Supporting Information

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SI Experimental Procedures

Protein Extraction, Western Blot, and Coimmunoprecipitation Assay. To detect *brassinosteroid insensitive 1* (*bri1*)-Ems-Suppressor 1 (BES1) proteins, 10-d-old seedlings grown on 1/2 MS agar plate were transferred to 6-well cell culture plates containing 1 mL of H_2O per well (10 seedlings per well). After overnight incubation, the seedlings were submerged with 1 mL of 2 μM brassinolide (BL) for 3 h, and ground to fine powder in liquid N2. The total proteins were extracted with 2× sample buffer and subjected for Western blot with an α-*bri1*-Ems-Suppressor (BES1) antibody (a kind gift from Y. Yin) to detect the phosphorylation status of BES1.

For coimmunoprecipitation (Co-IP) assay, 2×10^5 protoplasts transfected with indicated plasmids were lysed with 0.5 mL of extraction buffer (10 mM Hepes at pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% (vol/vol) glycerol, 0.5% Triton X-100, and protease inhibitor mixture from Roche). After vortexing vigorously for 30 s, the samples were centrifuged at 12,470 × *g* for 10 min at 4 °C. The supernatant was incubated with α-HA or α-FLAG antibody for 2 h and then with protein G-agarose beads (Roche) for another 2 h at 4 °C with gentle shaking. The beads were collected and washed three times with washing buffer (10 mM Hepes at pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.1% Triton X-100) and once with 50 mM Tris·HCl at pH 7.5. The immunoprecipitated proteins were analyzed by Western blot with an α-HA or α-FLAG antibody. For seedling Co-IP, approximate 15 g of leaf samples from 4-wk-old soil-grown plants were ground in liquid N2 and further ground in 10 mL of ice-cold extraction buffer 1 (20 mM Tris·HCl at pH 8.5, 150 mM NaCl, 1 mM EDTA, 20% glycerol, 1 mM PMSF, 20 mM NaF, 50 nM microcystin, and protease inhibitor mixture). Samples were centrifuged at 7,000 × *g* for 15 min at 4 °C. The resulting supernatants were further centrifuged at 100,000 × *g* for 2 h at 4 °C to precipitate the total membrane fraction. The pellet was resuspended in 1 mL buffer 2 (10 mM Tris·HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 20 mM NaF, and protease inhibitor mixture). The resulting fraction was used to perform Co-IP assay with the same procedures as protoplast Co-IP assay.

In Vitro Phosphorylation and Immunocomplex Kinase Assays. Expression of GST and MBP fusion proteins and affinity purification were performed as standard protocol. The protein concentration was determined with Nano Drop ND-1000 spectrophotometer and confirmed by the Bio-Rad Quick Start Bradford Dye Reagent. For in vitro kinase assay, kinase reactions were performed in 30 μL of kinase buffer (20 mM Tris·HCl at pH 7.5, 10 mM $MgCl_2$, 5 mM EGTA, 100 mM NaCl, and 1 mM DTT) containing 10 μg of fusion proteins with 0.1 mM cold ATP and 5 μCi of [^{32}P]-γ-ATP at room temperature for 3 h with gentle shaking. The reactions were stopped by adding 4× SDS loading buffer. The phosphorylation of fusion proteins was analyzed by autoradiography after separation with 10% SDS/PAGE. For immunocomplex kinase assays, 0.8-mL protoplasts at a density of 2×10^5/mL were transfected with 120 μg of plasmid DNA. The protoplasts were lysed with 0.5 mL of IP buffer (50 mM Tris·HCl at pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 2 mM NaF, 2 mM Na_3VO_3, 1% Triton, and protease inhibitor mixture). After centrifugation at 13,000 rpm for 10 min at 4 °C, the supernatant was incubated with an α-HA antibody for 2 h and then protein G-agarose beads for another 2 h at 4 °C with gentle shaking. The beads were collected and washed once with IP buffer and once with kinase buffer. The kinase reactions were performed in 20 μL of kinase buffer with 2 μg of GST fusion proteins, 0.1 mM cold ATP, and 5 μCi of [^{32}P]-γ-ATP at room temperature for 1 h with gentle shaking. The phosphorylation of GST and MBP fusion proteins was analyzed by 10% SDS/PAGE.

