THE
EMBO
JOURNAL

Manuscript EMBO-2009-72125

Phytaspase, a relocalisable cell death promoting plant protease with caspase specificity

Nina V. Chichkova, Jane Shaw, Raisa A. Galiullina, Georgina E. Drury, Alexander I. Tuzhikov, Sang Hyon Kim, Markus Kalkum, Teresa B. Hong, Elena N. Gorshkova, Lesley Torrance, Andrey B. Vartapetian and Michael Taliansky

Corresponding author: Michael Taliansky, Scottish Crop Research Institute

Review timeline:

Submission date:	06 August 2009
1st Editorial Decision:	23 September 2009
Revision received:	08 December 2009
2nd Editorial Decision	04 January 2010
Accepted:	04 January 2010

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 23 September 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. I apologise for the length of time it has taken to have your manuscript reviewed but in order to get the correct people to review the manuscript over the summer months means that we have to sometimes grant significant deadline extensions for referee reports. However, I have now received the third and final review and as you will see the referee reports provide mixed recommendations.

Referee #1 does not support publication and finds that several issues need to be resolved before the study can be properly assessed and also requires further experimentation to support the role of phytaspase in PCD. Referee #2 and #3 are more positive about the study but do require futher analysis, including importantly the localization of phytaspase. Given the interest in the study, should you be able to address these criticisms, we would 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 you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments.

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

Yours sincerely,

Editor
The EMBO Journal

© European Molecular Biology Organization

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Chichkova et al.
This manuscript reports on the characterisation of an apoplastic, PCD-related, serine dependent subtilase from tobacco and rice (named phytaphtase). It has a distinct tetrapeptid substrate specificty other CLPs, wiht VEID as a preferred substrate. Overexpression and downregulation through RNAi appears to facilitate and impair the hypersensitive response, respectively.
Remarks
1) Figure 1A: for the affinity purification bio-DEVD-CHO was used as a negative control. However in both tobacco and rice extracts a band of simila MW is visible. How to explain this ? Does it hint for aspecificity of the phytaspase ?
2) P7. The inactive S537A mutant appears to have a higher MW than the WT form, probably due to the abolishment of autoprocessing. This hypothesis is experimentally easy to validate. A mutational analysis of the TTHT motif could provide additional support as well.
3) Despite the fact that public databases were screened before designing the RNAi construct to avoid "off-target" effects, it cannot be garantueed that this is not the case -because of the unavailability of the full-genome sequence of tobacco-.
4) It is unclear how reproducible the results in Figure 4,5,6 are. How many independent transgenic lines (OX and RNAi) were tested ? Now it looks like only one transgenic event was tested. Is there a correlation between the transgene expression levels and stress resistance ?
5) A specific relocalisation from the apoplast to the cytoplasm during stress is suggested. As a negative control the apoplastic retainment of cathepsin B during stress is shown. However no details at all on the construct used and the experimental set-up are described. A more quantitative approach should be taken before firm statements on the relocalisation can be made.

Referee #2 (Remarks to the Author):

Since the first report of caspase-like protease activities in plants over a decade ago, their complexity and function in plant PCD remains unclear in spite of the report of caspase-like activity and cell death function for the vacuolar processing enzyme in tobacco and Arabidopsis. Biochemical identification of oat saspases, which appeared to be subtilisin-related proteases, by the Wolpert lab has not been followed up with functional analyses using molecular and genetic approaches. The present work by Chichkova fills this gap by carrying out a detailed molecular analysis coupled with reverse genetic approaches of a plant caspase-like protease.
Using affinity tagging approach, the authors followed up their earlier studies that initially described a TATDase activity in tobacco and successfully cloned the corresponding subtilisin-related protease with the same target specificity as the TATDase, which they named phytaspase. The predicted structure of phytaspase turned out to have similar characteristics as compared to the oat saspases first described in the Wolpert lab, although it completely lacks DEVDase activity. It would have been useful if the authors discuss more in detail the differences in substrate target specificity between saspases and phytaspase. This may clarify the utility of having additional names for these proteases.
The rest of the paper mainly followed with both overexpression and gene silencing approaches to ascertain the role of the cloned phytaspase. The data obtained are of good quality and largely supporting the role of this class of subtilisin as a positive regulator of HR cell death during TMV-N gene interaction as well as during abiotic stress-induced cell death upon high salt (NaCl) or MV treatments, both of which involved ROS production. Lastly, RFP tagging of phytaspase showed this protease is likely localized in the apoplast and upon activation of cell death, its localization pattern is altered. The model presented in which the localization of phytaspase is used as a regulatory mechanism to control its activity is intriguing and certainly deserves testing in the future.
In sum, this is an important contribution to the plant PCD field and it should be of broad interest as well, with respect to PCD in other eukaryotic model systems. Thus I think its publication in EMBO J. is highly appropriate. There are several points, however, that I would like the authors to clarify

