Supporting Information

Albrecht et al. 10.1073/pnas.1109921108

Comments on Fig. S8

To provide further support for the differential recruitment of BAK1 upon activation of the BRI1 or FLS2 receptor, a number of calibration experiments were performed. The first of these was to determine the total amount of tagged receptors in their respective lines. This was done by using quantitative Western blotting essentially as described previously (1). Briefly, a serial dilution series of YFP was run on the same gel as a serial dilution of a total extract of 7 d *BRI1p::BRI1-eGFP* expressing seedlings. YFP, GFP, and eGFP respond similarly to anti-BRI1 serum on Western blots. After blotting and incubation with GFP antiserum, the blots were developed with the ECL system and scanned in a Bio-Rad Imaging Lab system (Fig. S8*A*, *1*). The YFP data were used to obtain a linear fit (red line, Fig. S8*A*, *2*). Values for the total seedling extracts were then obtained for successive serial dilutions, excluding the highest and lowest values, and the average value was calculated. The resulting concentration of GFP was determined to be 17.5 pmol g^{-1} fresh weight (FW) for *BRI1p::BRI1-eGFP* transgenic lines. Multiple independent biological replicates revealed variation (Fig. S8*B*, *1* and *2*). This resulted in values of 17.5 ± 6.1 pmol g^{-1} FW for *BRI1p::BRI1-eGFP* transgenic lines (n = 14) and 8.0 ± 1.9 pmol g^{-1} FW for *FLS2p::FLS2-3myc-GFP* transgenic lines (n = 5).

These results show that the amount of input for the different coimmunoprecipitation experiments can vary approximately twofold. However, the amount of the two different receptors used here can clearly be distinguished from one another. The amount of BRI1-eGFP determined here is in agreement with the value of 2.1 ± 0.7 pmol g^{-1} FW reported for roots of the same *BRI1p::BRI1-eGFP* line (1), taking into account that the amount of GFP is approximately eightfold higher in seedling aerial parts than in roots.

The next series of calibration experiments were aimed to estimate the reproducibility of the immunoprecipitation (IP) step itself. In Fig. S8*C*, the amount of GFP left after incubation with anti-GFP beads is compared with the input, showing that for both receptors used, very little GFP remains in solution (~3%; n = 8). The amount of GFP that is recovered from the beads is then estimated from Western blots similar to the ones presented in Fig. 4*B* and expressed as percentage of the input (Fig. S8*D*, *1* and *2*). For BRI1-eGFP IP, the efficiency is 41 ± 5% (n = 7), and that for FLS2-3myc-GFP IP is 39 ± 4% (n = 4), showing good reproducibility. The GFP fusion proteins not recovered from the beads after IP are probably bound irreversibly or have been degraded during the incubation.

Finally, the efficiency of the various time-course co-IP experiments was compared. The results vary substantially between the individual experiments; therefore, a percentage of an arbitrarily set level was used to compare the contribution of BAK1 coimmunoprecipitated during the different treatments within the same experiment. The results confirm a large difference in the recruitment of BAK1 by BRI1 compared with FLS2 (Fig S8*E*). In general, less then 5% of BAK1 is being recruited by BRI1 compared with FLS2. Even at the longest incubation, no more than 10% of BAK1 was recruited by BRI1.

In conclusion, we believe the quantification experiments described here show that the IP procedure in different tagged lines is reproducible and of similar efficiency. Furthermore, we show that the amount of BRI1-eGFP is approximately twofold higher than the amount of FLS2-3myc-GFP in comparable lines grown under the same conditions. As the amount of BAK1 is unchanged in both lines, this implies that the large differences seen in recruitment of BAK1 by FLS2 compared with BRI1 cannot be attributed to a difference in level of the ligand-perceiving receptors.

