Title:
Danger peptide receptor signaling in plants ensures basal immunity upon pathogen-induced depletion of BAK1

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Running Title:
DAMP-mediated plant immunity on BAK1 depletion

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Abstract
Pathogens infect a host by suppressing defense responses induced upon recognition of microbe-associated molecular patterns (MAMPs). Despite this suppression, MAMP receptors mediate basal resistance to limit host susceptibility, via a process that is poorly understood. The *Arabidopsis* leucine-rich repeat (LRR) receptor kinase BAK1 associates and functions with different cell-surface LRR receptors for a wide range of ligands, including MAMPs. We report that BAK1 depletion is linked to defense activation through the endogenous PROPEP peptides (Pep epitopes) and their LRR receptor kinases PEPR1/PEPR2, despite critical defects in MAMP signaling. In *bak1*-knockout plants, PEPR elicitation results in extensive cell death and the prioritization of salicylate-based defenses over jasmonate-based defenses, in addition to elevated proligand and receptor accumulation. *BAK1* disruption stimulates the release of PROPEP3, produced in response to Pep application and during pathogen challenge, and renders PEPRs necessary for basal resistance. These findings are biologically relevant, since specific BAK1 depletion coincides with PEPR-dependent resistance to the fungal pathogen *Colletotrichum higginsianum*. Thus, the PEPR pathway ensures basal resistance when MAMP-triggered defenses are compromised by BAK1 depletion.
Main Text

Innate immunity based on a limited set of germ line-encoded receptors is fundamental for both plants and animals to recognize and respond to diverse microbes (Ronald & Beutler, 2010). Plants rely solely on innate immunity, which involves two tiers of functionally interlinked immune responses. The first is mediated by cell surface-localized pattern recognition receptors (PRRs) that sense molecular signatures typical of microbes, termed microbe-associated molecular patterns (MAMPs), including bacterial flagellin, elongation factor (EF)-Tu, peptidoglycans and fungal chitin (Boller & Felix, 2009; Macho & Zipfel, 2014). MAMP-triggered immunity (MTI) is typically insufficient to prevent infection by host-adapted pathogens that employ an array of virulence effectors to subvert PRR-mediated defenses. However, the second tier of plant immunity is triggered when these effectors are recognized. Effector-triggered immunity (ETI) leads to a robust and high-amplitude activation of immune responses that terminate pathogen growth, which is often accompanied by localized cell death. A large overlap in the defense outputs and signaling components leads to the notion that ETI is a magnified form of MTI (Cui et al, 2015; Jones & Dangl, 2006).

ETI is often induced upon recognition of effector-mediated modifications of a host target (Cui et al, 2015). Animal studies have also described pathogen effectors that elicit immune responses to protect the host (Stuart et al, 2013). Defense activation upon sensing pathogen effectors seems to represent a key principle in both plant and animal immunity.

In plants, substantial defenses are activated when susceptible hosts are challenged with virulent pathogens, and thereby limit host susceptibility. This response, known as basal resistance (Jones & Dangl, 2006), seems not to require the recognition of a specific pathogen effector, but is often enhanced when plants deficient in MAMP responses are exposed to pathogen effectors (Laluk et al, 2011; Li et al, 2014). These observations imply a link between basal resistance and pathogen effector actions. On the other hand, loss of individual MAMP receptors increases host susceptibility to virulent pathogens having a complete effector assembly (Zipfel et al, 2004, 2006; Willmann et al, 2011), indicating a critical role for MAMP recognition in basal resistance. However, it remains poorly understood how MAMP receptors mediate host resistance despite effector-mediated suppression of MTI signaling.

In Arabidopsis, the leucine-rich repeat (LRR) receptor kinases (RKs) FLS2 and EFR
recognize the bacterial MAMPs flagellin (flg22 epitope) and EF-Tu (elf18 epitope), respectively, and induce anti-bacterial immunity (Zipfel et al, 2004, 2006). Immediately after ligand binding, FLS2/EFR physically associate with the LRR-RK coreceptor BAK1, thereby offering a platform for defense signaling (Chinchilla et al, 2007; Heese et al, 2007; Sun et al, 2013). The FLS2/EFR-BAK1 complexes mediate phosphorylation of the receptor-like cytoplasmic kinases (RLCKs) BIK1 and related PBL proteins, which in turn dissociate from the receptor complexes to regulate downstream signaling (Lu et al, 2010; Zhang et al, 2010; Liu et al, 2013; Lin et al, 2014). These events are followed by a stereotypic set of cellular responses, including a rapid burst of Ca$^{2+}$ and reactive oxygen species (ROS), activation of Ca$^{2+}$-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs), cell wall remodeling, production of the phytohormones ethylene (ET) and salicylate (SA), and extensive transcriptional reprogramming (Boller & Felix, 2009; Macho & Zipfel, 2014).

Fine control of MAMP signaling is in part achieved through negative regulation within or in proximity to the PRR-BAK1 complexes. For instance, the LRR-RK BIR2 sequesters BAK1 from ligand-unbound FLS2 to avoid precocious signal activation (Halter et al, 2014). The BAK1-associated E3 ubiquitin ligases PUB12/PUB13 are recruited to the ligand-induced FLS2-BAK1 complex for ubiquitination and proteasomal degradation of the receptor (Lu et al, 2011). A ligand-induced decrease in FLS2 accumulation, apparently in association with receptor internalization, results in transient desensitization to flg22 before subsequent replenishment of the receptor (Robatzek et al, 2006; Smith et al, 2014). A subclass of protein phosphatase 2A dephosphorylate BAK1 to attenuate FLS2 signaling (Segonzac et al, 2014). However, it is less clear whether and how relief of negative regulation is linked to basal resistance during pathogen challenge.

MAMP signaling induces a subset of the soluble pro-peptide (PROPEP) family (carrying an immunogenic Pep epitope in their C termini), and then involves the LRR-RK Pep receptors PEPR1/PEPR2 (Huffakker et al, 2006; Krol et al, 2010; Ma et al, 2012; Tintor et al, 2013; Yamaguchi et al, 2006, 2010). The lack of N-terminal signal sequences for canonical secretion led to a model in which PROPEP-derived elicitors provide danger-associated molecular patterns (DAMPs) following their release upon membrane disintegration (Yamaguchi & Huffaker, 2011). Pep perception by PEPRs leads to MTI-hallmark outputs, largely through the aforementioned scheme of MTI
signaling (Yamaguchi & Huffaker, 2011; Liu et al, 2013). The PEPR pathway contributes to co-activation of SA and jasmonate (JA)/ET defenses (Ross et al, 2014) and to propagation of MAMP-triggered defense signaling (Ma et al, 2012; Flury et al, 2013; Tintor et al, 2013; Ross et al, 2014). These findings point to the importance of functional interactions between the FLS2/EFR and PEPR pathways as a critical step in MTI. However, despite increasing insight into the individual PRR pathways, the mechanisms underlying their functional interactions remain poorly understood.

Of note, FLS2, EFR and PEPRs all function with BAK1 in signal initiation. It remains to be determined whether BAK1 provides a node of functional convergence or is simply a common component in separate PRR pathways. Nevertheless, either scenario predicts that MTI will be vulnerable to pathogen assaults on BAK1. Indeed, BAK1 is a recurrent target in different plant hosts for structurally and functionally unrelated virulence-promoting effectors (Xin & He, 2013; Macho & Zipfel, 2015). However, bak1-knockout (KO) plants display almost intact or even enhanced post-invasion resistance against (hemi)biotrophic pathogens (Kemmerling et al, 2007), despite critical defects in a major branch of MTI signaling (Liebrand et al, 2014).

In addition to PRR signaling, BAK1 positively regulates brassinosteroid (BR) signaling and negatively regulates cell death (Liebrand et al, 2014). BAK1 acts as a coreceptor for the LRR-RK BR receptor BRI1 (Nam & Li, 2002; Li et al, 2002). BR signaling and MTI signaling antagonize each other (Albrecht et al, 2012; Belkhadir et al, 2012; Lin et al, 2013), but BAK1 is not rate-limiting between the two pathways (Albrecht et al, 2012). In cell death suppression, BAK1 acts together with the LRR-RK BIR1 and the membrane-associated copain-like BONZAI proteins BON1-BON3 (He et al, 2007; Kemmerling et al, 2007; Wang et al, 2011). Accordingly, bak1-KO plants exhibit enhanced cell death upon pathogen challenge, which is further enhanced by the loss of BKK1, the closest homolog of BAK1 (He et al, 2007; Kemmerling et al, 2007). Both bir1 and bon mutant plants display spontaneous cell death that is partially suppressed at high temperatures and by the loss of the lipase-like proteins EDS1/PAD4 or the nucleotide-binding LRR receptor (NLR) SNC1 (Yang et al, 2006; Gao et al, 2009). These findings suggest a link between cell death and resistance in bak1-KO plants, which remains to be explored.

