Danger peptide receptor signaling ensures plant basal immunity upon pathogen-induced depletion of BAK1

Kohji Yamada, Misuzu Yamashita-Yamada, Taishi Hirase, Tadashi Fujiwara, Kenichi Tsuda, Kei Hiruma and Yusuke Saijo

Corresponding author: Yusuke Saijo, Nara Institute of Science and Technology

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 May 2015

Thank you for submitting your manuscript entitled 'Danger peptide receptor signaling ensures plant basal immunity upon pathogen-induced depletion of BAK1'. I have now received reports of all referees, which are enclosed below.

As you will see, while the referees appreciate that Pep signaling plays an important role in the basal immune defense, they both think that your conclusions are not sufficiently supported by the data provided. I won't list all concerns here, as the reports, especially the one from referee #2, are clear and constructive. Importantly, the physiological significance derived from pathogen-induced downregulation of BAK1 is currently not convincing. Referee #2 furthermore delineates that the mechanism underlying the observed effects remains unclear, and that much further insight is needed for further consideration at The EMBO Journal.

However, given the interest into the topic and the constructive comments provided by the referees, I could offer to consider a revised version should you be able to substantiate your model along the lines suggested. Importantly, the data need to be conclusive, and further insight into the underlying mechanisms is needed. Referee #1 proposes to remove the data on BAK1 depletion upon infection, but this is in our view not an option. Rather, these data should be strengthened. A revision thus clearly demands a lot of work and time, as many of the experiments would have to be repeated and refined, and additional ones would have to be performed as well, with uncertain outcome. I can extend the revision time to 6 months maximum, should that be helpful.

Please note that a revised manuscript will be sent back to the original referees and that I would need
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strong support from them to consider publication here. Therefore, do consider your options carefully. If you see yourself in a position not to be able to address the concerns raised, then it is in your best interest to seek publication elsewhere at this stage.

I thank you in any case for the opportunity to consider your work for publication.

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REFEREE COMMENTS

Referee #1:

This manuscript presents a surprising new insight into a compensatory mechanism in Arabidopsis defense signaling. Specifically, the authors show that removal of the co-receptor kinase BAK1 (traditionally thought of as required for MAMP signaling) actually enhances signaling by endogenous "Danger Associated Molecular Patterns (DAMPs), even though BAK1 normally functions as a co-receptor in this signaling pathway. This enhancement of DAMP signaling is apparently due to substitution of BAK1 with a related kinase. Importantly, inactivation of BAK1 kinase activity does NOT enable this compensation to occur, presumably because the dead BAK1 still binds to the DAMP receptors (PEPR1 and PEPR2), blocking substitution. The manuscript presents a very large amount of data (19 figures!), and it can be a bit much to wade through, but the story is interesting and all of the data are relevant to the big picture. I have very few criticisms of the data (see minor points below), but I do take issue with some of the conclusions that the authors draw from these data. In particular, the authors attempt to strengthen the biological relevance of their data by showing that infection of Arabidopsis with Colletotrichum higginsianum leads to depletion of BAK1 levels. I do not find the immunoblot provided to support this claim (Fig. 8) very convincing, and furthermore, other data in this figure are inconsistent with their overall model. Specifically, based on their model, the bak1-4 mutation should enhance resistance to C.h. due to enhanced DAMP signaling, but they observe the opposite. My suggestion would be to drop Figure 8, and focus the discussion on the Pst data, which highlight the fact that no Pst effectors appear to eliminate BAK1, whereas other targets of Pst effectors do lead to target elimination (e.g. RIN4/AvrRpt2 and PBS1/AvrPphB), suggesting that there has been selection to maintain BAK1 protein, while inhibiting its function.

Minor points:
1) Some sentences are worded in a confusing manner. I suggest rewriting the following sentences to make their meaning clearer:

"Importantly, BAK1 disruption increases extracellular PROPEP3 release following pathogen effector-dependent generation, and renders PEPRs necessary for basal resistance."

"Of relevance, challenge with the fungal pathogen Colletotrichum higginsianum specifically reduces BAK1 accumulation, consistent with PEPR dependence of anti-fungal resistance."

"This response, known as basal resistance (Jones & Dangl, 2006), seems to occur without requiring the recognition of a specific pathogen effector, but is often enhanced by dysfunction of an immune layer(s) in a manner dependent on effectors"

"On the other hand, loss of single MAMP receptors increases host susceptibility to virulent pathogens of the whole effector assembly..."

"we discover that these three elements provide key regulatory layers in the production of the PROPEP proligands, a prerequisite for PEPR signaling activation."

2) The word 'tier' is misspelled as 'tire' throughout

3) 'under-phosphorylate' should be 'dephosphorylate'

4) On page 7, in the discussion about Figure 1A, I would not conclude that the kinase dead variant of bak1 suppressed Pep2 induced growth inhibition compared to WT. It looks just like WT.
5) On page 8, the statement "Consistent with this, Pep2, but not flg22, induced BIK1 phosphorylation and MAPK activation in bak1-KO plants (Figs 2A and EV1C)" is not supported by the data provided in Figure EV1C. In that figure, flg22 is clearly still activating MAPKs, albeit more weakly than Pep2.

