Loss of the common immune coreceptor BAK1 leads to NLR-dependent cell death

Yujun Wu[a], Yang Gao[a], Yanyan Zhan[a], Hong Kui[a], Hongyan Liu[a], Li Yan[a], Birgit Kemmerling[b], Jian-Min Zhou[c], Kai He[a,1], and Jia Li[a,1]

[a]Ministry of Education Key Laboratory of Cell Activities and Stress Adaptations, School of Life Sciences, Lanzhou University, Lanzhou 730000, China; [b]Department of Plant Biochemistry, Eberhard-Karls University, Tübingen 72076, Germany; and [c]State Key Laboratory of Plant Genomics, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

Plants utilize a two-tiered immune system consisting of pattern recognition receptor (PRR)-triggered immunity (PTI) and effector-triggered immunity (ETI) to defend themselves against pathogenic microbes. The receptor protein kinase BAK1 plays a central role in multiple PTI signaling pathways in *Arabidopsis*. However, double mutants made by *BAK1* and its closest paralog *BKK1* exhibit autoimmune phenotypes, including cell death resembling a typical nucleotide-binding leucine-rich repeat protein (NLR)-mediated ETI response. The molecular mechanisms of the cell death caused by the depletion of BAK1 and BKK1 are poorly understood. Here, we show that the cell-death phenotype of *bak1 bkk1* is suppressed when a group of *NLRs*, *ADR1s*, are mutated, indicating the cell-death of *bak1 bkk1* is the consequence of NLR activation. Furthermore, introduction of a *Pseudomonas syringae* effector HopB1, which proteolytically cleaves activated BAK1 and its paralogs via either gene transformation or bacterium-delivery, results in a cell-death phenotype in an *ADR1s*-dependent manner. Our study thus pinpoints that BAK1 and its paralogs are likely guarded by NLRs.

BAK1 | PRR | NLR | cell death | *Arabidopsis thaliana*

Plant innate immunity is a two-tiered immune system composed of pattern recognition receptor (PRR)-triggered immunity (PTI) and effector-triggered immunity (ETI) (1). PTI confers plants basal defense that allows resistance to most invading pathogens, whereas ETI is more often associated with hypersensitive response (HR), a type of programmed cell death.

To activate PTI, the cell surface-localized PRRs interact with pathogen-associated molecular pattern (PAMP) and subsequently initiate intracellular immune responses (2, 3). Many PRRs identified so far are receptor protein kinases (RKs) (2, 4). For example, FLAGELLIN-SENSING 2 (FLS2) and EF-Tu RECEPTOR (EFR), two leucine-rich repeat RKs (LRR-RKs), recognize flg22, a 22-amino acid peptide conserved among bacterial flagellin, and elf18, an 18-amino acid peptide conserved in EF-Tu, respectively (5, 6). BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1 (BAK1), a different LRR-RK, which was originally identified as a coreceptor of a brassinosteroid receptor BRI1 (7, 8), is able to interact with FLS2 or EFR when flg22 or elf18 is present (9, 10). BAK1, also named SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 3 (SERK3), belongs to a SERK subfamily, which contains five members in *Arabidopsis*. Unlike FLS2 or EFR that possesses a large extracellular domain (ECD) for a direct PAMP association, BAK1 contains only five LRRs in its ECD and is able to only interact with the new surfaces formed via the interaction of PRRs and their corresponding PAMPs. BAK1, therefore, is also considered as a coreceptor for multiple LRR-type PRRs. Structural assays indicated the ECD of BAK1 can directly recognize FLS2 and the C terminus of FLS2-bound flg22 (11). flg22 thus serves as molecular glue connecting the ECDs of FLS2 and BAK1. Consistently, *bak1* single mutants show significantly impaired flg22-mediated responses, indicating

BAK1 is essential for PAMP perception (9, 10). In general, PAMPs induce the interaction and transphosphorylation of PRRs with their coreceptors, initiating the downstream PTI cascades (2, 4).

Some microbial strains can deliver specific proteins, effectors, into host cells to repress PTI via disrupting key components in PTI signaling (12, 13). Intriguingly, plants have evolved additional immune receptors, originally termed resistance (R) proteins, to specifically recognize effectors, triggering faster and stronger immune responses, ETI (14). The majority of R proteins are nucleotide-binding leucine-rich repeat proteins (NLRs), containing either a Toll-interleukin 1-like receptor (TIR) domain or a coiled-coil (CC) domain at their N termini (15). Although some NLRs can directly associate with effectors to trigger ETI, a majority of NLRs detect effectors via monitoring the effector-targeting substrates, known as guardees or decoys. Modification of a guardee/decoy by an effector is guarded by NLRs that subsequently activate ETI (16). From a functional point of view, NLRs are thought to be composed of sensor NLRs and helper NLRs. Sensor NLRs can recognize specific effectors or guardees/decoys. Helper NLRs, on the other hand, cannot directly recognize effectors or guardees/decoys but

Significance

BAK1 plays a key role in multiple PRR-triggered immune signaling pathways. Double mutants generated by *BAK1* and its paralog *BKK1* show spontaneous cell death, which is not seen in any known *PRR* mutants. We discovered that the ADR1 class of helper nucleotide-binding leucine-rich repeat proteins (NLRs) is required for the autoimmune responses of *bak1 bkk1*. Knocking out three *ADR1s* can significantly suppress the cell death of *bak1-3 bkk1-1*, suggesting the autoimmune responses of *bak1 bkk1* are caused by NLR activation. Furthermore, expression of HopB1, an effector derived from *Pseudomonas syringae* that cleaves activated BAK1 and its paralogs, leads to cell death similar to *bak1 bkk1*, which requires *ADR1s*. Our results indicate BAK1 and its paralogs serve as guardees for NLRs.

are required for their corresponding sensor NLRs to trigger ETI (17, 18). Recent reports suggest an ACTIVATED DISEASE RESISTANCE 1 (ADR1) and its paralogs function as helper NLRs for several sensor NLRs, such as RPS2, RPP2, RPP4, CHS3, SNC1, and RRS1/RPS4 (19–22). The N-terminal CC domains of the ADR1 family members resemble an NLR protein RESISTANCE TO POWDERY MILDEW 8 (RPW8), and ADR1s are therefore termed as $CC_{RPW8(R)}$-NLR proteins, which may represent a separate class of NLRs (23). Another CC_R-NLR group, NRG1s (N REQUIREMENT GENE 1) also function as helper NLRs, downstream of TIR-type NLRs SNC1 and CHS3 (21). The *nrg adr1* sextuple mutant showed reduced disease resistance to *Pseudomonas syringae* pv. *maculicola* ES4326 compared with that of their parents, suggesting NRG1s and ADR1s play synergistic roles on basal defense (21). The detailed molecular mechanisms of helper NLRs in regulating immune responses are yet to be determined.