Here we show that the loss of BAK1 sensitizes PEPR signaling toward cell death, and results in reprogramming of PEPR-mediated defense outputs in favor of SA-related
resistance. This is accompanied by increased extracellular release of PROPEP3 upon pathogen challenge. Notably, selective BAK1 depletion occurs during challenge with the fungal hemibiotrophic pathogen *Colletotrichum higginsianum (Ch)*, and coincides with PEPR-dependent fungal resistance. Our findings indicate a critical role for PEPR-mediated DAMP signaling, which is stimulated and rewired when BAK1 is depleted, in plant immunity.

**RESULTS**

**Loss of BAK1 sensitizes PEPR-mediated signaling toward cell death**

Pursuing a molecular link between MAMP and PEPR pathways, we investigated a role of BAK1 in PEPR signaling. With transgenic plants expressing a functional PEPR1-Flag fusion in the *pepr1 pepr2* background (Fig EV1A), coimmunoprecipitation (coIP) analyses confirmed ligand-induced association of PEPR1-Flag and BAK1 (Fig EV1B), as previously deduced (Schulze et al., 2010).

Unexpectedly, however, Pep2-induced growth inhibition was greatly enhanced in *bak1-3* and *bak1-4* KO plants compared to the wild type (WT) (Figs 1A and EV2A). This phenotype was rescued by the introduction of a genomic BAK1 clone (Fig EV2B) and abolished in *bak1 pepr1 pepr2* plants (Figs 1A and EV2A), confirming that Pep sensitization upon *BAK1* disruption occurs through PEPRs. Of the BAK1-related SERK family members, single disruption of *BAK1* specifically enhanced Pep2-induced growth inhibition (Fig EV2C), correlated with enhanced cell death (He et al., 2007; Kemmerling et al., 2007). Indeed, cell death staining revealed that Pep2, but not flg22, induced extensive cell death in the roots and cotyledons of *bak1-4* plants, in a manner dependent on PEPRs (Figs 1B and EV3A). This demonstrates that PEPR-mediated pro-death signaling is sensitized upon *BAK1* disruption. Moreover, loss of PEPRs substantially suppressed the dwarfism of *bak1 bkk1* plants (Fig 1C), suggesting a contribution of endogenous PROPEP-PEPR signaling to cell death in the mutant. We thus conclude that PEPRs mediate one of the pro-death pathways that are suppressed by BAK1/BKK1.

To assess the specificity of the *bak1* effects, we tested Pep-induced growth inhibition in *lsd1c* plants, which display runaway cell death via EDS1 (Rustérucci et al., 2001). In contrast to *bak1* plants, *lsd1* plants retained WT-like Pep2 sensitivity (Fig EV3B). *EDS1* disruption did not affect Pep2-induced growth arrest in *bak1* plants (Fig EV3C). These
results suggest that PEPR-mediated pro-death signaling occurs independently of EDS1 and is specifically sensitized upon BAK1 disruption.

Enhanced cell death in bak1 plants was previously uncoupled from BR signaling (He et al, 2007; Kemmerling et al, 2007). Consistent with this, neither the reduction nor the sensitization of BR signaling in the presence of the bri1-301 allele (Nam & Li, 2002) or BES1-D (Albrecht et al, 2008), respectively, influenced the bak1 effects on Pep2-induced growth inhibition (Figs EV2D and EV2E). Our results thus rule out antagonism between BR and PRR signaling as the cause for the sensitization of Pep-induced cell death.

**Disruption, but not catalytic inactivation, of BAK1 sensitizes PEPR signaling**

To determine whether loss of BAK1 accumulation or kinase activity sensitizes PEPR signaling, we characterized the previously described bak1 alleles (Schwessinger et al, 2011; Ranf et al, 2012). Pep2-induced root growth inhibition was enhanced in the tested bak1-null alleles relative to the corresponding WT controls, while, in contrast, that in the bak1 alleles with a point substitution in the kinase domain, including a kinase-dead variant, was suppressed (Figs 1A, EV3E and EV3F). In the hypoactive bak1-5 allele (Schwessinger et al, 2011), Pep-induced growth inhibition and MAPK activation were almost abolished (Figs 1A and EV1C). These results are consistent with the established view that BAK1 positively regulates PEPR signaling (Roux et al, 2011). However, in contrast to catalytic inactivation of BAK1, our findings reveal that BAK1 depletion leads to the sensitization of PEPR-mediated pro-death signaling.

It is conceivable that another SERK member compensates for the absence of BAK1 in PEPR signaling but is virtually, if not completely, blocked in the presence of a kinase-inactive BAK1 variant. This model also explains the retention of PEPR function leading to growth inhibition in bak1 bkk1 plants (Fig 1C). We thus tested pairwise associations between PEPR1 and SERK members by coIP analyses in Nicotiana benthamiana leaves, following Agrobacterium-mediated co-expression of PEPR1-FLAG with individual SERK-HA proteins. In contrast to the preferential FLS2-BAK1 association (Roux et al, 2011), PEPR1-FLAG associated with the four tested SERK members in response to Pep2 (Fig 1D). Therefore, promiscuous employment of SERK members seems to underlie the tolerance of PEPR signaling to BAK1/BKK1 disruption.
PEPR signaling strictly requires BIK1 and PBL1

flg22, elf18 and Pep1 commonly induce phosphorylation of BIK1 and MAPKs, downstream of cognate PRR-BAK1 complexes (Lu et al, 2010; Zhang et al, 2010; Liu et al, 2013). PEPR1 directly phosphorylates BIK1, whereas FLS2 relies on BAK1 for BIK1 phosphorylation (Lu et al, 2010; Liu et al, 2013). Consistent with this, BIK1 phosphorylation and MAPK activation in response to Pep2 remain largely unaffected in bak1-KO plants, while those in response to flg22 were significantly reduced (Figs 2A and EV1C). By contrast, Pep responsiveness requires BIK1 and the closely related PBL1 (Liu et al, 2013). We verified that Pep2-induced MAPK activation and growth inhibition were reduced in bak1 bik1 pbl1 plants (Figs 2B and 2C), demonstrating that the authentic PEPR-SERK-RLCK module mediates sensitized Pep responses in the absence of BAK1.

Reprogramming of the PEPR-regulated transcriptome upon BAK1 disruption

To elucidate the bak1 effects on PEPR-regulated outputs, we performed a genome-wide microarray analysis for WT and bak1-3 seedlings exposed to Pep2. The loss of BAK1 had a larger effect on Pep2-regulated expression profiles at 10 h than at 2 h (Fig 3A). Cross-referencing Pep2-responsive genes in bak1 plants at 10 h with genes responsive to SA, methyl-JA and ET revealed an over-representation of SA- or JA-inducible genes among the Pep2 up- or down-regulated genes, respectively (Fig 3B; Table E1). Quantitative reverse transcription- (qRT-)PCR analyses confirmed that Pep2 induction of SA-related and -unrelated defense markers, PRI and NHL10, respectively, was enhanced, while in contrast that of a JA/ET marker, PDF1.2a, was suppressed in bak1-KO plants (Figs 3C and 3D). BIK1/PBL1 were again required for enhanced Pep2 induction of PRI and NHL10 in bak1 plants (Fig 3D), indicating that the authentic PEPR-BIK1/PBL1 signaling is rewired upon BAK1 disruption. Together, our results suggest that BAK1 disruption leads to the prioritized activation of these SA-related and -unrelated defenses at the cost of the JA-dependent defenses.

Of particular note, although SID2-mediated SA biosynthesis (Wildermuth et al, 2001) was required for PRI induction in response to Pep2, it was dispensable for cell death, growth arrest, NHL10 induction, and suppression of PDF1.2a induction in bak1-KO plants (Figs 3C and EV3D). These results point to the SA independence of
pro-death signaling and of suppressing JA/ET defense induction via PEPRs upon BAK1 disruption.

