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Peer Review File

Viral proteins resolve the virus-vector conundrum during hemipteran-mediated transmission by subverting salicylic acid signaling pathway

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript focuses on the function of viral proteins on the inhibition of the degradation of NPR3. I have the following comments.

1. How do the authors connect the degradation of NPR3 with plant defense against viral pathogens? It seems that the story is incomplete. The ultimate defense output should be plant defense genes. How do the viral proteins suppress plant defense by the inhibition of the degradation of NPR3? In other words, how these viral proteins suppress plant defense is still not clear.

2. I suggest the authors include a model in this manuscript to help the readers to have a clear understanding of the story.

3. Figure 1. Too many bar graphs. I suggest the authors move some of the data to supplemental figures.

4. In the introduction for NPR3, include the following reference [1].

Chang M, Zhao J, Chen H, Li G, Chen J, Li M, Palmer IA, Song J, Alfano JR, Liu F, et al.: PBS3 Protects EDS1 from Proteasome-Mediated Degradation in Plant Immunity. Mol Plant 2019, 12:678-688.

5. I suggest the authors measure NPR1 and EDS1 protein levels in wild type and the transgenic plants overexpressing the viral proteins.

6. Did the authors measure total SA or free SA or both. I suggest the authors measure both.

7. Line 59. Plant viral pathogens.

8. Line 63 change translocate to transmit.

9. Line 69 a vector, remove comma.

10. Line 69 to 71. The intricate dynamics of interactions among vectors, viruses, and plants play a pivotal role in determining the spread of viruses and, consequently, the occurrence of viral disease epidemics within agricultural ecosystems.

11. Line 107 remove putative

12. Figure 3. Change the title to TbCSB βC1 interacts with HSP90.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors analyzed how vector-borne plant viruses subvert host immunity to promote their infection during transmission. They found that whitefly feeding induced SA accumulation and plant defenses against whitefly-borne begomoviruses. βC1 encoded by betasatellites played a major role in the mitigation of SA-mediated defenses and interference of SA signaling pathway. They found that βC1 interacted with plant HSP90 proteins, which interacted with NbNPR3. βC1 suppressed SA-induced NbNPR3 degradation via HSP90. Two aphid-borne plant viruses similarly suppressed SA signaling by interfering with SA-induced NbNPR3 degradation through interacting with plant HSP90 proteins.

This story reveals viral countermeasures of insect feeding inducing plant antiviral defenses during insect-mediated virus transmission. However, some points require further investigation and clarification.

Major comments

1. Figure 6, was it reliable of the semi-in vitro protein extraction method followed by the study of SA and βC1 regulatory effects on NbNPR3 degradation be assessed? CHX and MG132 respectively inhibited the synthesis of proteins and the activity of the 26S proteasome. However, the status and activity of 26S proteasome within the extracted protein are unclear. Why did not authors consider an in vivo experimental approach, which could potentially offer more comprehensive insights?

2. It was lack of details about the establishment of viral infection and replication in plants after the exposure to virus-infected insects. Presently, it was difficult to believe whether the virus had just infected plants or had already replicated extensively. Did virus affect the regulation of the SA pathway?

Minor comments

1. Why was the dosages of SA treatment for tobacco and tomato plants in Figure 1 inconsistent?

2. Figure 1 is comprised exclusively of bar charts. For the clarity and simplification, I suggested authors to incorporate plant diagrams within these charts. Additionally, please merge similar datasets, for instance, intergrading Figures 1L and 1M into a single figure would improve data presentation.

3. Figure 1, it was unclear why NahG transgenic plants was shown. It would be helpful for readers in understanding their role this study.

4. Negative and positive controls should be added in Yeast two-hybrid assays.

5. It would be better to separate schematic representation into two discrete parts to clarify the effect of virus-infected and non-infected insect feeding on the SA pathway.

6. Line 33-35, activation of SA-regulated defenses did not always increase the performance of hemipteran insects. Revision is required here and in the Introduction section.