© European Molecular Biology Organization

and/or include additional experimental data before its acceptance:

1) In Figure 6A, last row, my understanding is that all samples have been treated with MV. I failed to see any signs of cell death in the "wt" sample after treatment with the empty vector. Shouldn't this "empty vector/MV" control be the same as that in the "MV only"? This is in glaring contrast to panel B, lower graph, in which this same sample is producing a large amount of ROS. This discrepancy needs to be resolved.

2) The last set of cell biological data using mRFP fusion is an important one for deriving a mechanistic model of phytaspase regulation. To me however, the interpretation that phytaspase is "relocalized" to the cytosol can benefit from additional confirmation data that may be obtained relatively easily by the authors. To my eyes, its appears that BFA treated samples and the PCD induced samples shared a lot of similarities with large aggregation "spots". It would be interesting to carry out western blot analyses before and after induction of cell death and in the presence or absence of BFA to examine the ratio of processed vs. unprocessed forms of phytaspase. If the authors can fractionate apoplastic fluids, cytosol and endomembrane fractions from the different samples for this western study, it would be really great for deciding whether it is truly relocalization of apoplastic into the cytoplasm or the suppression of secretion that is responsible for the change in cellular location of the fusion protein.

3) In their description of how the authors designed their RNAi vectors to avoid "off target" effects, it is implied that there are multiple subtilisin-like proteases that were "hit" when the datasets from various plant species were queried. It would be important to note how many different subtilisin genes may be targeted by these RNAi vectors at least for the tobacco and tomato databases. This information would be useful for interpretation of the specificity of this approach.

Upon incorporation of these changes, I believe this paper should be ready for publication in EMBO J.

Referee #3 (Remarks to the Author):

This manuscript describes a very interesting subtilisin-like protease that has caspase specificity and promotes cell death. The authors managed to identify the protease that binds to biotinylated TATD aldehyde, a sequence that was previously identified as the cleavage site in the Agrobacterium VirD2 protein that is cleaved during HR, resulting in detachment of its nuclear localization signal. The TATD-binding protein was identified from both rice and tobacco and was found to be a subtilase. The recombinant subtilase has similar substrate specificity and inhibitor sensitivity as the activity detected in plant extracts. RNAi (KD) lines and overexpression (OE) lines were generated and displayed altered VirD2 cleavage activity, demonstrating that they indeed identified the phytaspase. Interestingly, these lines also showed clear phenotypes under biotic and abiotic stress: OE promotes cell death and KD delays it. The ROS phenotype could be complemented with the rice subtilase. Finally, localisation studies indicate that this is a secreted enzyme that relocalizes to the cytoplasm during HR or ROS.
Overall, I find this a very interesting manuscript, and I have been convinced that this is the caspase they were looking for and that this caspase promotes cell death. There are, however, a few important issues that puzzle me.
1. All figures need a significant revision to improve readability, because many details are not in the figure or not even in the legend. For example, is fig1C detected with anti-GFP antibody? What does 1 and 0.2 mean in fig1D? Can you add a legend in fig2D? Please replace 1=WT, 2=OE and 3=KD in fig5. Why were treatments (e.g. 10uM MV on OE line) left out from this figure? Fig5C is copy-paste work: can I compare 75mM with 250mM NaCl? Probably not, so please add dash lines. Can you add some basic information to fig7A-E (e.g. 24hr BFA treatment)? At which timepoint after treatment was Fig7C and 7D taken?
2. I have a serious problem with the conclusion that the phytaspase relocalizes to the cytoplasm (e.g. in title). First, do the large granules in Fig7C and 7D represent the cytoplasm? It looks rather like large vesicles. A colocalization with a cytoplasmic marker would be necessary to verify this. Second, is this internalization or hampered secretion? To discriminate between these possibilities one would have to block translation (e.g. cyclohexamide) and monitor the internalization upon

