THE EMBO JOURNAL

Manuscript EMBO-2012-84303

The Arabidopsis PEPR pathway couples local and systemic plant immunity

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Review timeline:

Submission date:	21 December 2012
Editorial Decision:	11 February 2013
Revision received:	11 June 2013
Editorial Decision:	12 July 2013
Revision received:	08 October 2013
Editorial Decision:	18 October 2013
Revision received:	24 October 2013
Accepted:	31 October 2013

Editor: Karin Dumstrei

Transaction Report:

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

1st Editorial Decision 11 February 2013

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below. The referees also had access to the related manuscript that has been submitted and by now accepted elsewhere.

As you can see the referees find the link between ATPep signaling and systemic acquired signaling (SAR) interesting. However, they also raise a number of critical points: the lack of statistical analysis, the rational for some of the experiments and for why some genes were focused on is not clear, more mechanistic insight into how PERP trigger SAR is needed and the causal link between many of observations is not sufficiently sorted out. In addition, the referees also bring up the issue of your related submission both in the comments to the authors as well in follow up emails to me. The referees recognize that your related submission is a separate story, but also point out that that some of the figures in the related submission should have been included in the EMBO Journal submission. So clearly this is not an optimum situation and it would have been desirable had the submissions been handled in a different manner. I hope that you take this comment into consideration for future submissions.

Having taken all the points into consideration and recognizing that the related submission is for the most part a separate story on its own, I would like to invite you to submit a suitably revised manuscript should you be able to address the raised concerns in full with the inclusion of additional data. 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.

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://www.nature.com/emboj/about/process.html

REFEREE REPORTS

Referee #1

The manuscript by Ross et al. reports a novel role of PEPR receptors in systemic acquired resistance mediated by SA and JA. By gene expression profile analyses, the authors show that PROPEP2 and PROPEP3 expression is associated with persistent defenses induced by elf18. A comparison of PEP- and elf18-induced transcriptomes suggested that while SA-, JA, and ET-responsive genes were all enriched in elf18 and PEP treated seedlings, the PEP treatment appears to further activate subsets of JA-response genes. The PEP-induced disease resistance to Pseudomonas syringae requires SID2, but not DDE2 and EIN2, indicating that SA plays a primary role. Interestingly, the Pseudomonas-induced expression of PR2 and bacterial resistance in systemic leaves were significantly compromised in pepr1pepr2 double mutants. PROPEP2 and PROPEP3 proteins were found to accumulate in local leaves, but not systemic leaves, suggesting that PEPs act in local leaves to induce SAR. Application of PEPs to local leaves resulted in the accumulation of PR1 and PDF1.2a transcripts in systemic leaves. Furthermore, the PEP application allowed protection against Colletotrichum higginsianum in systemic leaves in a manner dependent on the JA signaling. The authors propose a model in which MAMP-triggered immunity induces PROPEP expression and PEPR activation in local leaves which subsequently generates systemic signals through ET, SA and JA pathways. Overall, the manuscript describes some highly important findings that are of interest to broad audience. However, as detailed below, several issues need to be resolved prior to publication.

1. The mechanisms for PROPEP induction following MAMP and Pst treatment are not clear. Are SA, ET and JA required for the induction?
2. Does PEP treatment result in SAR against Pseudomonas syringae?
3. The mechanism by which the PEPR pathway triggers SAR is not clear. The authors propose that the PEPR pathway trigger the production of systemic signals. It is necessary to show that petiole exudates from PEP-treated leaves are indeed active in SAR induction. Furthermore, it is necessary to test if the previously reported players in SAR, such as FMO1 and ALD1, are required for PEP-induced SAR.
4. Figure 2 showed that PROPEP2 overexpressed in plants can interact with PEPR1. This tells nothing about how PEPs get out of the cell or the nature of the ligand in plants.
5. Figure 4C, I think the authors over-interpreted the results. The results simply showed that JA is essential for PDF induction, SA antagonizes this, whereas ET enhances the induction.

Referee #2

This project initiated with the previous observation that rsw3, a mutant isolated in a genetic screen for Arabidopsis mutants affected in responses triggered by the bacterial MAMP elf18, was affected in late, but not early, elf18-induced responses and was highly susceptible to bacterial infection (Lu et al., PNAS 2009). Therefore, the central hypothesis of this work was that sustained immune responses triggered by elf18 were key to robust immunity. The authors concentrated their efforts on the characterization of the PROPE2 and PROPEP3 genes that encode putative precursors for endogenous peptides acting as DAMPs. It has been previously postulated that the perception of the Pep peptides by their corresponding receptors PEPR1 and PEPR2 would be part of an amplification system following MAMP perception (Huffaker and Ryan, PNAS 2007), but this hypothesis has never been addressed genetically and/or mechanistically. The bulk of this work has been therefore aimed at testing this hypothesis. In this manuscript, compelling evidence is given that the Pep/PEPR perception system is indeed required for the establishment of robust local immunity (as demonstrated with the infection with weakly virulent strains of Pseudomonas and Colletotrichum),

but also to systemic acquired resistance (SAR). While these findings are of high interest, critical points still need to be answered.

- It is not clear why the authors initially chose to focus specifically on the expression of PROPEP2 and PROPEP3 in the rsw3 mutant (Figure 1). Was it because these genes were found to be particularly down-regulated in rsw3 in microarray experiments? Please clarify.

- Unlike what is concluded by the authors, no causal link between sustained MTI response, PEPR1/2 and immunity has been demonstrated. The authors need to test if elf18-induced resistance is affected in pepr1 pepr2 plants?

- Similarly, no causal link between MAMP perception, PEPR1/2 and systemic immunity has been demonstrated. The crucial experiment is to test if SAR itself or at least systemic expression of marker genes is affected in pepr1 pepr2 plants upon treatment with elf18, as MAMPs are sufficient to induce SAR. The authors have only used AvrRpm1 (thereby inducing ETI) to trigger SAR in this manuscript.

- More importantly, the whole project leading to the demonstration that the Pep/PEPR system is important for induced local and systemic immunities upon MAMP perception was based on the original observation that rsw3 is impaired in the expression of PROPEP2 and PROPEP3. Yet, rsw3 is not impaired in responses triggered by the MAMP flg22 (Lu et al., PNAS 2009), potentially arguing against the generality of the proposed model. The authors must therefore also test alongside elf18, if the local and systemic immunities induced by flg22 are impaired in pepr1 pepr2 plants. If this is not the case, the authors should revise their model.

- The results presented in Figure S1 only show that Pep2 can induce PR1 and PR2 expression in both WT and rsw3 (ie. that Pep2-induced expression of PR1 and PR2 is not affected in rsw3). They do not allow the conclusion that "the increased supply of the PEPR ligand can alleviate or rescue the defects of EFR signalling in rsw3 plants".

- Figure 4: the ein2 mutant is affected in Pep2-induced defense gene expression and potentially Pep2/Pep3-induced resistance to Pseudomonas (which is difficult to judge in the absence of statistical analysis...). Given that ethylene perception is important for the expression of some PRRs (Boutrot et al., PNAS 2010), it seems essential to test if PEPR1 and PEPR2 are properly expressed in ein2, before trying to reach any conclusion on the role of ethylene in responses triggered by the activation of PEPR1/2.