6) Comparing Figures 3C and 5A, there is a huge difference in scales for fold induction of PR1, which makes it appear that PR1 is not induced in the bak1-4 mutant by Pep2 in Figure 5A, whereas it appears highly induced in Figure 3C. The authors should explicitly point out the difference in scales in these figures, and reason why the induction is so much higher in bon mutants (presumably much higher SA levels?)

7) In Figure 6A blot, is the lane labeled hrpS supposed to be labeled hrcC?

8) If Figure 8 is kept, I'd like to see some quantification of the signals for each band, averaged over the three replicates.

9) In the discussion, I do not understand the logic behind this statement:

"Given the differential requirements for an N-glycosylation-dependent ER quality control pathway between SOBIR1 and PEPR functions (Saijo, 2010; Sun et al, 2014; Tintor et al, 2013; Zhang et al, 2015), SOBIR1-dependent pathways are likely separate from (or, not required for) the PEPR pathway."

The differential requirement for the ER quality control pathway likely reflects different physical properties of the receptors themselves, not a different in their downstream signaling pathways.

Referee #2:

This manuscript provides interesting observations that loss of BAK1, an important co-receptor for immune receptor kinases, leads to increased sensitivity to Pep2, an endogenous defense-promoting peptide, and increased release of Propep protein upon pathogenic bacterial infection. Similarly, the autoimmune bon1 mutant also displays elevated sensitivity to Pep2. Loss of function bak1 and bon1 mutants are known to show increased basal resistance to bacterial and fungal pathogens. The authors show that this largely depends on Pep signaling. BAK1, BON1, and BIR1 are known to control cell death in Arabidopsis. The authors conclude that BAK1 and BON1 repress Propep release and propose that pathogen-induced depletion of BAK1 leads to increased Pep signaling, allowing plants maintain basal defense. Overall the findings that Pep signaling plays a significant role in basal defenses conferred by bak1 and bon1 mutants are novel, but the underlying mechanisms remain to be elucidated (as acknowledged by the authors). In addition, some of the conclusions are not fully warranted based on the data presented.

Major comments:
1. bak1 null mutants show increased Pep sensitivity. The authors show that this is associated with increased Propep production and secretion and PEPR stability in bak1 mutant. The bon1 mutant also shows increased Pep sensitivity. Another lesion-mimic mutant, lsd1, does not show this phenotype. The authors thus conclude that the control of Pep is specific to BAK1 and BON1, and cell death in bak1 and bon1 mutants results in Propep release. To this referee, it is not clear what causes the phenotype. It seems too early to assign BAK1 and BON1 as a specific mechanism here. What about other types of cell death? For example, activation of R proteins by defined Avr proteins leads to HR, does this similarly increase Pep sensitivity or release of Propep? While it is not known whether BAK1 is guarded by an R protein, the association of autoimmune phenotype with loss of BAK1 mutants but not kinase dead bak1 mutants is consistent with this possibility. The autoimmune phenotype in bon1 is known to be caused by R protein activation. Fig 6B shows a major role of type III system in Propep accumulation at late time points, and the strain carrying avrRpm1 seems to induce greater Propep accumulation. The authors suggest that this is caused by virulence effectors that may deplete BAK1. It is at least equally possible that this is caused by a weak recognition of some of the effectors by unknown R proteins. Also, ethylene is known to strongly induce pep
signaling. Does ethylene-induced senescence lead to Propep release? It would be helpful to define what really triggers Propep accumulation and release.

2. The authors suggest pathogen-induced depletion of BAK1 as a trigger of Pep signaling. However, the data presented do not appear to support this. Although AvrPtoB has been shown to cause BAK1 degradation, Pst challenge did not cause BAK1 depletion or Propep3 release (Fig E10 and page 14). In a time course study of Ch inoculation, only a single time point (5 dpi) showed slight reduction of BAK1 protein. At this late stage, it would be too late to mount an effective defense. It remains to be shown whether it is generally true that different pathogens induce BAK1 depletion. Thus the argument that Pep signaling is wired to sense BAK1 abundance is questionable.

