nature portfolio

Peer Review File

# Cleavage of Bcl-2-associated athanogene by metacaspase determines plant antiviral immunity

Corresponding Author: Professor Jian Ye

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study reports that whitefly infestation specifically upregulates metacaspase 4 (MC4). Through yeast two-hybrid assays, MC4 was found to interact with Bcl-2-associated athanogene 3 (BAG3). Knocking out BAG3 in tomato resulted in increased susceptibility to tomato yellow leaf curl virus (TYLCV). Further investigations showed that BAG3 is cleaved by MC4, and its N-terminal fragment (BAG3-N) induces cell death. Mechanistically, BAG3-N oligomerizes and localizes to the plasma membrane to trigger cell death. Notably, the TYLCV Rep protein interacts with BAG3-N, disrupting its oligomerization and thereby suppressing cell death induction. The findings that MC4-mediated cleavage of BAG3 generates a resistosome-like immune response provides a novel insight into plant immunity and anitiviral defense. The study is highly innovative, with well-organized writing, robust data, and represents excellent work. However, the conclusion that BAG3-N-induced cell death is dependent on Helper NLR NRCs remains to be further confirmed, as current evidence is insufficient to fully validate this claim.
Major Comments:
1. BAG3 is highly conserved across different species, including rice and maize, and BAG3-N from different species can induce cell death. The authors conclusion that BAG3-N relies on helper NLRs (NRCs) based on the observation that BAG3-N failed to induce cell death in nrc2/3 or nrc2/3/4 mutants. However, NRCs are absent in many other families, so the conclusion about NLR dependency in the title requires caution. Additionally, protein expression levels of BAG3-N in nrc2/3 or nrc2/3/4 mutants must be examined—if BAG3-N expression is significantly reduced in these helper NLR mutants, the inability to induce cell death could be due to its poor expression rather than NLR dependency.
2. Given that BAG3-N can form oligomers independently, it is plausible that BAG3-N itself forms a resistosome-like structure to induce cell death.
3. In Figure 2d, the bands for SlBAG1 and SlBAG13i are identical to the MOCK control. How was the specificity of the BAG3 band confirmed?
4. In Figure 4g, the subcellular localization of BAG3-G50A-N differs markedly from the wild-type (WT), with a distinct pattern. Soluble and plasma membrane fractions should be separated for further validation of localization.
5. In Figure 5b, to clarify whether the lack of cell death induced by BAG3-N in nrc2/3 or nrc2/3/4 mutants is due to reduced protein expression, Western blot analysis of BAG3-N levels in these mutants must be investigated.
6. In Figure 5d, no oligomerization bands for NRC2 were detected. If BAG3-N relies on NRC2/3/4, oligomerization of NRCs should be observable.

Reviewer #2

(Remarks to the Author)
Liang, Jiang et al. present a very intriguing story showing that MC4 can cleave BAG3, releasing it from autoinhibition and inhibiting viral replication. In addition, they provide data showing that certain viral proteins can bind and counter the cell death-causing portion of BAG3. The identification of a new metacaspase substrate in the context of cell death and immunity is very important, even more considering that both belong to families that has been long related to cell death processes, but whose mode of action is not very well established. The weaker part of the manuscript is that related to oligomerization, where the evidence provided is very weak. Another suboptimal point of the article is the general unclarity about the materials used and the derived conclusions. Since the authors use different combinations of plant species and proteins and a combination of transient and stable expression lines, this should be clearly stated for each of the experiments, to avoid

confusion. Also, the consequences of the presence of native MC4 and BAG3 proteins in N. benthamiana when performing transient assays with variants of Arabidopsis proteins should be clearly discussed. Also, the conditions and age of the plant should be included in the experiments, as it is not clear in the methods or the figure legends. Figure layout can be also significantly improved for clarity. Specific comments:

- INTRODUCTION: The introduction could be significantly improved. Right now, the different topics of the article are introduced in a somehow abrupt way. For example lines 73-76, make like a quite random link. In my opinion, it would make more sense to include in the introduction what is explained in the results section (lines 101-104 and 112-115). The link between the reported function of MC4 during wound responses in PROPEP1 maturation into Pep1 with the induction of PEPR1 during whitefly infestation is a nice link for the introduction that justifies the story.

- FIGURE 1:
o Panel b: to avoid confusion, p-values should be used here. The statistics used are not the most appropriate ones for the comparisons that are wanted to be made.
o Panel c: This immunoblot is very blurry and unclear, it looks like the transfer did not go very well. Can the authors present another replicate where the results are more clear? How many times was this immunoblot performed?
o Panel d: how was this quantification done? If immunoblots such as in panel c were used, this quantification is not valid.
o Does the virus activate MC4? Not only expression data, but also WB of MC4 should be provided, in line with the story.
o Perhaps as a supplementary figure, a phylogenetic tree including BAGs used in this study should be added. Include also the BAG3s from N. benthamiana.

- FIGURE 2:
o For non-virologists, include a better explanation of how to interpret the symptoms of infection with TYLCV i.e. yellowing/necrosis vs leaf curling and stunted growth.
o Since SlBAG3 is used, for consistency Arabidopsis BAG3 should be termed AtBAG3 throughout the article and in all variants. It would avoid confusion.
o Panel d: the authors claim an increase in SlBAG3 protein levels upon TYLCV infection, which I cannot see in the immunoblot. I rather see a decrease in all three bands (also in SlBAG1).
o How do the authors know that what is recognized is BAG1? What are the BAGs in tomato? More information about all BAGs present should be added (see comment before). For example, Supplementary Figure 2C including also Arabidopsis BAG3 should be transferred to main figure 2, because it is important information and helps clarifying. Does the anti-BAG3 antibody also recognize BAG3 from other species?
o Panels e and h could be transferred to supplementary data.
o In panels f and i, individual datapoints should be added.
o In panel b, p-values should be added.

- SUPPLEMENTARY FIGURE 4: This is an important dataset that should be transferred to main figure.

- SUPPLEMENTARY FIGURE 6: This is an important figure, because with these experiments the authors determine the minimal portion of AtBAG3 protein with cell death inducing capacity. However, there are several problems:
o The positive control, BAG3 did not work, as no cell death can be observed (see panel a).
o A scheme with all the truncations in relation to the entire protein and whether they induce cell death or not would be helpful to understand the experiment. Right now their conclusions based on the experiment are extremely confusing.

- FIGURE 3:
o Panel g: in the text, establish the link to the capacity to induce cell death in relation to MC4/MC4-CA that is shown in the figure. Add datapoints to the bars.
o AlphaFold should be also used to predict the interaction between MC4 and BAG3. Does it model the cleavage site of BAG3 to the catalytic domain of MC4.
o The interaction between MC4 and BAG3 should be shown. Considering that it is an active protease, the catalytically inactive version can be used instead. They should interact as well.
o How do the authors integrate the role of N. benthamiana native BAG3 in this story? When expressing BAG3 and MC4 from Arabidopsis in N. benthamiana to assess cell death and cleavage, there is native NbBAG3? Is NbBAG3 also cleaved by MC4? Regarding the inhibitory activity of BAG3-C over BAG3-N, wouldn´t native N. benthamiana NbBAG3-C be inhibiting transiently expressed BAG3-N in cell death assays if their model is correct? These possibilities need to be incorporated into the text, not to mislead the reader.

- FIGURE 4:
o An important piece of information here would be to show a co-immunoprecipitation experiment testing self-interaction of BAG3 with BAG3-K50A mutant (rather than BiFC).
o Panel e: as mentioned, the oligomerization data obtained by BN gel is not convincing. Either present other types of evidence or strongly reduce the claims associated to this dataset.
o In panel a, change the colors and labels of the structures, they cannot be distinguished well from the white background.