In Vitro Pull-Down Assay. Five micrograms of GST fusion proteins were preincubated with 5 μL of prewashed amylose agrose beads (New England Biolabs) in 150 μL of incubation buffer (10 mM Hepes at pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.5% Triton X-100) at 4 °C for 1 h with gentle shaking. After centrifuging at 13,000 rpm for 5 min, the supernatant was transferred and incubated with prewashed MBP fusion proteins immobilized on amylose-agarose beads at 4 °C for another 1 h. The beads were collected and washed four times with washing buffer and once with 50 mM Tris·HCl at pH 7.5. The immunoprecipitated proteins were analyzed by Western blot with an α-GST antibody.

Real-Time RT-PCR Analysis. Total RNA was isolated from leaves or seedlings with TRIzol Reagent (Invitrogen). First-strand cDNA was synthesized from 1 μg of total RNA with reverse transcriptase. Real-time RT-PCR analysis was carried out by using iTaq SYBR green Supermix (Bio-Rad) supplemented with reference dye (ROX) in an ABI GeneAmp PCR System 9700. Polyubiquitin 10 (*UBQ10*) was used as a control gene, and the expression of individual genes was normalized to the expression of *UBQ10*. The RT-PCR primer sequences are listed below.

Bimolecular Fluorescence Complementation Assay. The full-length Brassinosteroid Insensitive 1 (BRI1) or Botrytis-Induced Kinase 1 (BIK1) was subcloned into the modified bimolecular fluorescence complementation (BiFC) vectors (a kind gift from F. Rolland, Leuven, Belgium) with BamHI and StuI digestion. Two hundred microliters of protoplasts at a density of 2×10^5/mL were transfected with 40 μg of total DNA constructs. Fluorescent signals in the protoplasts were examined with confocal microscope (Leica Microsystems) 18 h after transfection. The filter sets used for excitation (Ex) and emission (Em) are as follows: GFP, 488 nm (Ex)/BP505 to 530 nm (Em); chlorophyll, 543 nm (Ex)/LP650 nm (Em); bright field, 633 nm. Signals were captured in multichannel mode, and images were analyzed and processed with Leica LAS AF Life and Adobe Photoshop.

Primers Used in This Study. Cloning primers (the restriction enzyme sites are underlined and start codon is italicized):

BRI1: 5′-CGGGATCC*ATG*AAGACTTTTTCAAGCTTC-3′ and 5′GAAGGCCTTAATTTTCCTTCAGGAAC-3′,

BRICD: 5′-CGGGATCC*ATG*AGAGAGATGAGGAAGAGACG-3′ and 5′-GAAGGCCTTAATTTTCCTTCAGGAAC-3′, and

BIK1 promoter: 5′-CCGCTCGAGCTCGAGATAGCGATGAGAGAGACAG-3′ and 5′-CGGGATCCCAAAGCTAAGAACAGATTC-3′.

Point mutation primers (the mutated sites are underlined):

BRI1CDKm: 5′-GCGCGGTGGCTATCGAGAAACTGATTCATG-3′ and 5′-CATGAATCAGTTTCTCGATAGCCACCGCGC-3′.

qRT-PCR primers:

UBQ10: 5′-AGATCCAGGACAAGGAAGGTATTC-3′ and 5′-CGCAGGACCAAGTGAAGAGTAG-3′,

CPD: 5′-TTGCTCAACTCAAGGAAGAG-3′ and 5′-TGATGTTAGCCACTCGTAGC-3′,

DWF4: 5′-CATAAAGCTCTTCAGTCACGA-3′ and 5′-CGTCTGTTCTTTGTTTCCTAA-3′, and

BR6OX: 5′-AAACCAAAGACTAAGATATGGGG-3′ and 5′-GAATATCAAGCATAGATTGCGG-3′.