- In Figure 6B, it is puzzling that the endogenous PROPEP2 and PROPEP3 could not been detected with the anti-PROPEP2 and anti-PROPEP3 in the corresponding pPROPEP-PROPEP transgenic lines generated. This should have been at least mentioned and discussed.

- How do the authors explain the local enhanced susceptibility of the pepr1 pepr2 plants? This is not integrated or discussed in their model

- All figures need statistical analysis.

Editorial points:
- The Introduction is poorly referenced with whole section and statements often having no reference or alternatively having only references to review instead of original research papers.
- Introduction and Discussion: cite Navarova et al. Plant Cell 2012 as a recent example of a link between MAMP perception and SAR, as MAMP perception induces the production of pipecolic acid, which appears to be an important mediator of SAR.
- The authors must mention and discuss the recent work from the Berkowitz laboratory (Ma et al., PNAS 2012) that showed an interdependency between responses triggered by MAMPs and Pep peptides.
- Add references for the delta avrpto/delta avrptoB and path-29 strains.
- Replace 'ROS spiking' by 'ROS burst'.
- bak1 mutants were also shown to be affected in Pep1-induced ethylene production (Roux et al., Plant Cell 2011).
- Regarding PEPR1-BAK1 association, also cite Postel et al., Eur J Cell Biol 2010, who showed that

PEPR1 interacts constitutively with BAK1 in yeast two-hybrid assays. Also, to be more correct, Schulze et al. JBC 2010 never conclusively demonstrated that "PEPR also undergoes ligand-induced BAK1 association upon Pep1 application". They only showed that a radioactive band migrating a similar size of PEPR1/2 could be detected in BAK1 immunoprecipitates after Pep1 treatment.
- Specify in Materials and Methods, which are the specific alleles of the different mutants used in the study.

Referee #3

This manuscript describes a between AtPep peptides (danger-associated molecular patterns, DAMPs) and systemic acquired resistance (SAR) in Arabidopsis. The idea that DAMP signaling enhances SAR is new and quite interesting. This is addressed in this study mainly through measurement of pathogen growth and/or global gene expression. This work also confirms and/or extends the previously shown interactions between BAK1 and PEPR1 and between PEPR1 and PROPEP2. Except for the SAR part, this manuscript appears to have substantial overlaps with two other pending papers, one from this lab and the other from another lab.

Major comments:

1. One of the most difficult aspects to interpret the results presented in the paper is the lack of statistical analysis for most figures, especially when two means have similar values or their standard deviations are high. This analysis must be performed for all figures to validate the authors' conclusions. Just to give two examples (but the authors should do this for all figures). The authors claim that there is no difference in SA levels presented in Fig. 5D. However, I can see that the SA level in systemic leaves is lower in the pepr1 pepr2 plants. Is this not statistically significant? The authors claim that Fig S3D shows PEPR-dependent root inhibition. However, the SD values seem to overlap (even though the means are different). How can this be statistically significant?

2. The authors claim that PROPEP2 (i.e., in contrast to the processed elicitor) could be recognized by PEPR1 in planta. This is based simply on co-IP result using transient co-expression of PROPEP2 and PEPR1 (Fig. 2). This claim requires more experimental support. In cell extracts used for co-IP, PROPEP2 and PEPR1, which may be in different cellular locations in vivo, are artificially mixed together. Cell-based imaging is needed.

3. The immunoblots for PROPEP2 (antiPROPEP2 and anti-GFP) in Fig 6B is of poor publication quality. It needs to be repeated. The 35S YFP sample lacks a band present in the WT which the authors claim is a non-specific band (marked with an asterisk). Also, there is a lower rectangle beneath each of the western blots for which there is no explanation as to what these bands represent.

4. Parts of this paper lack a logical flow; some experiments seem to be added to the paper just because they had been done. It seems that the microarray analyses of the effects on the jasmonate pathway (perhaps other pathways also) were split into two papers, making it uncomfortable to read.

Other comments:

1. Are Fig. S3B and S3C mentioned in the text? These should either be mentioned or removed from the supplementary data.

2. In Fig. 3 C, the authors should mention what is an acceptable p-value to say that there is an over-representation of genes involved in hormonal signaling in the microarray data.

3. In line 4 of paragraph 2 of page 3, the authors claim that "MTI provides functional links to effector-triggered immunity I do not understand what the authors are trying to state with this. Is it that MTI and ETI are interconnected? Or is it that the transcriptional responses are qualitatively similar but vary in the amplitude of the response?

4. In lines 8 - 9 of paragraph of page 4, the authors suggest that concomitant MAMP and DAMP detection could be a pattern of pathogenesis. I am not quite certain that so far there is evidence to make such a strong statement.

Overall, the connection between AtPep signaling and SAR is novel and interesting, but the lack of statistical analysis to support some of the major conclusions, overlaps between this manuscript and two other pending papers, and ambiguous reasons as to why several experiments were performed (writing needs improvement) are some of the main issues that make it uniquely difficult for me to make a specific recommendation at this point.

1st Revision - authors' response 11 June 2013

Point-to-point reply to the Referees' comments.
Manuscript EMBOJ-2012-84303

Referee #1

The manuscript by Ross et al. reports a novel role of PEPR receptors in systemic acquired resistance mediated by SA and JA. By gene expression profile analyses, the authors show that PROPEP2 and PROPEP3 expression is associated with persistent defenses induced by elf18. A comparison of PEP- and elf18-induced transcriptomes suggested that while SA-, JA, and ET-responsive genes were all enriched in elf18 and PEP treated seedlings, the PEP treatment appears to further activate subsets of JA-response genes. The PEP-induced disease resistance to Pseudomonas syringae requires SID2, but not DDE2 and EIN2, indicating that SA plays a primary role. Interestingly, the Pseudomonas-induced expression of PR2 and bacterial resistance in systemic leaves were significantly compromised in pepr1pepr2 double mutants. PROPEP2 and PROPEP3 proteins were found to accumulate in local leaves, but not systemic leaves, suggesting that PEPs act in local leaves to induce SAR. Application of PEPs to local leaves resulted in the accumulation of PR1 and PDF1.2a transcripts in systemic leaves. Furthermore, the PEP application allowed protection against Colletotrichum higginsianum in systemic leaves in a manner dependent on the JA signaling. The authors propose a model in which MAMP-triggered immunity induces PROPEP expression and PEPR activation in local leaves which subsequently generates systemic signals through ET, SA and JA pathways. Overall, the manuscript describes some highly important findings that are of interest to broad audience. However, as detailed below, several issues need to be resolved prior to publication.

1. The mechanisms for PROPEP induction following MAMP and Pst treatment are not clear. Are SA, ET and JA required for the induction?

Our reply:
In response to elf18 or flg22, it has been described that *PROPEP2* induction is impaired in *ein2* mutants, pointing to ET dependence (Tintor et al., 2013).

More importantly, we show in the revised manuscript (Figure S7) that the induction of both *PROPEP2* and *PROPEP3* is retained in *ein2*, *dde2*, and *sid2* plants upon challenges with *Pst* DC3000 *ΔhrpS* (triggering MTI) or *Pst* DC3000 *AvrRpm1* (triggering ETI). This points to the robustness for *PROPEP2*/*PROPEP3* induction during local defences against the bacterial pathogen.