7. Line 65, the compatibility among the three kinds of organisms determines…

8. Line 128-135, authors should examine the effect of SA spray on plant hormone levels and data should be shown.

9. Line 174, it would be better to explore how the βC1 mutation affected the infection of begomovirus-betasatellite complex. βC1 has many important functions in suppressing plant immunity and promoting virus accumulation in plants, such as suppressing RNA silencing. The changes of quantity may affect the function of begomovirus and betasatellite in plants, thereby leading to results presented in the manuscript.

10. Line 183, why PR1a and PR2, are used as marker genes, but not antiviral genes that were directly regulated by SA?

11. Line 229, all the domains of NbHSP90s are targeted by TbCSB βC1. This is interesting. However, it is important to determine a specific domain.

12. Line 233, TbCSV should be TbCSV+TbCSB complex.

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14. Line 329, …and TbCSB βC1 suppressed NbNPR3 degradation by interacting with NbHSP90s.

15. Line 335, why did authors test aphid-borne plant viruses, but not viruses transmitted by other insects. Discussion section required the reason why viral proteins acquired this function in longterm evolution.

16. Line 363, …their choice of host plants

17. Line 379, I am afraid that I could not agree with this statement. Only SA contents in a very early time point were tested. It is possible that SA played a role in the later stage of plant-virus interaction. While SA contents slightly increased, the expression of SA-controlled antiviral genes may be upregulated substantially.

18. Line 388-389, the point should be focused on whitefly infestation-induced SA signaling pathway.

19. Line 403-405, please explain other selection pressures that promoted the association between begomoviruses and betasatellites and compare them with the one authors identified.

20. Line 424-426, can the biological difference between NbNPR3 and AtNPR3 be explained by sequences? Some comments on sequence comparison will be helpful.

Point-to-point responses to reviewer comments

In response to the comments from the two Reviewers, we have edited the text and legends to accommodate their suggestions. We have revised Fig. 1 (moved some of the data from the original Figure 1 into supplementary Fig. S4 and added a schematic representation of experimental design) and Fig. 8 (schematic representation) to make these Figures more accessible to the reader. In addition, four experiments were performed to bolster our conclusion including: (1) determined the effect of whitefly infestation on SA and SAG (SA*O*-*β*-D-glucoside) content; (2) examined the effect of SA spray on the content of SA, JA and JA-Ile; (3) repeated yeast two-hybrid experiment to include positive controls; 4) Explored the effect of SA on NbNPR3 degradation and TbCSB βC1 on SA-induced NbNPR3 degradation in *in vivo* assay.

We thank both Reviewers for their thoughtful comments that have improved our manuscript. To facilitate reading, the comments from the Reviewers are copied in blue, followed by our response. The line numbers in the responses refer to those in the revised version of the manuscript with track changes.

REVIEWER COMMENTS Reviewer #1 (Remarks to the Author):

This manuscript focuses on the function of viral proteins on the inhibition of the degradation of NPR3. I have the following comments.

Authors' Response (AR): We appreciate your comments. Detailed responses to your comments are provided below.

1. How do the authors connect the degradation of NPR3 with plant defense against viral pathogens? It seems that the story is incomplete. The ultimate defense output should be plant defense genes. How do the viral proteins suppress plant defense by the inhibition of the degradation of NPR3? In other words, how these viral proteins suppress plant defense is still not clear.

AR: Thank you for pointing this out. We agree that the identification of plant defense genes whose expression were controlled by NPR3 and antagonized by viral proteins will make the story more complete. Currently, SA is known to induce resistance to viral replication, cell-to-cell movement, and systemic movement in plants (Murphy et al. 2020; Zhao and Li 2021). Yet the mechanisms of SA-induced antiviral resistance and the downstream defense gene products that directly target viruses to enact immunity are poorly characterized (Murphy et al. 2020). Identification of these antiviral genes is a large and independent study, which will be strongly facilitated by the discoveries that we report here. We monitored antiviral immunity by measuring the relative virus levels. We complemented this by using two *PR* genes (*PR1a* and *PR2)* to monitor the activation of the SA-signaling pathway, since the identity of the SA-induced antiviral genes are unknown at this moment. While *PR1a* and *PR2* do not seem to contribute to plant antiviral defenses, these genes are reliable and widely used as sentinels of the SA-signaling pathway (Chen et al. 2020; Murphy et al. 2020). In future endeavors, we will seek to identify and characterize these antiviral genes using experimental materials and knowledge obtained from this

study. Considering the significance of the point you raised, we have emphasized the points you raised in the Discussion section to clarify this issue. See lines 430-440.