SI Materials and Methods

Plant Materials and Growth. The mutant lines used in this study are Col-0/*bak1-4* (2), Ws-2/*bri1-9* (3), and Col-0/*bri1-301* (4). The transgenic line Ws-0/*FLS2p::FLS2-3myc-GFP* was previously described (5), as were the *rps5-2*/*BIK1p::BIK1-HA* line (6) and the Col-0/*BRI1p::BRI1-eGFP* line (7). Transgenic plants harboring the *35::BES1-GFP* gene were generated by using *Arabidopsis* Col-0 plants, and the *35S::BES1-GFP* binary construct was described previously (8). The Col-0/*BAK1p::BAK1-GFP* transgenic line was generated as follows: the 2-kb promoter region was directionally cloned with XhoI-SacII in a modified pDONRP4-P1r by using the following primers: BAK1p forward, 5′-CCGGCTCGAGGTCGTCATATTGAGAAGTCG-3′; and BAK1p reverse, 5′-TCCGCCGCGGTTTATCCTCAAGAGATTAAAAACAAACCC-3′. The *BAK1* gene was amplified from genomic DNA by using Phusion high-fidelity DNA polymerase (New England Biolabs) and the following primers: BAK1 forward, 5′-CACCATGGAACGAAGATTAATGATCCCTTGC-3′; and BAK1 reverse, 5′-TCTTGGACCCGAGGGGTATTCG-3′. The amplified fragment was directionally cloned into pENTR-D-TOPO (Invitrogen). These two constructs, together with pDONRP2r-P3-GFP and pK7m34GW, were recombined by using LR Clonase II Plus (Invitrogen), creating C-terminal *BAK1-GFP* gene fusion (9). The construct was verified by sequencing. This binary vector containing the *BAK1p::BAK1-GFP* construct was used to transform *Arabidopsis* Col-0 plants.

Arabidopsis plants used in this study were grown as one plant per pot at 20 °C to 21 °C with a 10-h photoperiod in environmentally controlled chambers. For *Arabidopsis* sterile seedlings, seeds were surface-sterilized and germinated on plates containing Murashige–Skoog medium (including vitamins; Duchefa) and 1% sucrose supplemented with 0.8% agar for the first 5 d at 22 °C and with a 16-h photoperiod. Seedlings were then pricked out in liquid Murashige–Skoog medium supplemented with 1% sucrose.

RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were treated with Turbo DNA-free DNase (Ambion) and quantified with a Nanodrop spectrophotometer (Thermo Scientific). First-strand cDNA was synthesized from 3 μg of RNA by using SuperScript RNA H-Reverse Transcriptase (Invitrogen) and an oligo(dT) primer, according to the manufacturer's instructions. cDNA were amplified in triplicate by quantitative PCR by using SYBR Green JumpStart Taq ReadyMix (Sigma) and the PTC-200 Peltier Thermal Cycler (MJ Research). The relative expression values were determined by using *U-box* as reference and the comparative Ct method ($2^{-\Delta\Delta Ct}$). Primers used for quantitative PCR are as follows: *SIRK*/*FRK1* (*At2g19190*) forward, 5′-ATCTTCGCTTGGAGCTTCTC-3′; and reverse, 5′-TGCAGCGCAAGGACTAGAG-3′ (10); *At2g17740* gene forward, 5′-TGCTCCATCTCTCTTTGTGC-3′; and reverse, 5′-ATGCGTTGCTGAAGAAGAGG-3′ (11); *U-box* (*At5g15400*) forward, 5′-TGCGCTGCCAGATAATACACTATT-3′; and reverse, 5′-TGCTGCCCAACATCAGGTT-3′ (10); *CPD* (*At5g05690*) forward, 5′-CCCAAACCACTTCAAAGATGCT-3′; and reverse, 5′-GGGCCTGTCGTTACCGAGTT-3′ (12).

Protein Extraction and IP Experiments. For Ws-0/*FLS2p::FLS2-3myc-GFP* and Col-0/*BAK1p::BAK1-GFP* transgenic lines, seedlings (2 g fresh weight) were treated with 1 μM flg22, elf18, and/or epiBL, ground in liquid nitrogen, and then extracted with buffer [50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton ×100; 1% (vol/vol) protease inhibitor mixture (Sigma)] added at 1 mL/g powder. Samples were centrifuged 15 min at 4 °C and 21,000 × *g*. Supernatants were adjusted to 4 mg/mL protein and incubated 4 h at 4 °C with 25 μL GFP-Trap coupled to agarose beads (Chromotek) with gentle agitation. Following incubation, the beads were collected and washed four times with the extraction buffer. SDS loading buffer was then added to the beads that were boiled for 10 min. Proteins were separated by SDS/PAGE 10% and further analyzed by Western blot using mouse monoclonal anti-GFP antibodies coupled to horseradish peroxidase (Miltenyi Biotec), rabbit polyclonal antiphosphothreonine antibodies (Zymed-Invitrogen) or rabbit polyclonal anti-BAK1 antibodies.