Minor comments:
1. Page 6, "...loss of PEPRs substantially restored the dwarfism of bak1 bkk1 plants" should be rephrased "...loss of PEPRs substantially suppressed the dwarfism of bak1 bkk1 plants".
2. Fig 1D, amounts of proteins in the input is highly variable. A better co-IP experiment is desirable.
3. Fig 4B, the accumulation and release of Propep3 in bak1-4 plants seems to be increased only modestly. A quantitation of band intensity would be helpful.
4. Fig 4B, Anti-GFP immunoblots seem to show greater difference between WT and bak1-4 plants than do anti-Propep3 immunooblots. Please explain.
5. Please indicate in the figure legend (Fig 4B) what is NT (non-transgenic?).
6. Fig 4, different time points were used. 10 h for A, D, and E, 1-24 h used in C, and 24 and 48 h were used in B. It makes hard to draw a conclusion whether it is the amount of Propep or increased PEPR stability contributes to the increased Pep sensitivity.
7. Fig 6B, there appears to be more Propep accumulation in the avrRpm1 sample at 10 h time point. Is this real? If it is, this would be an indication that R gene activation is involved.
8. Fig 6B, the Pst-induced Propep accumulation was not affected in bak1-4 plants, which argue against the proposal that pathogen-induced depletion sensitize Pep signaling.
9. Fig 6B, Not all bacterial MAMPs are sensed by PRRs in a BAK1-dependent manner. It is rather surprising to see a major defect in delta hrcC-induced Propep3 induction in bak1-4 plants. Please comment.
10. Please clearly indicate in Fig 6B-C legends that whether plants were inoculated, with what pathogen.
11. Fig 6C, while there is no difference in Propep3 accumulation between WT and bak1-4, there is a huge amount of release in bak1-4 at 48 h after inoculation. What is the level of cell death in bak1-4 at this stage?
12. Fig 7C, flg22-induced SA accumulation not affected bak1-4 plants. This seems odd, as BAK1 is required for the majority of flg22-induced responses. Does flg22 induce cell death in bak1-4 plants?
13. Fig E1, the title says "...BAK1 and related SERK family members" but the figures only shows BAK1.
14. Fig E2, please add SERKs to title. There is no WT control in Fig E2C.
15. Fig E4, why the numbers are much smaller in B. Were the seedlings measured at same stage?
16. Fig E5, the three panels seem to be unrelated experiments.
17. FigE9A, the immunoblot is of poor quality. Please replace with a better experiment.

1st Revision - authors' response 11 September 2015

Point-by-point replies to the reviewers' concerns

Referee #1:

This manuscript presents a surprising new insight into a compensatory mechanism in Arabidopsis defense signaling. Specifically, the authors show that removal of the co-receptor kinase BAK1 (traditionally thought of as required for MAMP signaling) actually enhances signaling by endogenous "Danger Associated Molecular Patterns (DAMPS), even though BAK1 normally functions as a co-receptor in this signaling pathway. This enhancement of DAMP signaling is apparently due to substitution of BAK1 with a related kinase. Importantly, inactivation of BAK1 kinase activity does NOT enable this compensation to occur, presumably because the dead BAK1 still binds to the DAMP receptors (PEPR1 and PEPR2), blocking substitution. The manuscript presents a very large amount of data (19 figures!), and it can be a bit much to wade through, but the story is interesting and all of the data are relevant to the big picture. I have very few criticisms of
the data (see minor points below), but I do take
issue with some of the conclusions that the authors draw from these data. In particular, the authors
attempt to strengthen the biological relevance of their data by showing that infection of Arabidopsis
with Colletotrichum higginsianum leads to depletion of BAK1 levels. I do not find the immunoblot
provided to support this claim (Fig. 8) very convincing, and furthermore, other data in this figure
are inconsistent with their overall model. Specifically, based on their model, the bak1-4 mutation
should enhance resistance to C.h. due to enhanced DAMP signaling, but they observe the opposite.
My suggestion would be to drop Figure 8, and focus the discussion on the Pst data, which highlight
the fact that no Pst effectors appear to eliminate BAK1, whereas other targets of Pst effectors do
lead to target elimination (e.g. RIN4/AvrRpt2 and PBS1/AvrPphB), suggesting that there has been
selection to maintain BAK1 protein, while inhibiting its function.

Our reply:
Thank you very much for the positive comments. In this revised manuscript, we have retained Fig 8
at the editor’s request. We present a new immunoblot in Fig. 8B, which very clearly shows a
decrease in BAK1 accumulation during Ch infection. Our data suggest that loss of BAK1 leads to
enhanced DAMP signaling via the PEPR pathway, which is critical for retaining basal resistance
despite MTI signaling defects in the absence of BAK1. However, this does not necessarily lead to
enhanced pathogen resistance in the bak1-4 background, as shown with flg22-induced bacterial resistance (Fig. 7B). We infer from these data that the overall basal resistance in bak1-KO plants
reflects the sum of reduced MAMP signaling (dependent on BAK1) and enhanced PEPR-DAMP
signaling. To clarify this point in the Arabidopsis-Ch interaction, we have added new data in the
revised Fig 8A (right) to show a critical role for BAK1 in the initial resistance to Ch invasion. This
indicates that BAK1 contributes to Ch resistance at an early phase (possibly MTI) in a manner
independent of PEPRs. However, PEPRs become critical in Ch resistance at a late phase, apparently
in association with BAK1 depletion during Ch infection. We have also added new text in the revised
discussion following your comments on the Pst effectors and target elimination, and reduced the
total number of figures by two in the revised manuscript.

Minor points:
1) Some sentences are worded in a confusing manner. I suggest rewriting the following sentences to
make their meaning clearer:

"Importantly, BAK1 disruption increases extracellular PROPEP3 release following pathogen
effector-dependent generation, and renders PEPRs necessary for basal resistance."