Our previous genetic studies revealed that *BAK1* is involved in a cell-death control pathway (24). Knocking out both *BAK1* and its closest paralog, *BAK1-LIKE 1 (BKK1)*, led to a spontaneous
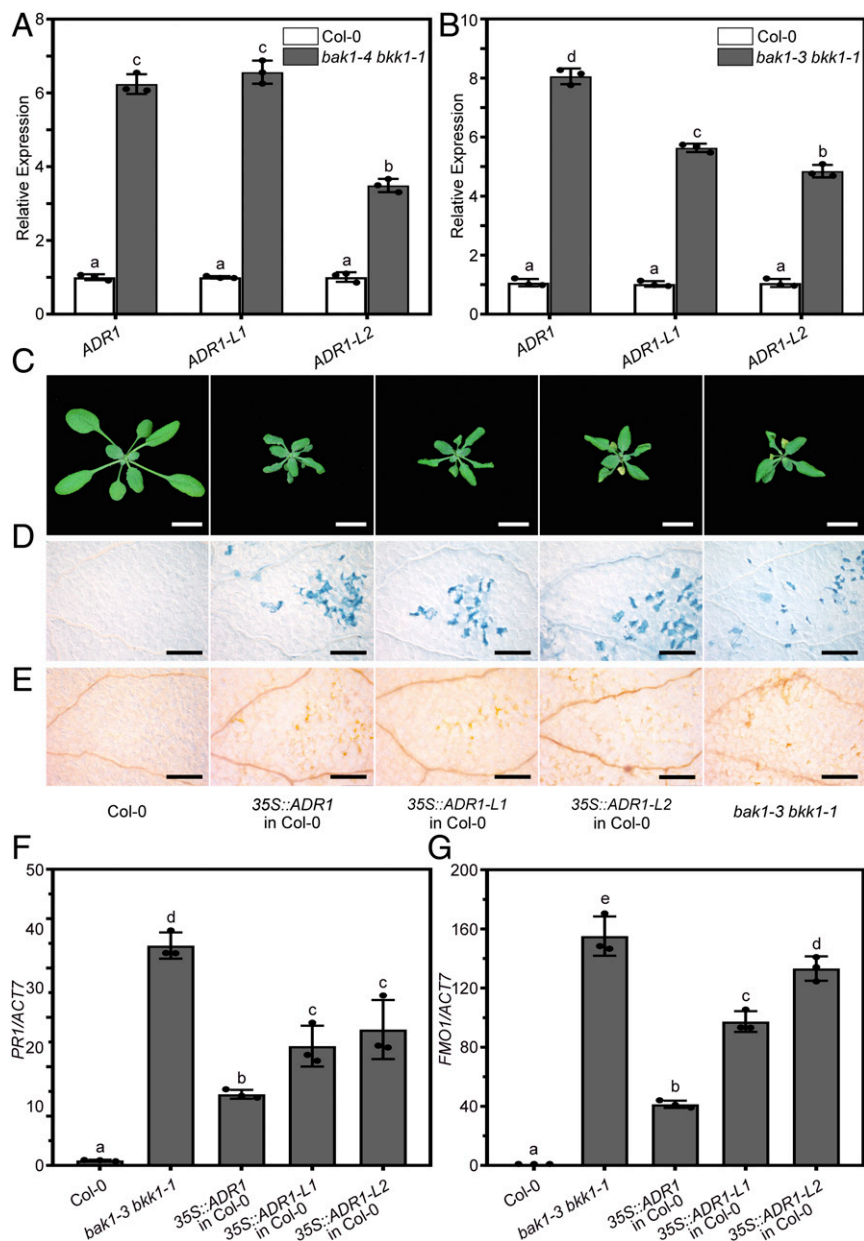
Fig. 1. *ADR1s are up-regulated in bak1 bkk1 double mutants. (A and B)* qRT-PCR assays indicate the expression levels of *ADR1*, *ADR1-L1*, and *ADR1-L2* are significantly increased in *bak1-4 bkk1-1 (A)* and *bak1-3 bkk1-1 (B)*. qRT-PCR was performed by using the total RNA from 8-d-old *bak1-4 bkk1-1* seedlings grown on 1/2 MS media or 2-wk-old *bak1-3 bkk1-1* plants grown in soil. *(C)* Overexpression of *ADR1s* in Col-0 results in autoimmune phenotypes similar to *bak1-3 bkk1-1*. Three-week-old plants grown in soil are presented. (Scale bars, 1 cm.) *(D and E)* Trypan blue staining *(D)* and DAB staining *(E)* assays indicate overexpression of *ADR1s* in Col-0 causes cell-death symptoms *(D)* and H_2O_2 accumulation *(E)* similar to *bak1-3 bkk1-1*. (Scale bars, 100 μm.) *(F and G)* *PR1 (F)* and *FMO1 (G)* are expressed in higher levels in *ADR1* overexpression lines and *bak1-3 bkk1-1*. qRT-PCR was performed by using the total RNA from 3-wk-old plants grown in soil. *ACT7* was used to normalize the transcript levels. Arbitrary units were used to show the relative abundance of transcript levels of *ADR1s*, *PR1*, and *FMO1* as compared to Col-0. Bars represent mean ± SD (*n* = 3). Different letters indicate a significant difference following one-way ANOVA with Tukey's multiple comparison test (*P* < 0.05).

cell-death phenotype even under a sterile culture condition (24–26). Although the role of BAK1 in regulating PTI is now well documented, the cell-death phenotype observed in *bak1 bkk1* is unlikely caused by the disruption of PTI responses (27). Previous studies indicated that knocking out a BAK1-associated *PRR* usually does not result in a cell-death phenotype in *Arabidopsis* (28). In addition, *bak1-5*, a dominant-negative mutant bearing a point mutation in *BAK1*, shows reduced PTI response compared to a *bak1-4* null mutant, but a *bak1-5 bkk1-1* double mutant is completely viable, again suggesting the cell death caused by loss of BAK1 and BAK1-mediated PTI are largely independent (29). NLR-mediated ETI activation is often accompanied by HR. Furthermore, like *snc1*, a gain-of-function mutant of an *NLR* gene, the autoimmune phenotypes of *bak1-3 bkk1-1* showed at 22 °C can be greatly suppressed by growing at 28 °C (*SI Appendix*, Fig. S1). We therefore hypothesized the HR-like cell death observed in *bak1 bkk1* is likely caused by the activation of NLR-mediated ETI rather than the reduction of PTI.

Here we report that *ADR1*s contribute to BAK1 depletion-triggered cell-death. The expression levels of *ADR1*s are dramatically up-regulated in *bak1 bkk1*. Knocking out *ADR1*s can significantly suppress the autoimmune responses, including cell death in *bak1-3 bkk1-1*, suggesting the cell-death phenotype of *bak1-3 bkk1-1* requires *NLR*s. Moreover, the expression of HopB1, a protease effector that targets BAK1 and other SERKs, not only caused impaired flg22-mediated immune responses but also resulted in the cell-death phenotype similar to *bak1 bkk1*. Furthermore, the HopB1-triggered cell-death symptom is also dependent on *ADR1*s. We conclude that the absence of BAK1 leads to the activation of NLRs, suggesting BAK1 is guarded by NLRs.

Results

Defense-Related Genes Are Up-Regulated in *bak1 bkk1*. To identify new components involved in cell death triggered upon BAK1 loss, we compared the global gene-expression profiles of the seedlings of WT Columbia-0 (Col-0) and *bak1-4 bkk1-1*, a double null mutant. *bak1-4 bkk1-1* starts to show a cell-death symptom a week after germination and is ultimately lethal even grown in sterilized culture media (24). We analyzed the differentially expressed genes in 7-d-old WT and *bak1-4 bkk1-1* plants by using an RNA-sequencing approach. Among 23,496 detected transcripts, we set a cutoff of change at twofold or greater with $P \leq 0.05$, which allowed us to identify 3,829 differentially expressed genes, including 1,848 up-regulated and 1,981 down-regulated ones in *bak1-4 bkk1-1* compared to WT (*SI Appendix*, Fig. S2*A* and Dataset S1). Gene ontology enrichment analyses showed that the genes associated with systemic acquired resistance, salicylic acid (SA) biosynthesis and signaling, cell death and HR, pathogen responses, and mitogen-activated protein kinase (MAPK) signaling were highly enriched in *bak1-4 bkk1-1* (*SI Appendix*, Fig. S2*B*). These results suggest that an autoimmune response is activated in *bak1-4 bkk1-1*.

To investigate whether the autoimmune phenotypes of *bak1-4 bkk1-1* are related to *NLR*-mediated responses, we analyzed the expression patterns of *NLR* genes and found a number of *NLR*s were up-regulated in *bak1-4 bkk1-1* (*SI Appendix*, Fig. S3). Since activated NLRs sometimes lead to the elevated transcriptional levels of their genes through a feedback loop, highly expressed *NLR*s might suggest the activation of the corresponding NLRs (30). We noticed three NLR subfamilies in which almost all their coding genes were highly up-regulated in *bak1-4 bkk1-1*. One of them is the ADR1 subfamily that was previously reported to function in multiple ETI signaling pathways. The second one was not reported before, and we named it a UNR1 (Uncharacterized NLR 1) subfamily. The third one is an RPS5 subfamily. RPS5, the founding member in this subfamily, recognizes the *P. syringae* effector AvrPphB (31). SUMM2, another member of the RPS5

subfamily, is required for the autoimmune phenotypes of two *MAPK* mutants, *mpk4* and *mekk1* (32). We thus tested the potential roles of the *NLR*s from these three subfamilies for their possible contribution to the cell death of *bak1 bkk1*.

