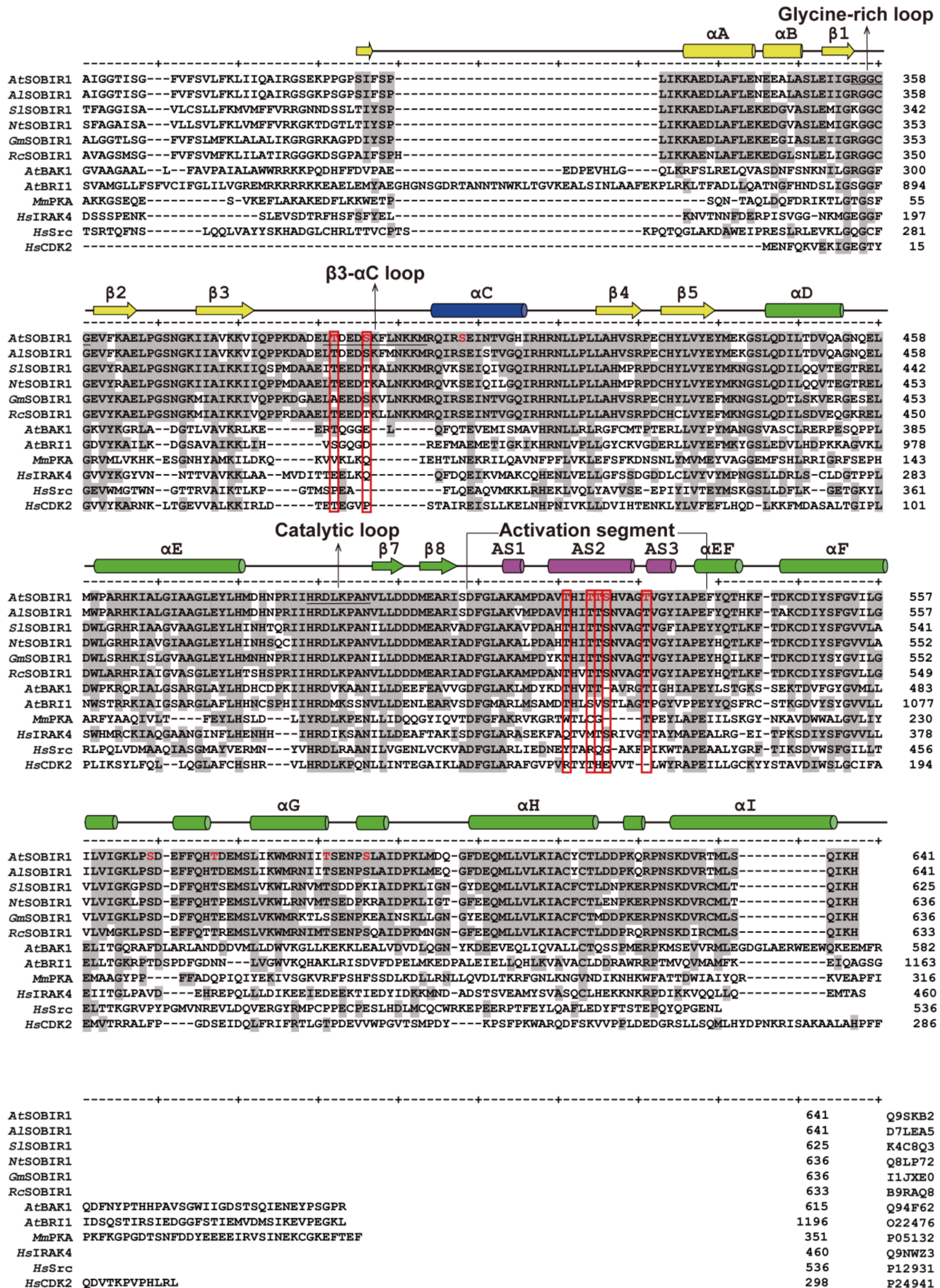

Figure S2. Amino acid sequence alignment of the kinase domains of SOBIR1 orthologs and selected plant and mammalian kinases. Related to Figure 1, Figure 3, and Figure 4

The Swiss-Prot ID is provided after each protein name. The secondary structure elements of SOBIR1-

KD are indicated above the sequences and colored as in the schematic diagram in Figure 1A. Phosphorylation sites identified in *Arabidopsis thaliana* SOBIR1-KD are highlighted in red. Conserved phosphorylation sites in the kinases are indicated in a red box. The glycine-rich loop, β3-αC loop, catalytic loop, and activation segment are labeled accordingly. *At*, *Arabidopsis thaliana*; *Al*, *Arabidopsis lyrata*; *Sl*, *Solanum lycopersicum*; *Nt*, *Nicotiana tabacum*; *Gm*, *Glycine max*; *Rc*, *Ricinus communis*; *Mm*, *Mus musculus*; *Hs*, *Homo sapiens*.