- SUPPLEMENTARY FIGURE 9
o The authors claim that N-terminal domains of BAG3 from Marchantia and Physcomitrium do not trigger cell death in N. benthamiana, but in the case of Marchantia, there is cell death. In any case, trypan blue stainings for a and d should be provided.
o Data points in c and f should be provided.

- FIGURE 5:
o In panel c, NbNRC2 and NbNRC3 alone (without BAG3-N) should be included as well as controls.
o In panel d the dimer and tetramers should be pointed out in the image.
o In associated Supplementary Fig. 11, indicate the tag of the proteins in the western blot labels, for clarity.

- FIGURE 6:
o Supplementary Fig. 12 should be part of main Fig. 6. This dataset is important and should include the following: Viral proteins expressed without BAG3-N as controls. Quantification of cell death of all viral proteins +/- BAG3-N by ion leakage. Co-immunoprecipitation of viral proteins with BAG3-N to be able to claim that the interaction BAG3-N-Rep is specific.
o In line 342 the claim “Rep specifically binds to BAG3-N” should be modulated, as co-IP doesn´t necessarily mean interaction, but rather association.
o Is residue K50 of BAG3-N also necessary for its interaction with Rep? This should be tested by co-IP to claim that it mimics the interaction pattern of BAG3-C.
o In panel h, again, the quality of the experiment is poor and the claim that Rep disrupts the oligomeric state of BAG3-N is based on very weak evidence. This claim should be removed or better experimental evidence provided.

- DISCUSSION:
o The discussion includes new results and information that should be transferred to the results section (Supplementary Fig. 13 and lines 565 to 569)
o The model presented in Supplementary Fig. 14 should be better explained in the text. In the figure, the role of BAG3-C should be integrated in the model.
o I´m not sure that BAG3-N can be compared to a resistosome-like complex assembly, since it will probably not be able to form pores in the membrane.
o In the discussion and model, concepts taken from results in different species are mixed. Through most of the paper Arabidopsis BAG3-N is used in transient assays in N. benthamiana. What happens with the native BAG3-N of N. benthamiana? Does it have no function?
o Line 584: “recognition” is perhaps not the best word here, given the evidence.

Other comments:
- Figure layout: panels should be arranged in a correlative order and cited so in the text.
- Add data points to all figures, and p-values
- Small panels: difficult to see images and to read labels
- Color choice and font size of labels makes them sometimes unreadable, especially the N. benthamiana ones.
- Considering what is known about their mode of action and characteristic and as reported in various articles, metacaspases cannot be considered caspase-like proteases (line 22)
- Lines 63-64: “Metacaspases have been shown to be involved in immune-related processes”. Add references showing that.
- Line 65: a connection between metacaspases and NLRs has been established recently in doi: 10.1038/s44319-025-00426-4
- Line 71: cite also doi: 10.1105/tpc.15.00626
- Add to every figure legend how many times was every experiment repeated and to the figures all the points.
- Line 117: Figure 1c-d should be cited at the end of the line.
- Line 122-123: what do the authors mean by “MC4 may switch substrate specificity to enhance plant immune response”?
- Line 233: “Self-interaction is a critical mechanism in the activation among cell death regulators”. The authors open this section with this sentence and then cite the ZAR1 article. I think they are making a general case from a particular one. Either they cite more articles supporting this point or rephrase the sentence to fit the reference.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have now addressed most of my questions. The revised manuscript can now be acceppted by NC.

Reviewer #2

(Remarks to the Author)
In the new version of the manuscript the authors have addressed all questions I had and modified their claims so that it better reflects the data presented.

**Reviewer 1:**

This study reports that whitefly infestation specifically upregulates metacaspase 4 (MC4). Through yeast two-hybrid assays, MC4 was found to interact with Bcl-2-associated athanogene 3 (BAG3). Knocking out BAG3 in tomato resulted in increased susceptibility to tomato yellow leaf curl virus (TYLCV). Further investigations showed that BAG3 is cleaved by MC4, and its N-terminal fragment (BAG3-N) induces cell death. Mechanistically, BAG3-N oligomerizes and localizes to the plasma membrane to trigger cell death. Notably, the TYLCV Rep protein interacts with BAG3-N, disrupting its oligomerization and thereby suppressing cell death induction. The findings that MC4-mediated cleavage of BAG3 generates a resistosome-like immune response provides a novel insight into plant immunity and anitiviral defense. The study is highly innovative, with well-organized writing, robust data, and represents excellent work. However, the conclusion that BAG3-N-induced cell death is dependent on Helper NLR NRCs remains to be further confirmed, as current evidence is insufficient to fully validate this claim.

Response:

We sincerely thank the reviewer for the thoughtful and encouraging evaluation of our work.

We fully acknowledge the reviewer's concern regarding the conclusion that BAG3-N-induced cell death is dependent on helper NLRs (NRCs). In response, we have carefully revised the title, abstract, and main text to remove definitive language regarding NRCs dependency and now present this as a potential mechanism that warrants further investigation.

To address this issue experimentally, we performed additional immunoblot analyses and oligomerization assays. These revealed that BAG3-N protein expression is significantly reduced in *nrc2/3* and *nrc2/3/4* mutants, and that co-expression of BAG3-N does not induce the canonical oligomeric activation of NRC2. These findings suggest that BAG3-N-induced cell death may proceed independently of NRC2 hexameric resistosome formation. Nonetheless, we recognize that the exact molecular mechanism

remains to be fully elucidated.

We greatly appreciate the reviewer's insights, which have helped us refine both the scientific conclusions and the overall clarity of the manuscript.

Major Comments:

1. BAG3 is highly conserved across different species, including rice and maize, and BAG3-N from different species can induce cell death. The authors conclusion that BAG3-N relies on helper NLRs (NRCs) based on the observation that BAG3-N failed to induce cell death in nrc2/3 or nrc2/3/4 mutants. However, NRCs are absent in many other families, so the conclusion about NLR dependency in the title requires caution. Additionally, protein expression levels of BAG3-N in nrc2/3 or nrc2/3/4 mutants must be examined—if BAG3-N expression is significantly reduced in these helper NLR mutants, the inability to induce cell death could be due to its poor expression rather than NLR dependency.

Response:

Thank you for your insightful comments.

We agree that the presence or absence of NRCs in different species complicates a generalized conclusion regarding NLR dependency. To address this, we have revised **the title** and **relevant sections** to remove the term "NLR-dependent" and clarify the scope of our conclusion.

To further assess whether the lack of cell death in *nrc2/3* and *nrc2/3/4* mutants is due to impaired NRC function or reduced BAG3-N protein levels, we examined BAG3-N expression across multiple genetic backgrounds, including WT, *eds1*, *nrc4*, *nrc2/3*, and *nrc2/3/4*. Western blot analyses revealed that BAG3-N protein accumulation is markedly decreased in the *nrc2/3* and *nrc2/3/4* mutants compared to WT **(Supplementary Fig. 14a)**. This finding suggests that the diminished cell death observed in these mutants may be at least partially attributable to reduced BAG3-N protein stability or accumulation, rather than a direct reliance on NRCs for signaling.

We have now incorporated this data and revised our interpretation accordingly to more accurately reflect the experimental observations and avoid overextension of the conclusions **(Lines 329-341)**.

2. Given that BAG3-N can form oligomers independently, it is plausible that BAG3-N itself forms a resistosome-like structure to induce cell death.

Response:

We appreciate the reviewer's insightful suggestion regarding the potential of BAG3-N to form a resistosome-like structure independently.