2. Does PEP treatment result in SAR against Pseudomonas syringae?

Our reply:
Under our conditions we were able to see a 1-log reduction in the growth of *Pst* DC3000 in systemic leaves three times, but failed to see such a reduction twice. In the present manuscript, we would like to leave this issue without making a clear conclusion.

3. *The mechanism by which the PEPR pathway triggers SAR is not clear. The authors propose that the PEPR pathway trigger the production of systemic signals. (If so,) It is necessary to show that petiole exudates from PEP-treated leaves are indeed active in SAR induction. Furthermore, it is necessary to test if the previously reported players in SAR, such as FMO1 and ALD1, are required for PEP-induced SAR.*

Our reply:

At present, it is understood that different systemic signals contributes differentially to SAR in a context-dependent manner (Dempsey and Klessig, 2012). Here we show that local Pep application triggers systemic activation of the JA and SA markers *PDF1.2* and *PR1*, respectively, without detectable *PROPEP2*/*PROPEP3* activation or PEOPEP3-Venus accumulation in systemic leaves. We infer from these results that local PEPR signalling contributes to the production of systemic signals.

In the revised manuscript, we have added new data that Pep-induced systemic activation of *PDF1.2* (but not *PR1*) is still retained in the absence of the previously defined SAR regulators FMO1, ALD1, and NPR1. The uncoupling of the JA- and SA-related outputs in these mutants points to a role for PEPR signalling in the co-activation of separate immune branches in systemic tissues. However, future studies will be required to reveal more details in the mechanisms by which localized PEPR signalling promotes systemic immunity.

Unfortunately, we were unable to show significant activity of the petiole exudates from Pep-treated leaves for defence activation under our conditions. The activity of Pep-triggered systemic signals might not be retained through the petiole exudate preparation procedures. In the revised manuscript, we thus avoid the use of the term SAR to define Pep-induced systemic immunity. Nevertheless, we believe that our model best explains all our data.

4. Figure 2 showed that PROPEP2 overexpressed in plants can interact with PEPR1. This tells nothing about how PEPs get out of the cell or the nature of the ligand in plants.

Our reply:
Considering the comments form the Referee #3, we decided not to present these data in the revised manuscript. We agree with this Referee that the mechanisms by which PEPs get out of the cell and the nature of the ligand in plants remain to be shown. We would like to address these unsolved questions in future studies.

5. Figure 4C, I think the authors over-interpreted the results. The results simply showed that JA is essential for PDF induction, SA antagonizes this, whereas ET enhances the induction.

Our reply:
We agree with the Referee, and revised the text accordingly.

Referee #2

This project initiated with the previous observation that rsw3, a mutant isolated in a genetic screen for Arabidopsis mutants affected in responses triggered by the bacterial MAMP elf18, was affected in late, but not early, elf18-induced responses and was highly susceptible to bacterial infection (Lu et al., PNAS 2009). Therefore, the central hypothesis of this work was that sustained immune responses triggered by elf18 were key to robust immunity. The authors concentrated their efforts on the characterization of the PROPE2 and PROPEP3 genes that encode putative precursors for endogenous peptides acting as DAMPs. It has been previously postulated that the perception of the Pep peptides by their corresponding receptors PEPR1 and PEPR2 would be part of an amplification system following MAMP perception (Huffaker and Ryan, PNAS 2007), but this hypothesis has never been addressed genetically and/or mechanistically. The bulk of this work has been therefore aimed at testing this hypothesis. In this manuscript, compelling evidence is given that the Pep/PEPR perception system is indeed required for the establishment of robust local immunity (as demonstrated with the infection with weakly virulent strains of Pseudomonas and Colletotrichum), but also to systemic acquired resistance (SAR). While these findings are of high interest, critical points still need to be answered.

1. It is not clear why the authors initially chose to focus specifically on the expression of PROPEP2 and PROPEP3 in the rsw3 mutant (Figure 1). Was it because these genes were found to be particularly down-regulated in rsw3 in microarray experiments? Please clarify.

Our reply:
We thank the referee for excellent summary of our work. We indeed aimed to test the model proposed by Ryan and Huffaker in 2007, and therefore selected these two genes in the beginning of the present study. We clearly stated our major aim in the revised manuscript.

2. Unlike what is concluded by the authors, no causal link between sustained MTI response, PEPR1/2 and immunity has been demonstrated. The authors need to test if elf18-induced resistance is affected in pepr1 pepr2 plants?

Our reply:
We have shown that elf18-induced bacterial resistance is indeed impaired in *pepr1 pepr2* in Tintor et al., 2013. This provides evidence for PEPR function in MTI.

3. Similarly, no causal link between MAMP perception, PEPR1/2 and systemic immunity has been demonstrated. The crucial experiment is to test if SAR itself or at least systemic expression of marker genes is affected in pepr1 pepr2 plants upon treatment with elf18, as MAMPs are sufficient to induce SAR. The authors have only used AvrRpm1 (thereby inducing ETI) to trigger SAR in this manuscript.

Our reply:
We show in the revised manuscript that flg22-induced systemic gene expression is reduced in *pepr1 pepr2* plants (Fig. 4E).

4. More importantly, the whole project leading to the demonstration that the Pep/PEPR system is important for induced local and systemic immunities upon MAMP perception was based on the original observation that rsw3 is impaired in the expression of PROPEP2 and PROPEP3. Yet, rsw3 is not impaired in responses triggered by the MAMP flg22 (Lu et al., PNAS 2009), potentially arguing against the generality of the proposed model. The authors must therefore also test alongside elf18, if the local and systemic immunities induced by flg22 are impaired in pepr1 pepr2 plants. If this is not the case, the authors should revise their model.

Our reply:

In the revised manuscript, we refer to Ma et al., 2012 for a decrease in flg22-induced local defence responses of *pepr1 pepr2* plants. As commented above, we also show new data that flg22-induced systemic activation of *PR* genes is reduced in *pepr1 pepr2* plants.

5. The results presented in Figure S1 only show that Pep2 can induce PR1 and PR2 expression in both WT and rsw3 (ie. that Pep2-induced expression of PR1 and PR2 is not affected in rsw3). They do not allow the conclusion that "the increased supply of the PEPR ligand can alleviate or rescue the defects of EFR signalling in rsw3 plants".

Our reply:
Thanks for pointing out our overstatement. In the revised text, we state as follows. “These results imply that the increased supply of the PEPR ligand can alleviate or rescue the defects of these EFR signalling outputs in *rsw3* plants.”

6. Figure 4: the ein2 mutant is affected in Pep2-induced defense gene expression and potentially Pep2/Pep3-induced resistance to Pseudomonas (which is difficult to judge in the absence of statistical analysis...). Given that ethylene perception is important for the expression of some PRRs (Boutrot et al., PNAS 2010), it seems essential to test if PEPR1 and PEPR2 are properly expressed in ein2, before trying to reach any conclusion on the role of ethylene in responses triggered by the activation of PEPR1/2.

Our reply:
We have described in Tintor et al. 2013 that *PEPR1* expression is retained while in contrast *FLS2* expression is greatly reduced in *ein2* mutant plants. We referred to this work in the revised discussion.

7. In Figure 6B, it is puzzling that the endogenous PROPEP2 and PROPEP3 could not been

detected with the anti-PROPEP2 and anti-PROPEP3 in the corresponding pPROPEP-PROPEP transgenic lines generated. This should have been at least mentioned and discussed.