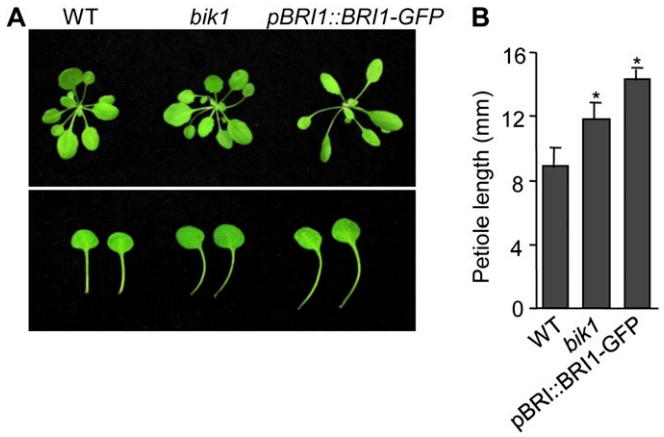

Fig. S1. The *bik1* mutant plants have moderately elongated and curling petioles, reminiscent of *pBRI1::BRI1-GFP* plants. (*A*) The aerial parts (*Upper*) and detached second pair of leaves (*Lower*) of 4-wk-old WT, *bik1* mutant, and *pBRI1::BRI1-GFP* transgenic plants. (*B*) The average petiole length of second pair of leaves of WT, *bik1* mutant, and *pBRI1::BRI1-GFP* transgenic plants. The data are shown as mean ± SE from at least 20 4-wk-old plants. Asterisk indicates a significant difference with $P < 0.05$ compared with data from WT plants.

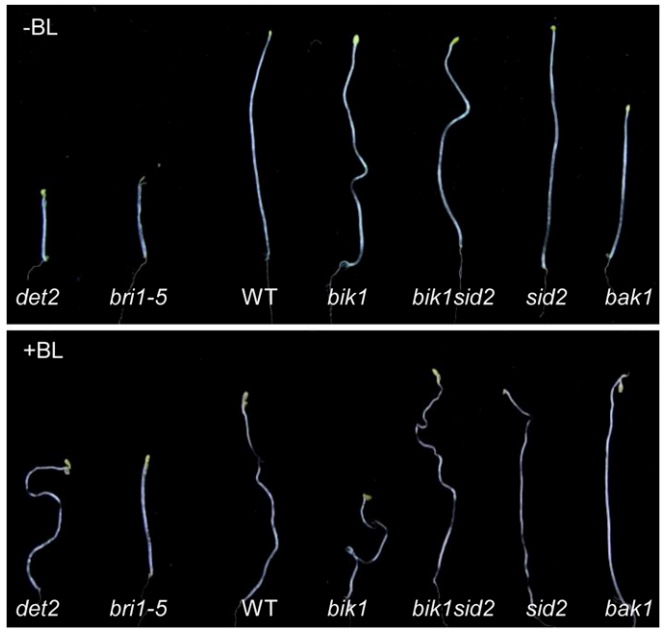

Fig. S2. The dark grown *bik1* mutant exhibited twisted hypocotyls. The *Arabidopsis* seedlings of the indicated genotypes were grown in the dark for 7 d in the absence (*Upper*) or presence of 50 nM BL (*Lower*).