Up-Regulation of *ADR1*s Is the Key for the Cell Death Triggered upon BAK1 Loss. We first confirmed the expression patterns of all aforementioned candidate *NLR*s in *bak1-3 bkk1-1*, in which the transcription level of *BAK1* is significantly reduced and that of *BKK1* is absent. *bak1-3 bkk1-1*, showing obvious autoimmune phenotypes including cell-death when grown in soil, is completely fertile, making it an ideal double mutant for genetic analyses (24, 33). qRT-PCR results confirmed that almost all gene members in these three *NLR* subfamilies were up-regulated in *bak1-4 bkk1-1* and *bak1-3 bkk1-1* (Fig. 1 *A* and *B* and *SI Appendix*, Fig. S4). Next, we tried to reduce the expression of these *NLR*s in *bak1-3 bkk1-1* by using an RNAi approach. For each subfamily, DNA fragments conserved among the gene members were cloned into an RNAi binary vector *pBIB-BASTA-35S-GWRNAi* and transformed into *bak1-3 bkk1-1*. qRT-PCR results indicated the expression levels of most gene members in the three subfamilies were dramatically decreased in corresponding RNAi transgenic plants compared to *bak1-3 bkk1-1* (*SI Appendix*, Fig. S5).

The autoimmune phenotypes were significantly suppressed in *ADR1* RNAi in *bak1-3 bkk1-1* plants, whereas, *UNR1* or *RPS5* RNAi in *bak1-3 bkk1-1* showed no obvious phenotypic difference from *bak1-3 bkk1-1* (*SI Appendix*, Fig. S6 *A–C*). Consistently, the transcription levels of a defense marker gene *PR1* and a defense and cell-death marker gene *FMO1* were strongly decreased in the *ADR1* RNAi plants but not in *UNR1* or *RPS5* RNAi lines compared to *bak1-3 bkk1-1* (*SI Appendix*, Fig. S6 *D* and *E*). To further understand whether *SUMM2* is involved in the cell-death of *bak1-3 bkk1-1*, we generated a *summ2 bak1-3 bkk1-1* triple mutant. Our results indicated that although *summ2* was able to partially suppress the cell-death phenotype of *mekk1* or *mkp4*, it cannot suppress that of *bak1-3 bkk1-1* (*SI Appendix*, Fig. S7*A*). Trypan blue and DAB staining assays also suggest that *SUMM2* may not contribute to the autoimmune responses of *bak1-3 bkk1-1* (*SI Appendix*, Fig. S7 *B* and *C*). Consistently, the expression levels of *PR1* and *FMO1* were not decreased in *summ2 bak1-3 bkk1-1* compared to *bak1-3 bkk1-1* (*SI Appendix*, Fig. S7 *D* and *E*).

ADR1-mediated ETI signaling requires both SA and *EDS1* (34). Similarly, the cell-death phenotype of *bak1 bkk1* was partially inhibited when endogenous SA was depleted or an *EDS1* mutation was introduced (25, 26). Previous study indicated that the mutation of *ADR1-L2* was able to suppress the cell-death of *lsd1*, a lesion-mimic mutant showing a runaway cell-death phenotype under the treatment of an SA analog benzothiadiazole (34). More importantly, we found that reduced expression of *ADR1*s could suppress the cell death in *bak1-3 bkk1-1*. We therefore set to investigate the potential roles of *ADR1*s in regulating the cell-death of *bak1 bkk1*.

The *ADR1* family contains three members: *ADR1*, *ADR1-LIKE 1* (*ADR1-L1*), and *ADR1-LIKE 2* (*ADR1-L2*) (20, 35). Overexpression of *ADR1*, *ADR1-L1*, or *ADR1-L2* in Col-0 resulted in a dwarfed phenotype with compacted and curved rosette leaves and cell-death (Fig. 1*C*). Trypan blue and DAB staining assays also indicated the cell death and H_2O_2 accumulation were significantly triggered in the overexpression lines (Fig. 1 *D* and *E*). *PR1* and *FMO1* were highly expressed in these transgenic lines (Fig. 1 *F* and *G*). These results demonstrate that enhanced expression of *ADR1*s leads to an autoimmune phenotype similar to *bak1-3 bkk1-1*.

Knocking Out *ADR1*s Suppresses the Cell-Death Phenotype of *bak1-3 bkk1-1*. We next isolated the previously reported T-DNA insertion lines for all three *ADR1*s (19). RT-PCR analyses confirmed
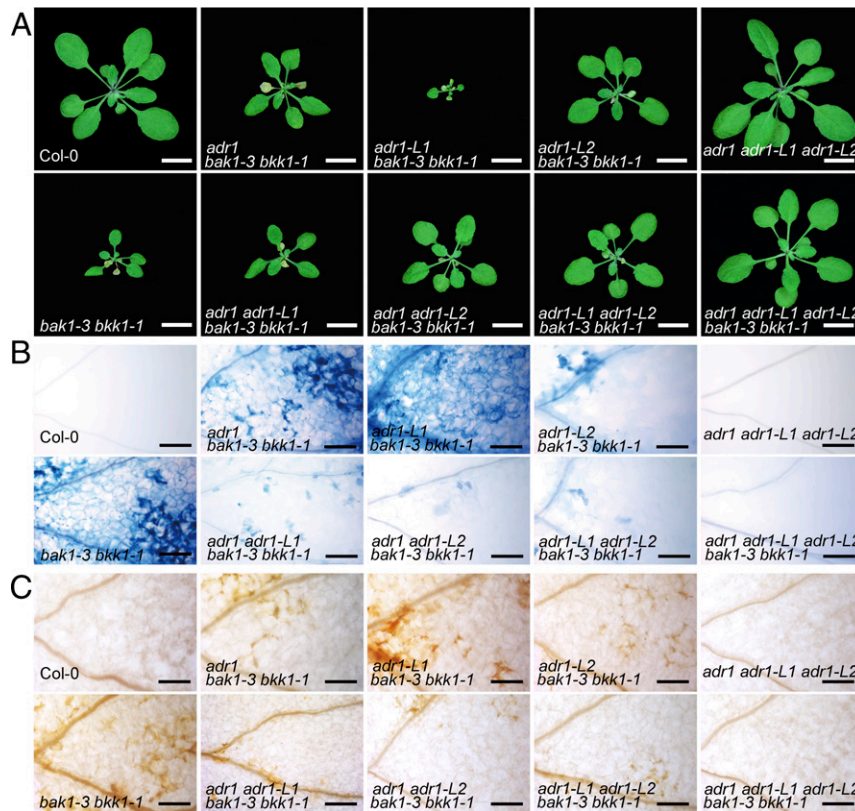
Fig. 2. The autoimmune phenotypes of *bak1-3 bkk1-1* require *ADR1*s. (*A*) Mutation in *ADR1* or *ADR1-L2* weakly suppresses the cell death of *bak1-3 bkk1-1*. Mutation in *ADR1-L1* enhances the cell-death symptoms of *bak1-3 bkk1-1*. *adr1*s *bak1-3 bkk1-1* quadruple mutants show additional cell-death suppression compared to *adr1*s *bak1-3 bkk1-1* triple mutants. The *adr1* triple mutant strongly suppresses the autoimmune phenotypes of *bak1-3 bkk1-1*. Three-week-old plants grown in soil are presented. (Scale bars, 1 cm.) (*B* and *C*) Trypan blue staining (*B*) and DAB staining (*C*) assays indicate the cell-death symptoms and H_2O_2 accumulation in the plants presented in *A*. Two-week-old plants grown in soil were analyzed. (Scale bars, 100 μm.)

that *adr1*, *adr1-L1*, and *adr1-L2* are true null mutants (*SI Appendix*, Fig. S8*A*). *adr1*, *adr1-L1*, *adr1-L2*, and *adr1 adr1-L1 adr1-L2* plants do not exhibit any defective phenotypes, similar to Col-0 (*SI Appendix*, Fig. S8 *B–D*). Compared to *bak1-3 bkk1-1*, the autoimmune phenotypes, including cell-death, accumulation of H_2O_2, and increased expression levels of *PR1* in *adr1-L2 bak1-3 bkk1-1* and *adr1 bak1-3 bkk1-1* were partially suppressed (Fig. 2 and *SI Appendix*, Fig. S9). *adr1-L1 bak1-3 bkk1-1* showed an enhanced cell-death phenotype (Fig. 2). To understand why *adr1-L1 bak1-3 bkk1-1* showed enhanced autoimmune phenotypes, we analyzed the expression levels of all *ADR1*s in three different *adr1*s *bak1-3 bkk1-1* triple mutants. qRT-PCR result showed that loss-of-function of *ADR1-L1* caused a compensatory increased expression of *ADR1* and *ADR1-L2* in *adr1-L1 bak1-3 bkk1-1* (*SI Appendix*, Fig. S10). These results are consistent with an earlier report showing that *adr1* or *adr1-L2* suppressed the autoimmune responses of *snc1* (20). In contrast, *snc1 adr1-L1* double mutants showed enhanced phenotypes compared to *snc1* due to compensatory expression of *ADR1* and *ADR1-L2* (20). To verify the aforementioned phenotypes of the *adr1*s *bak1-3 bkk1-1* triple mutants, genomic sequences of *ADR1*s were cloned into a binary vector (modified from *pFAST-G01*) and transformed into the corresponding *adr1*s *bak1-3 bkk1-1* triple plants. The resulting transgenic lines showed the phenotypes similar to *bak1-3 bkk1-1* (*SI Appendix*, Fig. S11).