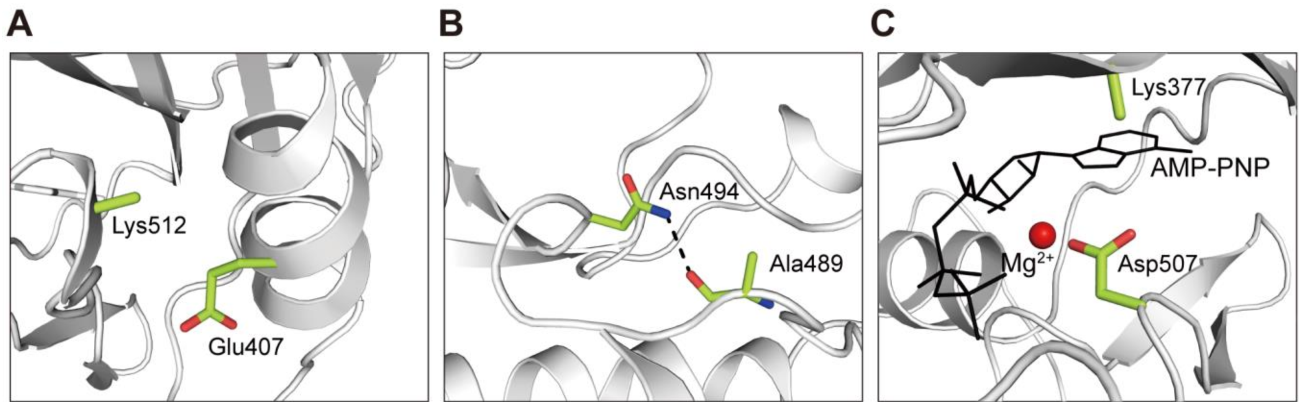

Figure S3. Interaction between Ala489 and Asn494 that stabilizes the Src-like inactive conformation of SOBIR1-KD^{D489A}. Related to Figure 2

The residues stabilizing the Src-like inactive conformation are highlighted as lemon sticks. Salt bridges formed by Ala489 and Asn494 (**B**) are presented as black dashed sticks. The Lys512 (**A**) and Lys377 (**C**) side chains cannot be seen due to low resolution, which were supposed to form salt bridges with Glu407 and Asp507, respectively. AMP-PNP is presented as black sticks, and the Mg^{2+} ion is indicated by a red sphere.

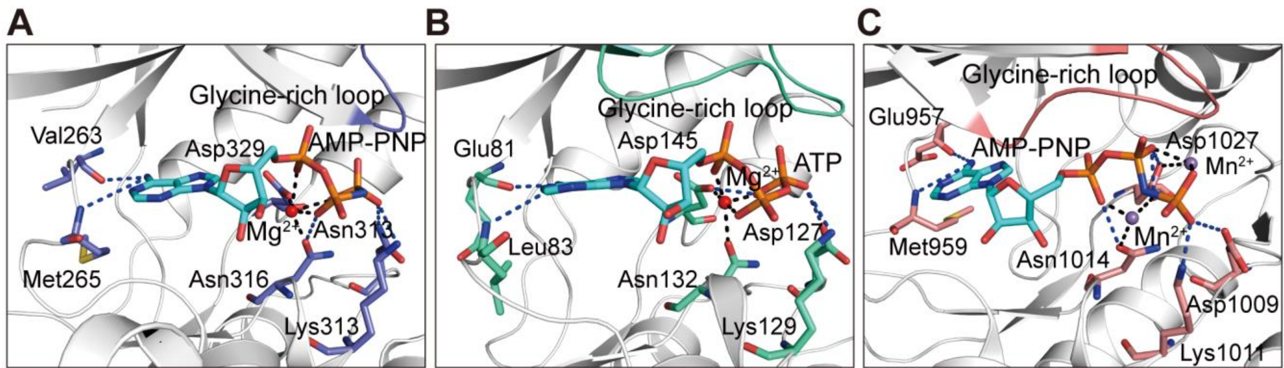

Figure S4. Conformations of the nucleotide-binding sites of IRAK4 (PDB code: 6EGF), CDK4 (PDB code: 1HCK), and BRI1 (PDB code: 5LPV). Related to Figure 2

