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The Arabidopsis CERK1-associated kinase PBL27 connects chitin perception to MAPK activation

Kenta Yamada, Koji Yamaguchi, Tomomi Shirakawa, Hirofumi Nakagami, Akira Mine, Kazuya Ishikawa, Masayuki Fujiwara, Mari Narusaka, Yoshihiro Narusaka, Kazuya ichimura, Yuka Kobayashi, Hidenori Matsui, Yuko Nomura, Mika Nomoto, Yasuomi Tada, Yoichiro Fukao, Tamo Fukamizo, Kenichi Tsuda, Ken Shirasu, Naoto Shibuya and Tsutomu Kawasaki

Corresponding author: Tsutomu Kawasaki, Kindai University

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(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.)

Pre-Decision Consultation 05 April 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please find enclosed the comments of three reviewers whom we had asked to evaluate the manuscript.

As you will see, while the referees consider that your study is potentially interesting, referees 2 and 3 conclude that the work is too preliminary at this stage. More data are required to support the proposed pathway. Referee 1 is more positive, but also raises concerns that overlap with the issues noted by referee 2 and 3.

Therefore, before taking a decision, I think it would be most productive if you could provide me upfront with a point-by-point response to the raised criticisms in order to see how you would address them.

Please send me the point-by-point draft by reply email - I am looking forward to receiving it before making a formal editorial decision. Thank you very much.

REFEREE REPORTS

Referee #1

(Report for Author)
Summary
Deciphering how plant PRR complexes activate downstream substrates to initiate distinct immune responses in response to MAMP perception is critical to our understanding of plant innate immunity. This manuscript reveals a direct link between Arabidopsis PBL27 and MAPKKK5 that is required to activate MAPK cascades in response to chitin. The authors show that PBL27 interacts with the C-terminal domain of MAPKKK5 in Y2H assays, and with the full-length protein in vivo. Furthermore, they show that PBL27 phosphorylates MAPKKK5 C-terminus in vitro, as well as its kinase domain in the presence of the chitin receptor CERK1. In turn, MAPKKK5 phosphorylates MKK4/5 in vitro. Consistently, mapkkk5 mutants were impaired in chitin-triggered MAPK activation, and subsequently in transcription reprograming, callose deposition and resistance against a pathogenic fungus.

The findings in this manuscript are very interesting, and the quality and amount of data are impressive. There are however a few points were I think improvements could be made in order to substantiate the authors' claims.

Major points

1. To test the specificity of the proposed pathway, the authors should test whether MAPKKK5 is required for MAPK activation in response to flg22 and/or elf18.

2. Fig 5E: a control must be included to show that the observed dissociation between PBL27 and MAPKKK5 is not simply due to the activated immune state of plant cells. Thus, the authors should test whether flg22 or elf18 would similarly (or not) lead to such dissociation

3. One of the major points of this manuscript is the ability of PBL27 to phosphorylate MAPKKK5 and activate MPKs in response to chitin. Accordingly, the authors propose that PBL27 is able to phosphorylate several residues in the C-terminus of MAPKKK5 in vitro (Fig. 6). However, whether this action is specific to PBL27 or just results from in vitro phosphorylation of MAPKKK5 remains to be determined given that it is impossible to judge from the data presented in Fig. 6B whether these residues are phosphorylated in a PBL27-dependent manner. I suggest the authors test side-by-side whether BIK1 can also phosphorylate MAPKK5 in vitro. This would be an adequate control since BIK1 is not required for chitin-induced MPK activation and does not interact with MAPKKK5 in vivo.

4. The authors propose a possible phospho-signaling pathway consisting of CERK1-PBL27-MAPKKK5-MKK4/MKK5-MPK3/6. A nice link between MAPKKK5 is provided with in vitro phosphorylation assays. However, no data supporting the interaction between MAPKKK5 and MKK4/5 is provided in vivo. Testing this by co-IP in Nb or protoplasts, or by BiFC would greatly support their model.

Minor points

5. In the text, the authors mention that 319 genes show reduced induction or suppression in mapkkk5 compared to WT, whereas the legend of Sup Fig 11 states "Genes showing reduced induction in mapkkk5 compared to wild type were selected (319 genes)". This is confusing and not accurate. Please correct accordingly.

6. In order to determine MAPKKK5 subcellular localization, the authors isolated soluble and microsomal protein fractions, and observed the localization of MAPKKK5-GFP transiently expressed in N. benthamiana under the microscope. The authors conclude that MAPKKK5 is both cytosolic and PM-associated. Although this is most likely the case (especially taking into account

the later BiFC experiments), there are some issues with both experiments that prevent the reader from confidently reaching the same conclusion:

a) In Fig 3C no control for a known PM-localized protein is provided. A negative control is provided with GFP but the signal is so low in total and soluble fractions that it is difficult to conclude about its true absence in the microsomal fraction. In Sup Fig 13, a control is provided by using anti-EAP1; however, no reference or further experiments are provided for its validity as a soluble/microsomal marker protein. I suggest the authors repeat this experiment (for both MAPKKK5 and K375M) including a known PM-localized protein, for example. Also, a Ponceau staining of the membrane should be provided in this case.

b) From the images presented in Fig 3D it is difficult to conclude whether MAPKKK5 is associated with the PM. It would be nice if the authors could show a zoomed in section and perform plasmolysis and/or co-localization with a known PM-localized protein.

7. pMAPKKK5::MAPKKK5_6xA-FLAG transgenic plants are used to assess the role of MKKK5 phosphorylation in MPK activation. However, expression of MAPKKK5_6xA-FLAG could not be detected in these plants. The authors honestly refer to this in the text and provide additional experiments with the same construct transiently expressed in protoplasts and Nb leaves, suggesting that MAPKKK5_6xA-FLAG still accumulates and has a similar localization to the WT protein. While I value this effort, this does not show that the transgenic pMAPKKK5::MAPKKK5_6xA-FLAG plants are actually expressing MAPKKK5. I propose to the authors to perform an anti-FLAG IP on these lines, which should enrich MAPKKK5_6xA-FLAG to detectable levels. If expression cannot still be addressed maybe an RT-PCR could be included to show that MAPKKK5_6xA-FLAG is at least expressed at the mRNA level.

Alternatively, the authors could test whether MAPK activation is affected in mapkkk5 protoplasts transfected with MAPKKK5/MAPKKK5_6xA.

8. The authors show in Fig 2 that mkkk5 mutants are compromised in certain immune outputs (callose deposition, transcription reprogramming, and immunity against Alternaria). However, chitin-induced ROS burst is not affected in mkkk5 mutants (Sup Fig 10). This is a very interesting result that further supports the previous notion that MPK activation and ROS production are independent events. In my opinion, Sup Fig10 should be incorporated in Fig 2 in order to highlight this point. Similarly, Sup Fig 12 should have its place in the main text.
In turn, many other main figures could be easily switched to the supplemental data without great loss to the read. This is the case for Figs 3, 4A and 5A, which display important observations but are not essential for the main conclusions.

9. The MS spectra relative to Fig 6B should be provided as supplementary data.

10. I would suggest amending the title to "The Arabidopsis CERK1-associated kinase PBL27 connects chitin perception to MAPK activation".

11. p 4, line 21: Nuhse et al., 2007 and Zhang et al., 2007 are not the most appropriate references for this sentence. As done previously in the Introduction, rather cite a comprehensive review.

12. p 5, lines 5-16: move this paragraph before mentioning that it is currently unclear how MAPKs are activated downstream of FLS2 and EFR.

13. p 5, line 28: "These results suggested that OsRLCK185...". Also mention OsRLCK176.

14. p 13, line 31: Ao et al., 2014 is not an appropriate reference here.

15. p 14, line 4: it is not correct to state that it is unknown which RLCK(s) connect CERK1-mediated chitin perception to RBOHD activity, as it was shown previously that BIK1 and BIK1-mediated phosphorylation of RBOHD are required for chitin-induced ROS production (Zhang et al., 2010; Kadota et al., 2014).

Referee #2

(Report for Author)
General comment.
The manuscript of Yamada et al. addresses the chitin-dependent activation of MAPKKK5 via the RLCK PBL27. It demonstrates that mpkkk5 mutants are impaired in MAPK activation after treatment with chitin. PBL27 can phosphorylate MAPKKK5 at the plasma membrane, and this phosphorylation is dependent on PBL27 phosphorylation by the CERK1 chitin pattern recognition receptor (PRR). As such a direct molecular link between plant PRRs and MAPK activation has not been described before, the manuscript is certainly of interest to plant and animal researchers. Since MAPKKK5 is very lowly expressed in vivo, the manuscript focuses primarily on in vitro approaches whose interpretation requires some caution.

Main Issues
- Figure 2: 339 genes: it would be nice to have a list of genes different in the mkkk5 mutant.
- Figs. 3A & 4E: The tomato MAPKKK SlMKKKα also causes cell death when overexpressed in N. Benthamiana (del Pozo et al., 2004 EMBO J.). Could the authors comment on how this might relate to MAPKKK5? For example, is it possible that MKKK5 levels are homeostatically limited as the protein may be a positive regulator of cell death? In that case, to study the protein in Arabidopsis maybe the authors could try to make the lines in an eds1 or ndr1 background, in case the cause of the cell death is some R protein mediated pathway (reviewed in Rodriguez et al. 2015 FEBS J). Another suggestion would be to check the level of PBL27 in the mkkk5 mutant background (mkkk5/PBL27-HA).
- Fig. 3C: MAPKKK5 is more abundant in the soluble fraction than in the microsomal fraction. Could the authors clarify why they focus on the microsomal fraction for the co-IP in Fig. 4 instead of the soluble fraction? The authors do not comment much on the possible reason for membrane localization of the MKKK5.
- Fig. 3D: the authors show the localization of wild-type MAPKKK5 and see signal in the cytosol and plasma membrane, but no plasmolysis is done to support the claim of membrane localization (while this is done in Fig. 4D for the mutant MKKK5).
- Fig. 4C: it seems strange that MKKK5 is not enriched by IP, could authors explain this?
- Fig. 5: the authors speculate that PBL27 is degraded in the presence of MKKK5. Could this be tested by adding MG132 to inhibit proteasome-dependent degradation? This would at least give an indication of a mechanism by which PBL27 disappears.
- Fig. 8: shows that MAPKKK5 can phosphorylate MKK4/MKK5, however they do not show that this interaction is chitin dependent. MAPK modules have been shown to be activated after PAMP treatments and abiotic stress, so they cannot conclude that this pathway is strictly chitin-dependent.
- The authors do not mention the MEKK1, MKK1/2, MPK4 pathway (reviewed in Rasmussen et al. 2012 Front Plant Sci.). The western blots of phosphorylated MPKs very clearly show at least 3 MAPKs (3, 6 & 4), although only 3 & 6 are mentioned (for example Figs. 1C&D and 7A). Since MPK4 phosphorylation is also reduced in mapkkk5 mutants, is the MEKK1, MKK1/2, MPK4 pathway not also affected in mapkkk5 ? If so, what implications does this have?
- A simple control experiment of MPK phosphorylation by western blot in the mapkkk5 mutants upon flg22 or elf18. If this is not affected, then it would fit the conclusion that MAPKKK5 acts downstream of CERK1.

Specific Issues
- Fig. 3B: Could the authors explain why the MAPKKK5 protein is running at a different molecular weight at 36 hpi than 24 hpi?
- Fig. 8B, C E: Is the MKKK5 kinase domain C-terminal running at a higher molecular weight than the kinase+C-terminal?
- Fig. 8B: in the legend it should be 'CERK1-dependent' not 'depedenet'
Supplementary Figures: the activation of MPK3/6 in SF3, SF9, SF15 appears very different between wild-types. Why is it so variable?
- Supp Fig 6A: the semi quantitative rtPCR showing increased MAPKKK5 transcript levels upon chitin treatment is not very convincing. It could be performed as qPCR and preferably with more than one time point, since three hours seems quite late.

- Supp Fig. 12. the statistically significant differences are indicated by letter. However, for the BON3 in the WT, both the mock and the chitin treated samples are given an "a", although they appear significantly different. Maybe the chitin treated sample Col should be b, or c if it is also significant different from the two chitin treated mutants.