As noted in the Discussion **(Lines 402-413)**, BAG3-N exhibits a β-sheet-rich region and oligomerization potential, both features reminiscent of proteins involved in higher-order immune complexes (Bi et al., 2021; Wang et al., 2023; Liu et al., 2024). These observations led us to speculate that BAG3-N could act as a structural scaffold for a resistosome-like complex. However, we wish to clarify that this remains a hypothetical model at this stage, as our current study does not provide direct structural evidence to confirm such an assembly.

We are actively pursuing this hypothesis through ongoing structural and biochemical investigations. Preliminary results from BN-PAGE and size-exclusion chromatography support the formation of higher-order BAG3-N oligomers **(Fig. 4e and R1)**. However, it remains unclear whether these assemblies are formed solely by BAG3-N or involve additional host components. This is an active area of investigation, and we have initiated cryo-EM analysis to further dissect the architecture and potential functional relevance of these complexes.

Given that these structural studies are still in progress and fall beyond the scope of the current manuscript, we have revised the Discussion to frame this as a plausible but unproven direction and explicitly highlight it as a key question for future research.

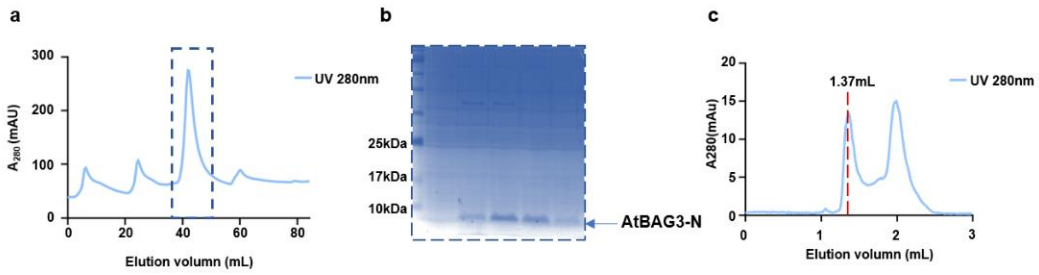


**Fig. R1 Purification and SEC analysis of BAG3-N protein. (a)** Affinity chromatography purification. The boxed region indicates the elution peak containing the target protein. A280,

absorbance at 280 nm. **(b)** SDS-PAGE analysis of the eluted fractions from affinity chromatography. The gel shows the presence of a single major band corresponding to the expected molecular weight of the target protein, confirming successful purification. **(c)** Size-exclusion chromatography (SEC) profile of the purified protein. The elution profile indicates the oligomeric state of the protein based on the elution volume, compared to molecular weight standards.

Reference:

Bi, G. et al. The ZAR1 resistosome is a calcium-permeable channel triggering plant immune signaling. *Cell*, 2021, 184(13), 3528-3541.

Wang, W. et al. WeiTsing, a pericycle-expressed ion channel, safeguards the stele to confer clubroot resistance. *Cell*, 2023, 186(12), 2656-2671.

Liu, F. et al. Activation of the helper NRC4 immune receptor forms a hexameric resistosome. *Cell*, 2024, 187(18), 4877-4889.

3. In Figure 2d, the bands for SlBAG1 and SlBAG13i are identical to the MOCK control. How was the specificity of the BAG3 band confirmed?

Response:

We sincerely thank the reviewer for raising this important concern regarding the specificity of the bands originally shown in Figure 2d **(now presented in Fig. 2h)**.

While initial band assignment relied on predicted molecular weight matching SlBAG1/3/3l, we agree this alone is insufficient. To rigorously validate the identity of the SlBAG1/3/3l band, we performed additional Western blot experiments using the *Slbag1/3/3l* triple mutant lines (*Slbag1/3/3l* #3and #18). Notably, the bands corresponding to the expected size of SlBAG1/3/3l were completely absent in both lines. These results provide strong genetic evidence for the specificity of the detected band and indicate minimal antibody cross-reactivity under our experimental conditions **(Lines 166-172)**.

4. In Figure 4g, the subcellular localization of BAG3-G50A-N differs markedly from the

wild-type (WT), with a distinct pattern. Soluble and plasma membrane fractions should be separated for further validation of localization.

Response:

We thank the reviewer for this constructive suggestion.

To further validate the subcellular localization differences between BAG3-N and the BAG3$^{K50A}$-N mutant, we performed subcellular fractionation assays to separate cytosolic and plasma membrane fractions. As shown in the newly added panel in **Fig. 4h**, BAG3-N is predominantly localized to the plasma membrane, whereas BAG3$^{K50A}$-N is distributed in both the cytosolic and membrane fractions. This result confirms that the BAG3$^{K50A}$-N mutation alters the subcellular localization of BAG3-N, consistent with the imaging data presented in Fig. 4g **(Lines 280-287)**.

5.In Figure 5b, to clarify whether the lack of cell death induced by BAG3-N in nrc2/3 or nrc2/3/4 mutants is due to reduced protein expression, Western blot analysis of BAG3-N levels in these mutants must be investigated.

Response:

We appreciate the reviewer's constructive suggestion.

To directly address this point, we performed immunoblot analysis of BAG3-N protein levels in *nrc2/3* and *nrc2/3/4* mutants, alongside WT, *eds1*, and *nrc4*. The results showed a clear reduction of BAG3-N accumulation in *nrc2/3* and *nrc2/3/4* backgrounds **(Supplementary Fig. 14a)**.

We have incorporated these data and revised the corresponding text to reflect this observation **(Lines 329-332)**.

6. In Figure 5d, no oligomerization bands for NRC2 were detected. If BAG3-N relies on NRC2/3/4, oligomerization of NRCs should be observable.

We thank the reviewer for highlighting this important point.

To further assess whether BAG3-N triggers NRC2/3/4 activation in planta, we repeated the oligomerization assay multiple times **(Fig 5d)**. Consistently, NRC2 formed only detectable dimeric and tetrameric species, and no hexameric assemblies were

observed upon BAG3-N expression. The resting state of NRC2 forms homodimers or tetramers, while its activated form adopts a hexameric structure (Ma et al., 2024; Madhuprakash et al., 2024). This result suggests that, although NRC2 may be present, it does not adopt an activated oligomeric state in response to BAG3-N. Thus, BAG3-N-induced cell death likely proceeds independently of the canonical oligomerization-based activation of NRC2.

We have revised the title, abstract, and main text **(Lines 28-30, 335-341 and 434-436)** to avoid overstating NRC dependency and to clarify that BAG3-N-induced cell death does not depend on the oligomeric activation of NRC2.

Reference:

Ma, S. et al. Oligomerization-mediated autoinhibition and cofactor binding of a plant NLR. *Nature*, 2024, 632(8026), 869-876.

Madhuprakash, J. et al. A disease resistance protein triggers oligomerization of its NLR helper into a hexameric resistosome to mediate innate immunity. *Science Advances*, 2024, 10(45), eadr2594.

**Reviewer 2:**

Liang, Jiang et al. present a very intriguing story showing that MC4 can cleave BAG3, releasing it from autoinhibition and inhibiting viral replication. In addition, they provide data showing that certain viral proteins can bind and counter the cell death-causing portion of BAG3. The identification of a new metacaspase substrate in the context of cell death and immunity is very important, even more considering that both belong to families that has been long related to cell death processes, but whose mode of action is not very well established. The weaker part of the manuscript is that related to oligomerization, where the evidence provided is very weak. Another suboptimal point of the article is the general unclarity about the materials used and the derived conclusions. Since the authors use different combinations of plant species and proteins and a combination of transient and stable expression lines, this should be clearly stated for each of the experiments, to avoid confusion. Also, the consequences of the presence of native MC4 and BAG3 proteins in *N. benthamiana* when performing transient assays with variants of Arabidopsis proteins should be clearly discussed. Also, the conditions and age of the plant should be included in the experiments, as it is not clear in the methods or the figure legends. Figure layout can be also significantly improved for clarity.