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"This response, known as basal resistance (Jones & Dangl, 2006), seems to occur without requiring
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"we discover that these three elements provide key regulatory layers in the production of the
PROPEP proligands, a prerequisite for PEPR signaling activation."

2) The word 'tier' is misspelled as 'tire' throughout
3) 'under-phosphorylate' should be 'dephosphorylate'

Our reply:
We have revised the wording to clarify the meaning of these sentences, and made the two
corrections.

4) On page 7, in the discussion about Figure 1A, I would not conclude that the kinase dead variant
of bak1 suppressed Pep2 induced growth inhibition compared to WT. It looks just like WT.

Our reply:
We have added the data for WT plants, which were obtained side by side with the other plants, in the right panel of Fig 1A in the revised manuscript. Our statistical analysis supports our conclusion that the Pep2-induced root growth inhibition in WT plants was suppressed in the presence of the kinase-dead variant of BAK1.

5) On page 8, the statement "Consistent with this, Pep2, but not flg22, induced BIK1 phosphorylation and MAPK activation in bak1-KO plants (Figs 2A and EV1C)" is not supported by the data provided in Figure EV1C. In that figure, flg22 is clearly still activating MAPKs, albeit more weakly than Pep2.

Our reply:
We have revised the text to make it clear that BIK1 phosphorylation and MAPK activation in response to Pep2 were less affected by bak1-4 mutation than those in response to flg22.

6) Comparing Figures 3C and 5A, there is a huge difference in scales for fold induction of PR1, which makes it appear that PR1 is not induced in the bak1-4 mutant by Pep2 in Figure 5A, whereas it appears highly induced in Figure 3C. The authors should explicitly point out the difference in scales in these figures, and reason why the induction is so much higher in bon mutants (presumably much higher SA levels?)

Our reply:
As you point out, Pep2-induced PR1 induction was much higher in bon mutant plants than in bak1-4 plants. We have stated this clearly and noted the difference in scales between Figs 3C and 5A in the revised text. However, in Fig 5A, we still detected a significant increase of PR1 induction in bak1-4 plants as compared to WT plants. Unlike bon mutants, bak1-KO plants do not constitutively display immune activation. Constitutive SA-related defense responses in non-elicited bon plants (Yang et al., Plant J., 2006) seem to facilitate massive PR1 expression following Pep2 application. We have revised the text accordingly.

7) In Figure 6A blot, is the lane labeled hrpS supposed to be labeled hrcC?

Our reply:
This figure has been removed. PROPEP3-Venus induction at early and late phases of Pst challenge is essentially shown in Figs 6B and 6E in the revised manuscript.

8) If Figure 8 is kept, I'd like to see some quantification of the signals for each band, averaged over the three replicates.

Our reply:
For this revision, we repeated this experiment three times more, and we present representative immunoblots in the revised Fig 8B (left), with the band intensities quantified.

9) In the discussion, I do not understand the logic behind this statement:

"Given the differential requirements for an N-glycosylation-dependent ER quality control pathway between SOBIR1 and PEPR functions (Saijo, 2010; Sun et al, 2014; Tintor et al, 2013; Zhang et al, 2015), SOBIR1-dependent pathways are likely separate from (or, not required for) the PEPR pathway."

The differential requirement for the ER quality control pathway likely reflects different physical properties of the receptors themselves, not a different in their downstream signaling pathways.

Our reply:
In the revised text, we state: “Previous studies showed 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.”

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Referee #2:

This manuscript provides interesting observations that loss of BAK1, an important co-receptor for immune receptor kinases, leads to increased sensitivity to Pep2, an endogenous defense-promoting peptide, and increased release of PROPEP3 protein upon pathogenic bacterial infection. Similarly, the autoimmune bon1 mutant also displays elevated sensitivity to Pep2. Loss of function bak1 and bon1 mutants are known to show increased basal resistance to bacterial and fungal pathogens. The authors show that this largely depends on Pep signaling. BAK1, BON1, and BIR1 are known to control cell death in Arabidopsis. The authors conclude that BAK1 and BON1 repress PROPEP3 release and propose that pathogen-induced depletion of BAK1 leads to increased Pep signaling, allowing plants maintain basal defense. Overall the findings that Pep signaling plays a significant role in basal defenses conferred by bak1 and bon1 mutants are novel, but the underlying mechanisms remain to be elucidated (as acknowledged by the authors). In addition, some of the conclusions are not fully warranted based on the data presented.