Referee #3

(Report for Author)
This manuscript by Yamada et al describes an Arabidopsis MAPKKK5 mutant required for chitin response. Knock-out MAPKKK5 mutant shows reduced chitin-induced MAPK activation, callose deposition and disease resistance to Alternaria brassicicola, suggesting a role of MAPKKK5 in the chitin-induced MAPK cascade. Based on PBL27 interaction with and phosphorylation of MAPKKK5, and MAPKKK5 phosphorylation of MKK4/5 in vitro, the authors propose the CERK1-PBL27-MAPKKK5-MKK4/5-MPK3/6 pathway. While the biochemical function of MAPKKK5 remains unclear, the genetic data support a role of MAPKKK5 in chitin-MAPK signaling pathway. This is a significant finding.

My major concern is that not all the data are consistent with the conclusion. For Chitin MAPK activation assays, by authors' results (Figure 1C/D, Figure 7A, Supplemental Figure 9), MPK6/3/4/11 activities were all compromised by MAPKKK5 in chitin-induced MAPK activation assays, so there should be in vitro and in vivo evidence provided that MAPKKK5 interacts with and phosphorylates MKK1/2/4/5. Does the phosphorylation state and activity of MKK1/2/4/5 change in mapkkk5, pbl27 and cerk1 mutants? And what is the biological relevance of this phosphorylation? Can the putative residues within MKK1/2/4/5 that are phosphorylated by MAPKKK5 be identified and mutated, and then shown that this affects MKK1/2/4/5 function in vivo? as MKK1/2 and MKK4/5 are the direct upstream regulators of MPK4 and MPK3/6, respectively. Moreover, the response to chitin in MKK1/2 and MKK4/5 mutants should also be tested. These are very important experiments if the authors want to conclude that MAPKKK5 links PBL27 with the MAPK module.

Other comments:
1. For the experiment "Expression of MAPKKK5 was induced by chitin", a control treatment iw required, because we don't know the effect of circadian rhythms on expression of MAPKKK5, and a qRT-PCR is also recommended to be performed.
2. Supplemental Figure 7 should change to a higher resolution picture.
3. For RNA data, it is recommended to give a list of differentially expressed genes. And more genes need to be tested by qRT-PCR, only two genes is not enough.

Pre-Decision Consultation – Author Response 07 April 2016

Thank you very much for handling our manuscript. We thank the reviewers for their careful review as well as constructive comments. We will respond all points raised by Rev #1 and Rev #2. However, we understood but respectfully disagree with some of the comments raised by Rev #3, because some experiments requested by the reviewer are technically difficult by available methods and materials. Rev #3 requested to analyze chitin-induced MAPK activation using the MAPKK mutant lines, mkk1/mkk2 and mkk4/mkk5. However, since both mutants are known to be seedling lethal because of pleiotropic developmental defects, it is difficult to analyze it. In fact, none of papers concerning PAMPs-induced MAPK activation used these mutant lines. In addition, It is technically difficult to test the phosphorylation state and activity of MKK1/2/4/5 change in mapkkk5, pbl27 and cerk1 mutants by available methods. Therefore, instead, we will analyze whether MAPKKK5 phosphorylates MKK1/2/4/5 in planta using transient expression system using tobacco. We hope that the editor agrees that such additional experimentation of MKK regulation, which was requested by Rev #3, is beyond the scope of this already comprehensive study that deciphered how plant PRR complexes activate downstream substrates to initiate distinct immune responses in response to MAMP perception as pointed by Rev #1.

A point-by-point response addressing the reviewers' criticisms is below.

Referee #1

Summary
Deciphering how plant PRR complexes activate downstream substrates to initiate distinct immune responses in response to MAMP perception is critical to our understanding of plant innate immunity. This manuscript reveals a direct link between Arabidopsis PBL27 and MAPKKK5 that is required to activate MAPK cascades in response to chitin. The authors show that PBL27 interacts with the C-terminal domain of MAPKKK5 in Y2H assays, and with the full-length protein in vivo. Furthermore, they show that PBL27 phosphorylates MAPKKK5 C-terminus in vitro, as well as its kinase domain in the presence of the chitin receptor CERK1. In turn, MAPKKK5 phosphorylates MKK4/5 in vitro. Consistently, mapkkk5 mutants were impaired in chitin-triggered MAPK activation, and subsequently in transcription reprograming, callose deposition and resistance against a pathogenic fungus.

The findings in this manuscript are very interesting, and the quality and amount of data are impressive. There are however a few points were I think improvements could be made in order to substantiate the authors' claims.

Major points

1. To test the specificity of the proposed pathway, the authors should test whether MAPKKK5 is required for MAPK activation in response to flg22 and/or elf18.

(Response) We have already analyzed the MAPK activation in response to flg22 and elf18. We found that the mapkkk5 mutation enhanced the flg22 or elf18-induced MAPK activation with unknown reason. These results suggest antagonistic regulation of MAPK activation between different PRR signaling.
Initially, we planned to report the result in future paper after elucidating the molecular mechanism. However, we will add the data in the manuscript according to the comment.

2. Fig 5E: a control must be included to show that the observed dissociation between PBL27 and MAPKKK5 is not simply due to the activated immune state of plant cells. Thus, the authors should test whether flg22 or elf18 would similarly (or not) lead to such dissociation.

(Response) We will analyze whether flg22 or elf18 induces disassociation between PBL27 and MAPKKK5.

3. One of the major points of this manuscript is the ability of PBL27 to phosphorylate MAPKKK5 and activate MPKs in response to chitin. Accordingly, the authors propose that PBL27 is able to phosphorylate several residues in the C-terminus of MAPKKK5 in vitro (Fig. 6). However, whether this action is specific to PBL27 or just results from in vitro phosphorylation of MAPKKK5 remains to be determined given that it is impossible to judge from the data presented in Fig. 6B whether these residues are phosphorylated in a PBL27-dependent manner. I suggest the authors test side-by-side whether BIK1can also phosphorylate MAPKK5 in vitro. This would be an adequate control since BIK1 is not required for chitin-induced MPK activation and does not interact with MAPKKK5 in vivo.

(Response) We will analyze whether BIK1 phosphorylates MAPKKK5 in vitro.

4. The authors propose a possible phospho-signaling pathway consisting of CERK1-PBL27-MAPKKK5-MKK4/MKK5-MPK3/6. A nice link between MAPKKK5 is provided with in vitro phosphorylation assays. However, no data supporting the interaction between MAPKKK5 and MKK4/5 is provided in vivo. Testing this by co-IP in Nb or protoplasts, or by BiFC would greatly support their model.

(Response) We will carry out BiFC to show the in vivo interaction between MAPKKK5 and MKK4/5.

Minor points

5. In the text, the authors mention that 319 genes show reduced induction or suppression in mapkkk5 compared to WT, whereas the legend of Sup Fig 11 states "Genes showing reduced induction in mapkkk5 compared to wild type were selected (319 genes)". This is confusing and not accurate. Please correct accordingly.

(Response) We will correct it according to the reviewer's comment.

6. In order to determine MAPKKK5 subcellular localization, the authors isolated soluble and microsomal protein fractions, and observed the localization of MAPKKK5-GFP transiently expressed in N. benthamiana under the microscope. The authors conclude that MAPKKK5 is both cytosolic and PM-associated. Although this is most likely the case (especially taking into account the later BiFC experiments), there are some issues with both experiments that prevent the reader from confidently reaching the same conclusion:
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(Response) We will re-analyze it using a known PM-localized protein marker.

b) From the images presented in Fig 3D it is difficult to conclude whether MAPKKK5 is associated with the PM. It would be nice if the authors could show a zoomed in section and perform plasmolysis and/or co-localization with a known PM-localized protein.

(Response) We will re-analyze it using plasmolysis.

7. pMAPKKK5::MAPKKK5_6xA-FLAG transgenic plants are used to assess the role of MKKK5 phosphorylation in MPK activation. However, expression of MAPKKK5_6xA-FLAG could not be detected in these plants. The authors honestly refer to this in the text and provide additional experiments with the same construct transiently expressed in protoplasts and Nb leaves, suggesting that MAPKKK5_6xA-FLAG still accumulates and has a similar localization to the WT protein. While I value this effort, this does not show that the transgenic pMAPKKK5::MAPKKK5_6xA-FLAG plants are actually expressing MAPKKK5. I propose to the authors to perform an anti-FLAG IP on these lines, which should enrich MAPKKK5_6xA-FLAG to detectable levels. If expression cannot still be addressed maybe an RT-PCR could be included to show that MAPKKK5_6xA-FLAG is at least expressed at the mRNA level. Alternatively, the authors could test whether MAPK activation is affected in mapkkk5 protoplasts transfected with MAPKKK5/MAPKKK5_6xA.

(Response) We have already test the enrichment of MAPKKK5-GFP or FLAG by IP according to the method (Kadota et al. Methods Mol Biol, 1363, 133-144 (2016). However, in spite of repeatedly experiments we could not detect the proteins. We have already indicated the mRNA level of MAPKKK5_6xA (Supplemental Fig 14B, Fig 7C).

8. The authors show in Fig 2 that mkkk5 mutants are compromised in certain immune outputs (callose deposition, transcription reprogramming, and immunity against Alternaria). However, chitin-induced ROS burst is not affected in mkkk5 mutants (Sup Fig 10). This is a very interesting result that further supports the previous notion that MPK activation and ROS production are independent events. In my opinion, Sup Fig10 should be incorporated in Fig 2 in order to highlight this point. Similarly, Sup Fig 12 should have its place in the main text. In turn, many other main figures could be easily switched to the supplemental data without great loss to the read. This is the case for Figs 3, 4A and 5A, which display important observations but are not essential for the main conclusions.

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Referee #2

General comment.
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Main Issues
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(Response) We will add the list according to the comment.

- Figs. 3A & 4E: The tomato MAPKKK SlMKKKa also causes cell death when overexpressed in N. Benthamiana (del Pozo et al., 2004 EMBO J.). Could the authors comment on how this might relate

to MAPKKK5? For example, is it possible that MKKK5 levels are homeostatically limited as the protein may be a positive regulator of cell death? In that case, to study the protein in Arabidopsis maybe the authors could try to make the lines in an eds1 or ndr1 background, in case the cause of the cell death is some R protein mediated pathway (reviewed in Rodriguez et al. 2015 FEBS J). Another suggestion would be to check the level of PBL27 in the mkkk5 mutant background (mkkk5/PBL27-HA).

(Response) We will analyze whether mapkkk5 mutation affects R-protein mediated cell death.

- Fig. 3C: MAPKKK5 is more abundant in the soluble fraction than in the microsomal fraction. Could the authors clarify why they focus on the microsomal fraction for the co-IP in Fig. 4 instead of the soluble fraction? The authors do not comment much on the possible reason for membrane localization of the MKKK5.
(Response) When we used total proteins for Co-IP, we could not detect the interaction between PBL27 and MAPKKK5. Because the results of the BiFC experiment indicated the plasma membrane interaction of these protein, we used the membrane fraction to enrich both proteins. We will comment it in the text.

- Fig. 3D: the authors show the localization of wild-type MAPKKK5 and see signal in the cytosol and plasma membrane, but no plasmolysis is done to support the claim of membrane localization (while this is done in Fig. 4D for the mutant MKKK5).

(Response) We will re-analyze it using plasmolysis according to the comment.

- Fig. 4C: it seems strange that MKKK5 is not enriched by IP, could authors explain this?

(Response) We used 1/10 input for immunoblot. We will correct the figure.

- Fig. 5: the authors speculate that PBL27 is degraded in the presence of MKKK5. Could this be tested by adding MG132 to inhibit proteasome-dependent degradation? This would at least give an indication of a mechanism by which PBL27 disappears.

(Response) We will test it according to the comment.

- Fig. 8: shows that MAPKKK5 can phosphorylate MKK4/MKK5, however they do not show that this interaction is chitin dependent. MAPK modules have been shown to be activated after PAMP treatments and abiotic stress, so they cannot conclude that this pathway is strictly chitin-dependent.

(Response) As pointed out by the reviewer, it is possible that MAPKKK5 regulates MKK4 and MKK5 in other defense signaling and abiotic signaling. Therefore, we will add the comment in the text.

- The authors do not mention the MEKK1, MKK1/2, MPK4 pathway (reviewed in Rasmussen et al. 2012 Front Plant Sci.). The western blots of phosphorylated MPKs very clearly show at least 3 MAPKs (3, 6 & 4), although only 3 & 6 are mentioned (for example Figs. 1C&D and 7A). Since MPK4 phosphorylation is also reduced in mapkkk5 mutants, is the MEKK1, MKK1/2, MPK4 pathway not also affected in mapkkk5 ? If so, what implications does this have?