Our reply:

We have tried hard but found it more difficult to detect the endogenous PROPEP3 at least under these assay conditions. We mentioned this in the revised text. Due to the poor quality in our immunoblot analysis as pointed by the Referee #3, we remove the data for PROPEP2-Venus in the revised manuscript.

8. How do the authors explain the local enhanced susceptibility of the pepr1 pepr2 plants? This is not integrated or discussed in their model

Our reply:
We added discussions in the revised manuscript regarding possible causes for the enhanced local susceptibility of *pepr1 pepr2* plants to the tested hemi-biotrophic pathogens, including the defects of *pepr1 pepr2* plants in MAMP-induced outputs described in Ma et al., 2012 and Tintor et al., 2013, and the loss of PEPR-mediated co-activation of JA/ET- and SA-branches.

- All figures need statistical analysis.

Our reply:
We have done statistical analysis in the revised manuscript.

Editorial points:
- The Introduction is poorly referenced with whole section and statements often having no reference or alternatively having only references to review instead of original research papers.
- Introduction and Discussion: cite Navarova et al. Plant Cell 2012 as a recent example of a link between MAMP perception and SAR, as MAMP perception induces the production of pipecolic acid, which appears to be an important mediator of SAR.
- The authors must mention and discuss the recent work from the Berkowitz laboratory (Ma et al., PNAS 2012) that showed an interdependency between responses triggered by MAMPs and Pep peptides.
- Add references for the delta avrpto/delta avrptoB and path-29 strains.
- Replace 'ROS spiking' by 'ROS burst'.
- bak1 mutants were also shown to be affected in Pep1-induced ethylene production (Roux et al., Plant Cell 2011).

- Regarding PEPR1-BAK1 association, also cite Postel et al., Eur J Cell Biol 2010, who showed that PEPR1 interacts constitutively with BAK1 in yeast two-hybrid assays. Also, to be more correct, Schulze et al. JBC 2010 never conclusively demonstrated that "PEPR also undergoes ligand-induced BAK1 association upon Pep1 application". They only showed that a radioactive band migrating a similar size of PEPR1/2 could be detected in BAK1 immunoprecipitates after Pep1 treatment.

Our reply:
We cited these references in the revised manuscript, following these suggestions. We did not cite the last one, as we remove the PEPR1-BAK1 coIP data following suggestions by the Referee #3.

- Specify in Materials and Methods, which are the specific alleles of the different mutants used in the study.

Our reply:
We provide the information in the revised manuscript.

Referee #3

This manuscript describes a between AtPep peptides (danger-associated molecular patterns, DAMPs) and systemic acquired resistance (SAR) in Arabidopsis. The idea that DAMP signaling

enhances SAR is new and quite interesting. This is addressed in this study mainly through measurement of pathogen growth and/or global gene expression. This work also confirms and/or extends the previously shown interactions between BAK1 and PEPR1 and between PEPR1 and PROPEP2. Except for the SAR part, this manuscript appears to have substantial overlaps with two other pending papers, one from this lab and the other from another lab.

Major comments:

1. One of the most difficult aspects to interpret the results presented in the paper is the lack of statistical analysis for most figures, especially when two means have similar values or their standard deviations are high. This analysis must be performed for all figures to validate the authors' conclusions. Just to give two examples (but the authors should do this for all figures). The authors claim that there is no difference in SA levels presented in Fig. 5D. However, I can see that the SA level in systemic leaves is lower in the pepr1 pepr2 plants. Is this not statistically significant? The authors claim that Fig S3D shows PEPR-dependent root inhibition. However, the SD values seem to overlap (even though the means are different). How can this be statistically significant?

Our reply:
We have confirmed our conclusions with statistical analyses in the revised manuscript.

2. The authors claim that PROPEP2 (i.e., in contrast to the processed elicitor) could be recognized by PEPR1 in planta. This is based simply on co-IP result using transient co-expression of PROPEP2 and PEPR1 (Fig. 2). This claim requires more experimental support. In cell extracts used for co-IP, PROPEP2 and PEPR1, which may be in different cellular locations in vivo, are artificially mixed together. Cell-based imaging is needed.

Our reply:
We performed coIP analysis with a mixture of cell extracts from the cells expressing either PROPEP2, PEPR1 or BAK1. Indeed, PROPEP2-PEPR1 coIP was detected to a lesser degree in this coIP. This suggests that PROPEP2-PEPR1 coIP can occur from cell-free extracts. However, our pilot studies also suggest that PROPEP2-induced PEPR1-BAK1 coIP is dependent on the co-expression of all three proteins in the same leaves, as we have never detected PEPR1-BAK1 coIP in a mixture of cell extracts on separate expression of the two proteins in different leaves. This makes it unlikely that ligand-induced PEPR1-BAK1 interaction occurs in cell-free extracts under our conditions. However, we agree with the Referee that this claim deserves and requires more supportive evidence.

As the Referee suggests, cell imaging would be ideal to validate this conclusion. However, this verification per se deserves and requires a full project to be pursued in years of time.

Also considering the comment 4 below, we do not present these data and would like to reserve concluding this issue in the present manuscript.

3. The immunoblots for PROPEP2 (antiPROPEP2 and anti-GFP) in Fig 6B is of poor publication quality. It needs to be repeated. The 35S YFP sample lacks a band present in the WT which the authors claim is a non-specific band (marked with an asterisk). Also, there is a lower rectangle beneath each of the western blots for which there is no explanation as to what these bands represent.

Our reply:
Despite our intensive efforts, we were unable to eliminate a non-specific band that very closely migrates with the band for PROPEP2-Venus, which has hampered us to make an unambiguous conclusion. We thus remove the data for PROPEP2-Venus and reserve this issue for future studies. However, we believe that the present datasets sufficiently support our model in which PROPEP2/3 induction is essentially restricted to the local leaves during pathogen-induced SAR.

4. Parts of this paper lack a logical flow; some experiments seem to be added to the paper just because they had been done. It seems that the microarray analyses of the effects on the jasmonate pathway (perhaps other pathways also) were split into two papers, making it uncomfortable to read.

Our reply:
In the revised manuscript, we have put more emphasis on a role of the PEPR pathway in linking MTI to systemic immunity, by meeting the requests from the Referee #1. As for microarray analysis data, Tintor et al. 2013 described ET (EIN2)-dependent EFR-regulons, whilst this paper compares between PEPR- and EFR-mediated regulons.

Other comments:

1. Are Fig. S3B and S3C mentioned in the text? These should either be mentioned or removed from the supplementary data.

Our reply:
We did in the revised manuscript.

2. In Fig. 3 C, the authors should mention what is an acceptable p-value to say that there is an over-representation of genes involved in hormonal signaling in the microarray data.

Our reply:
We revised our conclusions in the revised manuscript such that SA-responsive genes were similarly over-represented in elf18- and Pep2-treated samples, whilst the over-representation of ET- and JA-responsive genes were much greater in Pep2-treated samples compared to elf18-treated samples.

3. In line 4 of paragraph 2 of page 3, the authors claim that "MTI provides functional links to effector-triggered immunity ..." I do not understand what the authors are trying to state with this. Is it that MTI and ETI are interconnected? Or is it that the transcriptional responses are qualitatively similar but vary in the amplitude of the response?

