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GRIM REAPER peptide binds to receptor kinase PRK5 to trigger cell death in Arabidopsis

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Transaction Report:

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

Editor: Andrea Leibfried

1st Editorial Decision 17 April 2014

Thank you for submitting your manuscript entitled 'Arabidopsis GRIM REAPER functions through metacaspase processing and receptor-ligand interaction'. I have now received all reports from the referees, which are enclosed below.

As you can see, both referees appreciate your data and conclusions very much. However, they propose some amendments of the text, also to make sure that your manuscript will be well received by a broad audience. Referee #2 furthermore proposes some additional experiments to better support your data, all of which are clearly outlined in the reports.

Given the clear comments provided, I would like to invite you to submit a revised version of the manuscript, addressing the concerns of the referees. Please feel free to contact me in case of any questions regarding the revision.

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

REFEREE COMMENTS

Referee #1:

The authors have previously described the secreted small Arabidopsis protein GRIM REAPER (GRI) as a trigger of cell death (Wrzaczek et al., 2009). In this excellent new manuscript, the authors demonstrate that a small region of GRI of 11 amino acids is sufficient to induce cell death and revealed the leucine-rich repeat receptor-like kinase PRK5 as a receptor for GRI that perceives the GRI-derived peptide at the nanomolar range. Furthermore, they very nicely characterize GRI's cleavage, which depends on the cysteine-dependent protease AtMC9. In addition to elegant genetic-based results, the authors use a wide-range of biochemical techniques to document GRI cleavage by AtMC9 and the perception of the GRI-derived peptide by PRK5. These make the present study a very solid and convincing piece of work, which certainly merits publication in The EMBO Journal.

My only comments concern the writing, which could be improved at places to improve the understanding and impact of the study:

- The title should be more specific and should reflect the known role of GRI in cell death induction. For example, it could be "Cell death induced by Arabidopsis GRIM REAPER depends on AtMC9-mediated cleavage and perception by the receptor kinase PRK5".

- The authors should provide more background information on GRI in the Introduction.

- p5, line 76-79: please mention the names of the tomato RLKs that were shown previously to interact with LeSTIG1.

- There is no Fig. S2A-C.

- To further substantiate that PRK5 is expressed in tissues/organs other than pollen, the authors may want to display in a supplementary figure expression profiles retrieved from the public databases eFP Browser and Genevestigator.

- Please define what is the 'internalization/endocytosis motif' present in the C-terminus of PRK5.

- As the authors comment on the non-conservation of critical residues in the PRK5 kinase domain, it would be informative to show a multiple alignment with other kinases.

- In the Discussion, when discussing about know examples of atypical kinases whose function is known, the authors should also include BIR2 (Halter et al., Curr Biol 2014).

- At several occasions, the referencing to a previous publication or figure should me moved after the statement that actually refers to it.

- Finally, the authors should discuss in more details how GRI-mediated cell death may be triggered. Indeed, while the current study elegantly shows how GRI gets cleaved and the resulting peptide recognized by PRK5, a major remaining question is when/under which condition(s) this cell death-inducing process is occurring during the life of the plants. The authors mentioned that GRI processing/recognition might be ROS-mediated. Actually, all experiments performed in this study were performed upon infiltration of GRI or derived peptides. So, do the authors assume that the wounding response triggered by the infiltration generates enough ROS that is sufficient to trigger GRI processing? This point could be made clearer in the Discussion.

Referee #2:

The authors describe that the pro-cell death peptide GRI undergoes METACASPASE-9 (AtMC9)-mediated processing for the generation of an active form that can induce ROS-dependent cell death