BES1 Phosphorylation. Total proteins were extracted as described for transgenic lines in the previous section by using 10-d-old seedlings treated with 1 μM flg22 and/or 1 μM epiBL for the indicated times. The phosphorylation status of BES1-GFP was determined based on its electrophoretic mobility on SDS/PAGE, followed by immunodetection with mouse monoclonal anti-GFP antibodies coupled to horseradish peroxidase (Miltenyi Biotec). The immunoblotting experiment was performed by using 20 μg of proteins, which were separated on Mini-PROTEAN TGX 4% to 15% (Bio-Rad).

BIK1 Phosphorylation. To prepare samples for examining the accumulation of BIK1-HA, protein extraction was performed as described in the next section. Proteins were separated by SDS/PAGE 12% and further analyzed by Western blot by using mouse polyclonal anti-HA antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology).

In Vitro Phosphorylation Assay. Two-week-old WT Col-0 seedlings were treated with 1 μM flg22 and/or epiBL and ground in liquid nitrogen. Proteins were extracted with 0.5 volume/weight of buffer [50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 10% glycerol; 1 mM EDTA; 5 mM DTT; 1% (vol/vol) protease inhibitor mixture (Sigma); 1% (vol/vol) Nonidet P-40, 2.5 mM Na_3VO_4, 50 nM calyculin A, 1 mM PMSF, 10 mM NaF, 5 mM Na_2MoO_4]. Samples were centrifuged 15 min at 4 °C and 21,000 × *g*. Supernatants were filtered and adjusted to 2 to 3 mg/mL protein; 1.5 mL were incubated with gentle agitation for 4 h at 4 °C in the presence of 20 μL TrueBlot anti-rabbit Ig IP beads (eBioscience) and either 15 μL anti-BAK1 antibodies or 15 μL anti-FLS2 antibodies. Beads were washed twice with washing buffer 1 (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 1% Nonidet P-40) and once with washing buffer 2 (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 M NaCl, 1% Nonidet P-40). Anti-BAK1 immunoprecipitates were washed once with kinase buffer 1 [20 mM Hepes, pH 7.4, 15 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT, 1 μM ATP] and anti-FLS2 immunoprecipitates were washed with kinase buffer 2 (50 mM Tris-HCl, pH 7.4, 2 mM $MnCl_2$, 1 mM DTT, 1 μM cold ATP). Immunoprecipitates were finally incubated 30 min at 30 °C and under vigorous shaking with 30 μL of their respective kinase buffers supplemented with radioactive [^{32}P]γ-ATP (183 kBq; Perkin-Elmer). The reactions were stopped by addition of 10 μL of NuPAGE 4× LDS sample buffer (Invitrogen) in presence of 1× reducing agent and denatured for 10 min at 70 °C. Proteins were separated by SDS/PAGE 10% and analyzed by Western blot by using rabbit polyclonal anti-FLS2 antibodies or anti-BAK1 antibodies. Immunoblots were subsequently immersed in methanol to denature the antibodies. The membranes were subject to autoradiography by using a FLA5000 PhosphorImager (Fuji).