Our reply:
We revised the text to state that MTI and ETI are functionally interconnected.

4. In lines 8 - 9 of paragraph of page 4, the authors suggest that concomitant MAMP and DAMP detection could be a pattern of pathogenesis. I am not quite certain that so far there is evidence to make such a strong statement.

Our reply:
This is a proposed model. We clearly mentioned so in the revised text.

Overall, the connection between AtPep signaling and SAR is novel and interesting, but the lack of statistical analysis to support some of the major conclusions, overlaps between this manuscript and two other pending papers, and ambiguous reasons as to why several experiments were performed (writing needs improvement) are some of the main issues that make it uniquely difficult for me to make a specific recommendation at this point.

Our reply:
We have addressed these points in the revised manuscript as outlined above.

2nd Editorial Decision 12 July 2013

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referees # 2 and 3.

As you can see below, both referees still have significant concerns with the study. Referee #1 is not convinced that the advance and insight provided is sufficient to consider publication here given recent studies that have come out on this topic. In addition, the referee raises concerns regarding the narrative of the paper and inconsistencies in how some of the experiments were carried out. Referee #3 still has issues with the lack of statistical tests for many of the experiments. This was an issue that was brought up initially as well.

Regarding the issue of novelty that referee #2 brings up. As the paper from the Zhou group came out while your paper was under revision and as the referees had access to your Tintor et al. paper during initial review, we will not take these papers into consideration. However significant work is still needed. We usually allow only one round of revision, but I can offer in this case to give you one more opportunity to address the raised concerns should you be able to address them in full with the inclusion of additional experiments. I should also add that as some of the issues raised concern the conclusiveness of the findings reported that I therefore can't guarantee the outcome on another revision, just you are aware of this upfront.

Please also make sure that you discuss the recent papers that have appeared on this topic in a good manner.

I hope that you find these comments helpful.

REFEREE REPORTS

Referee #2

Several major points raised by myself or the reviewers remain unanswered, or the authors have decided simply to remove previously unclear data. More importantly, part of the novelty was taken away by the recent publication by this group, as well by the Zhou group, that the Pep/PEPR perception system is required for sustained local immune responses and resistance to pathogens. This is clearly illustrated by the fact that the authors often refer to Tintor et al. (2013) to reply to points raised by the reviewers. Therefore, while I find interesting that the Pep/PEPR perception system is required for systemic immunity (which is truly the novelty of this manuscript), I would recommend publication of these results in a more specialized journal.

Specific points

- The overall logic of this manuscript is still not clear to me (also pointed out by Reviewer 3. Indeed, it starts with the analysis of rsw3 mutant, which was shown by the previous authors (Lu et al., PNAS 2009) to be unaffected in responses triggered by flg22. So, since the authors clearly state in their rebuttal and the revised manuscript that their main aim was to test the hypothesis previously proposed by Ryan and Huffaker (2007), I would suggest to omit the results related to rsw3, and simply start their story as a logical follow-up of the Tintor et al. (2013) paper, stating that they wanted to further evaluate the role of the Pep/PEPR perception system in immunity.
For example, the way the results paragraph on the role of PEPRs in basal resistance is written seems now outdated given the recent publications of an increased susceptibility of pepr1/2 to Pst DC3000 (Ma et al., 2012; Tintor et al., 2013) and to Botrytis cinerea (Liu et al., 2013).
On a similar note, it is surprising that the authors did not update the Introduction based on the recent demonstration that the Pep/PEPR perception system plays a role in local immunity (Tintor et al., PNAS 2013; Liu et al., PNAS 2013). They obviously cite these publications, but only briefly by stating that "Genetic interactions between MAMP and PEPR signaling pathways have been documented supporting this model (Flury et al., 2013; Liu et al., 2013; Ma et al., 2012; Tintor et al., 2013"; while in fact, at least Liu et al. (2013) provides mechanistic insights into how these pathways are interconnected, and the cumulative evidence presented in the 4 papers cited does not allow the authors to write anymore that such model has been proposed, but not proven.

- In Fig. 4E, why did the authors used flg22, while elf18 was used otherwise throughout this study?

- Page 14: If the authors do not show that PEPR1/2 are required for flg22- or elf18-induced immunity to Pst, they cannot write as a paragraph title that: "The PEPR pathway couples MTI activation with systemic immunity". They should rather write: "The PEPR pathway couples MAMP perception with systemic gene expression".

- It is confusing that the authors used Ch path-29 to perform the systemic immunity assays in Fig. 6D, while all other experiments related to systemic immunity are performed with Pst strains or bacteria-derived MAMPs.

- The revised title lacks clarity. The authors should refer specifically to endogenous Pep elicitors, as 'the endogenous elicitor-mediated signaling' is too vague and also implies that the Pep/PEPR perception system is the only signaling pathway in Arabidopsis triggered by endogenous elicitors, which is highly unlikely.

Referee #3

The manuscript is improved. However, a number of issues still remain.

1. The authors responded to my comment of lack of statistical analysis in several Figures. However, there are still several issues. When a single experiment shows MULTIPLE comparisons, a correction for a T-test should be employed (e.g. Tukey HSD test) or, at the very least, correct the level of significance using a Bonferroni correction for multiple comparisons. This needs to be corrected throughout the entire article (Figs. 1B, 1C, 3B, 4B and S6). Also, the experiments that look at gene expression by qRT-PCR have no statistics (Figs 1C, 3C, 4A, 4D, 6A, 6B, 6C, 6D, 7, S1, S3, S5 and S7) nor the ion leakage experiment (Fig. S4). Please add them to ALL the experiments.

2. The authors still did not address the lack of explanation in the legend for Figure 5B of the lower rectangle beneath the western blot (I assume it represents the loading control). Please add it to the legend. Also, the sample loading for the p35S::YFP construct expressed in N. benthamiana was much lower than that of the Arabidopsis samples, as judged from the lack of presence of a band in the lower rectangle.

3. Abstract: ... Here we show that sustained PROPEP2/PRROPEP3 induction is associated with effective activation of EFR- triggered immunity ...
Perhaps a claim like this requires a more definite proof. For example, the authors could provide results from an protection assay with an elf-18 pre-treatment on WT and pepr1 pepr2 plants.

4. Page 7: ... Thus, the increased supply of the PEPR ligand can alleviate or rescue the defects of these EFR signalling outputs in rsw3 plants, ...

Part of the conclusion drawn from Figure S1 may be incorrect. There is no signaling defect shown for PR-1 and PR-2; the reduced signaling shown in Fig. 1A is after elf18 treatment, whereas that shown in Fig. S1 is after Pep2. Please explain.

5. Please describe in the materials and methods how the gene ontology analyses were performed.

6. Page 10: ...revealed that SA-responsive genes were similarly over-represented in the PEPR- and EFR-regulons, whilst the overrepresentation of ET- and JA-responsive genes was much greater in the PEPR-regulons compared to the EFR-regulons (Figure 2C, Table S4).

What is the basis to make this claim? Is it based on the p-values observed on Fig. 2C or on the number of genes on each category from elf18- and Pep2-treated plants?