**Loss of BAK1 reinforces the PEPR pathway at both the proligand and receptor levels**

PEPR-mediated PROPEP2/PROPEP3 activation is thought to provide positive feedback for defense signal amplification (Yamaguchi & Huffaker, 2011). Pep2-induced PROPEP2/PROPEP3 activation was enhanced in bak1-KO plants (Fig 4A). With anti-GFP and anti-PROPEP3 antibodies (raised against both N- and C-terminal fragments of PROPEP3; Ross et al, 2014), we traced Pep2 induction of a functional PROPEP3-Venus fusion protein, driven by the native DNA regulatory sequences (Ross et al, 2014). To determine whether PROPEP3-Venus is released from the cell, we examined an extracellular protein fraction recovered from the surrounding liquid medium. Both antibodies detected a specific signal that is nearly of the predicted full-length size (approximately 10.4 + 27 kDa) in both in planta and extracellular fractions (Fig 4B). The PROPEP3-Venus form was produced and released into the extracellular space in response to Pep2, to a greater degree in bak1-4 plants compared to WT plants (Fig 4B). Although differences were less pronounced in anti-PROPEP3 immunoblots, our results indicate that Pep-induced PROPEP3 release is increased in the absence of BAK1. Under our conditions, we failed to detect endogenous PROPEP3 or a small, possibly processed form of PROPEP3-Venus. These data suggest that PROPEP3 can be released without extensive processing.

We next tested whether BAK1 disruption influences PEPR accumulation. PEPR1-FLAG transgenic plants recapitulated the observed bak1 effects on PRI and PDF1.2a induction in response to Pep2, without significantly affecting steady-state receptor accumulation (Figs EV4A and EV4B). In the presence of BAK1, Pep1 but not flg22 application leads to a transient decline of PEPR1-FLAG accumulation by ~50% within 5 h (Fig 4C), without significantly lowering the transcript levels (Fig EV4C). In the absence of BAK1, however, the receptor accumulation remained unaltered over the tested time course after Pep1 application (Fig 4D). Ligand-induced PEPR1-FLAG down-regulation was also blocked in the presence of the proteasome inhibitor MG132 (Rock et al, 1994), or the endocytosis inhibitors Wortmannin (Emans et al, 2002) and Tyrphostin A23 (Banbury et al, 2003) (Fig 4E). These results suggest that Pep1 induces
the proteasomal degradation of PEPR1, in association with BAK1-dependent internalization, as previously described for FLS2 (Chinchilla et al, 2007; Smith et al, 2014). Stabilization of a ligand-elicited receptor pool may prolong Pep responses in bak1-KO plants.

**BAK1 and BON proteins control the balance of SA- and JA-related outputs in PEPR signaling**

We further addressed the mechanisms by which BAK1 disruption influences the balance between different PEPR-mediated defense outputs. Of the tested bak1 serk double mutants, Pep2 induction of PRI and NHL10 was further enhanced in bak1 bkk1 plants (Fig EV2F), pointing to a correlation between cell death de-repression (He et al, 2007) and defense reprogramming.

This prompted us to assess whether loss of BON proteins recapitulates the bak1-like cell death phenotype. In bon1 bon2 and bon1 bon3 plants, Pep2 induction of PRI and NHL10 was much further enhanced, while that of PDF1.2 was lowered, compared to WT plants (Fig 5A). Pep2-induced PRI induction was much higher in bon mutant plants than in bak1-4 plants (see the difference in scales between Figs 3C and 5A), which may be attributable to the previously described constitutive sensitization of SA-related defenses in bon plants (Yang et al., 2006; Fig 5A). Pep2-induced growth inhibition was also stronger in bon, bon1 bon2 and bon1 bon3 plants than in WT plants (Fig 5B). In good accordance, the dwarfism of bon1 plants was again alleviated in the absence of PEPRs (Fig 5C), pointing to a contribution of endogenous PROPEP-PEPR signaling to cell death in plants lacking BON1. Our results thus indicate that the removal of the BAK1/BON pathway redirects PEPR signaling to the potentiation of SA-related defenses and cell death. We further showed that BAK1 accumulation was retained in bon1 bon2 and bon1 bon3 plants (Fig 5D), excluding the possibility that the bon phenotype is caused by reduced BAK1 accumulation.

**Virulence effector-dependent production of PROPEP prolignands**

To assess the biological relevance of our findings, we traced the production and possible extracellular release of PROPEP prolignands after pathogen challenge. Of PROPEP1-PROPEP6, PROPEP3 and PROPEP2 were massively induced upon challenge with the bacterial phytopathogen *Pseudomonas syringae* pv. *tomato* (Pst)
DC3000 (Figs 6A and EV5A). There were two phases of PROPEP2/PROPEP3 induction over the time course tested, although the first (2 h post inoculation (hpi)) was less pronounced for PROPEP2 (Fig 6A). The second phase (24 hpi) was almost abolished (or, possibly, much delayed) following challenge with Pst DC3000 ΔhrcC, which is deficient in type III effector (T3E) secretion (Yuan & He, 1996), as well as following flg22 application (Figs 6A and EV5B). Our results thus suggest that the bacterial MAMPs and virulence effectors, respectively, greatly contribute to the first and second phases of the proligand induction at the mRNA level.

PROPEP2/PROPEP3 induction in response to flg22 and Pst DC3000 ΔhrcC was lowered in bak1-KO plants (Figs EV5B and EV5C). A BAK1-dependent receptor(s) may predominantly contribute to PROPEP3 production in response to bacterial MAMPs, as previously shown for stomatal closure during bacterial challenge (Zeng and He, 2010). However, WT-like PROPEP2/PROPEP3 induction was restored in bak1 plants after Pst DC3000 inoculation (Fig EV5D). These data suggest that BAK1 is required for MAMP-dependent induction of the proligands, but becomes dispensable for effector-dependent induction during bacterial challenge. PEPRs per se were dispensable for PROPEP2/PROPEP3 induction in response to Pst DC3000 in both WT and bak1-4 backgrounds (Fig EV5D), pointing to robustness of the proligand production.

Our immunoblot analysis revealed that PROPEP3-Venus protein accumulation largely reflected PROPEP3 induction as described above. In bak1-4 plants, early PROPEP3-Venus accumulation was reduced in response to Pst DC3000 ΔhrcC, but it was unaffected in response to Pst DC3000 (Fig 6B). These results confirm the BAK1 independence of PROPEP3 production in plants exposed to bacterial T3Es.

PROPEP3-Venus accumulation was sustained for at least 48 h in response to Pst DC3000, and was largely indistinguishable between WT and bak1-4 plants (Fig 6C). Unlike in Pep-treated plants (Fig 4B), we detected two small forms of PROPEP3-Venus, in addition to the most abundant, apparently full-length form, at 24 and 48 h after inoculation (Fig 6C left). Whether they were produced inside or outside the cell remains to be determined. Of particular note, in the extracellular fraction, the smallest form (~30 kDa) of PROPEP3-Venus predominantly accumulated during bacterial challenge (Fig 6C right). Extracellular release of PROPEP3-Venus was again increased in bak1-4 plants, despite no significant increase in the proligand production in planta (Fig 6C). We observed enhanced cell death in bak1-4 plants, compared to WT plants, following
Pst DC3000 challenge (Fig 6D), as described previously (He et al, 2007; Kemmerling et al, 2007). These results suggest that, in the absence of BAK1, enhanced cell death leads to an increase in the extracellular release of the proligand (Fig 6C).

Together with this, the previously described role for PEPR in coupling ETI with systemic immunity (Ross et al, 2014) prompted us to test whether cell death associated with ETI also stimulates PROPEP3 release. We observed massive cell death and high PROPEP3-Venus accumulation 48 h after inoculation with an avirulent strain of Pst DC3000, AvrRpm1 (Figs 6D and 6E left). As well as during basal resistance to Pst DC3000 (Fig 6E right), we again detected the two small forms, in addition to the apparently full-length form, of PROPEP3-Venus. PROPEP3-Venus was also released during ETI, predominantly in the smallest form. Given the retention of the Venus epitope in the released small form (Fig 6F), PROPEP3-Venus likely underwent N-terminal truncation following bacterial challenge as deduced previously (Huffaker et al, 2006).