Detailed binding modes of AMP-PNP in the nucleotide-binding pockets of IRAK4 (**A**), CDK4 (**B**), and BRI1 (**C**). The nucleotide-interacting residues of IRAK4, CDK4, and BRI1 are presented as slate, green cyan, and salmon sticks and labeled accordingly. Hydrogen bonds related to the interaction with AMP-PNP and Mg^{2+}/Mn^{2+} are presented as blue or black dashed lines. Mn^{2+} and Mg^{2+} are indicated by purple and red spheres, respectively.

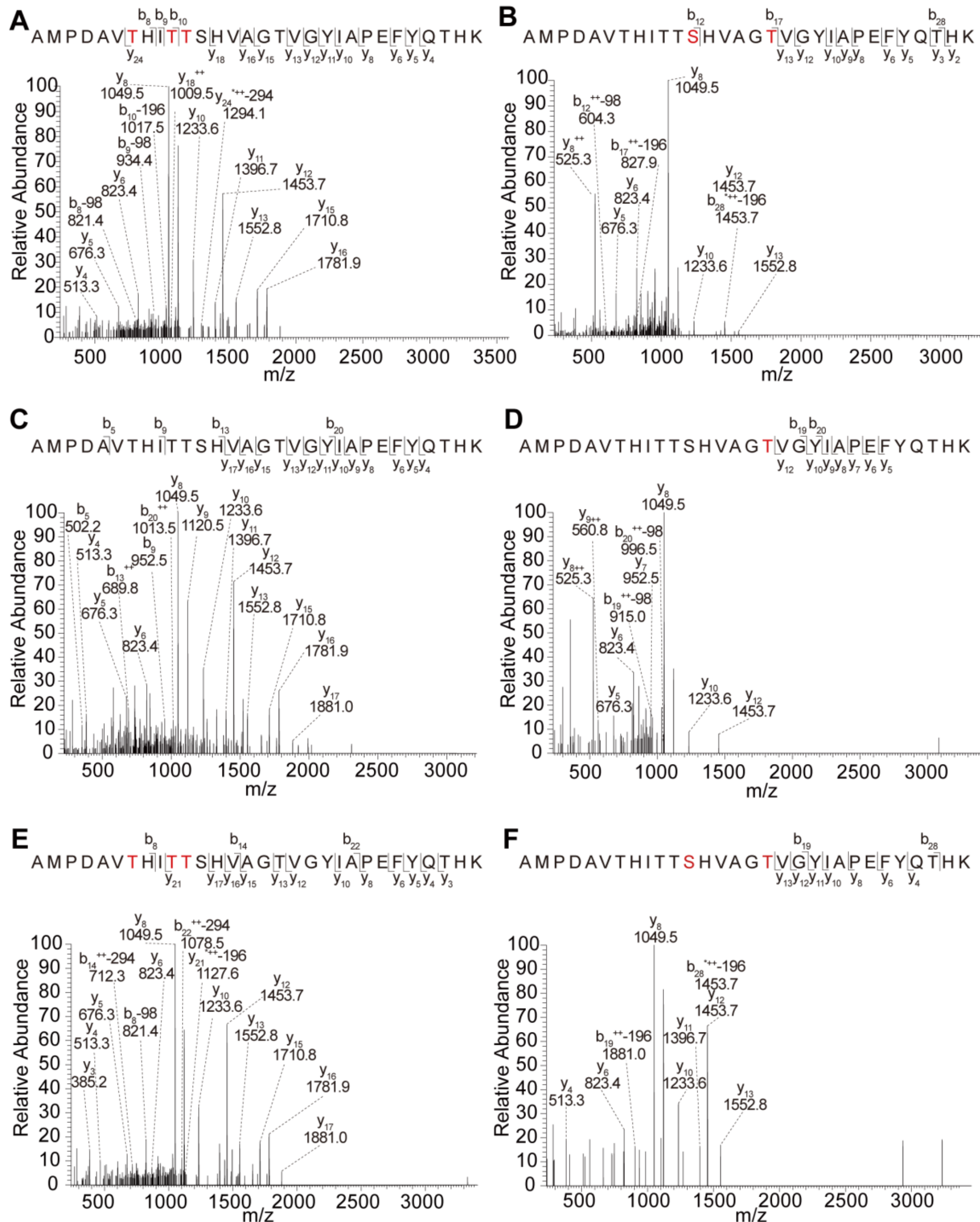

Figure S5. LC-MS/MS spectra revealing the *in vitro* phosphorylation sites in the SOBIR1 activation segment. Related to Figure 3 and Table 2

