

Perception of viral infections and initiation of antiviral defense in rice

Corresponding Author: Professor Yi Li

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

Using rice as a model the study by Huang et al describes the untangling of a signaling cascade, which is triggered within rice plants upon perception of viral coat proteins and which involves a RING Ub-ligase, i.e. RBR, that targets a conserved repressor of jasmonate signaling, i.e. NINJA, for degradation. The resultant decrease in NINJA protein levels triggers the jasmonate response leading to enhanced resistance to the virus. The authors support this model mainly by using the RSV virus and its CP9 coat protein as the viral effector. Through a set of final additional experiments with coat proteins from the RDV virus they support that the proposed model may be a common mechanism to mount viral defense, at least in rice. The concept of the study is interesting and the story and disclosed signaling cascade follows a logical path. Yet, there are still several caveats, overstatements, inconsistencies and conflicting data that need further attention and experimentation. Furthermore, overall, the study as currently presented suffers somewhat from a tunnel vision and several aspects related with potential broader physiological roles have been neglected.

Major

1/ Lines 108-111 & Fig.1a-c. The authors have conducted an IP-MS with CP as a bait, which allowed them to retrieve OsRBRL as a prey. However, the resulting proteomics data are nowhere shown, hence it is difficult to assess how specific or broad the potential CP interactome is. As such it is also unclear as to why the authors decided to focus on RBRL. It is imperative to include and release the full proteomics data, including a prey list and the statistical analysis supporting their selection. That is a minimal standard and good common practice for such type of data. The same applies to the IP-MS with RBRL as a bait (lines 217-219 and ED Fig. 4a-b), which allowed to retrieve NINJA. Again, there is no hint on possible specificity.

2/ Lines 121-123 & ED Fig.2b. Although working with truncated protein fragments indeed may give inconclusive results in some cases, this is nonetheless a worrying result. I advise the authors to include additional and proper negative controls, as just free nLUC or cLUC are typically no longer accepted as proper negative controls. Inclusion of a nuclear-localized protein as a negative control is the minimal. See for instance the study by Kudla et al for the design of good BiFC and assays (Kudla J, Bock R. Lighting the Way to Protein-Protein Interactions: Recommendations on Best Practices for Bimolecular Fluorescence Complementation Analyses. Plant Cell. 2016 May;28(5):1002-8.).

3/ Lines 128-131 & Fig.1k. I would like to see more information on the potential broader role of the important regulator that RBRL seems to be. In their current transcriptome analysis, the authors exclusively focus on genes involved in JA biosynthesis and signaling. I agree it is logic to focus the qPCR analysis on that within the concept of the study, but it is as important/relevant to determine how specific the role of OsRBRL is in rice immunity (and/or other processes). As such, an RNA-seq analysis should be included, at least of wildtype and *rbrl* KO rice plants, in absence and presence of the virus (see also my comment #4). The same applies to the transcriptome analysis of the OsNINJA3 KO lines (lines 283-284 and Fig. 5d). Again, there is no hint on possible broader effects of loss of OsNINJA3 function, either in viral defense or JA signaling (see also my comment #7).

4/ Lines 135-137 & ED Fig.2d. This is a wrong conclusion. Initiation of JA biosynthesis in *rbrl* mutant plants is not defective but can be considered at maximum attenuated, given you still see a ca 10-fold induction in JA levels following viral infection (if one can rely on the absolute JA contents shown in the graphs). The data of Fig.1j and ED Fig.2d should be presented in different way, in one figure, which may require additional experimentation. Furthermore, a corresponding transcriptome analysis needs to be conducted, again including both mock and virus treatments in the two backgrounds.

4/ Lines 165-170 & Fig.2e-f. I am not certain if I get the outcome of this experiment. To me it looks contradictory to the

5/ Lines 179-183 & ED Fig.3b. This is the most conflicting dataset within the study. Further down in the manuscript the authors demonstrate that OsRBRL targets 3 out of the 4 OsNINJAs encoded in the rice genome. The only OsNINJA that is not targeted by OsRBRL is OsNINJA4, which is exactly the ortholog of the NINJAs found in all dicots. Conversely, given that NINJA is typically encoded by only 1 gene in most dicot species, it is plausible to assume that dicots do not possess orthologs for OsNINJA1-3 (see also my note on the AFPs further down this comment). Hence, in this regard, it is not intuitive to assume OsRBRL can promote derepression of AGO18pro::LUC activity in the transactivation assays in *Nicotiana benthamiana* leaves, which only harbor the ortholog of OsNINJA4. Given that the authors conclude that such NINJAs cannot be bound or targeted by OsRBRL (see ED Fig.4d), what would then be the target of OsRBRL in *Nicotiana benthamiana*? If this result is to be trusted, then it indicates OsRBRL may have more targets, which could be potentially pinpointed in the non-disclosed RBRL IP-MS prey list. Could these elusive additional targets then be the AFP orthologs from *N. benthamiana* (which could be plausible if one looks at the phylogenetic tree in ED Fig.4c)? Yet, neither that would be an obvious assumption given that, at least in *Arabidopsis*, AFPs are not involved in JA signaling nor can bind to the JAZ proteins. Hence increased OsRBRL-mediated degradation of *N. benthamiana* AFPs may not have any effect on derepression of AGO18pro::LUC activity.

6/ Lines 233-245 & Fig.4e-f & ED Fig.5a. FLAG-GUSA is not sufficient as a negative control for the YFP-NINJA3 degradation assays. An additional needed control is YFP-OsNINJA4, at least for the assays conducted via transient expression, such as those shown in the semi in vivo system in *N. benthamiana* leaves and in the rice protoplasts. This will not only corroborate the findings shown in the binary interaction assays in Fig.4a-b, but also further down those obtained in the phenotypic analysis of the *ninja3* mutant lines, and as such will further illustrate and/or support the evolutionary/physiological uniqueness/relevance of the emergence of this other NINJA clade in monocot species, which seems to be evolutionary more similar to the AFPs from dicots (of note, which are thus presumably not involved in JA signaling).

7/ Lines 281-295 & Fig.5c-j & lines 372-376. Though the resulting data prove that the authors to some extent made a correct choice, I don't fully understand the rationale behind the decision to exclusively target OsNINJA3 for CRISPR/Cas9 editing. It is plausible to assume that OsNINJA1&2 would also be OsRBRL targets and hence that redundancy would exist. In this regard, I think the statement that there is little redundancy in the OsNINJAs during rice antiviral defense is too premature. Such a strong statement should be supported by additional experimentation, requiring generation of triple *osninja123* mutants and assessing their resilience to viral infections. It cannot be excluded that such a triple mutant may even be more resistant to viral infections than the single *ninja3* mutant, thus supporting the opposite conclusion of redundancy. Likewise, as already indicated above, I feel the transcriptome analysis could be extended to an omic-wide level by RNA-seq analysis of the *ninja3* mutant line, and as such go beyond the currently very targeted analysis, to allow assessing the specificity and role of OsNINJA3 in JA signaling and antiviral responses. Furthermore, given that the authors claim they want to understand the role of OsNINJA3 in these 2 responses, that should be done in the absence/presence of viral infection and/or exogenous JA treatment.

8/ The authors followed quite an extensive path to unravel a novel viral protein sensing cascade. Yet, what has not been addressed in this manuscript is the molecular mechanism by which CP would stimulate the RBR E3 Ub-ligase activity by binding it. Indeed, this is a very interesting element in their model as it is potentially a type of molecular effect which may be quite unique, as far as I am aware. Are there other examples in the field of virology where viral coat field proteins are bound by E3 Ub ligases, and if so, does this also result in altered ubiquitination activities? Would CP stimulate interaction between OsRBRL and OsNINJA(s)? Or would it promote OsRBRL ubiquitination activity? The experiment that I miss here is an assay where you show that CP itself stimulates RBRL-mediated degradation or ubiquitination of OsNINJA3. This could be for instance achieved in analogous assays as those shown in Fig.4e or ED Fig.5a, but then including combinations of OsNINJA3 +/-RBRL +/-CP. But perhaps the authors can come up with additional suggestions.

Minor

A/ Lines 76-79 can be omitted. The statement that the mechanism underlying initiation of JA biosynthesis has not been fully elucidated falls short on the many discoveries made and insights already generated in this aspect.

B/ Lines 112-113 & ED Fig.1a. With regard to my comment #5, it would also be informative to include more dicot RBRLs in the phylogenetic tree in ED Fig.1a.

C/ Lines 189-190 & Fig.3a. I am not that familiar with in vitro ubiquitination assays, but I was wondering whether it is common to observe an ubiquitin-ladder in the control sample devoid of the E3 Ub ligase (-RBRL in lane 3 in this case)?

D/ Lines 198-200 & Fig.3c. I realize these are different types of assays, yet it is remarkable to see the extreme instability of the OsRBRL(RBR) construct in Fig.3c whereas that same construct was successfully used to get detectable protein in the LCI assays in ED Fig.2b.

E/ Lines 225-228 & Fig.4a-c & ED Fig.4d. It would be informative to also map the domain of OsRBRL with which the OsNINJAs interact (including the appropriate negative controls, see also my comment #2).

F/ Line 277. Though obviously plausible, I am not certain whether this statement is supported by experimental data? Has interaction of OsNINJAs with OsTPLs been experimentally demonstrated in other studies?

G/ Lines 327-330. I wonder whether it would be relevant to also include measurements of AO-mediated generation of ROS as a parameter to assess the viral response in the mutant *osrbrl* and *osninja* backgrounds?

Referee #2

(Remarks to the Author)

The authors identified a novel jasmonic acid (JA) signaling activation pathway comprising the E3 ligase RBRL and the NINJA3 module, triggered by viral infection. Overall, it is an interesting and comprehensive study that is easy to follow. The novel aspects of the paper include the perception of CP by RBRL and the establishment of a new signaling cascade. It is

pity that there is no evidence documenting RBRL as a sensor for CP, apart from the induction of RBRL by CP, which is a crucial aspect of this paper. Additionally, some key claims are overstated and not well supported by current data. Moreover, appropriate controls are lacking in many experiments.

General comments:

There is no strong evidence documents RBRL as a sensor for CP.

The authors aim to explore the nuclear signaling pathway triggered by the CP protein, and they have identified the interactor RBRL. However, the interaction assays used in the current draft do not strongly support this idea, as they did not use the specific nuclear fraction. Despite having nuclear-enriched IP-MS, unfortunately they lacked a control. It is also important to examine the subcellular localization and co-localization of RBRL and CP protein. To conclusively determine whether the signaling activation occurs from the nucleus rather than the cytosol, it is necessary to conduct nuclear exclusion experiments with RBRL to test viral responses. With the scenario, the biological significance of CP/CP-RBRL nuclear location should be clearly addressed, because they speculated that nucleus-localized CP might be a signaling factor used by plants to perceive viral infection.

It is interesting that the authors identified a potential E3 as a CP sensor, they should put more effort on this aspect: they should determine the specificity of RBRL perceiving CP, how about the ligand binding affinity? whether other conserved CPs of viruses are also perceived by RBRL? How CP activates RBRL to degrade OsNINJA3 only due to induce/degrade RBRL? It also looks that CP promotes NINJA3 degradation with a high threshold (see below).

The data indicate that RBRL constitutively regulates JA production; however, it is still unknown whether it is required for RSV-induced JA production. To address this concern, the author should measure JA concentrations in *rbrl* and RBRL OE plants before and after RSV infection to test whether the induced production of JA will be affected. A similar issue is observed with other signaling components throughout this manuscript, as indicated in the specific comments.

The data that CP OE-mediated antiviral responses partially depend on RBRL raises the possibility that RBRL-like protein could play a redundant function role with RBRL. Given that RBRL-like proteins have lower expression levels, it makes sense that it didn't appear in the IP-MS. Although the data show that RBRL-like protein is not required for the de-repression of OsAGO18 expression, it is possible that other signaling components might be regulated by RBRL-like protein. At this stage, I think it is necessary to test CP interaction with the RBRL-like proteins, and assessing antiviral responses in CP OE/*rbrl* *rbrl*-like will strengthen the conclusion.

It would be premature to conclude that CP is not a substrate of RBRL based on the current data. At least, an in vitro ubiquitination assay should be conducted to confirm this conclusion. Did CP was ubiquitinated in vitro? Should use another control E3 that ubiquitylates and degrades CP, how about RBRL-like?

Actually, Fig 3e somehow suggests that YFP-CP undergoes degradation when co-incubated with RBLB. Autoinhibition and CP-activation is not sufficient to support RBRL as a CP sensor. How CP binding relieves RBRL autoinhibition? Again, including a negative control is essential or use RBRL-like protein?. It look

Specific comments:

Does CP directly induce RBRL? Should do ChIP-PCR or in vivo activation.

Please check all protein expression levels in all LCI assays.

-Fig. 1b

Lack of molecular size of WB image.

CP band in nucleoplasm input of CP OE looks different.

Uninfected rice as a negative control for CP detection.

Why the CP-IP band in the CP OE plant in Fig. 1b showed a different molecular size?

Why didn't the CP protein show enrichment after IP compared to the input?

The exact time of the RSV treatment for the plants should be indicated in the legend.

-Fig. 1c

It is not a good way to conduct IP-MS without any proper controls. Using nuclear-localized proteins or different fractions of CP protein as control will be more convincing.

-Fig. 1e

check the protein expression.

-Extended data 1

adjust the scale to reflect RBRL-like expression levels, as it appears to be reduced upon RSV infection.

-Fig. 2

One-way ANOVA should be used to test the significant difference between different genotypes (d and g).

-Extended data 3b

check all protein expression levels.

Line 204-205

Is there any evidence to show that the E3 ligase activity of RBRL is affected by CP? The degradation assay showing the promotion of RBRL degradation by CP unlikely supports this.

-Fig. 3e

YFP, but not EV, should be used as a negative control.

CP promotes RBRL degradation is not convincing, should include a negative control.

Line 210-211

provide the data supporting the claim that “we did not detect ubiquitinated CP in wild-type NPB or RBRL-OE rice plants infected with RSV”.

-Extended data 4a and b

It is weird that NPB and RBRL OE showed similar protein band signatures with CBB staining after IP. Additionally, there is no specificity band of RBRL in the gel with CBB. Better to present all potential interactors and their unique peptide sequences.

-Line 226

Change “confirmed” to “examined”.

-Fig. 4a

check the protein expression of NINJA4.

-Fig. 4d

How about NINJA3 protein levels before and after RSV infection in *rbrl*?

-Fig. 4e

NINJA4 would be a more suitable negative control than EV.

-Fig. 5b

How about NINJA3 protein levels in CP OE/*rbrl*?

-Extended data Fig 5c

CP-1,-2 did not obviously promote NINJA3 degradation only CP-5 and -10 worked? Too high threshold?

-Extended data Fig 7B

P2 and P9 was degraded by RBRL?

Referee #3

(Remarks to the Author)

In this paper, the authors present data to provide a mechanistic basis on how RSV CP promotes JA accumulation and induction of AGO18 through MYB transcription factor. Based on the data shown, the authors conclude that RBRL E3 ligase interacts with RSV CP that degrades NINJA3 transcriptional repressor to promote JA responsive genes for antiviral defense. Results presented are interesting but need additional evidence to show that some of the process described occurs during natural RSV infection of rice.

Specific comments:

1. Authors identified RBRL using CP overexpressing plants through IP-MS. MS methods and data presented lack the standard that is routinely followed. The methods section also only includes description for how they identified RBRL interacting proteins but not CP. Authors state that they identified RBRL and show scores just for CP and RBRL in Fig. 1c table. How did they actually do the MS of IP sample shown in Fig. 1c gel? Is RBRL the only interactor of CP? Similarly for RBRL data in Extended Data Fig. 4B – how were these experiments done? Is NINJA3 the only interactor identified from RBRL IP-MS?

2. CP and RBRL interaction: What is the difference between Fig. 1d and Extended Data Fig. 2a? In Extended Data Fig. 2a – the interaction between CP and RBRL seems to be very weak. Since authors have CP and RBRL antibodies; they should use these antibodies to show that this interaction occurs during natural infection of RSV in rice. This is important to put the data in perspective with natural infection in rice.

3. Fig. 2: CP OE lines: How do authors rule out classical CP-mediated resistance (DOI: 10.1073/pnas.89.20.9865) in these experiments?

4. Authors state in lines 188-189: “To understand the molecular function of RBRL, we performed in vivo self-ubiquitination

assays (utilizing the plant ubiquitination cascade reconstituted in bacteria)". If it is done in bacteria – how can this be in vivo? In vivo – in bacteria or in plants? The reference should be provided and methods should clearly described in the methods section on this.

5. Fig. 2d-h data: The data presented is hard to interpret. Authors state that RSV CP promotes RBRL degradation. At the same time RSV infection induces RBRL at the RNA and protein level. If this is the case why there is increased RBRL in Fig. 2g when CP is high during virus infection? If CP promotes RBRL activation and degradation, then one would expect to see less RBRL in Fig 2g. Therefore, it is important to show that the ubiquitination of RBRL occurs in vivo in rice and CP promotes its activation RBRL. Since authors have RBRL antibodies, can they show RBRL is indeed ubiquitinated in rice? Then what is the consequence of this ubiquitination in RSV infected samples? This will provide direct evidence that RBRL targets RSV CP, and this promotes RBRL E3 activity in rice during natural RSV infection.

6. Fig. 4: Authors nicely show that NINJA3 is ubiquitinated during RSV infection using NINJA3 antibodies. Authors could use the same antibodies to show in rice during RSV interaction, NINJA3 indeed interacts with RBRL and this interaction promotes NINJA3 degradation during RSV infection in rice. Similar experiments could be done using *rbrl* and RBRL OE lines. These experiments will demonstrate that in natural infection, NINJA3 is degraded through RBRL.

7. Fig. 6: The data shown indicate that RBRL OE lines show enhanced resistance to RDV. Based on this the authors conclude that "RBRL mediates a broad spectrum of natural antiviral defenses". Difficult to make this conclusion. Is the enhanced resistance due to indirect effect because of increased JA and JA responsive genes in RBRL OE lines? Do RDV P2 and P9 localize to the nucleus? RDV P2 has been shown to hijack auxin signaling and GA, how does this intertwine with JA data shown here?

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have conducted a substantial effort in an attempt to address all of the comments that I had raised during my first review. This involved a considerable number of additional experimentation, for which I acknowledge the authors. In most cases, the novel experimentation and data were satisfactory in the sense that they filled the caveats spotted and mitigated the inconsistencies put forward. That did not only regard my comments but, as far as I can judge, also most of the comments of the other reviewers. I have a few concerns/comments left, raised by the novel data, which I'd like the authors to address.

1/ The authors have provided many valuable control data, yet in several cases only in 'Response Figures', not included in the manuscript. Particularly for Response Figures #1 and 2, I would like to see these data incorporated in the final manuscript (as ED or SI data).