7. Page 10: ... Moreover, Pep2 triggers far greater PDDF1.2a induction as compared to elf18 or flg22 (Figure S3A)
As mentioned before, without statistical analysis, this conclusion is not well supported. It also seems to be only about 3-fold greater the induction by Pep2 than by flg22 (and therefore, not a far greater induction as implied in the text) and no induction by elf18.
8. Are pepr1-1 pepr2-3 plants more resistant to bacterial infection when mock-inoculated? (please see figure 3B; this is contrast to what is observed for figure 4B).

9. Page 12: ... conferred by SID2 and PAD4 substantially restored Pep2-induction of the two genes in ein2 pad4 sid2 plants, despite the ET signalling dysfunction (Figure 3C)...
No such claim can be made without proper statistical analyses of the data.

10. Anti-PROPEP2 antibody shown in Fig S6D does not appear to be used throughout the text. If so, please remove it from the manuscript.

11. Page 16: ... PROPEP2 and PROPEP3 were induced without a significant decrease in all these mutants, when challenged with a Pst DC3000 hrpS ...
The data seem variable. Again, please perform proper statistics to support the claim.

2nd Revision - authors' response 08 October 2013

Point-to-point reply to the Reviewers' comments
Manuscript EMBO J 2012-84303

Referee #2

Several major points raised by myself or the reviewers remain unanswered, or the authors have decided simply to remove previously unclear data. More importantly, part of the novelty was taken away by the recent publication by this group, as well by the Zhou group, that the Pep/PEPR perception system is required for sustained local immune responses and resistance to pathogens. This is clearly illustrated by the fact that the authors often refer to Tintor et al. (2013) to reply to points raised by the reviewers. Therefore, while I find interesting that the Pep/PEPR perception system is required for systemic immunity (which is truly the novelty of this manuscript), I would recommend publication of these results in a more specialized journal.

Our reply:
In this revision, we have addressed major points raised as outlined below.

In sum, we refer to recent studies in details, including ours (Tintor et al, 2013) and others' regarding the role for the PEPR pathway in MTI and basal defences at the pathogen challenge sites. A series of these publications in 2012-2013 point to the existence of high attention in the research field to this putative DAMP pathway. However, to our best knowledge, the mechanisms by which the PEPR pathway promotes local immunity still remain poorly understood. In this respect, our genome-wide transcriptome data obtained from Pep2-treated plants (in comparison with elf18-treated plants) gain important insight into the question, which would be highly valuable for the society.

Moreover, as the reviewer pointed out, we also provide evidence for the role for the PEPR pathway in systemic immunity. In this revision, we further strengthen our findings to support this important claim.

Specific points

- The overall logic of this manuscript is still not clear to me (also pointed out by Reviewer 3. Indeed, it starts with the analysis of rsw3 mutant, which was shown by the previous authors (Lu et al., PNAS 2009) to be unaffected in responses triggered by flg22. So, since the authors clearly state in their rebuttal and the revised manuscript that their main aim was to test the hypothesis previously proposed by Ryan and Huffaker (2007), I would suggest to omit the results related to rsw3, and simply start their story as a logical follow-up of the Tintor et al. (2013) paper, stating that they wanted to further evaluate the role of the Pep/PEPR perception system in immunity.
For example, the way the results paragraph on the role of PEPRs in basal resistance is written seems now outdated given the recent publications of an increased susceptibility of pepr1/2 to Pst DC3000 (Ma et al., 2012; Tintor et al., 2013) and to Botrytis cinerea (Liu et al., 2013).
On a similar note, it is surprising that the authors did not update the Introduction based on the recent demonstration that the Pep/PEPR perception system plays a role in local immunity (Tintor et al., PNAS 2013; Liu et al., PNAS 2013). They obviously cite these publications, but only briefly by stating that "Genetic interactions between MAMP and PEPR signaling pathways have been documented supporting this model (Flury et al., 2013; Liu et al., 2013; Ma et al., 2012; Tintor et al., 2013"; while in fact, at least Liu et al. (2013) provides mechanistic insights into how these pathways are interconnected, and the cumulative evidence presented in the 4 papers cited does not allow the authors to write anymore that such model has been proposed, but not proven.

Our reply:

In the revised manuscript, we have cited these publication updates in details and reserved the data for basal immunity to *Pst* for our future paper, following this reviewer's suggestion. However, as the super-susceptibility of *pepr1 pepr2* plants to *Colletotrichum higginsianum* is new and helpful for the society, we include the data in a supplemental figure.

In the revised manuscript, we start with our data for the robustness of *PROPEP2*/*PROPEP3* induction upon *Pst* challenges, as an implication for the PEPR function in immunity to a wide range of pathogens. In addition, the data obtained with *rsw3* plants also gave us a starting point for this study. We therefore include the *rsw3* data in the revised Figure 1. Importantly, by referring to the recent publications in details, we hope the revised manuscript avoids a misimpression that the PEPR pathway merely acts downstream of EFR.

Strictly speaking, Liu et al 2013 showed that Pep-induced resistance via PEPRs is effective against *Botrytis cinerea*, but did not clearly conclude the requirement for PEPRs in basal immunity to this pathogen in the absence of exogenous Pep application. Considering the broad-spectrum resistance that can be ultimately achieved by enforced activation of pattern-triggered immunity, we believe that the lowered immunity in non-Pep-pretreated *pepr1 pepr2* plants is valuable and should be noted.

- In Fig. 4E, why did the authors used flg22, while elf18 was used otherwise throughout this study?

Our reply:
Mishina and Zeier 2007 showed that flg22 application triggers systemic immune responses, but did not mention whether or not elf18 application is effective. In our pilot experiments, flg22 was more potent and consistent than elf18 in inducing systemic immune responses. Therefore, we used this MAMP in this experiment.

As replied above, we hope our data for *PROPEP2*/*PROPEP3* induction upon *Pst ΔhrcC* (in the revised Figure 1) could also justify the use of flg22 as a model MAMP.

- Page 14: If the authors do not show that PEPR1/2 are required for flg22- or elf18-induced immunity to Pst, they cannot write as a paragraph title that: "The PEPR pathway couples MTI activation with systemic immunity". They should rather write: "The PEPR pathway couples MAMP perception with systemic gene expression".

Our reply:
We revised the paragraph title as suggested.

However, we note that Ma et al 2012 and Tintor et al 2013 have shown that PEPRs are required for full activation of flg22- and elf18-induced immunity to *Pst*, respectively.

- It is confusing that the authors used Ch path-29 to perform the systemic immunity assays in Fig. 6D, while all other experiments related to systemic immunity are performed with Pst strains or bacteria-derived MAMPs.

Our reply:
Please note that the other systemic immunity assays were conducted to test avirulent pathogen-induced systemic immunity, whilst the one in Fig. 6D was to test Pep-induced systemic immunity.

As stated in the point-to-point reply on our previous revision, under our conditions we were unable to draw a clear conclusion as to whether systemic immunity conferred by local Pep application is effective against the bacterial pathogen. Instead, robust *PDF1.2* activation in the systemic leaves (Figure 6) and earlier studies by Hiruma et al (2011) prompted us to use the *Ch* strain in assessing Pep-induced systemic immunity.

Unfortunately, chitin, the best characterized fungal MAMP, was much less potent to trigger immune responses on the whole plants under our conditions. To our knowledge, there are no other MAMPs relevant and available for *Ch*.

- The revised title lacks clarity. The authors should refer specifically to endogenous Pep elicitors, as

'the endogenous elicitor-mediated signaling' is too vague and also implies that the Pep/PEPR perception system is the only signaling pathway in Arabidopsis triggered by endogenous elicitors, which is highly unlikely.