1. van Esse GW, et al. (2011) Quantification of the Brassinosteroid Insensitive1 receptor in planta. *Plant Physiol* 156:1691–1700.
2. Chinchilla D, et al. (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448:497–500.
3. Noguchi T, et al. (1999) Brassinosteroid-insensitive dwarf mutants of Arabidopsis accumulate brassinosteroids. *Plant Physiol* 121:743–752.
4. Xu W, Huang J, Li B, Li J, Wang Y (2008) Is kinase activity essential for biological functions of BRI1? *Cell Res* 18:472–478.
5. Robatzek S, Chinchilla D, Boller T (2006) Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis. *Genes Dev* 20:537–542.
6. Zhang J, et al. (2010) Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector. *Cell Host Microbe* 7:290–301.
7. Geldner N, Hyman DL, Wang X, Schumacher K, Chory J (2007) Endosomal signaling of plant steroid receptor kinase BRI1. *Genes Dev* 21:1598–1602.
8. Yin Y, et al. (2002) BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. *Cell* 109:181–191.
9. Karimi M, Bleys A, Vanderhaeghen R, Hilson P (2007) Building blocks for plant gene assembly. *Plant Physiol* 145:1183–1191.
10. Boutrot F, et al. (2010) Direct transcriptional control of the *Arabidopsis* immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1. *Proc Natl Acad Sci USA* 107:14502–14507.
11. He P, et al. (2006) Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in *Arabidopsis* innate immunity. *Cell* 125:563–575.
12. Goda H, Shimada Y, Asami T, Fujioka S, Yoshida S (2002) Microarray analysis of brassinosteroid-regulated genes in Arabidopsis. *Plant Physiol* 130:1319–1334.

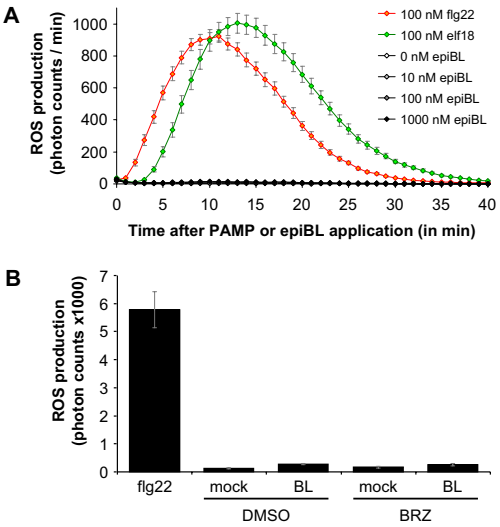


Fig. S1. Exogenous epiBL does not induce reactive oxygen species (ROS) in leaf discs (*A*) not pretreated or (*B*) pretreated with the brassinosteroid (BR) biosynthesis inhibitor brassinazole (BRZ). (*A*) Oxidative burst triggered by 100 nM flg22 (in 0.005% ethanol), 100 nM elf18 (in 0.005% ethanol), or different concentrations of epiBL (in 0.005% ethanol) in Col-0 leaf discs. ROS production is measured during 40 min after treatment and presented in photon counts per minute. The corresponding total photon counts are given in Fig. 1*B*. (*B*) Oxidative burst triggered by 1 μM flg22 (in 0.005% ethanol, 0.025% DMSO), mock (0.005% ethanol), or 1 μM epiBL (in 0.005% ethanol) in leaf discs, from 5-wk-old Col-0 plants, pretreated for 16 h with mock (0.025% DMSO) or 2.5 μM BRZ (in 0.025% DMSO). ROS production is presented as total photon counts during 40 min of treatment. Values are mean ± SE (n = 20).

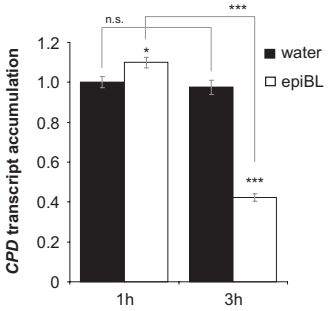


Fig. S2. Five-week old plants are responsive to exogenous epiBL. Quantitative RT-PCR analysis of *CPD* expression in leaf discs of 5-wk-old Col-0 plants treated with water or 1 μM epiBL for 1 or 3 h. Transcript levels are normalized to the *U-box* housekeeping gene and are presented as relative to 1 h treatment with water. Values are mean ± SD (n = 3; *P < 0.05 and ***P < 0.001).

