THE EMBO JOURNAL

Manuscript EMBO-2010-75572

The Arabidopsis peptide KISS OF DEATH is an inducer of Programmed Cell Death

Robert Blanvillain, Bennett Young, Yao-Min Cai, Valerie Hecht, Fabrice Varoquax, Valérie Delorme, Jean-Marc Lancelin, Michel Delseny, Patrick Gallois

Corresponding author: Patrick Gallois, University of Manchester

Review timeline:

Submission date:	05 August 2010
Editorial Decision:	08 September 2010
Revision received:	07 December 2010
Editorial Decision:	15 December 2010
Revision received:	23 December 2010
Accepted:	05 January 2011

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 08 September 2010

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by two referees and their comments to the authors are provided below. Both referees find the study interesting, but also indicate that some further analysis is needed to consider publication here. Their concerns are listed below. There are two points that in particular should be addressed in order for further consideration here and that is that the expression analysis of the KOD needs to be provided and some insight into how its expression is regulated is needed. Should you be able to address the concerns raised in full then we would be consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This is an important manuscript because it reports the discovery of a new regulator of programmed cell death in plants. This is important because there are relatively few well characterized regulators of programmed cell death in plants to date. The authors provide convincing that the KISS OF DEATH GENE (KOD) is necessary for cell death and that experimental ectopic or overexpression results in ectopic cell death resulting in developmental abnormalities.

The key observations are:

1. kod mutants have delayed or decreased cell death. This is well characterized in the suspensor and they show that cell death is defective in the root hair mutants.
2. The KOD proteins is a soluble cytosolic peptide
3. Expression of KOD is sufficient to induce cell death in cultured tobacco cells.
4. Induction of KOD in Arabidopsis induced caspase-3 that is required for cell death and induced mitochondrial depolarization. This demonstrates its position in the regulatory cascade that regulates cell death in plants.

General points to be addressed:
It would be good to know where the JOD gene is expressed in the plant. Is it restricted to the suspensor or does it control the death of other cells such as root hairs. Are there other data to show where this gene is expressed? Are promoter GFP fusions or in situ hybridization data available?

It would be good to have the localization of the peptide verified in Arabidopsis.

Minor points:
134: Is it a hydrophobic peptide. It might be worth discussing this at this point.
146:" We first scored the cytology of Col-0 embryos" - should this not read "we scored the phenotypes of Col-0 embryos"

Referee #2 (Remarks to the Author):

Programmed cell death is fundamental to development and defense mechanisms in plants. Authors identified novel factor, KISS OF DEATH (KOD), which encoding a 25 aa peptide, and activates a several cell death pathway in Arabidopsis.
The findings are very important, and useful for many researchers. However, the direct linking of natural PCD, not artificial overexpression system, is little bit poor.

Detailed comments
1. Using 276S line, GUS expression analysis in other plant tissues should be done. Authors described the KOD mutant reduced cell death of heat shocked- root hairs, and over expression caused death in leaves. The native KOD gene expression should be checked in such tissues and PCD inducing conditions.
2. P5, L124 and Fig.1F; Which primers in Supplemental Table were used for the RT-PCR ? DNA bands in Fig1F exhibit GFP. If so, why you can say KOD::GUS fusion was expressed ?
3. Fig.1G and Fig1H; Are these experiment done in WT plant or in 276S ? Describe more clearly.
4. Fig.S3A; Why no data of WT plant ?
5. KOD affects the suspensor cell death, but it may be not so crucial as a pro-death factor, because suspensor cell death occurs without KOD in the mutant with some time-lag.
6. Is the KOD expression induced by heat shock or pathogen infection? Such data should be necessary to show the involvement of KOD gene in native plant cell death events.

1st Revision - authors' response 07 December 2010

Please find here our reply to the referees' comments. It was a pleasure to read that the referees were supportive of the novelty and interest of our work on the KOD gene. We have tried hard to address all the issues that were pointed at, have carried out additional experiments and added new data to improve our manuscript.

Referee #1 (Remarks to the Author):

This is an important manuscript because it reports the discovery of a new regulator of Programmed Cell Death in plants. This is important because there are relatively few well characterized regulators of programmed cell death in plants to date. The authors provide convincing that the KISS OF DEATH GENE (KOD) is necessary for cell death and that experimental ectopic or overexpression results in ectopic cell death resulting in developmental abnormalities.