Response:

We sincerely thank the reviewer's thoughtful and constructive comments, as well as for recognizing the novelty and significance of our findings.

**Regarding BAG3-N oligomerization**, we appreciate the reviewer's concern about the strength of the evidence. To address this, we conducted additional analyses using both Blue Native PAGE (BN-PAGE) and Size Exclusion Chromatography (SEC). The BN-PAGE experiments, performed under identical conditions for full-length AtBAG3 and AtBAG3-N, revealed high molecular weight complexes exclusively in the AtBAG3-N, suggesting that the N-terminal region alone drives oligomer formation. To further validate this, we purified recombinant AtBAG3-N and analyzed it using SEC on a Superdex 200 3/150 column. The elution peak at ~1.37 mL corresponds to a molecular weight well above the monomeric form, consistent with the BN-PAGE results. While these SEC data strongly support oligomerization, they were obtained in a heterologous system under non-native conditions.

To reflect this limitation, we have also softened the claims about oligomerization in the revised manuscript and reframed the discussion accordingly **(Lines 269-272)**. We would also like to clarify that BN-PAGE is a widely accepted approach to infer oligomeric states in the absence of high-resolution structural data (Hu et al., 2020; Yu et al.,2024; Wang et al., 2025). However, we acknowledge the importance of direct structural validation, which we are currently pursuing as part of an ongoing effort.

**Concerning experimental clarity**, we fully agree that clear identification of plant species, expression systems, and protein variants is essential for interpretability. In the revised manuscript, we have carefully annotated each experiment with specific information about the host species (*Nicotiana benthamiana*, *Arabidopsis thaliana*, or *Solanum lycopersicum*), the source and form of BAG3 proteins used, and whether transient or stable expression was employed. This information has been included in both the figure legends and the Methods section. We have also updated the Methods to include the growth conditions and developmental stage of plants used in each assay.

**To improve overall clarity and presentation**, we have restructured several multi-panel figures to enhance readability. Redundant panels were consolidated, font sizes standardized, and schematic diagrams added where appropriate to better guide the reader through complex experimental setups.

Once again, we thank the reviewer for their insightful feedback, which has substantially improved the clarity, rigor, and interpretability of our study.

Reference:

Hu, M. et al. Bacterial effectors induce oligomerization of immune receptor ZAR1 in vivo. *Molecular Plant*, 2020, 13(5), 793-801.

Yu, H. et al. Activation of a helper NLR by plant and bacterial TIR immune signaling. *Science*, 2024, 386(6728), 1413-1420.

Wang, M. et al. The plant immune receptor SNC1 monitors helper NLRs targeted by a bacterial effector. *Cell Host & Microbe*, 2025, 31(11), 1792-1803.

Specific comments:

- INTRODUCTION: The introduction could be significantly improved. Right now, the different topics of the article are introduced in a somehow abrupt way. For example, lines 73-76, make like a quite random link. In my opinion, it would make more sense to include in the introduction what is explained in the results section (lines 101-104 and 112-115). The link between the reported function of MC4 during wound responses in PROPEP1 maturation into Pep1 with the induction of PEPR1 during whitefly infestation is a nice link for the introduction that justifies the story.

Response:

We thank the reviewer for this insightful suggestion.

In the revised manuscript, we have restructured the Introduction to improve the logical flow between topics. Specifically, we have clarified the role of MC4 in PROPEP1 cleavage and Pep1 production, and incorporated the connection between PEPR1 induction and whitefly infestation (previously described in the Results section, lines 101-115 of the original version) to better justify our hypothesis and overall rationale. These revisions help establish a more coherent narrative leading to our investigation of BAG3 and its potential role in linking MC4 activity to plant antiviral immunity **(Lines 61-69)**.

- FIGURE 1:

o Panel b: to avoid confusion, p-values should be used here. The statistics used are not the most appropriate ones for the comparisons that are wanted to be made.

Response:

We thank the reviewer for this helpful comment and agree that using *P*-values can enhance the clarity of statistical interpretation.

In Fig. 1b, we compared the expression levels of nine *Arabidopsis MC*s under three treatment conditions (mock, whitefly infestation, and TYLCV infection). One-way ANOVA followed by Tukey's multiple range test was performed to evaluate pairwise differences. To present the results concisely, we annotated the bar plot using letter groupings based on adjusted *P*-values **(Fig. 1b)**.

We fully agree that displaying exact *P*-values can increase clarity, but given the number of comparisons, including all values directly on the plot would reduce its readability. To

balance transparency and clarity, we have included all *P*-values in the **Source Data** file. Additionally, we have revised the figure legend to explicitly mention the statistical method used **(Lines 809-814)**.

o Panel c: This immunoblot is very blurry and unclear, it looks like the transfer did not go very well. Can the authors present another replicate where the results are more clear? How many times was this immunoblot performed?

Response:

Thank you very much for your valuable comments and suggestions.

We acknowledge that the image quality of the immunoblot in panel c could be improved. To address this, we have repeated the experiment and replaced the panel with a clearer replicate **(Fig. 1d)**. The new immunoblot displays sharper band signals and improved resolution, confirming the original findings.

Regarding the number of replicates, this experiment has been repeated more than three times independently, and consistent results were obtained in each case, supporting the robustness of our observations. We have also updated the figure legend to include this information **(Lines 820-823)**. To further illustrate this consistency, an additional biological replicate is presented in the new panel **(Fig. R1)**.

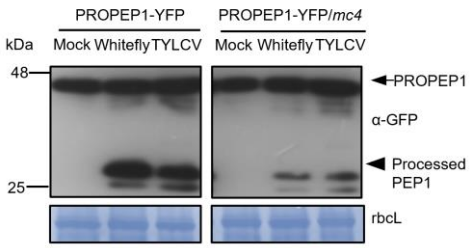


**Fig. R1 Whitefly infestation promotes the processing of PROPEP1-YFP and TYLCV infection disturbs this process.** Protein detection was performed using anti-GFP antibody. CBB stained bands of the rbcL were used as a loading control. Three biological replicates were conducted.

o Panel d: how was this quantification done? If immunoblots such as in panel c were used, this quantification is not valid.

Response:

We thank the reviewer for raising this important point.

To clarify, the quantification originally shown in panel d was based on three independent biological replicates, each assessed by immunoblotting. The immunoblot previously presented in panel c was one representative replicate, but we agree that its image quality was suboptimal and may have led to confusion regarding the validity of the quantification.

To improve clarity and rigor, we have replaced the original blot with a higher-quality replicate, and now moved the quantification data to **Supplementary Fig. 1b**. The quantification shown in Supplementary Fig. 1b was derived from three independent experiments, and not from the individual blot presented in Fig. 1d. Band intensities were measured using ImageJ, with background subtraction and normalization to the internal loading control. All raw blot images and numerical data are provided in the **Source Data file** to ensure transparency.

o Does the virus activate MC4? Not only expression data, but also WB of MC4 should be provided, in line with the story.

Response:

Thank you for your valuable suggestion.

In response to your comment, we performed additional Western blot analyses to assess MC4 activation. Specifically, we expressed MC4-Flag in *N. benthamiana* plants under mock and TYLCV-infected conditions. A marked increase in the self-cleaved MC4 band was observed in TYLCV-infected samples, indicative of enhanced MC4 activation upon viral infection. These new results and their quantification have been incorporated into the revised manuscript **(Fig. 1c, Supplementary Fig. 1a, and lines 107-109)**.

o Perhaps as a supplementary figure, a phylogenetic tree including BAGs used in this study should be added. Include also the BAG3s from N. benthamiana.

Response:

We thank the reviewer for this helpful suggestion.