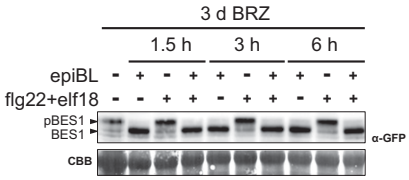


Fig. S3. Activation of BAK1 following perception of multiple PAMPs does not enhance BR signaling. EpiBL-induced mobility shift caused by the dephosphorylation of the BR target transcriptional regulator BES1-GFP was examined after the indicated times of seedling treatment using anti-GFP antibodies. *35S::BES1-GFP* seedlings were pretreated with 2.5 μM of the BR biosynthesis inhibitor BRZ for 3 d, then treated with 1 μM epiBL and/or a combination of flg22 and elf18 (1 μM each). Blot stained with colloidal brilliant blue (CBB) is presented to show equal loading.

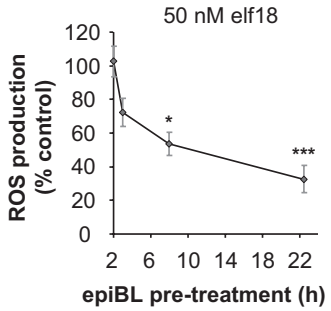


Fig. S4. BR perception inhibits ROS signaling triggered by elf18. Effect of pretreatment with 1 μM epiBL for the indicated time on ROS triggered by 50 nM elf18. Total ROS production during 40 min of treatment is expressed as percentage of elf18-treated Col-0 without epiBL pretreatment. Values are mean ± SE (n = 20; $^{*}P < 0.05$ and $^{***}P < 0.001$).

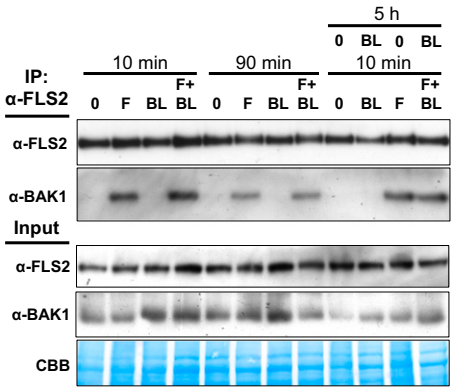


Fig. S5. EpiBL treatment did not affect the amount of BAK1 recruited by FLS2. IP of FLS2 in Col-0 seedlings treated with 1 μM flg22 (F) and/or epiBL (BL) for 10 min or 90 min (with or without 5 h epiBL pretreatment). Coimmunoprecipitated proteins were further analyzed by using anti-FLS2 or anti-BAK1 antibodies. The blot stained with CBB is presented to show equal loading.

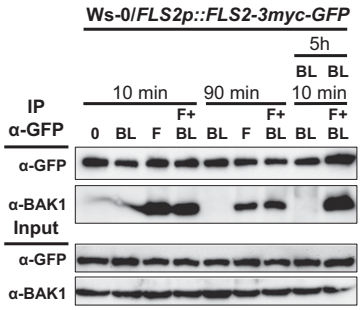


Fig. S6. EpiBL treatment did not affect the amount of BAK1 recruited by FLS2-3myc-GFP. IP of GFP in Ws-0/*FLS2p::FLS2-3myc-GFP* seedlings treated with 1 μM flg22 (F) and/or epiBL (BL) for 10 min or 90 min (with or without 5 h epiBL pretreatment). Coimmunoprecipitated proteins were further analyzed by using anti-GFP and anti-BAK1 antibodies.