2/ Figures ED3 and ED10. It is not clear on which gene sets the GO analysis is performed on. That is the case for 3e, 3j and 10d, which respectively refer to the DEGs shown in 3c, 3h and 10b. In each case it is not clear however whether the GO analysis is for instance done only on the DEG sets in the overlap of the Venn diagrams, or of all DEGs combined or on DEGS of either mock or RSV transcriptomes. This should be indicated in the legend.

3/ Fig 1m. I agree with the general impressions that increases in JA levels seem less in the *rbrl* backgrounds but proper statistics need to be conducted to verify the significance of the genotype X treatment effect and really support the robustness of the conclusion. Furthermore, on line 123, the term 'is essential' should be replaced by 'contributes' as there is clearly still a 2-fold increase in JA levels in the *rbrl* background, indicating that other factors/processes contribute as well and RBRL is not the only player.

4/ ED Fig 4. I agree the data in the rice protoplast provide results consistent with the hypotheses. Yet, I firmly disapprove the practice of omission of puzzling data. That equals inappropriate scientific conduct in my opinion. The authors thereby also avoid to respond to my concern. Nonetheless, given the substantial efforts done by the authors to address my comments, I tend to agree that the analogous, yet puzzling, findings obtained should perhaps not be seen anymore as something conflicting. Yet, keeping proper scientific conduct in mind, I would suggest to include both the old (bentha leaves) and new (rice protoplast) data in a new ED Fig 4. That will provide future readers a full view on the experimentation conducted by the authors and potential inspiration for (other) researchers, interested in following-up of the findings presented in this manuscript.

5/ ED fig 9e. The findings presented in this panel are puzzling. Why would one actually be able to see poly-ubiquitinated NINJA in RSV-infected rice plants? That is quite unusual, as far as I know, given that poly-ubiquitinated proteins are typical proteasomal targets? Based on the immunoblots shown, it appears even that there is no degradation of NINJA by RBRL in rice whatsoever, but actually full conversion and even stabilization to poly-ubiquitinated NINJA? That would implicate a need for a revisit of the model proposed by the authors? Or is this experiment conducted in the presence of MG132? This is not indicated in the legend and I also do not think it is the case, given one can see degradation of NINJA in mock conditions in the RBRL-OE line. Along those lines, do the authors then observe poly-ubiquitinated NINJA in the experiment shown in ED Fig 9b?

6/ I find the rationales/speculations raised by the authors on my inquiry about the possible molecular mechanism by which CP would stimulate the RBR E3 Ub-ligase activity worthwhile to be included in the discussion in the manuscript. Particularly, because a similar inquiry was raised by another referee as well.

Referee #2

(Remarks to the Author)

The authors have largely addressed my comments with additional experiments, which provide essential support of their conclusions in the revised version.

I have only two comments:

1. CP degrades and also promotes RBRL activation. How the two opposite functions work or are coordinated mechanistically? They should at least discuss this. In addition, the Figure 6 model is not straight and informative.
2. I noticed that the master JA signaling suppressors, JAZs, were also strongly induced in Extended Figure 14a, I am wondering whether JAZs are targeted by CP-RBRL and/or how JAZ are actively involved in the pathway.

Referee #3

(Remarks to the Author)

In the revised version, the authors have performed additional experiments and addressed many of the issues raised in the previous review cycle.

One issue that still has some issue is the ubiquitination of OsNINJA3 by OsRBRL in vivo during virus infection that the authors should provide explanation. The extended Data Fig. 9e: Overall the top blots are grainy and overexposed compared to other blots in 9e. In the mock-treated RBRL OE, NINJA3 is very low. One could argue that this is due to RBRL-mediated degradation. However, the ubiquitinated form of NINJA3 is not present in these lanes. Also, a high molecular weight protein band present in RSV infected samples is absent in mock with RBRL OE. In RSV infected NPB and RBRL OE – typically ubiquitination should have some laddering but authors detected a high molecular weight band. Furthermore, the lower bands are absent in the NPB lane with RSV. Authors should explain what these results imply in terms of NINJA3 degradation by OsRBRL.

Response Fig. 12: Not sure how authors conclude that RBRL is self-ubiquitinated in NPB infected with RSV. There is increased accumulation in RSV treated but there is no ubiquitinated laddering one should observe if it is ubiquitinated.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have addressed all of my remaining concerns adequately. I particularly like to acknowledge them for their answers and efforts to respond to my comments #4 and #5.

As in the previous review round, I find the response figures #1 and #2 very informative (and convincing), so I would encourage to include these as well in the final manuscript.

Referee #2

(Remarks to the Author)

I appreciate the efforts of the authors to address my concerns raised during the second round of review. I now agree that their conclusions are well supported. I have no more concern.

Zuhua He

Referee #3

(Remarks to the Author)

The authors have addressed the issues raised in the last review.

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Referees' comments:

Referee #1 (Remarks to the Author):

Using rice as a model the study by Huang *et al* describes the untangling of a signaling cascade, which is triggered within rice plants upon perception of viral coat proteins and which involves a RING Ub-ligase, i.e. RBR, that targets a conserved repressor of jasmonate signaling, i.e. NINJA, for degradation. The resultant decrease in NINJA protein levels triggers the jasmonate response leading to enhanced resistance to the virus. The authors support this model mainly by using the RSV virus and its CP9 coat protein as the viral effector. Through a set of final additional experiments with coat proteins from the RDV virus they support that the proposed model may be a common mechanism to mount viral defense, at least in rice.

The concept of the study is interesting and the story and disclosed signaling cascade follows a logical path. Yet, there are still several caveats, overstatements, inconsistencies and conflicting data that need further attention and experimentation. Furthermore, overall, the study as currently presented suffers somewhat from a tunnel vision and several aspects related with potential broader physiological roles have been neglected.

Response: We thank the reviewer for the detailed review and specific suggestions to improve our manuscript. We greatly appreciate and have carefully considered each of these comments and suggestions. We have provided point-by-point responses to the comments below with relevant excerpts for ease of review.

Major

1/ Lines 108-111 & Fig.1a-c. The authors have conducted an IP-MS with CP as a bait, which allowed them to retrieve OsRBRL as a prey. However, the resulting proteomics data are nowhere shown, hence it is difficult to assess how specific or broad the potential CP interactome is. As such it is also unclear as to why the authors decided to focus on RBRL. It is imperative to include and release the full proteomics data, including a prey list and the statistical analysis supporting their selection. That is a minimal standard and good common practice for such type of data. The same applies to the IP-MS with RBRL as a bait (lines 217-219 and ED Fig. 4a-b), which allowed to retrieve NINJA. Again, there is no hint on possible specificity.

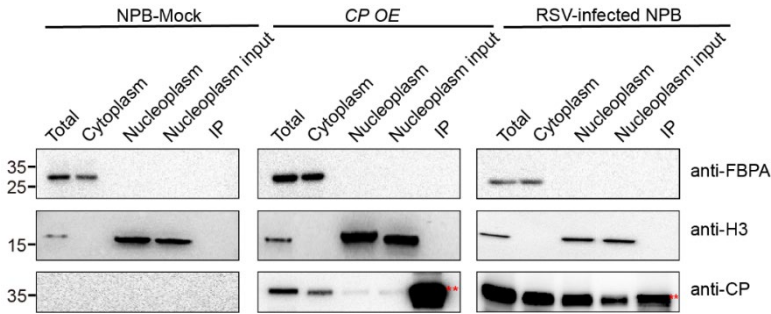
Response: Thank you very much for your insightful comments. We apologize for not providing the raw IP-MS data in the original manuscript. We have now included the

raw IP-MS data with CP as the bait in our revised manuscript, Supplementary Table 1. Additionally, we conducted IP-MS using NPB-Mock materials as a negative control (please see the revised Fig. 1b). We focused on OsRBRL because it is in the prey lists of both *CP OE* and RSV-infected NPB samples. Therefore, we believe that this interaction between CP and RBRL is more convincing and interesting. We also repeated the IP-MS with OsRBRL as the bait (please see the revised Extended Data Fig. 7b) and added the raw IP-MS data to Supplementary Table 4. OsNINJA3 was in the prey list of RBRL IP-MS, consistent with reports that NINJA proteins are important regulators of JA signaling (Pauwels *et al.*, 2010 Nature). We hope these data address your concerns regarding the focus on RBRL and NINJA.

References:

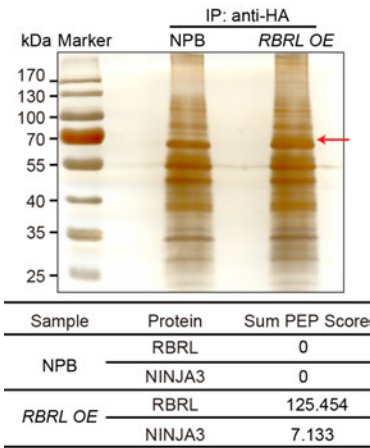
Pauwels, L. *et al.* NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature 464, 788-791 (2010).

Fig. 1b



Revised Fig. 1b. IP-MS assays conducted with the nuclear fraction of mock-inoculated NPB plants, *CP OE* plants and RSV-infected NPB plants. FBPA served as a cytoplasmic marker, and histone H3 served as a nuclear marker. IP, immunoprecipitation.

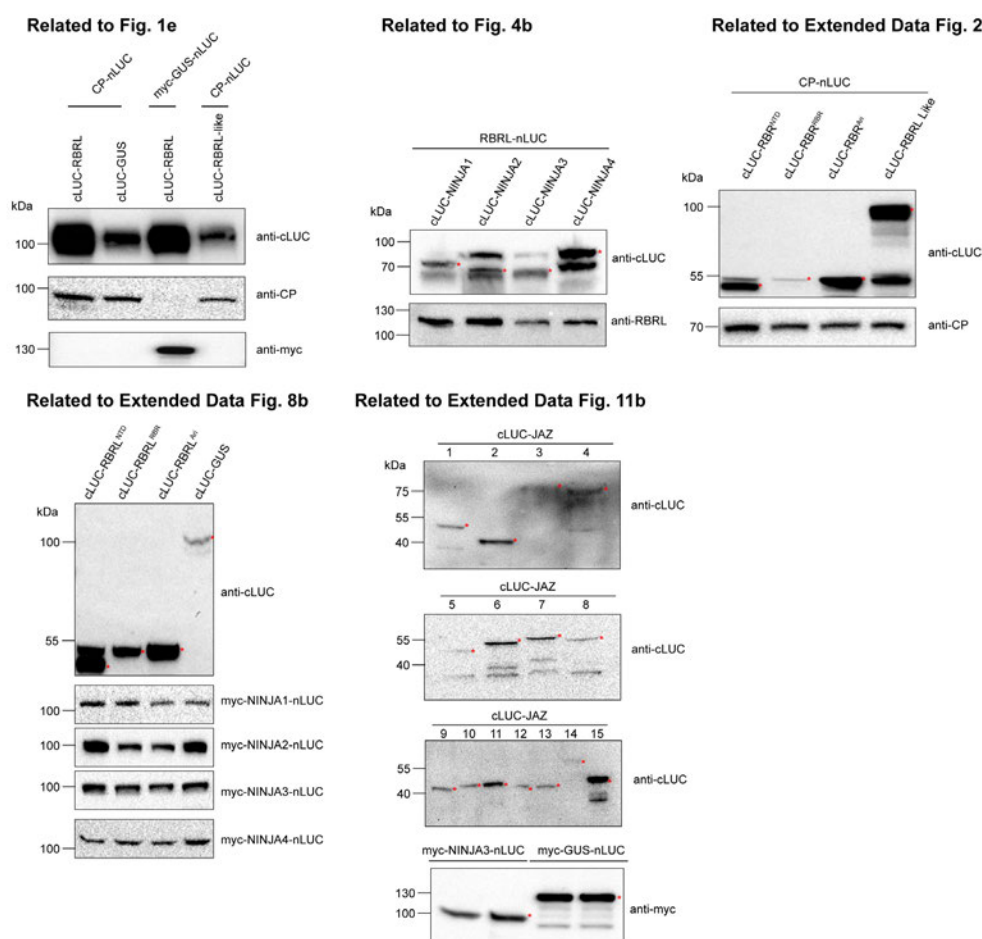
Extended Data Fig. 7b



Extended Data Fig. 7b. HA-RBRL was immunopurified with anti-HA antibodies from NPB and *RBRL OE* rice plants, and copurified proteins were analyzed by SDS-PAGE followed by silver staining. Scores of the RBRL protein and its interacting protein NINJA3 identified by mass spectrometry in different samples. Red arrows indicate the specific RBRL band.

2/ Lines 121-123 & ED Fig.2b. Although working with truncated protein fragments indeed may give inconclusive results in some cases, this is nonetheless a worrying result. I advise the authors to include additional and proper negative controls, as just free nLUC or cLUC are typically no longer accepted as proper negative controls. Inclusion of a nuclear-localized protein as a negative control is the minimal. See for instance the study by Kudla *et al* for the design of good BiFC and assays (Kudla J, Bock R. Lighting the Way to Protein-Protein Interactions: Recommendations on Best Practices for Bimolecular Fluorescence Complementation Analyses. Plant Cell. 2016 May;28(5):1002-8.).

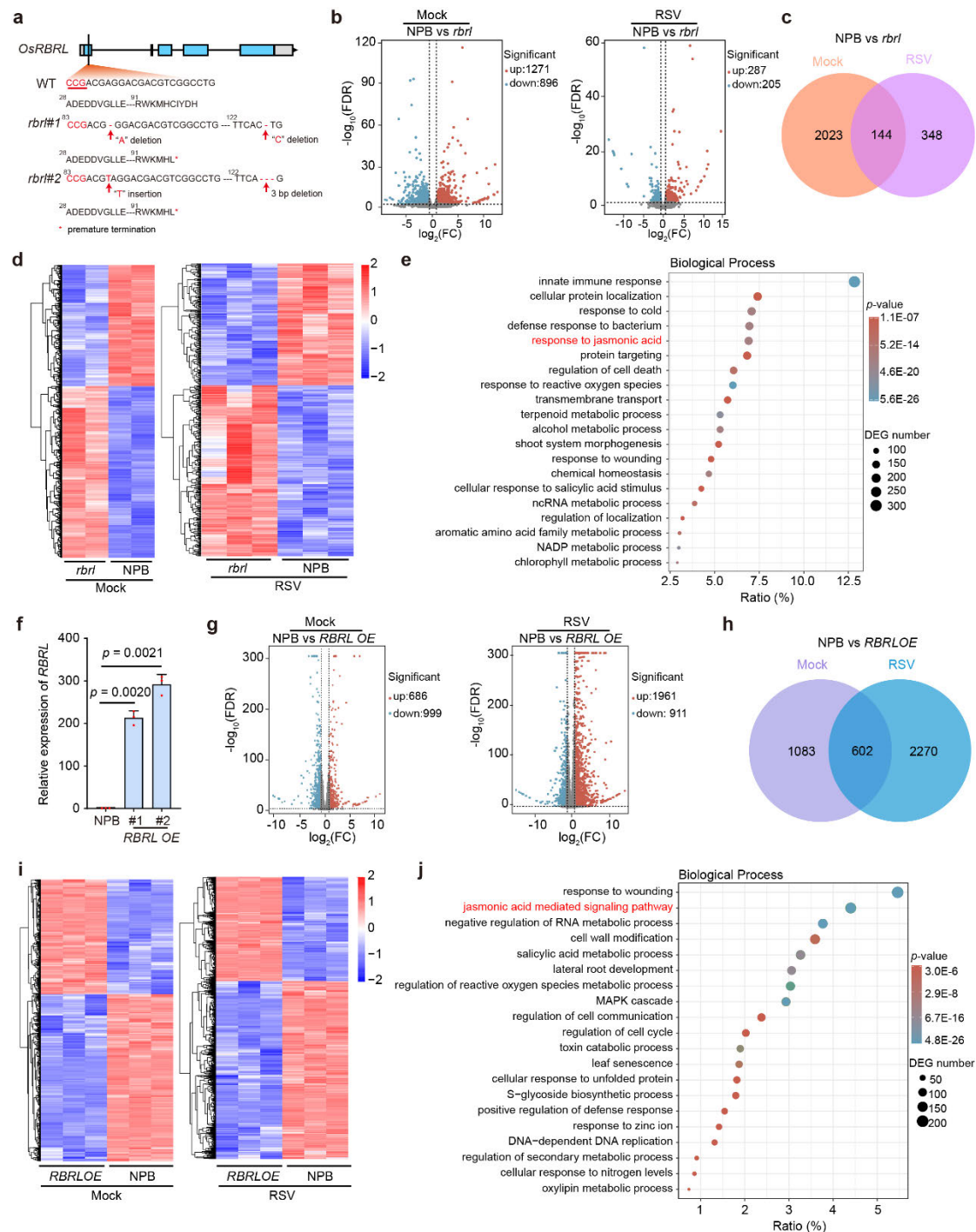
Response: Thank you for the valuable suggestion. We have replaced the negative controls in all the LCI experiments. Specifically, we used RBRL-like-nLUC, NINJA4-nLUC, GUS-nLUC and cLUC-GUS as negative controls in different LCI experiments. We also determined target protein expression in all these experiments (please see Response Fig. 1 below) to confirm that these proteins were correctly expressed.



Response Fig. 1. All the proteins in the corresponding LCI assays were correctly expressed. Protein detection of different combinations in LCI assays. The indicated proteins were detected via the corresponding antibodies. The red asterisks indicate the target protein bands.