through the membrane-localized receptor PRK5. They provide genetic evidence that PRK5 but not PRK4 is required for cell death (ion leakage) triggered upon GRIp65-84 application. In vitro kinase assays suggest that PRK5 is not an active kinase. In vitro ligand-receptor binding studies show that GRIp65-84 binding to the plant membrane proteins occurs in a PRK5-dependent manner. MC9-mediated cleavage activity was shown in vitro for GRI25-168. Exogenous application of GRIp31-96, which can be produced in gri1 mutant (Wrzaczek and Kangasjärvi et al 2009 PNAS), induces cell death in WT plants but not in prk5 or atmc9 mutant plants. GRIp65-84 and GRIp68-97 induced cell death in atmc9 but not in prk5 mutant plants. These results support the model above. This work seems to follow up their previous studies on GRI (Wrzaczek and Kangasjärvi et al 2009 PNAS), and gain novel insight into the activation mechanism for the signalling peptide. Given the limited knowledge about signalling peptide processing systems in plants, the present study could be appealing to the society, if the following concerns are correctly addressed. A major concern is, the biological significance of their findings, whether and how AtMC9 and PRK5 contribute to GRI's biological functions described previously, has not been clearly addressed or discussed in the present manuscript. In addition, the manuscript lacks convincing or kind explanations in many important details listed below.

The readers have to see the reference Wrzaczek and Kangasjärvi et al 2009 PNAS) for known biological functions of GRI, as little information is provided in the text. The authors must provide a brief summary for the phenotype of gri1 mutants and how GRI could influence cell death and cell death-related processes, and then provide comprehensive discussions on the basis of their previous and present studies in the biological context. For this, additional experimentation seems to be needed.

In my view, to the broad readership of the journal, "GRI has sequence similarity to Stig1" or "PRK5 is the first plant system to demonstrate that an atypical, kinase-inactive, RLK can act as a primary receptor for peptide ligand recognition" (Line 242) would be less interesting.

The authors need to assess a possible role for AtMC9 and PRK5 in the biological processes previously assigned to GRI in their own studies (Wrzaczek et al., 2009).

(1) Cell death triggered upon GRI31-96 application was dependent on SID2 and AtRbohD. Does the identified MC9-cleaved product(s) overcome the requirements for SID2 (SA) or AtRbohD?

(2) The growth of Pst DC3000 and Pst DC3000 AvrRpt2 was reduced in gri mutant plants. What are the bacterial growth phenotypes in prk5 and atmc9 mutant plants?

These feasible experiments and thoughtful discussions would greatly help the authors strengthen the biological significance of their findings.

More detailed comments:

Line 31: Pep1 is not smaller than 20 aa.
Line 35: Not just "suggested". Cleavage of CLE peptides has been described.

Figs 1E and F are hard to see. Where is the prc5-2 sample?

The retention of GRI-induced ion leakage in prc5 mutants is not compatible with the model. The authors need to provide an explanation to reconcile the discrepancy.

Is PRK5-CFP used for localization biologically functional?

Page 6 should be separated into two paragraphs on the line 114.

Why do the authors say "PRK5 closely resembles an active kinase based on sequence", while "critical AAs in kinase subdomains VIb and VII (Stone and Walker, 1995) are not conserved in PRK5"? The sequence information rather implies that PRK5 is kinase-inactive.

In Fig 2G, the amounts seem to be much greater for the PRK5 H500D A520G variant than for the "kinase-negative" samples. Also, a truncated form(s) seems to be more abundant for the kinase-negative samples. Have the authors excluded the possibilities that these also influence the apparently lower kinase activity of these "negative" samples?

Fig 3A, which bands in the bottom stained gel correspond to the indicated proteins? Western analysis with GST antibodies should help.
Although the authors say "GRI31-168 is weaker in the binding to PRK5 ectodomain", the lowered recovery for PRK5 ectodomain seems to be correlated with the amounts of GST-GRI31-168 used.

Fig SI7 does not seem to support "atmc9 displayed slightly less
181 elevated ion leakage induced by XXO compared to wild type plants (Fig. S7)" (Line 180) with statistical significance.

Line 210: "the binding of 210 radioactively labelled 125I-Y-GRIp65-84 " to what?

In Fig SI1, the authors should use upper case for amino acid sequences.
In Fig SI2, what is the basis for "low levels"? It is not surprising to see that the mRNA levels are lower than Actin levels for the majority of the genes tested.
In Fig SI3C, what do the green circles represent?
In Fig SI5, there is no explanation for the identity of the proteins bands seen on the blot and stained gel and the basis for the identification.
In Fig SI6A, what is the basis for the identification of the asterisk band as MBP-GRI25-168? A band(s) of the same size is also seen in the absence of AtMC9.
The resolution of Fig SI6B is too low.