References

Chen, J., Clinton, M., Qi, G., Wang, D., Liu, F. & Fu, Z. Q. Reprogramming and remodeling: transcriptional and epigenetic regulation of salicylic acid-mediated plant defense. Journal of Experimental Botany 71, 5256-5268 (2020). https://doi.org/10.1093/jxb/eraa072

Murphy, A. M., Zhou, T. & Carr, J. P. An update on salicylic acid biosynthesis, its induction and potential exploitation by plant viruses. Current Opinion in Virology 42, 8-17 (2020). https://doi.org/10.1016/j.coviro.2020.02.008

Zhao, S. & Li, Y. Current understanding of the interplays between host hormones and plant viral infections. PLoS Pathogens 17, e1009242 (2021). https://doi.org/10.1371/journal.ppat.1009242

2. I suggest the authors include a model in this manuscript to help the readers to have a clear understanding of the story.

AR: Thank you for the comment. We have included a model in the manuscript to facilitate understanding of the story (Fig. 8).

3. Figure 1. Too many bar graphs. I suggest the authors move some of the data to supplemental figures.

AR: Thank you for pointing this out. We have moved results on whitefly-ToLCCNV+tomato pathosystem to supplementary information (Fig. S4). Additionally, we have added an illustration of experimental design in Fig. 1a so that readers can better understand our logic flow.

4. In the introduction for NPR3, include the following reference [1].

Chang M, Zhao J, Chen H, Li G, Chen J, Li M, Palmer IA, Song J, Alfano JR, Liu F, et al.: PBS3 Protects EDS1 from Proteasome-Mediated Degradation in Plant Immunity. Mol Plant 2019, 12:678-688.

AR: Thank you for the comment. We have added this article in the introduction for AtNPR3. See lines 483-484.

5. I suggest the authors measure NPR1 and EDS1 protein levels in wild type and the transgenic plants overexpressing the viral proteins.

AR: Thank you for the comment. AtNPR1 and AtEDS1 are important positive regulators of the SA-signaling pathway, and their function is indeed modulated by the negative regulator AtNPR3. In this study, we focused on the function of NbNPR3 and its modulation by HSP90 and its control of antiviral defenses. With due respect to the Reviewer, we feel that our story is already complex. The addition of upstream regulators NPR1 and EDS1 would add additional complexity to the

manuscript that might distract our readers from our focus on antiviral immunity. In future endeavors, we will explore the modulation of NbNPR1 and NbEDS1 by NbNPR3 and viral proteins, and the biological significance of these modulations.

6. Did the authors measure total SA or free SA or both. I suggest the authors measure both.

AR: Thank you for the comments. In our initial submission, we only measured free SA, as well as JA and JA-Ile. In response to your comment, we repeated whitefly infestation experiment and in this experiment profiled the changes in SA and SAG (SA*O*-*β*-D-glucoside), the major SA conjugate in tobacco plants (Lee et al 1995). These data appear in Figs. S1c-d and lines 134-137 in the main text. The content of both SA and SAG increased significantly upon whitefly infestation.

Reference

Lee, H. I., Leon, J. & Raskin, I. Biosynthesis and metabolism of salicylic acid. Proceedings of the National Academy of Sciences of the United States of America 92, 4076-4079 (1995). https://doi.org/10.1073/pnas.92.10.4076

7. Line 59. Plant viral pathogens.

AR: Thank you for the comment. After reading your comment, we realize that many (if not most) viruses in ecosystems are not threats. Instead, they may be neutral or beneficial. We have revised this sentence accordingly. See line 62.

8. Line 63 change translocate to transmit.

AR: Thank you for the comment. We have revised accordingly. See line 66.

9. Line 69 a vector, remove comma.

AR: Thanks for the comment. We have revised accordingly. See line 73.

10. Line 69 to 71. The intricate dynamics of interactions among vectors, viruses, and plants play a pivotal role in determining the spread of viruses and, consequently, the occurrence of viral disease epidemics within agricultural ecosystems.

AR: Thanks for the comment. We have revised accordingly. See lines 73-75.