The cell-death symptoms of three quadruple mutants, *adr1 adr1-L1 bak1-3 bkk1-1*, *adr1-L1 adr1-L2 bak1-3 bkk1-1*, and *adr1 adr1-L2 bak1-3 bkk1-1*, were further suppressed compared to the *adr1*s *bak1-3 bkk1-1* triple mutants. We subsequently

generated a quintuple mutant *adr1 adr1-L1 adr1-L2 bak1-3 bkk1-1* in which the autoimmune phenotypes were dramatically suppressed to a WT-like level (Fig. 2). We next tested whether the rescued phenotypes of *adr1 adr1-L1 adr1-L2 bak1-3 bkk1-1* are caused by increased *BAK1* transcripts or elevated BAK1 protein abundance. qRT-PCR analyses failed to detect the increased expression of *BAK1* in *adr1 adr1-L1 adr1-L2 bak1-3 bkk1-1* compared to *bak1-3 bkk1-1* (*SI Appendix*, Fig. S12*A*). Immunoblotting analyses using an α-BAK1 antibody showed that the BAK1 protein level was not altered in *adr1 adr1-L1 adr1-L2 bak1-3 bkk1-1* compared to *bak1-3 bkk1-1* (*SI Appendix*, Fig. S12*B*). In summary, our genetic results indicated the cell-death phenotype of *bak1 bkk1* requires *ADR1*s.

flg22-Mediated PTI Response Is Impaired but ADR1s-Mediated ETI Is Enhanced in *bak1-3 bkk1-1*. To further dissect the functions of *BAK1* and *ADR1*s in disease resistance, 3-wk-old Col-0, *adr1 adr1-L1 adr1-L2*, *bak1-3 bkk1-1*, and *adr1 adr1-L1 adr1-L2 bak1-3 bkk1-1* plants were challenged with various strains of a bacterial pathogen *P. syringae* pv. *tomato* (*Pto*) DC3000. The *adr1* triple mutant showed slightly reduced resistance to WT *Pto* DC3000 compared to Col-0 (Fig. 3 *A* and *B*), consistent with the results from a previous report (19). *bak1-3 bkk1-1* exhibited enhanced resistance to *Pto* DC3000, which is consistent with the enhanced defenses in this mutant (Fig. 3 *A* and *B*). *adr1 adr1-L1 adr1-L2 bak1-3 bkk1-1* plants, however, are more susceptible to *Pto* DC3000 compared to *bak1-3 bkk1-1*, indicating that the autoimmunity of *bak1-3 bkk1-1* was suppressed in the quintuple
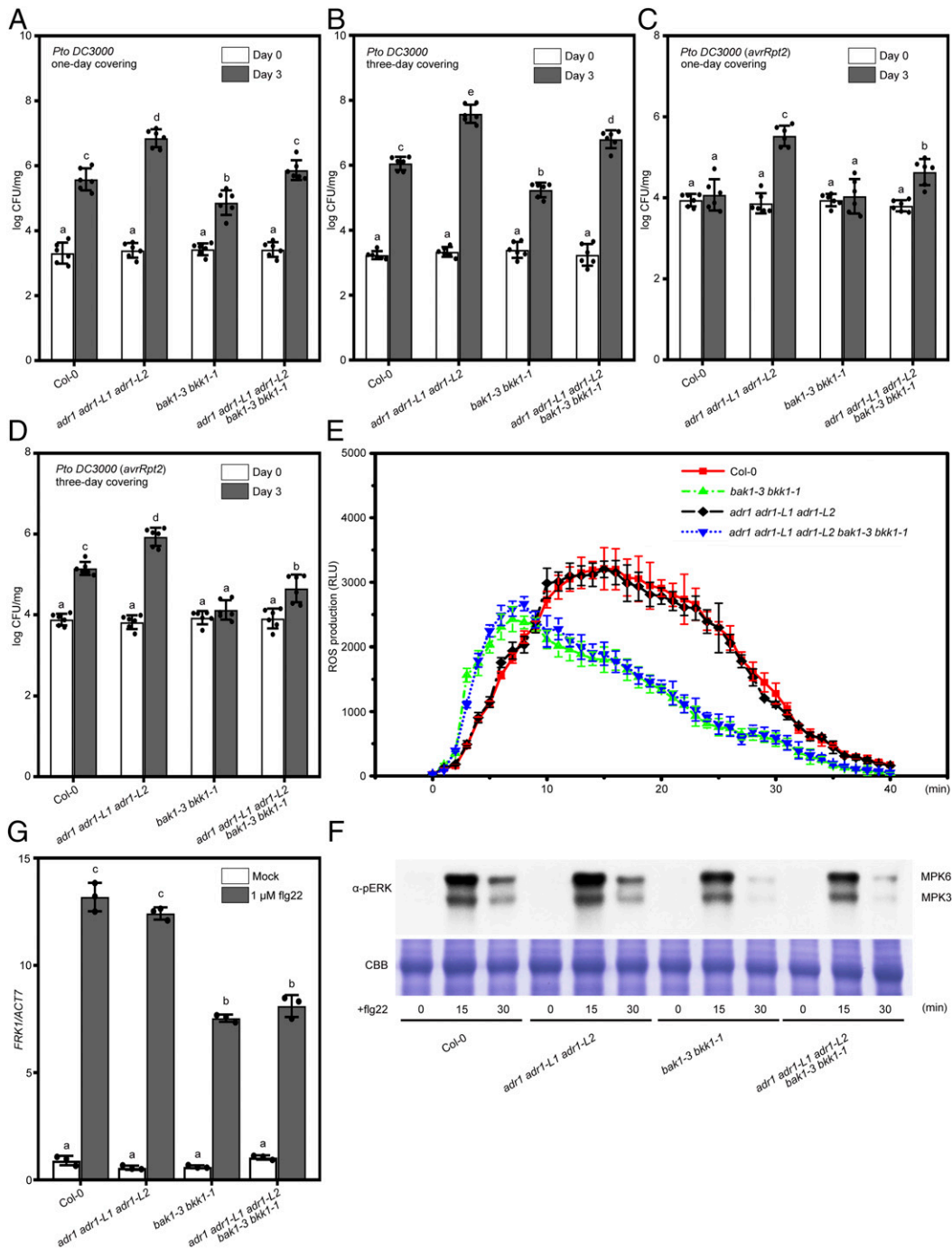
Fig. 3. *bak1-3 bkk1-1* shows enhanced effector-triggered responses and impaired PAMP-triggered responses. (*A* and *B*) Three-week-old plants were treated with *Pto DC3000* and covered for 1 d (*A*) or 3 d (*B*). Bacterial growth was assessed at 0- and 3-d postinoculation (dpi). The *adr1* triple mutant is slightly susceptible while *bak1-3 bkk1-1* shows enhanced resistance to *Pto* DC3000. The *adr1* triple mutant partially restores the response of *bak1-3 bkk1-1* to *Pto* DC3000. Bars represent mean ± SD (*n* = 6). (*C*) Three-week-old plants were treated with *Pto DC3000* (*avrRpt2*) and covered for 1 d. Bacterial growth was assessed at 0 and 3 dpi. Col-0 and *bak1-3 bkk1-1* show resistance to *Pto DC3000* (*avrRpt2*). The *adr1* triple mutant is susceptible to *Pto DC3000* (*avrRpt2*). The *adr1* triple mutant partially suppresses the resistance of *bak1-3 bkk1-1* to *Pto* DC3000 (*avrRpt2*). Bars represent mean ± SD (*n* = 6). (*D*) Three-week-old plants were treated with *Pto DC3000* (*avrRpt2*) and covered for 3 d. Bacterial growth was assessed at 0 and 3 dpi. Col-0 shows susceptibility but *bak1-3 bkk1-1* shows resistance to *Pto DC3000* (*avrRpt2*). The *adr1* triple mutant partially suppresses the resistance of *bak1-3 bkk1-1* to *Pto* DC3000 (*avrRpt2*). Bars represent mean ± SD (*n* = 5). (*E*) Oxidative burst upon flg22 treatment is reduced in *bak1-3 bkk1-1* and *adr1 adr1-L1 adr1-L2 bak1-3 bkk1-1* compared to Col-0 and the *adr1* triple mutant. ROS production was measured as relative light units (RLU) in a luminol-based assay. Values are mean ± SD (*n* = 5). (*F*) Col-0 and the *adr1* triple mutant show similar MAPK activation upon flg22 treatment. *bak1-3 bkk1-1* and *adr1 adr1-L1 adr1-L2 bak1-3 bkk1-1* show similar MAPK activation upon flg22 treatment. MAPK activation was analyzed by immunoblotting with an α-pERK antibody. The control for protein loading is shown by Coomassie brilliant blue (CBB). (*G*) flg22-mediated signaling is intact in the *adr1* triple mutant. *FRK1* expression was determined 1 h after treatment with 1 μM flg22. qRT-PCR was performed by using the total RNA from 7-d-old seedling. *ACT7* was used to normalize the transcript levels. Arbitrary units were used to show the relative abundance of *FRK1* transcript levels as compared to Col-0. Bars represent mean ± SD (*n* = 3). Different letters indicate a significant difference following one-way ANOVA with Tukey's multiple comparison test ($P < 0.05$).