Our reply:
Sorry, but a wrong title stayed on the previous version of the manuscript PDF file sent to the review. We have revised the title accordingly.

Referee #3

The manuscript is improved. However, a number of issues still remain.

1. The authors responded to my comment of lack of statistical analysis in several Figures. However, there are still several issues. When a single experiment shows MULTIPLE comparisons, a correction for a T-test should be employed (e.g. Tukey HSD test) or, at the very least, correct the level of significance using a Bonferroni correction for multiple comparisons. This needs to be corrected throughout the entire article (Figs. 1B, 1C, 3B, 4B and S6). Also, the experiments that look at gene expression by qRT-PCR have no statistics (Figs 1C, 3C, 4A, 4D, 6A, 6B, 6C, 6D, 7, S1, S3, S5 and S7) nor the ion leakage experiment (Fig. S4). Please add them to ALL the experiments.

Our reply:
With statistical analysis we confirmed statistical significance for all the datasets shown. Statistical analysis was conducted as stated in the revised materials and methods. To meet this request, we have repeated some of the qRT-PCR and pathogen inoculation experiments for the last 3 months, and now present the conclusions obtained from combined datasets of independent biological replicates in the revised manuscript.

2. The authors still did not address the lack of explanation in the legend for Figure 5B of the lower rectangle beneath the western blot (I assume it represents the loading control). Please add it to the legend. Also, the sample loading for the p35S::YFP construct expressed in N. benthamiana was much lower than that of the Arabidopsis samples, as judged from the lack of presence of a band in the lower rectangle.

Our reply:
We included the explanation in the figure legend. That is for loading control.
We include the sample derived from p35S::YFP in *N. benthaminana* just to show the position of free YFP, which is indicated by an asterisk in the lower blot probed with anti-GFP antibodies. Thus, it does not have to be equally loaded.

3. Abstract: ... Here we show that sustained PROPEP2/PRROPEP3 induction is associated with effective activation of EFR- triggered immunity ...
Perhaps a claim like this requires a more definite proof. For example, the authors could provide results from an protection assay with an elf-18 pre-treatment on WT and pepr1 pepr2 plants.

Our reply:
We agree with this reviewer. In the revised text we refer to Tintor et al., 2013 that *pepr1 pepr2* plants are reduced in elf18-induced bacterial resistance. However, we agree with the Reviewer 2 that this is not a major novel issue any more.
Therefore, we instead emphasize the robustness of *PROPEP2/PROPEP3* induction upon pathogen challenges against the perturbations of salicylate (SA), jasmonate (JA), and ethylene pathways.

4. Page 7: ... Thus, the increased supply of the PEPR ligand can alleviate or rescue the defects of these EFR signalling outputs in rsw3 plants, ...

Part of the conclusion drawn from Figure S1 may be incorrect. There is no signaling defect shown for PR-1 and PR-2; the reduced signaling shown in Fig. 1A is after elf18 treatment, whereas that shown in Fig. S1 is after Pep2. Please explain.

Our reply:

We like to say that PEPR-mediated activation of these genes is essentially retained in rsw3 plants. We revised the text accordingly.

5. Please describe in the materials and methods how the gene ontology analyses were performed.

Our reply:
We described it in the revised materials and methods.

6. Page 10: ...revealed that SA-responsive genes were similarly over-represented in the PEPR- and EFR-regulons, whilst the overrepresentation of ET- and JA-responsive genes was much greater in the PEPR-regulons compared to the EFR-regulons (Figure 2C, Table S4).

What is the basis to make this claim? Is it based on the p-values observed on Fig. 2C or on the number of genes on each category from elf18- and Pep2-treated plants?

Our Reply:
The p-Values in Fig 2C were calculated by comparing between the ratio of gene numbers for SA-responsive genes/elf18-responsive genes and that for SA-responsive genes/the genes of detectable expression.

7. Page 10: ... Moreover, Pep2 triggers far greater PDDF1.2a induction as compared to elf18 or flg22 (Figure S3A)
As mentioned before, without statistical analysis, this conclusion is not well supported. It also seems to be only about 3-fold greater the induction by Pep2 than by flg22 (and therefore, not a far greater induction as implied in the text) and no induction by elf18.

Our reply:
We confirmed the statistical significance, and stated "Pep2 triggers greater PDF1.2a induction …" in the revised text.

8. Are pepr1-1 pepr2-3 plants more resistant to bacterial infection when mock-inoculated? (please see figure 3B; this is contrast to what is observed for figure 4B).

Our reply:

We have repeated the assays, but have not seen the statistical significance.

Please note that bacterial growth in local leaves (after water pretreatment) was tested in Figure 3B, whilst that in systemic leaves (after localized water pretreatment) was tested in Figure 4B. It cannot be ruled out that this might cause slight differences.

9. Page 12: ... conferred by SID2 and PAD4 substantially restored Pep2-induction of the two genes in ein2 pad4 sid2 plants, despite the ET signalling dysfunction (Figure 3C)...
No such claim can be made without proper statistical analyses of the data.

11. Page 16: ... PROPEP2 and PROPEP3 were induced without a significant decrease in all these mutants, when challenged with a Pst DC3000 hrpS ...
The data seem variable. Again, please perform proper statistics to support the claim.

Our reply:
We confirmed statistical significance.

10. Anti-PROPEP2 antibody shown in Fig S6D does not appear to be used throughout the text. If so, please remove it from the manuscript.

Our Reply:
It was done.

3rd Editorial Decision 18 October 2013

Thank you for submitting your revised manuscript. Your study has now been re-reviewed by referee #3. As you can see the referee still has some remaining issues and hesitations. I have discussed the revision with my colleagues and we have come to the conclusion that we will accept the paper for publication here.

However there are some issues that have to be resolved before acceptance here. Please see referee comments below.

Statistical analysis: please go though the figures and make sure that you compare the right treatments. See for example figure 3C. In cases where you use only 2 biological replicates, please don't use SD. Modify the figure to display the value of both samples and not the average

Referee point #2: Do you need to show the rsw3 mutant in this figure. If you feel so please explain in point-by-point response, if not then you can remove this from the figure

Referee point #2 and 3: Please explain in point-by-point response the rational for adding/keeping the data in the figures.

Once we get these last issues fixed, then we will proceed with acceptance of the paper for publication here.

Please don't hesitate to contact me if you have any questions.

REFEREE REPORT

Referee #3

As mentioned before, the key findings of this article are that the Pep2/3 peptides provide systemic resistance in Arabidopsis against P. syringae and Colletotrichum, which is correlated with systemic marker gene activation, and that this requires Pepr1/2 (Figure 4A, 4B, 4E, these figures repeat themselves for Figure 5, but using instead markers for jasmonate (JA) instead of salicylic acid (SA) and a different pathogen). Another finding (already shown for ethylene (ET) and JA in the Tintor et al. paper for local leaves) is that gene activation by Pep2/3 is differentially affected by compromising SA, JA and ET hormone pathways.