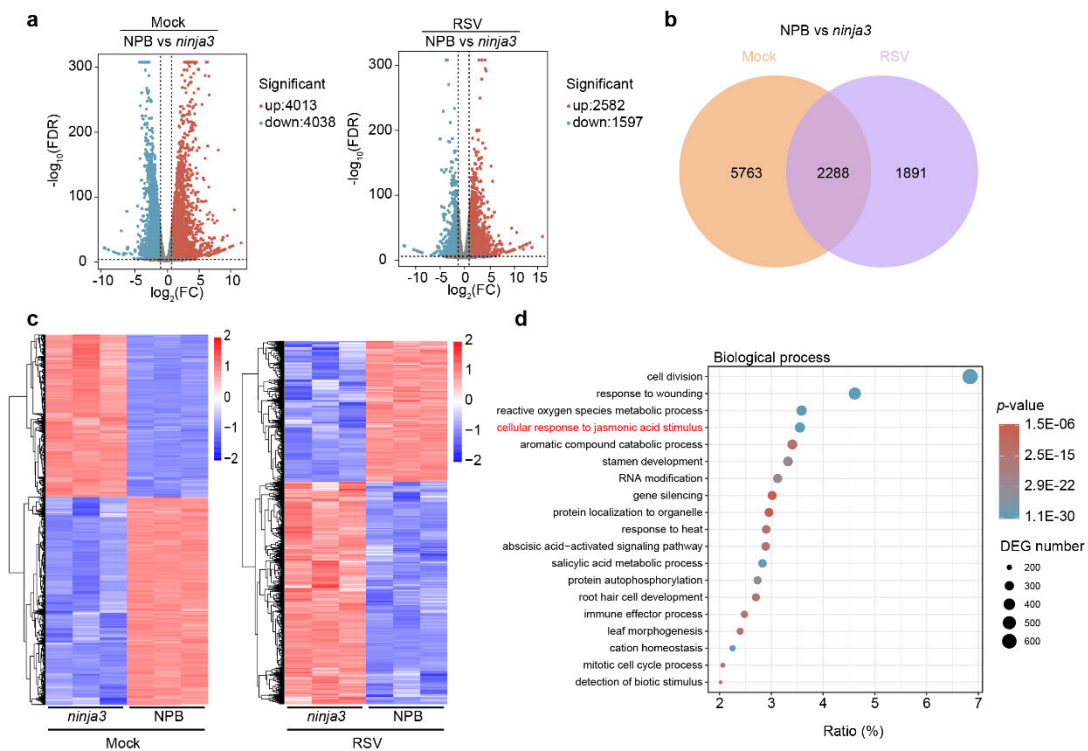
3/ Lines 128-131 & Fig.1k. I would like to see more information on the potential broader role of the important regulator that RBRL seems to be. In their current transcriptome analysis, the authors exclusively focus on genes involved in JA biosynthesis and signaling. I agree it is logic to focus the qPCR analysis on that within the concept of the study, but it is as important/relevant to determine how specific the role of OsRBRL is in rice immunity (and/or other processes). As such, an RNA-seq analysis should be included, at least of wildtype and *rbrl* KO rice plants, in absence and presence of the virus (see also my comment #4). The same applies to the transcriptome analysis of the OsNINJA3 KO lines (lines 283-284 and Fig. 5d). Again, there is no hint on possible broader effects of loss of OsNINJA3 function, either in viral defense or JA signaling (see also my comment #7).

Response: Thank you for the insightful suggestions. We conducted transcriptome sequencing of all WT, *rbrl* and *RBRL* OE plants with or without RSV infection (Revised Extended Data Fig. 3). GO analysis of the differentially expressed genes in the WT, *rbrl* mutant and *RBRL* OE plants revealed that genes involved in the JA response pathway were significantly enriched. Additionally, several other pathways, such as the response to wounding, stimulus and ROS, were also enriched, suggesting a broader role of OsRBRL in regulating plant development and the defense response. On the basis of our RT-qPCR quantification of the expression of genes related to JA biosynthesis and signaling and the differential JA contents in the corresponding plant materials, we focused on the role of OsRBRL in regulating the JA pathway upon viral infection. We also performed transcriptome sequencing of both WT and *ninja3* mutant plants with or without RSV infection (Revised Extended Data Fig. 10). Consistently, the differentially expressed genes were enriched in biological processes related to the wound response, JA signaling and ROS pathways (Revised Extended Data Fig. 10).



Revised Extended Data Fig. 3. Transcriptome analysis of *OsRBRL*-related rice plants with or without RSV infection. **a**, Generation of *rbrl*-knockout (CRISPR/Cas9) lines. The guide RNA (gRNA) sequence targeting *OsRBRL* is indicated, and the protospacer-adjacent motif (PAM) is highlighted in red. In *rbrl* line #1, an 'A' deletion and a 'C' deletion in the gRNA target site resulted in premature translational termination of *OsRBRL*. In *rbrl* line #2, a 'T' insertion and a 3-bp deletion in the gRNA target site resulted in premature termination of the translation of *OsRBRL*. **b**, Volcano plots representing the fold changes in the expression of differentially expressed genes (DEGs) in the NPB versus *rbrl* comparisons with or without RSV infection (FDR < 0.01, fold change ≥ 2.0). **c**, Venn diagram

representing DEGs regulated by *OsRBRL*. **d**, Hierarchical clustered heatmap of 2167 DEGs (1271 upregulated genes and 896 downregulated genes, left) and 492 DEGs (287 upregulated genes and 205 downregulated genes, right). **e**, Gene Ontology (GO) analysis showing the top 20 enrichment terms of the DEGs shown in (c). **f**, qRT–PCR analysis of the *OsRBRL* expression level in the NPB and *RBRL* OE plants. Statistical analysis was performed via two-sided Student's *t* tests, and all *p* values are shown in the figure. The data are the means $\pm$ SDs. *n* = 3 independent biological samples. **g**, Volcano plots representing the fold change in expression of the DEGs in the NPB versus *RBRL* OE comparisons with or without RSV infection (FDR < 0.01, fold change $\geq$ 2.0). **h**, Venn diagram representing DEGs regulated by *OsRBRL*. **i**, Hierarchical clustered heatmap of 1685 DEGs (686 upregulated genes and 999 downregulated genes, left) and 2872 DEGs (1961 upregulated genes and 911 downregulated genes, right). **j**, GO analysis showing the top 20 enrichment terms of the DEGs shown in (h).



Revised Extended Data Fig. 10. Transcriptome analysis of NPB and *ninja3* with or without RSV infection. **a**, Volcano plots representing the fold change in the expression of the DEGs in the NPB versus *ninja3* comparisons with or without RSV infection (FDR < 0.01, fold change $\geq$ 2.0). **b**, Venn diagram representing DEGs regulated by *OsNINJA3*. **c**, Hierarchical clustered heatmap of 8051 DEGs (4013 upregulated genes and 4038 downregulated genes, left) and 4449 DEGs (2582 upregulated genes and 1597 downregulated genes, right). **d**, Gene Ontology (GO) analysis showing the top 20 enrichment terms of the DEGs shown in (b).

4/ Lines 135-137 & ED Fig.2d. This is a wrong conclusion. Initiation of JA biosynthesis in *rbrl* mutant plants is not defective but can be considered at maximum attenuated, given you still see a ca 10-fold induction in JA levels following viral infection (if one can rely on the absolute JA contents shown in the graphs). The data of Fig.1j and ED Fig.2d should be presented in different way, in one figure, which may require additional experimentation. Furthermore, a corresponding transcriptome analysis needs to be conducted, again including both mock and virus treatments in the two backgrounds.

Response: Thank you for your valuable advice. We apologize for the improper explanation of the *rbrl* mutant. As you suggested, we have remeasured the JA contents in the relevant rice lines with or without RSV infection and presented the results in the revised Fig. 1l and 1m as you suggested. Through this analysis, we found that the JA content was lower in the *rbrl* mutant but greater in the *RBRL OE* line than in the NPB rice plants. Specifically, after RSV infection, the JA content increased by 5-fold in the NPB rice, whereas the JA content increased by only slightly less than 2-fold in the *rbrl* mutant, and substantially increased by 6-fold in the *RBRL OE* lines. These results are consistent with the transcriptome data.

Fig. 1l

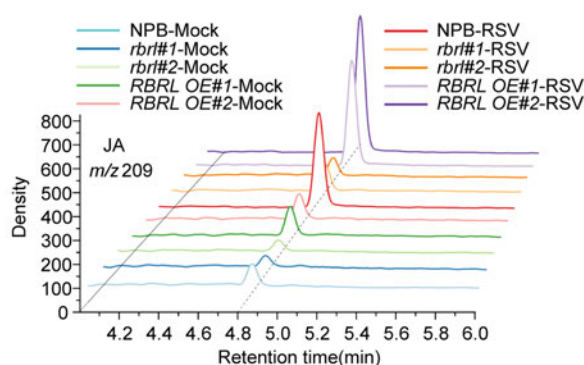
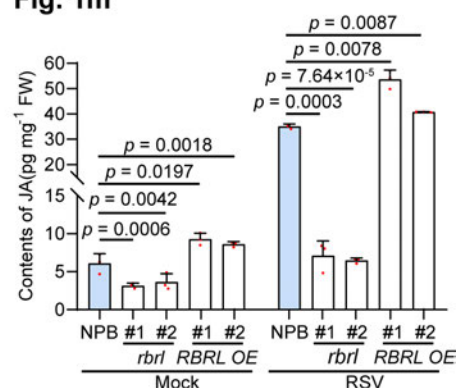


Fig. 1m



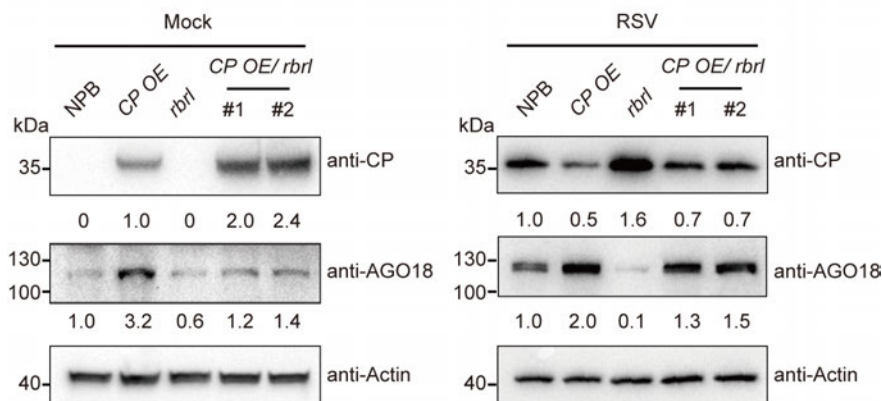
Revised Fig. 1l-m. JA content was lower in the *rbrl* mutant but greater in the *RBRL OE* line than in the NPB rice plants. LC-MS analysis (l) and quantification (m) of the JA concentrations in the NPB, *rbrl*, and *RBRL OE* plants with or without RSV infection. Rice plants were collected at 3 wpi.

5/ Lines 165-170 & Fig.2e-f. I am not certain if I get the outcome of this experiment. To me it looks contradictory to the proposed model. If CP is sensed by RBRL, leading to the activation of JA biosynthesis, then why and how would CP overexpression complement viral sensitivity of an *rbrl* mutant plant?

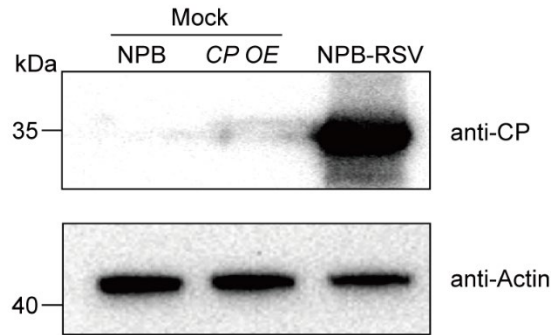
Response: Thank you for this insightful comment. To make these results easier to understand, we have added the results from the immunoblotting analysis of CP and

AGO18 levels in mock-inoculated wild-type NPB, *CP OE*, *rbrl* and *CP OE/rbrl* plants in the revised Fig. 2h. The results indicated that in the absence of viral infection, even though the expression level of CP in *CP OE/rbrl* plants (overexpressing *CP* in the *rbrl* mutant background) is higher than that in *CP OE* plants (overexpressing *CP* in NPB plants), the accumulation level of AGO18 in *CP OE/rbrl* plants is still lower than that in *CP OE* plants. This result suggested that the deletion of *RBRL* indeed attenuated the CP-mediated AGO18 expression, which is consistent with the result in RSV-infected plants. Compared with *CP OE*, *CP OE/rbrl* resulted in a weakened CP-mediated antiviral immune response. As the RSV CP accumulated to a much higher level under RSV infection than that in *CP OE* plants (Response Fig. 2), we propose that a low dose of CP is sufficient to be sensed by RBRL for initiating the JA-AGO18 antiviral signaling, while over accumulated CP after RSV infection may also activate AGO18 expression by interacting with other factors to exert rice antiviral response. That is why *CP OE/rbrl* plants partially complemented the susceptible phenotype of the *rbrl* mutants upon RSV infection. Taken together, these results suggested that the CP-mediated activation of host antiviral defense was mainly but not entirely dependent on RBRL. As you suggested in comment #1, in this regard, we cannot exclude the possibility that other CP-interacting factors may also contribute to this process. We have added this description to the revised manuscript as follows: “A comparison of the *CP OE/rbrl* lines with the *rbrl* mutant lines revealed that overexpression of *CP* partially rescued the sensitive phenotypes of the *rbrl* mutant plants after RSV infection, suggesting that natural antiviral signaling is initiated in rice plants not entirely, but mainly through OsRBRL.”

Fig. 2h



Revised Fig. 2h. Immunoblotting analysis of the RSV CP and AGO18 levels in the specified plants. All the mock-inoculated rice plants were 6-week-old and all the RSV-infected plants were collected at 4 wpi.



Response Fig. 2. RSV CP accumulated to a much higher level under RSV infection than that in *CP OE* plants. Immunoblotting analysis of the RSV CP levels in the specified plants. All the mock-inoculated rice plants were 6-week-old and all the RSV-infected plants were collected at 4 wpi.

6/ Lines 179-183 & ED Fig.3b. This is the most conflicting dataset within the study. Further down in the manuscript the authors demonstrate that OsRBRL targets 3 out of the 4 OsNINJAs encoded in the rice genome. The only OsNINJA that is not targeted by OsRBRL is OsNINJA4, which is exactly the ortholog of the NINJAs found in all dicots. Conversely, given that NINJA is typically encoded by only 1 gene in most dicot species, it is plausible to assume that dicots do not possess orthologs for OsNINJA1-3 (see also my note on the AFPs further down this comment). Hence, in this regard, it is not intuitive to assume OsRBRL can promote derepression of AGO18pro::LUC activity in the transactivation assays in *Nicotiana benthamiana* leaves, which only harbor the ortholog of OsNINJA4. Given that the authors conclude that such NINJAs cannot be bound or targeted by OsRBRL (see ED Fig.4d), what would then be the target of OsRBRL in *Nicotiana benthamiana*? If this result is to be trusted, then it indicates OsRBRL may have more targets, which could be potentially pinpointed in the non-disclosed RBRL IP-MS prey list. Could these elusive additional targets then be the AFP orthologs from *N. benthamiana* (which could be plausible if one looks at the phylogenetic tree in ED Fig.4c)? Yet, neither that would be an obvious assumption given that, at least in Arabidopsis, AFPs are not involved in JA signaling nor can bind to the JAZ proteins. Hence increased OsRBRL-mediated degradation of *N. benthamiana* AFPs may not have any effect on derepression of AGO18pro::LUC activity.

Response: Thank you for the insightful comments. We apologize for the writing mistakes in our original manuscript. We performed these dual-luciferase assays in both

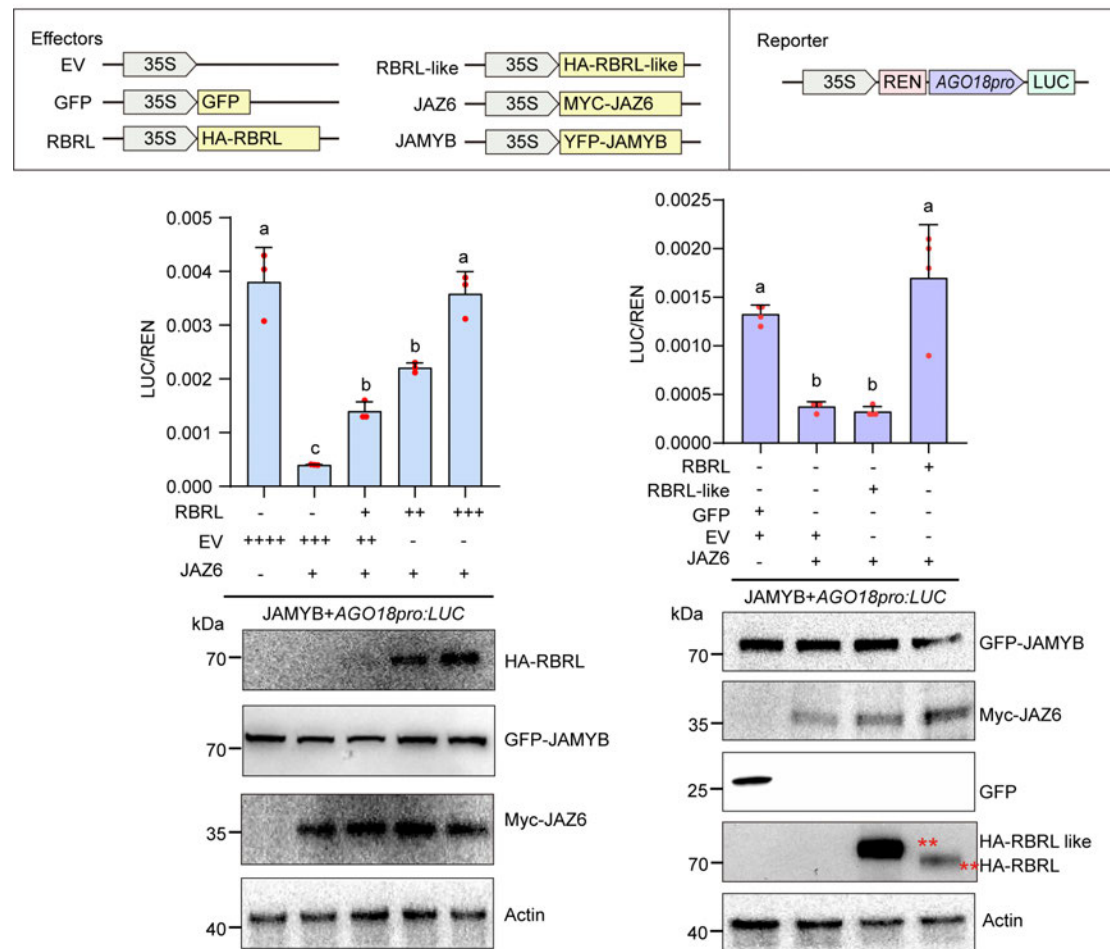
N. benthamiana leaves and rice protoplasts, and the results were consistent. In the original manuscript, we presented the results in *N. benthamiana* leaves. To further confirm this result, we repeated these assays in rice protoplasts to verify the expression of these indicated proteins (revised Extended Data Fig. 4b). These results showed that OsRBRL promoted the derepression of *AGO18pro:LUC* activity in transactivation assays in both *N. benthamiana* leaves and rice protoplasts. To avoid confusing readers, we displayed the results in rice protoplasts in the revised manuscript and Extended Data Fig. 4b. To address the question that NINJA in *Nicotiana benthamiana* is not bound or targeted by OsRBRL and thus that OsRBRL cannot promote derepression of *AGO18pro:LUC* activity in the transactivation assays in *Nicotiana benthamiana* leaves, we first reconstructed the phylogenetic tree of NINJAs from rice and other species, and the results revealed that AFP proteins from dicots and NINJA1/2/3/4 from rice belong to different subclades, whereas AFP3 from *Arabidopsis* (*Arabidopsis thaliana*) and A0A1S3Z6S5 from tobacco (*Nicotiana tabacum*) belong to one subclade, and they are very close to the OsNINJA1/2/3 subclades. In addition, Hong *et al.* reported that the *Arabidopsis afp2* mutant presents a phenotype that is very similar to that of the *jaz-D* decuple mutant, suggesting that AFP may be involved in the regulation of the JA signaling pathway (Hong *et al.*, 2020, PNAS). Third, the understanding and functions of NINJA proteins from monocots are very limited. In this study, we revealed the negative role of OsNINJA3 in regulating the rice defense response against RSV through repression of JA signaling. In particular, an RBR-type E3 ligase mediates the degradation of OsNINJA3. These findings are novel and enrich the research on the molecular function and turnover mechanism of NINJA proteins from monocots. We have added a description of the functions of NINJAs and AFPs in the revised manuscript as follows: “To fully characterize this interaction, we conducted a phylogenetic analysis using the full-length protein sequences of OsNINJA3 and NINJA family proteins from the indicated plant species (revised Extended Data Fig. 7c). The results revealed that the NINJA1/2/3 proteins of rice (*O. sativa* subsp. *japonica*) belong to the same subclade as most NINJA proteins in monocotyledonous plants, including maize (*Zea mays*) and wheat (*Triticum aestivum*). Intriguingly, this subclade is closely related to the subclade of ABA INSENSITIVE FIVE (ABI5) BINDING PROTEINS (AFPs) found in dicot plants such as *Arabidopsis* (*Arabidopsis thaliana*), tobacco (*Nicotiana tabacum*), and soybean (*Glycine max*) (Extended Data Fig. 7c). Additionally, the rice NINJA4 and maize NINJA7/8 proteins fall within the same

subclade as the NINJA proteins from Arabidopsis and tobacco (Extended Data Fig. 7c).
 “...the *afp2* mutant presented phenotypes similar to those of the Arabidopsis *jaz-D* (decuple *JAZ* mutant)⁴², indicating that AFPs in dicotyledonous plants may also be involved in the regulation of JA signaling.”