Major comments:
1. bak1 null mutants show increased Pep sensitivity. The authors show that this is associated with increased PROPEP3 production and secretion and PEPR stability in bak1 mutant. The bon1 mutant also shows increased Pep sensitivity. Another lesion-mimic mutant, lsd1, does not show this phenotype. The authors thus conclude that the control of Pep is specific to BAK1 and BON1, and cell death in bak1 and bon1 mutants results in PROPEP3 release. To this referee, it is not clear what causes the phenotype. It seems too early to assign BAK1 and BON1 as a specific mechanism here. What about other types of cell death? For example, activation of R proteins by defined Avr proteins leads to HR, does this similarly increase Pep sensitivity or release of PROPEP3? While it is not known whether BAK1 is guarded by an R protein, the association of autoimmune phenotype with loss of BAK1 mutants but not kinase dead bak1 mutants is consistent with this possibility. The autoimmune phenotype in bon1 is known to be caused by R protein activation. Fig 6B shows a major role of type III system in PROPEP3 accumulation at late time points, and the strain carrying avrRpm1 seems to induce greater PROPEP3 accumulation. The authors suggest that this is caused by virulence effectors that may deplete BAK1. It is at least equally possible that this is caused by a weak recognition of some of the effectors by unknown R proteins. Also, ethylene is known to strongly induce pep signaling. Does ethylene-induced senescence lead to PROPEP3 release? It would be helpful to define what really triggers PROPEP3 accumulation and release.

Our reply:
First of all, we do not intend to claim that BAK1 depletion is the only trigger for the sensitization of PEPR-mediated resistance or PROPEP3 release. WT-like Pep sensitivity in lsd1 plants and increased Pep sensitivity in bak1-KO or bon mutants points to a specific correlation of Pep sensitization with bak1 and bon mutants. These data show that not all types of cell death in different “enhanced cell death” mutants are linked to Pep sensitization. We emphasize that this claim does not exclude the existence of another trigger for PROPEP3 release. Following your suggestion, in the revised Figs 6D-F we present new data that PROPEP3 release occurs during RPM1-mediated ETI, in association with massive cell death. The lack of significant BAK1 depletion (Fig EV8) strengthens the notion stated above. Due to HR cell death and the lack of a PEPR-specific defense marker, we are not able to test whether Pep sensitivity is enhanced by ETI. Nevertheless, the observed PROPEP3 release following Pst AvrRpm1 inoculation is consistent with the previously described role of PEPRs in systemic acquired resistance (Ross et al., 2014), pointing to active engagement of the PEPR pathway following ETI.

Unexpectedly, our new experiments led to the detection of a small form of PROPEP3-Venus in the extracellular fraction following inoculation with Pst DC3000 and Pst DC3000 AvrRpm1. By contrast, the apparently full-length form predominantly accumulated in the extracellular fraction following Pep2 or Pep1 application. We infer from these results that PROPEP3-Venus release occurs without processing, but that a processed form of PROPEP3 predominantly accumulates in the extracellular space during ETI or basal resistance to the bacteria. The precise sequences and role of this small PROPEP3 form remain to be determined. We do not know how to reconcile the apparent discrepancies regarding bacterium-induced release of PROPEP3-Venus between the first and revised manuscripts. The previous and present data were obtained before and after the transition of our lab from MPIPZ, Germany to NAIIST, Japan. The first author also independently reproduced the present data at another lab in Kyoto University, Japan. Our results might be affected by
unrecognized differences in the experimental conditions. We are convinced of the new data in the revised manuscript, which have been reproduced 5 times under our current conditions. Future studies will be required to resolve these open questions. Nevertheless, our new results demonstrate that PROPEP3 release occurs during (or after) challenge with virulent and avirulent Pst strains, even when BAK1 accumulation is unaffected. In the revised text, we clearly state that BAK1 depletion is not essential for, but positively influences, PEPR-mediated resistance and PROPEP3 release.

Although we agree that studies on PROPEP3 release in other biological contexts, such as ET-induced senescence, are interesting, they will require another large volume of work to allow us to make a clear conclusion in another experimental setting. We would therefore like to explore these possibilities in future studies.

2. The authors suggest pathogen-induced depletion of BAK1 as a trigger of Pep signaling. However, the data presented do not appear to support this. Although AvrPtoB has been shown to cause BAK1 degradation, Pst challenge did not cause BAK1 depletion or Propep3 release (Fig E10 and page 14). In a time course study of Ch inoculation, only a single time point (5 dpi) showed slight reduction of BAK1 protein. At this late stage, it would be too late to mount an effective defense. It remains to be shown whether it is generally true that different pathogens induce BAK1 depletion. Thus the argument that Pep signaling is wired to sense BAK1 abundance is questionable.

Our reply:
As stated above, our conclusion is that BAK1 depletion is not the only trigger for Pep sensitization and PROPEP3 release. Our additional experiments have revealed that AvrRpm1-triggered ETI also leads to PROPEP3 release without significant BAK1 depletion. Nevertheless, our data argue for a positive role of BAK1 depletion in Pep sensitization (for cell death and resistance) and PROPEP3 release. When BAK1 accumulation is significantly reduced, as seen with bak1-KO mutant plants or with Ch-challenged plants (see the revised Fig 8B, showing lowered BAK1 accumulation at 4 and 5 dpi with Ch), we detected an increase of PEPR-mediated cell death and/or defenses. As for Pst-challenged plants, we wonder if the referee’s comments may over-simplify the published data. Although AvrPtoB was shown to have BAK1 ubiquitination activity in vitro (Gohre et al., Current Biology, 2008), whether BAK1 ubiquitination leads to its degradation remains unclear. In addition to our work, a previous study by another group failed to detect a significant alteration in BAK1 steady-state levels during Pst DC3000 challenge (Nicaise et al., EMBO J., 2012). The lack of substantial BAK1 depletion and the retention of basal bacterial resistance in pepr1 pepr2 BAK1 (+) plants support our conclusion that BAK1 depletion is closely associated with active engagement of the PEPR pathway.