mutant (Fig. 3 *A* and *B*). ADR1s function as helper NLRs for the sensor NLR, RPS2, which recognizes the bacterial effector AvrRpt2 (19). ADR1s are required to initiate AvrRpt2-triggered ETI activation. When treated with *Pto* DC3000 (*avrRpt2*) followed by 1-d covering to keep humidity, Col-0 showed resistance to bacterial infection and the *adr1* triple mutant was more susceptible (Fig. 3*C*). *bak1-3 bkk1-1* plants are resistant to *Pto* DC3000 (*avrRpt2*), similar to Col-0. Since both Col-0 and *bak1-3 bkk1-1* showed resistance to *Pto* DC3000 (*avrRpt2*) under this covering condition, we cannot distinguish the difference of resistance between Col-0 and *bak1-3 bkk1-1*. It was reported that increasing humidity can enhance the susceptibility of plants to pathogens (36). We therefore increased the covering time from 1 d to 3 d after the plants were treated with *Pto* DC3000 (*avrRpt2*). Under the altered condition, Col-0 showed slightly enhanced susceptibility to *Pto* DC3000 (*avrRpt2*) but *bak1-3 bkk1-1* was still resistant to *Pto* DC3000 (*avrRpt2*) (Fig. 3*D*). We thus concluded that ETI mediated by ADR1s is likely activated in *bak1-3 bkk1-1*. Under both aforementioned covering conditions after bacterial treatments, *adr1 adr1-L1 adr1-L2 bak1-3 bkk1-1* showed enhanced susceptibility to *Pto* DC3000 (*avrRpt2*) compared to *bak1-3 bkk1-1* (Fig. 3 *C* and *D*), indicating ADR1s contribute to the resistance of *bak1-3 bkk1-1* to *Pto* DC3000 (*avrRpt2*). Our results also showed that *adr1 adr1-L1 adr1-L2 bak1-3 bkk1-1* is more resistant than the *adr1* triple mutant to *Pto* DC3000 (*avrRpt2*). Considering that additional NLRs besides ADR1s could be activated in *bak1-3 bkk1-1* and may partially contribute to the autoimmune phenotypes of *bak1-3 bkk1-1*, we speculate that the defense responses activated by additional NLRs other than ADR1s in *adr1 adr1-L1 adr1-L2 bak1-3 bkk1-1* are responsible for its resistance to *Pto* DC3000 (*avrRpt2*).

Both the burst of reactive oxygen species (ROS) and MPK3/6 activities can be quickly triggered in plants after PAMPs are recognized by PRRs. We next analyzed the PAMP-mediated responses in various plant lines by detecting ROS burst and MPK3/6 activities upon the treatment of flg22. *bak1-3 bkk1-1* showed partially reduced ROS accumulation and MPK3/6 activation compared to Col-0. *bak1-3 bkk1-1* exhibited ROS accumulation and MPK3/6 activities similar to *adr1 adr1-L1 adr1-L2 bak1-3 bkk1-1*, and *adr1 adr1-L1 adr1-L2* displayed ROS accumulation and MPK3/6 activities similar to Col-0 upon the treatment of flg22 (Fig. 3 *E* and *F*). In addition, we analyzed the expression of *FRK1*, a marker gene for PTI signaling, in the different genetic backgrounds upon flg22 treatment. qRT-PCR results indicated that the flg22-mediated PTI response in *adr1 adr1-L1 adr1-L2* was similar to that in Col-0. The PTI responses in both *bak1-3 bkk1-1* and *adr1 adr1-L1 adr1-L2 bak1-3 bkk1-1*, however, were dampened compared to Col-0 (Fig. 3*G*). These results demonstrate that flg22-triggered PTI responses are partially impaired in *bak1-3 bkk1-1*, and ADR1s are not involved in flg22-mediated PTI responses. Although PTI responses are partially impaired, *bak1-3 bkk1-1* still showed enhanced resistance to *Pto* DC3000 and *Pto* DC3000 (*avrRpt2*), suggesting the elevated disease resistance in *bak1-3 bkk1-1* is most likely caused by the activation of ADR1s.

Expression of *HopB1* Mimics the Autoimmune Responses of *bak1-3 bkk1-1*. Given the fact that knocking out or significantly knocking down *BAK1* and *BKK1* leads to NLR-dependent immune responses, we next investigated the biological significance of NLR activation upon depletion of *BAK1* and its paralogs, *SERK*s. To promote full pathogenicity in the host, microbial pathogens deliver effectors to plant cells to shut down PTI signaling by attacking key components in PTI. The effectors HopF2 and AvrPtoB were found to associate with and disrupt BAK1 (37, 38). In a previous report, we identified a *Pto* DC3000-derived protease HopB1 that specifically cleaves flg22-activated

BAK1 and other SERKs (39). We generated transgenic plants harboring estrogen inducible *HopB1-FLAG* in Col-0 (Est-*HopB1-FLAG* in Col-0). Upon treatment with estradiol to induce the expression of *HopB1* for 2 wk, Est-*HopB1-FLAG* in Col-0 exhibited a phenotype with slightly more compacted rosette leaves compared to Col-0 (Fig. 4*A* and *SI Appendix*, Fig. S13). Because HopB1 was found to only cleave flg22-activated BAK1 (39), we used flg22 to activate BAK1. When treated with both estradiol and flg22 for 2 wk, Est-*HopB1-FLAG* in Col-0 showed a striking cell-death symptom reminiscent of *bak1 bkk1* (Fig. 4*A* and see *SI Appendix*, Fig. S13). As a control, the abundance of BAK1 in Col-0 was not noticeably affected by the treatments of estradiol alone, flg22 alone, or estradiol plus flg22 for 2 wk. In Est-*HopB1-FLAG* in Col-0, the abundance of BAK1 was not changed upon the treatments of estradiol or flg22 alone but was significantly decreased when both estradiol and flg22 were applied for 2 wk. BAK1 abundance was analyzed by using an α-BAK1 antibody and the induced HopB1 was detected by using an α-FLAG antibody (Fig. 4*B*). The treatment with estradiol and flg22 for 2 wk not only resulted in cell death, but also H_2O_2 accumulation, and up-regulation of *PR1* and *FMO1* in Est-*HopB1-FLAG* in Col-0 (Fig. 4 *C* and *D* and *SI Appendix*, Fig. S14). In addition, *HopB1* expression further enhanced the cell-death symptom of *bak1-3 bkk1-1* in the presence of flg22 (*SI Appendix*, Figs. S15 and S16). These data suggest cleavage of BAK1 and other SERKs by HopB1 triggers cell death in plants, mimicking the *bak1 bkk1* double mutant.