(Response) As pointed by the reviewer, it appears that the activation level of MPK4 was also reduced in the mapkkk5. Therefore, will test the interaction between MAPKKK5 and MKK1/MKK2. If we will detect the interaction, it is possible that MAPKKK5 may also regulate MKK1/MKK2-MPK4 signaling in addition to MEKK1. We will discuss it in the text.

- A simple control experiment of MPK phosphorylation by western blot in the mapkkk5 mutants upon flg22 or elf18. If this is not affected, then it would fit the conclusion that MAPKKK5 acts downstream of CERK1.

(Response) We have already analyzed the MAPK activation in response to flg22 and elf18. We found that the mapkkk5 mutation enhanced the flg22 or elf18-induced MAPK activation with unknown reason. These results suggest antagonistic regulation of MAPK activation between different PRR signaling. Initially, we planed to report the result in future paper after elucidating the molecular mechanism. However, we will add the data in the manuscript according to the comment.

Specific Issues
- Fig. 3B: Could the authors explain why the MAPKKK5 protein is running at a different molecular weight at 36 hpi than 24 hpi?
(Response) We considered that MAPKKK5 may be modified during cell death program.

- Fig. 8B, C E: Is the MKKK5 kinase domain C-terminal running at a higher molecular weight than the kinase+C-terminal?

(Response) Thank you for your notice. In Fig 8C, we used GST-MAPKKK5-C. We will correct it.

- Fig. 8B: in the legend it should be 'CERK1-dependent' not 'depedenet'
(Response) We will correct it.

Supplementary Figures: the activation of MPK3/6 in SF3, SF9, SF15 appears very different between wild-types. Why is it so variable?

(Response) In our hands, timing of chitin-induced MAPK activation is slightly different among the experiments. Therefore, we evaluated the MAPK activation by their phosphorylation levels.

- Supp Fig 6A: the semi quantitative rtPCR showing increased MAPKKK5 transcript levels upon chitin treatment is not very convincing. It could be performed as qPCR and preferably with more than one time point, since three hours seems quite late.

(Response) We will analyze it by qPCR using several time points according to the comment.

- Supp Fig. 12. the statistically significant differences are indicated by letter. However, for the BON3 in the WT, both the mock and the chitin treated samples are given an "a", although they appear significantly different. Maybe the chitin treated sample Col should be b, or c if it is also significant different from the two chitin treated mutants.

(Response) We will correct it.

Referee #3

This manuscript by Yamada et al describes an Arabidopsis MAPKKK5 mutant required for chitin response. Knock-out MAPKKK5 mutant shows reduced chitin-induced MAPK activation, callose deposition and disease resistance to Alternaria brassicicola, suggesting a role of MAPKKK5 in the chitin-induced MAPK cascade. Based on PBL27 interaction with and phosphorylation of MAPKKK5, and MAPKKK5 phosphorylation of MKK4/5 in vitro, the authors propose the CERK1-PBL27-MAPKKK5-MKK4/5-MPK3/6 pathway. While the biochemical function of MAPKKK5 remains unclear, the genetic data support a role of MAPKKK5 in chitin-MAPK signaling pathway.

This is a significant finding.

My major concern is that not all the data are consistent with the conclusion. For Chitin MAPK activation assays, by authors' results (Figure1C/D, Figure 7A, Supplemental Figure 9), MPK6/3/4/11 activities were all compromised by MAPKKK5 in chitin-induced MAPK activation assays, so there should be in vitro and in vivo evidence provided that MAPKKK5 interacts with and phosphorylates MKK1/2/4/5. Does the phosphorylation state and activity of MKK1/2/4/5 change in mapkkk5, pbl27 and cerk1 mutants? And what is the biological relevance of this phosphorylation? Can the putative residueswithin MKK1/2/4/5 that are phosphorylated by MAPKKK5 be identified and mutated, and then shown that this affects MKK1/2/4/5 function in vivo? As MKK1/2 and MKK4/5 are the direct upstream regulators of MPK4 and MPK3/6, respectively. Moreover, the response to chitin in MKK1/2 and MKK4/5 mutants should also be tested. These are very important experiments if the authors want to conclude that MAPKKK5 links PBL27 with the MAPK module.

(Response) As pointed by the reviewer, it appears that the activation level of MPK4 was also reduced in the mapkkk5. Therefore, will test the interaction between MAPKKK5 and MKK1/MKK2. If we will detect the interaction, it is possible that MAPKKK5 may also regulate MKK1/MKK2-MPK4 signaling. We also can test whether MAPKKK5 phosphorylates MKK1/2 in vitro. However, because mkk1/2 mutant or mkk4/5 mutant is known to be seedling lethal, we can not test whether MKK1/2 and MKK4/5 mutations affects chitin response and whether the putative residues within MKK1/2/4/5 that are phosphorylated by MAPKKK5 affects MKK1/2/4/5 function in vivo. In addition, It is also technically difficult to test the phosphorylation state and activity of MKK1/2/4/5 change in mapkkk5, pbl27 and cerk1 mutants by available methods and materials. Therefore, in stead, we will analyze whether MAPKKK5 phosphorylates MKK1/2/4/5 in planta using transient expression system using tobacco. We hope that the editor agrees that such additional experimentation is beyond the scope of this already comprehensive study.

Other comments:
1. For the experiment "Expression of MAPKKK5 was induced by chitin", a control treatment iw required, because we don't know the effect of circadian rhythms on expression of MAPKKK5, and a qRT-PCR is also recommended to be performed.

(Response) We will analyze it using several time points according to the comment.

2. Supplemental Figure 7 should change to a higher resolution picture.

(Response) We will change the picture according to the comment.

3. For RNA data, it is recommended to give a list of differentially expressed genes. And more genes need to be tested by qRT-PCR, only two genes is not enough.

(Response) We will add the list and the data of additional genes.

1st Editorial Decision 07 April 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. I attach once more the referee comments below.

Given the referees' comments and the outline you have already provided on how to address the concerns, I would like to invite you to submit a revised version of the manuscript.
I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. We generally allow three months as standard revision time. I can extend this to six months, should that be helpful.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

As a matter of policy, competing manuscripts published during the revision period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting the revision deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

Summary
Deciphering how plant PRR complexes activate downstream substrates to initiate distinct immune responses in response to MAMP perception is critical to our understanding of plant innate immunity. This manuscript reveals a direct link between Arabidopsis PBL27 and MAPKKK5 that is required to activate MAPK cascades in response to chitin. The authors show that PBL27 interacts with the C-terminal domain of MAPKKK5 in Y2H assays, and with the full-length protein in vivo. Furthermore, they show that PBL27 phosphorylates MAPKKK5 C-terminus in vitro, as well as its kinase domain in the presence of the chitin receptor CERK1. In turn, MAPKKK5 phosphorylates MKK4/5 in vitro. Consistently, mapkkk5 mutants were impaired in chitin-triggered MAPK activation, and subsequently in transcription reprograming, callose deposition and resistance against a pathogenic fungus.
The findings in this manuscript are very interesting, and the quality and amount of data are impressive. There are however a few points were I think improvements could be made in order to substantiate the authors' claims.

Major points

1. To test the specificity of the proposed pathway, the authors should test whether MAPKKK5 is required for MAPK activation in response to flg22 and/or elf18.

2. Fig 5E: a control must be included to show that the observed dissociation between PBL27 and MAPKKK5 is not simply due to the activated immune state of plant cells. Thus, the authors should test whether flg22 or elf18 would similarly (or not) lead to such dissociation

3. One of the major points of this manuscript is the ability of PBL27 to phosphorylate MAPKKK5 and activate MPKs in response to chitin. Accordingly, the authors propose that PBL27 is able to phosphorylate several residues in the C-terminus of MAPKKK5 in vitro (Fig. 6). However, whether this action is specific to PBL27 or just results from in vitro phosphorylation of MAPKKK5 remains to be determined given that it is impossible to judge from the data presented in Fig. 6B whether these residues are phosphorylated in a PBL27-dependent manner. I suggest the authors test side-by-side whether BIK1 can also phosphorylate MAPKK5 in vitro. This would be an adequate control since BIK1 is not required for chitin-induced MPK activation and does not interact with MAPKKK5 in vivo.

4. The authors propose a possible phospho-signaling pathway consisting of CERK1-PBL27-MAPKKK5-MKK4/MKK5-MPK3/6. A nice link between MAPKKK5 is provided with in vitro phosphorylation assays. However, no data supporting the interaction between MAPKKK5 and

MKK4/5 is provided in vivo. Testing this by co-IP in Nb or protoplasts, or by BiFC would greatly support their model.

Minor points

5. In the text, the authors mention that 319 genes show reduced induction or suppression in mapkkk5 compared to WT, whereas the legend of Sup Fig 11 states "Genes showing reduced induction in mapkkk5 compared to wild type were selected (319 genes)". This is confusing and not accurate. Please correct accordingly.

6. In order to determine MAPKKK5 subcellular localization, the authors isolated soluble and microsomal protein fractions, and observed the localization of MAPKKK5-GFP transiently expressed in N. benthamiana under the microscope. The authors conclude that MAPKKK5 is both cytosolic and PM-associated. Although this is most likely the case (especially taking into account the later BiFC experiments), there are some issues with both experiments that prevent the reader from confidently reaching the same conclusion:
a) In Fig 3C no control for a known PM-localized protein is provided. A negative control is provided with GFP but the signal is so low in total and soluble fractions that it is difficult to conclude about its true absence in the microsomal fraction. In Sup Fig 13, a control is provided by using anti-EAP1; however, no reference or further experiments are provided for its validity as a soluble/microsomal marker protein. I suggest the authors repeat this experiment (for both MAPKKK5 and K375M) including a known PM-localized protein, for example. Also, a Ponceau staining of the membrane should be provided in this case.
b) From the images presented in Fig 3D it is difficult to conclude whether MAPKKK5 is associated with the PM. It would be nice if the authors could show a zoomed in section and perform plasmolysis and/or co-localization with a known PM-localized protein.

7. pMAPKKK5::MAPKKK5_6xA-FLAG transgenic plants are used to assess the role of MKKK5 phosphorylation in MPK activation. However, expression of MAPKKK5_6xA-FLAG could not be detected in these plants. The authors honestly refer to this in the text and provide additional experiments with the same construct transiently expressed in protoplasts and Nb leaves, suggesting that MAPKKK5_6xA-FLAG still accumulates and has a similar localization to the WT protein. While I value this effort, this does not show that the transgenic pMAPKKK5::MAPKKK5_6xA-FLAG plants are actually expressing MAPKKK5. I propose to the authors to perform an anti-FLAG IP on these lines, which should enrich MAPKKK5_6xA-FLAG to detectable levels. If expression cannot still be addressed maybe an RT-PCR could be included to show that MAPKKK5_6xA-FLAG is at least expressed at the mRNA level.
Alternatively, the authors could test whether MAPK activation is affected in mapkkk5 protoplasts transfected with MAPKKK5/MAPKKK5_6xA.

8. The authors show in Fig 2 that mkkk5 mutants are compromised in certain immune outputs (callose deposition, transcription reprogramming, and immunity against Alternaria). However, chitin-induced ROS burst is not affected in mkkk5 mutants (Sup Fig 10). This is a very interesting result that further supports the previous notion that MPK activation and ROS production are independent events. In my opinion, Sup Fig10 should be incorporated in Fig 2 in order to highlight this point. Similarly, Sup Fig 12 should have its place in the main text.
In turn, many other main figures could be easily switched to the supplemental data without great loss to the read. This is the case for Figs 3, 4A and 5A, which display important observations but are not essential for the main conclusions.

9. The MS spectra relative to Fig 6B should be provided as supplementary data.

10. I would suggest amending the title to "The Arabidopsis CERK1-associated kinase PBL27 connects chitin perception to MAPK activation".

11. p 4, line 21: Nuhse et al., 2007 and Zhang et al., 2007 are not the most appropriate references for this sentence. As done previously in the Introduction, rather cite a comprehensive review.

12. p 5, lines 5-16: move this paragraph before mentioning that it is currently unclear how MAPKs

are activated downstream of FLS2 and EFR.

13. p 5, line 28: "These results suggested that OsRLCK185...". Also mention OsRLCK176.

14. p 13, line 31: Ao et al., 2014 is not an appropriate reference here.