Reference:

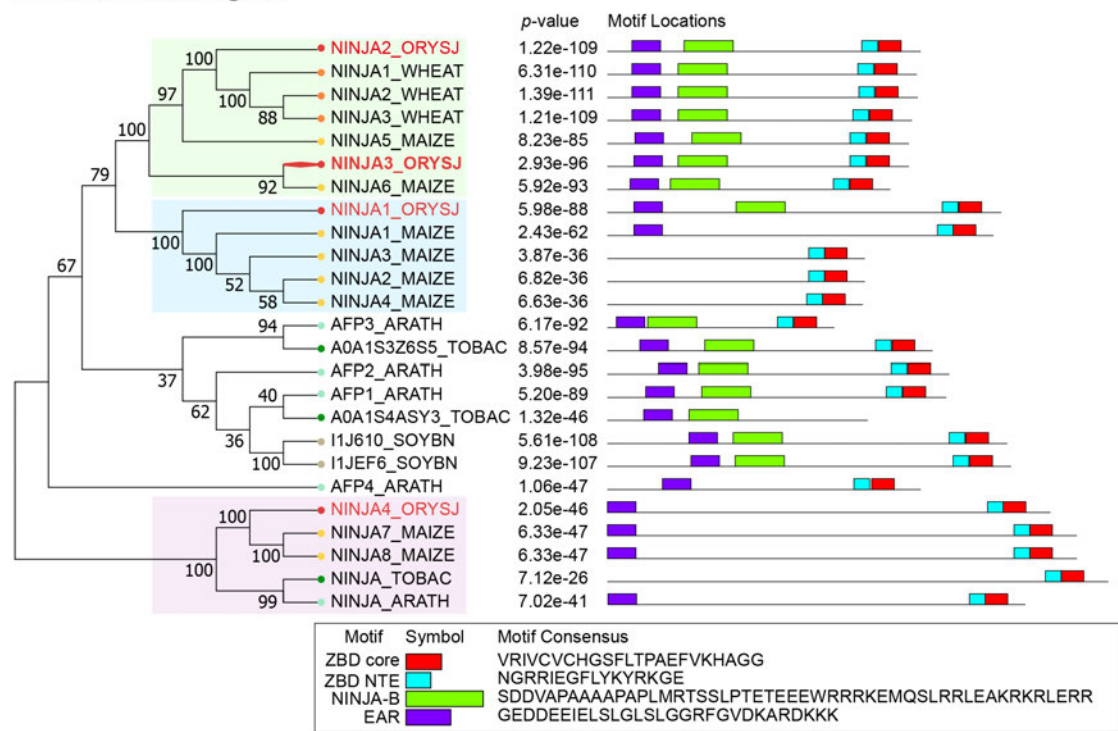
Hong, S. A.-O. *et al.* Heterologous microProtein expression identifies LITTLE NINJA, a dominant regulator of jasmonic acid signaling. *Proc Natl Acad Sci U S A* 117, 26197-26205 (2020).

Extended Data Fig. 4b



Revised Extended Data Fig. 4b. OsRBRL promotes the transcription of OsAGO18 in rice protoplasts. Dual-luciferase assays in rice protoplasts. A schematic diagram of the constructs is shown in the upper panel. The activities of firefly luciferase (LUC) and Renilla luciferase (REN) were measured sequentially, and the LUC/REN ratio was calculated as the final transcriptional activity. EV: empty vector. Statistical analysis was performed via one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test (different letters represent significantly different groups; $p < 0.05$).

Extended Data Fig. 7c



Revised Extended Data Fig. 7c. Phylogenetic analysis of NINJA proteins in rice and other plant species. Rice NINJA proteins are highlighted in red. Conserved motif analysis was performed using the online software program MEME (<http://meme-suite.org/tools/meme>).

7/ Lines 233-245 & Fig.4e-f & ED Fig.5a. FLAG-GUSA is not sufficient as a negative control for the YFP-NINJA3 degradation assays. An additional needed control is YFP-OsNINJA4, at least for the assays conducted via transient expression, such as those shown in the semi in vivo system in *N. benthamiana* leaves and in the rice protoplasts. This will not only corroborate the findings shown in the binary interaction assays in Fig.4a-b, but also further down those obtained in the phenotypic analysis of the *ninja3* mutant lines, and as such will further illustrate and/or support the evolutionary/physiological uniqueness/relevance of the emergence of this other NINJA clade in monocot species, which seems to be evolutionary more similar to the AFPs from dicots (of note, which are thus presumably not involved in JA signaling).

Response: Thank you for the insightful suggestion. We have added YFP-NINJA4 as a negative control for the YFP-NINJA3 degradation assays in the revised Fig. 4e-f and Extended Data Fig. 9a, as you suggested. The results showed that NINJA4 was not degraded by RBRL, further suggesting that different NINJA members may play distinct roles during plant growth and defense regulation. Additionally, we added the RBRL-like protein as a negative control for RBRL, as shown in Extended Data Fig. 9a.

Gradually increasing the expression of the RBRL-like protein did not induce the degradation of NINJA3. We wish to point out that FLAG-GUSA was not used as a negative control in these experiments; instead, it served as an internal control to standardize expression levels across different combinations in *N. benthamiana* on the basis of previously published studies.

Fig. 4e

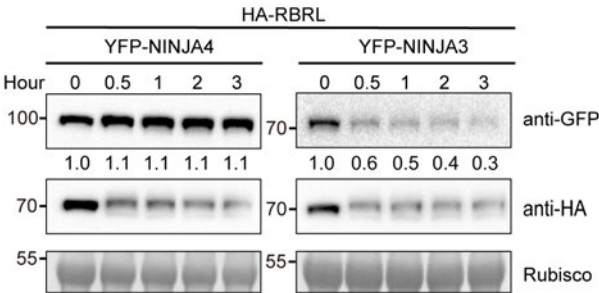
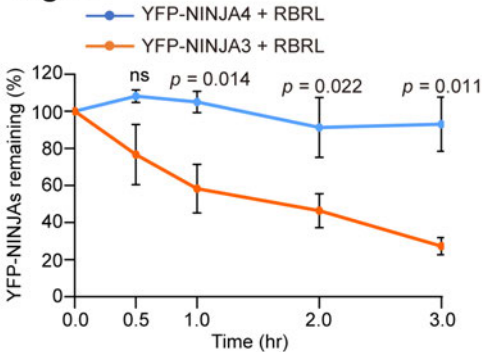
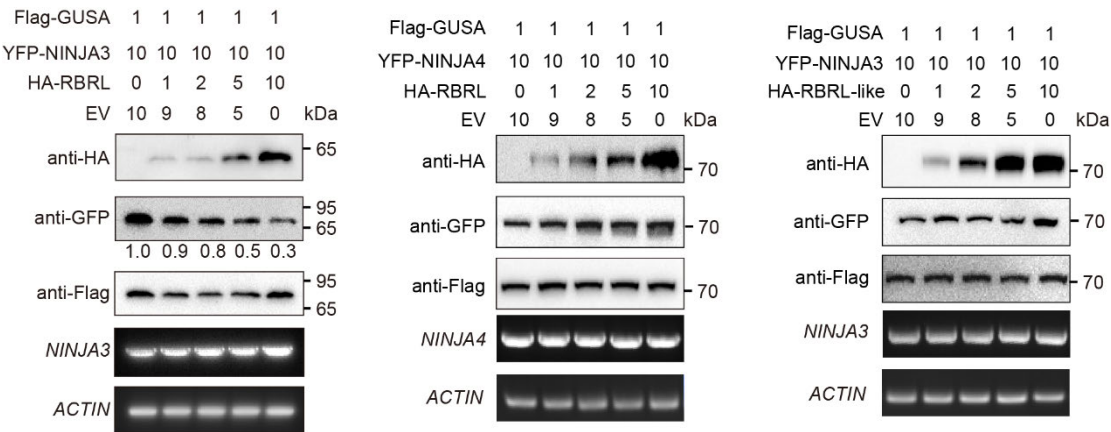


Fig. 4f



Revised Fig. 4e-f. RBRL targets NINJA3 for degradation. **e**, Time course of RBRL-promoted NINJA3 degradation. YFP-NINJA4 served as a negative control. Ponceau S staining (bottom panel) of the Rubisco protein served as a loading control. **f**, The intensities of the bands shown in (**e**) were quantified with ImageJ software. Relative values were calculated via comparison with the values at 0 hours (set to 1.0). Statistical analysis was performed via two-sided Student's *t* tests, and all *p* values are shown in the figure. The data are the means $\pm$ SDs. *n* = 3 independent biological samples. ns, not significant.

Extended Data Fig. 9a



Revised Extended Data Fig. 9a. RBRL targets NINJA3 for degradation. The *in vivo* degradation of NINJA3 was assessed by determining the YFP-NINJA3 protein level via coinfiltration experiments with increasing amounts of HA-RBRL. Flag-GUSA served as the internal control. The mRNA expression levels of the target genes *OsNINJA3* and *ACTIN* were analyzed via RT-PCR. The numbers at the top denote the ratios of the concentrations of *Agrobacterium* used for coinfiltration. YFP-NINJA4 (middle panel) and HA-RBRL-like (right panel) served as negative controls.

8/ Lines 281-295 & Fig.5c-j & lines 372-376. Though the resulting data prove that the authors to some extent made a correct choice, I don't fully understand the rationale behind the decision to exclusively target OsNINJA3 for CRISPR/Cas9 editing. It is plausible to assume that OsNINJA1&2 would also be OsRBRL targets and hence that redundancy would exist. In this regard, I think the statement that there is little redundancy in the OsNINJAs during rice antiviral defense is too premature. Such a strong statement should be supported by additional experimentation, requiring generation of triple *osninja123* mutants and assessing their resilience to viral infections. It cannot be excluded that such a triple mutant may even be more resistant to viral infections than the single *ninja3* mutant, thus supporting the opposite conclusion of redundancy. Likewise, as already indicated above, I feel the transcriptome analysis could be extended to an ome-wide level by RNA-seq analysis of the *ninja3* mutant line, and as such go beyond the currently very targeted analysis, to allow assessing the specificity and role of OsNINJA3 in JA signaling and antiviral responses. Furthermore, given that the authors claim they want to understand the role of OsNINJA3 in these 2 responses, that should be done in the absence/presence of viral infection and/or exogenous JA treatment.

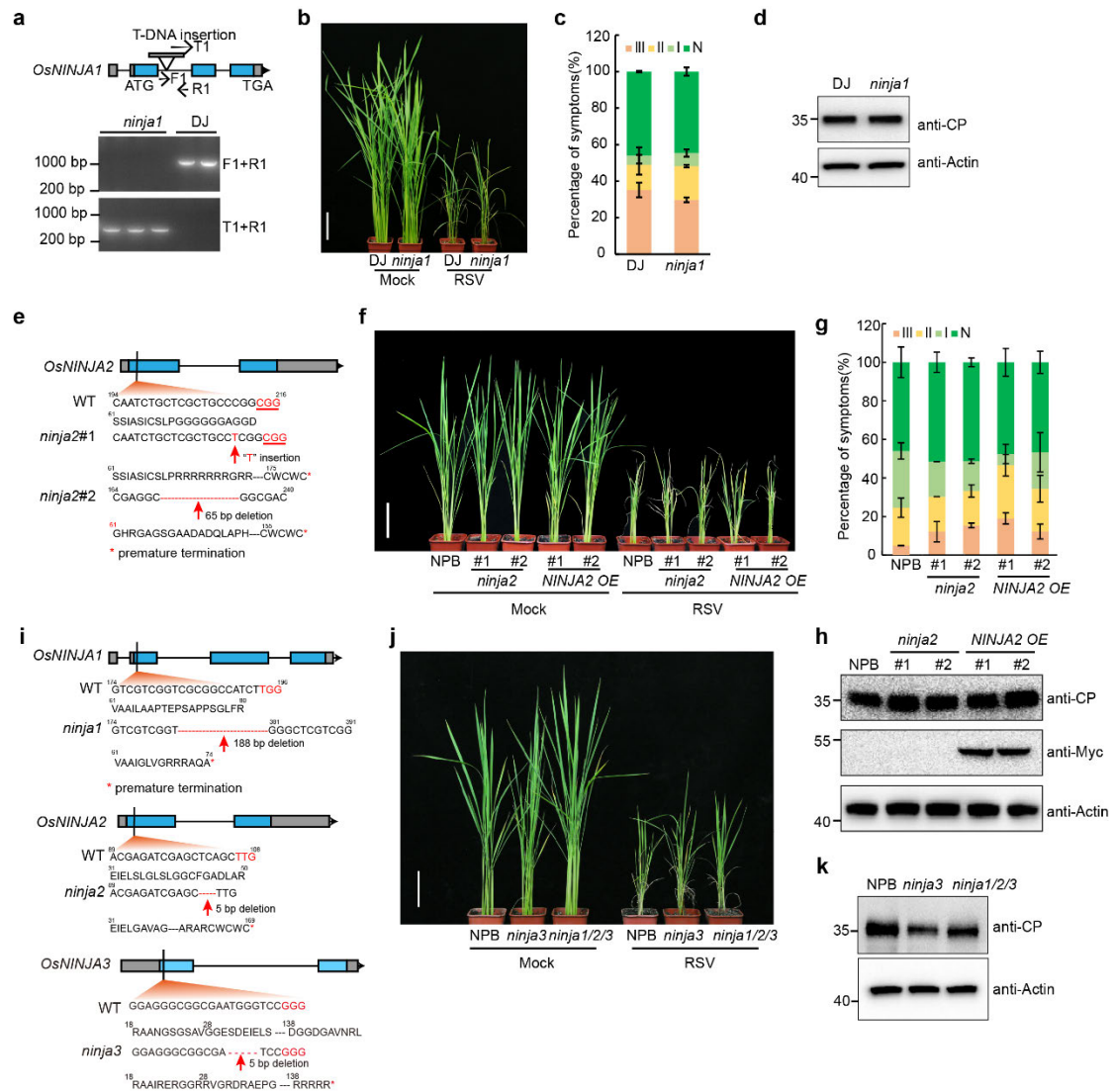
Response: Thank you for your insightful comments. We generated both *ninja1* and *ninja2* single mutants and *ninja1/2/3* triple mutants and conducted RSV infection experiments. We found that neither *ninja1* nor *ninja2* exhibited the strong antiviral phenotype observed in the *ninja3* mutant, with no significant difference compared to the wild-type NPB controls (revised Extended Data Fig. 12.) Therefore, we believe that different NINJA proteins may play different roles in regulating plant growth and defense responses.

To further confirm these results, we also inoculated *ninja1/2/3* triple mutants. We found that the disease symptoms and CP accumulation levels of the triple mutants were similar to those of the *ninja3* single mutant (revised Extended Data Fig. 12.). As you suggested, we also performed RNA-seq analysis on the *ninja3* mutant with or without RSV infection. The results revealed that the JA signaling pathway was significantly activated in the *ninja3* mutant compared with NPB (please see the response to comment #3), which is consistent with previous RT-qPCR results.

We have included these results in the revised Extended Data Fig. 12 and added a description in the revised manuscript as follows:

"Given that OsRBRL interacts with OsNINJA1, OsNINJA2 and OsNINJA3, to verify

the roles of OsNINJA1 and OsNINJA2 in rice antiviral defense, we also obtained a *ninja1* mutant (*modd-2*)⁴⁴, which lacks *OsNINJA1* expression due to a T-DNA insertion (Extended Data Fig. 12a), along with *OsNINJA2* knockout mutants via CRISPR/Cas9 (Extended Data Fig. 12e) and *OsNINJA2* overexpression (*NINJA2 OE*) lines (using a construct encoding a MYC-tag fused to the N-terminus of OsNINJA2) via transgenic methods. In response to natural RSV infection, the disease symptoms of the *ninja1* mutants were similar to those of the wild-type Dongjing (DJ) plants (Extended Data Fig. 12b, c and Supplementary Table 3). Compared with that in DJ plants, CP accumulation in *ninja1* mutants was not significantly different (Extended Data Fig. 12d). Compared with the wild-type NPB plants, the *ninja2* mutant and *NINJA2 OE* rice plants presented similar disease symptoms and CP accumulation (Extended Data Fig. 12f-h and Supplementary Table 3). We also generated *ninja1/2/3* triple mutants in the *ninja3* single-mutant background via CRISPR/Cas9 (Extended Data Fig. 12i). The resistance of the *ninja1/2/3* triple mutants to RSV infection was comparable to that of the *ninja3* single mutant (Extended Data Fig. 12j, k). These data indicated that, unlike *OsNINJA3*, *OsNINJA1* and *OsNINJA2* are not major players in rice antiviral defense against RSV.”



Revised Extended Data Fig. 12. NINJA1 and NINJA2 are not involved in rice antiviral immunity. **a**, Characterization of *ninja1* T-DNA insertion mutants. Schematic representation of the *OsNINJA1* gene and the position of the T-DNA insertion. F1, R1, and T1 represent the positions of the two primer pairs used to identify whether T-DNA insertion had occurred. **b**, Images showing mock-inoculated or RSV-infected Dongjing (DJ) and *ninja1* plants. Scale bar, 10 cm. **c**, Percentages of RSV-infected DJ and *ninja1* plants with various disease symptom grades. **d**, Immunoblotting analysis of the RSV CP levels in the specified plants. **e**, Generation of *ninja2*-knockout (CRISPR/Cas9) lines. The guide RNA (gRNA) sequence targeting *OsNINJA2* is indicated, and the protospacer-adjacent motif (PAM) is highlighted in red. In *ninja2* line #1, a ‘T’ insertion in the gRNA target site resulted in premature translational termination of *OsNINJA2*. In *ninja2* line #2, a 65-bp deletion in the gRNA target site resulted in premature termination of the translation of *OsNINJA2*. **f**, Images showing mock-inoculated or RSV-infected NPB, *ninja2* and *NINJA2* OE plants.