However, we have finally detected PROPEP3 release during Pst DC3000 challenge in WT plants, albeit more weakly than in bak1-4 plants (Fig 6C). It remains to be determined whether this PROPEP3 release is dependent on AvrPtoB. Nevertheless, this indicates that PROPEP3 release occurs without substantial BAK1 depletion, and also that it is increased in the absence of BAK1. Taking these considerations together, we clearly state in the revised manuscript that BAK1 depletion is not essential for, but stimulates, PROPEP3 release.

As for the role of BAK1 depletion in PEPR-mediated Ch resistance, we show in the revised Fig 8A that BAK1 plays a critical role in Ch invasion resistance at an early phase (3 dpi) before BAK1 depletion becomes apparent, and that PEPRs are not required for this early resistance. However, as shown in Fig 8B, PEPRs are required to restrict Ch growth at a later phase (5 dpi) in BAK1 (+) plants as well as in bak1-KO plants. These results again point to a close association between Ch-induced BAK1 depletion and PEPR-dependent fungal resistance.

Determining whether different pathogens induce BAK1 depletion will require further studies, and is not within the scope of this manuscript.

Minor comments:
1. Page 6, "...loss of PEPRs substantially restored the dwarfism of bak1 bkk1 plants" should be rephrased "...loss of PEPRs substantially suppressed the dwarfism of bak1 bkk1 plants".
Our reply:

We have corrected this phrase.

2. Fig 1D, amounts of proteins in the input is highly variable. A better co-IP experiment is desirable.

Our reply:

In practical terms, it is very challenging to equalize the input protein levels among different samples in transient gene expression systems. Importantly, the ratio between the bait PEPR1 levels and the co-precipitated SERK member levels points to our conclusion.

3. Fig 4B, the accumulation and release of Propep3 in bak1-4 plants seems to be increased only modestly. A quantitation of band intensity would be helpful.

Our reply:

We present the relative intensities of the PROPEP3-Venus band in WT and bak1-4 plants in the revised Fig 4B.

4. Fig 4B, Anti-GFP immunoblots seem to show greater difference between WT and bak1-4 plants than do anti-Propep3 immunoblots. Please explain.

Our reply:

What caused the apparent differences in the immunoblots between the two antibodies remains unclear. Anti-PROPEP3 antibodies are more sensitive than anti-GFP antibodies under our conditions. The signal obtained with anti-PROPEP3 may thus be partially saturated in bak1-4 plants. Given the retention of the signal size, it is very unlikely that our analyses were complicated by truncation of PROPEP3-Venus. Importantly, both results point to enhanced production and release of PROPEP3 in bak1-4 plants.

5. Please indicate in the figure legend (Fig 4B) what is NT (non-transgenic?).

Our reply:

NT represents non-transgenic plants, as indicated in the revised manuscript.

6. Fig 4, different time points were used. 10 h for A, D, and E, 1-24 h used in C, and 24 and 48 h were used in B. It makes hard to draw a conclusion whether it is the amount of Propep or increased PEPR stability contributes to the increased Pep sensitivity.

Our reply:

We chose these different time points because they are optimal for showing the changes in PROPEP3/2 induction (at 10 h, A), PROPEP3-Venus production and release (at 24 and 48 h, B), and PEPR steady-state levels (at 5 and 10 h in C and D, at 10 h in E) in response to Pep2. These data point to a close correlation between the proligand and receptor levels and Pep sensitivity in bak1-KO plants. We infer from these data that the increases in the proligand and receptor levels and reprogramming of PEPR signaling downstream of the receptor collectively contribute to the increased Pep sensitivity. Future studies will be required to unambiguously determine whether and the extent to which these individual elements contribute to the overall Pep sensitization.

7. Fig 6B, there appears to be more Propep accumulation in the avrRpm1 sample at 10 h time point. Is this real? If it is, this would be an indication that R gene activation is involved.

Our reply:

Although there does appear to be an increase in PROPEP3-Venus accumulation at 10 h after Pst AvrRpm1 inoculation, our quantification revealed that there was not. In the revised manuscript, we have replaced this figure with new Figs 6E and F and discussed only differences in the band patterns, which may reflect processing of the proligand.

8. Fig 6B, the Pst-induced Propep accumulation was not affected in bak1-4 plants, which argue against the proposal that pathogen-induced depletion sensitize Pep signaling.

Our reply:
Our results suggest not that proligand production (Fig 6B) but that proligand release (revised Fig 6C) is enhanced in bak1-4 plants during Pst DC3000 challenge; this release likely contributes to PEPR signaling sensitization.