11. Line 107 remove putative

AR: Thanks for the comment. We have removed putative. See line 113.

12. Figure 3. Change the title to TbCSB βC1 interacts with HSP90.

AR: Thank you for the advice. We have changed the title of Figure 3 to TbCSB βC1 interacts with *N. benthamiana* HSP90s (NbHSP90s) to clarify the figure's content. See line 947.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors analyzed how vector-borne plant viruses subvert host immunity to promote their infection during transmission. They found that whitefly feeding induced SA accumulation and plant defenses against whitefly-borne begomoviruses. βC1 encoded by betasatellites played a major role in the mitigation of SA-mediated defenses and interference of SA signaling pathway. They found that βC1 interacted with plant HSP90 proteins, which interacted with NbNPR3. βC1 suppressed SA-induced NbNPR3 degradation via HSP90. Two aphid-borne plant viruses similarly suppressed SA signaling by interfering with SA-induced NbNPR3 degradation through interacting with plant HSP90 proteins.

This story reveals viral countermeasures of insect feeding inducing plant antiviral defenses during insect-mediated virus transmission. However, some points require further investigation and clarification.

AR: Thank you for the comments. Detailed responses to your comments are provided below.

Major comments

1. Figure 6, was it reliable of the semi-in vitro protein extraction method followed by the study of SA and βC1 regulatory effects on NbNPR3 degradation be assessed? CHX and MG132 respectively inhibited the synthesis of proteins and the activity of the 26S proteasome. However, the status and activity of 26S proteasome within the extracted protein are unclear. Why did not authors consider an in vivo experimental approach, which could potentially offer more comprehensive insights?

AR: Thank you for pointing this out. We agree that sometimes semi-*in vivo* assays will not reflect what happens in the plants. More specifically, it is possible that the status and activity of 26S proteasome within the extracted proteins may differ from that in live plants.

In response to your comments, we have repeated the most important findings of this study *in vivo*. We determined SA-induced NbNPR3 degradation and the ability of viral proteins to interfere with SA-induced NbNPR3 degradation *in vivo*. The results were added (see Figs. 6b and e, and lines 338-345 in the main text). Data obtained in the *in vivo* degradation assay nicely recapitulate the findings obtained in the semi-*in vivo* assay. This has strengthened our conclusions.

2. It was lack of details about the establishment of viral infection and replication in plants after the exposure to virus-infected insects. Presently, it was difficult to believe whether the virus had just infected plants or had already replicated extensively. Did virus affect the regulation of the SA pathway?

AR: Thank you for the comment. We tested viral quantities in systematic leaves (not inoculated leaves) at 10 days post inoculation when the viruses have extensively replicated, moved systematically, and viral symptoms have fully developed. We have added this clarification in the

text (lines 127-129).

As for the regulation of SA-signaling pathway by viruses, in the initial submission we presented the change of SA content induced by virus infection. We found that begomoviruses alone did not significantly affect SA content, and begomovirus+betasatellite marginally increased SA content (originally Fig. S3, now Fig. S5). In response to your comments, we have further determined the changes in the expression of SA-sentinel genes *PR1a* and *PR2* induced by virus infection and found that both TbCSV and TbCSV+TbCSB induced the expression of these genes. These results were added in the main text (lines 188-193) and Figs. S5d-e.

Minor comments

1. Why was the dosages of SA treatment for tobacco and tomato plants in Figure 1 inconsistent?

AR: Thank you for pointing this out. In our preliminary trails, we found 1.0 mM SA was able to induce plant defenses against begomoviruses in tobacco, but not in tomato plants. We thus increased the SA concentration for the treatment on tomato. We have added a sentence to explain why different SA concentrations were used. See lines 622-624.

2. Figure 1 is comprised exclusively of bar charts. For the clarity and simplification, I suggested authors to incorporate plant diagrams within these charts. Additionally, please merge similar datasets, for instance, intergrading Figures 1L and 1M into a single figure would improve data presentation.

AR: Thank you for pointing this out. When reading your comment and the third comment from Reviewer 1, we realize that too much information was presented in Fig. 1 of the original manuscript. Therefore, to simplify our presentation, we moved results on whitefly-ToLCCNV+tomato pathosystem to supplementary information (current Fig. S4). Additionally, we added an illustration of experimental design in Fig. 1a so that readers can better understand our logic flow.