HR/MV treatment. Fig7A seems to suggest that epidermal cells contain chloroplasts. Is it? How does the dsRED control behave in these assays?
3. The TMV-induced HR assay (fig4) could be more convincing. Panels in A are not that clear and seem to represent different regions of a leaf. Also magnification bars are missing. The phenotype should be supported by quantitative data (lesion sizes). Second, TMV quantification e.g. by Northern blot, could be used to show that the phytaspase plays a role in pathogen resistance.
4. The complementation with the rice phytaspase is certainly very neat, but why was this only done on MV-induced phenotypes, and not on the TMV assay? Is the complementation absent with a catalytically dead rice phytaspase?
5. Phytaspase is definitely an important enzyme, but not the only protease having caspase-activity in plants. This should be acknowledged e.g. in the conclusion.

1st Revision - authors' response 08 December 2009

Referee 1

1. Figure 1A: for the affinity purification bio-DEVD-CHO was used as a negative control. However in both tobacco and rice extracts a band of similar MW is visible. How to explain this ? Does it hint for aspecificity of the phytaspase ?

We have performed additional experiments using a 'DMSO only' control in parallel to bio-DEVD-CHO and bio-TATD-CHO assays. Results obtained demonstrate that some background binding of phytaspase seen in bio-DEVD-CHO sample is largely due to non-specific binding of phytaspase to the resin. Specifically: protein samples were incubated with either bio-TATD-CHO, or bio-DEVD-CHO (both dissolved in DMSO), or with equivalent amounts of DMSO only. Then samples were applied onto avidin resin, washed, and the retarded material was eluted with biotin and electrophoresed. Note that phytaspase was retarded to some minor extent even without peptide aldehyde added (for an unknown reason). bio-DEVD-CHO does not add much to this binding, whereas bio-TATD-CHO does. Therefore, it hints not at aspecificity of phytaspase towards peptide aldehydes but to non-specific absorption of the enzyme to the resin. These data have now been included in Supplementary Information (Supplementary Figure 1). Also, a high peptide aldehyde concentration (0.4 mM) was used in these experiments to increase the yield of the purified enzyme - this could also contribute to non-specific binding. We have now included these details briefly in the 'Materials and methods' Section.

2. P7. The inactive S537A mutant appears to have a higher MW than the WT form, probably due to the abolishment of autoprocessing. This hypothesis is experimentally easy to validate. A mutational analysis of the TTHT motif could provide additional support as well.

We have now determined the N-terminal region of the S537A mutant by Edman degradation sequencing which corresponded to the N-terminus of the proenzyme indicating that no prodomain elimination occurred. This suggests that prodomain processing of phytaspase occurs autocatalytically (page 3, lines 3 -14). In support of this notion, we have mutated D117 residue immediately preceding the TTHT motif in phytaspase. The newly constructed phytaspase D117A mutant displayed impaired processing, i.e. its MW was identical to that of the S537A mutant and was distinctly higher than that of the mature wt form. Figure 1E illustrating this finding has been added to the manuscript and appropriate additions have been made to the 'Results' Section (page 5, lines 20-21).

3. Despite the fact that public databases were screened before designing the RNAi construct to avoid "off-target" effects, it cannot be guaranteed that this is not the case -because of the unavailability of the full-genome sequence of tobacco.

We agree with the reviewer that "off-target" effect cannot be excluded during RNAi transgenic silencing of the phytaspase gene since full-genome sequence of tobacco is not available. However, to ensure that phenotypic effects observed during RNAi were due to specific silencing of the phytaspase gene we performed a functional complementation assay showing that heterologous expression of the active rice phytaspase in the RNAi transgenic leaves prior to stress treatments led

to restoration of the wild-type phenotype Moreover, according to the comment of reviewer 3, we have shown that the inactive rice enzyme (S535A mutant) was unable to complement deficiency of tobacco phytaspase in RNAi plants (page 10, lines 14-23; page 11, lines 20-24; Figures 4 and 6).

4. It is unclear how reproducible the results in Figure 4,5,6 are. How many independent transgenic lines (OX and RNAi) were tested ? Now it looks like only one transgenic event was tested. Is there a correlation between the transgene expression levels and stress resistance?