The key observations are:

1. kod mutants have delayed or decreased cell death. This is well characterized in the suspensor and they show that cell death is defective in the root hair mutants.
2. The KOD proteins is a soluble cytosolic peptide
3. Expression of KOD is sufficient to induce cell death in cultured tobacco cells.
4. Induction of KOD in Arabidopsis induced caspase-3 that is required for cell death and induced mitochondrial depolarization. This demonstrates its position in the regulatory cascade that regulates cell death in plants.

General points to be addressed:
It would be good to know where the kOD gene is expressed in the plant. Is it restricted to the suspensor or does it control the death of other cells such as root hairs. Are there other data to show where this gene is expressed? Are promoter GFP fusions or in situ hybridization data available?

We provide Q-PCR data that indicate that in addition to expression in seeds and roots there is also expression at a reduced level in other tissues (Fig1 H). We have also added data showing that KOD is induced by treatment that induce PCD such as the pathogen Pseudomonas syringae, H2O2 and heat (Fig1 i-L).
We have previously made two promoter::GUS fusion constructs: one with 1,5kb of promoter sequence and one with 2.5 kb. The 1.5 kb lines expressed GUS only in seeds and in root tips while the 2.5 kb only expressed GUS strongly in the suspensor with weak expression in endosperm. Comparing the GUS pattern with Q-PCR data, we concluded that the promoter GUS fusion did recapitulate the GUS expression seen in the promoter trap line but did not recapitulate the expression pattern of the gene as shown by RT-PCR. This is possibly because there is a yet unidentified transcription enhancer 3' of the gene that is not included in the construct. Because the promoter fusion results do not add anything to the 276S trap line we did not include the results.
With regards to in situ hybridization, we spent at least six months trying to obtain a signal. One of us (FV) even spent several weeks in a lab experienced with in situ hybridization, but this approach remained unsuccessful. This is possibly because of a low expression level or/and of the small size of the transcript.

It would be good to have the localization of the peptide verified in Arabidopsis.

The peptide was found localised in the cytosol in onion cells and tobacco cells. The sub-cellular localization of KOD::GFP was verified in the dex-inducible Arabidopsis lines and did not have any discrete pattern and was equivalent to the data using onion cells. This has been added in the text, line 242.

Minor points:
134: Is it a hydrophobic peptide. It might be worth discussing this at this point.

KOD is predicted to be an amphiphilic peptide and we have added this info in line 148 and added a helical representation in figS1. One could expect this property to target the peptide to a membrane,

however we have no experimental evidence of this. It may rather be the amphyphilic nature of KOD is relevant to protein-protein interaction or self assembly as a multimer.

146:" We first scored the cytology of Col-0 embryos" - should this not read "we scored the phenotypes of Col-0 embryos"

Corrected as suggested

Referee #2 (Remarks to the Author):

Programmed cell death is fundamental to development and defense mechanisms in plants. Authors identified novel factor, KISS OF DEATH (KOD), which encoding a 25 aa peptide, and activates a several cell death pathway in Arabidopsis.
The findings are very important, and useful for many researchers. However, the direct linking of natural PCD, not artificial overexpression system, is little bit poor.

As suggested further on by this referee, we have now included expression data showing that KOD expression is induced by abiotic and biotic inducers of pcd: H2O2, heat shock and P. syringae (Fig 1 I-L), thereby strengthening the correlation with PCD. In addition, it is worth considering that the P9S mutation is an excellent control for our over expression studies. P9S result in a reduced cell death in both the mutant line and in over expression experiments. This in our mind validates the over expression data.

Detailed comments
1. Using 276S line, GUS expression analysis in other plant tissues should be done. Authors described the KOD mutant reduced cell death of heat shocked- root hairs, and over expression caused death in leaves. The native KOD gene expression should be checked in such tissues and PCD inducing conditions.

The request of this referee for more expression data was addressed using QPCR. The results show KOD expression in roots and induction of KOD following heat shock in leaves and in young seedlings used for the root hair assay. This is presented in figure 1. The experiment suggested with line 276S has been carried out: GUS histochemistry was carried out in all tissues of Line 276S. 276S only expresses GUS in the suspensor and not in tissues that are positive for KOD using QPCR. In addition, GUS expression was not inducible in GUS negative tissue e.g. by heat shock. In conclusion, unfortunately, the 276S line does not recapitulate the native gene expression possibly because the T-DNA insert is just behind the stop, possibly taking away the influence of a 3' transcription enhancer.