**PEPRs are required for basal resistance in bak1-knockout plants**

We next assessed a role for the PEPR pathway in pathogen resistance in the presence or absence of BAK1. pepr1 pepr2 BAK1 (+) plants were essentially indistinguishable from WT plants in their basal resistance to the virulent Noco2 strain of the oomycete phytopathogen *Hyaloperonospora arabidopsis* (Hpa) (Fig 7A), as shown for post-invasion resistance to Pst DC3000 (Ross et al, 2014). The growth of Hpa Noco2 was reduced in bak1 plants as described previously (Kemmerling et al, 2007), but was substantially permitted in bak1 pepr1 pepr2 plants (Fig 7A). We also assessed post-invasion resistance to Pst DC3000 in these plants following leaf infiltration of the bacteria, under conditions in which a contribution of leaf surface immunity can be essentially ignored. The growth of the bacteria was limited in (mock-treated) bak1 plants, but was again increased in bak1 pepr1 pepr2 plants (Fig 7B). These results indicate a critical role for PEPRs in basal resistance in the absence of BAK1.

flg22-induced resistance, which is entirely dependent on FLS2 (Zipfel et al, 2004), was largely unaffected in bak1-4 and pepr1 pepr2 plants under our conditions (Fig 7B). Notably, however, flg22-induced resistance almost collapsed in bak1-4 pepr1 pepr2 plants (Fig 7B). Correlated with this, flg22-induced SA production and PRI activation
remained unaffected in bak1-4 and pepr1 pepr2 plants, but both were lowered in bak1-4 pepr1 pepr2 plants (Figs 7C and 7D), without a significant decrease in steady-state FLS2 accumulation (Fig EV5E). It should be noted that even bak1-KO plants displayed reduced but detectable PROPEP2/PROPEP3 induction in response to flg22 (Fig EV5B). We infer from these results that, once weakened, FLS2 signaling is linked to PEPR signaling, which then sustains SA-based defenses in the absence of BAK1. Consistent with this model, we found that SID2 was required for bacterial resistance in bak1-4 plants, with or without flg22 treatment (Fig 7E). Moreover, loss of BAK1 increased bacterial resistance even in the sid2 background (Fig 7E), pointing to an enhancement of SA-independent defense in bak1-KO plants. Whether this also requires PEPRs remains to be determined.

We further showed that elf18-triggered PRI and FRK1 induction was elevated in bak1-4 plants compared to WT plants, yet the bak1 effects were abolished in the absence of PEPRs (Fig EV5F). This was also the case for PRI induction upon Pst DC3000 ΔhrcC inoculation in bak1-4 plants (Fig EV5G). These results suggest that, upon BAK1 disruption, the PEPR pathway not only reinstates suppressed MAMP receptor pathways but also boosts those that are less affected.

BAK1 depletion during Colletotrichum higginsianum challenge renders PEPRs essential in anti-fungal resistance

Our findings above would be of relevance if BAK1 depletion occurred during pathogen challenge. BAK1 accumulation was retained following challenge with Pst DC3000 and Pst DC3000 AvrRpm1 (Fig EV5H), consistent with WT-like basal resistance and ETI of pepr1 pepr2 BAK1 (+) plants (Fig 7B mock; Ross et al, 2014), respectively. However, we found that Ch resistance was significantly lowered in pepr1 pepr2 BAK1 (+) plants at 5 days post inoculation (dpi) (Fig 8A left). This could be accounted for if the cellular supply of BAK1 were lowered during Ch resistance. Indeed, the accumulation of BAK1, but not of PEPR1-FLAG or MPK3, was substantially reduced 4 days after Ch challenge (Fig 8B). This seems to occur at the post-transcriptional level, given the unaltered BAK1 mRNA accumulation (Fig 8B). The specific decrease in BAK1 protein accumulation precludes the possibility that it merely reflects a consequence of necrotrophic cell death caused by Ch infection. Therefore, our results indicate that BAK1 depletion indeed occurs during Ch infection, and thereby
renders the PEPR pathway necessary for basal resistance.

We further revealed that bak1-4 plants enhanced both the rate of successful Ch invasion and fungal growth (assessed by lesion size) (Fig 8A), pointing to the existence of a BAK1-dependent step in anti-fungal resistance. By contrast, loss of PEPRs did not increase fungal invasion rate, which was determined at 3 dpi (Fig 8A right), before BAK1 was substantially depleted (Fig 8B). These results suggest that Ch-induced BAK1 depletion contributes to the suppression of invasion resistance but results in PEPR-dependent post-invasion resistance. Importantly, we again observed that loss of PEPRs further reduced overall fungal resistance in the absence of BAK1 (Fig 8A left). This strengthens our contention that the PEPR pathway plays a critical role in basal resistance in plants depleted of BAK1.

Discussion

As a shared co-receptor for different PRRs, BAK1 may represent an Achilles heel in plant immunity. A key question involves how plants display effective resistance in response to or in the presence of pathogen assaults on BAK1. This is of crucial relevance in light of the range of pathogen effectors that target the co-receptor. The present study reveals that the PEPR pathway is engaged and rewired in response to MAMPs, pathogen effectors and BAK1 depletion, and thereby leads to basal resistance (Fig 9).

In Arabidopsis-Pst compatible interactions, we discovered that these three elements collectively contribute to the production of the PROPEP proligands, a prerequisite for PEPR signaling activation. As shown with PROPEP2/PROPEP3 and PROPEP3-Venus, an initial MAMP-triggered induction is followed by further induction dependent on bacterial T3Es. On the other hand, sustained PROPEP2/PROPEP3 induction was previously detected in seedlings treated with elf18 (Tintor et al, 2013). The apparent discrepancy may be attributable to differences in the MAMPs and/or plant tissues used. Nevertheless, the present data show that Pst virulence effectors increase the production of the proligands as compared to their production in response to MAMPs alone. This seems not merely to reflect vigorous bacterial growth, given the potent PROPEP3-Venus production despite the termination of bacterial growth in ETI (Belkhadir et al, 2004). It is also notable that effector-dependent PROPEP2/PROPEP3 induction overcomes the requirement for BAK1 in MAMP-dependent induction. This
likely underlies the engagement of the PEPR pathway in basal resistance in the absence of BAK1. Moreover, PEPR-independent PROPEP production on exposure to bacterial effectors further increases robustness of the proligand production. This may enable non-cell-autonomous PEPR signaling from cells displaying damaged and impaired receptor function to the surrounding cells.

Remarkably, PROPEP3 release during *Pst* basal resistance is increased upon BAK1 disruption, apparently in association with elevated cell death, demonstrating that at least PROPEP3 (or its derivatives) provides a DAMP, indicative of membrane disintegration associated with pathogen-induced BAK1 depletion. This presents, for the first time, compelling evidence that the PEPR pathway acts in DAMP sensing and signaling during plant immunity. Given the previously described PEPR requirement for MAMP responses in sterile seedlings (Ma et al., 2012; Tintor et al., 2013), PROPEP release should occur to some extent upon exposure to MAMPs alone. The present study illustrates that, following MAMP-dependent induction, pathogen effectors and BAK1 depletion additively enhance the generation and release of the proligands. Furthermore, predominant accumulation of a putatively processed small form of PROPEP3-Venus in the extracellular space following exposure to bacterial effectors (or bacterial pathogens) implies the existence of an additional regulatory step in the activation of PEPR ligands. Of particular note, this form was apparently absent in plants treated with Pep1 or Pep2, pointing to an association between its formation and pathogen challenge. Future studies will be required to determine the identity and biological significance of this apparently processed form(s) of PROPEP3, and also the possible involvement of a protease produced by the modified host or the invading bacteria. Nevertheless, such stepwise reinforcement of extracellular PROPEP supply is reminiscent of the danger hypothesis, in which sensing host damage (DAMPs) associated with pathogen infection, in addition to MAMPs, is critical for robust defense activation (Stuart et al., 2013). It should be also noted that release of the small PROPEP3 form into the extracellular space also occurs without BAK1 depletion during and/or after ETI. Our results thus clearly show that BAK1 depletion is not essential for PROPEP3 release, but positively influences this important step during basal resistance. ETI activation, possibly in association with cell death, may offer an alternative to BAK1 depletion for the stimulation of the DAMP pathway.

At present, it remains unclear whether PROPEP processing facilitates PEPR
recognition. Nonetheless, we show that Pep-induced PROPEP3 generation and subsequent release is also strengthened upon 

\textit{BAK1} disruption, in a manner dependent on PEPRs. This predicts that if active ligands are supplied in the absence of BAK1, PEPR signaling leads to cell death and is maintained by positive auto-feedback, thereby sustaining basal resistance.

We further revealed that BAK1 depletion and catalytic inactivation, respectively, lead to sensitization and desensitization of PEPR-mediated cell death and defense responses. Given the differential requirements for BAK1 kinase activity between cell death and PRR signaling (Schwessinger et al, 2011), BAK1 kinase suppression is likely to block PEPR signaling without de-repressing pro-death signaling. In agreement with this, different bacterial effectors have been reported to target BAK1 and/or PRR partners through physical associations (Macho & Zipfel, 2015). AvrPto inhibits FLS2/EFR kinase activity and may also block PRR-BAK1 associations (Shan et al, 2008; Xiang et al, 2008, 2011). AvrPtoB is able to suppress FLS2-BAK1 association and kinase activity of BAK1 (Cheng et al, 2011; Shan et al, 2008). HopAO1 phosphatase reverses critical phosphorylation on a tyrosine residue of EFR that is conserved in many RKs including FLS2, BAK1 and PEPR1/PEPR2 (Macho et al, 2014). On the other hand, HopU1 mono-ADP ribosyltransferase reduces FLS2/EFR accumulation by inhibiting specific binding of the RNA-binding protein GRP7 to 

\textit{FLS2/EFR} but not \textit{BAK1} transcripts (Nicaise et al, 2013). Although it is possible that AvrPtoB-mediated BAK1 ubiquitination (Goehre et al, 2008) leads to BAK1 degradation, the unaltered BAK1 accumulation and the low PROPEP3-Venus release that we observed in \textit{BAK1} (+) plants during \textit{Pst} challenge imply that \textit{Pst} predominantly inhibits BAK1/PRR activity without significantly affecting BAK1 abundance. This strategy may have been selected to minimize de-repression of PEPR and parallel DAMP signaling, given that \textit{Pst} indeed eliminates other host targets by T3Es, e.g., PBS1 by AvrPphB and RIN4 by AvrRpt2 (Kim et al, 2005; Shao et al, 2003).