Mass spectra for the SOBIR1-KD activation segment in different states. The wild-type protein (**A, B**) was recombinantly expressed in *E. coli*. The PP2Cα-dephosphorylated proteins (**C**) were generated by incubating 10 μM SOBIR1-KD and 1 μM GST-PP2Cα in the kinase reaction buffer at 25 °C for 1 h. Proteins were purified with GST columns, followed by gel filtration chromatography (Superdex 200

HR 10/30 column). Autophosphorylated SOBIR1-KD (**D**) was generated by incubating 10 μM dSOBIR1-KD with 1 mM ATP and 10 mM Mg^{2+} at 25 °C for 3 h. BAK1-phosphorylated SOBIR1-KD (**E**) was generated by incubating 10 μM dSOBIR1-KD and 1 μM GST-BAK1-CD in the kinase reaction buffer at 25 °C for 1 h. Each LC-MS/MS spectrum presents the ions produced by the collision-induced dissociation of the intact peptides. Predominant b and y product ion peaks are labeled and product ions eliciting neutral mass losses of H_3PO_4 (98 Da) are also indicated. The * and ++ indicate the ions that lost ammonia (−17 Da) and the doubly protonated ions, respectively. The phosphorylated residues in each peptide are highlighted in red.

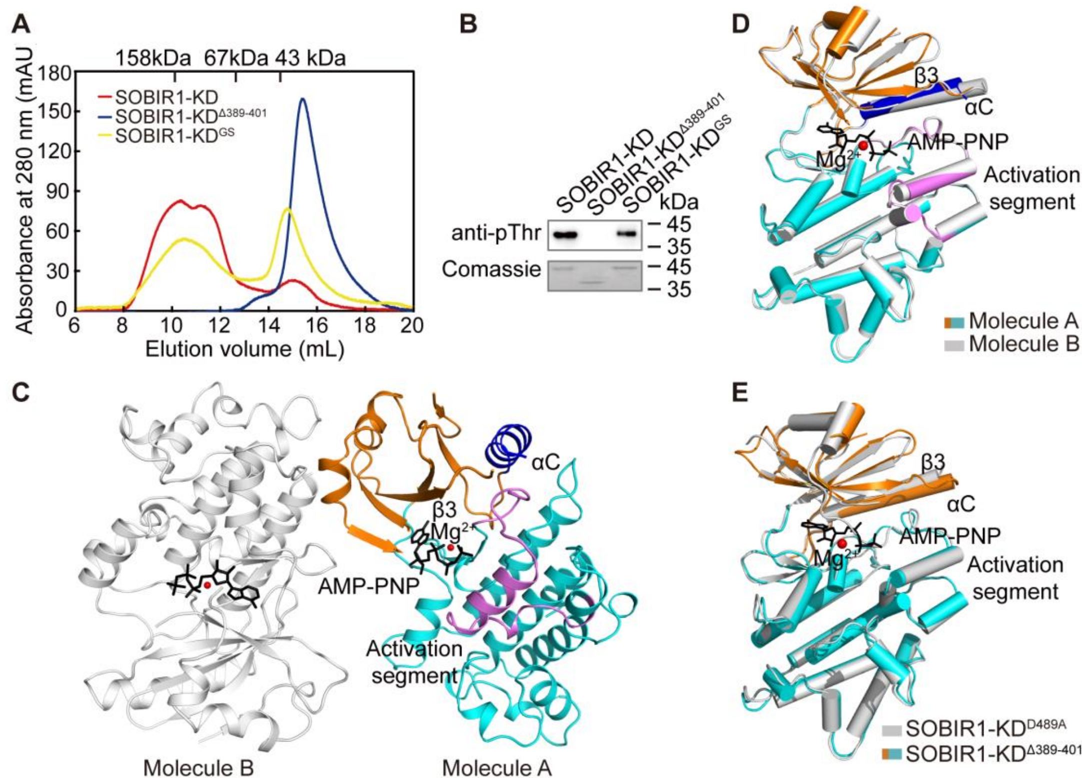

Figure S6. Overall structure of SOBIR1-KD$^{Δ389-401}$. Related to Figure 4 and Table 1

(A) Determination of the oligomerization states based on size exclusion chromatography. Wild-type SOBIR1-KD and SOBIR1-KD^{GS} mutant eluted as multimers, whereas the deletion of the β3-αC loop (SOBIR1-KD$^{Δ389-401}$) apparently resulted in a monomeric kinase domain. Elution volumes of the protein standards are indicated.