However, PROPEP3 induction per se can also be seen as an output of PEPR signaling. Although Pep-induced PROPEP3 accumulation was enhanced in bak1-4 plants (Fig 4B), Pst-induced PROPEP3 accumulation was not enhanced (Fig 6B). We show in the revised manuscript that PEPRs are not required for PROPEP3 induction following bacterial challenge (Fig EV6D). Thus, PROPEP3 induction is not specifically attributable to PEPR signaling triggered upon Pst challenge. It seems likely that PEPR-independent proligand generation confers robustness to the PEPR system, which may enable non-cell-autonomous PEPR signaling in response to the ligands released from damaged/infected cells.

9. Fig 6B, Not all bacterial MAMPs are sensed by PRRs in a BAK1-dependent manner. It is rather surprising to see a major defect in delta hrcC-induced Propep3 induction in bak1-4 plants. Please comment.

Our reply:
Although different MAMPs are able to induce stomatal closure, it was shown that FLS2 predominantly contributes to the stomatal response during Pst challenge (Zeng and He, Plant Physiol., 2010). Likewise, it is conceivable that FLS2 or any other BAK1-dependent PRR(s) predominantly contributes to MAMP-induced PROPEP3 production during Pst challenge. We have incorporated this interpretation into the revised text.

10. Please clearly indicate in Fig 6B-C legends that whether plants were inoculated, with what pathogen.

Our reply:
We have done this.

11. Fig 6C, while there is no difference in Propep3 accumulation between WT and bak1-4, there is a huge amount of release in bak1-4 at 48 h after inoculation. What is the level of cell death in bak1-4 at this stage?

Our reply:
As shown in the revised Fig 6D, trypan blue staining revealed an increase in the stained regions in bak1-4 plants as compared to WT plants at 48 hpi with Pst DC3000, pointing to a positive role of cell death in the extracellular release of PROPEP3-Venus. We present a new immunoblot in the revised Fig 6C, showing increased release of the small PROPEP3-Venus form in bak1-4 plants compared to WT plants. Under our new conditions (after our lab transition, as noted above), we no longer observe such huge differences in the PROPEP3-Venus release levels between bak1-4 plants and WT plants. Nevertheless, our new results point to the same conclusion that PROPEP3 release following Pst DC3000 challenge is enhanced in bak1-4 plants.

12. Fig 7C, flg22-induced SA accumulation not affected bak1-4 plants. This seems odd, as BAK1 is required for the majority of flg22-induced responses. Does flg22 induce cell death in bak1-4 plants?

Our reply:
As Referee #1 pointed out, flg22-induced responses, including MAPK activation and BIK1 phosphorylation, were significantly reduced but not fully suppressed in bak1-4 plants. Our genetic evidence reveals that PEPRs are required for maximal SA accumulation and bacterial resistance of bak1-4 plants in response to flg22. We infer from these results that once the PEPR pathway is activated following the residual signal emanating from FLS2, PEPR-mediated signaling (sensitized toward SA-related defenses) leads to SA accumulation and basal resistance. By contrast, our data show that PEPRs are not required for flg22-induced SA accumulation or bacterial resistance in the presence of BAK1. Loss of BAK1 seems to render the PEPR pathway necessary for flg22-induced SA accumulation and resistance.
Our trypan blue staining did not detect cell death in flg22-treated plants (Fig EV3A). It is unclear whether cell death (below the detection limit) is involved in PEPR signaling activation in bak1-4 plants in response to flg22. Future studies will be required to elucidate the mechanism that links FLS2-triggered signaling to the PEPR pathway in the absence of BAK1.

13. Fig E1, the title says "...BAK1 and related SERK family members" but the figures only shows BAK1.

Our reply:
We have corrected the title.

14. Fig E2, please add SERKs to title. There is no WT control in Fig E2C.

Our reply:
In Fig EV2C, we compared Pep-triggered root growth inhibition between bak1-4 plants and the tested serk mutant plants. The name of "serk" has been in the figure title. Could this be fine?

15. Fig E4, why the numbers are much smaller in B. Were the seedlings measured at same stage?

Our reply:
The seedlings were measured at the same stage and the same time in Figs E4A and B, as these experiments were carried out in parallel. The differences may be attributable to differences in the transgenic backgrounds between the two mutant groups.

16. Fig E5, the three panels seem to be unrelated experiments.

Our reply:
To trace PEPR1 accumulation under the tested conditions in Figs 4C-E, we used the PEPR1-FLAG plants. In the three panels of Fig EV5, we present supplementary and related information regarding the PEPR1-FLAG lines used, to support our conclusions obtained in Figs 4C-E.

17. FigE9A, the immunoblot is of poor quality. Please replace with a better experiment.

Our reply:
We have done so.

2nd Editorial Decision 07 October 2015

Thank you for submitting your revised manuscript for our consideration. Your manuscript has now been seen once more by the original referees (see comments below), and I am happy to inform you that they are both broadly in favor of publication, pending satisfactory minor revision.

I would therefore like to ask you to address referee #1’s remaining concern and to tone down the claims in the abstract to address referee #2’s points. To address referee #2’s concerns I think it suffices to delete the ‘pathogen-induced depletion of’ in line 6 in your abstract. I think the title may remain as is though.