Scale bar, 10 cm. **g**, Percentages of RSV-infected NPB, *ninja2* and *NINJA2* OE plants with various disease symptom grades. **h**, Immunoblotting analysis of the RSV CP levels in the specified plants. **i**, Generation of *ninja1/2/3*-knockout (CRISPR/Cas9) lines. The guide RNA (gRNA) sequences targeting the indicated *OsNINJAs* are indicated, and the protospacer-adjacent motifs (PAMs) are highlighted in red. A 188-bp deletion in *NINJA1*, a 5-bp deletion in *NINJA2* and a 5-bp deletion in *NINJA3* resulted in premature translational termination of OsNINJA1, OsNINJA2 and OsNINJA3. **j**, Images showing mock-inoculated or RSV-infected NPB, *ninja3* and *ninja1/2/3* plants. Scale bar, 10 cm. **k**, Immunoblotting analysis of the RSV CP levels in the specified plants. Rice plants were all collected at 4 wpi.

9/ The authors followed quite an extensive path to unravel a novel viral protein sensing cascade. Yet, what has not been addressed in this manuscript is the molecular mechanism by which CP would stimulate the RBR E3 Ub-ligase activity by binding it. Indeed, this is a very interesting element in their model as it is potentially a type of molecular effect which may be quite unique, as far as I am aware. Are there other examples in the field of virology where viral coat field proteins are bound by E3 Ub ligases, and if so, does this also result in altered ubiquitination activities? Would CP stimulate interaction between OsRBRL and OsNINJA(s)? Or would it promote OsRBRL ubiquitination activity? The experiment that I miss here is an assay where you show that CP itself stimulates RBRL-mediated degradation or ubiquitination of OsNINJA3. This could be for instance achieved in analogous assays as those shown in Fig.4e or ED Fig.5a, but then including combinations of OsNINJA3 +/-RBRL +/-CP. But perhaps the authors can come up with additional suggestions.

Response: Thank you for the insightful advice. Currently, there are very few reports on viral capsid proteins binding to E3 ligases. One of the reports revealed that a **RING-type E3 ligase**, TRIM5, binds to the HIV capsid protein and subsequently enhances E3 ubiquitin ligase activity to activate innate immune signaling (Pertel *et al.*, 2011 Nature; Fletcher *et al.*, 2018 Cell Host & Microbe). However, the process of activity changes in **RBR-type E3 ligases** is still unclear, although in 2016, Scott *et al.* reported that HHARI is activated upon binding to neddylated Cullin-RING ligase (CRL) complexes (Scott *et al.*, 2016 Cell). In our study, to examine the role of RSV CP in enhancing the ubiquitination activity of RBRL, we determined ubiquitination activity via RBRL assays in a bacterial system, and in these assays, we included combinations in

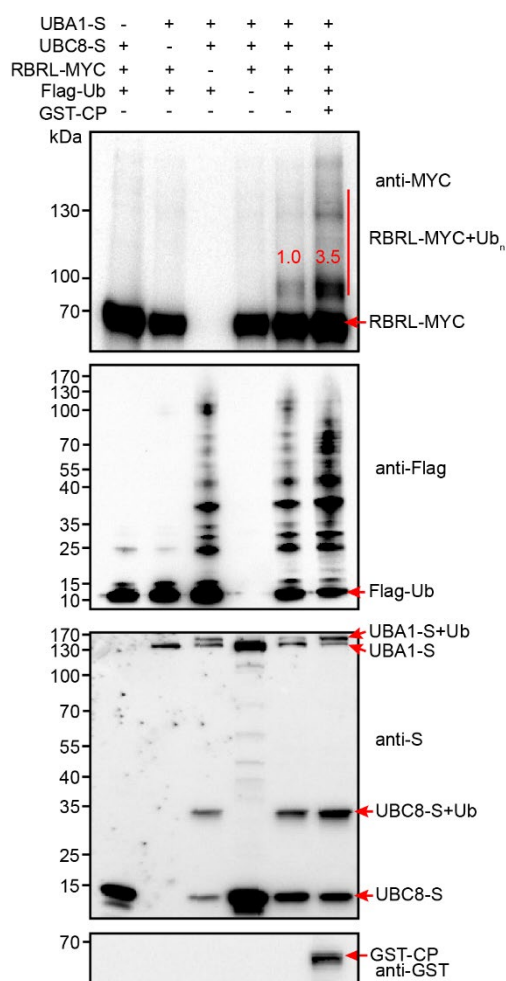
the absence or presence of CP, as mentioned in the revised Fig. 3e and Extended Data Fig. 9d. The results showed that the presence of CP significantly enhanced the ubiquitination activities of RBRL and increased the substrate ubiquitination levels of NINJA3. Additionally, we performed analogous assays, as shown in revised Extended Data Fig. 9c. As shown in the revised Extended Data Fig. 9c, gradually increasing the expression level of CP promoted the degradation of NINJA3 but not that of NINJA4.

Previous structural studies of human HHARI and the mammalian homolog of ari-2 (TRIAD) protein, orthologs of OsRBRL, have shown that binding by some protein partners or ligands could release their autoinhibition by opening the mask of the Ariadne domain on the RING2 active site (Scott *et al.*, 2016 Cell; Spratt *et al.*, 2014 Biochem. J.). Therefore, we presume that CP releases the autoinhibition of OsRBRL, possibly through interaction with OsRBRL, thus exposing the active site.

References:

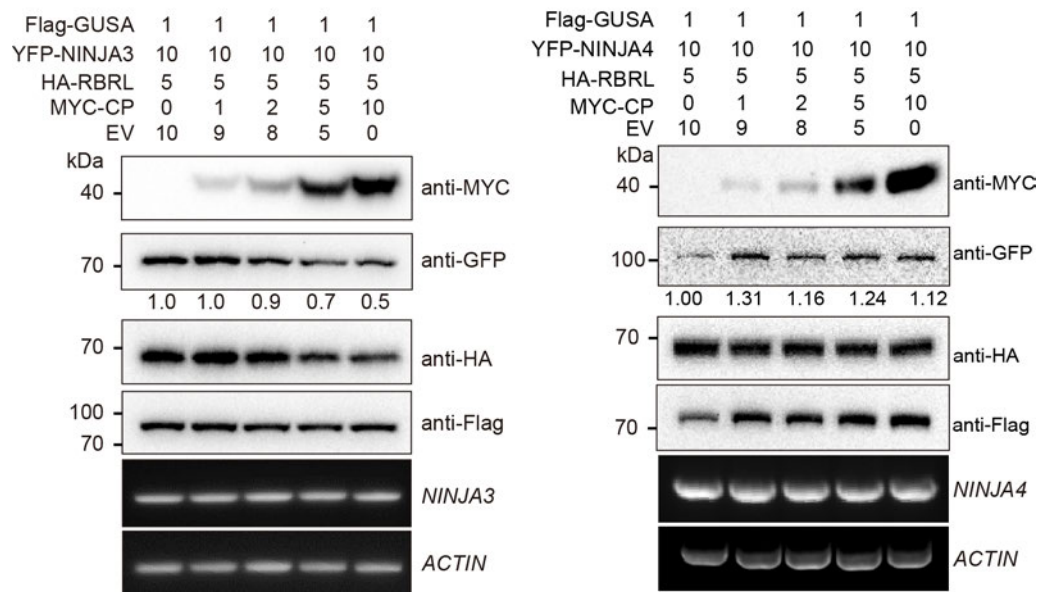
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Fig. 3e



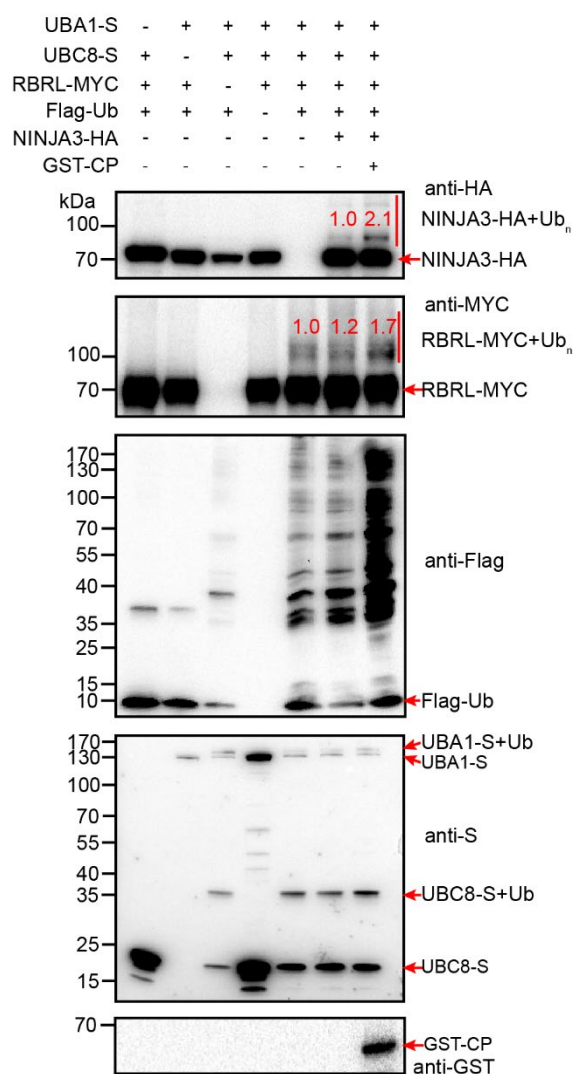
Revised Fig. 3e. CP enhanced the ubiquitin ligase activity of OsRBRL. CP enhanced the ubiquitin ligase activity of OsRBRL. The bacterial lysates from *E. coli* strains expressing UBA1-S, UBC8-S, Flag-Ub, RBRL-MYC and GST-CP or from strains lacking one of these components were analyzed by immunoblotting with an anti-MYC antibody (upper panel), an anti-Flag antibody (middle panel), an anti-S antibody (lower panel) and an anti-GST antibody (bottom panel).

Extended Data Fig. 9c



Revised Extended Data Fig. 9c. RSV CP promoted the proteolysis of NINJA3 but not that of NINJA4. The *in vivo* degradation of OsNINJA3 was conducted by determining the YFP-NINJA3 protein level via coinfiltration experiments with increasing amounts of MYC-CP. Flag-GUSA was used as the internal control. The mRNA expression levels of the target genes *OsNINJA3* and *ACTIN* were analyzed via RT-PCR. The numbers at the top indicate the ratio of the concentrations of *Agrobacterium* used in coinfiltration. YFP-NINJA4 (right panel) served as a negative control.

Extended Data Fig. 9d



Revised Extended Data Fig. 9d. CP promotes the degradation of NINJA3 by increasing the activity of OsRBRL. The bacterial lysates from *E. coli* strains expressing UBA1-S, UBC8-S, Flag-Ub, RBRL-MYC, MBP-NINJA3-HA and GST-CP or from strains lacking one of these components were analyzed by immunoblotting with an anti-HA antibody to detect the ubiquitination of OsNINJA3.

Minor

A/ Lines 76-79 can be omitted. The statement that the mechanism underlying initiation of JA biosynthesis has not been fully elucidated falls short on the many discoveries made and insights already generated in this aspect.

Response: Thank you very much for this suggestion. We have revised this sentence to reflect the current state of knowledge in this field as follows: “Although considerable

C/ Lines 189-190 & Fig.3a. I am not that familiar with in vitro ubiquitination assays, but I was wondering whether it is common to observe an ubiquitin-ladder in the control sample devoid of the E3 Ub ligase (-RBRL in lane 3 in this case)?

Response: Thank you. These ubiquitination assays were conducted according to the methods of previous studies (Han *et al.*, 2017 Plant J.). We have added the detailed procedure of this experiment to the “METHODS” section. It is normal to observe ubiquitin ladders in the control sample without E3 ligase (-RBRL in Lane 3). E1 and E2 enzymes can undergo autoubiquitination in the presence of ubiquitin molecules, leading to the formation of ubiquitin ladders. Importantly, the lanes enriched with E3 ligase (RBRL) presented significantly stronger ubiquitination bands, indicating the specific ability of RBRL to increase ubiquitination.

Reference:

Han, Y. *et al.* Reconstitution of the plant ubiquitination cascade in bacteria using a synthetic biology approach. Plant J. 91, 766-776 (2017).

METHODS section:

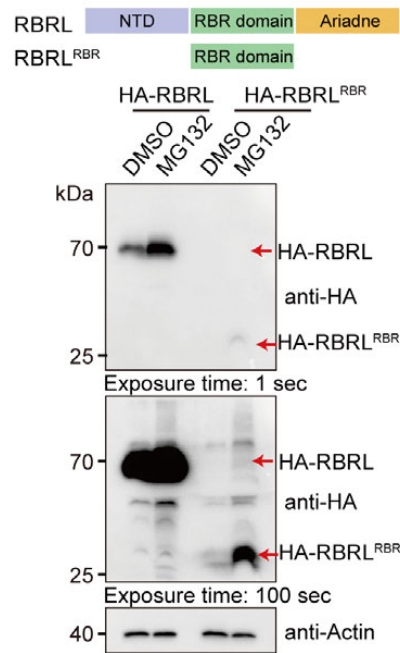
Protein expression and ubiquitination assay in *E. coli*

The protein expression and ubiquitination assays conducted in the *Escherichia coli* strain BL21 (DE3) were performed according to previous studies⁴³. Briefly, different combinations of the indicated expression vectors were transformed into the *E. coli* strain BL21 (DE3), and the strains were cultured in Luria–Bertani (LB) liquid medium at 37°C. Then, 0.5 mM IPTG was added to the medium when the absorbance at 600 nm reached 0.4–0.6 to induce the expression of the target proteins. The bacteria were further cultured at 28°C for 10–12 hours and then stored at 4°C overnight. The crude proteins were extracted and analyzed via western blotting with the corresponding antibodies.

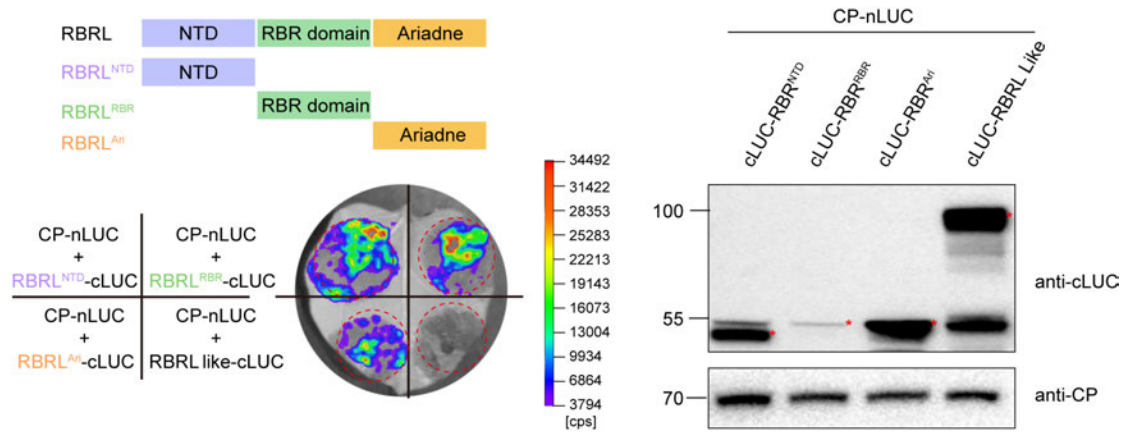
D/ Lines 198-200 & Fig.3c. I realize these are different types of assays, yet it is remarkable to see the extreme instability of the OsRBRL(RBR) construct in Fig.3c whereas that same construct was successfully used to get detectable protein in the LCI assays in ED Fig.2b.

Response: Thank you for this observation. RBRL^{RBR} is indeed highly unstable and difficult to detect at the protein level. As shown in the revised Fig. 3c, it is still detectable at low levels, which likely explains why we were able to capture the interaction in the LCI assays. Additionally, we determined that the protein expression of RBRL^{RBR} in the LCI assays was relatively low (please see Response Fig. 3 below).

Fig. 3c



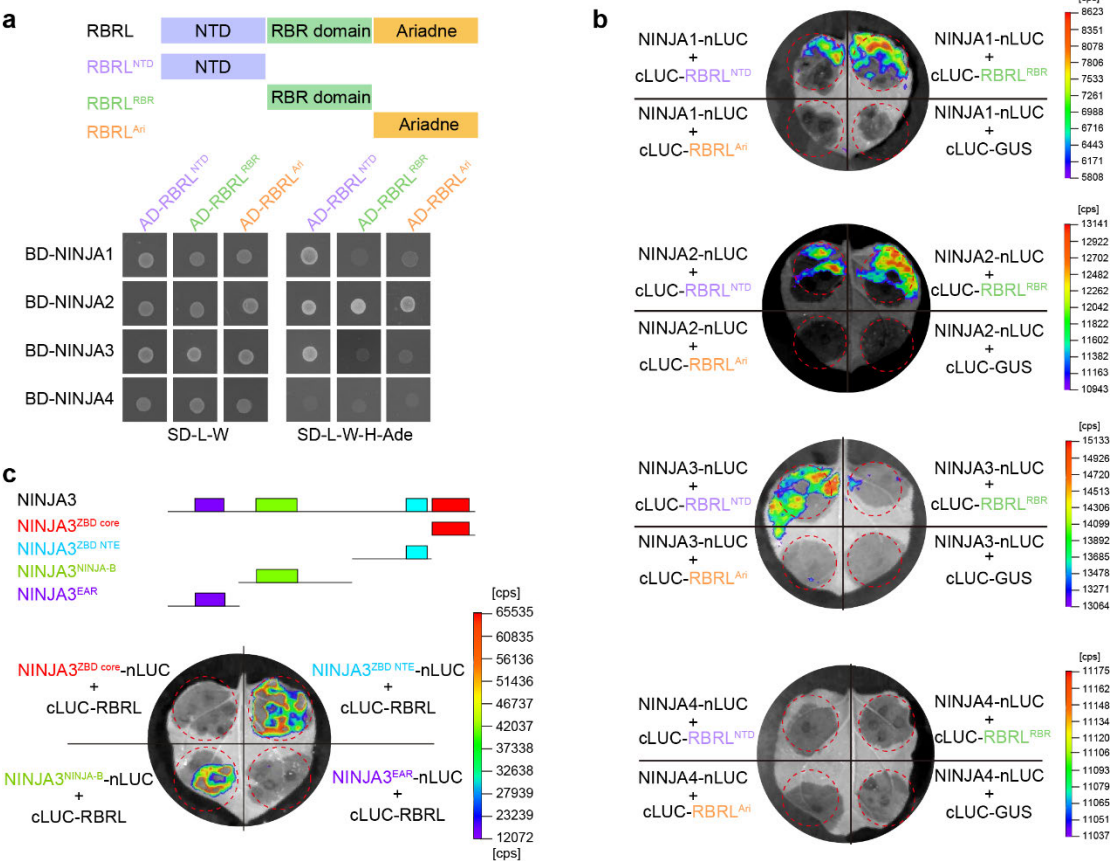
Revised Fig. 3c. Removing the NTD and Ariadne domains of OsRBRL resulted in instability of OsRBRL^{RBR} when the gene was expressed independently in plant cells and proteasome inhibitor MG132 inhibited the degradation of the full-length OsRBRL and the RBR domain of OsRBRL.