(B) Phosphorylation states of the wild-type SOBIR1-KD as well as the SOBIR1-KD$^{Δ389-401}$ and SOBIR1-KD^{GS} mutants.

(C) Schematic representation of two molecules within one asymmetric unit of SOBIR1-KD$^{Δ389-401}$. N-lobe, C-lobe, αC, and activation segment of molecule A are colored in orange, cyan, blue, and violet, respectively, whereas molecule B (left) is presented in gray. The bound AMP-PNPs are presented as black sticks, and Mg^{2+} ions are indicated by red spheres.

(D) Molecules A and B superimposed in one asymmetric unit.

(E) Comparison between the SOBIR1-KD^{D489A} and SOBIR1-KD$^{Δ389-401}$ structures. SOBIR1-KD^{D489A} is presented in gray, whereas SOBIR1-KD$^{Δ389-401}$ is presented in orange (N-lobe) and cyan (C-lobe). The bound AMP-PNP and Mg^{2+} ion of SOBIR1-KD$^{Δ389-401}$ are presented as a black stick and a red sphere, respectively.

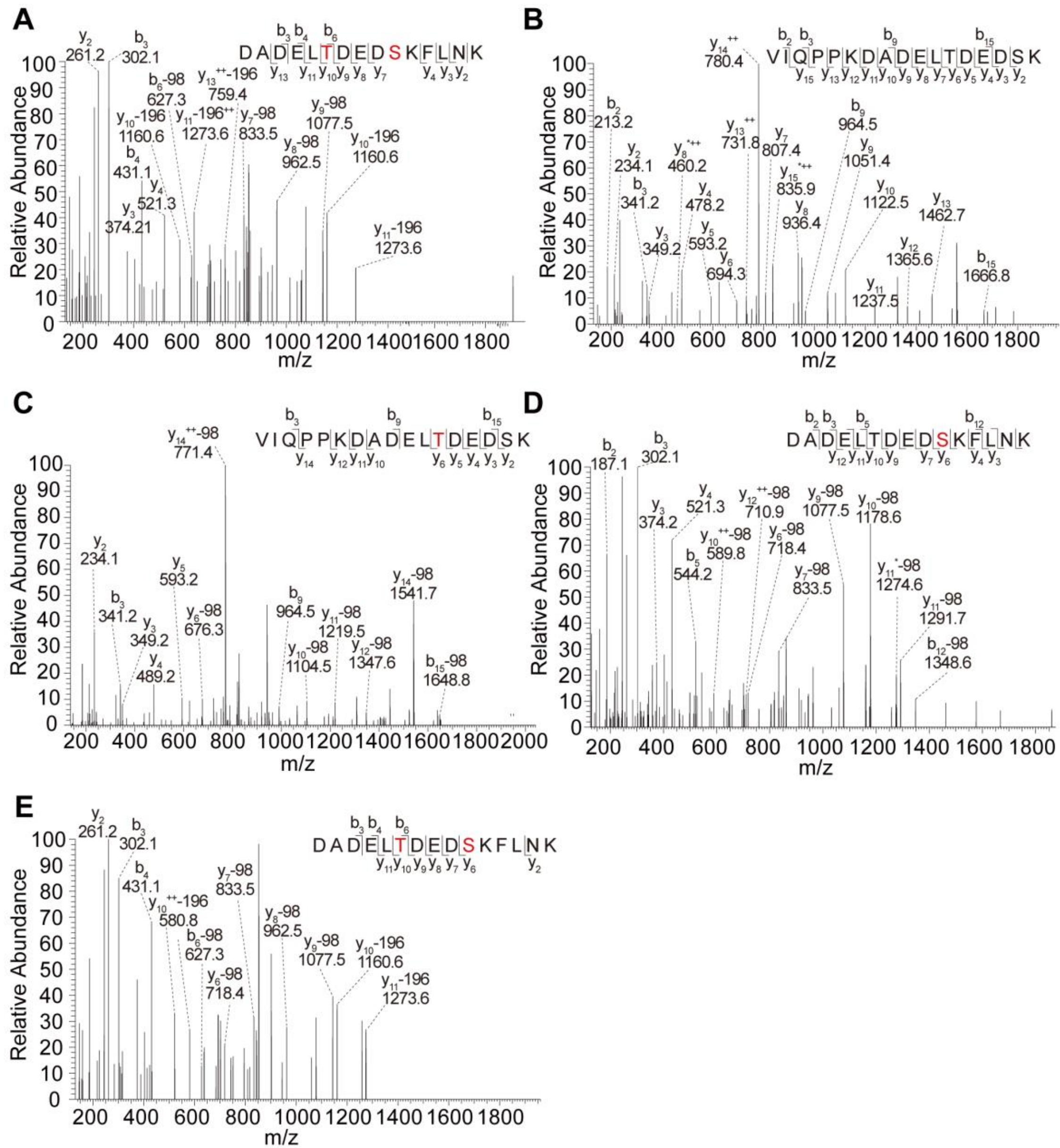

Figure S7. LC-MS/MS spectra revealing the *in vitro* phosphorylation sites in the SOBIR1 β3-αC loop. Related to Figure 4 and Table 2