Data (including photographs) were typical of all generated KD and OE transgenic lines, which were tested in at least three experiments with three replicates in each. The quantitative data are means ± SD from three experiments with three independent replicates in each as now indicated in legends for Figures 4, 5 and 6. Numbers of tested transgenic lines have now been included in the text (page 9, lines 3-8). Phytaspase expression levels did not differ significantly between any of the tested KD and OE lines respectively, so correlation between the transgene expression and stress resistance could not be investigated within the groups of KD or OE transgenic plants, but it was shown between the KD, wt and OE plants.

5. A specific relocalisation from the apoplast to the cytoplasm during stress is suggested. As a negative control the apoplastic retainment of cathepsin B during stress is shown. However no details at all on the construct used and the experimental set-up are described. A more quantitative approach should be taken before firm statements on the relocalisation can be made.

Details on the cathepsin B construct and experimental set-up were published by Gilroy et al, 2007. The reference and brief description of the experimental design have now been included in the manuscript (page 13, lines 14 -17, Figure 8E legend). Quantification of the levels of accumulation of mRFP in apoplast vs intracellular space has now been done using ImageJ software (Abramoff et al. 2004) and the data have been included in the text (page 12, lines 21-23; page 13, lines 11-14)

Referee 2

Discuss more in detail differences in substrate specificity between saspases and phytaspase to clarify the utility of additional names.

The differences between saspases and phytaspase have now been discussed in more detail in Conclusions (page 15, lines 7-12)

1. In Figure 6A, last row, my understanding is that all samples have been treated with MV. I failed to see any signs of cell death in the "wt" sample after treatment with the empty vector. Shouldn't this "empty vector/MV" control be the same as that in the "MV only"? This is in glaring contrast to panel B, lower graph, in which this same sample is producing a large amount of ROS. This discrepancy needs to be resolved.

The wrong sample was shown by mistake as wt in "the empty vector/MV" in the former Figure 6A. However, in compliance with the comment of reviewer 3 we have now replaced this panel with "inactive S535A rice enzyme panel" (see new Figure 6)

2. The last set of cell biological data using mRFP fusion is an important one for deriving a mechanistic model of phytaspase regulation. To me however, the interpretation that phytaspase is "relocalized" to the cytosol can benefit from additional confirmation data that may be obtained relatively easily by the authors. It would be interesting to carry out western blot analyses before and after induction of cell death and in the presence or absence of BFA to examine the ratio of processed vs. unprocessed forms of phytaspase. If the authors can fractionate apoplastic fluids, cytosol and endomembrane fractions from the different samples for this western study, it would be really great.

Such experiments have been done and the results confirming phytaspase relocalisation from the apoplast to inside the cell are included in the manuscript (Figure 7 and page 12, line 24 - page 13, line 5; page 13, line 22 - page 14, line 2). See also response to comment 2 of reviewer 3.

3. It would be important to note how many different subtilisin genes may be targeted by these RNAi

vectors at least for the tobacco and tomato databases. This information would be useful for interpretation of the specificity of this approach.

This information has now been included (page 8, lines 21-25). The specificity of this approach has also been demonstrated by functional complementation tests using wt rice phytaspase which have now been supplemented by the additional control, inactive S535A rice phytaspase (see Figures 4 and 6)

Refereee 3.

1. All figures need a significant revision to improve readability.
Is Fig. 1C detected with anti-GFP antibody?

This is Coommassie Blue staining. This has now been included into the Figure 1 Legend.

What does 1 and 0.2 mean in Fig. 1D?

These are the relative amounts of recombinant phytaspases assayed for proteolytic activity and this has now been explained in the Figure 1 legend.

Can you add a legend in Fig. 2D?

Done. We have also enlarged designations in Figure 1B, C, D and in Figure 2 C, D to improve readability.

Please replace 1=WT, 2=OE and 3=KD in fig5.

Done

Why were treatments (e.g. 10uM MV on OE line) left out from Figure5?

Overproduction of phytaspase in transgenic lines in OE lines significantly accelerated PCD-related processes induced by 2.5 M MV (and 75 mM NaCl) (Figure 5A-C). However, no significant differences in bleaching and cell death between wild-type and silenced KD transgenic plants were detected at these concentrations. Therefore, to discriminate between the wild-type and KD lines, higher concentrations of stress inducers were required (10 M MV and 250 mM NaCl). That is why 10 M MV / 250 mM NaCl were not used for OE lines, and 2.5 M MV / 75 mM NaCl were not used for KD lines.

Fig5C is copy-paste work: can I compare 75mM with 250mM NaCl? Probably not, so please add dash lines.