3. Figure 1, it was unclear why NahG transgenic plants was shown. It would be helpful for readers in understanding their role this study.

AR: Thank you for pointing this out. We have added a sentence describing *NahG* and *NahG*-transgenic plants in the Result section so as to facilitate reading. See lines 160-161.

4. Negative and positive controls should be added in Yeast two-hybrid assays.

AR: Thank you for the comment. We have added positive controls in yeast two-hybrid assays (see Figs. 3a, 5a, 5e, 7e and 7f).

5. It would be better to separate schematic representation into two discrete parts to clarify the

effect of virus-infected and non-infected insect feeding on the SA pathway.

AR: Thank you for the comment. We have modified our model and its presentation has been enhanced by providing two separate biological scenarios. Rather than illustrating response to viruliferous vs nonviruliferous whiteflies, we focused on the comparison of vector-borne viruses that express SA-suppressive proteins (i.e. βC1, HC-Pro and 2b) vs those do not. We believe the new organization of the model emphasizes the key discoveries of our work.

6. Line 33-35, activation of SA-regulated defenses did not always increase the performance of hemipteran insects. Revision is required here and in the Introduction section.

AR: Thank you for pointing this out. After reading your comments, we conducted a literature survey and found that in some cases the activation of SA-regulated defenses will decrease aphid performance. We have deleted this sentence to shorten the Abstract as per journal formatting guideline. We have also revised related contents in the Introduction section. See lines 35-36 and 86.

7. Line 65, the compatibility among the three kinds of organisms determines…

AR: Thank you for the comment. We have revised accordingly. See lines 68-69.

8. Line 128-135, authors should examine the effect of SA spray on plant hormone levels and data should be shown.

AR: Thank you for pointing this out. We have measured the contents of SA, JA and JA-Ile using control and SA-sprayed plants. The data were added as Fig. S3 and a sentence was added in the Result section (lines 146-148). It should be noted that in SA-sprayed plants, many SA molecules stay on the surface of leaves. We have clarified this point in the added sentence.

9. Line 174, it would be better to explore how the βC1 mutation affected the infection of begomovirus-betasatellite complex. βC1 has many important functions in suppressing plant immunity and promoting virus accumulation in plants, such as suppressing RNA silencing. The changes of quantity may affect the function of begomovirus and betasatellite in plants, thereby leading to results presented in the manuscript.

AR: Thank you for the comment. βC1 mutation may alter the infection of begomovirus-betasatellite complex. Actually, we demonstrated this in our previous study (Wu YJ et al. 2023, reference 25 in this manuscript). We agree that the βC1 mutation may induce the changes in virus accumulation and thus indirectly leads to results presented in the manuscript. This is the reason we constructed βC1-transgenic plants to unambiguously determine the role of βC1. Based on results obtained from transgenic plants, we believe the function of βC1 described in this manuscript is genuine.

10. Line 183, why PR1a and PR2, are used as marker genes, but not antiviral genes that were directly regulated by SA?

AR: Thank you for pointing this out and Reviewer 1 made a similar comment. We agree that it will be better if we used antiviral genes whose expression were directly regulated by SA as marker genes. However, currently these defense genes are not known (Murphy et al. 2020). We thus used *PR1a* and *PR2*, two reliable and widely-used downstream genes of the SA-signaling pathways, as the indicator of the activation status of the SA-signaling pathways (Chen et al. 2020; Murphy et al. 2020). We have added a paragraph to clarify this issue as per your comment and the comment from another Reviewer. See lines 430-440.

Reference

Chen, J., Clinton, M., Qi, G., Wang, D., Liu, F. & Fu, Z. Q. Reprogramming and remodeling: transcriptional and epigenetic regulation of salicylic acid-mediated plant defense. Journal of Experimental Botany 71, 5256-5268 (2020). https://doi.org/10.1093/jxb/eraa072

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11. Line 229, all the domains of NbHSP90s are targeted by TbCSB βC1. This is interesting. However, it is important to determine a specific domain.