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we should have received the revised version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

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REFEREE REPORTS

Referee #1:

The authors have largely addressed the issues I raised in my first review. I appreciate the addition of
quantitative data for the various immunoblots. The authors should describe in their materials and methods how this quantification was performed. One other minor point is that I remain confused as to why the bak1 null mutation does not have a larger effect on flg22 signaling (Figure 7). How do these findings compare to prior work on bak1 null mutants? Are the authors 100% confident that they have genotyped these plants correctly?

Referee #2:

The authors have done a very good job in revising the manuscript to address reviewers' comments. The findings that the BAK1 depletion is linked to release and sensitivity to Pep are significant as they support that BAK1 is monitored by the PEPR DAMP signaling pathway. The authors have provided better data to show that Ch infection indeed leads to BAK1 depletion at 4-5 days post inoculation. However, the infection rate and lesion size were measured at 3 days post inoculation. More over, it is not determined whether the depletion at 4-5 days post Ch inoculation co-incites with increased release of Pep. The vast majority of experiments were conducted with Pst, which does not appear to cause depletion in BAK1. Thus, the "pathogen-induced depletion" of BAK1 remains under-developed in the current work. While it is ok to discuss the biological significance of BAK1 depletion-enhanced Pep release and sensitivity, it is not appropriate to state that in the title and abstract.

2nd Revision - authors’ response

Point-by-point replies to the reviewers’ concerns

The Editor

Your manuscript has now been seen once more by the original referees (see comments below), and I am happy to inform you that they are both broadly in favor of publication, pending satisfactory minor revision.

I would therefore like to ask you to address referee #1’s remaining concern and to tone down the claims in the abstract to address referee #2’s points. To address referee #2’s concerns I think it suffices to delete the 'pathogen-induced depletion of' in line 6 in your abstract. I think the title may remain as is though.

Our reply: Thank you very much for your positive evaluation of our manuscript for publication. Having depleted ‘pathogen-induced’ in Line 6, we state in the revised abstract ‘We report that BAK1 depletion is linked to ---’.

Referee #1: The authors have largely addressed the issues I raised in my first review. I appreciate the addition of quantitative data for the various immunoblots. The authors should describe in their materials and methods how this quantification was performed. One other minor point is that I remain confused as to why the bak1 null mutation does not have a larger effect on flg22 signaling (Figure 7). How do these findings compare to prior work on bak1 null mutants? Are the authors 100% confident that they have genotyped these plants correctly?

Our reply: We have described our quantification procedure in the revised manuscript.

In bak1 null mutant plants, as you noted in your first review comments, flg22-induced responses, including MAPK activation and BIK1 phosphorylation, were significantly reduced but not fully suppressed. Moreover, in the presence of a hypoactive bak1-5 allele, flg22-induced outputs were previously shown to be lower than in a null bak1-4 allele (Schwessinger et al, 2011). These data point to the partial retention of FLS2 signaling (at least for these outputs) in the absence of BAK1. Consistent with this, a previous coIP analysis detected flg22-induced association of FLS2 with the
other SERK members, albeit more weakly than with BAK1 (Roux et al, 2011). In the present study, our genetic evidence reveals that PEPRs are required for the retention of flg22 signaling in bak1 null mutants. We infer from these findings that the residual flux of FLS2 signaling is sufficient to engage PEPR signaling in bak1 null mutants, which in turn retains SA production, PR-1 activation and basal resistance, as shown in Figure 7.

We are 100% confident of the genotype for the plants used.

Referee #2:

*The authors have done a very good job in revising the manuscript to address reviewers' comments. The findings that the BAK1 depletion is linked to release and sensitivity to Pep are significant as they support that BAK1 is monitored by the PEPR DAMP signaling pathway. The authors have provided better data to show that Ch infection indeed leads to BAK1 depletion at 4-5 days post inoculation. However, the infection rate and lesion size were measured at 3 days post inoculation.*

**Our reply:**
We wonder if you might have misunderstood our data. In Figure 8A left and right, shown are the lesion size and invasion rate of Ch that were determined at 5 dpi and 3 dpi, respectively. Our data indicate that pepr1 pepr2 and WT plants were indistinguishable in Ch resistance at the early phase, but pepr1 pepr2 plants became more susceptible than WT plants at the late phase, at which BAK1 depletion was apparent. To avoid confusion, we have clearly stated in the revised text that the lesion size was determined at 5 dpi.

*More over, it is not determined whether the depletion at 4-5 days post Ch inoculation co-incites with increased release of Pep. The vast majority of experiments were conducted with Pst, which does not appear to cause depletion in BAK1. Thus, the "pathoge-induced depletion" of BAK1 remains underdeveloped in the current work. While it is ok to discuss the biological significance of BAK1 depletion-enhanced Pep release and sensitivity, it is not appropriate to state that in the title and abstract.*

**Our reply:**
We agree that we have not determined whether BAK1 depletion leads to increased release of PROPEP3 during Ch resistance. We have thus followed your comments and the editor’s recommendation, and revised the abstract as stated above.