English editing would improve the readability of the text (which has many typos).

1st Revision - authors' response 04 August 2014

Referee #1:

The authors have previously described the secreted small Arabidopsis protein GRIM REAPER (GRI) as a trigger of cell death (Wrzaczek et al., 2009). In this excellent new manuscript, the authors demonstrate that a small region of GRI of 11 amino acids is sufficient to induce cell death and revealed the leucine-rich repeat receptor-like kinase PRK5 as a receptor for GRI that perceives the GRI-derived peptide at the nanomolar range. Furthermore, they very nicely characterize GRI's cleavage, which depends on the cysteine-dependent protease AtMC9. In addition to elegant genetic-based results, the authors use a wide-range of biochemical techniques to document GRI cleavage by AtMC9 and the perception of the GRI-derived peptide by PRK5. These make the present study a very solid and convincing piece of work, which certainly merits publication in The EMBO Journal.

My only comments concern the writing, which could be improved at places to improve the understanding and impact of the study

- The title should be more specific and should reflect the known role of GRI in cell death induction. For example, it could be "Cell death induced by Arabidopsis GRIM REAPER depends on AtMC9-mediated cleavage and perception by the receptor kinase PRK5".

We have changed the title but had to make adjustments in order to stay within the character limit.

- The authors should provide more background information on GRI in the Introduction.

We have added an additional paragraph to the introduction containing background information on GRI.

- p5, line 76-79: please mention the names of the tomato RLKs that were shown previously to interact with LeSTIG1.

We have added the names of the tomato RLKs.

- There is no Fig. S2A-C.

We have corrected this. The information is now shown in Fig. S2.

- To further substantiate that PRK5 is expressed in tissues/organs other than pollen, the authors may want to display in a supplementary figure expression profiles retrieved from the public databases eFP Browser and Genevestigator.

We have added *PRK5* expression data from eFP browser as supplementary information (Fig. S3 – S8).

- Please define what is the 'internalization/endocytosis motif' present in the C-terminus of PRK5.

We have defined the putative internalization motif in the figure legend and also added the residues for other domains in the legend for Fig. 2 as well as in the manuscript text.

- As the authors comment on the non-conservation of critical residues in the PRK5 kinase domain, it would be informative to show a multiple alignment with other kinases.

We agree with the referee and have added a sequence alignment using kinase domains of several RLKs with known enzymatic activity and examples of inactive kinases (for example BIR2; see comment below) as Fig. 2G (subdomains VIb and VII; the alignment of the complete kinase domains is shown in Fig. S11A).

- In the Discussion, when discussing about know examples of atypical kinases whose function is known, the authors should also include BIR2 (Halter et al., Curr Biol 2014).

We thank the reviewer for the suggestion. We have included the reference and also included BIR2 in the sequence alignments (see comment above and Fig. 2G/Fig. S11A).

- At several occasions, the referencing to a previous publication or figure should me moved after the statement that actually refers to it.

We have corrected this throughout the manuscript.

- Finally, the authors should discuss in more details how GRI-mediated cell death may be triggered. Indeed, while the current study elegantly shows how GRI gets cleaved and the resulting peptide recognized by PRK5, a major remaining question is when/under which condition(s) this cell death-inducing process is occurring during the life of the plants. The authors mentioned that GRI processing/recognition might be ROS-mediated. Actually, all experiments performed in this study were performed upon infiltration of GRI or derived peptides. So, do the authors assume that the wounding response triggered by the infiltration generates enough ROS that is sufficient to trigger GRI processing? This point could be made clearer in the Discussion.

We have improved the discussion to address the reviewer's comment. In addition, we provide a new supplementary figure showing a model (Fig. S19) to clarify our discussion of the results.