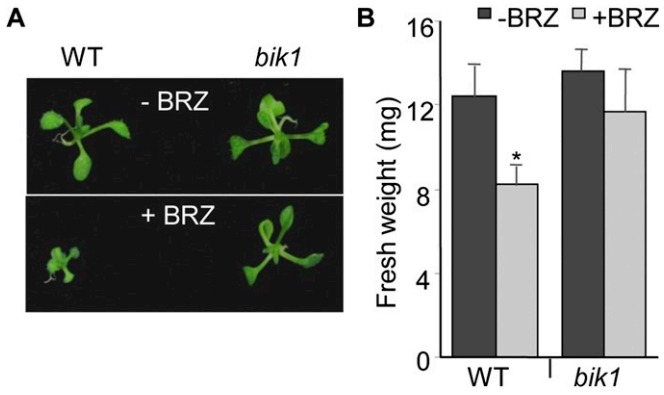

Fig. S3. Insensitivity of *bik1* mutant to Brassinazole-Resistant (BRZ) treatment. (*A*) The phenotype of WT and *bik*1 mutant grown in 2 μM BRZ for 14 d. (*B*) The fresh weight of WT and *bik*1 mutant in the absence or presence of BRZ. The data are shown as mean ± SE from at least 25 seedlings. Asterisk indicates a significant difference with $P < 0.05$ compared with data without BRZ treatment.

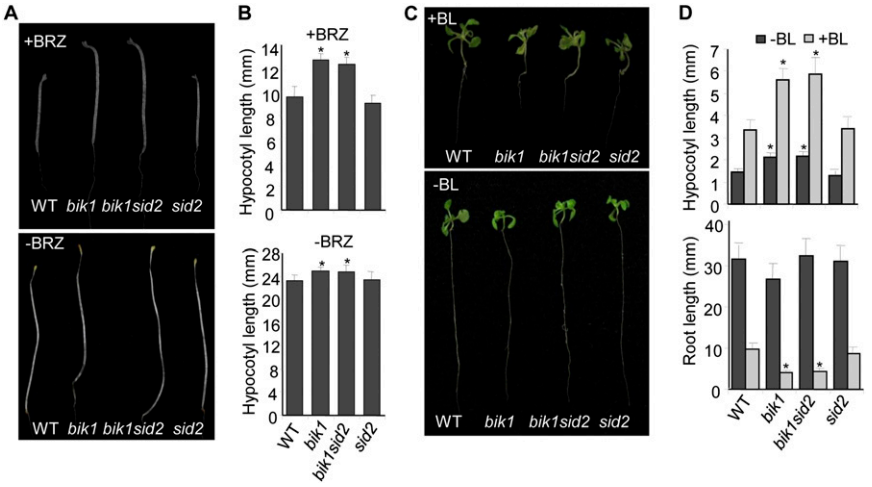

Fig. S4. The brassinosteroid (BR) hypersensitivity of *bik1* mutant is SA independent. (*A*) Eight-day-old dark-grown seedlings of WT, *bik1*, *bik1sid2*, and *sid2* plants in the absence or presence of 2 μM BRZ. (*B*) The average hypocotyl length of WT, *bik1*, *bik1sid2*, and *sid2* seedlings. (*C*) Fourteen-day-old seedlings of WT, *bik1*, *bik1sid2*, and *sid2* plants in the absence or presence of 100 nM BL under the constant light. (*D*) The average hypocotyl and root length of WT, *bik1*, *bik1sid2*, and *sid2* seedlings. The data are shown as mean ± SE from at least 20 seedlings. Asterisk indicates a significant difference with $P < 0.05$ compared with data from WT seedlings.

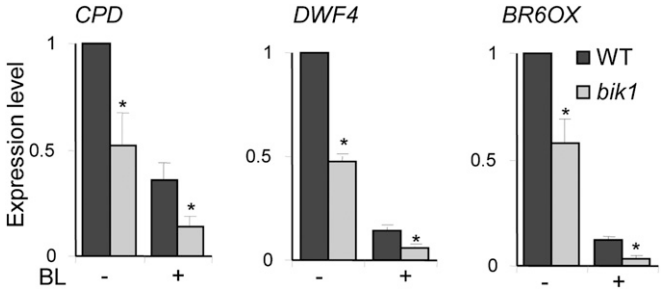

Fig. S5. BR-regulated gene expression in WT and *bik1* plants. The leaves of 4-wk-old plants were treated with 2 μM BL or H_2O control for 3 h, and the samples were collected for quantitative RT-PCR (qRT-PCR) analysis. The expression of *CPD* (*Left*), *DWF4* (*Center*), and *BR6OX* (*Right*) was normalized to the expression of *UBQ10*. The data are shown as the mean ± SE from three independent biological replicates. Asterisk indicates a significant difference with $P < 0.05$ compared with data from WT plants.