Response Fig. 3. LCI assays showing that the RSV CP interacts with the N-terminal domain (NTD), RBR domain, and Ariadne domain of OsRBRL (left, revised Extended Data Fig. 8c). cLUC-RBRL-like served as a negative control. cps, signal counts per second. Expression analysis of the corresponding proteins in different combinations (right). The proteins were detected via the indicated antibodies.

E/ Lines 225-228 & Fig.4a-c & ED Fig.4d. It would be informative to also map the domain of OsRBRL with which the OsNINJAs interact (including the appropriate negative controls, see also my comment #2).

Response: Thank you for your suggestion. We replaced the negative controls and investigated the interaction domains between OsNINJA3 and OsRBRL. Yeast two-hybrid and LCI assays revealed that the N-terminal domain (NTD) and RBR domain of RBRL interact primarily with NINJA1/2/3 (please see the revised Extended Data Fig. 8a). Furthermore, the NINJA-B and ZBD NTE regions but not the EAR motif of NINJA3 interact with RBRL (please see the revised Extended Data Fig. 8c). We have included these results in the revised manuscript as follows: “Yeast two-hybrid (Y2H) and LCI assays further demonstrated that the full-length NINJA1/2/3 proteins interacted mainly with the NTD and RBR domains of RBRL (Extended Data Fig. 8a, b). Additionally, the full-length RBRL protein interacted with the ZBD NTE motif- and NINJA-B motif-containing domains of the NINJA1/2/3 proteins (Extended Data Fig. 8c).”



Revised Extended Data Fig. 8. Functional domains involved in the interaction between OsRBRL and OsNINJA3. **a**, Y2H assays showing that the N-terminal domain (NTD) of OsRBRL primarily interacts with OsNINJA1/2/3. OsNINJA4 served as a negative control. cps, signal counts per second. **b**, LCI assays showing that the N-terminal domain

(NTD) of OsRBRL primarily interacts with OsNINJA1/2/3. cLUC-GUS served as a negative control. cps, signal counts per second. c, LCI assays showing that the NINJA-B and ZBD NTE regions but not the EAR motif of OsNINJA3 interact with OsRBRL. cps, signal counts per second.

F/ Line 277. Though obviously plausible, I am not certain whether this statement is supported by experimental data? Has interaction of OsNINJAs with OsTPLs been experimentally demonstrated in other studies?

Response: Thank you for your questions. The interaction between OsNINJAs and TOPLESS/TOPLESS-RELATED (TPL/TPR)-type corepressors has been demonstrated in previous studies. Tang *et al.* demonstrated via yeast two-hybrid and coimmunoprecipitation assays that NINJA1 interacted with TOPLESS-RELATED 3 (TPR3) (Tang *et al.*, 2016 Plant Cell). Another study showed that NINJA1 interacted with TPR1 in plant cells (Kashihara *et al.*, 2019 Plant Physiol.). We have revised the manuscript and added such descriptions and citations to make it clearer for readers.

“Previous studies have shown that OsNINJA1 can interact with TPR1/3 to inhibit downstream gene expression^{43,44}. The formation of complexes with OsJAZ proteins allows OsNINJA3 to inhibit JA-responsive gene expression by recruiting the corepressor TPL/TPR.”

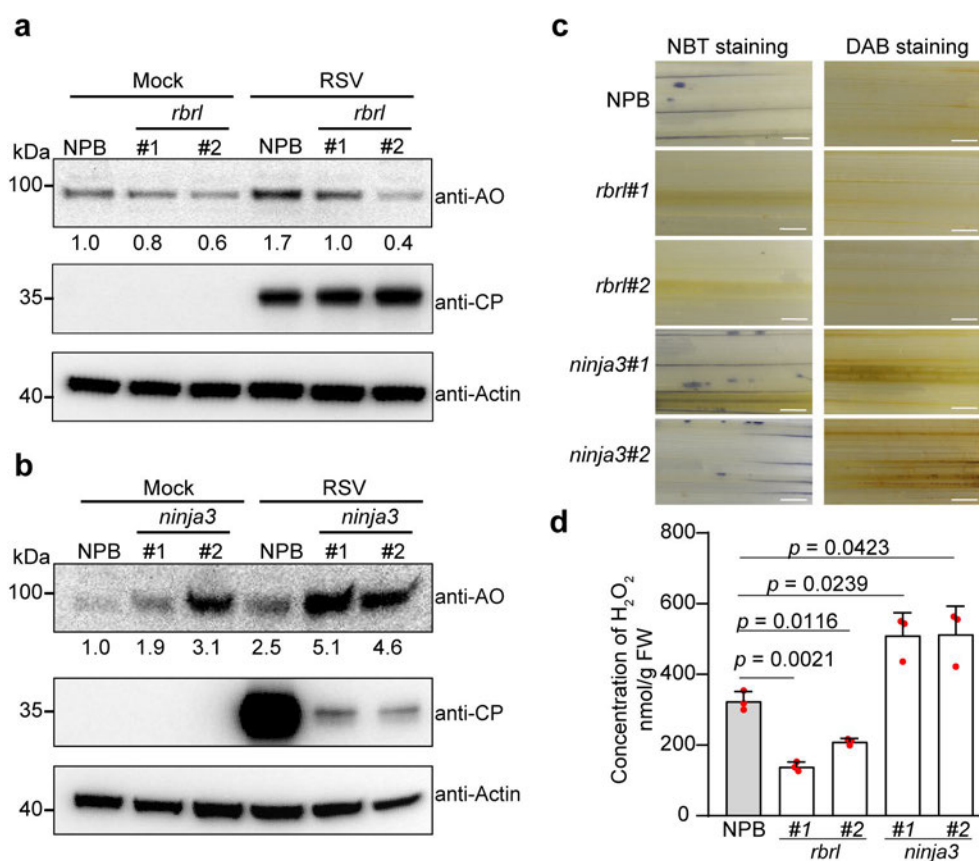
References:

Tang, N. *et al.* MODD mediates deactivation and degradation of OsbZIP46 to negatively regulate ABA signaling and drought resistance in rice. Plant Cell 28, 2161-2177 (2016).
Kashihara, K. *et al.* Overexpression of OsNINJA1 negatively affects a part of OsMYC2-mediated abiotic and biotic responses in rice. J. Plant Physiol. 232, 180-187 (2019).

G/ Lines 327-330. I wonder whether it would be relevant to also include measurements of AO-mediated generation of ROS as a parameter to assess the viral response in the mutant *osrbrl* and *osninja* backgrounds?

Response: We appreciate your suggestions. We determined the AO protein levels and ROS levels in the indicated rice lines. The results revealed that AO protein levels were higher in the *ninja3* mutant rice lines but lower in the *rbrl* mutant lines compared with those in the WT (please see the revised Extended Data Fig. 13a and 13b). Similarly, the ROS levels were greater in the *ninja3* mutants and lower in the *rbrl* mutants than in the WT (please see the revised Extended Data Fig. 13c and 13d). These data further support that AO-generated ROS are a parameter for assessing the viral response in the *rbrl* and *ninja3* mutant backgrounds, which is also consistent with our model. We

have included these data in the revised manuscript as follows: “Previous studies have shown that OsAGO18 releases OsAO through competitive binding of miR528 with OsAGO1, thereby increasing ROS levels to defend against viruses. We thus measured the levels of OsAO protein and ROS in wild-type NPB, *rbrl* and *ninja3* mutant plants. OsAO levels were reduced in the *rbrl* mutants (Extended Data Fig. 13a) and increased in the *ninja3* mutants (Extended Data Fig. 13b) compared with the wild-type NPB plants. Consistently, the ROS levels were lower in the *rbrl* mutants and higher in the *ninja3* mutants (Extended Data Fig. 13c, d).”



Revised Extended Data Fig. 13. The OsRBRL-OsNINJA3 module regulates the accumulation of downstream AO and ROS. **a**, Immunoblotting analysis of the AO levels in mock-inoculated and RSV-infected NPB and *rbrl* plants. **b**, Immunoblotting analysis of the AO levels in mock-inoculated and RSV-infected NPB and *ninja3* plants. **c**, *In situ* detection of leaf ROS levels via DAB and NBT staining in the indicated rice plants. **d**, Quantification of H₂O₂ levels in the indicated rice plants. Statistical analysis was performed via two-sided Student's *t* tests, and all *p* values are shown in the figure. The data are the means $\pm$ SDs. *n* = 3 independent biological samples.

Referee #2 (Remarks to the Author):

The authors identified a novel jasmonic acid (JA) signaling activation pathway comprising the E3 ligase RBRL and the NINJA3 module, triggered by viral infection. Overall, it is an interesting and comprehensive study that is easy to follow. The novel aspects of the paper include the perception of CP by RBRL and the establishment of a new signaling cascade. It is pity that there is no evidence documenting RBRL as a sensor for CP, apart from the induction of RBRL by CP, which is a crucial aspect of this paper. Additionally, some key claims are overstated and not well supported by current data. Moreover, appropriate controls are lacking in many experiments.

Response: We thank the reviewer for carefully reading our manuscript and recognizing the novelty of our work. We are especially thankful for the important suggestions, such as the addition of appropriate controls, which greatly improved our manuscript. We have addressed these potential limitations of our findings; please see our point-by-point response below.

General comments:

There is no strong evidence documents RBRL as a sensor for CP.

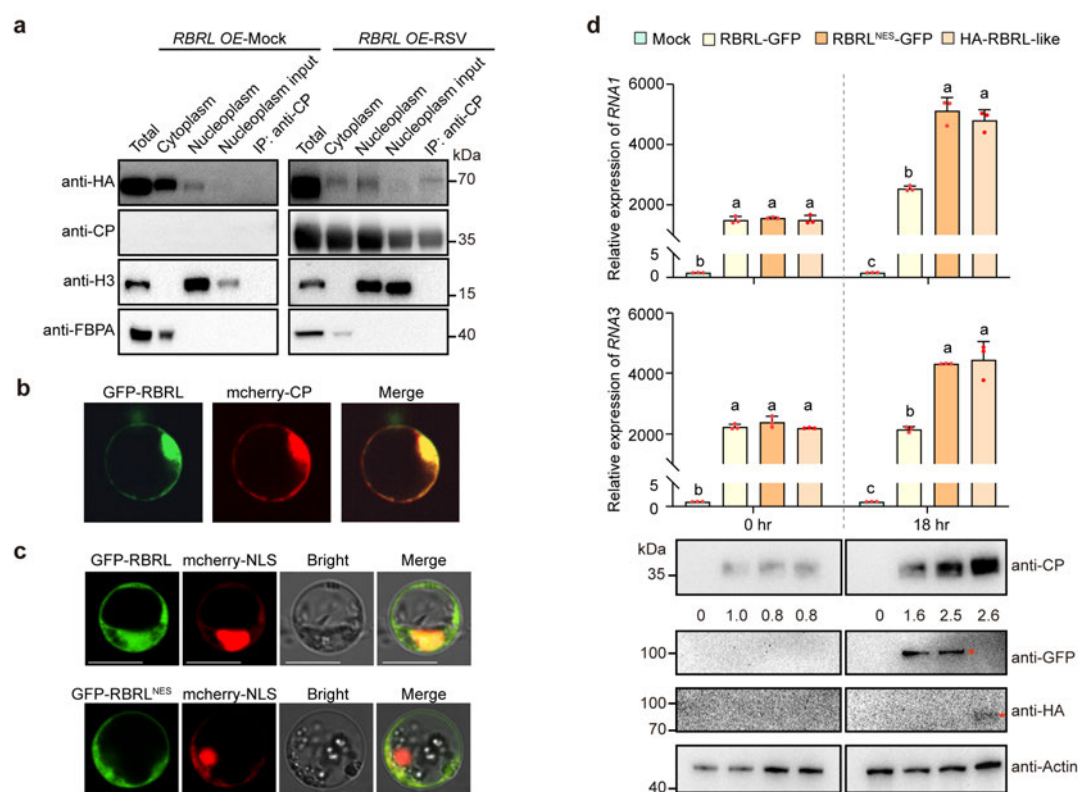
The authors aim to explore the nuclear signaling pathway triggered by the CP protein, and they have identified the interactor RBRL. However, the interaction assays used in the current draft do not strongly support this idea, as they did not use the specific nuclear fraction. Despite having nuclear-enriched IP-MS, unfortunately they lacked a control. It is also important to examine the subcellular localization and co-localization of RBRL and CP protein. To conclusively determine whether the signaling activation occurs from the nucleus rather than the cytosol, it is necessary to conduct nuclear exclusion experiments with RBRL to test viral responses. With the scenario, the biological significance of CP/CP-RBRL nuclear location should be clearly addressed, because they speculated that nucleus-localized CP might be a signaling factor used by plants to perceive viral infection.

Response: Thank you for these insightful comments. We understand these concerns about the subcellular localization of CP and RBRL, especially the interaction between CP and RBRL. As you suggested, we have added the NPB-mock sample as a negative control for the nuclear-enriched IP-MS and added raw IP-MS data to Supplementary Table 1. Additionally, we performed nuclear–cytoplasmic fractionation experiments using *RBRL*-overexpressing plants with or without RSV infection, followed by co-IP

assays. We found that CP interacted with RBRL in the nuclear fraction (please see the revised Extended Data Fig. 5a). We also examined the protein subcellular localization of RBRL and CP in rice protoplasts (please see revised Extended Data Fig. 5b). Our results revealed that RBRL and CP exhibit similar subcellular localizations. In addition, we constructed a GFP-tagged RBRL^{NES} mutant isoform (containing a nuclear export signal) and expressed it in rice protoplasts to assess the antiviral response (Wang *et al.*, 2014 Nat. Commun.). The results revealed that the GFP-RBRL^{NES} protein localized to the cytoplasm and that the virus accumulated more than that expressed GFP-RBRL protein (please see Extended Data Fig. 5c and 5d).

References:

Wang, Q. *et al.* STV11 encodes a sulphotransferase and confers durable resistance to rice stripe virus. Nat. Commun. 5, 4768 (2014).



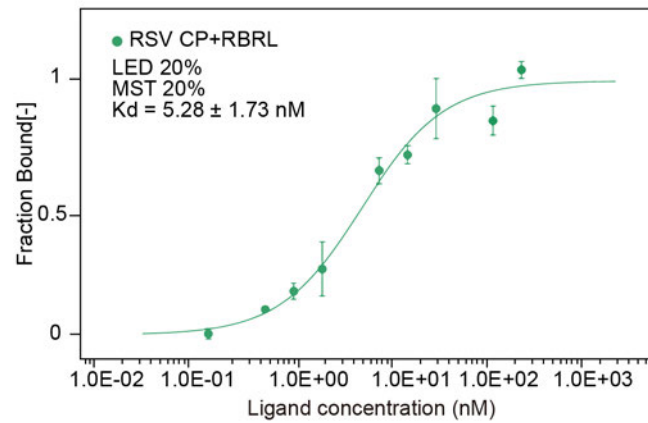
Revised Extended Data Fig. 5. OsRBRL colocalizes with CP in rice, and its biological function depends on its nuclear localization. **a**, IP assays were conducted with the nuclear fraction of mock-inoculated and RSV-infected *RBRL* OE plants. FBPA served as a cytoplasmic marker, and histone H3 served as a nuclear marker. Mock-inoculated *RBRL* OE plants served as a negative control. **b**, Subcellular localization of GFP-RBRL and mCherry-CP in rice protoplasts. Scale bars, 10 μ m. **c**, RBRL^{NES} was observed to be localized in the cytoplasm via confocal microscopy (scale bars, 10 μ m). **d**, qRT-PCR analysis (upper panel) of RSV RNAs accumulation in the rice protoplast transfected with

RBRL-GFP, *RBRL^{NES}-GFP* or *HA-RBRL-like*, together with RSV. Immunoblotting analysis (lower panel) of RSV CP accumulation, *RBRL-GFP*, *RBRL^{NES}-GFP* and *HA-RBRL-like* expression in the indicated samples. Mock, the protoplasts did not transfect with RSV. Data are shown as means $\pm$ SDs. $n = 3$ independent biological samples. Statistical analysis was performed via one-way analysis of variance (ANOVA) with Tukey's multiple comparison test (different letters represent significantly different groups; $p < 0.05$).

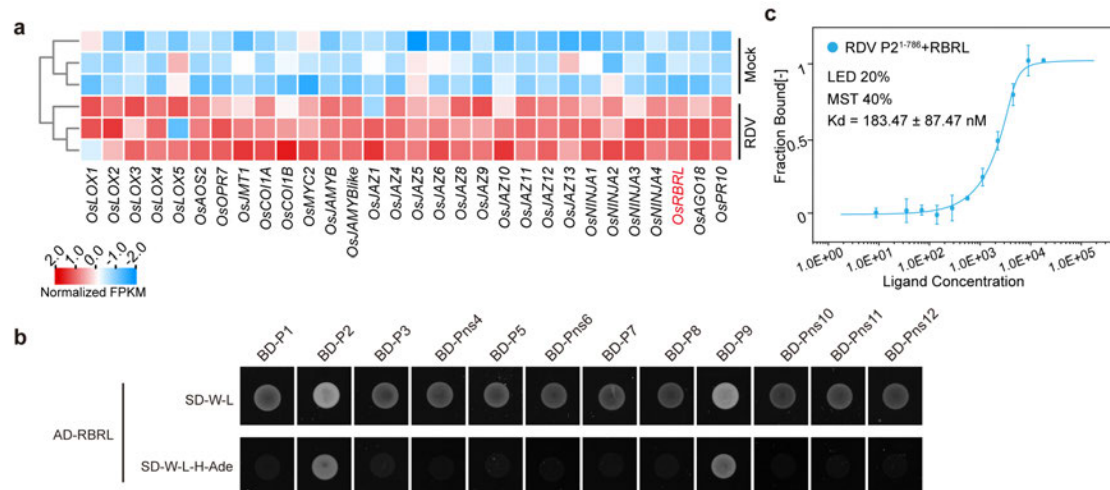
It is interesting that the authors identified a potential E3 as a CP sensor, they should put more effort on this aspect: they should determine the specificity of RBRL perceiving CP, how about the ligand binding affinity? whether other conserved CPs of viruses are also perceived by RBRL? How CP activates RBRL to degrade OsNINJA3 only due to induce/degrade RBRL? It also looks that CP promotes NINJA3 degradation with a high threshold (see below).