Referee #2:

The authors describe that the pro-cell death peptide GRI undergoes METACASPASE-9 (AtMC9)-mediated processing for the generation of an active form that can induce ROS-dependent cell death through the membrane-localized receptor PRK5. They provide genetic evidence that PRK5 but not PRK4 is required for cell death (ion leakage) triggered upon GRIp65-84 application. In vitro kinase assays suggest that PRK5 is not an active kinase. In vitro ligand-receptor binding studies show that GRIp65-84 binding to the plant membrane proteins occurs in a PRK5-dependent manner. MC9-

mediated cleavage activity was shown in vitro for GRI25-168. Exogenous application of GRIp31-96, which can be produced in gri1 mutant (Wrzaczek and Kangasjärvi et al 2009 PNAS), induces cell death in WT plants but not in prk5 or atmc9 mutant plants. GRIp65-84 and GRIp68-97 induced cell death in atmc9 but not in prk5 mutant plants. These results support the model above. This work seems to follow up their previous studies on GRI (Wrzaczek and Kangasjärvi et al 2009 PNAS), and gain novel insight into the activation mechanism for the signalling peptide. Given the limited knowledge about signalling peptide processing systems in plants, the present study could be appealing to the society, if the following concerns are correctly addressed. A major concern is, the biological significance of their findings, whether and how AtMC9 and PRK5 contribute to GRI's biological functions described previously, has not been clearly addressed or discussed in the present manuscript. In addition, the manuscript lacks convincing or kind explanations in many important details listed below.

The readers have to see the reference Wrzaczek and Kangasjärvi et al 2009 PNAS) for known biological functions of GRI, as little information is provided in the text. The authors must provide a brief summary for the phenotype of gri1 mutants and how GRI could influence cell death and cell death-related processes, and then provide comprehensive discussions on the basis of their previous and present studies in the biological context. For this, additional experimentation seems to be needed.

We have added a short section on GRI to the introduction; please also see our comment above in response to reviewer #1.

In my view, to the broad readership of the journal, "GRI has sequence similarity to Stig1" or "PRK5 is the first plant system to demonstrate that an atypical, kinase-inactive, RLK can act as a primary receptor for peptide ligand recognition" (Line 242) would be less interesting.

We did not completely understand the point but we have revised the statements to better integrate with the rest of the text.

The authors need to assess a possible role for AtMC9 and PRK5 in the biological processes previously assigned to GRI in their own studies (Wrzaczek et al., 2009).

(1) Cell death triggered upon GRI31-96 application was dependent on SID2 and AtRbohD. Does the identified MC9-cleaved product(s) overcome the requirements for SID2 (SA) or AtRbohD?

We have performed the experiment and added the data (Fig. S16B). The 11aa peptide-induced cell death is still dependent on SA and ROS produced by RBOHD.

(2) The growth of Pst DC3000 and Pst DC3000 AvrRpt2 was reduced in gri mutant plants. What are the bacterial growth phenotypes in prk5 and atmc9 mutant plants?

We have assessed bacterial growth in *prk5* and *atmc9*. The results are shown in the new Fig. S18. While bacterial growth of virulent *Pseudomonas syringae* DC3000 is reduced in *gri*, *prk5* and *atmc9* show a wild type-like phenotype (Fig. S18). In the 2009 publication (Wrzaczek et al. 2009, PNAS) growth of avirulent *Pseudomonas syringae* DC3000 AvrRpt2was similar in Col-0 and *gri* plants, this is consistent with our new results (Fig. S18).

These feasible experiments and thoughtful discussions would greatly help the authors strengthen the biological significance of their findings.

We have included the experiments requested by the reviewer (please see our response to the comments above).

More detailed comments:

Line 31: Pep1 is not smaller than 20 aa.

We have corrected this.

Line 35: Not just "suggested". Cleavage of CLE peptides has been described.

The reviewer is correct. We have corrected this and rephrased our statement.

Figs 1E and F are hard to see. Where is the prc5-2 sample?

We have replaced the line graph with a bar graph to improve clarity of the figure. Thus, figures 1E and 1F have been replaced with a single Fig. 1E.

The retention of GRI-induced ion leakage in prc5 mutants is not compatible with the model. The authors need to provide an explanation to reconcile the discrepancy.

We do not completely understand the reviewer's statement. In our opinion GRI-derived peptides induce elevated ion leakage in wild type plants whereas removal of the receptor should reduce ion leakage to the background level as it is shown in Fig. 1C.

Is PRK5-CFP used for localization biologically functional?