15. p 14, line 4: it is not correct to state that it is unknown which RLCK(s) connect CERK1-mediated chitin perception to RBOHD activity, as it was shown previously that BIK1 and BIK1-mediated phosphorylation of RBOHD are required for chitin-induced ROS production (Zhang et al., 2010; Kadota et al., 2014).

Referee #2:

General comment.
The manuscript of Yamada et al. addresses the chitin-dependent activation of MAPKKK5 via the RLCK PBL27. It demonstrates that mpkkk5 mutants are impaired in MAPK activation after treatment with chitin. PBL27 can phosphorylate MAPKKK5 at the plasma membrane, and this phosphorylation is dependent on PBL27 phosphorylation by the CERK1 chitin pattern recognition receptor (PRR). As such a direct molecular link between plant PRRs and MAPK activation has not been described before, the manuscript is certainly of interest to plant and animal researchers. Since MAPKKK5 is very lowly expressed in vivo, the manuscript focuses primarily on in vitro approaches whose interpretation requires some caution.

Main Issues
- Figure 2: 339 genes: it would be nice to have a list of genes different in the mkkk5 mutant.
- Figs. 3A & 4E: The tomato MAPKKK SlMKKKα also causes cell death when overexpressed in N. Benthamiana (del Pozo et al., 2004 EMBO J.). Could the authors comment on how this might relate to MAPKKK5? For example, is it possible that MKKK5 levels are homeostatically limited as the protein may be a positive regulator of cell death? In that case, to study the protein in Arabidopsis maybe the authors could try to make the lines in an eds1 or ndr1 background, in case the cause of the cell death is some R protein mediated pathway (reviewed in Rodriguez et al. 2015 FEBS J). Another suggestion would be to check the level of PBL27 in the mkkk5 mutant background (mkkk5/PBL27-HA).
- Fig. 3C: MAPKKK5 is more abundant in the soluble fraction than in the microsomal fraction. Could the authors clarify why they focus on the microsomal fraction for the co-IP in Fig. 4 instead of the soluble fraction? The authors do not comment much on the possible reason for membrane localization of the MKKK5.
- Fig. 3D: the authors show the localization of wild-type MAPKKK5 and see signal in the cytosol and plasma membrane, but no plasmolysis is done to support the claim of membrane localization (while this is done in Fig. 4D for the mutant MKKK5).
- Fig. 4C: it seems strange that MKKK5 is not enriched by IP, could authors explain this?
- Fig. 5: the authors speculate that PBL27 is degraded in the presence of MKKK5. Could this be tested by adding MG132 to inhibit proteasome-dependent degradation? This would at least give an indication of a mechanism by which PBL27 disappears.
- Fig. 8: shows that MAPKKK5 can phosphorylate MKK4/MKK5, however they do not show that this interaction is chitin dependent. MAPK modules have been shown to be activated after PAMP treatments and abiotic stress, so they cannot conclude that this pathway is strictly chitin-dependent.
- The authors do not mention the MEKK1, MKK1/2, MPK4 pathway (reviewed in Rasmussen et al. 2012 Front Plant Sci.). The western blots of phosphorylated MPKs very clearly show at least 3 MAPKs (3, 6 & 4), although only 3 & 6 are mentioned (for example Figs. 1C&D and 7A). Since MPK4 phosphorylation is also reduced in mapkkk5 mutants, is the MEKK1, MKK1/2, MPK4 pathway not also affected in mapkkk5 ? If so, what implications does this have?
- A simple control experiment of MPK phosphorylation by western blot in the mapkkk5 mutants upon flg22 or elf18. If this is not affected, then it would fit the conclusion that MAPKKK5 acts

downstream of CERK1.

Specific Issues
- Fig. 3B: Could the authors explain why the MAPKKK5 protein is running at a different molecular weight at 36 hpi than 24 hpi?
- Fig. 8B, C E: Is the MKKK5 kinase domain C-terminal running at a higher molecular weight than the kinase+C-terminal?
- Fig. 8B: in the legend it should be 'CERK1-dependent' not 'depedenet'
Supplementary Figures: the activation of MPK3/6 in SF3, SF9, SF15 appears very different between wild-types. Why is it so variable?
- Supp Fig 6A: the semi quantitative rtPCR showing increased MAPKKK5 transcript levels upon chitin treatment is not very convincing. It could be performed as qPCR and preferably with more than one time point, since three hours seems quite late.
- Supp Fig. 12. the statistically significant differences are indicated by letter. However, for the BON3 in the WT, both the mock and the chitin treated samples are given an "a", although they appear significantly different. Maybe the chitin treated sample Col should be b, or c if it is also significant different from the two chitin treated mutants.

Referee #3:

This manuscript by Yamada et al describes an Arabidopsis MAPKKK5 mutant required for chitin response. Knock-out MAPKKK5 mutant shows reduced chitin-induced MAPK activation, callose deposition and disease resistance to Alternaria brassicicola, suggesting a role of MAPKKK5 in the chitin-induced MAPK cascade. Based on PBL27 interaction with and phosphorylation of MAPKKK5, and MAPKKK5 phosphorylation of MKK4/5 in vitro, the authors propose the CERK1-PBL27-MAPKKK5-MKK4/5-MPK3/6 pathway. While the biochemical function of MAPKKK5 remains unclear, the genetic data support a role of MAPKKK5 in chitin-MAPK signaling pathway. This is a significant finding.
My major concern is that not all the data are consistent with the conclusion. For Chitin MAPK activation assays, by authors' results (Figure 1C/D, Figure 7A, Supplemental Figure 9), MPK6/3/4/11 activities were all compromised by MAPKKK5 in chitin-induced MAPK activation assays, so there should be in vitro and in vivo evidence provided that MAPKKK5 interacts with and phosphorylates MKK1/2/4/5. Does the phosphorylation state and activity of MKK1/2/4/5 change in mapkkk5, pbl27 and cerk1 mutants? And what is the biological relevance of this phosphorylation? Can the putative residues within MKK1/2/4/5 that are phosphorylated by MAPKKK5 be identified and mutated, and then shown that this affects MKK1/2/4/5 function in vivo? as MKK1/2 and MKK4/5 are the direct upstream regulators of MPK4 and MPK3/6, respectively. Moreover, the response to chitin in MKK1/2 and MKK4/5 mutants should also be tested. These are very important experiments if the authors want to conclude that MAPKKK5 links PBL27 with the MAPK module.
Other comments:
1. For the experiment "Expression of MAPKKK5 was induced by chitin", a control treatment iw required, because we don't know the effect of circadian rhythms on expression of MAPKKK5, and a qRT-PCR is also recommended to be performed.
2. Supplemental Figure 7 should change to a higher resolution picture.
3. For RNA data, it is recommended to give a list of differentially expressed genes. And more genes need to be tested by qRT-PCR, only two genes is not enough.

1st Revision - authors' response 15 July 2016

Response to reviewers’ comments.

We thank the reviewers for careful review as well as constructive comments. According to the reviewers’ suggestions, we have thoroughly revised the manuscript.
Below, we provide a detailed point-by-point response addressing the reviewers’ criticisms.

Referee #1

Deciphering how plant PRR complexes activate downstream substrates to initiate distinct immune responses in response to MAMP perception is critical to our understanding of plant innate immunity. This manuscript reveals a direct link between Arabidopsis PBL27 and MAPKKK5 that is required to activate MAPK cascades in response to chitin. The authors show that PBL27 interacts with the C-terminal domain of MAPKKK5 in Y2H assays, and with the full-length protein in vivo. Furthermore, they show that PBL27 phosphorylates MAPKKK5 C-terminus in vitro, as well as its kinase domain in the presence of the chitin receptor CERK1. In turn, MAPKKK5 phosphorylates MKK4/5 in vitro. Consistently, mapkkk5 mutants were impaired in chitin-triggered MAPK activation, and subsequently in transcription reprograming, callose deposition and resistance against a pathogenic fungus.
The findings in this manuscript are very interesting, and the quality and amount of data are impressive. There are however a few points were I think improvements could be made in order to substantiate the authors' claims.

We thank the reviewer for careful review as well as positive and constructive comments.

Major points
1. To test the specificity of the proposed pathway, the authors should test whether MAPKKK5 is required for MAPK activation in response to flg22 and/or elf18.

According to the reviewer's comment, we analyzed the MAPK activation in response to flg22. New data indicated that the *mapkkk5* mutation enhanced the flg22-induced MAPK activation, suggesting that MAPKKK5 may negatively regulate flg22-induced MAPK activation. We added the data in Fig 1E. (Page 9, line 1-4)

2. Fig 5E: a control must be included to show that the observed dissociation between PBL27 and MAPKKK5 is not simply due to the activated immune state of plant cells. Thus, the authors should test whether flg22 or elf18 would similarly (or not) lead to such dissociation.

We thanks for the advise. We examined whether flg22 treatment induces disassociation between and MAPKKK5. New data indicated that the interaction was not affected by treatment with flg22. We added the data in Fig 4F. (Page12, line 7 – 8)

3. One of the major points of this manuscript is the ability of PBL27 to phosphorylate MAPKKK5 and activate MPKs in response to chitin. Accordingly, the authors propose that PBL27 is able to phosphorylate several residues in the C-terminus of MAPKKK5 in vitro (Fig. 6). However, whether this action is specific to PBL27 or just results from in vitro phosphorylation of MAPKKK5 remains to be determined given that it is impossible to judge from the data presented in Fig. 6B whether these residues are phosphorylated in a PBL27-dependent manner. I suggest the authors test side-by-side whether BIK1 can also phosphorylate MAPKK5 in vitro. This would be an adequate control since BIK1 is not required for chitin-induced MPK activation and does not interact with MAPKKK5 in vivo.

We agree with the reviewer's comment. We analyzed in vitro phosphorylation of MAPKKK5 by BIK1. New data indicate that BIK1 did not phosphorylate any domains of MAPKKK5, although BIK1 phosphorylated the N-terminal domain of RbohD as previously reported by Kadota et al. We added the data in Fig 5B. (Page 12, line 14 – 16)

4. The authors propose a possible phospho-signaling pathway consisting of CERK1-PBL27-MAPKKK5-MKK4/MKK5-MPK3/6. A nice link between MAPKKK5 is provided with in vitro phosphorylation assays. However, no data supporting the interaction between MAPKKK5 and MKK4/5 is provided in vivo. Testing this by co-IP in Nb or protoplasts, or by BiFC would greatly support their model.

According to the reviewer's comment, we analyzed in vivo interaction between MAPKKK5 and MKK4 / MKK5 by BiFC assay using Arabidopsis protoplast. The data indicate that MAPKKK5 interacts with MKK4 and MKK5 mainly in cytosol. We added the data in Fig 8A. (Page 15, line 7 – 11)

Minor points
5. In the text, the authors mention that 319 genes show reduced induction or suppression in mapkkk5 compared to WT, whereas the legend of Sup Fig 11 states "Genes showing reduced induction in mapkkk5 compared to wild type were selected (319 genes)". This is confusing and not accurate. Please correct accordingly.

We thank you for noticing this. We corrected the legend of Appendix Figure S5.

6. In order to determine MAPKKK5 subcellular localization, the authors isolated soluble and microsomal protein fractions, and observed the localization of MAPKKK5-GFP transiently expressed in N. benthamiana under the microscope. The authors conclude that MAPKKK5 is both cytosolic and PM-associated. Although this is most likely the case (especially taking into account the later BiFC experiments), there are some issues with both experiments that prevent the reader from confidently reaching the same conclusion:
a) In Fig 3C no control for a known PM-localized protein is provided. A negative control is provided with GFP but the signal is so low in total and soluble fractions that it is difficult to conclude about its true absence in the microsomal fraction. In Sup Fig 13, a control is provided by using anti-EAP1; however, no reference or further experiments are provided for its validity as a soluble/microsomal marker protein. I suggest the authors repeat this experiment (for both MAPKKK5 and K375M) including a known PM-localized protein, for example. Also, a Ponceau staining of the membrane should be provided in this case.

According to the reviewer's comment, we re-analyzed subcellular localization of MAPKKK5 and MAPKKK5^{K375M} using an PIP1 antibody as a known PM-localized protein marker. We added the data in Fig 3 A and D. We also provided the reference of the EAP1 antibody in Materials and Methods.

b) From the images presented in Fig 3D it is difficult to conclude whether MAPKKK5 is associated with the PM. It would be nice if the authors could show a zoomed in section and perform plasmolysis and/or co-localization with a known PM-localized protein.