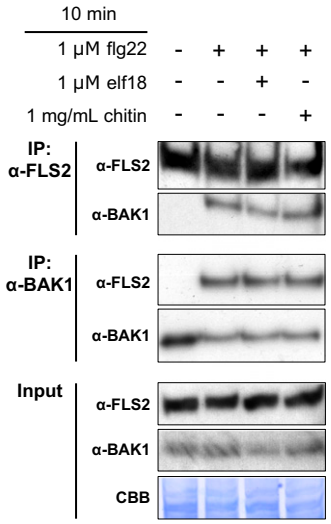
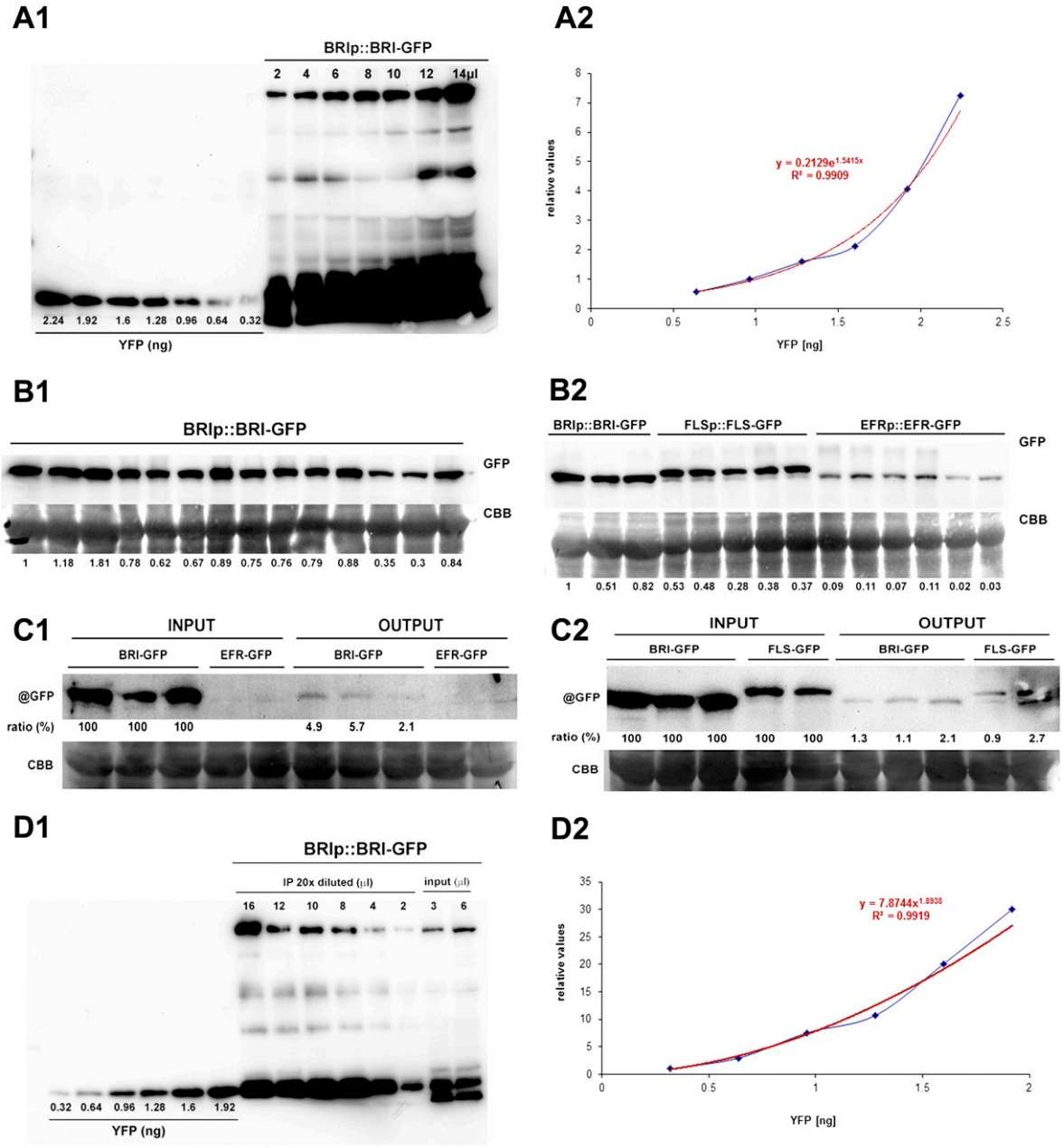


Fig. S7. The amount of BAK1 available for recruitment by both FLS2 and EFR is not limiting. IP of FLS2 and BAK1 in Col-0 seedlings treated with 1 μM flg22, 1 μM elf18, or 1 mg/mL chitin for 10 min. Coimmunoprecipitated proteins were further analyzed by using anti-FLS2 anti-BAK1 antibodies. The blot stained with CBB is presented to show equal loading.