We showed the cell-death phenotype of *bak1-3 bkk1-1* requires *ADR1*s. We next investigated whether ADR1s also contribute to *HopB1*-induced immune responses. Transgenic plants harboring estrogen inducible *HopB1* in *adr1 adr1-L1 adr1-L2* (Est-*HopB1-FLAG* in the *adr1* triple mutant) were generated. In comparison with those of Est-*HopB1-FLAG* in Col-0, the cell-death phenotype of Est-*HopB1-FLAG* in the *adr1* triple mutant is significantly suppressed when treated with both estradiol and flg22 for 2 wk (Fig. 4*A* and *SI Appendix*, Figs. S13 and S14). Immunoblotting analyses indicated that in both Est-*HopB1-FLAG* in the *adr1* triple mutant and Est-*HopB1-FLAG* in Col-0 plants, treatments with estradiol and flg22 for 2 wk all caused dramatic reduction of BAK1 to an equivalent level (Fig. 4*B*). Those results indicated the cell death caused by HopB1-induced BAK1 cleavage is ADR1s-dependent. In addition, *HopB1* expression in the presence of flg22 resulted in increased expression of *PR1* and *FMO1* in Col-0, which was largely inhibited in the *adr1* triple mutant (Fig. 4 *C* and *D*). Moreover, in the presence of flg22, the induced expression of *HopB1* in Col-0 caused elevated expression of *ADR1*s, suggesting HopB1-mediated cleavage of BAK1 and other SERKs leads to the activation of ADR1s (Fig. 4*E*).

To examine whether HopB1 affects PTI responses in which BAK1 plays an essential role, we tested MPK3/6 activation after BAK1 was activated by flg22 and *HopB1* was induced by estradiol. We pretreated Est-*HopB1-FLAG* in Col-0 and in the *adr1* triple mutant with estradiol for 24 h to induce the expression of *HopB1*. We then applied flg22 to activate BAK1, which led to a dramatic decrease of BAK1 abundance in both types of transgenic plants after a 15-min treatment (*SI Appendix*, Fig. S17A). Accordingly, MPK3/6 activation upon flg22 treatment was significantly suppressed in Est-*HopB1-FLAG* in Col-0 and in the *adr1* triple mutant, indicating flg22-mediated PTI signaling is repressed when *HopB1* is induced (*SI Appendix*, Fig. S17A). Similarly, analyses of *FRK1* expression also showed *HopB1* expression caused reduced flg22-mediated PTI response in Col-0 and the *adr1* triple mutant (*SI Appendix*, Fig. S17B). These results suggest *HopB1* expression dampened PTI responses in which ADR1s are not involved.
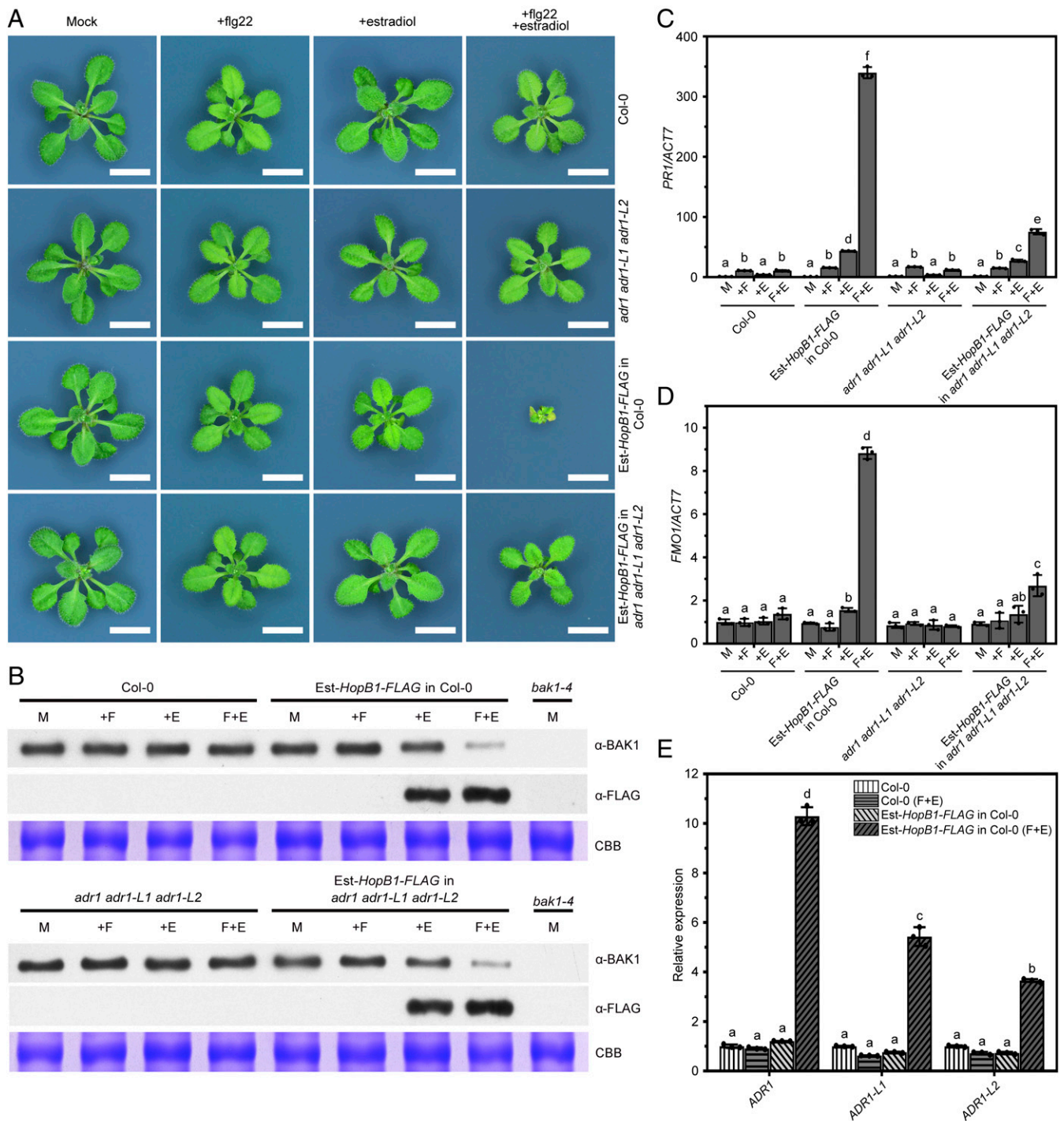
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Fig. 4. Estrogen-induced *HopB1* expression causes ADR1s-dependent immune responses. (*A*) Estrogen-induced *HopB1* expression in Col-0 leads to a striking cell-death phenotype upon flg22 treatment. HopB1-induced cell death is significantly inhibited in the *adr1* triple mutant. Three-week-old plants grown on 1/2 MS media supplemented with or without estradiol and/or flg22 are presented. (Scale bars, 0.5 cm.) (*B*) The abundance of BAK1 and induced HopB1 is analyzed by immunoblotting. The abundance of BAK1 was detected using an α-BAK1 antibody. BAK1 protein is significantly reduced in *HopB1* transgenic plants when treated with both estradiol and flg22 for 2 wk. The HopB1 protein was detected using an α-FLAG antibody. HopB1 protein is induced in *HopB1* transgenic plants when estradiol is applied. Coomassie brilliant blue (CBB) staining for a duplicated SDS/PAGE gel was used to show equal loading. (*C* and *D*) *HopB1* expression causes up-regulation of *PR1* (*C*) and *FMO1* (*D*) in Col-0, which is significantly suppressed in the *adr1* triple mutant in the presence of flg22. qRT-PCR was performed by using the total RNA from 3-wk-old plants grown on 1/2 MS media supplemented with estradiol and/or flg22. *ACT7* was used to normalize the transcript levels. Arbitrary units are used to show the relative abundance of *PR1* and *FMO1* transcript levels as compared to Col-0. Bars represent mean ± SD ($n = 3$). (*E*) *HopB1* expression up-regulates *ADR1*s in the presence of flg22. qRT-PCR was performed by using the total RNA from 3-wk-old plants grown on 1/2 MS media supplemented with or without estradiol and flg22. *ACT7* was used to normalize the transcript levels. Arbitrary units were used to show the relative abundance of transcript levels of *PR1*, *FMO1*, and *ADR1*s as compared to Col-0. Bars represent mean ± SD ($n = 3$). Different letters indicate a significant difference following one-way ANOVA with Tukey's multiple comparison test ($P < 0.05$). E, estradiol; F, flg22; M, mock. Three biological replicates were conducted and similar results were obtained. Here are the representative results.