According to the reviewer's comment, we re-analyzed it by plasmolysis. We added new data in Fig 3B.

7. pMAPKKK5::MAPKKK5_6xA-FLAG transgenic plants are used to assess the role of MKKK5 phosphorylation in MPK activation. However, expression of MAPKKK5_6xA-FLAG could not be detected in these plants. The authors honestly refer to this in the text and provide additional experiments with the same construct transiently expressed in protoplasts and Nb leaves, suggesting that MAPKKK5_6xA-FLAG still accumulates and has a similar localization to the WT protein. While I value this effort, this does not show that the transgenic pMAPKKK5::MAPKKK5_6xA-FLAG plants are actually expressing MAPKKK5. I propose to the authors to perform an anti-FLAG IP on these lines, which should enrich MAPKKK5_6xA-FLAG to detectable levels. If expression cannot still be addressed maybe an RT-PCR could be included to show that MAPKKK5_6xA-FLAG is at least expressed at the mRNA level. Alternatively, the authors could test whether MAPK activation is affected in mapkkk5 protoplasts transfected with MAPKKK5/MAPKKK5_6xA.

We have already tested the enrichment of MAPKKK5-GFP or FLAG by IP according to the method (Kadota et al. Methods Mol Biol, 1363, 133-144 (2016)). However, in spite of repeatedly experiments we could not detect the proteins. Therefore, we indicate the mRNA level of MAPKKK5_6xA (Appendix Figure S4). (Page 8, line 19 – 21)

8. The authors show in Fig 2 that mkkk5 mutants are compromised in certain immune outputs (callose deposition, transcription reprogramming, and immunity against Alternaria). However, chitin-induced ROS burst is not affected in mkkk5 mutants (Sup Fig 10). This is a very interesting result that further supports the previous notion that MPK activation and ROS production are independent events. In my opinion, Sup Fig10 should be incorporated in Fig 2 in order to highlight this point. Similarly, Sup Fig 12 should have its place in the main text.
In turn, many other main figures could be easily switched to the supplemental data without great loss to the read. This is the case for Figs 3, 4A and 5A, which display important observations but are not essential for the main conclusions.

We thanks for the advise. According to the comment, we added the result of chitin-induced ROS production in main text (Fig 2B). In addition, four figures concerning MAPKKK5-induced cell death in Nb leaves were moved to Figure EV3.

9. The MS spectra relative to Fig 6B should be provided as supplementary data.

According to the reviewer's comment, we added the MS spectra data in Appendix Figure S5.

10. I would suggest amending the title to "The Arabidopsis CERK1-associated kinase PBL27 connects chitin perception to MAPK activation".

We thanks for the advise. We changed the title according to the comment.

11. p 4, line 21: Nuhse et al., 2007 and Zhang et al., 2007 are not the most appropriate references for this sentence. As done previously in the Introduction, rather cite a comprehensive review.

We changed the references according to the comment. The refs were replaced by a review paper (Macho & Zipfel Mol Cell 2014). (Page 4, line 21)

12. p 5, lines 5-16: move this paragraph before mentioning that it is currently unclear how MAPKs are activated downstream of FLS2 and EFR.

We modified the manuscript according to the comment.

13. p 5, line 28: "These results suggested that OsRLCK185...". Also mention OsRLCK176.

We modified the manuscript according to the comment. (Page 6, line 20)

14. p 13, line 31: Ao et al., 2014 is not an appropriate reference here.

We thanks for your notice. The ref was replaced by Li et al Cell Host & Microbe (2014).(Page 17, line 24)

15. p 14, line 4: it is not correct to state that it is unknown which RLCK(s) connect CERK1-mediated chitin perception to RBOHD activity, as it was shown previously that BIK1 and BIK1-mediated phosphorylation of RBOHD are required for chitin-induced ROS production (Zhang et al., 2010; Kadota et al., 2014).

We thanks for your notice. We corrected it in the revised version. (Page 18, line 4 – 5).

Referee #2
General comment.
The manuscript of Yamada et al. addresses the chitin-dependent activation of MAPKKK5 via the RLCK PBL27. It demonstrates that mpkkk5 mutants are impaired in MAPK activation after treatment with chitin. PBL27 can phosphorylate MAPKKK5 at the plasma membrane, and this phosphorylation is dependent on PBL27 phosphorylation by the CERK1 chitin pattern recognition receptor (PRR). As such a direct molecular link between plant PRRs and MAPK activation has not been described before, the manuscript is certainly of interest to plant and animal researchers. Since MAPKKK5 is very lowly expressed in vivo, the manuscript focuses primarily on in vitro approaches whose interpretation requires some caution.

Main Issues
- Figure 2: 339 genes: it would be nice to have a list of genes different in the mkkk5 mutant.

We thanks for the advice. We added the list in Table EV1.

- Figs. 3A & 4E: The tomato MAPKKK SlMKKKα also causes cell death when overexpressed in N. Benthamiana (del Pozo et al., 2004 EMBO J.). Could the authors comment on how this might relate

to MAPKKK5? For example, is it possible that MKKK5 levels are homeostatically limited as the protein may be a positive regulator of cell death? In that case, to study the protein in Arabidopsis maybe the authors could try to make the lines in an eds1 or ndr1 background, in case the cause of the cell death is some R protein mediated pathway (reviewed in Rodriguez et al. 2015 FEBS J). Another suggestion would be to check the level of PBL27 in the mkkk5 mutant background (mkkk5/PBL27-HA).

In addition to tomato MAPKKK SlMKKKα, over-expression of tobacco MEKK family is known to induce cell death in plant (Hashimoto et al. BMC Plant Biol 2012). Since expression of constitutively active forms of MKK4 and MKK5 also causes cell death, we consider that ectopic activation of MAPK cascades likely results in cell death phenotype. To examine whether MAPKKK5 is involved in R-gene-mediated HR cell death, we inoculated the *mapkkk5* plant with Pst DC3000: avrRpm1 and Pst DC3000: avrRpt2. We observed same levels of cell death between WT and *mapkkk5*, indicating that MAPKKK5 is not involved in RPM1- and RPS2-mediated HR.

- Fig. 3C: MAPKKK5 is more abundant in the soluble fraction than in the microsomal fraction. Could the authors clarify why they focus on the microsomal fraction for the co-IP in Fig. 4 instead of the soluble fraction? The authors do not comment much on the possible reason for membrane localization of the MKKK5.

When we used total proteins for Co-IP, we could not detect the interaction between PBL27 and MAPKKK5. Because the results of the BiFC experiment indicated that these proteins interact with each other at the plasma membrane, we used the membrane fraction to enrich both proteins. We added the reason in the text. (Page 11, line 4 – 8)

- Fig. 3D: the authors show the localization of wild-type MAPKKK5 and see signal in the cytosol and plasma membrane, but no plasmolysis is done to support the claim of membrane localization (while this is done in Fig. 4D for the mutant MKKK5).

According to the reviewer's comment, we re-analyzed it by plasmolysis. We added the data in Fig 3B.

- Fig. 4C: it seems strange that MKKK5 is not enriched by IP, could authors explain this?

We thanks for your notice. "Input" was not suitable for the figure. We modified the figure (Fig 3E).

- Fig. 5: the authors speculate that PBL27 is degraded in the presence of MKKK5. Could this be tested by adding MG132 to inhibit proteasome-dependent degradation? This would at least give an indication of a mechanism by which PBL27 disappears.

According to the reviewer's comment, we analyzed whether MG132 affects the protein levels of PBL27 and MAPKKK5. Degradation of both PBL27 and MAPKKK5 was suppressed by MG132, suggesting that the protein levels of these proteins were regulated in the proteasome dependent manner. We added the data in Fig 4C. (Page 13, line 23 – Page 14, line 1)

- Fig. 8: shows that MAPKKK5 can phosphorylate MKK4/MKK5, however they do not show that this interaction is chitin dependent. MAPK modules have been shown to be activated after PAMP treatments and abiotic stress, so they cannot conclude that this pathway is strictly chitin-dependent.

We analyzed in vivo interaction between MAPKKK5 and MKK4/MKK5 by BiFC. The BiFC signals were detected without chitin treatment, indicating that the interaction occurs at least in absence of chitin (Fig 8A). So far, although we have not examine whether mapkkk5 affects other responses, as suggested by the reviewer, it is possible that MAPKKK5 plays roles in other defense signaling and abiotic signaling.

- The authors do not mention the MEKK1, MKK1/2, MPK4 pathway (reviewed in Rasmussen et al. 2012 Front Plant Sci.). The western blots of phosphorylated MPKs very clearly show at least 3 MAPKs (3, 6 & 4), although only 3 & 6 are mentioned (for example Figs. 1C&D and 7A). Since MPK4 phosphorylation is also reduced in mapkkk5 mutants, is the MEKK1, MKK1/2, MPK4 pathway not also affected in mapkkk5 ? If so, what implications does this have?

As pointed by the reviewer, chitin-induced MPK4 activation was also reduced in *mapkkk5*. Although MPK4 is known to be activated through MKK1 and MKK2, our new data indicate that MAPKKK5 did not phosphorylate the activation loops of MKK1 and MKK2 (Figure EV5). To examine whether MKK1 and MKK2 are activated downstream MAPKKK5 in vivo, we transiently expressed MKK1 and MKK2 in protoplasts of wild or *mapkkk5* with or without chitin treatment, and analyzed activation of each MAPKK by *in vitro* phosphorylation of MPK4 using immunoprecipitated MAPKKs. However, the reasonable results were not obtained even when we did same experiments using MKK4 and MKK5. We think the possibility that transient expression of MKKs may mask the MKKs phosphorylated by MAPKKK5. Therefore, we thought that the method did not work. In addition, overexpression of MAPKKK5 in protoplasts induced activation of all of MPK3, MPK4, and MPK6 with or without each of MKK1/MKK2/MKK4/MKK5. Transient expression of MEKK1 is also known to activate the MAPKs, but the result was not consistent with genetic result using *mekk1* mutant (Ichimura et al. JBC 2006). Therefore, it seems that transient expression of MAPKKKs is not suitable to examine specificities between MAPKKKs and activation of MKKs. Thus, we could not elucidate that MAPKKK5 regulates MKK1 and MKK2 using available methods. In addition to these explanations, we discusses about a possibility that MAPKKK5 may cooperate with MEKK1 in chitin signaling in the text (Page 18, line 17 - 22).

- A simple control experiment of MPK phosphorylation by western blot in the mapkkk5 mutants upon flg22 or elf18. If this is not affected, then it would fit the conclusion that MAPKKK5 acts downstream of CERK1.

According to the reviewer's comment, we analyzed the MAPK activation in response to flg22. We found that the *mapkkk5* mutation enhanced the flg22-induced MAPK activation, suggesting that MAPKKK5 may negatively regulate flg22-induced MAPK activation. We added the data in Fig 1E. (Page 9, line 1 -4)

Specific Issues
- Fig. 3B: Could the authors explain why the MAPKKK5 protein is running at a different molecular weight at 36 hpi than 24 hpi?

We considered a possibility that post-translational modification of MAPKKK5 may occur during cell death program (Figure EV3 B). However, we have not identified the modification yet.

- Fig. 8B, C E: Is the MKKK5 kinase domain C-terminal running at a higher molecular weight than the kinase+C-terminal?

Thank you for your notice. We incorrectly wrote the protein name in the previous manuscript. "MAPKKK5-C" in Fig 8C was "GST-MAPKKK5-C". We corrected it (Fig 7C in the revised manuscript).

- Fig. 8B: in the legend it should be 'CERK1-dependent' not 'depedenet'

Thank you for your notice. We corrected it.

Supplementary Figures: the activation of MPK3/6 in SF3, SF9, SF15 appears very different between wild-types. Why is it so variable?

In our hands, timing of chitin-induced MAPK activation is slightly different among the experiments. Therefore, we evaluated the MAPK activation by their phosphorylation levels.

- Supp Fig 6A: the semi quantitative rtPCR showing increased MAPKKK5 transcript levels upon chitin treatment is not very convincing. It could be performed as qPCR and preferably with more than one time point, since three hours seems quite late.

According to the reviewer's comment, we analyzed expression of MAPKKK5 by real- time qPCR using several time points. We added the data in Figure EV2 A. (Page 8, line 1)

- Supp Fig. 12. the statistically significant differences are indicated by letter. However, for the BON3 in the WT, both the mock and the chitin treated samples are given an "a", although they appear significantly different. Maybe the chitin treated sample Col should be b, or c if it is also significant different from the two chitin treated mutants.