Response: Thank you for these important comments. To explore the binding affinity between CP and RBRL, we conducted a microscale thermophoresis (MST) experiment and found that their dissociation constant (Kd) was 5.28 nM (please see the revised Extended Data Fig. 2c), indicating a strong binding affinity between RBRL and CP. In addition, our preliminary findings suggest that RBRL may also recognize the RDV capsid protein P2, as they interact with each other, and RDV infection also induces the expression of *RBRL* and activation of the JA pathway (please see the revised Extended Data Fig. 14a and 14b). The binding affinity between RBRL and P2 was also detected through MST experiment (please see the revised Extended Data Fig. 14c). In this study, we found that CP increased the E3 ligase activity of RBRL and promoted the degradation of its substrate, NINJA3. In the revised Fig. 3e and Extended Data Fig. 9d, we added CP to the bacterial degradation system to further confirm this conclusion. Finally, regarding the concern of whether CP promotes NINJA3 degradation at a high threshold, we should explain that the numbers for CP in Extended Data Fig. 9c do not represent the actual concentrations but rather the proportion of *Agrobacterium* used for the transient expression of CP. The maximum proportion of CP is 10/26 of the total, which is less than half of the total system. Therefore, the promotion of NINJA3 degradation by CP does not require a very high concentration.

Extended Data Fig. 2c

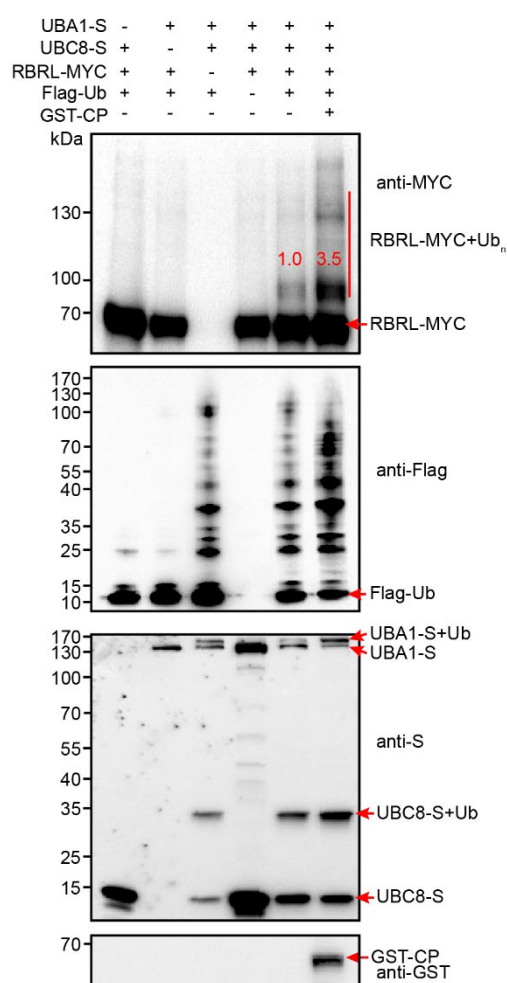


Revised Extended Data Fig. 2c. MST assays showing the binding affinity between RSV CP and OsRBRL. The data are the means $\pm$ SDs. $n = 3$ independent biological samples.



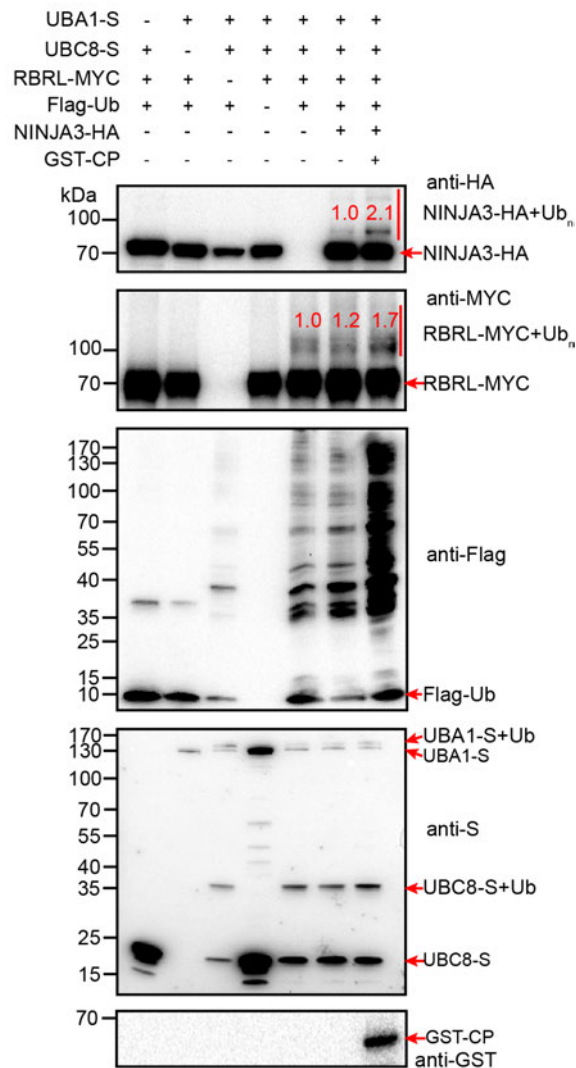
Revised Extended Data Fig. 14a-c. **a**, RDV infection induces the expression of *RBRL* and activation of the JA pathway. Expression analysis of genes related to JA synthesis and JA signaling in mock-inoculated and RDV-infected wild-type Zhonghua11 rice plants via transcriptome sequencing data from a previous study. The numbers are the Z scores of the FPKM values for each gene. FPKM, fragments per kilobase of transcript per million. **b**, Analysis of the interactions between RDV proteins and OsRBRL via Y2H assays. OsRBRL interacts with the capsid proteins of RDV. **c**, MST assays showing the binding affinity between RDV P2 N-terminus and OsRBRL. The data are the means $\pm$ SDs. $n = 3$ independent biological samples.

Fig. 3e



Revised Fig. 3e. CP enhanced the ubiquitin ligase activity of OsRBRL. The bacterial lysates from *E. coli* strains expressing UBA1-S, UBC8-S, Flag-Ub, RBRL-MYC and GST-CP or from strains lacking one of these components were analyzed by immunoblotting with an anti-MYC antibody (upper panel), an anti-Flag antibody (middle panel), an anti-S antibody (lower panel) and an anti-GST antibody (bottom panel).

Extended Data Fig. 9d



Revised Extended Data Fig. 9d. CP promotes the degradation of NINJA3 by increasing the activity of OsRBRL. The bacterial lysates from *E. coli* strains expressing UBA1-S, UBC8-S, Flag-Ub, RBRL-MYC, MBP-NINJA3-HA and GST-CP or from strains lacking one of these components were analyzed by immunoblotting with an anti-HA antibody to detect the ubiquitination of OsNINJA3.

The data indicate that RBRL constitutively regulates JA production; however, it is still unknown whether it is required for RSV-induced JA production. To address this concern, the author should measure JA concentrations in *rbrl* and RBRL OE plants before and after RSV infection to test whether the induced production of JA will be affected. A similar issue is observed with other signaling components throughout this manuscript, as indicated in the specific comments.

Response: Thank you for this suggestion. We measured JA concentrations in *rbri* mutant and *RBRL*-overexpressing plants with and without RSV infection as suggested (please see the revised Fig. 1l and 1m). Our results revealed that in the mock condition, the JA levels in the *rbri* mutants were significantly lower than those in the NPB plants, whereas the JA levels were greater in the *RBRL* OE plants. After RSV infection, the JA levels in the *rbri* mutants did not significantly increase, whereas the JA levels significantly increased in the NPB or *RBRL* OE plants. These findings indicate that *RBRL* indeed influences JA production upon RSV infection.

Fig. 1l

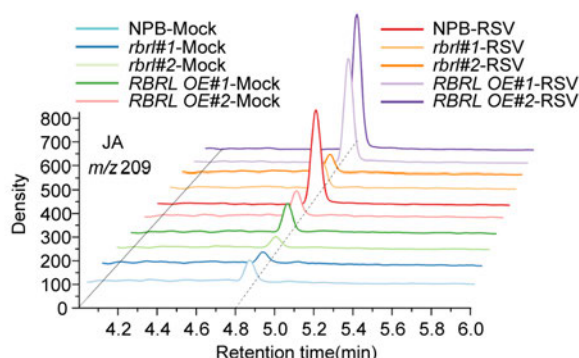
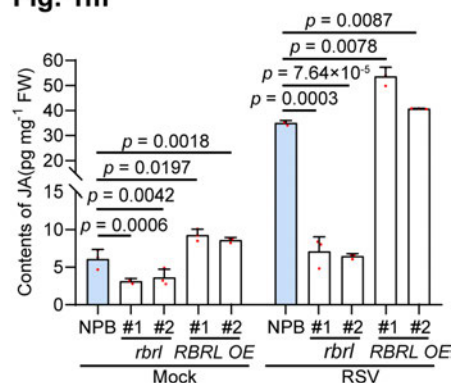


Fig. 1m

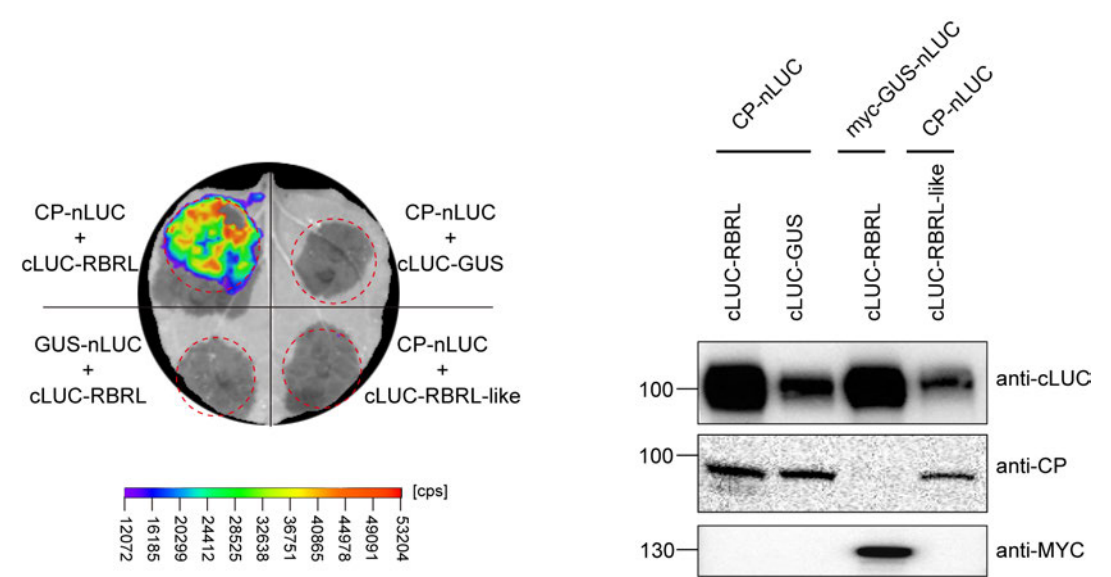


Revised Fig. 1l-m. JA content was lower in the *rbri* mutant but greater in the *RBRL* OE line than in the NPB rice plants. LC-MS analysis (l) and quantification (m) of the JA concentrations in the NPB, *rbri*, and *RBRL*-OE plants with or without RSV infection. Rice plants were collected at 3 wpi.

The data that CP OE-mediated antiviral responses partially depend on *RBRL* raises the possibility that *RBRL*-like protein could play a redundant function role with *RBRL*. Given that *RBRL*-like proteins have lower expression levels, it makes sense that it didn't appear in the IP-MS. Although the data show that *RBRL*-like protein is not required for the de-repression of *OsAGO18* expression, it is possible that other signaling components might be regulated by *RBRL*-like protein. At this stage, I think it is necessary to test CP interaction with the *RBRL*-like proteins, and assessing antiviral responses in CP OE/*rbri* *rbri*-like will strengthen the conclusion.

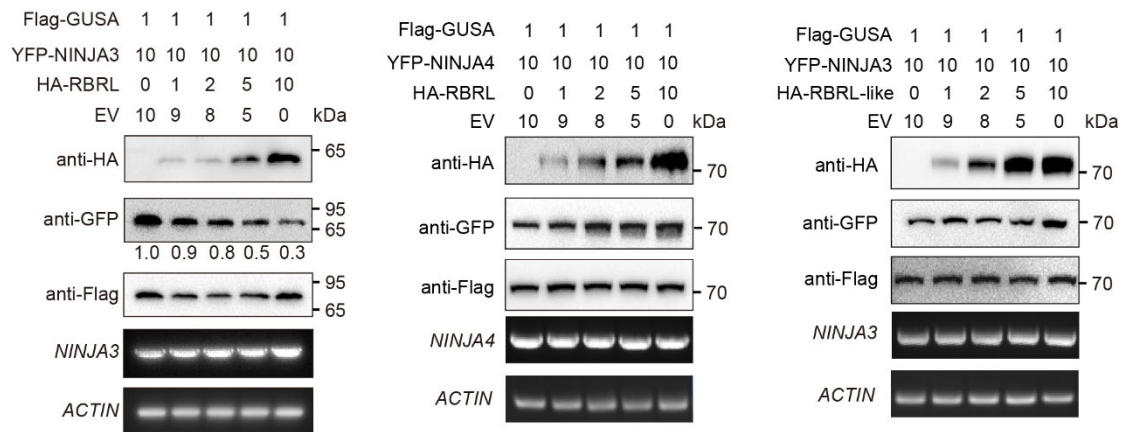
Response: Thank you for your valuable comments. We tested the interaction between *RBRL*-like and CP, as suggested, and found that *RBRL*-like does not interact with RSV CP (please see the Response Fig. 4). Furthermore, our degradation experiments revealed that gradually increasing the expression of *RBRL*-like proteins had no effect on the stability of *NINJA3* (please see the revised Extended Data Fig. 9a). These results suggested that *RBRL*-like does not interact with CP and cannot target *NINJA3*.

for degradation. Furthermore, we analyzed the RNA-seq data generated from this study. The results revealed that the expression of RBRL-like was very low in both the absence and presence of RSV (please see the revised Extended Data Fig. 1c). Taken together, these data demonstrate that RBRL-like proteins may have different roles than RBRL. We also obtained *rbrl/rbrl-like* double mutants and tested the antiviral responses of the double mutants. The results revealed that the double mutants presented symptoms similar to those of the *rbrl* single mutants (please see Response Fig. 5), which further suggested that the RBRL-like protein has little redundant function with RBRL during the antiviral response of rice to RSV. We have revised the manuscript as follows: “In the LCI assays, we also showed that CP did not interact with OsRBRL-like (Fig. 1e).”

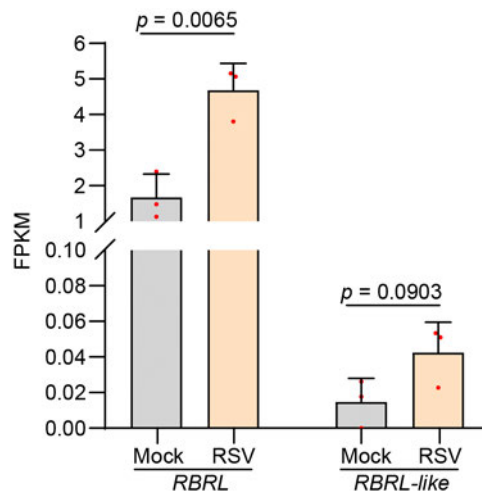


Response Fig. 4. LCI assays demonstrating the interaction between RSV CP and OsRBRL (left, revised Fig. 1e). cLUC-GUS and GUS-nLUC served as negative controls. cps, signal counts per second. Expression analysis of the corresponding proteins in different combinations (right). The proteins were detected via the indicated antibodies.

Extended Data Fig. 9a



Revised Extended Data Fig. 9a. RBRL targets NINJA3 for degradation. The *in vivo* degradation of NINJA3 was assessed by determining the YFP-NINJA3 protein level via coinfiltration experiments with increasing amounts of HA-RBRL. Flag-GUSA served as the internal control. The mRNA expression levels of the target genes *OsNINJA3* and *ACTIN* were analyzed via RT-PCR. The numbers at the top denote the ratios of the concentrations of *Agrobacterium* used for coinfiltration. YFP-NINJA4 (middle panel) and HA-RBRL-like (bottom panel) served as negative controls.



Revised Extended Data Fig. 1c. Expression analysis of *OsRBRL* and *OsRBRL-like* via transcriptome sequencing (RNA-seq) data. FPKM, fragments per kilobase of exon per million reads mapped. Statistical analysis was performed via two-sided Student's *t* tests, and all *p* values are shown in the figure. The data are the means ± SDs. *n* = 3 independent biological samples.

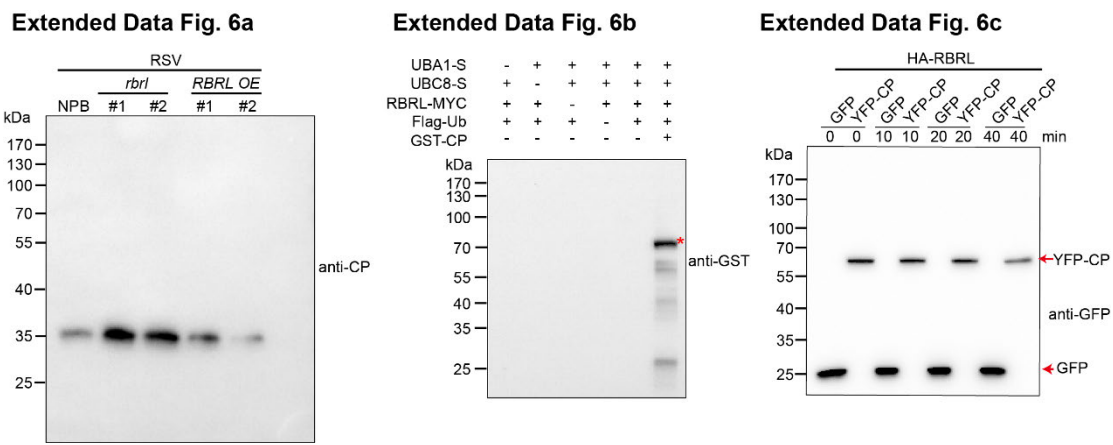
RESPONSE FIG. 5 REDACTED

It would be premature to conclude that CP is not a substrate of RBRL based on the current data. At least, an in vitro ubiquitination assay should be conducted to confirm this conclusion. Did CP was ubiquitylated in vitro? Should use another control E3 that ubiquitylates and degrades CP, how about RBRL-like?