In response, we have generated a phylogenetic tree including all BAGs used in this study,

as well as the BAG3 homologs from *N. benthamiana*. The resulting tree, now presented as **Supplementary Fig. 2**, provides an evolutionary framework for interpreting the relationships and conservation among BAGs, particularly those functionally characterized in our work.

- FIGURE 2:

o For non-virologists, include a better explanation of how to interpret the symptoms of infection with TYLCV i.e. yellowing/necrosis vs leaf curling and stunted growth.

Response:

We thank the reviewer for this helpful suggestion. To improve clarity for non-specialist readers, a clarifying sentence explaining that necrosis is typically associated with localized defense responses, while leaf curling and stunted growth indicate systemic infection and impaired immunity (Moshe et al., 2016; Cao et al., 2024). This addition has been included in the revised manuscript **(Lines 154-156)**.

Reference:

Moshe, A. et al. Tomato plant cell death induced by inhibition of HSP90 is alleviated by Tomato yellow leaf curl virus infection. *Molecular Plant Pathology*, 2016, 17(2), 247-260.

Cao, X. et al. Tomato yellow leaf curl virus: Characteristics, influence, and regulation mechanism. *Plant Physiology and Biochemistry*, 2024, 213, 108812.

o Since SlBAG3 is used, for consistency Arabidopsis BAG3 should be termed AtBAG3 throughout the article and in all variants. It would avoid confusion.

Response:

We agree with the reviewer's suggestion.

To ensure consistency and avoid confusion, we have revised the manuscript to refer to Arabidopsis BAG3 as "AtBAG3" throughout the main text, figure legends, and supplementary information.

o Panel d: the authors claim an increase in SlBAG3 protein levels upon TYLCV infection,

which I cannot see in the immunoblot. I rather see a decrease in all three bands (also in SlBAG1).

Response:

We thank the reviewer for the careful and insightful observation regarding Panel d.

We agree that the apparent changes in the intensity of the three SlBAG1/3/3l bands are subtle and not statistically significant. Accordingly, we have revised the manuscript to tone down any quantitative claims about these bands **(Lines 166-172)**. Instead, we emphasize the more robust and reproducible observation—the appearance of a novel ~15 kDa cleavage product (indicated by the arrow), which is consistently detected only in TYLCV-infected plants and absent in both mock-treated controls and the *Slbag1/3/3l* mutant lines **(Fig. 2h)**. We believe this band represents the key experimental insight from this panel.

o How do the authors know that what is recognized is BAG1? What are the BAGs in tomato? More information about all BAGs present should be added (see comment before). For example, Supplementary Figure 2C including also Arabidopsis BAG3 should be transferred to main figure 2, because it is important information and helps clarifying. Does the anti-BAG3 antibody also recognize BAG3 from other species?

Response:

We appreciate the reviewer's insightful suggestion regarding the identification of the band recognized in Figure 2 and the importance of distinguishing among BAG family members. To rigorously determine whether the band detected in Figure 2d (now presented as **Fig. 2h**) corresponds to SlBAG1, SlBAG3, or SlBAG3l, we performed additional Western blot analyses using two independently generated Slbag1/3/3l triple mutant lines (*Slbag1/3/3l* #3 and #18). As shown in the revised **Fig. 2h**, the band at the expected molecular weight (~38 kDa for SlBAG1, ~36.7 kDa for SlBAG3, and ~31.4 kDa for SlBAG3l) was completely absent in both lines. This complete loss of signal strongly supports the specificity of the antibody and confirms that the detected bands in WT samples represent SlBAG1, SlBAG3, and SlBAG3l.

To further clarify the BAG gene family in tomato, we performed a comprehensive sequence analysis and identified 11 BAG genes in the tomato genome. We have now included a

phylogenetic tree comparing BAG3 homologs involved in this study in **Supplementary Fig. 2**, as suggested by the reviewer. Furthermore, Supplementary Fig. 2C, which includes alignment with *Arabidopsis* BAG3, has been transferred to the main **Fig. 2d**, as this comparative analysis provides essential information.

Regarding the antibody used in this study: the anti-BAG3 antibody was originally raised against AtBAG3. We validated its cross-reactivity in *Arabidopsis* transgenic lines, tomato knockout mutants, and TYLCV-infected tomato samples. In all cases, the antibody consistently recognized bands at the expected molecular weights, indicating its effectiveness in detecting AtBAG3 and SlBAG1/3/3l proteins.

o Panels e and h could be transferred to supplementary data.

Response:

Thank you for this suggestion.

Panels e and h have been moved to the **Supplementary Fig. 6a and b**.

o In panels f and i, individual datapoints should be added.

Response:

Thank you for this suggestion.

We have updated panels f and i to include individual data points **(now presented as Fig. 2i and k)**.

o In panel b, p-values should be added.

Response:

We appreciate the reviewer's suggestion.

*P*-values have now been added directly to panel b to enhance statistical clarity and interpretability **(now presented as Fig. 2f)**.

- SUPPLEMENTARY FIGURE 4: This is an important dataset that should be transferred to main figure.

Response:

We thank the reviewer for highlighting the importance of this dataset.

In response, we have transferred the content of Supplementary Figure 4 to the main figures as **Fig. 2a-c** in the revised manuscript. The corresponding figure legend and relevant text have been updated accordingly **(Lines 140-146 and 835-846)**.

- SUPPLEMENTARY FIGURE 6: This is an important figure, because with these experiments the authors determine the minimal portion of AtBAG3 protein with cell death inducing capacity. However, there are several problems:

o The positive control, BAG3 did not work, as no cell death can be observed (see panel a).

Response:

We appreciate the reviewer's careful observation.

We clarify that full-length AtBAG3 was not intended as a positive control, but served as a reference construct to evaluate the cell death activity of its truncated variants. Consistent with our prior findings, full-length AtBAG3 does not induce cell death, thereby providing a baseline for comparison. This design allowed us to identify the specific region of AtBAG3 responsible for triggering cell death **(Fig 2g and Supplementary Fig. 8)**.

o A scheme with all the truncations in relation to the entire protein and whether they induce cell death or not would be helpful to understand the experiment. Right now their conclusions based on the experiment are extremely confusing.

Response:

We thank the reviewer for the helpful suggestion. To clarify the relationship between the truncation constructs and the observed cell death phenotypes, we have added a schematic summary showing all AtBAG3 truncations relative to the full-length protein **(Supplementary Fig. 8c)**. The schematic also indicates whether each construct induces cell death or not, based on the phenotypic results shown in Supplementary Fig. 8a and 8b. We believe this addition improves the clarity and interpretability of the experiment **(Lines190-197)**.

- FIGURE 3:

o Panel g: in the text, establish the link to the capacity to induce cell death in relation to MC4/MC4-CA that is shown in the figure. Add datapoints to the bars.

Response:

We thank the reviewer for this helpful suggestion.

In the main text, we now more explicitly state the functional distinction between wild-type MC4 and its catalytically inactive mutant $MC4^{C139A}$. Specifically, we note that $MC4^{C139A}$ failed to cleave AtBAG3 and did not trigger cell death upon co-expression, highlighting the requirement of MC4 enzymatic activity for AtBAG3-induced cell death **(Lines 233-237)**.

In addition, we have added individual data points to each bar to improve clarity and transparency, as suggested by the reviewer **(now presented as Fig. 3i)**.

o AlphaFold should be also used to predict the interaction between MC4 and BAG3. Does it model the cleavage site of BAG3 to the catalytic domain of MC4.

Response:

Thank you for the insightful comment.