AR: Thank you for pointing this out. Yes, it is interesting that all the three domains of NbHSP90s are targeted by TbCSB βC1. There is precedent in the literature for more than one HSP90 domain interacting with its client proteins (e.g. Cha et al. 2017 Nature Communications DOI: 10.1038/s41467-016-0014-9). Further dissection of the HSP90 domains in controlling NPR3 stability is a big and future endeavor. In response to the Reviewer's comments, we have modified the Discussion section (lines 504-507). In the revision we emphasize that βC1 may manipulate the function of multiple NbHSP90 domains.

12. Line 233, TbCSV should be TbCSV+TbCSB complex.

AR: Thank you for pointing this out. We have changed TbCSV to TbCSV+TbCSB complex. See line 261.

13. Line 324, various

AR: Thank you for pointing this out. We have corrected the error. See line 362.

14. Line 329, …and TbCSB βC1 suppressed NbNPR3 degradation by interacting with NbHSP90s.

AR: Thank you for the comment. We have revised accordingly. See lines 367-368.

15. Line 335, why did authors test aphid-borne plant viruses, but not viruses transmitted by other

insects. Discussion section required the reason why viral proteins acquired this function in longterm evolution.

AR: Aphid-borne plant viruses were chosen because they are widespread and of substantial economic significance. We have added this rationale in the text (lines 378-379). We have added a sentence to highlight the reason why viral proteins acquired this function in the long-term evolution (lines 410-411).

16. Line 363, …their choice of host plants

AR: Thank you for the comment. We have revised accordingly. See line 404.

17. Line 379, I am afraid that I could not agree with this statement. Only SA contents in a very early time point were tested. It is possible that SA played a role in the later stage of plant-virus interaction. While SA contents slightly increased, the expression of SA-controlled antiviral genes may be upregulated substantially.

AR: Thank you for pointing this out. As per your second major comment, we tested the expression of SA downstream genes (*PR1a* and *PR2*) in three kinds of *N. benthamiana* plants: pBINPLUS, TbCSV and TbCSV+TbCSB. Indeed, TbCSV infection did not significantly induce SA accumulation after 10 days post infection, but it did substantially upregulate the expression of SA downstream genes. Similarly, TbCSV+TbCSB slightly induced SA accumulation, and the complex dramatically upregulated the expression of SA downstream genes (Figs. S5c-e). Therefore, we agree that the original statement is problematic. We have thus revised this sentence according to the updated results. See lines 422-424.

18. Line 388-389, the point should be focused on whitefly infestation-induced SA signaling pathway.

AR: Thank you for pointing this out. We have revised this sentence to highlight the induction of SA-signaling pathway by whitefly infestation. See lines 443-444.

19. Line 403-405, please explain other selection pressures that promoted the association between begomoviruses and betasatellites and compare them with the one authors identified.

AR: Thank you for pointing this out. We have added some sentences to clarify these selection pressures and compare them with the one identified in this study. See lines 459-466.

20. Line 424-426, can the biological difference between NbNPR3 and AtNPR3 be explained by sequences? Some comments on sequence comparison will be helpful.

AR: Thank you for raising this point. We have added the alignment between NbNPR3 and AtNPR3 protein sequences in the supplementary information (Fig. S12c). The two sequences diverged substantially in all regions. Note that an EAR-like motif in C-terminus was present in

both NbNPR3 (VDLNEVP) and AtNPR3 (VDLNETP) (underlined in Fig. 12c). We have revised the text to incorporate this information (lines 299-301). Due to the substantial sequence divergences, we are afraid that at this moment, we can not correlate the biological difference between NbNPR3 and AtNPR3 with sequence divergence.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed all my concerns.

Reviewer #2 (Remarks to the Author):

I am pleased to see that authors made effort to reply to my concerns and to improve the manuscript. Most points that I raised in my previous comments have been clarified. In the current version, the overall quality of the manuscript has been notably improved. Still, there are a few points that sill could be improved. Suggestions and comments to this revised version of the manuscript are listed below.

1. E3 ubiquitin ligases play a crucial role in the degradation of proteins via the 26S proteasome pathway by transferring ubiquitin molecules to target proteins. While HSP90 primarily functions as a molecular chaperone, assisting in the proper folding and stability of other proteins, it does not typically possess E3 ubiquitin ligase activity on its own. However, HSP90 may interact with certain E3 ubiquitin ligases, thereby indirectly participating in the ubiquitination process.