In contrast, \textit{Ch} infection is accompanied by a substantial decrease in BAK1 accumulation, which likely serves to overcome BAK1-dependent defenses. However, this also seems to result in the stimulation of PEPR-mediated post-invasion defenses. This model well illustrates the observed separation between PEPR-dependent and -independent steps during basal \textit{Ch} resistance. Future studies will address whether and how a fungal effector(s) mediates specific BAK1 depletion.
This study reveals that BAK1 depletion and consequent dysfunction of BAK1/BON-mediated control leads to active engagement and rewiring of the PEPR pathway toward cell death and anti-biotrophic resistance (Fig 9). Previous genetic studies showed that pro-death pathways, distinct from the PEPR pathway in SA and EDS1/PAD4 dependence, are derepressed when BIR1, BIR2 or BON function is compromised (Gao et al., 2009; Halter et al., 2014; Yang et al., 2006). At least one such pathway requires the LRR-RK SOBIR1 (Gao et al., 2009), which functions with different LRR receptor-like proteins to mount anti-fungal resistance (Liebrand et al, 2014; Gust & Felix, 2014). However, it was previously shown that PEPR function is retained in the absence of an N-glycosylation-dependent ER quality control (QC) pathway, while in contrast SOBIR1 function is impaired (Saijo, 2010; Sun et al, 2014; Tintor et al, 2013; Zhang et al, 2015), indicating that SOBIR1 function is not required for PEPR function. These findings point to the notion that a wide array of defense pathways, differing in ER QC requirements, is under BAK1/BON-mediated attenuation. This is in good agreement with the hypothesis that the disintegration of BAK1/BON complexes is sensed and linked to robust defense activation (Gao et al, 2009; Halter et al, 2014). Whether and how these separate defense pathways become engaged in resistance following pathogen-induced PEPR requires further investigation.

Mounting evidence indicates that defects in different MTI components, including BAK1, BIK1, the NADPH oxidase RbohD, MPK4 and the callose synthase PMR4, enhance basal resistance against virulent (hemi-)biotrophic pathogens (Nishimura et al, 2003; Kim et al, 2005; Zhang et al, 2010; Laluk et al, 2011; Zhang et al, 2012; Kadota et al, 2014; Roux et al, 2015). Studies on natural variation in Arabiopsis-Hpa interactions point to the prevalence of intermediate resistance based on interactions between multiple host-pathogen components (Krasileva et al, 2011). Although BAK1 depletion results in PEPR-mediated basal resistance, strict BIK1 requirement excludes the DAMP pathway from the resistance activated in bik1 plants (Zhang et al, 2010; Laluk et al, 2011). Multilayered engagement of different DAMP pathways, possibly in response to effector-mediated interference in MAMP signaling, is likely to underlie basal resistance against virulent pathogens.

**Materials and Methods**
**Plant materials and growth conditions**

WT plants used were *Arabidopsis thaliana* Col-0 unless otherwise stated. *pepr1-1* (SALK_059281) and *pepr2-3* (SALK_098161), *bak1-3* and *bak1-4* (Chincilla et al, 2007), *bak1-7, bak1-8, bak1-10* (in the Aeq cyt background), *bak1-13, bak1-14* and *bak1-15* (in the Aeq vmd background) (Ranf et al, 2012), *bak1-5, BAK1pro:BAK1/bak1-4* and BAK1pro:BAK1(D416N)/bak1-4 (Schwessinger et al, 2011), *sid2-1* (Wildermuth et al, 2001), *eds1-2* and *lsdlc* (Rustérucci et al, 2001), *fis2* (Zipfel et al, 2004), *bon1-1, bon2-2, and bon3-3* (Yang et al, 2006), *serk1-1, serk1-3, serk2-2, bkk1-1, serk5-1* and 35Spro:BES1D/serk1-3 bak1-3 (Albrecht et al, 2008), *bri1-301* (Nam and Li, 2002) and *PROPEP3pro:PROPEP3-Venus* (Ross et al, 2014) were used. *PROPEP3pro:PROPEP3-Venus/bak1-4* was generated by crossing. Plants were grown on soil or 0.5 x Murashige and Skoog (MS) medium containing 25 mM sucrose, under 10 h Light/14 h Dark or 12 h Light/12 h Dark, respectively, unless otherwise stated.

**Plant transformation**

The genomic DNA sequences of the *PEPR1* (At1g73080) loci including the intergenic and coding regions were inserted into the binary vector pAM-PAT for expressing their C-terminal FLAG fusions, and then introduced into pepr1 pepr2 plants via *Agrobacterium*-mediated transformation.

**Microbial materials**

The ΔhrcC (Yuan & He, 1996) and *AvrRpm1* (Debener et al, 1991) strains of *Pseudomonas syringae pv. tomato* (Pst) DC3000 (Dong et al, 1991), Noco2 strain (Parker et al, 1993) of *Hyaloperonospora arabidopsidis* (*Hpa*), and IMI 349063A strain (O'Connell et al, 2004) of *Colletotrichum higginsianum* (*Ch*) were used in this study.

**Bioassay for pattern-triggered responses**

For root growth assays, 2-day-old seedlings were treated with 100 nM Pep2 in liquid medium for 7 days, before measuring root length or staining with Evans blue or lactophenol trypan blue. For MAPK assays, 10-day-old seedlings were treated for the indicated times with 1 μM Pep2 or *flg22*. Proteins were extracted in an extraction buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 10 mM NaF, 25 mM
beta-glycerophosphate, 2 mM sodium orthovanadate, 10% glycerol, 2 mM DTT, 1 mM PMSF, 1% (v/v) P9599 protease inhibitor cocktail (Sigma). The supernatants were recovered after centrifugation at 17,000 g for 15 minutes at 4 °C, and then subjected to immunoblot analysis on 10% SDS-PAGE with rabbit anti-p44/42 MAPK antibodies (Cell Signaling). For qRT-PCR analysis, 10-day-old seedlings were exposed to the indicated elicitors for the indicated times, unless otherwise stated. The PCR primers used were described previously (Ross et al, 2014; Tintor et al, 2013).

Pathogen inoculation assays

For flg22-induced resistance assays, rosette leaves of 4-week-old plants were infiltrated with 1 µM flg22 or water (mock) for 24 h, before infiltration with Pst DC3000 at 10⁵ cfu/ml. Inoculated plants were kept in a covered container for 3 days before harvesting the leaves for bacterial quantification. For Hpa inoculation, 2-week-old seedlings spray-inoculated with Noco2 strain spores (5 x 10⁴ spores/ml) were were incubated at high humidity at 18 °C for 6 days until the spores were suspended in water for counting with a hemocytometer. For Ch assays, 5 µl of spores at 2.5 x 10⁵ spores/ml of the IMI 349063A strain grown on Mathur’s medium (2.8 g/l glucose, 1.2 g/l MgSO₄/7H₂O, 2.7 g/l KH₂PO₄, 2.2 g/l mycological Peptone, 3% Bacto Agar) were dropped onto leaves of 4-week-old plants. Inoculated plants were incubated at high humidity for 5 days. The lesion diameter of inoculated leaves was determined with Adobe Photoshop in photo images.

To measure the invasion rates of Ch on Arabidopsis leaves, 2 µl of Ch spore suspension (5 x 10⁵ spores/ml) was drop-inoculated onto the cotyledons of 10-day-old seedlings. The cotyledons were mounted in water under a coverslip, with the inoculated surface facing the objective lens. The invasion rate (%) was calculated at 3 dpi by the following numerical formula: (the number of conidia with formation of invasive hypha) / (the number of appressoria) x 100 (Hiruma et al, 2011).