To address this point, we used AlphaFold-Multimer to predict the potential interaction between AtMC4 and AtBAG3. The predicted structure shows that several residues on AtBAG3 are involved in the interaction interface with MC4, specifically:

- AtBAG3 R127 with MC4 D202
- AtBAG3 E133 with MC4 D200
- AtBAG3 Q201 with MC4 T100

These interaction sites are located near the R122 region of AtBAG3, which is putatively recognized as the cleavage site by MC4. Structurally, the predicted interface is also in proximity to the catalytic domain of MC4, which includes Cys139 and His86 (Zhu et al., 2020), consistent with the known active site residues.

Although AlphaFold does not explicitly model enzymatic cleavage events, the spatial positioning of the predicted interaction interface supports a substrate-binding mode that places the cleavage site near the catalytic center. This suggests that AtBAG3 could indeed be a physiological substrate of AtMC4, and the interaction likely precedes cleavage.

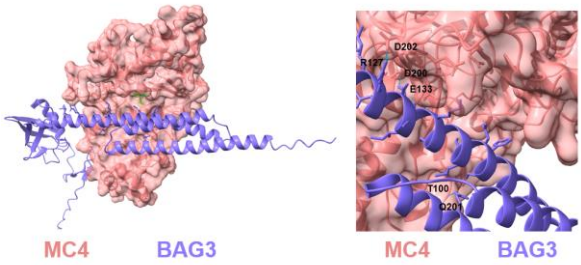


**Fig. R2 The interaction model between MC4 (pink) and AtBAG3 (purple) was predicted using AlphaFold 3.** The stick model represents the potential interaction sites between MC4 and AtBAG3; this region is predicted with a high confidence score.

Reference:

Zhu, P. et al. Structural basis for $Ca^{2+}$-dependent activation of a plant metacaspase. *Nature communications*, 2020, 11(1), 2249.

o The interaction between MC4 and BAG3 should be shown. Considering that it is an active protease, the catalytically inactive version can be used instead. They should interact as well.

Response:

We thank the reviewer for this valuable suggestion.

As requested, we examined the interaction between MC4 and AtBAG3 using the catalytically inactive mutant of MC4 ($MC4^{C139A}$). Co-IP assays confirmed that AtBAG3 interacts with the $MC4^{C139A}$ mutant, demonstrating that their interaction is independent of MC4's protease activity. The results have been included in the revised manuscript **(Fig. 1f)**, and the corresponding text in the Results and Methods sections has been updated accordingly **(Lines 131-133 and 498-499)**.

o How do the authors integrate the role of N. benthamiana native BAG3 in this story? When expressing BAG3 and MC4 from Arabidopsis in N. benthamiana to assess cell death and cleavage, there is native NbBAG3? Is NbBAG3 also cleaved by MC4? Regarding the inhibitory activity of BAG3-C over BAG3-N, wouldn´t native N. benthamiana NbBAG3-C be

inhibiting transiently expressed BAG3-N in cell death assays if their model is correct? These possibilities need to be incorporated into the text, not to mislead the reader.

Response:

We appreciate the reviewer's insightful comments regarding the potential involvement of native *N. benthamiana* BAG3 homologs in our transient expression assays.

To address these concerns, we performed additional control experiments and and included relevant clarifications in the revised manuscript.

When co-expressing Arabidopsis BAG3 (AtBAG3) and Arabidopsis MC4 in *N. benthamiana*, we observed robust cell death **(Fig. 3d)**. In contrast, co-expression of AtBAG3 with a control protein (GUS), or expression of MC4 with an empty vector, failed to induce cell death **(Fig. 3d)**, and no AtBAG3 cleavage was detected under these conditions **(Fig. 3f)**. These findings suggest that NbBAG3 homologs are not sufficient to induce cell death in this system.

To assess potential contributions from native *N. benthamiana* BAG3 homologs, we identified three candidate genes—*NbBAG1*, *NbBAG3*, and *NbBAG3-like (NbBAG3l)*—by sequence alignment. We then simultaneously silenced these genes using virus-induced gene silencing (VIGS) **(Supplementary Fig. 10a)**. Silencing had no observable effect on AtBAG3/MC4-induced cell death phenotypes **(Supplementary Fig. 10b)**, further confirming that endogenous *Nb*BAG3 homologs do not mediate or suppress the observed cell death.

We further examined whether native NbBAG3-C could suppress AtBAG3-N-induced cell death. Upon transient expression of AtBAG3-N alone or with an empty vector, clear cell death was consistently observed in *N. benthamiana* **(Fig.2g and 3a)**, indicating that endogenous NbBAG3-C does not exert an inhibitory effect on AtBAG3-N-induced cell death under these experimental conditions.

Taken together, these data support the conclusion that the cell death is specifically attributable to the heterologous interaction between AtBAG3 and MC4, and is independent of native *N. benthamiana* BAG3 homologs or their cleavage products. To ensure clarity and avoid potential misinterpretation, we have incorporated this discussion and relevant references into the revised manuscript **(Lines 207-209 and 225-233)**.

- FIGURE 4:

o An important piece of information here would be to show a co-immunoprecipitation experiment testing self-interaction of BAG3 with BAG3-K50A mutant (rather than BiFC).

Response:

We thank the reviewer for this insightful suggestion.

We performed Co-IP assays to assess the self-interaction of wild-type AtBAG3-N and K50A mutant. As shown in **Fig.4d and Supplementary Fig. 11a**, AtBAG3-N efficiently self-interacts, whereas the AtBAG3$^{K50A}$-N substantially diminishes this interaction. The data have been incorporated into the revised manuscript, and the corresponding **Results** have been updated accordingly **(Lines 263-265)**.

o Panel e: as mentioned, the oligomerization data obtained by BN gel is not convincing. Either present other types of evidence or strongly reduce the claims associated to this dataset.

Response:

We thank the reviewer for this valuable comment. We acknowledge that BN-PAGE offers limited resolution for determining precise oligomeric states.

In our BN-PAGE experiments, we included both full-length AtBAG3 and AtBAG3-N. Interestingly, high-molecular-weight complexes were observed exclusively for AtBAG3-N, but not for the full-length protein **(Fig. R3)**, suggesting that the N-terminal region has the intrinsic potential to mediate oligomerization.

To strengthen our conclusion, we performed size-exclusion chromatography (SEC) using purified AtBAG3-N, which consistently eluted at a molecular weight exceeding that of the monomer, suggesting the presence of oligomers.

In response, we have substantially revised the text, referring to the BN-PAGE results as indicative rather than definitive, and clarifying that the observed high-molecular-weight bands are consistent with, but do not conclusively demonstrate, oligomerization **(Lines 269-272)**.

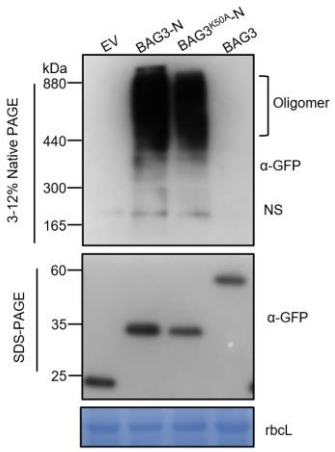


**Fig. R3 Oligomerization analysis of AtBAG3 and AtBAG3-N by BN-PAGE.** Square brackets point to oligomers. NS, nonspecific bands (upper image). The protein expression level and loading control were detected by SDS-PAGE and rbcL, respectively (bottom image).

o In panel a, change the colors and labels of the structures, they cannot be distinguished well from the white background.

Response:

We thank the reviewer for this helpful suggestion.

In response, we adjusted the color scheme and labeling in panel a to enhance contrast and improve visibility against the white background. The revised figure is now presented as **Fig. 4a** in the updated manuscript.

- SUPPLEMENTARY FIGURE 9

o The authors claim that N-terminal domains of BAG3 from Marchantia and Physcomitrium do not trigger cell death in N. benthamiana, but in the case of Marchantia, there is cell death. In any case, trypan blue stainings for a and d should be provided.