Bacterium-Delivered HopB1 Triggers ETI Responses in an ADR1s-Dependent Manner. To further exclude the interference of the effectors other than HopB1 in the bacterial strain *Pto* DC3000, we used a *Pseudomonas fluorescens Pf0-1* strain that has no effectors and can only trigger PTI. When sprayed with *P. fluorescens-EV (Pf0-1-EV)*, Col-0 and *adr1* triple mutant showed similar resistance, while *eds1* and *pad4* exhibited increased susceptibility, indicating that PTI confers plant resistance in a manner dependent on EDS1 and PAD4 but independent of ADR1s (Fig. 5*A*). When sprayed with *P. fluorescens-HopB1 (Pf0-1-HopB1)*, the bacterium grew to higher levels in the *adr1* triple mutant compared to Col-0, and the *adr1* triple mutant was as susceptible as to *eds1* and *pad4*, indicating an ADR1-dependent immunity triggered by HopB1 (Fig. 5*B*). The *Pf0-1-HopB1* strain grew to much higher levels than the *Pf0-1 EV* strain on the *adr1* triple mutant, indicating a profound role of HopB1 in virulence (Fig. 5 *A* and *B*). On Col-0 plants, the *Pf0-1-HopB1* strain grew only slightly more than the *Pf0-1 EV* strain, which reflect an outcome of combined effect of the HopB1-triggered susceptibility and a HopB1-triggered immunity in normal plants. Consistent with the aforementioned antibacterial resistance, *PR1* and *FMO1* showed a modest induction in Col-0 and *adr1* triple mutant when inoculated with the *Pf0-1 EV* strain compared to mock treatment (Fig. 5 *C* and *D*). This induction is abolished in *eds1* and *pad4* plants. When inoculated with *Pf0-1-HopB1,* a strong induction of *PR1* and *FMO1* was observed only in Col-0, which was significantly reduced in the *adr1* triple (Fig. 5 *C* and

D). When injected with *Pf0-1-HopB1*, the leaves of Col-0 showed a clear cell-death symptom which was clearly reduced in *adr1* triple (Fig. 5 *E* and *F*), indicating that the bacterially delivered HopB1 can indeed trigger cell death in a manner dependent on ADR1s.

Discussion

It has been more than a decade since we first reported that *bak1 bkk1* exhibited a cell death phenotype (24). Significant efforts have been made to elucidate mechanisms leading to such an unexpected phenotype. Genetic analyses identified a number of proteins that are involved in the cell-death control of *bak1 bkk1*, including an SA biosynthetic enzyme (SID2), components regulating ETI signaling and SA biosynthesis (EDS1 and PAD4), a nucleoporin subunit protein (SBB1), a regulator mediating endoplasmic reticulum quality control (STT3a), and two calcium ion channels (CNGC19/20) (24, 40–42). But the interrelationships among these proteins are not well understood. Especially the early events leading to BAK1-depletion triggered cell death are not elucidated.

In this study, we demonstrate that BAK1 is likely guarded by an ADR1-dependent NLR. First, the cell-death of *bak1 bkk1* resembles the phenotype of NLR-mediated autoimmune responses. Second, genetic analyses indicated that *ADR1*s are required for the autoimmune phenotypes of *bak1 bkk1*. Third, the increased disease resistance of *bak1-3 bkk1-1* to *Pto* DC3000
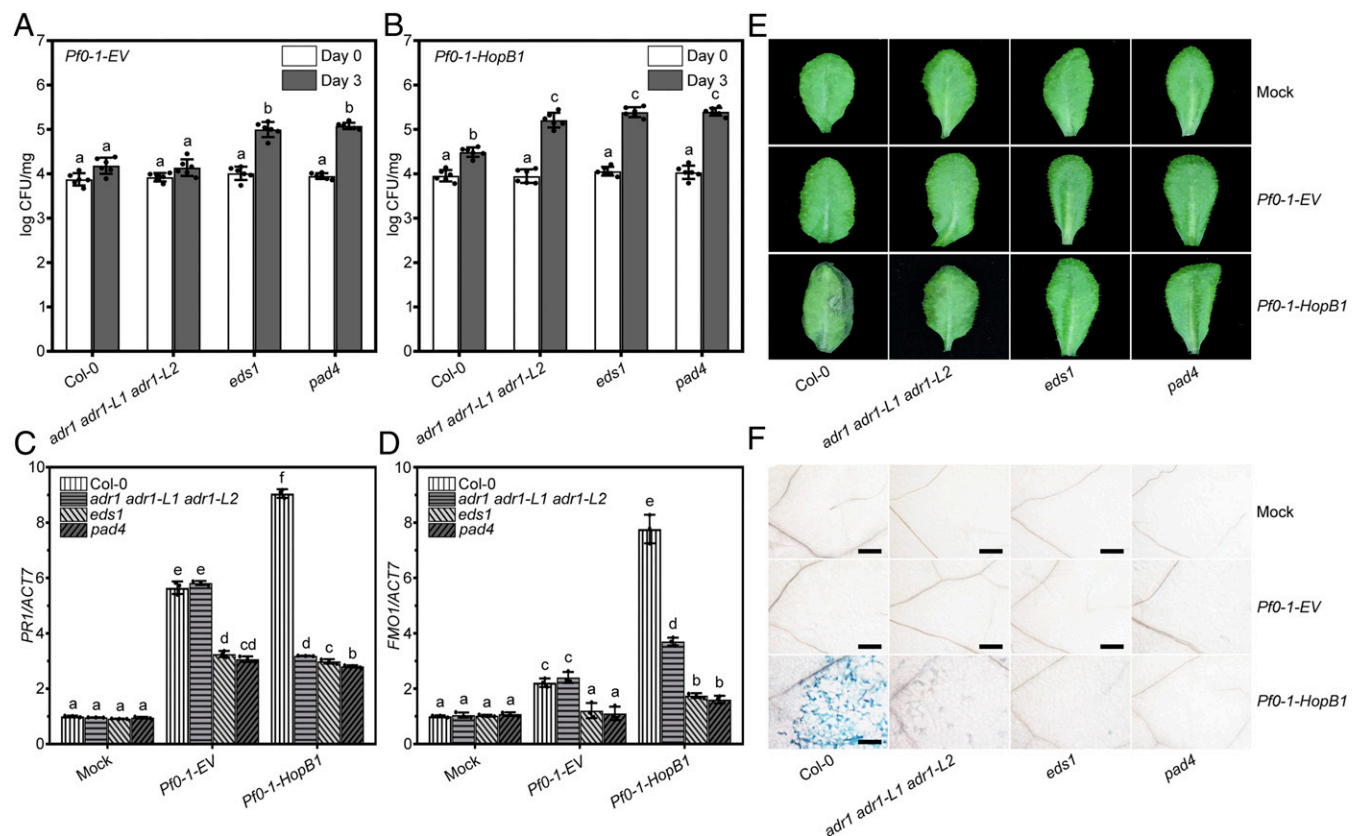
Fig. 5. Bacterium-delivered HopB1 triggers ADR1s-dependent ETI responses. (*A* and *B*) Three-week-old plants were sprayed with *Pf0-1-EV* (*A*) and *Pf0-1-HopB1* (*B*). Bacterial growth was assessed at 0 and 3 dpi. Compared to Col-0, the *adr1* triple mutant shows similar resistance to *Pf0-1-EV*, but is more susceptible to *Pf0-1-HopB1*. Bars represent mean ± SD (*n* = 6). (*C* and *D*) *Pf0-1-EV* treatment causes similar levels of up-regulation of *PR1* (*C*) and *FMO1* (*D*) in Col-0 and in the *adr1* triple mutant. Compared to Col-0, *PR1* (*C*) and *FMO1* (*D*) expression is significantly suppressed in the *adr1* triple mutant upon the treatment of *Pf0-1-HopB1*. qRT-PCR was performed by using the total RNA from 3-wk-old plants grown in soil after *Pf0-1-EV* or *Pf0-1-HopB1* injected and incubated for 10 h. *ACT7* was used to normalize the transcript levels. Arbitrary units are used to show the relative abundance of *PR1* and *FMO1* transcription levels as compared to Col-0. Bars represent mean ± SD (*n* = 3). (*E*) Injection of *Pf0-1-HopB1* induces clear cell-death phenotype in Col-0 but clearly reduced in *adr1* triple mutant. (*F*) Trypan blue staining assays indicate the cell-death phenotypes of Col-0 after *Pf0-1-HopB1* injection are suppressed in the *adr1* triple mutant. (Scale bars, 200 μm.) Three biological replicates were conducted and similar results were obtained. Here are the representative results. Different letters indicate a significant difference following one-way ANOVA with Tukey's multiple comparison test (*P* < 0.05).