Quantitative RT-PCR (qRT-PCR) analysis

Ten-day-old seedlings or 4-week-old plants were treated or syringe-infiltrated with the indicated peptides or bacterial strains, and then harvested after the indicated times. Total RNA was isolated from the plant samples using TRI reagent following the manufacturer’s instructions (Ambion). Total RNA was reverse-transcribed using an
oligo(dT) primer and reverse transcriptase (Roche). Quantitative PCR was performed with the Bio-Rad iQ5 multicolor real-time PCR detection system (Bio-Rad). The expression levels of genes of interest were normalized relative to those of a reference gene, At4g26410 (Czechowski et al., 2005). *PR1, NHL10* and *PDF1.2a* genes were used as defense markers for salicylic acid (SA)-dependent induction, SA-independent induction and jasmonic acid/ethylene-dependent induction, respectively (Bousocq et al., 2010; Koornneef et al., 2008). Representative results of two or more independent experiments with three biological replicates each are shown, unless otherwise stated.

**Microarray analysis**

Microarray analysis was performed essentially as described previously (Ross et al., 2014), with ten-day-old seedlings (WT, bak1-3 and pepr1 pepr2) exposed to 1 µM Pep2 for 2 and 10 h.

Hierarchical clustering analysis was conducted on 2214 genes that exhibited significant changes (more than 2-fold, q < 0.01) in bak1-3 plants 10 h after Pep2 application, compared to mock-treated bak1-3 plants. Based on the expression patterns, these genes were classified into six clusters (Table E1): Cluster 1 represents genes that are specifically up-regulated in bak1-3 plants (314 genes); Cluster 2 represents those whose up-regulation was enhanced in bak1-3 plants (633 genes); Cluster 3 represents those that are equally or less up-regulated in bak1-3 plants (52 genes); Cluster 4 represents those whose down-regulation was enhanced in bak1-3 plants (284 genes); Cluster 5 represents those that are equally down-regulated between WT and bak1-3 plants (66 genes); and Cluster 6 represents those that are specifically down-regulated in bak1-3 plants (865 genes). The cross-referenced datasets were Exp. ID AT-00391, Exp. ID AT-00113, and Exp. ID AT-00013 in Genevestigator v3, which were also obtained in GeneChip ATH1-121501 Genome Array.

**Transient gene expression in* Nicotiana benthamiana***

*Agrobacterium tumefaciens* GV3101 strains were grown in YEP medium with appropriate antibiotics. Cultures were spun down and resuspended in infiltration buffer (10 mM MgCl2, 10 mM MES-KOH pH 5.5) to OD600 = 0.1. Suspensions of *Agrobacterium* strains carrying cauliflower mosaic virus (CaMV) 35S promoter (35Spro)::PEPR1-FLAG (pAMPAT), 35Spro:SERK-HA (pGWB14) (Roux et al., 2011)
were mixed at a 1:1 ratio, and then syringe-infiltrated into 3-week-old *N. benthamiana* leaves. Leaves were harvested 2 days after inoculation.

**Protein extraction from plant tissues for immunoblot analysis**

Unless otherwise stated, protein extracts were prepared by incubating ground frozen tissues in lysis buffer [50 mM Tris-HCl pH 7.5, 2% SDS, 2 mM DTT, 1 mM AEBSF, 1% (v/v) P9599 protease inhibitor cocktail (Sigma)] for 20 min at room temperature. The supernatants were recovered after centrifugation at 17,000 g for 15 min, and then subjected to immunoblot analysis with the indicated antibodies.

**Co-immunoprecipitation (co-IP) assay**

Plant materials were ground in liquid nitrogen, and lysed in extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM DTT, 1 mM PMSF (Sigma), 1% (v/v) P9599 protease inhibitor cocktail, 0.5% (v/v) IGEPAL CA-630 (Sigma)). Samples were cleared by centrifugation at 8,000 g for 15 minutes at 4 °C, and subsequently filtered using a mesh with pore diameter 75 µm. The supernatants were incubated with anti-Flag M2 antibody (See below) on ice for 10 minutes, and then rotated with protein G beads (Dynabeads, Life Technologies) for 3 h at 4°C. Following three washing steps, the recovered beads were boiled in SDS sample buffer. The eluates were subjected to immunoblot analysis.

**Immunoblot analysis**

Anti-GFP (B-2), anti-FLAG (M2), anti-HA (3F10) and anti-p44/42 MAPK antibodies were purchased from Santa Cruz Biotechnology, Sigma, Roche and Cell Signaling, respectively. Anti-PROPEP3 antibodies raised against both N- and C-terminal fragments of PROPEP3 were described previously (Ross et al., 2014). Anti-BAK1 and anti-FLS2 antibodies were generated in rabbits using C-terminal peptides of BAK1 (amino acid residues 600-614, DSTSQIENEYPSGPR) and FLS2 (amino acid residues 1159-1173, KANSFREDRNEDREV) as antigens, respectively.

Molecular weight markers used were Prestained protein marker broad range (Nacalai Tesque in Figs 6C and 6F), Protein ladder one triple-color (Nacalai Tesque in Fig 6E) and Precision plus protein dual-color standards (Bio-Rad in Figs 4B and 6B). Quantification of immunoblots was performed with ImageJ (http://rsb.info.nih.gov/ij/)
or CSAnalyzer4 (ATTO). Band intensities were normalized with the values of Ponceau S stained bands, and then relative band intensities were calculated with that of the corresponding controls (1.0).

**BIK1 phosphorylation assay**

Protoplasts were prepared from expanded rosette leaves of 4-week-old *Arabidopsis* plants, according to the previously published method (Yoo et al, 2007). Ten μg of the 35Spro:BIK1-HA construct (pAMPAT) was introduced into a 100 μl cell suspension at 2.5 x 10^5 cells / ml. Six hours after transfection, 1 μM of Pep2 or flg22 was applied for 10 minutes. Immunoblot analysis for BIK1-HA was conducted as described above for MAPK assay, except that rat anti-HA antibody (3F10, Roche) was used.

**Extracellular PROPEP3-Venus detection assay**

Five-day-old seedlings grown on MS agar plates under continuous light conditions were transferred to MS liquid medium. Six days later, the medium was replaced with either fresh medium for Pep2 application or with water for bacterial inoculation. One day later, the seedlings were exposed to 1 μM Pep2 for the indicated times, or to the indicated bacterial strains for 20 min. For bacterial inoculation, the inoculated seedlings were washed with water twice, and then were further incubated in water for the indicated times. The media were collected and filtered (pore diameter of 0.22 μm) to remove bacteria. Proteins were concentrated from the filtered media with Strataclean resin (Agilent Technologies). After recovery by centrifugation, resins were boiled in SDS sample buffer before separation by SDS-PAGE. Seedling tissues were also subjected to immunoblot analysis.

**Salicylate (SA) measurement**

SA was extracted from 100 mg plant leaves in 1 ml chloroform/methanol/water (1:2:0.3) containing 160 pmol 2-hydroxybenzoic-3,4,5,6-d4 acid (SA-d4; Campro Scientific) as an internal standard, and then measured as described previously (Ross et al, 2014).

**Statistical analysis**
The following models were fit to the cycle threshold (Ct) values (for qRT-PCR) or log$_2$-transformed root length (RL) with the lmer function in the lme4 package or the lm function in the R environment: Ctgr = GTgt+Rr+egtr (Figures 3C, 3D, 4A, 5A, 7D, EV2F, EV5B, EV5C and EV5F); Ctg = Gg+Rr+egr (Figure EV5G); RLgr = GTgt+Rr+egtr (Figures EV2B and EV2D); RLgt = GTgt+egt (Figures EV2C and EV2E), where fixed factors; G, genotype factors; GT, genotype:treatment interactions; T, treatment factors, and random factors; R, independent replicate; e, residual. The mean estimates of the fixed factors were used as the modeled Ct values or RL. Differences between estimated means were compared in two-tailed t-tests. For the t-tests, the standard errors appropriate for the comparisons were calculated with the variance and covariance values obtained from the model fittings.

Expanded View Information

Expanded View Information includes five figures and one table.

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Author contributions
KY and YS conceived the study. KY, MYY, TH, TF and KH developed and performed the experiments. KY, TH, TF, KT and KH analyzed the data. KY and YS wrote the manuscript with contribution from the other authors.

Conflict of interest
The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. BAK1 disruption sensitizes PEPR signaling toward cell death.

(A and B) Root length (averages ± SD, n ≥ 15) (A) and cell death staining of cotyledons and primary root tips (B) of Arabidopsis seedlings that were exposed to 100 nM Pep2 for 7 days, 2 days after germination. BAK1 (D416N) is a kinase-dead variant. (C) Overview of 3-week-old plants. (D) Pep2 induces PEPR1-FLAG association with SERK1-, SERK2-, BKK1-, and BAK1-HA in N. benthamiana. Experiments were repeated at least three times, with the same conclusions. IP and IB denote immunoprecipitation and immunoblotting with the indicated antibodies, respectively. NT indicates non-transformed plants. (*, p < 0.05 in two-tailed tests compared to the differences (± Pep2) from the corresponding values of WT plants.)