The wild-type (**A**), PP2Cα-dephosphorylated (**B**), autophosphorylated (**C, D**), and BAK1-phosphorylated (**E**) SOBIR1-KDs were generated as described in the Supplemental Figure 5 legend. Each LC-MS/MS spectrum presents the collection of ions produced by the collision-induced dissociation of the intact peptides. Predominant b and y product ion peaks are labeled and product ions eliciting neutral mass losses of H_3PO_4 (98 Da) are also indicated. The * and ++ indicate the ions that lost ammonia (−17 Da) and the doubly protonated ions, respectively. The phosphorylated residues in each peptide are highlighted in red.

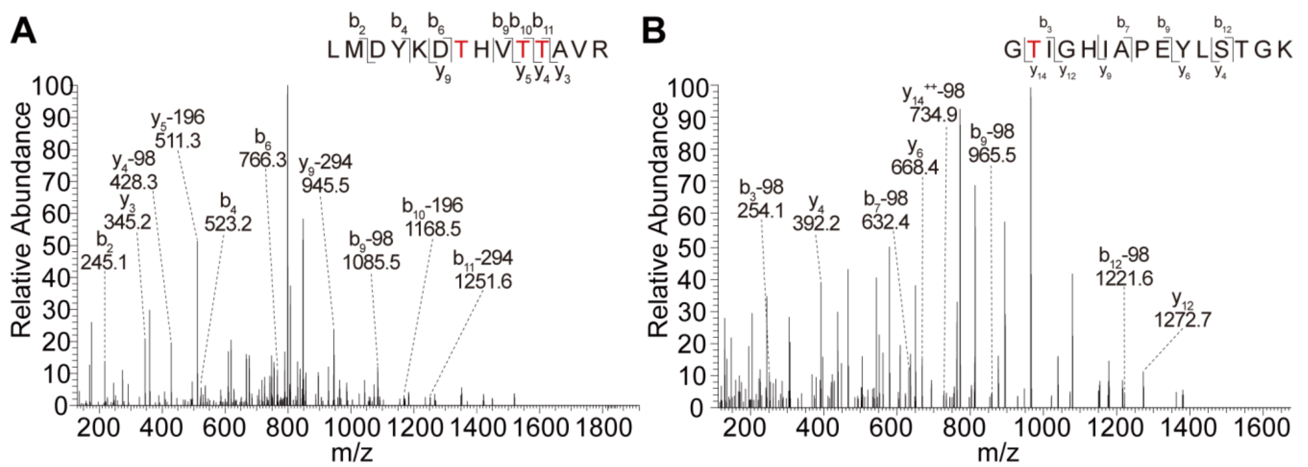

Figure S8. LC-MS/MS spectra revealing the *in vitro* phosphorylation sites of SOBIR1-phosphorylated BAK1. Related to Figure 5 and Table 2

The reaction was completed by incubating 10 μM GST-BAK1-CD^{D434N} and 1 μM SOBIR1-KD at 25 °C for 1 h. Each LC-MS/MS spectrum presents the collection of ions produced by the collision-induced dissociation of the intact peptides. Predominant b and y product ion peaks are labeled and product ions eliciting neutral mass losses of H_3PO_4 (98 Da) are also indicated. The * and ++ indicate ions that lost ammonia (−17 Da) and doubly protonated ions, respectively. Phosphorylated residues in each peptide are highlighted in red.