I would like to ask if you considered the possibility that E3 ubiquitin ligases might interact with the HSP90-NPR3 complex, facilitating NPR3 degradation. Moreover, viral proteins might potentially weaken this process via competitive binding to the complex or influencing the recruitment of E3 ubiquitin ligases. Could you please provide relevant experimental data or discuss this potential mechanism in the Discussion section?

2. The images of all BIFC experiments showed weak fluorescence, particularly in Figures S8 and S9. To enhance the readability of results, please provide enlarged images of the fluorescence interaction areas or optimize the imaging conditions.

Point-to-point responses to reviewer comments

In response to the comments from the two Reviewers, we have added some discussions on how βC1 inhibits NbNPR3 degradation by interacting with NbHSP90s. In addition, we have repeated some bimolecular fluorescence complementation (BiFC) experiments and replaced BiFC images that show weak fluorescence.

We thank both Reviewers for their approval of our previous revisions and comments that have improved our manuscript. To facilitate reading, the comments from the Reviewers are copied in blue, followed by our response. The line numbers in the responses refer to those in the revised version of the manuscript with track changes.

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed all my concerns.

Authors' Response (AR): Thanks you for approving our revision.

Reviewer #2 (Remarks to the Author):

I am pleased to see that authors made effort to reply to my concerns and to improve the manuscript. Most points that I raised in my previous comments have been clarified. In the current version, the overall quality of the manuscript has been notably improved. Still, there are a few points that sill could be improved. Suggestions and comments to this revised version of the manuscript are listed below.

AR: Thank you for approving our revision and comments. Below, we provide detailed responses to your feedback.

1. E3 ubiquitin ligases play a crucial role in the degradation of proteins via the 26S proteasome pathway by transferring ubiquitin molecules to target proteins. While HSP90 primarily functions as a molecular chaperone, assisting in the proper folding and stability of other proteins, it does not typically possess E3 ubiquitin ligase activity on its own. However, HSP90 may interact with certain E3 ubiquitin ligases, thereby indirectly participating in the ubiquitination process. I would like to ask if you considered the possibility that E3 ubiquitin ligases might interact with the HSP90-NPR3 complex, facilitating NPR3 degradation. Moreover, viral proteins might potentially weaken this process via competitive binding to the complex or influencing the recruitment of E3 ubiquitin ligases. Could you please provide relevant experimental data or discuss this potential mechanism in the Discussion section?

AR: Thank you for pointing this out. In our study, we found that βC1 inhibits SA-induced NbNPR3 degradation by interacting with NbHSP90s. Therefore, we propose two possibilities based on how βC1 may affect the interaction between NbNPR3 and the ubiquitin system, as well as the role of NbHSP90s. We agree with your suggestion that there may be a third scenario. It is possible that HSP90 interacts with certain E3 ubiquitin ligases, leading to the ubiquitination of HSP90 and subsequent proteasomal degradation of the HSP90-NbNPR3 complex. Furthermore,

the binding of βC1 to HSP90 may interfere with the recruitment of E3 ubiquitin ligases, thereby inhibiting the ubiquitination of HSP90 and the degradation of the HSP90-NbNPR3 complex. We have added these points to the text in lines 480-490.

2. The images of all BIFC experiments showed weak fluorescence, particularly in Figures S8 and S9. To enhance the readability of results, please provide enlarged images of the fluorescence interaction areas or optimize the imaging conditions.

AR: Thank you for the comment. We agree that some of the BiFC images showed weak fluorescence. As a result, we have thus repeated several experiments and replaced these images. Specifically, we re-examined the interactions in the following combinations: TbCSB βC1+NbHSP90s (for Fig. S8), TbCSB βC1+NbHSP90-2-MD, TbCSB βC1+NbHSP90-10-ND and TbCSB βC1+NbHSP90-10-MD (for Fig. S9), as well as NbHSP90s+NbHSP90s (for Fig. 11). Please refer to the updated Fig. S8, S9 and S11 in the Supplementary Information, which are available in both with track change and clean versions.