Figure 2. BIK1-dependent sensitization of PEPR signaling in the absence of BAK1.

(A) Immunoblot analysis for BIK1-HA in Arabidopsis protoplasts exposed to Pep2 or flg22 for 10 min. Arrowheads indicate phosphorylated BIK1-HA. (B) Immunoblot analysis for Pep2-triggered MAPK activation. Ponceau S-stained loading controls are shown (bottom). (C) Root length (averages ± SD, n ≥ 15) of Arabidopsis seedlings that were exposed to 100 nM Pep2. (*, p < 0.05 in two-tailed tests compared to the differences (± Pep2) from the corresponding values of bak1-3 plants. Two independent experiments were combined for statistical analysis.)

Figure 3. BAK1 disruption leads to reprogramming of PEPR–mediated defense outputs.

(A) Scatter plots for log2 fold changes of gene expression in WT plants versus bak1-3 plants treated with Pep2 for 2 and 10 h. The regression of these scatter plots is indicated by the red lines. (B) Hierarchical analysis of genes exhibiting a >2-fold change in expression level in bak1-3 plants after Pep2 application for 10 h in a whole-genome microarray analysis. Using Genevestigator v3, these genes were separately cross-referenced to public databases to show their expression responses to salicylate (SA), methyl-jasmonate (JA), or ethylene (ET). (C and D) qRT-PCR analysis of defense-related genes in 10-day-old seedlings exposed to 0.5 µM Pep2 for 10 h. Data are averages (±SD) of three biological replicates. (*, p < 0.05 in two-tailed tests compared to the corresponding bak1-4 and bak1-3 values in C and D, respectively.)
Relative cycle threshold (Ct) values of two independent experiments were combined for statistical analysis.

**Figure 4. PROPEP proligand production and PEPR1 accumulation following Pep application are reinforced in the absence of BAK1.**

(A) qRT-PCR analysis of *PROPEP* genes in 10-day-old seedlings exposed to 0.5 µM Pep2 for 10 h. *, p < 0.05 in two-tailed tests compared to the corresponding WT values. Relative Ct values of two independent experiments with three biological replicates each were combined for statistical analysis. Data are averages (± SD) of three biological replicates. (B) Anti-GFP (upper) and anti-PROPEP3 (middle) immunoblot analysis of PROPEP3-Venus in seedlings submerged in liquid medium and treated with (+) or without (-) 0.5 µM Pep2 for the indicated times. The positions of the molecular weight markers are shown on the left. Arrowheads and asterisks indicate PROPEP3-Venus and cross-reactive bands, respectively. (C, D and E) Immunoblot analysis of PEPR1-FLAG in 10-day-old seedlings following Pep1 or flg22 application. (C and D) Plants were exposed to 1 µM Pep1 or flg22 for the indicated times. (E) Plants were pre-exposed to MG132 (50 µM), Wortmannin (Wm) (30 µM) and Tyrphostin A23 (TyrA23) (100 µM) for 1 h before being exposed to Pep1 for 10 h. The numbers below the immunoblots represent relative intensities of the PROPEP-Venus (B) and PEPR1-FLAG (C-E) bands normalized to the backgrounds in the Ponceau S-stained loading controls (bottom), with the band/background values in WT plant extracts 24 h (upper) and 0 h (middle) after Pep2 application, respectively, set as 1.0.

**Figure 5. Reprogramming of PEPR-mediated defense outputs upon disruption of BAK1/BON-mediated cell death suppression.**

(A) qRT-PCR analysis of defense-related genes in 10-day-old seedlings exposed to 0.5 µM Pep2 for 10 h. *, p < 0.05 in two-tailed tests compared to the corresponding WT values. Relative Ct values of two independent experiments with three biological replicates each were combined for statistical analysis. Data are averages (± SD) of these biological replicates. (B) Root length (averages ± SD, n ≥ 15) of *Arabidopsis* seedlings exposed to 100 nM Pep2. (*, p < 0.05 in two-tailed tests compared to the differences (± Pep2) from the corresponding values of WT plants. Two independent experiments were
combined for statistical analysis.) (C) Fresh weight (averages ± SD, n ≥ 25) of 2-week-old plants grown on soil. (*, p < 0.05 in two-tailed tests compared to the corresponding values of WT plants. ■, p < 0.05 in two-tailed tests. Two independent experiments were combined for statistical analysis.) (D) Immunoblot analysis for BAK1 in 10-day-old seedlings with or without 0.5 µM Pep1 for 24 h. The numbers below the upper panel represent relative intensities of the BAK1 band normalized to the backgrounds in the Ponceau S-stained loading controls, with the value in non-elicited WT plants set as 1.0.

Figure 6. PROPEP production and release during bacterial resistance.
(A) qRT-PCR analysis in 4-week-old plant leaves challenged with the indicated P. syringae pv. tomato (Pst) DC3000 strains for the indicated times. Data are averages (± SD) of three biological replicates. (B) Anti-GFP immunoblot analyses in seedlings challenged with Pst DC3000 (DC3000) and Pst DC3000 ΔhrcC (ΔhrcC) for the indicated times. (C) Anti-PROPEP3 immunoblot analysis of PROPEP3-Venus (upper) in plant tissues (expressed in plants) and in the extracellular fraction (released into medium), 24 h and 48 h after the inoculation with (+) or without (-) Pst DC3000. Lower panels indicate Ponceau S-stained loading controls. The numbers below the upper panel represent relative intensities of the PROPEP3-Venus band normalized to the backgrounds in the Ponceau S-stained loading controls. NT denotes non-transgenic WT plants. (D) Trypan blue cell death staining of Arabidopsis seedlings challenged with Pst DC3000 or Pst DC3000 AvrRpm1 (AvrRpm1) for 48 h. (E) Anti-PROPEP3 immunoblot analyses in the seedlings exposed to the indicated Pst DC3000 strains or 1 µM Pep1 for 48 h. (F) Anti-GFP immunoblot analysis for the extracellular fraction of the seedlings exposed to Pst DC3000 AvrRpm1 for 48 h. Arrowheads and arrows indicate an apparently full-length and processed form of PROPEP3-Venus, respectively. Asterisks indicate cross-reactive bands. The positions of the molecular weight markers are shown on the left of the immunoblots.

Figure 7. PEPRs are required to mount SA-dependent basal resistance in the absence of BAK1.
(A) Growth of H. arabidopsidis (Hpa) Noco2 in 2-week-old plants. Spores were counted at 6 days post inoculation (dpi). Results are averages ± SE of three biological
replicates. (*, p < 0.05 in two-tailed tests compared to the corresponding values of Col plants. ■, p < 0.05 in two-tailed tests between the indicated values. Three independent experiments were combined for statistical analysis.) (B) Growth of syringe-infiltrated Pst DC3000 in rosette leaves of 4-week-old plants pretreated with water (Mock) or 1 µM flg22 for 24 h. Bacterial titers were determined at 3 dpi. Results are averages ± SE of three independent replicates. (*, p < 0.05, **, p < 0.01 in two-tailed tests compared to the corresponding values of WT plants. ▲, p < 0.01 in two-tailed tests compared to the differences (± flg22) from the corresponding values of WT plants. Three independent experiments were combined for statistical analysis.) (C) Total SA levels were determined in the rosette leaves of 4-week-old plants with or without 1 µM flg22 application for 24 h. Data are averages (±SD) of material from three independent plants. The experiments were repeated twice, with similar results. (▲, p < 0.01 in two-tailed tests compared to the differences (± flg22) from the corresponding values of WT plants. Two independent experiments were combined for statistical analysis.) (D) qRT-PCR analysis of the PR1 gene in 4-week-old plants exposed to 1 µM flg22. Data are averages (±SD) of three biological replicates. (*, p < 0.05 in two-tailed tests compared to the corresponding WT values. Relative Ct values of four independent experiments were combined for statistical analysis.) (E) Growth of syringe-infiltrated Pst DC3000 in rosette leaves of 4-week-old plants pretreated with water (Mock) or 1 µM flg22 for 24 h. Bacterial titers were determined at 3 dpi. Results are averages ± SE of three independent replicates. (**, p < 0.01, ***, p < 1.0e-10 in two-tailed tests compared to the corresponding values of WT plants. ▲, p < 0.01 in two-tailed tests compared to the differences (± flg22) from the corresponding values of WT plants. Three independent experiments were combined for statistical analysis.)