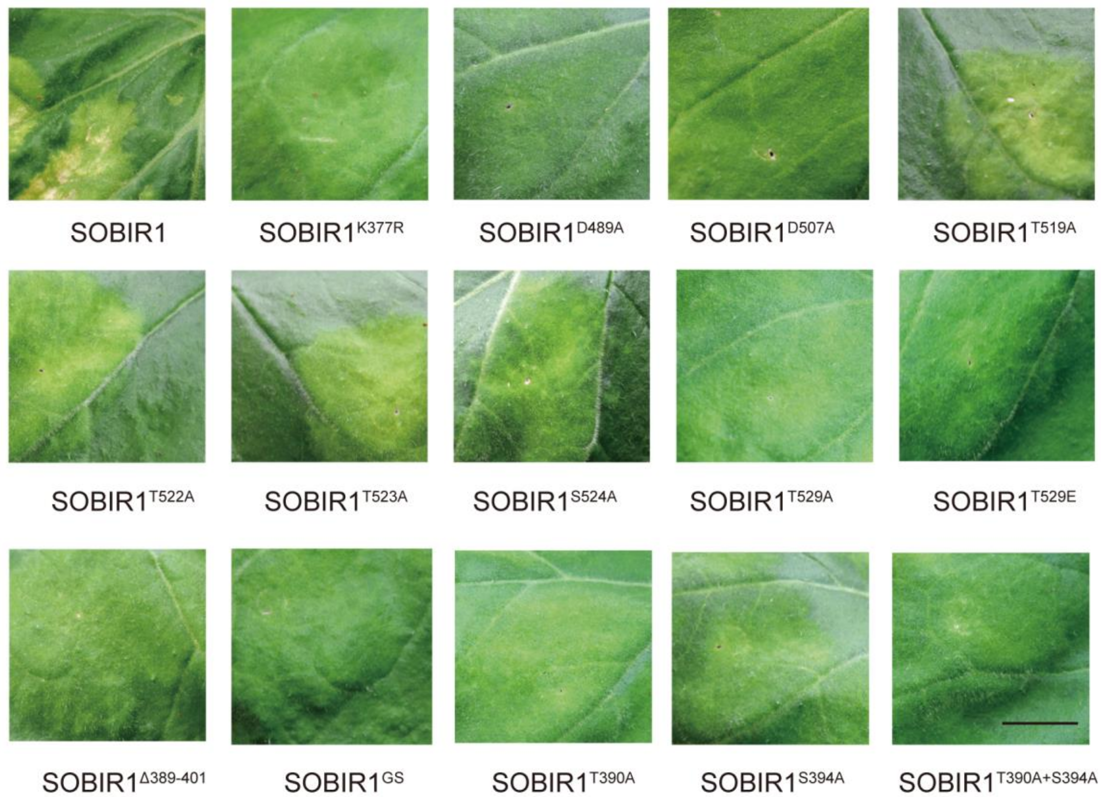

Figure S9. Functional analysis of SOBIR1-KD phosphorylation sites or segment *in planta*. Related to Figure 6 and Figure 7

*At*SOBIR1 and various mutants fused to C-terminally enhanced green fluorescent protein were transiently expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression (agroinfiltrations). Photographs were taken at 4 days postinfiltration. All assays were performed three times and a representative photograph is shown. Scale bar = 0.5 cm.

Supplemental Table 1. Data collection and refinement statistics

	SOBIR1-KD[D489A]	SOBIR1-KD[Δ389-401]
Data collection		
Space group	$P2_1$	$P2_1$
Cell dimensions		
a, *b*, *c* (Å)	67.6, 50.9, 105.4	72.4, 50.7, 93.1
α, β, γ (°)	90°, 95.3°, 90°	90°, 106.6°, 90°
Resolution (Å)	50.00-2.90 (3.00-2.90) [a]	50.00-2.90 (3.00-2.90)
No. of measured reflections	101722	71544
No. of unique reflections	16161	14273
R_{sym} or R_{merge}	0.080(0.430)	0.072 (0.411)
$I / \sigma I$	19.9 (2.5)	14.6 (2.2)
Completeness (%)	99.8 (99.1)	98.1 (95.5)
Redundancy	6.3 (5.2)	5.0 (4.4)
Refinement statistics		
Resolution (Å)	43.39-2.89	28.63-2.91
No. reflections	16148	14267
R_{work} / R_{free} (%)	21.7/23.9	22.0/24.7
No. atoms		
Protein	4443	4535
Ligand/ion	64	62
Water	0	0
B-factors ($Å^2$)		
Protein	94.5	58.9
Ligand/ion	82.1	52.3
Water	0	0
R.m.s. deviations		
Bond lengths (Å)	0.010	0.010
Bond angles (°)	0.74	0.71
Ramachandran plot statistics		
Most favored (%)	97.59	97.77
Allowed (%)	2.41	2.23
Disallowed (%)	0	0

[a]All data sets were collected from a single crystal.