We re-analyzed expression of MAPKKK5-regulated genes using real-time PCR, and show the data in Fig 2F.

Referee #3
This manuscript by Yamada et al describes an Arabidopsis MAPKKK5 mutant required for chitin response. Knock-out MAPKKK5 mutant shows reduced chitin-induced MAPK activation, callose deposition and disease resistance to Alternaria brassicicola, suggesting a role of MAPKKK5 in the chitin-induced MAPK cascade. Based on PBL27 interaction with and phosphorylation of MAPKKK5, and MAPKKK5 phosphorylation of MKK4/5 in vitro, the authors propose the CERK1-PBL27-MAPKKK5-MKK4/5-MPK3/6 pathway. While the biochemical function of MAPKKK5 remains unclear, the genetic data support a role of MAPKKK5 in chitin-MAPK signaling pathway. This is a significant finding.
My major concern is that not all the data are consistent with the conclusion. For Chitin MAPK activation assays, by authors' results (Figure 1C/D, Figure 7A, Supplemental Figure 9), MPK6/3/4/11 activities were all compromised by MAPKKK5 in chitin-induced MAPK activation assays, so there should be in vitro and in vivo evidence provided that MAPKKK5 interacts with and phosphorylates MKK1/2/4/5. Does the phosphorylation state and activity of MKK1/2/4/5 change in mapkkk5, pbl27 and cerk1 mutants? And what is the biological relevance of this phosphorylation? Can the putative residues within MKK1/2/4/5 that are phosphorylated by MAPKKK5 be identified and mutated, and then shown that this affects MKK1/2/4/5 function in vivo? as MKK1/2 and MKK4/5 are the direct upstream regulators of MPK4 and MPK3/6, respectively. Moreover, the response to chitin in MKK1/2 and MKK4/5 mutants should also be tested. These are very important experiments if the authors want to conclude that MAPKKK5 links PBL27 with the MAPK module.

We thank the reviewer for careful review as well as constructive comments. As pointed by this reviewer, chitin-induced activation of MPK4 was reduced in *mapkkk5*. The result suggests that MAPKKK5 may regulate MKK1 and MKK2 that are known to function as MAPKKs for MPK4. In fact, the BiFC experiment indicates interaction between MAPKKK5 and MKK2. Therefore, we analyzed whether MAPKKK5 phosphorylates MKK1 and MKK2. However, MAPKKK5 did not phosphorylate MKK1 (Fig EV5A). Although MAPKKK5 weakly phosphorylated MKK2, the phosphorylation sites was not the activation loop of MKK2. Thus, in contrast to MKK4 and MKK5, the *in vitro* phosphorylation assay did not support the idea that MAPKKK5 regulates MKK1 and MKK2.

According to reviewer’s comment, we tried to analyze whether MKK1 and MKK2 are activated downstream of MAPKKK5 *in vivo*. Since the antibodies for MKK1 and MKK2 are not available, we transiently expressed MKK1 and MKK2 in protoplasts of wild or *mapkkk5* with or without chitin treatment, and analyzed activation of each MAPKK by *in vitro* phosphorylation of MPK4 using immunoprecipitated MAPKKs. However, the reasonable results were not obtained even when we did same experiments using MKK4 and MKK5. We think the possibility that transient expression of MKKs may mask the MKKs phosphorylated by MAPKKK5. Therefore, we thought that the method did not work.

In addition, overexpression of MAPKKK5 in protoplasts induced activation of all of MPK3, MPK4, and MPK6 with or without each of MKK1/MKK2/MKK4/MKK5. Transient expression of MEKK1 is also known to activate the MAPKs, but the result was not consistent with genetic result using *mekk1* mutant (Ichimura et al. JBC 2006). Therefore, it seems that transient expression of MAPKKKs is not suitable to examine specificities between MAPKKKs and activation of MKKs. Furthermore, *mkk1*/*mkk2* and *mkk4*/*mkk5* exhibit lethal phenotype. Therefore, we would like the reviewer to understand that it is difficult to analyze the specificities between MAPKKKs and MAPKKs using available tools. Although this reviewer requested many experiments concerning detailed regulation of MKKs, these information have been not obtained even by MEKK1, the well-investigated MAPKKK (For example, whether MEKK1 phosphorylates MKK1 and MKK2 remains to be analyzed). We hope that the editor agrees that such a experimentation is beyond the scope of this already comprehensive study that deciphered how plant PRR complexes activate downstream

substrates to initiate distinct immune responses in response to MAMP perception as pointed by Rev #1.

Other comments:
1. For the experiment "Expression of MAPKKK5 was induced by chitin", a control treatment iw required, because we don't know the effect of circadian rhythms on expression of MAPKKK5, and a qRT-PCR is also recommended to be performed.

According to the reviewer's comment, we analyzed expression of MAPKKK5 using qRT-PCR (Fig EV2).

2. Supplemental Figure 7 should change to a higher resolution picture.

We changed the picture (Figure EV2 D) according to the comment.

3. For RNA data, it is recommended to give a list of differentially expressed genes. And more genes need to be tested by qRT-PCR, only two genes is not enough.

We thanks for the advice. We added the list of differentially expressed genes in Table EV1. In addition, we showed the qRT-PCR data of four genes in Fig 2F.

2nd Editorial Decision 23 August 2016

Thank you for submitting your revised manuscript to us. All original referees have assessed it again and I copy their comments below.

As you will see, there are still some issues that need your attention. Some of them can be addressed by changes to the text (most points of referees #1 and #2), but a few need further experimental data and validation.

I would thus like to invite you to provide a point-by-point response to the criticisms and to revise your manuscript again to address the issues raised. Note that I don't expect you to solve the issue raised by referee #3 regarding the downstream targets of MAPKKK5 (point 4 of this referee's report).

REFEREE REPORTS

Referee #1:

The revised version of this manuscript addresses most of the points previously raised. In particular, it now shows that MAPKKK5 is specifically phosphorylated in vitro by PBL27, but not by BIK1. Moreover, a link between MAPKKK5 and the downstream MKK4/5 is now provided by BiFC interaction in planta, and by phosphorylation assays in vitro. This greatly improved the manuscript and altogether the data are consistent with the model that CERK1-PBL27-MAPKKK5-MKK4/5-MPK3/6 constitute a signaling module during chitin perception. This is a major advance for the field of plant immune signaling, and thus I highly recommend its swift publication.
There are, however, some points that could not be completely addressed (or raised additional questions), which I feel should be made clear and better discussed in the manuscript (see below). I don't think additional experiments are required at this stage.

1. Specificity of MAPKKK5 during chitin and flg22 signaling
According to the reviewer's comment, we analyzed the MAPK activation in response to flg22. New data indicated that the mapkkk5 mutation enhanced the flg22-induced MAPK activation, suggesting that MAPKKK5 may negatively regulate flg22-induced MAPK activation. We added the data in Fig 1E. (Page 9, line 1-4)
This is an interesting result, although somewhat unexpected. While it shows that MAPKKK5 functions as a positive regulator specifically during chitin perception, it suggests that MAPKKK5

might have different roles on other pathways. This is reminiscent of the recent work by Mithoe et al. (EMBO rep 2016), where MAPKKK7 negatively regulates MPK6 during flg22 signaling.
In turn, the enhanced MPK activation could also be part of a compensation mechanism, where the absence of MAPKKK5 somehow increases the abundance/access of other MAPKKKs that activate MPKs during flg22 signaling. Similarly, this could be caused by enhanced levels of FLS2 protein in mapkkk5 mutants. This last point could be easily tested using an anti-FLS2 antibody.
The authors should at least include a discussion of the points mentioned above in their manuscript.

2. Activation of MPK4

As pointed by Reviewer 3, the link between MPK4 activation and MAPKKK5 remains unknown. However, I don't think this is the major point to be addressed by this interesting and already quite comprehensive manuscript. Sure it raises more questions, but its merit in providing a physical link between a PRR and (at least) one MAPK cascade is of uttermost interest for the scientific community.
Thus, while the experiments suggested by Reviewer 3 are certainly pertinent (although technically challenging), I believe they should be left for a new project aimed at characterising the different MKKs in response to PAMP treatments.

Regarding the discussion about MPK4 activation on Page 19, I believe that it is within the realm of possibilities that a MAPKK other than MKK1/2 could phosphorylate MPK4 during chitin signaling (in a MAPKKK5-dependent manner). This could be included in the discussion.
I also agree that MAPKKK5 could cooperate with MEKK1, as the authors mention.

Specific points and suggestions:

1. On Fig 4 there seems to be a problem with the WB labelling. I believe MAPKKK5 is present in both lanes. The "-" should be corrected to "+".

2. Page 3 line 9 (abstract)
Chitin receptor complex CERK1-LYK5 instead of CERK1/LYK5.

3. Page 6 line 24
MAPKKK5 at the plasma membrane...

4. Page 7 line 1
PBL2 phosphorylates MAPKKK5 in a CERK1-dependent...

5. Page 7 line 12
These results raised the possibility that PBL27 directly transmits CERK1 activation to MAPKKKs, the starting modules of MAPK cascades.

6. Page 7 line 17
Space between immunity and the reference.

7. Page 14 line 13
These results suggest that PBL27-mediated phosphorylation (...) MAPKKK5 is dependent on CERK1.

8.Page 14 line 23
Because phosphorylation of the activation loop...

9. On Fig EV5A, MBP-MKK1 is used instead of the GST fusions used for the other MKKs. What is the reason for this? Should it be included in the text? In any case, the use of MBP-MKK4 as a control shows the phosphorylation assay works with MBP fusions.

10. Page 16 line 20
However, the interaction with full length MAPKKK5 could not be observed in the yeast two hybrid and in vitro experiments; instead only the C-terminal...

11. Page 17 line 6
...by PBL2 in a CERK1-dependent manner.

Referee #2:

IMPORTANT COMMENTS TO REBUTTAL ARE CAPITALIZED BELOW. IN ADDITION - In Figure 4C, the panel forMKKK5-GFP the + and - have to be swopped around for the figure to make sense. And on p14 they refer to PBK27 twice, instead of PBL27.
Referee #2
Main Issues
- Figure 2: 339 genes: it would be nice to have a list of genes different in the mkkk5 mutant.
We thanks for the advice. We added the list in Table EV1.
OK
- Figs. 3A & 4E: The tomato MAPKKK SlMKKKα also causes cell death when overexpressed in N. Benthamiana (del Pozo et al., 2004 EMBO J.). Could the authors comment on how this might relate to MAPKKK5? For example, is it possible that MKKK5 levels are homeostatically limited as the protein may be a positive regulator of cell death? In that case, to study the protein in Arabidopsis maybe the authors could try to make the lines in an eds1 or ndr1 background, in case the cause of the cell death is some R protein mediated pathway (reviewed in Rodriguez et al. 2015 FEBS J). Another suggestion would be to check the level of PBL27 in the mkkk5 mutant background (mkkk5/PBL27-HA).
In addition to tomato MAPKKK SlMKKKα, over-expression of tobacco MEKK family is known to induce cell death in plant (Hashimoto et al. BMC Plant Biol 2012). Since expression of constitutively active forms of MKK4 and MKK5 also causes cell death, we consider that ectopic activation of MAPK cascades likely results in cell death phenotype. To examine whether MAPKKK5 is involved in R-gene-mediated HR cell death, we inoculated the mapkkk5 plant with Pst DC3000: avrRpm1 and Pst DC3000: avrRpt2. We observed same levels of cell death between WT and mapkkk5, indicating that MAPKKK5 is not involved in RPM1- and RPS2-mediated HR.
IT APPEARS THAT THE AUTHOR'S DO NOT UNDERSTAND THE PROBABILITY THAT CELL DEATH IN PLANTS OVER-EXPRESSING KINASE ACTIVE FORMS OF MAPKKK5 IS DUE TO R PROTEIN ACTIVATION. THIS IS A PITY AS THE CELL DEATH AND RELATED ASSAYS ARE FAIRLY PROMINENT IN THE MANUS. AND EVIDENCE FOR R PROTEIN ACTIVATION VIA EDS1/NDR1 IN THE N BENTH SYSTEM HAS BEEN DOCUMENTED (LIU ET AL 2002 PLANT J; TRAN ET AL. 2016 VIROLOGY). IN ADDITION THE AUTHOR'S COMMENT 'WE OBSERVED SAME LEVELS OF CELL DEATH BETWEEN WT AND MAPKKK5, INDICATING THAT MAPKKK5 IS NOT INVOLVED IN RPM1- AND RPS2-MEDIATED HR' IS HARDLY REASSURING. THIS SHOULD EASILY BE CHECKED BY INCLUDING A VIGS OF EDS1 IN BENTH AND/OR MENTIONING THIS POSSIBILITY IN THE MS (SEE RELATED COMMENT BELOW.
- Fig. 3C: MAPKKK5 is more abundant in the soluble fraction than in the microsomal fraction. Could the authors clarify why they focus on the microsomal fraction for the co-IP in Fig. 4 instead of the soluble fraction? The authors do not comment much on the possible reason for membrane localization of the MKKK5.
When we used total proteins for Co-IP, we could not detect the interaction between PBL27 and MAPKKK5. Because the results of the BiFC experiment indicated that these proteins interact with each other at the plasma membrane, we used the membrane fraction to enrich both proteins. We added the reason in the text. (Page 11, line 4 - 8)
OK
- Fig. 3D: the authors show the localization of wild-type MAPKKK5 and see signal in the cytosol and plasma membrane, but no plasmolysis is done to support the claim of membrane localization (while this is done in Fig. 4D for the mutant MKKK5).
According to the reviewer's comment, we re-analyzed it by plasmolysis. We added the data in Fig 3B.
OK
- Fig. 4C: it seems strange that MKKK5 is not enriched by IP, could authors explain this?
We thanks for your notice. "Input" was not suitable for the figure. We modified the figure (Fig 3E).
OK