E

Amount of BAK1 immunoprecipitated in BRI-GFP IP versus FLS or EFR IP at different time points (%)								
	Time curve on crude extract				Time curve on microsomal extract extract			
	(BRI/FLS) 10	(BRI/FLS) 90	(BRI/EFR) 10	(BRI/EFR) 90	(BRI/FLS) 10	(BRI/FLS) 90	(BRI/EFR) 10	(BRI/EFR) 90
	0.85	1.51	0.56	0.57	3.58	1.90	1.42	0.13
	0.32	2.36	0.17	0.62	2.62	9.69	7.02	5.58
average (%)	0.58	1.93	0.37	0.59	3.10	5.80	4.22	2.85

Amount of BAK1 immunoprecipitated in BRI-GFP IP versus FLS or EFR IP at different time points (%)				
	(BRI/FLS) 10	(BRI/FLS) 90	(BRI/EFR) 10	(BRI/EFR) 90
	0.85	1.51	0.56	0.57
	0.32	2.36	0.17	0.62
	3.58	1.90	1.42	0.13
	2.62	9.69	7.02	5.58
average (%)	1.84	3.87	2.29	1.72
st dev	1.32	3.38	2.77	2.23

Fig. S8. Quantitative Western analysis. (*A*) *1*, Example of a quantitative Western blot showing the different amounts of purified YFP and the serial dilutions of a total extract from *BRI1p::BRI1-eGFP* transgenic seedlings. *2*, Linear fit of the calibration curve values as presented by Lab Imager software. (*B*) *1*, Multiple biological repeats of total protein extracts of *BRI1p::BRI1-eGFP* seedlings analyzed by Western blotting using anti-GFP serum. The values below the lanes show the relative values obtained compared with the first lane. CBB staining shows equal loading. *2*, Multiple biological repeats of total protein extracts of *BRI1p::BRI1-eGFP* seedlings (first three lanes), *FLS2p::FLS2-3myc-GFP* seedlings (next five lanes) and *efr-1/EFRp::EFR-eGFP-HA* (last six lanes) analyzed by Western blotting and probed with anti-GFP serum. The values below the lanes show the relative values obtained compared with the first lane. CBB staining shows equal

Legend continued on following page

loading. (*C*, *1* and *2*) Comparison between the amount of GFP present in the IP input of *BRI1p::BRI1-eGFP* (BRI1-GFP), *FLS2p::FLS2-3myc-GFP* (FLS2-GFP) and *efr-1/EFRp::EFR-eGFP-HA* (EFR-GFP) seedlings and remaining in solution (i.e., output). The values below the lanes represent the input in percentages of the input set at 100 arbitrary units. CBB staining shows equal loading. (*D*) *1* and *2*, Example of quantitative IP experiment using *BRI1p::BRI1-eGFP* seedlings. On each blot, corresponding to one IP experiment, the YFP standard serial dilutions, serial dilution of the IP, and the input are analyzed and the corresponding values calculated from the linear fit shown in *2* according to the description in *A*. (*E*) Quantification of the amount of BAK1 coimmunoprecipitated with BRI1-eGFP in the BRI1p::BRI1-eGFP line expressed as a percentage of the amount of BAK1 coimmunoprecipitated with FLS2-3myc-GFP in the FLS2p::FLS2-3myc-GFP line or with EFR-eGFP-HA in the *efr-1/EFRp::EFR-eGFP-HA* line. The FLS2-GFP and EFR-GFP IPs were used as the 100% value. All samples were run on the same blot. The experiments represent a subset of the ones shown in Fig. 4*B*, but the seedlings were now treated with both flg22 and BR simultaneously as described for the corresponding WT seedling experiment shown in Fig. 4*A*. Both FLS2 and BRI1 receptors are activated by cotreatment with flg22 and epiBL for the indicated times (10 and 90 min after application of the ligands). The values were calculated form IP experiments such as shown in *D, 1*, and quantified as in *D*, *2*. The results of two independent experiments are presented, performed on either total or microsomal extracts.