[b]Values in parentheses are for highest-resolution shell.

Supplemental Table 2. Phosphorylated sites identified by LC-MS/MS

Proteins	Identified Sites
SOBIR1-KD	**Thr390, Ser394**, Ser406, **Thr519, Thr522, Thr523, Ser524, Thr529**, Ser566, Thr573, Thr587, Ser592
PP2Cα-dephosphorylated SOBIR1	no phosphorylation sites
autophosphorylated dSOBIR1-KD	**Thr390, Ser394**, Ser406, **Thr529**
BAK1-phosphorylated dSOBIR1-KD	**Thr390, Ser394**, Ser406, Thr410, **Thr519, Thr522, Thr523, Ser524, Thr529**, Ser592
SOBIR1-phosphorylated BAK1-CD^{D434N}	Thr324, **Thr446, Thr449, Thr450, Thr455**, Ser595, Ser602, Thr603, Ser604

Proteins are listed in the first column and the position of the phosphorylated residues corresponding to the proteins are shown in the second column. Phosphorylated sites of proteins identified in the β3-αC loops or the activation segments are highlighted in black bold.

Supplemental Table 3. Primers used in this study

Mutants	Primers	Sequence (5' to 3')
Full length	F1	TGCTCTAG AATGGCTGTTCCCACGGGAAGCG
	R1	CGGGGTAC CCTAGTGCTTGATCTGGGACAACATG
Kinase domain	F2	CGCCATATG TCCATATTCAGCCCATTGATC
	R2	CCGCTCGAG CTAGTGCTTGATCTGGGACAAC
T519A	F3	CAATGCCAGATGCAGTCGCGCATATTACAACCTCGCATG
	R3	CATGCGAGGTTGTAATATGCGCGACTGCATCTGGCATTG
T522A	F4	GATGCAGTCACACATATTGCGACCTCGCATGTTGCAGGTAC
	R4	GTACCTGCAACATGCGAGGTCGCAATATGTGTGACTGCATC
T523A	F5	GCAGTCACACATATTACAGCGTCGCATGTTGCAGGTACTG
	R5	CAGTACCTGCAACATGCGACGCTGTAATATGTGTGACTGC
S524A	F6	GTCACACATATTACAACCGCGCATGTTGCAGGTACTG
	R6	CAGTACCTGCAACATGCGCGGTTGTAATATGTGTGAC
T529A	F7	GCATGTTGCAGGTGCGGTGGGATACATAG
	R7	CTATGTATCCCACCGCACCTGCAACATGC
T529E	F8	GCATGTTGCAGGTGAAGTGGGATACATAG
	R8	CTATGTATCCCACTTCACCTGCAACATGC
△389-401	F9	GATCCAACCGCCTAAAGACGCCGATGAAAGGCAAATTAGATCCGAGATCA
	R9	CTGTGTTGATCTCGGATCTAATTTGCCTTTCATCGGCGTCTTTAGGCGGTT
GS	F10	GCCTAAAGACGCCGATGAAGGUAGCGGUAGCGGUAGCGGUAGCGGUAG CGGUAGGCAAATTAGATCCGAG
	R10	CTCGGATCTAATTTGCCTACCGCTACCGCTACCGCTACCGCTACCGCTACC TTCATCGGCGTCTTTAGGC
T390A	F11	GACGCCGATGAACTAGCGGATGAAGATTCCAAG
	R11	CTTGGAATCTTCATCCGCTAGTTCATCGGCGTC
S394A	F12	GAACTAACAGATGAAGATGCGAAGTTTCTGAACAAG
	R12	CTTGTTCAGAAACTTCGCATCTTCATCTGTTAGTTC
T390A+S394A	F13	GCCGATGAACTAGCGGATGAAGATGCGAAGTTTCTG
	R13	CAGAAACTTCGCATCTTCATCCGCTAGTTCATCGGC

Note: The underline represents the mutagenic position.