- Fig. 5: the authors speculate that PBL27 is degraded in the presence of MKKK5. Could this be tested by adding MG132 to inhibit proteasome-dependent degradation? This would at least give an indication of a mechanism by which PBL27 disappears.
According to the reviewer's comment, we analyzed whether MG132 affects the protein levels of PBL27 and MAPKKK5. Degradation of both PBL27 and MAPKKK5 was suppressed by MG132, suggesting that the protein levels of these proteins were regulated in the proteasome dependent manner. We added the data in Fig 4C. (Page 13, line 23 - Page 14, line 1)
OK
- Fig. 8: shows that MAPKKK5 can phosphorylate MKK4/MKK5, however they do not show that this interaction is chitin dependent. MAPK modules have been shown to be activated after PAMP treatments and abiotic stress, so they cannot conclude that this pathway is strictly chitin-dependent.
We analyzed in vivo interaction between MAPKKK5 and MKK4/MKK5 by BiFC. The BiFC signals were detected without chitin treatment, indicating that the interaction occurs at least in absence of chitin (Fig 8A). So far, although we have not examine whether mapkkk5 affects other responses, as suggested by the reviewer, it is possible that MAPKKK5 plays roles in other defense signaling and abiotic signaling.
OK, ALTHOUGH IT SHOULD BE CHECKED WHETHER THE AUTHOR'S BIFC EXPERIMENTS IN GENERAL MEET THE CONTROL REQUIREMENTS OUTLINED RECENTLY IN THE PLANT CELL COMMENTARY OF KUDLA & BOCK (OUTLINED IN THEIR FIG. 1D-F AND TEXT).
- The authors do not mention the MEKK1, MKK1/2, MPK4 pathway (reviewed in Rasmussen et al. 2012 Front Plant Sci.). The western blots of phosphorylated MPKs very clearly show at least 3 MAPKs (3, 6 & 4), although only 3 & 6 are mentioned (for example Figs. 1C&D and 7A). Since MPK4 phosphorylation is also reduced in mapkkk5 mutants, is the MEKK1, MKK1/2, MPK4 pathway not also affected in mapkkk5 ? If so, what implications does this have?
As pointed by the reviewer, chitin-induced MPK4 activation was also reduced in mapkkk5. Although MPK4 is known to be activated through MKK1 and MKK2, our new data indicate that MAPKKK5 did not phosphorylate the activation loops of MKK1 and MKK2 (Figure EV5). To examine whether MKK1 and MKK2 are activated downstream MAPKKK5 in vivo, we transiently expressed MKK1 and MKK2 in protoplasts of wild or mapkkk5 with or without chitin treatment, and analyzed activation of each MAPKK by in vitro phosphorylation of MPK4 using immunoprecipitated MAPKKs. However, the reasonable results were not obtained even when we did same experiments using MKK4 and MKK5. We think the possibility that transient expression of MKKs may mask the MKKs phosphorylated by MAPKKK5. Therefore, we thought that the method did not work. In addition, overexpression of MAPKKK5 in protoplasts induced activation of all of MPK3, MPK4, and MPK6 with or without each of MKK1/MKK2/MKK4/MKK5. Transient expression of MEKK1 is also known to activate the MAPKs, but the result was not consistent with genetic result using mekk1 mutant (Ichimura et al. JBC 2006). Therefore, it seems that transient expression of MAPKKKs is not suitable to examine specificities between MAPKKKs and activation of MKKs. Thus, we could not elucidate that MAPKKK5 regulates MKK1 and MKK2 using available methods. In addition to these explanations, we discusses about a possibility that MAPKKK5 may cooperate with MEKK1 in chitin signaling in the text (Page 18, line 17 - 22).
Vaguely OK, but entirely open ended...
- A simple control experiment of MPK phosphorylation by western blot in the mapkkk5 mutants upon flg22 or elf18. If this is not affected, then it would fit the conclusion that MAPKKK5 acts downstream of CERK1.
According to the reviewer's comment, we analyzed the MAPK activation in response to flg22. We found that the mapkkk5 mutation enhanced the flg22-induced MAPK activation, suggesting that MAPKKK5 may negatively regulate flg22-induced MAPK activation. We added the data in Fig 1E. (Page 9, line 1 -4)
OK, BUT NOTE THAT THIS WOULD SUPPORT THE POSSIBILITY THAT OVER-EXPRESSION OF KINASE ACTIVE FORMS OF MAPKKK5 WOULD INHIBIT FLG22-INDUCED MAPK ACTIVATION WHICH WOULD TRIGGER THE ACTIVATION OF R PROTEINS GUARDING COMPONENTS OF THE MAPK CASCADES SUCH AS THAT INVOLVING MPK4 (ZHANG ET AL 2012 CELL HOST MICROBE). AGAIN (SEE ABOVE), THIS SHOULD EASILY BE CHECKED BY INCLUDING A VIGS OF EDS1 IN BENTH AND/OR MENTIONING THIS POSSIBILITY IN THE MS.
Specific Issues
- Fig. 3B: Could the authors explain why the MAPKKK5 protein is running at a different molecular weight at 36 hpi than 24 hpi?

We considered a possibility that post-translational modification of MAPKKK5 may occur during cell death program (Figure EV3 B). However, we have not identified the modification yet.
OK
- Fig. 8B, C E: Is the MKKK5 kinase domain C-terminal running at a higher molecular weight than the kinase+C-terminal?
Thank you for your notice. We incorrectly wrote the protein name in the previous manuscript. "MAPKKK5-C" in Fig 8C was "GST-MAPKKK5-C". We corrected it (Fig 7C in the revised manuscript).
OK
- Fig. 8B: in the legend it should be 'CERK1-dependent' not 'depedenet'
Thank you for your notice. We corrected it.
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Supplementary Figures: the activation of MPK3/6 in SF3, SF9, SF15 appears very different between wild-types. Why is it so variable?
In our hands, timing of chitin-induced MAPK activation is slightly different among the experiments. Therefore, we evaluated the MAPK activation by their phosphorylation levels.
OK
- Supp Fig 6A: the semi quantitative rtPCR showing increased MAPKKK5 transcript levels upon chitin treatment is not very convincing. It could be performed as qPCR and preferably with more than one time point, since three hours seems quite late.
According to the reviewer's comment, we analyzed expression of MAPKKK5 by real- time qPCR using several time points. We added the data in Figure EV2 A. (Page 8, line 1)
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- Supp Fig. 12. the statistically significant differences are indicated by letter. However, for the BON3 in the WT, both the mock and the chitin treated samples are given an "a", although they appear significantly different. Maybe the chitin treated sample Col should be b, or c if it is also significant different from the two chitin treated mutants.
We re-analyzed expression of MAPKKK5-regulated genes using real-time PCR, and show the data in Fig 2F.
OK

Referee #3:

The authors have provided additional experiments to improve the manuscript but several points still require attention:

Major points:

1. The specificity of the interaction of PBL27 with MAPKKK5 is a key issue. In Y2H assays, only the C-terminal part interacts. However, the kinase domain of MAPKKK3 shows interaction with PBL27 but was not investigated further. Given that the Y2H are not completely satisfying, the BiFC data are very important to prove specificity and therefore need some further controls with related MAPKKKs, including MAPKKK3.
In this respect, it is not clear why the authors show in Fig. 3E and 3F only interaction with the mutant MAPKKK5 protein. The interaction should be shown with the wild type MAPKKK5.

2. From the experiments shown in Fig. 4C, it is clear that the MAPKKK5 protein is under strong control of protein stability. However, this tool should allow to perform most of the experiments that were currently only carried out with mutant or partial versions with the full length MAPKKK5 protein.

3. Another major point is the ability of PBL27 to phosphorylate MAPKKK5. Here, the authors carried out in vitro phosphorylation assays of PBL27 with different parts of MAPKKK5 as substrate and found phosphorylation only with the C-terminal peptide region. No particular phosphorylation motifs were retrieved questioning the specificity of the kinase reaction. Since the GST tags on the MAPKKK5 protein might be inhibiting the access of PBL27 to the phosphorylation sites, tag-free recombinant MAPKKK5 should be used and the experiments should also include the full length MAPKKK5 protein.

4. Since MAPKKK5 is a putative MAPKKK, the question of its downstream targets is essential. Since MAPKKK5 mutants are unable to activate MPK3, 4 and 6 in response to chitin, the authors tested the ability of MAPKKK5 to interact and phosphorylate the respective upstream MAPKKs MKK1, 2, 4 and 5. However, they investigated by BiFC the interaction of the four MAPKKs with the inactive MAPKKK5 version only, revealing interaction of MAPKKK5 with MKK2, 4 and 5. Interaction should be tested with the MAPKKK5 full length and at least in Y2H with all ten MAPKKs.

5. Phosphorylation assays were carried out with MAPKKK5-KD only, although kinase domains of MAPKKKs alone are well known to lose their specificity, possibly explaining why among the MAPKKs, only MKK4 and 5 were found to be phosphorylated in their activation loops, although MKK2 was also phosphorylated. Here, it would be necessary to carry out the phosphorylation assays using the full length MAPKKK5 proteins.

Minor points:

1. The transcriptome data of mapkkk5 should be compared to other chitin-related data sets for their overlap to allow the statement:"These results point to the significance of MAPKKK5-mediated signaling in chitin-triggered transcriptional reprogramming."

2. It should be clarified why BON3 does not appear in the list of differentially expressed genes?

Overall, the genetic data clearly show that MAPKKK5 is an upstream regulator of the chitin-induced MPK3,4 and 6 pathways
but the biochemical mechanism of its action lacks in vivo evidence. Given the problems to provide such direct evidence, the indirect in vitro evidence still needs to be substantially refined.

2nd Revision - authors' response 26 August 2016

We thank the reviewers for careful review as well as constructive comments. According to the reviewers' suggestions, we have thoroughly revised the manuscript. Below, we provide a detailed point-by-point response addressing the reviewers' criticisms.

Referee #1:

The revised version of this manuscript addresses most of the points previously raised. In particular, it now shows that MAPKKK5 is specifically phosphorylated in vitro by PBL27, but not by BIK1. Moreover, a link between MAPKKK5 and the downstream MKK4/5 is now provided by BiFC interaction in planta, and by phosphorylation assays in vitro. This greatly improved the manuscript and altogether the data are consistent with the model that CERK1-PBL27-MAPKKK5-MKK4/5-MPK3/6 constitute a signaling module during chitin perception. This is a major advance for the field of plant immune signaling, and thus I highly recommend its swift publication.
There are, however, some points that could not be completely addressed (or raised additional questions), which I feel should be made clear and better discussed in the manuscript (see below). I don't think additional experiments are required at this stage.

We thank the reviewer for positive comments.