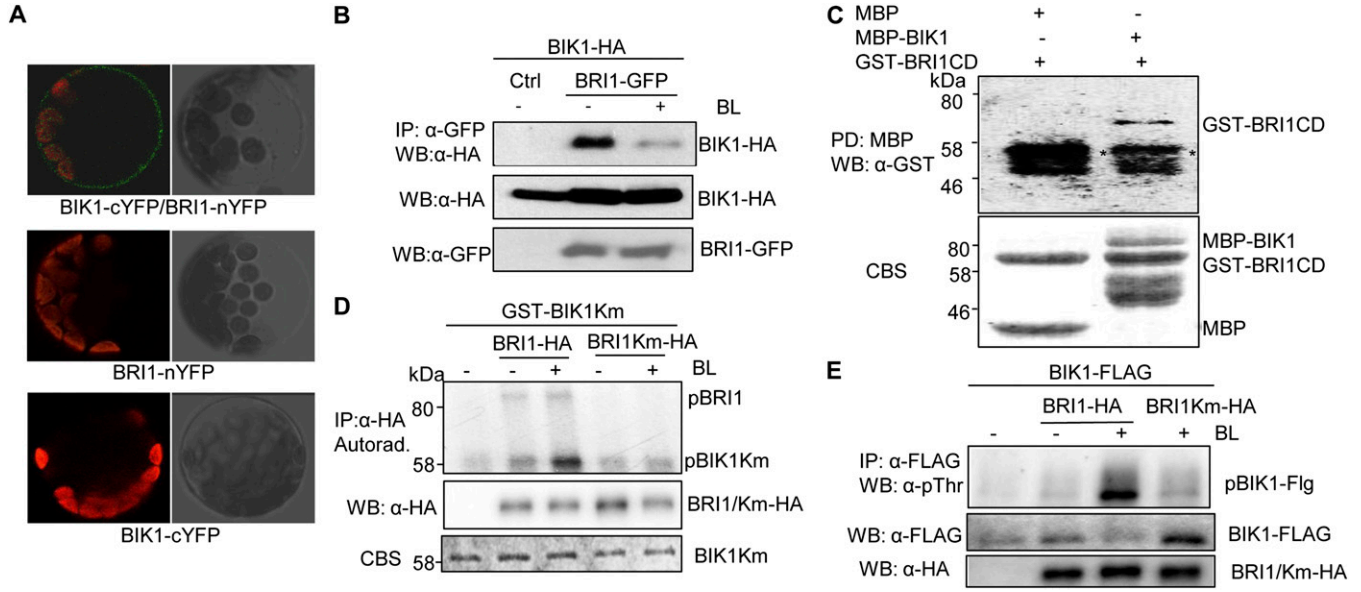

Fig. S6. BIK1 and BRI1 interaction and phosphorylation. (*A*) BIK1 interacts with BRI1 with BiFC assay in *Arabidopsis* protoplasts. The various BiFC constructs were transfected into protoplasts and the cells were observed under a confocal microscope. (*B*) BIK1-BRI1 association and BL-induced dissociation in *Nicotiana benthamiana*. Two-week-old *N. benthamiana* was inoculated with *Agrobacteria* carry 35S::BIK1-HA and pBRI1::BRI1-GFP. Two days after inoculation, the leaves were treated with 2 μM BL for 3 h. Leaves (1.1 mg) for each example were collected for Co-IP and immunoblot. (*C*) MBP-BIK1 pulls down GST-BRI1CD. An in vitro pull-down assay was performed with MBP or MBP-BIK1 immobilized on amylose-agarose beads as bait (PD:MBP) against GST-BRI1CD fusion proteins. BRI1CD was detected by Western blot with an α-GST antibody, and the protein loading control was shown by CBS. Asterisk indicates nonspecific bands with α-GST antibody. (*D*) BL treatment enhances BRI1, but not BRI1Km-mediated phosphorylation on BIK1. BRI1-HA or BRI1Km-HA was expressed in WT protoplasts for 10 h followed by 2 μM BL treatment for 2 h. BRI1-HA or BRI1Km-HA proteins were immunoprecipitated with an α-HA antibody and subjected to an in vitro kinase assay with GST-BIK1Km proteins as substrates (*Top*). *Middle* shows the BRI1-HA and BRIKm-HA expression, and *Bottom* shows GST-BIK1Km proteins. (*E*) BL-induced BIK1 phosphorylation by BRI1. BIK1-FLAG was coexpressed with BRI1-HA or BRI1Km-HA in protoplasts, and incubated for 12 h before treated with 2 μM BL for 3 h. BIK1 proteins were immunoprecipitated with an α-FLAG–agarose beads, separated by 10% SDS/PAGE, and immunoblotted with an α-pThr–HRP antibody.