(*avrRpt2*) relative to WT is ADR1-dependent. Fourth, cleavage of activated SERKs by either transgenic expression of a bacterial effector protein HopB1 or bacterium-delivered HopB1 led to a cell-death phenotype similar to *bak1 bkk1*, which is also ADR1-dependent. These results demonstrate both the cell death and increased disease resistance phenotypes of *bak1-3 bkk1-1* rely on the activation of ADR1s.

It was previously proposed that many plant autoimmune responses are caused by inappropriate activation of NLRs (43). For example, MAP kinases MEKK1 and MPK4 are two downstream components of the FLS2-BAK1–mediated PTI signaling. The activities of MEKK1 and MPK4 are guarded by an NLR protein, SUMM2. The *summ2* mutant can partially suppress the autoimmune phenotypes of *mekk1* or *mpk4*, indicating a surveillance system guards the downstream components of PTI (32). Whether PRRs or their coreceptors are guarded by NLRs is largely unknown. Mutations in RK PRRs, such as *FLS2* and *EFR*, do not show any autoimmune phenotypes, suggesting RK PRRs are unlikely guarded by NLRs. BAK1, as a shared coreceptor, plays a key role in multiple PTI pathways, making it an ideal target for microbial effectors. It is an efficient strategy for plants to trigger much stronger defense responses to eliminate microbes if BAK1 is attacked (Fig. 6).

A recent study showed mutations in *PEP-RECEPTORs* (*PEPRs*), encoding the receptors of Pep peptides, could partially inhibit the autoimmune responses of *bak1-3 bkk1-1* (44). Our results suggest ADR1s-mediated cell death in *bak1-3 bkk1-1* may be independent of PEPR-mediated immune responses. For example, compared to *pepr1 pepr2*, the *adr1* triple mutant showed greater reduction of cell death caused by *bak1-3 bkk1-1* mutations (*SI Appendix*, Fig. S18). It was reported that Pep2 treatment can significantly inhibit root growth (44). The root inhibition by Pep2 in *adr1* triple mutant is similar to that in WT (*SI Appendix*, Fig. S19A). In addition, the expression levels of *PROPEP2* and *PROPEP3*, encoding Pep proligands, are moderately increased in Col-0 and dramatically elevated in *bak1-3 bkk1-1* upon Pep2 treatment (*SI Appendix*, Fig. S19 *B* and *C*). The expression levels of *PROPEPs* in *adr1* triple mutant were

similar to those in WT regardless of the treatment of Pep2, demonstrating ADR1s are not involved in PEPR-mediated immune signaling.

bak1 bkk1 is not a naturally existing double mutant. To validate the biological significance of the cell death observed in the double mutant, we studied the consequence when BAK1 and other SERKs are attacked by effectors from bacteria. HopB1 is a protease effector derived from *P. syringae*. Previous analyses indicated that HopB1 can directly interact with FLS2 and cleave flg22-activated SERKs to promote virulence when plants are infected by *Pto* DC3000 (39). While a transient induction of *HopB1* transgene expression leads to increased susceptibility to *Pto hrcC⁻* bacteria (39), a prolonged induction of the *HopB1* transgene in the presence of flg22 for 2 wk was found to activate immune responses in this study. As the prolonged treatment of estradiol and flg22 is expected to cause greater depletion of BAK1 and other SERKs, it is possible that a threshold of BAK1 and other SERKs must be reached before the activation of defenses. We propose that the protein levels of SERKs are monitored by an unknown NLR and that this NLR is activated once the SERK protein levels of BAK1 and other SERKs are below certain threshold.

It should be noted that HopB1 naturally delivered from *Pto* DC3000 does not trigger measurable ETI or cell death, as the strain is fully virulent on *Arabidopsis*. One plausible explanation is that there may exist an effector that masks HopB1-triggered ETI. This scenario is well supported by our experiments with the *Pf0-1-HopB1*, which does not carry any other effectors (Fig. 5).

Another recent study showed that an effector AvrRps4 can be recognized by sensor NLRs, RPS4/RRS1, together with helper ADR1s (45). Can HopB1, as an effector, also be recognized by an unknown sensor NLR and helper ADR1s? Inducible expression of *AvrRps4* did not trigger HR-like phenotypes. Without flg22 treatment, expression of *HopB1* also did not induce autoimmune responses. Based on these results, we cannot exclude the possibility that ADR1s may contribute to HopB1 recognition. First, HopB1-triggered cell death is flg22-dependent, indicating the cell death triggered by *HopB1* expression likely involves
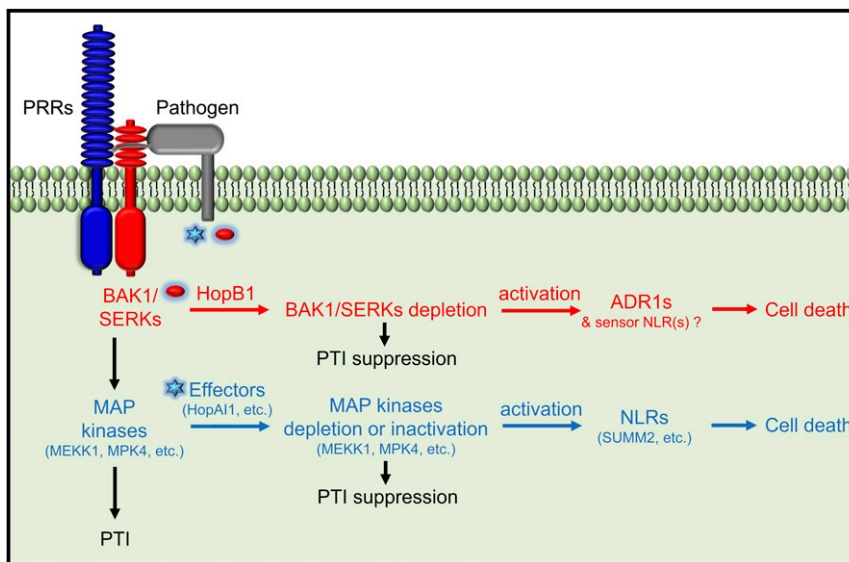

Fig. 6. A model to show BAK1 is directly or indirectly guarded by NLR-mediated signaling. BAK1 acts as a coreceptor for LRR-type PRRs, by sensing PAMPs, through MAPK kinase cascades to positively regulates PTI signaling. When pathogens infect plants, pathogen-delivered effectors can target PTI components to promote virulence. Plants have evolved NLR proteins to monitor the situation of corresponding targets, either the downstream MAP kinases or the coreceptor BAK1/SERKs. Different targets are usually attacked by their corresponding effectors, their specific NLRs are then activated, leading to cell-death phenotypes. In *bak1 bkk1*, both *BAK1* and *BKK1* are absent, similar to the depletion of BAK1/SERKs by effectors such as HopB1, NLRs (such as ADR1s) can be constitutively activated, leading to spontaneous cell-death phenotypes even under sterile growth conditions.

flg22-related components, such as BAK1. Second, we failed to detect the interaction between ADR1s and HopB1 (*SI Appendix*, Fig. S20). Therefore, we conclude HopB1 is unlikely recognized by ADR1s.

To adapt to ever-changing environments and maximize their chances of survival, plants have evolved sophisticated mechanisms to coordinate growth and defense. PTI activation allows plants to protect themselves against most invading pathogens. NLR-mediated signaling pathways, the stronger and damage-causing immune responses, need to be repressed during normal growth and development. When BAK1 and other SERKs are attacked by effectors, however, the depletion of BAK1 and other SERKs is detected and NLR-mediated defense responses are initiated at the cost of reduced growth. BAK1 and other SERKs are activated upon PAMP perception and positively regulates PTI responses. BAK1 and other SERKs also serve as guardees by NLRs. Depletion of BAK1 and other SERKs result in the activation of an unknown sensor NLR (NLRs), which acts upstream of ADR1s to activate immune responses including cell death (Fig. 6).

Materials and Methods

The detailed information about plant materials, plant growth and treatment conditions, Trypan blue staining, DAB staining, gene-expression analyses, plasmid construction, generation of transgenic plants, pathogen infection assays, immunoblotting, and oxidative burst measurement are described in *SI Appendix, SI Materials and Methods*.

Data Availability. All of the data discussed in this study can be found either in the main text, *SI Appendix*, and Dataset S1.

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