Added.

Can you add some basic information to fig7A-E (e.g. 24hr BFA treatment)? At which timepoint after treatment was Fig7C and 7D taken?

Added (see new Figure 8 legend). We have also added new Figure 7 showing results of subcellular fractionation and Western blot assay of phytaspase-mRFP. All the details required are included in the Figure 7 legend.

2. I have a serious problem with the conclusion that the phytaspase relocalizes to the cytoplasm (e.g. in title). First, do the large granules in Fig7C and 7D represent the cytoplasm? It looks rather like large vesicles. A colocalization with a cytoplasmic marker would be necessary to verify this.

To verify relocalisation of phytaspase during stress conditions we have used the additional approach suggested by reviewer 2 based on subcellular fractionation of phytaspase-mRFP before and after PCD. Although using this approach we did confirm relocalisation of phytaspase from the apoplast to inside the cell (principally into intracellular soluble fraction S30 enriched by cytoplasmic proteins) we have now toned down our conclusion about relocalization of phytaspase to the cytoplasm.

Instead we have described this process as relocalization from the apoplast to inside the cell (intracellular fraction) without detailing intracellular compartments. The more detailed intracellular localisation of the mature phytaspase during PCD (as indicated in the text, page 14, lines 13-14) will be a major task for the future which will need an extensive separate investigation (involving the use of various subcellular markers).

Second, is this internalization or hampered secretion? To discriminate between these possibilities one would have to block translation (e.g. cyclohexamide) and monitor the internalization upon HR/MV treatment.

Done (page 14, lines 3-13; new Figure 7; Supplementary Figure 3)

Fig7A seems to suggest that epidermal cells contain chloroplasts. Is it? How does the dsRED control behave in these assays?

Yes, this Figure (currently 8A) shows that epidermal cells contain chloroplasts [in agreement with previously published results (Wright et al., Traffic 2007, 8: 21-31; Escobar et al., Plant Cell 2003, 15: 1507-1523; Gilroy et al., Plant Journal 2007, 52: 1-13.

How does the dsRED control behave in these assays?

Two new panels have now been added to Figure 8 (F and G) to show distribution of free mRFP in tobacco cells before and after PCD.

3. The TMV-induced HR assay (fig4) could be more convincing. Panels in A are not that clear and seem to represent different regions of a leaf. Also magnification bars are missing. The phenotype should be supported by quantitative data (lesion sizes).

The figure has been modified in accordance with the referee's suggestion. Quantitative data and magnification bars have been added (page 10, lines 6-10, Figure 4)

Second, TMV quantification e.g. by Northern blot, could be used to show that the phytaspase plays a role in pathogen resistance.

TMV accumulation has been quantified by ELISA (see Table 1) to show that phytaspase is involved in the N gene-mediated resistance to TMV (page 10, line 24 -page 11, line 4).

4. The complementation with the rice phytaspase is certainly very neat, but why was this only done on MV-induced phenotypes, and not on the TMV assay? Is the complementation absent with a catalytically dead rice phytaspase?

The functional complementation assay using rice phytaspase has now been done on TMV-mediated PCD (Figure 4; page 10, lines 14-19). Catalytically inactive S535A phytaspase has now also been used (which did not complement phytaspase-deficiency during TMV- and MV- induced PCD (Figures 4 and 6, page 10, lines 19-23; page 11, lines 23-24)

5. Phytaspase is definitely an important enzyme, but not the only protease having caspase-activity in plants. This should be acknowledged e.g. in the conclusion.

Done (page 14, line 23 - page 15, line 7)

2nd Editorial Decision 04 January 2010

Thank you for submitting your revised version of your manuscript to EMBO J. It has now been seen by two of the original referees who recommend publication. I am happy to inform you that the manuscript is accepted and you will receive the official letter in the next day or so. I would like to draw your attention to a typo noted by

Ref #3 that should be corrected in the proofs.

Sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2

The authors adequately addressed all the issues that I have raised in the review of the first version of the manuscript. I thus recommend its publication in the EMBO J.

Referee #3

All the issues I had have been adequately resolved in this revised version. As far as I could read, this also holds for the other reviewer(s). Only one typo: 'D' in line 6 on page 35 should read 'G'. I fully support publication of this manuscript and I would like to congratulate the authors with the excellent work they have delivered in such a short timespan.

© European Molecular Biology Organization