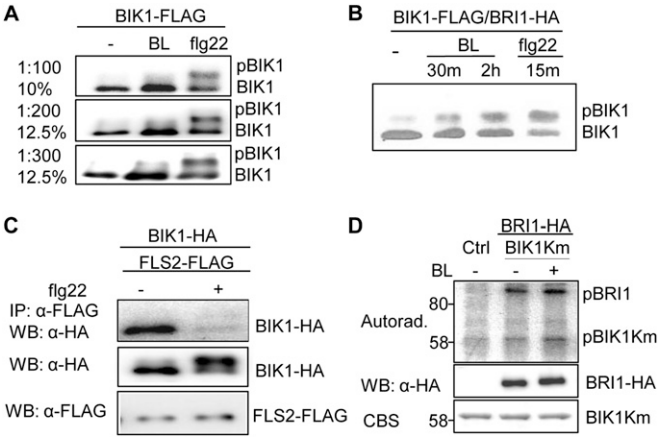

Fig. S7. BRI1 phosphorylates BIK1. (*A*) BL and flg22 induce BIK1 phosphorylation. The protoplasts were transfected with BIK1-FLAG and incubated for 6 h before 2 μM BL treatment for 2 h or 1 μM flg22 treatment for 15 min. The samples were collected for SDS/PAGE with indicated acrylamide concentration at different ratio of bisacrylamide to acrylamide. (*B*) Overexpression BRI1 promotes BL-induced BIK1 phosphorylation. The protoplasts were cotransfected with BIK1-FLAG and BRI-HA, and incubated for 6 h before 2 μM BL treatment for 30 min or 2 h, or 1 μM flg22 treatment for 15 min. The samples were collected for SDS/PAGE with 10% acrylamide at a ratio of 1:37.5 for bisacrylamide to acrylamide. (*C*) flg22-induced Flagellin Sensing 2 (FLS2)-BIK1 dissociation in WT protoplasts. The protoplasts were coexpressed with BIK1-HA and FLS2-FLAG and incubated for 6 h before1 μM flg22 treatment for 15 min. Co-IP was carried out with an α-FLAG antibody (IP: α-FLAG), and the proteins were analyzed by using Western blot with α-HA antibody. (*D*) BRI1-associated kinase 1 (BAK1)-independent BL-induced BRI1 phosphorylation on BIK1. BRI1-HA was expressed in *bak1-4* protoplasts for 10 h followed by 2 μM BL treatment for 2 h. BRI1-HA proteins were immunoprecipitated with α-HA antibody and subjected to an in vitro kinase assay with GST-BIK1Km proteins as substrates.