Figure 8. BAK1 is depleted during C. higginsianum infection.

(A) Lesion sizes (left) were determined 5 days after C. higginsianum (Ch) inoculation. Invasion rates (right) of Ch on Arabidopsis leaves were determined 3 days after spore inoculation. An average of 3 replicates is shown (±SD) (left) and (±SE) (right). (■, p < 0.05 in two-tailed tests between the indicated samples. *, p < 0.05 in two-tailed tests compared to the corresponding WT values. Two independent experiments were combined for statistical analysis.) (B) Immunoblot (left) and qRT-PCR (right) analyses following Ch challenge for the indicated times (dpi). The numbers below the
immunoblots represent relative intensities of the indicated bands normalized to the backgrounds, with the values at 0 dpi set as 1.0. Experiments were performed three times with the same conclusions.

**Figure 9. A model for PEPR-mediated basal resistance following the removal of BAK1/BON-mediated control.**

Following BAK1 depletion, possibly by pathogen effector actions, MAMP-triggered outputs including induction of PROPEPs are weakened. However, effector-dependent PROPEP induction compensates for low amounts of MAMP-induced PROPEPs in the absence of BAK1. Cell death spread following dysfunction of BAK1/BON-mediated control probably facilitates release of cytosolic PROPEPs into the extracellular space. Moreover, in the absence of BAK1/BON1 complexes, PEPR signaling is strengthened by the stabilization of the receptor and by the potentiation of SA-related defenses toward anti-biotrophic resistance.
Figure E1. Functional significance of BAK1 and related SERK family members in PEPR signaling.

(A) qRT-PCR analysis of PEPR1 in 10-day-old Arabidopsis wild-type (WT) seedlings and those expressing PEPR1-FLAG under the cauliflower mosaic virus 35S promoter in the pepr1 pepr2 background (PEPR1-FLAG pepr1 pepr2). Data are averages (± SD) of three biological replicates (left). Immunoblot analysis of Pep2-induced MAPK activation in Arabidopsis pepr1 pepr2 plants (right). Equal loading was verified with Ponceau S staining (bottom).

(B) PEPR1-FLAG associates with BAK1 in Arabidopsis following Pep2 application for the indicated times. IB and IP denote immunoblotting and immunoprecipitation with the indicated antibodies, respectively.

(C) Immunoblot analysis with anti-phosphorylated p44/p42 MAPK antibody in 10-day-old seedlings exposed to 0.5 µM Pep2 or flg22 for the indicated times. Equal loading was verified with Ponceau S staining.

Figure E2. Pep-induced root growth inhibition in serk mutant plants.

(A) bak1-4 plants are more sensitive to Pep2 than WT plants in root growth inhibition, in a manner dependent on PEPRs.

(B) Complementation of the Pep hypersensitive phenotype of bak1-4 plants by the introduction of a genomic BAK1 DNA clone. (*, p < 0.01 in two-tailed tests compared to the differences (± Pep2) from the corresponding values of bak1-4 plants. Log2 values of two independent experiments were combined for statistical analysis.)

(C) Of the SERK genes tested, single BAK1 disruption has the largest effect in increasing Pep sensitivity. (*, p < 0.01 in two-tailed tests compared to the differences (± Pep2) from the corresponding values of bak1-4 plants. Log2 values were used for statistical analysis.)

(D) Pep2-induced root growth inhibition in the presence of bri1-301. (*, p < 0.01 in two-tailed tests compared to the differences (± Pep2) from the corresponding values of bri1-301 plants. Log2 values of two independent experiments (n ≥ 15 each) were combined for statistical analysis.)

(E) Pep2-induced root growth inhibition of serk1-3 bak1-3 plants in the presence or absence of a constitutive BR activator, BES1D-GFP. (*, p < 0.01 in two-tailed tests compared to the differences (± Pep2) from the corresponding values of WT plants.
Log2 values were used for statistical analysis.

(F) Simultaneous BKK1 disruption enhances Pep2 induction of PRI and NHL10 in bak1-knockout plants. qRT-PCR analysis of defense-related genes in 10-day-old seedlings exposed to 0.5 µM Pep2 for 10 h. Results are averages ±SD. (*, p < 0.01 in two-tailed tests. Relative cycle threshold (Ct) values of two independent experiments with three biological replicates each were combined for statistical analysis.)

(A-E) Root length of 9-day-old seedlings was determined following 100 nM Pep2 application for 7 days. Results are averages ± SD (n ≥ 15).

**Figure E3.** Pep-induced cell death in bak1 mutant plants.

(A) Evans blue staining for dead cells in primary root tips treated with flg22 at 100 nM.
(B) WT and lsd1c plants were indistinguishable in Pep2-induced root growth inhibition.
(C) Characterization of bak1-3 eds1-2 plants.
(D) Characterization of bak1-4 sid2-1 plants. Trypan blue staining for dead cells in cotyledons (left).
(B-D) Pep2-induced root growth inhibition (averages ± SD, n ≥ 15) after treatment with Pep2 at 100 nM. n.s. denotes non-significant differences (p > 0.05 in two-tailed tests). The differences (± Pep2) were compared between the indicated samples.
(E) Pep2-induced root growth inhibition was sensitized in bak1-null alleles.
(F) Pep2-induced root growth inhibition was suppressed in bak1 alleles with point substitutions in the kinase domain.

Results are averages ± SD (n ≥ 15). Root length of 9-day-old seedlings was determined following 100 nM Pep2 application for 7 days. (*, p < 0.01 in two-tailed tests compared to the differences (± Pep2) from the corresponding values of Aeq cyt (A) or Aeq vmd (B) plants. Two independent experiments were combined for statistical analysis.)

**Figure E4.** PEPR1-FLAG plants in the presence or absence of BAK1.

(A) qRT-PCR analysis of defense-related genes in 10-day-old PEPR1-FLAG pep1 pepr2 and PEPR1-FLAG bak1-4 seedlings (as described in Figure E1A) exposed to 0.5 µM Pep2 for 10 h. Data are averages (±SD) of three biological replicates. (*, p < 0.01 in two-tailed tests compared to the corresponding WT values.)
(B) Immunoblot analysis of PEPR1-FLAG and BAK1 in 10-day-old seedlings. Equal loading was verified with Ponceau S staining (bottom). The numerals below the immunoblot represent relative band intensities for the PEPR1-FLAG signal with the value of that in PEPR1-FLAG plants as 1.0.
(C) qRT-PCR analysis of PEPR1 expression following 1 µM Pep1 application for the indicated times in PEPR1-FLAG pepr1 pepr2 plants. Data are averages (± SD) of three biological replicates.

Figure E5. Role for BAK1 in the activation of the PEPR pathway in response to bacterial challenge and MAMPs.
(A) qRT-PCR analysis of PROPEPs in 4-week-old plants after syringe-infiltrated Pst DC3000 challenge.
(B) qRT-PCR analysis of PROPEP2 and PROPEP3 expression in 4-week-old plants following 1 µM flg22 application for the indicated times. (*, p < 0.05 in two-tailed tests compared to the corresponding WT values. Relative Ct values of three independent experiments were combined for statistical analysis.)
(C) qRT-PCR analysis of PROPEP2/PROPEP3 expression in 4-week-old plant leaves 2 h after syringe infiltration with Pst ΔhrcC at 10^7 cfu/ml. (*, p < 0.05, in two-tailed tests compared to the corresponding WT values. Relative Ct values of two independent experiments with three biological replicates were combined for statistical analysis.)
(D) qRT-PCR analysis of PROPEP2/PROPEP3 expression in 4-week-old plant leaves 24 h after syringe infiltration with Pst DC3000 at 10^7 cfu/ml. n.s. denotes non-significant differences (p > 0.05 in two-tailed tests). Data are averages (± SD) of three biological replicates (A-D).
(E) Immunoblot analysis of FLS2 in the rosette leaves of 4-week-old plants. Equal loading was verified with Ponceau S staining (bottom).
(F and G) qRT-PCR analysis of PR1 in 4-week-old plants exposed to 1 µM elf18 or challenged with Pst ΔhrcC for 24 h. Data are averages (±SD) of three biological replicates. (*, p < 0.05 in two-tailed tests compared to the corresponding WT values. Relative Ct values of two independent experiments with three biological replicates were combined for statistical analysis.)
(H) Seedlings were exposed to suspensions of Pst DC3000 and Pst DC3000 AvrRpm1 at 10^8 cfu/ml for 20 min, and then washed twice before further plant culturing in fresh
sterilized water. Seedlings harvested at the indicated times were subjected to immunoblot analysis with anti-BAK1 antibodies. Equal loading was verified with Ponceau S staining (bottom).