Response:

We thank the reviewer for this observation.

To clarify, the cell death phenotype induced by *Marchantia polymorpha* BAG3-N was markedly weaker compared to the positive control (AtBAG3-N), and comparable to the negative control. This aligns with our original conclusion that it does not strongly trigger cell

death. To address this point further, we repeated these experiments multiple times independently and observed no statistically significant cell death relative to the control. As requested, the newly included trypan blue staining images now visually confirm these observations and are shown in **Supplementary Fig. 12a and 12d**.

o Data points in c and f should be provided.

Response:

We thank the reviewer for pointing this out.

The individual data points for panels c and f have now been included in **Supplementary Fig. 12c** and **12f** to enhance transparency and facilitate comparison across replicates.

- FIGURE 5:

o In panel c, NbNRC2 and NbNRC3 alone (without BAG3-N) should be included as well as controls.

Response:

We thank the reviewer for this important suggestion.

To assess whether NbNRC2 or NbNRC3 alone could induce cell death, we co-expressed each gene with an empty vector in *N. benthamiana*. Neither NbNRC2 nor NbNRC3 alone triggered any visible cell death, as shown in **Fig. 5c** and **Supplementary Fig. 14b**.

o In panel d the dimer and tetramers should be pointed out in the image.

Response:

We thank the reviewer for this helpful suggestion.

The dimer and tetramer bands have now been annotated in **Fig. 5d** to improve clarity and facilitate interpretation.

o In associated Supplementary Fig. 11, indicate the tag of the proteins in the western blot labels, for clarity.

Response:

As suggested, we have specified the protein tags (FLAG- or GFP-tagged) in the western

blot labels now presented in **Supplementary Fig. 14** (previously Supplementary Fig. 11) to clarify protein identity and improve interpretability. The corresponding figure legend has also been updated accordingly **(Lines 1156-1162)**.

- FIGURE 6:

o Supplementary Fig. 12 should be part of main Fig. 6. This dataset is important and should include the following: Viral proteins expressed without BAG3-N as controls. Quantification of cell death of all viral proteins +/- BAG3-N by ion leakage. Co-immunoprecipitation of viral proteins with BAG3-N to be able to claim that the interaction BAG3-N- Rep is specific.

Response:

We thank the reviewer for this constructive and detailed suggestion.

In response, we have incorporated the former Supplementary Fig. 12 into the main manuscript as **Fig. 6a**, and have performed the additional experiments as requested.

1) We have performed the necessary control experiments by expressing each viral protein in the absence of AtBAG3-N and protein expressions were shown in **Supplementary Fig. 15**.
2) Ion leakage assays were performed to quantify cell death induced by each viral protein with or without AtBAG3-N, presented in revised **Fig. 6b**.
3) To assess specificity, we conducted Co-IP assays between AtBAG3-N and each viral protein. Only Rep co-immunoprecipitated with AtBAG3-N, supporting the specificity of this interaction **(Fig. 6c)**.

Corresponding changes have been made to the **main text** to reflect these additions (**Lines 367-370**).

o In line 342 the claim “Rep specifically binds to BAG3-N” should be modulated, as co-IP doesn´t necessarily mean interaction, but rather association.

Response:

We thank the reviewer for this important clarification.

We agree that Co-IP indicates protein association, not necessarily direct binding. Accordingly, we have revised the sentence in **lines 367-370** to reflect this distinction and

more accurately represent the nature of the experimental evidence.

o Is residue K50 of BAG3-N also necessary for its interaction with Rep? This should be tested by co-IP to claim that it mimics the interaction pattern of BAG3-C.

Response:

We thank the reviewer for this important suggestion.

We would like to clarify that residue K50 is specifically required for the self-association of BAG3-N, but not for its interaction with BAG3-C.

To test whether K50 is also necessary for BAG3-N interaction with Rep, we performed Co-IP assays using BAG3$^{K50A}$-N and Rep. As shown in **Fig. R4**, the BAG3$^{K50A}$-N mutant retains its ability to bind Rep, indicating that this interaction is K50-independent. These results suggest that although Rep mimics the functional role of BAG3-C in suppressing BAG3-N activity, but does not engage BAG3-N through the same interface used in N-N interaction.

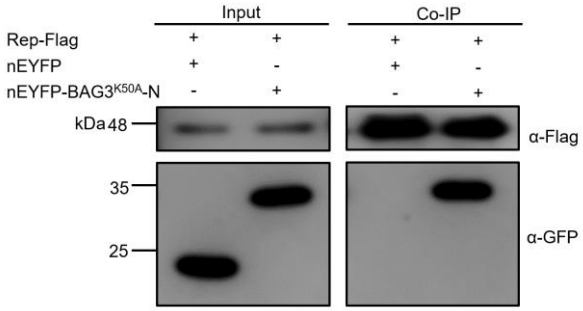


**Fig. R4 Co-IP analysis of nEYFP-AtBAG3$^{K50A}$-N and Rep-Flag *in vivo*.** Total protein was extracted from *N. benthamiana* leaves transiently expressing *35S: nEYFP-AtBAG3$^{K50A}$-N* together with *35S: Rep-Flag*. Flag-trap beads were used to precipitate the interaction complex, and anti-GFP (α-GFP) and anti-Flag (α-Flag) antibodies were used for detection. The experiment was repeated independently three times with similar results.

o In panel h, again, the quality of the experiment is poor and the claim that Rep disrupts the oligomeric state of BAG3-N is based on very weak evidence. This claim should be removed or better experimental evidence provided.

Response:

We thank the reviewer for this constructive comment.

We agree that the data presented in the original panel h did not adequately support the conclusion regarding Rep-mediated disruption of BAG3-N oligomerization. We have therefore removed this panel and the associated claim from the revised manuscript.

- DISCUSSION:

o The discussion includes new results and information that should be transferred to the results section (Supplementary Fig. 13 and lines 565 to 569)

Response:

We thank the reviewer for pointing this out.

We agree that these data represent new experimental results and are more appropriately presented in the Results section. Accordingly, the corresponding text (previously lines 565-569) has been moved to **lines 287-290** of the Results section.

In addition, Supplementary Fig. 13 has been reassigned as **Supplementary Fig. 16**, and is now cited accordingly in the revised manuscript **(lines 376-381)**.

o The model presented in Supplementary Fig. 14 should be better explained in the text. In the figure, the role of BAG3-C should be integrated in the model.

Response:

We thank the reviewer for this valuable suggestion.

In response, we have revised the main text to provide a more detailed explanation of the working model, now presented as **Supplementary Fig. 17**. Specifically, we have now integrated the role of BAG3-C, which functions as a negative regulatory element under resting conditions. As described in the revised text **(Lines 428-439)**, BAG3 adopts an autoinhibited conformation via intramolecular interactions between BAG3-N and BAG3-C, thereby preventing premature immune activation.

This autoinhibitory mechanism is supported by co-expression experiments, structural modeling, Co-IP assays, and BiFC assays **(Fig. 3a-c and Supplementary Fig. 9)**. Together, these revisions clarify the mechanistic framework of the model and more accurately represent how BAG3 integrates metacaspase activity with antiviral immune signaling.

o I´m not sure that BAG3-N can be compared to a resistosome-like complex assembly, since it will probably not be able to form pores in the membrane.

Response:

We thank the reviewer for this insightful comment.

In the discussion, we speculated that BAG3-N oligomers exhibit oligomerization behavior reminiscent of resistosome assemblies, based on their membrane localization and higher-order oligomerization. However, we fully agree that the lack of pore-forming activity represents a fundamental mechanistic difference.