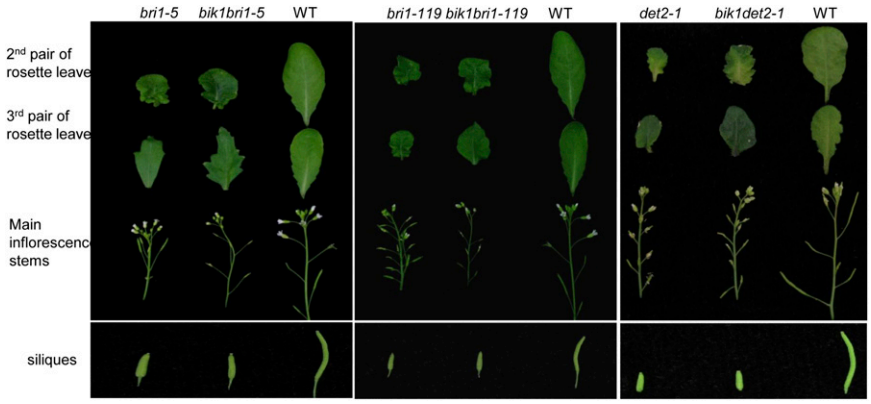

Fig. S8. Leaves, stems and siliques of 8-wk-old *bik1bri1-5, bik1bri1-119*, and *bik1det2* mutants. The second and third pairs of rosette leaves of *bik1bri1-5, bik1bri1-119*, and *bik1det2* double mutants are bigger than the corresponding single mutants. The inflorescence and siliques of *bik1bri1-5, bik1bri1-119*, and *bik1det2* double mutants still resemble *bik1* mutant.

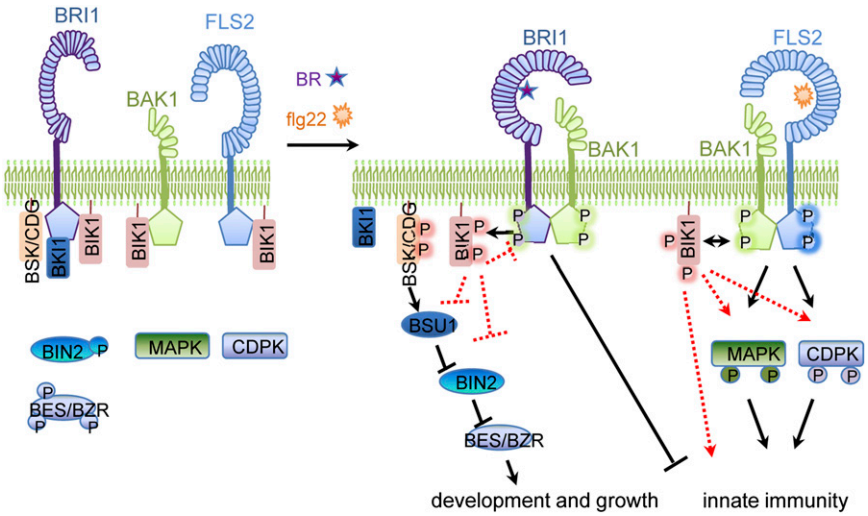

Fig. S9. A model of BIK1-mediated inverse modulation of flagellin and BR signaling. In the absence of flagellin (flg22) and low level of BR, BIK1 associates with FLS2, BRI1, and BAK1 in an inactive state. BR binding to BRI1 leads to BRI1 activation, which in turn phosphorylates BIK1. The phosphorylated BIK1 is released from BRI1 complex and suppresses BR-mediated plant development and growth. BIK1 negatively regulates BR signaling possibly through modulating the activity of BRI1 or downstream components, such as *bri1* Suppressor 1 and Brassinosteroid Insensitive 2 or unknown components (red dotted lines). In flagellin signaling, flg22 binding to FLS2 leads to recruitment and activation of BAK1, which phosphorylates BIK1. The phosphorylated BIK1 is able to transphosphorylate FLS2/BAK1 complex and results in the release of BIK1 from the complex to positively regulate flagellin-mediated plant immunity. BIK1 may function upstream or independent of MAPK and CDPK cascades to activate plant innate immunity (red dotted arrows).