2. P5, L124 and Fig.1F; Which primers in Supplemental Table were used for the RT-PCR? DNA bands in Fig1F exhibit GFP. If so, why you can say KOD::GUS fusion was expressed ?

The primers selected are now specified in the figure legend and in text from line 120. The primers used in Fig 1F were primer oexTi15 in KOD and primer oGUSj in the GUS sequence. The DNA bands amplified correspond therefore to a KOD::GUS transcriptional fusion and not GFP.

3. Fig.1G and Fig1H; Are these experiment done in WT plant or in 276S ? Describe more clearly.

Experiments were done in WT; this information added in figure 1 and in its legend.

4. Fig.S3A; Why no data of WT plant as opposed to hetero?

KOD amplifies in both wild type and heterozygous lines. It just happened that a kod homozygous line and a KOD/kod heterozygous line were adjacent on the gel. We agree it is more usual to show data using Wt rather than a segregant heterozygous line but I cannot see anything wrong with this. This has been explained in the legend to clarify.

5. KOD affects the suspensor cell death, but it may be not so crucial as a pro-death factor, because suspensor cell death occurs without KOD in the mutant with some time-lag.

We agree with the referee's point with the caveat that we cannot tell whether suspensor PCD is delayed in all suspensors or inhibited in part of the suspensor population scored. The root hair system is much clearer. This partial suppression may indicate that there is an additional componant(s) promoting cell death. It remains that the experiments still suggest KOD is a novel regulator of PCD and that is what we claim in our title. We have taken into account this comment in the text.

6. Is the KOD expression induced by heat shock or pathogen infection? Such data should be necessary to show the involvement of KOD gene in native plant cell death events.

This was a good suggestion that we followed. The results clearly show induction of KOD in both suggested conditions. For heat shock we used the same seedlings and the same treatment as for the root-hair-PCD assay. This is presented in figure 1.

2nd Editorial Decision 15 December 2010

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #2 to review the revised paper and I have now received the comments back. As you can see below, the referee appreciates the introduced changes. I am therefore very pleased to accept the paper for publication in the EMBO Journal. Referee #2 has a few minor suggestions that I would like to ask you incorporate in a final revision. Once we receive the revision, we will proceed with its acceptance. When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review. 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 initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Yours sincerely,
Editor
The EMBO Journal

———

REFEREE REPORTS:

Referee #2 (Remarks to the Author):

The revised manuscript is a greatly improved over the original submission. The authors have carefully and thoughtfully responded to the comments of the reviewers.
There are some minor suggestion I would like to make to the authors;

1) The first sentence of Fig.5 legend "KOD, mode of action" is strange.
2) In the text, "P-small 9-S" is used. However, P9S "P-large 9-S" is used in the Fig1E, Fig2A, and Fig2B.
3) In Fig1K and Fig1L, 15 min and 30 min are shown as 15" and 30". Fifteen minutes should be shown as 15'. Usually, the 15" is 15 sec.
4) Fig3D; Font sizes of "Evans" and "Blue" are different.
5) Fig.5C; Titles in the X-axis are not aligned.

2nd Revision - authors' response 23 December 2010

Please find here our reply to the referee's comments. We have implemented all the minor changes suggested by referee 2.

Referee #2 (Remarks to the Author):

The revised manuscript is a greatly improved over the original submission. The authors have carefully and thoughtfully responded to the comments of the reviewers. There are some minor suggestions I would like to make to the authors;

1) The first sentence of Fig.5 legend "KOD, mode of action" is strange.
Changed to 'Fig. 5. KOD-induced PCD pathway'

2) In the text, "P-small 9-S" is used. However, P9S "P-large 9-S" is used in the Fig1E, Fig2A, and Fig2B.
Changed in text from P9S to P9S.

3) In Fig1K and Fig1L, 15 min and 30 min are shown as 15" and 30". Fifteen minutes should be shown as 15'. Usually, the 15" is 15 sec.
It was already set as 15' but the small print size made it difficult to differentiate 15' from 15". To avoid confusion we have changed the labelling to '15m' and '30m', which is more visible. 'Min' was too long to fit under the graph.

4) Fig3D; Font sizes of "Evans" and "Blue" are different.
Corrected

5) Fig.5C; Titles in the X-axis are not aligned.
Corrected