To avoid overinterpretation, we have revised the text to remove the term “resistosome-like” and clarify that the comparison is structural rather than functional **(Lines 404-410)**.

o In the discussion and model, concepts taken from results in different species are mixed. Through most of the paper Arabidopsis BAG3-N is used in transient assays in N. benthamiana. What happens with the native BAG3-N of N. benthamiana? Does it have no function?

Response:

We thank the reviewer for raising this important point.

Indeed, most of our functional analyses were performed using AtBAG3 in *N. benthamiana* as a heterologous expression system. This approach was chosen due to the well-established utility of *N. benthamiana* as a model for transient expression.

To assess the potential role of endogenous BAG3 homologs in *N. benthamiana*, we identified NbBAG1, NbBAG3, and NbBAG3-like via sequence alignment and simultaneously silenced them using virus-induced gene silencing (VIGS). Silencing of these genes had no detectable effect on AtBAG3/MC4-induced cell death, suggesting that endogenous NbBAG3 proteins do not influence the observed phenotype under our assay conditions **(Supplementary Fig. 10)**.

While these results indicate that NbBAG3 is not required for AtBAG3-N-mediated activity, the intrinsic function of NbBAG3-N itself remains to be elucidated. Given this, we have refrained from incorporating NbBAG3 into our working model.

To avoid overgeneralization, we have revised the discussion to explicitly state that our conclusions are derived from AtBAG3, and that the relevance to native NbBAG3 remains to be determined **(Supplementary Fig. 17 and lines 428-439)**.

o Line 584: “recognition” is perhaps not the best word here, given the evidence.

Response:

We appreciate the reviewer’s insightful comment.

We agree with the reviewer that "recognition" may not be the best term here. In response, we have removed this term and revised the sentence **(now presented in lines 380–381)** to more cautiously interpret the observation.

Other comments:

- Figure layout: panels should be arranged in a correlative order and cited so in the text.

Response:

We thank the reviewer for this valuable suggestion.

In the revised version of the manuscript, we have carefully rearranged the figure panels to follow a logical and correlative order that better reflects the progression of the results. In addition, we have revised the main text to ensure that each panel is cited in the correct sequence and context.

These changes have been made to improve the clarity and readability of the manuscript.

- Add data points to all figures, and p-values

Response:

We sincerely appreciate the reviewer’s thoughtful and constructive suggestions regarding our figures.

In response, we have added individual data points to all relevant figures to better reflect data distribution and variability.

Regarding the *P*-values, we fully agree with the importance of presenting statistical details. However, after careful consideration, we believe that displaying all pairwise *P*-values directly on the figures would significantly compromise figure clarity, particularly in cases

with multiple comparisons. To ensure both transparency and readability, we have included all detailed statistical results, including exact *P*-values, in the **Source Data** files associated with each figure. This ensures full accessibility of the data while maintaining the clarity of visual presentation.

We hope this solution is acceptable and we are grateful for your understanding.

- Small panels: difficult to see images and to read labels

Response:

We thank the reviewer for pointing this out.

In response, we have enlarged the panels in the revised figures to enhance image visibility and label readability, thereby improving the overall clarity and accessibility of the data.

- Color choice and font size of labels makes them sometimes unreadable, especially the N. benthamiana ones.

Response:

We appreciate the reviewer's comment.

To improve readability, we have adjusted the color scheme and increased the font size of all figure labels, including those for *N. benthamiana*, in the revised figures.

- Considering what is known about their mode of action and characteristic and as reported in various articles, metacaspases cannot be considered caspase-like proteases (line 22)

Response:

We thank the reviewer for this important clarification.

We agree that metacaspases can not be described as caspase-like proteases (Minina et al, 2020). Accordingly, we have revised the sentence in the introduction **(Line 21)**.

Reference:

Minina, E. A. *et al*. Classification and nomenclature of metacaspases and paracaspases: no more confusion with caspases. *Molecular cell*, 2020, 77(5), 927-929.

- Lines 63-64: “Metacaspases have been shown to be involved in immune-related processes”. Add references showing that.

Response:

We appreciate the reviewer’s comment.

We have now cited relevant studies to support the statement on metacaspase involvement in plant immune responses, now presented in **line 67** of the revised manuscript.

- Line 65: a connection between metacaspases and NLRs has been established recently in doi: 10.1038/s44319-025-00426-4

Response:

We thank the reviewer for highlighting this important and timely study.

We have now cited this reference in **line 68** to acknowledge the recently established mechanistic link between metacaspases and NLR-mediated immune signaling.

- Line 71: cite also doi: 10.1105/tpc.15.00626

Response:

We appreciate the reviewer’s suggestion.

The recommended reference has been added **at line 73** of the revised manuscript to support the described role of BAG family proteins in plant immunity.

- Add to every figure legend how many times was every experiment repeated and to the figures all the points.

Response:

We thank the reviewer for this important comment.

In response, we have revised all figure legends to include the number of biological replicates performed for each experiment. This information is now clearly indicated to improve transparency and reproducibility.

In addition, we have added individual data points to all relevant figures to accurately reflect data variability and distribution. These changes were made throughout the revised manuscript to enhance the clarity and rigor of data presentation.

- Line 117: Figure 1c-d should be cited at the end of the line.

Response:

We thank the reviewer for the suggestion.

We have corrected the citation accordingly, and Figure 1c-d is now cited at the end of the sentence in **line 115**.

- Line 122-123: what do the authors mean by “MC4 may switch substrate specificity to enhance plant immune response”?

Response:

We thank the reviewer for pointing out this important issue.

By “MC4 may switch substrate specificity to enhance plant immune response”, we refer to our observation that MC4 cleaves different substrates depending on the biotic stress context.

Under whitefly infestation alone, MC4 cleaves PROPEP1 to release Pep1, a damage-associated molecular pattern that triggers immune responses. Under combined whitefly herbivory and TYLCV infection, although MC4 expression is further induced, its cleavage of PROPEP1 is markedly reduced. Instead, MC4 cleaves BAG3 under these conditions, releasing its N-terminal domain, which induces cell death to enhance plant immune response.

This shift in substrate preference—from PROPEP1 to BAG3—suggests that MC4 reconfigures its substrate repertoire under distinct immune contexts to fine-tune defense responses. To clarify this interpretation, we have added an explanatory sentence to the revised manuscript (now in **lines 119–123**).

- Line 233: “Self-interaction is a critical mechanism in the activation among cell death regulators”. The authors open this section with this sentence and then cite the ZAR1 article. I think they are making a general case from a particular one. Either they cite more articles supporting this point or rephrase the sentence to fit the reference.

Response:

We thank the reviewer for this important observation.

To address this, we have added additional references supporting the role of self-interaction in the activation of diverse cell death regulators, including NLRs and other immune-related proteins (Liu et al., 2024; Wang et al., 2023; Jacob et al., 2021; Madhuprakash et al., 2024; Bi et al., 2019). These citations, now included in **lines 254-255**, strengthen the foundation for our general statement and broaden its relevance beyond the ZAR1 example.

Reference:

Liu, F. et al. Activation of the helper NRC4 immune receptor forms a hexameric resistosome. *Cell*, 2024, 187(18), 4877-4889.

Wang, W. et al. WeiTsing, a pericycle-expressed ion channel, safeguards the stele to confer clubroot resistance. *Cell*, 2023, 186(12), 2656-2671.

Jacob, P. et al. Plant “helper” immune receptors are $Ca^{2+}$-permeable nonselective cation channels. *Science*, 2021, 373(6553), 420-425.

Madhuprakash, J. et al. A disease resistance protein triggers oligomerization of its NLR helper into a hexameric resistosome to mediate innate immunity. *Science Advances*, 2024, 10(45), eadr2594.

Bi, G. et al. The ZAR1 resistosome is a calcium-permeable channel triggering plant immune signaling. *Cell*, 2021, 184(13), 3528-3541.