Regarding my comments on the proper use of statistics, t-tests were performed on all the data in the revised manuscript and the authors corrected the statistical analyses for multiple comparisons. However, for some figures, the comparisons being made are not straightforward, since they compared the induced resistance but never compared if the mock levels were the same (an example of this is Figure 3C). Also, it is stated that the qRT-PCR for each treatment in figure 3A and 3C was performed for at least 2 biological replicates and standard errors were shown. However, a standard error should at the very least have 3 biological data points to be calculated.

1) There is repetition of data previously shown in other articles, particularly local activation of marker genes by the Pep pathway. It is true that the genes chosen were different from those on their PNAS article (and some belong to different pathways), but this does not warrant so many figures dealing with gene expression (plus a microarray experiment). Examples of this issue are:

2) Figure 1 shows local activation of PROPEP2 and 3 by elf18, which appeared already in Figure 4B and 4C of Tintor et al. The use of the rsw3 mutant does not shed new light since this mutant was not used anymore throughout the article.

3) The analysis of Figure 2 is new, but the data presented had partially been shown in Figure 3 for elf18 and Figure S5 for Pep2 of Tintor et al.

4) Expression for PR1 in Figure 3A and its reduction in the pepr1/2 mutant after Pep2 treatment was already shown in Figure 4D of Tintor et al or, as they mention, in an article from a different group

from 2007.

The article lacks a mechanistic explanation as to why systemic immunity is compromised. They show the phenotype and the lack of gene induction, but not how this might be occurring. The article has redundant gene expression experiments, and draws correlations about the connection between gene expression and the final phenotype, which does not guarantee causation.

3rd Revision - authors' response 24 October 2013

Point-to-point reply (#EMBOJ 2012-84303-R1)

Thank you for submitting your revised manuscript. Your study has now been re-reviewed by referee #3. As you can see the referee still has some remaining issues and hesitations. I have discussed the revision with my colleagues and we have come to the conclusion that we will accept the paper for publication here.

However there are some issues that have to be resolved before acceptance here. Please see referee comments below.

Statistical analysis: please go though the figures and make sure that you compare the right treatments. See for example figure 3C. In cases where you use only 2 biological replicates, please don't use SD. Modify the figure to display the value of both samples and not the average

Our reply:
In Fig 3C, we combined the data from multiple independent experiments under the same conditions, of which each contains a partially overlapping but different set of samples. With the power of the linear model, we can analyze those datasets together and calculate the mean value, the standard errors and consequently the p-values.

Following your comments, after removing the samples of only two replicates, we keep the data of 3 or more replicates in the revised Fig 3C. Also, to reduce the data redundancy between different figures, we also remove the data of *ein2* from Fig 3A (which are now shown only in Fig 3C). We edited the main text accordingly.

Regarding the comparisons, we believe that in the context of this manuscript it is best to discuss the mRNA levels in the plants exposed to Pep application or pathogen challenges, for 3 reasons: (1) The phenotypes of the WT and mutant plants elicited with these stimuli are in question; (2) The mRNA levels for these genes are much lower in non-elicited WT plants than in Pep-treated WT plants; (3) Our conclusions remain unaffected for the role of the tested hormones in the elevation of these marker transcripts in the elicited plants, even if the basal expression levels of these genes are reduced in the tested mutant plants.

To be sure, we clearly stated in the revised text that we compared the transcript levels in the elicited plants in these figures.

Referee point #2: Do you need to show the rsw3 mutant in this figure. If you feel so please explain in point-by-point response, if not then you can remove this from the figure.

Our reply:
Following our previous work (Lu et al, 2009), the absence of sustained elf18-induced elevation for both *PROPEP2* and *PROPEP3* transcripts in *rsw3* plants implied a possible role for the PEPR pathway in the amplification of MAMP-triggered signaling, and thus prompted us to pursue this study. To clarify a major motive for the present work, we like to keep this initial finding in the revised manuscript. Please see below for what we like to claim by including the data in *rsw3* plants in this figure.

Referee point #2 and 3: Please explain in point-by-point response the rational for adding/keeping the data in the figures.

Our reply:
In Tintor et al, we showed *PROPEP2*/*PROPEP3* induction in response to elf18 in WT plants at one time point. In this study, by comparing between WT and *rsw3* plants for *PROPEP2*/*PROPEP3* induction in the time course, we can see that not initial but sustained induction of *PROPEP2*/*PROPEP3* is impaired in *rsw3* plants. Together with Lu et al, the results imply a role for the PEPR pathway in coupling an initial phase and a late phase during MTI signalling.

In Tintor et al, we used the microarray data for elf18-responsive transcriptome in WT plants as the control to compare with the data in *ein2* plants. Here we use the same data for elf18-responsive transcriptome as the control to compare with Pep2-responsive transcriptome. We need to show the control.

In Fig S5 of Tintor et al, part of the microarray data in Pep2-treated WT plants only for the pre-selected "EIN2-depenedent elf18-responsive" genes was shown. In this study, the whole microarray data in Pep2-treated WT plants are compared with the corresponding data in elf18-treated WT plants.

Likewise, we compared previously described microarray datasets with our own data in Figures 2B and C.

We need to use/show these previously described data for the comparison.

Referee #3

As mentioned before, the key findings of this article are that the Pep2/3 peptides provide systemic resistance in Arabidopsis against P. syringae and Colletotrichum, which is correlated with systemic marker gene activation, and that this requires Pepr1/2 (Figure 4A, 4B, 4E, these figures repeat themselves for Figure 5, but using instead markers for jasmonate (JA) instead of salicylic acid (SA) and a different pathogen). Another finding (already shown for ethylene (ET) and JA in the Tintor et al. paper for local leaves) is that gene activation by Pep2/3 is differentially affected by compromising SA, JA and ET hormone pathways.

Regarding my comments on the proper use of statistics, t-tests were performed on all the data in the revised manuscript and the authors corrected the statistical analyses for multiple comparisons. However, for some figures, the comparisons being made are not straightforward, since they compared the induced resistance but never compared if the mock levels were the same (an example of this is Figure 3C). Also, it is stated that the qRT-PCR for each treatment in figure 3A and 3C was performed for at least 2 biological replicates and standard errors were shown. However, a standard error should at the very least have 3 biological data points to be calculated.

Our reply:
Please see our comments above.

1) There is repetition of data previously shown in other articles, particularly local activation of marker genes by the Pep pathway. It is true that the genes chosen were different from those on their PNAS article (and some belong to different pathways), but this does not warrant so many figures dealing with gene expression (plus a microarray experiment). Examples of this issue are:

Our reply:
As commented above, we reduced redundancy in the revised manuscript. We believe that a minimum degree of data redundancy, *e.g.* for the controls, is rather helpful to validate our experimental conditions.

2) Figure 1 shows local activation of PROPEP2 and 3 by elf18, which appeared already in Figure 4B and 4C of Tintor et al. The use of the rsw3 mutant does not shed new light since this mutant was not used anymore throughout the article.

3) The analysis of Figure 2 is new, but the data presented had partially been shown in Figure 3 for elf18 and Figure S5 for Pep2 of Tintor et al.

Our reply:
Please see our comments above.

4) Expression for PR1 in Figure 3A and its reduction in the pepr1/2 mutant after Pep2 treatment was already shown in Figure 4D of Tintor et al or, as they mention, in an article from a different group from 2007.

Our reply:
In Figure 3A, the data in *pepr1 pepr2* plants are presented as a negative control to show the background levels for Pep2-induced *PR1* expression under our conditions.