1. Specificity of MAPKKK5 during chitin and flg22 signaling
This is an interesting result, although somewhat unexpected. While it shows that MAPKKK5 functions as a positive regulator specifically during chitin perception, it suggests that MAPKKK5 might have different roles on other pathways. This is reminiscent of the recent work by Mithoe et al. (EMBO rep 2016), where MAPKKK7 negatively regulates MPK6 during flg22 signaling. In turn, the enhanced MPK activation could also be part of a compensation mechanism, where the absence of MAPKKK5 somehow increases the abundance/access of other MAPKKKs that activate MPKs during flg22 signaling. Similarly, this could be caused by enhanced levels of FLS2 protein in mapkkk5 mutants. This last point could be easily tested using an anti-FLS2 antibody. The authors should at least include a discussion of the points mentioned above in their manuscript.

We thank the reviewer for constructive comments. According to the comment, we discussed them in the MS (Page 18, line 10 – page 19 line 4), because we do not have the anti-FLS2 antibody.

2. Activation of MPK4
As pointed by Reviewer 3, the link between MPK4 activation and MAPKKK5 remains unknown. However, I don't think this is the major point to be addressed by this interesting and already quite comprehensive manuscript. Sure it raises more questions, but its merit in providing a physical link between a PRR and (at least) one MAPK cascade is of uttermost interest for the scientific community. Thus, while the experiments suggested by Reviewer 3 are certainly pertinent (although technically challenging), I believe they should be left for a new project aimed at characterising the different MKKs in response to PAMP treatments.

We thank for your understanding.

Regarding the discussion about MPK4 activation on Page 19, I believe that it is within the realm of possibilities that a MAPKK other than MKK1/2 could phosphorylate MPK4 during chitin signaling (in a MAPKKK5-dependent manner). This could be included in the discussion.
I also agree that MAPKKK5 could cooperate with MEKK1, as the authors mention.

We thank for the advise. According to the comment, we added the discussion in the MS (Page 19, line 17-18).

Specific points and suggestions:
1. On Fig 4 there seems to be a problem with the WB labelling. I believe MAPKKK5 is present in both lanes. The "-" should be corrected to "+".

We thank for your notice. We corrected Fig 4C.

2. Page 3 line 9 (abstract)
Chitin receptor complex CERK1-LYK5 instead of CERK1/LYK5.

We thank for your advice. We changed it.

3. Page 6 line 24
MAPKKK5 at the plasma membrane...

We thank for your advice. We corrected it.

4. Page 7 line 1
PBL2 phosphorylates MAPKKK5 in a CERK1-dependent...

We thank for your advice. We corrected it.

5. Page 7 line 12
These results raised the possibility that PBL27 directly transmits CERK1 activation to MAPKKKs, the starting modules of MAPK cascades.

We thank for your advice. We corrected it.

6. Page 7 line 17
Space between immunity and the reference.

We thank for your advice. We corrected it.

7. Page 14 line 13
These results suggest that PBL27-mediated phosphorylation (...) MAPKKK5 is dependent on CERK1.
We thank for your advice. We changed it.

8.Page 14 line 23
Because phosphorylation of the activation loop...
We thank for your advice. We corrected it.

9. On Fig EV5A, MBP-MKK1 is used instead of the GST fusions used for the other MKKs. What is the reason for this? Should it be included in the text? In any case, the use of MBP-MKK4 as a control shows the phosphorylation assay works with MBP fusions.

Since GST-MKK1 was the same size as GST-MAPKKK5-KD, we used MBP-tag for MKK1. The result (Fig EV5A) that MBP-MKK4 was phosphorylated at same level with GST-MKK4 by GST-MAPKKK5 KD indicated that this assay worked. We added the information in the figure legend.

10. Page 16 line 20
However, the interaction with full length MAPKKK5 could not be observed in the yeast two hybrid and in vitro experiments; instead only the C-terminal...
We thank for your advice. We changed it.

11. Page 17 line 6
...by PBL2 in a CERK1-dependent manner.
We thank for your advice. We changed it.

Referee #2:

1) In Figure 4C, the panel forMKKK5-GFP the + and - have to be swopped around for the figure to make sense. And on p14 they refer to PBK27 twice, instead of PBL27.

We thank for your notice. We corrected them.

*2) It appears that the authors do not understand the probability that cell death in plants over-expressing kinase active forms of MAPKKK5 is due to R protein activation. This is a pity as cell death and related assays are fairly prominent in the manus and evidence for R protein activation via EDS1/NDR1 in the N benth system has been documented (LIU et al 2002 PLANT J; TRAN et al 2016 VIROLOGY). In addition, the authors comment " we observed same levels of cell death between WT and mapkkk5, indicating that MAPKKK5 s not involved in RPM1- and RPS-2mediated HR" is hardly reassuring. This should easily be checked by including a VIGS of EDS1 in N bentha and / or mentioning this possibility in the MS (See related comment belo*w).

We thank for your advice. We misunderstood the comment. We added the discussion concerning a possibility that cell death induced by over-expression of MAPKKK5 would be caused by R protein activation in the MS (Page 19, line 5 – 10). We could not test a VIGS of EDS1, because we do not have experimental system for VIGS.

3) Although it should be checked whether the author's BiFC experiments in general meet the control requirements outlined recently in the Plant Cell commentary of Kudla & Bock (Outlined in the Fig 1D-F and text).

We thank for your advice. We checked the guidelines for avoiding artefactual interactions described in the Plant Cell commentary. We used BIK1 as a BiFC control for PBL27. Since BIK1 and PBL27 belong to same VII subfamily of RLCK and both proteins are localized at the plasma membrane, BIK1 is likely the appropriate control. In Fig8, we used the GUS protein for the control, because it shows cytoplasmic localization as MKKs do. In addition, because MKK1 did not interact with MAPKKK5, MKK1 is the appropriate control for interaction of MAPKKK5 with MKK2, MKK4, and MKK5.

4) The mapkkk5 mutation enhanced the flg22-induced MAPK activation, suggesting that MAPKKK5 may negatively regulate flg22-induced MAPK activation. This would support the possibility that over-expression of kinase active forms of MAPKKK5 would inhibit flg22-induced MAPK activation which would trigger the activation of R proteins guarding components of the MAPK cascades such

as that involving MPK4 (ZHANG et al 2012 CELL HOST MICROBE). again (See above), This should easily be checked by including a VIGS of eDS1 in N benta and / or mentioning this possibility inn the MS.

We thank for your advice. As mentioned above, we added the discussion concerning a possibility that cell death induced by over-expression of MAPKKK5 may be caused by R protein activation in the MS (Page 19, line 5 – 10).

Referee #3:

1. The specificity of the interaction of PBL27 with MAPKKK5 is a key issue. In Y2H assays, only the C-terminal part interacts. However, the kinase domain of MAPKKK3 shows interaction with PBL27 but was not investigated further. Given that the Y2H are not completely satisfying, the BiFC data are very important to prove specificity and therefore need some further controls with related MAPKKKs, including MAPKKK3.

In this respect, it is not clear why the authors show in Fig. 3E and 3F only interaction with the mutant MAPKKK5 protein. The interaction should be shown with the wild type MAPKKK5.

We thank the reviewer for careful review.

As shown in Fig 3C, co-expression of PBL27 and WT-MAPKKK5 in Nb leaves strongly reduced both protein levels and induced cell death. Therefore, we were not able to use WT-MAPKKK5 for the experiments of Fig 3E and 3F.

Although PBL27 interacts with the kinase domain of MAPKKK3, the *mapkkk3* mutation did not affect chitin-induced MAPK activation. Therefore, we did not do further experiments. However, it is possible that the PBL27 – MAPKKK3 module may function in other signaling pathways. Although we focused on MAPKKK5 in the MS because MAPKKK3 is not involved in chitin-induced MAPK activation, further experiment will reveal the role of the PBL27 – MAPKKK3 module in plant signaling.

The reviewer also requested the BiFC experiment for interaction of PBL27 with additional 20 MEKK family as the controls of BiFC. As pointed out by the reviewer 2, BiFC sometime produces false positive and false negative results. Therefore, we do not think that BiFC analyses requested by the reviewer are useful to prove the specificity of interaction between PBL27 and MAPKKK5. Rather, we think that it will be useful to identify new MAPKKKs interacted with PBL27.

2. From the experiments shown in Fig. 4C, it is clear that the MAPKKK5 protein is under strong control of protein stability. However, this tool should allow to perform most of the experiments that were currently only carried out with mutant or partial versions with the full length MAPKKK5 protein.

We thank for your advice. As shown in Fig 4C, PBL27-HA and MAPKKK5-GFP were accumulated by MG132, suggesting that the protein levels of PBL27 and MAPKKK5 are regulated in the proteasome pathway. However, we do not know whether these accumulated proteins have biological activities. Therefore, we think that it is difficult to interpret the data obtained using MG132.

3. Another major point is the ability of PBL27 to phosphorylate MAPKKK5. Here, the authors carried out in vitro phosphorylation assays of PBL27 with different parts of MAPKKK5 as substrate and found phosphorylation only with the C-terminal peptide region. No particular phosphorylation motifs were retrieved questioning the specificity of the kinase reaction. Since the GST tags on the MAPKKK5 protein might be inhibiting the access of PBL27 to the phosphorylation sites, tag-free recombinant MAPKKK5 should be used and the experiments should also include the full length MAPKKK5 protein.

We thank for your comment. BIK1 belongs to same subfamily of RLCK as PBL27. So far, the phosphorylation sites of RbohD by BIK1 have been identified (Kadota et al. Mol Cell 2014). However, no particular phosphorylation motifs have been identified. Therefore, comprehensive

study of phosphorylation sites of RLCK family would be required to identify the phosphorylation motifs.

In Fig 7B, we used tag-free MAPKKK5-KD:C. In this assay, PBL27 did not phosphorylate MAPKKK5-KD:C in absence of CERK1, but the PBL27 protein phosphorylated by CERK1 could phosphorylate MAPKKK5-KD:C. Thus, the phosphorylation of MAPKKK5 by PBL27 is dependent on CERK1.

We could not purify full length MAPKKK5, because the protein was not expressed in E. coli expression system. However, based upon the data of MAPKKK5-KD:C, we could speculate a possible regulatory mechanism of full length MAPKKK5.

4. Since MAPKKK5 is a putative MAPKKK, the question of its downstream targets is essential. Since MAPKKK5 mutants are unable to activate MPK3, 4 and 6 in response to chitin, the authors tested the ability of MAPKKK5 to interact and phosphorylate the respective upstream MAPKKs MKK1, 2, 4 and 5. However, they investigated by BiFC the interaction of the four MAPKKs with the inactive MAPKKK5 version only, revealing interaction of MAPKKK5 with MKK2, 4 and 5. Interaction should be tested with the MAPKKK5 full length and at least in Y2H with all ten MAPKKs.

We thank for your comment. As pointed out by the reviewer1, we would like the reviewer to understand that comprehensive characterization of the different MKKs in chitin signaling is beyond the scope of this already comprehensive study.

5. Phosphorylation assays were carried out with MAPKKK5-KD only, although kinase domains of MAPKKKs alone are well known to lose their specificity, possibly explaining why among the MAPKKs, only MKK4 and 5 were found to be phosphorylated in their activation loops, although MKK2 was also phosphorylated. Here, it would be necessary to carry out the phosphorylation assays using the full length MAPKKK5 proteins.

As mentioned above, full length MAPKKK5 protein could not be purified by the recombinant protein expression systems that we tested. In general, other domains than the kinase domains are considered to be involved in their specificity. However, the kinase domain of MAPKKK5 strongly phosphorylates MKK4 and MKK5, and no or very weak phosphorylation of MKK1 or MKK2, respectively, were detected. The data imply that the kinase domain of MAPKKK5 may contribute to determine the specificity.

Minor points:
1. The transcriptome data of mapkkk5 should be compared to other chitin-related data sets for their overlap to allow the statement:"These results point to the significance of MAPKKK5-mediated signaling in chitin-triggered transcriptional reprogramming."

We thank for your advice. We changed the statement to “These results indicate that MAPKKK5 regulates chitin-triggered transcriptional reprogramming”.

2. It should be clarified why BON3 does not appear in the list of differentially expressed genes?

We thank for your notice. Gene number of BON3 in Fig 2 F was not correct. We corrected it.

3rd Editorial Decision 30 August 2016

Thank you for submitting your revised manuscript to us. I appreciate the introduced changes and the point-by-point response, and I am happy to accept your manuscript for publication in the EMBO Journal.

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This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

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The data shown in figures should satisfy the following conditions:

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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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