This is not a trivial question and we have not tested this in detail. This would require a rather extensive effort to generate transgenic plants and analyze them. However, binding of radiolabelled Y-GRIp$^{65-84}$ to PRK5-c-myc (Fig. 3E) suggests that addition of a tag to the C-terminus of the receptor does not impair ligand binding.

Page 6 should be separated into two paragraphs on the line 114

We have separated the paragraphs.

Why do the authors say "PRK5 closely resembles an active kinase based on sequence", while "critical AAs in kinase subdomains VIb and VII (Stone and Walker, 1995) are not conserved in PRK5"? The sequence information rather implies that PRK5 is kinase-inactive.

The reviewer is correct. We chose to phrase this more cautiously since it is virtually impossible to test kinase activity under every possible condition, thus we can only conclude that PRK5 did not show detectable enzymatic kinase activity under the conditions tested (under the same conditions we were able to detect phosphorylation activity by PRK5$^{H500D\ A520G}$).

We have added a sequence alignment using kinase domains of several RLKs with known enzymatic activity and examples of inactive kinases (for example BIR2; see response to comment by referee #1) as Fig. 2G (subdomains VIb and VII; the alignment of the complete kinase domain is shown in the supplementary information Fig. S11A).

In Fig 2G, the amounts seem to be much greater for the PRK5 H500D A520G variant than for the "kinase-negative" samples. Also, a truncated form(s) seems to be more abundant for the kinase-negative samples. Have the authors excluded the possibilities that these also influence the apparently lower kinase activity of these "negative" samples?

We have performed the assay several times and exposed the radioactive gel for various lengths of time. We have swapped Fig. 2H and Fig. S11D where the protein amounts match better. We cannot rule out low residual kinase activity of wild type PRK5, however, kinase activity of reverted PRK5$^{H500D\ A520G}$ is considerably stronger.

Fig 3A, which bands in the bottom stained gel correspond to the indicated proteins? Western analysis with GST antibodies should help.

We have indicated the bands in the picture. In addition, we have added a figure with Western analysis of the GST-tagged proteins using anti-GST antibody as well as anti-GRI (directed against an epitope in the C-terminal region of GRI) and anti-GRI-peptide (directed against an epitope in the N-terminal region of GRI) antibodies (Fig. S12).

Although the authors say "GRI31-168 is weaker in the binding to PRK5 ectodomain", the lowered recovery for PRK5 ectodomain seems to be correlated with the amounts of GST-GRI31-168 used.

We have toned down our statement in the manuscript emphasizing that binding of $GRIp^{31-96}$ is stronger to PRK5 rather than PRK4.

Fig SI7 does not seem to support "atmc9 displayed slightly less elevated ion leakage induced by XXO compared to wild type plants (Fig. S7)" (Line 180) with statistical significance.

We have clarified this in the manuscript.

Line 210: "the binding of 210 radioactively labelled 125I-Y-GRIp65-84 " to what?

We have corrected this statement in the manuscript.

In Fig SI1, the authors should use upper case for amino acid sequences.

We have corrected this throughout the manuscript (Fig. S1 as well as Fig. 4C).

In Fig SI2, what is the basis for "low levels"? It is not surprising to see that the mRNA levels are lower than Actin levels for the majority of the genes tested.

The reviewer is correct that the majority of genes will be expressed in lower amounts compared to Actin. However, we intended to emphasize that *PRK5* transcript was detectable, albeit in small amounts, in *Arabidopsis* leaves. We have added *PRK5* expression profiles based on eFP browser as supplementary information (Fig. S3 – S8; please also see response to comment by referee #1).

In Fig SI3C, what do the green circles represent?

The green circles (in the revised manuscript Fig. S9B and Fig. S11C) highlight the amino acids in the catalytic core of PRK5 that were restored to match the consensus protein kinase domain. We have added a clarification to the figure legend.

In Fig SI5, there is no explanation for the identity of the proteins bands seen on the blot and stained gel and the basis for the identification.

Fig. S5 (in the revised manuscript Fig. S11D) contains an autoradiograph from a radiokinase assay and a Coumassie-stained gel, but no (Western) blot. We have added explanations to the figure and the legend.

In Fig SI6A, what is the basis for the identification of the asterisk band as MBP-GRI25-168? A band(s) of the same size is also seen in the absence of AtMC9.

Yes, there is a weak, broad, band visible in the picture (in the revised manuscript Fig. S13A) which however does not migrate exactly as the band in the $MBP\text{-}GRI^{25-168}$ and AtMC9 sample. This is an artifact that is quite frequently present due to the staining method. In addition, HPLC-MS analysis of MC9 cleavage of biochemically pure $GRIp^{31-96}$ (used in infiltration assays), showed no proteolysis in the absence of MC9.

The resolution of Fig SI6B is too low.

Unfortunately the resolution (in the revised manuscript Fig. S13B) is limited by the export of the image from the MS analysis software. Therefore we are unable to improve image quality.

English editing would improve the readability of the text (which has many typos).

A native English speaker has read and corrected the final version of the manuscript.

2nd Editorial Decision 15 August 2014

Thank you for submitting your revised manuscript to us. It has now been seen by referee #2 again, whose comments are enclosed. As you will see, the referee now supports publication here, pending satisfactory minor revision.

The referee thinks that a negative control of your in vitro binding assay would be good to be included. In case you have such data at hand, please incorporate them into your manuscript. Otherwise please discuss alternative possibilities in your text as outlined by the referee. Please also address the second point raised by the referee.

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

REFEREE COMMENTS

Referee #2:

I am happy with their revisions except the following points.

The authors provide compelling evidence that RPK5 is responsible for (the majority of) the ligand-binding activity in Arabidopsis. Given this, the in vitro binding data (Fig 3A) would be a key to conclude that RPK5 is a receptor for GRI-derived peptides. However, the experiments lack a negative control for the (ecto-domain of) receptor candidate. It is still possible that GRI peptides non-specifically bind to any proteins tested in this assay. Strictly speaking, without concrete in vitro data, it cannot be ruled out that another true receptor (in the RPK5 complex co-immunoprecipitated with RPK5) directly binds the ligand(s), which requires RPK5-mediated assistance. The authors need to ensure the in vitro data with a proper negative control and/or mention these possibilities in the text.

The interpretation/discussion for the results Fig S18 (page 10 lines 228-230) sounds inappropriate. One clear conclusion is that PRK5 and AtMC9 are dispensable for resistance against the Pst strains tested. To test the relevance of the present findings in this setting, one needs to test possible requirements for PRK5 and AtMC9 in the gri mutant background (or compare loss-of-function phenotypes between GRI, PRK5, and AtMC9). The authors must provide such explanations/discussions in the text, and could address the question in the future.

2nd Revision - authors' response 20 October 2014

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We agree that this is a very relevant suggestion by the reviewer. Thus, we have added the requested negative control (shown in Figure S12F) using the extracellular domain of FLS2. We have also included a sequence alignment of PRK5 and PRK4 (Figure S12E) to illustrate the high sequence identity and similarity between the two receptors. The high sequence similarity between the two PRKs is a likely explanation for the observed binding of GST-GRI and GST-GRI$^{31-96}$ to the ectodomain of PRK4. We have added a statement explaining this to our results section. However, we did not detect binding of GST-GRI or GST-GRI$^{31-96}$ to the ectodomain of FLS2, the negative control. Therefore, we conclude that GRI and derived peptides preferentially bind to the ectodomain of PRK5.

The interpretation/discussion for the results Fig S18 (page 10 lines 228-230) sounds inappropriate. One clear conclusion is that PRK5 and AtMC9 are dispensable for resistance against the Pst strains tested. To test the relevance of the present findings in this setting, one needs to test possible requirements for PRK5 and AtMC9 in the gri mutant background (or compare loss-of-function phenotypes between GRI, PRK5, and AtMC9). The authors must provide such explanations/discussions in the text, and could address the question in the future.

We realize that our explanation might have been misleading. We have clarified our description and interpretation of the pathogen phenotypes of *prk5* and *atmc9* and hope that the reviewer is satisfied with our changes.

3rd Editorial Decision 21 October 2014

I am happy with the introduced changes in the revised version of your manuscript and I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