Response: Thank you for these comments. We detected the CP protein levels in *rbrl* mutants and *RBRL OE* with RSV infection and detected only a single CP band, suggesting that CP did not undergo protein modification (Extended Data Fig. 6a). Additionally, our ubiquitination assays revealed that CP is not ubiquitinated by RBRL (please see revised Extended Data Fig. 6b). Besides, we conducted semi-*in vivo* degradation experiments and found that RBRL does not degrade CP (please see Extended Data Fig. 6c). On the basis of these results, we conclude that CP is not a substrate of RBRL. The RBRL-like protein does not interact with CP, suggesting that CP is not a substrate of the RBRL-like protein.

We have revised the manuscript as follows: “However, we did not detect ubiquitinated CP in wild-type NPB or *RBRL OE* rice plants infected with RSV (Extended Data Fig. 6a). Additionally, no ubiquitinated CPs were observed in the degradation assays performed in

either the bacterial system or *Nicotiana benthamiana* (*N. benthamiana*) leaves (Fig. 3e, f). Collectively, these data suggested that the substrate of RBRL is another factor and that the interaction between CP and RBRL promotes the degradation of the substrate of RBRL and triggers downstream antiviral immune responses.”



Revised Extended Data Fig. 6a-c. **a**, Full gel graph of the immunoblotting analysis of CP in the indicated plants; no ubiquitinated bands of CP were detected. **b**. Full gel graph of the immunoblotting analysis of CP in the bacterial ubiquitination system (related to Fig. 3e); no ubiquitinated bands of CP were detected. **c**. Full gel graph of the immunoblotting analysis of CP in the semi-*in vivo* degradation experiments (related to Fig. 3f); no ubiquitinated bands of CP were detected.

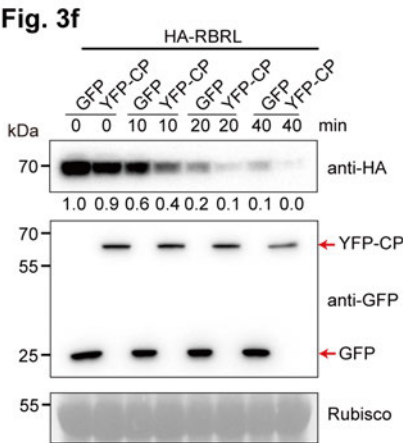
Actually, Fig 3e somehow suggests that YFP-CP undergoes degradation when co-incubated with RBRL. Autoinhibition and CP-activation is not sufficient to support RBRL as a CP sensor. How CP binding relieves RBRL autoinhibition? Again, including a negative control is essential or use RBRL-like protein?. It look

Response: Thank you for these questions. As suggested, we have replaced the negative control with GFP in the revised Fig. 3f (Fig. 3e in the original manuscript) and added the full gel graph of the immunoblotting analysis of CP in the Extended Data Fig. 6c. The result indicates that CP but not the control GFP promoted the degradation of RBRL, and CP did not undergo degradation when co-expressed with RBRL. Previous studies have shown that RBR-type E3 ubiquitin ligases commonly possess autoinhibitory domains, and the release of this autoinhibitory domain typically involves interaction with a specific protein or posttranslational modification. In the case of Parkin and HOIP, the truncated forms of the proteins are active, whereas the full-length proteins are inactive. The underlying mechanism involves the binding of the N-terminal

domain of Parkin between the IBR and RING2 domains to lock the E3 ligase in an inactive conformation. *In vivo*, Parkin is activated through phosphorylation by the PINK1 kinase (Kane *et al.*, 2014 J. Cell Biol.). Moreover, the HOIP protein is activated by interaction with the LUBAC complex (Duda *et al.*, 2013 Structure). By analyzing the protein sequences between RBRL and its homologs, we found that RBRL contains conserved domains and motifs. In addition, RBRL^{RBR}, the truncated form of RBRL, is extremely unstable compared with the full-length protein, which also indicates that the RBRL protein undergoes autoinhibition (revised Fig. 3c). Our appended data further showed that adding CP to the degradation system significantly increased the E3 ligase activity of RBRL and promoted the degradation of its substrate NINJA3 (revised Extended Data Fig. 9c, d). Besides, the binding affinities between RBRL and viral capsid proteins (RSV CP and RDV P2) are strong (revised Extended Data Fig. 2c and 14c). Therefore, we speculate that the interaction between CP and RBRL similarly alters the conformation of RBRL's autoinhibitory domain, thereby activating its E3 activity. We presume that RBRL is a sensor of CP on the basis of our findings that, upon viral infection, RBRL E3 ligase activity is released by interactions with CP, subsequently leading to the degradation of NINJA3 and the activation of downstream JA signaling and antiviral RNAi. However, the precise mechanisms by which CP releases the E3 ligase activity of RBRL require further study.

References:

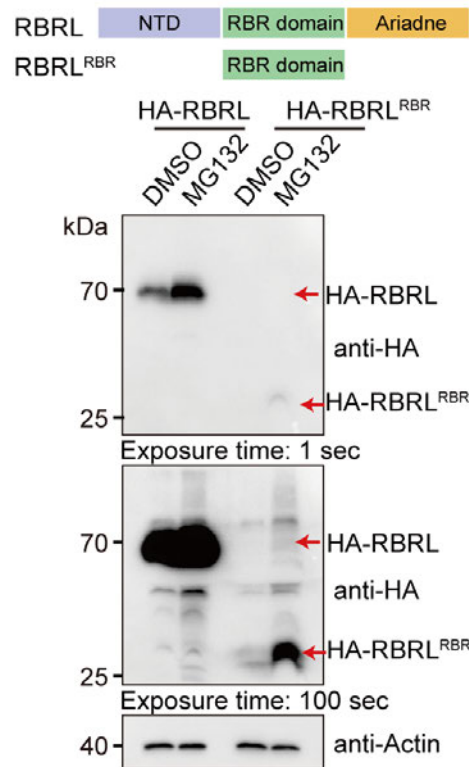
Kane, L. A. *et al.* PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. J. Cell Biol. 205, 143-153 (2014).
Duda, David M. *et al.* Structure of HHARI, a RING-IBR-RING ubiquitin ligase: autoinhibition of an Ariadne-family E3 and insights into ligation mechanism. Structure 21, 1030-1041 (2013).
Scott, D. C. *et al.* Two distinct types of E3 ligases work in unison to regulate substrate ubiquitylation. Cell 166, 1198-1214 (2016).



Revised Fig. 3f. Time course of CP-promoted OsRBRL self-degradation. The OsRBRL

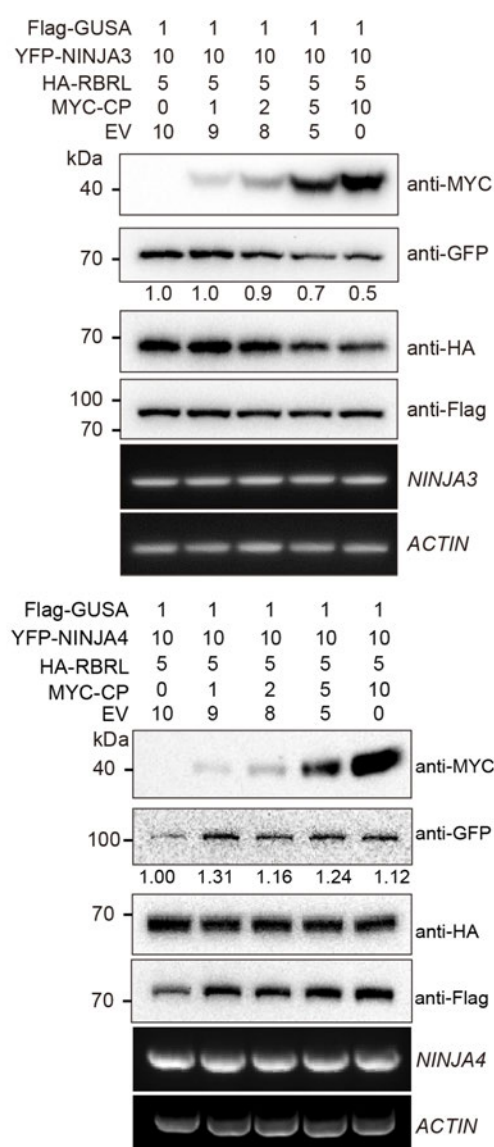
degradation assay was performed by mixing cell extracts from separately infiltrated GFP, YFP-CP and HA-RBRL samples, and GFP served as a negative control. Ponceau S staining (bottom panel) of the Rubisco protein is shown as a loading control.

Fig. 3c

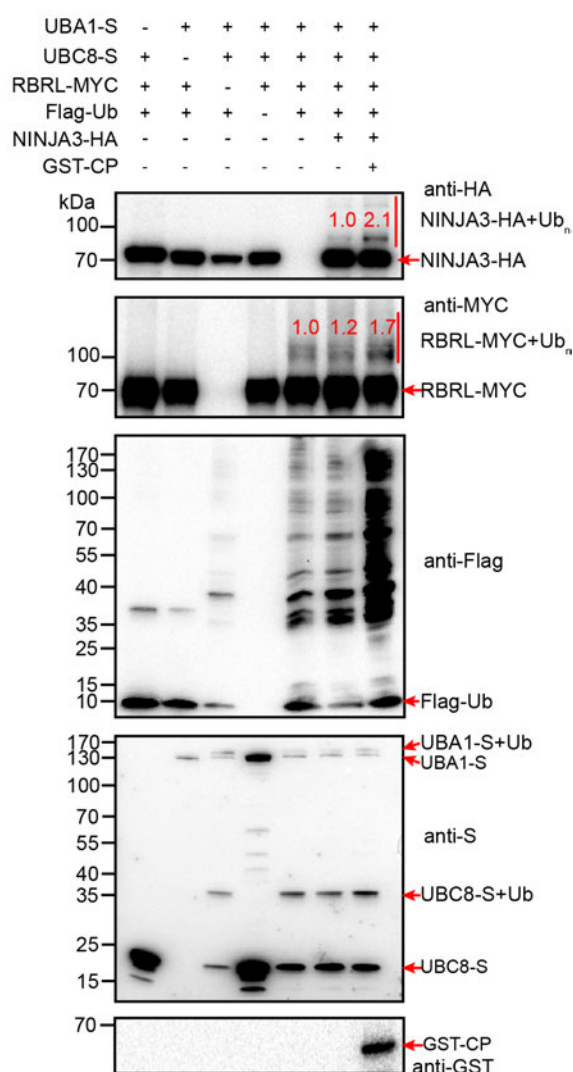


Revised Fig. 3c. Removing the NTD and Ariadne domains of OsRBRL resulted in instability of OsRBRL^{RBR} when the gene was expressed independently in plant cells and proteasome inhibitor MG132 inhibited the degradation of the full-length OsRBRL and the RBR domain of OsRBRL.

Extended Data Fig. 9c

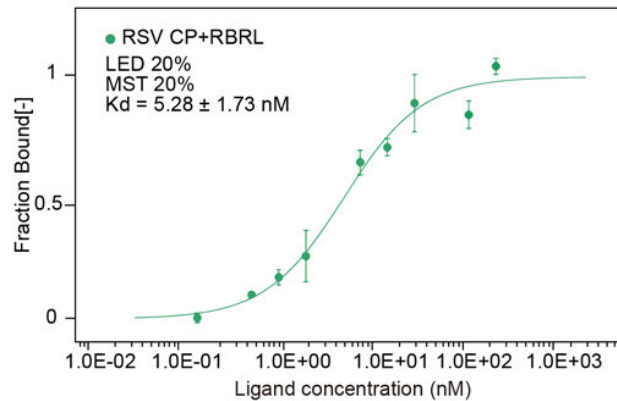


Extended Data Fig. 9d



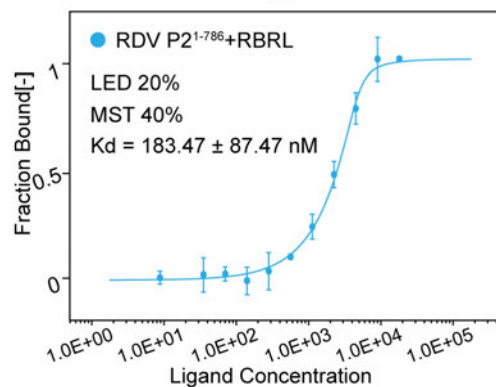
Revised Extended Data Fig. 9c and d. RSV CP promoted the proteolysis of NINJA3 but not that of NINJA4 by increasing the activity of RBRL. c, The *in vivo* degradation of OsNINJA3 was conducted by determining the YFP-NINJA3 protein level via coinfiltration experiments with increasing amounts of MYC-CP. Flag-GUSA was used as the internal control. The mRNA expression levels of the target genes *OsNINJA3* and *ACTIN* were analyzed via RT-PCR. The numbers at the top indicate the ratio of the concentrations of *Agrobacterium* used in coinfiltration. YFP-NINJA4 (bottom panel) served as a negative control. **d,** CP promotes the degradation of NINJA3 by OsRBRL. The bacterial lysates from *E. coli* strains expressing UBA1-S, UBC8-S, Flag-Ub, RBRL-MYC, MBP-NINJA3-HA and GST-CP or from strains lacking one of these components were analyzed by immunoblotting with an anti-HA antibody to detect the ubiquitination of OsNINJA3.

Extended Data Fig. 2c



Revised Extended Data Fig. 2c. MST assays showing the binding affinity between RSV CP and OsRBRL. The data are the means $\pm$ SDs. $n = 3$ independent biological samples.

Extended Data Fig. 14c



Revised Extended Data Fig. 14c. MST assays showing the binding affinity between RDV P2 N-terminus (RDV P2¹⁻⁷⁸⁶) and OsRBRL. The data are the means $\pm$ SDs. $n = 3$ independent biological samples.

Specific comments:

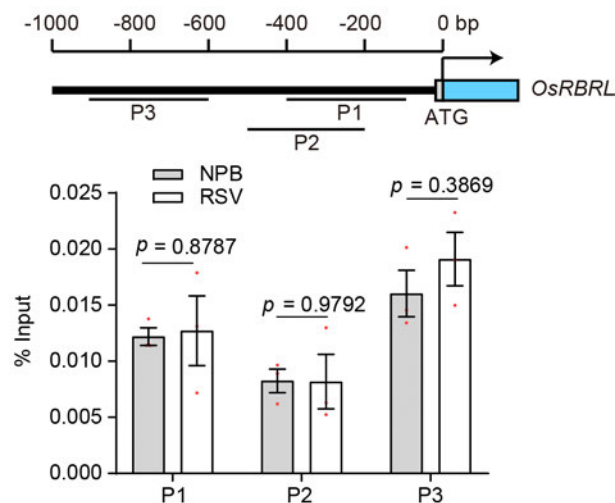
Does CP directly induce RBRL? Should do ChIP-PCR or in vivo activation.

Response: Thank you for your insightful comment. Upon analyzing the protein sequence of CP and reviewing studies on viral CPs, we hypothesize that CP does not directly bind to the *RBRL* promoter to activate *RBRL* expression. Instead, this process likely involves a specific transcription factor. Recent studies on the interaction between viral coat proteins and DNA have focused mainly on the role of capsid proteins in viral assembly (Sarker *et al.*, 2016 Nat. Commun.). The functional annotations of the RSV CP in the UniProt database revealed that CP exhibited only RNA-binding activity but not DNA-binding activity. We also determined whether CP can directly bind to the

RBRL promoter in mock-inoculated or RSV-infected NPB plants by ChIP-PCR as suggested. The results showed that CP did not bind the promoter of *RBRL* (please see Response Fig. 6 below).

Reference:

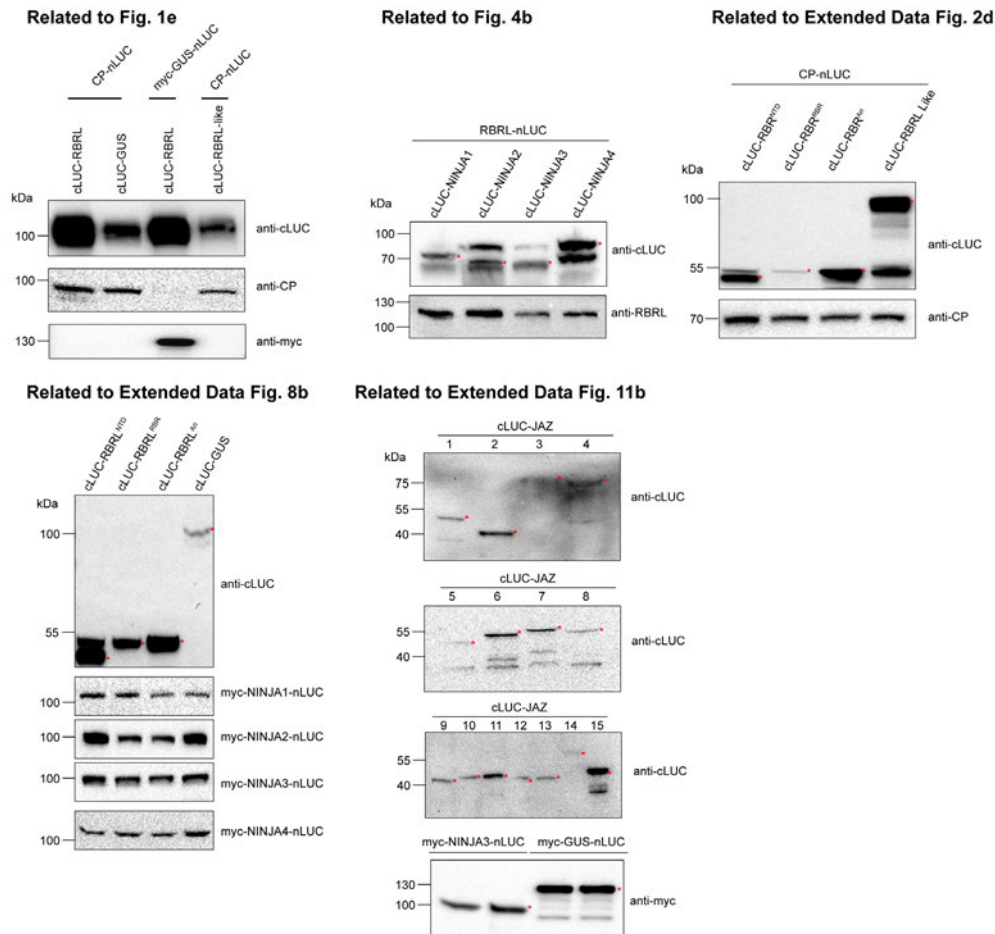
Sarker, S. A.-O. *et al.* Structural insights into the assembly and regulation of distinct viral capsid complexes. Nat. Commun. 7, 13014 (2016).



Response Fig. 6. ChIP-qPCR analyses. RSV CP did not directly promote the transcription of *OsRBRL*. Chromatin from mock-inoculated or RSV-infected NPB plants was immunoprecipitated with an anti-CP antibody and amplified with promoter-specific primers. Statistical analysis was performed via two-sided Student's *t* tests, and all *p* values are shown in the figure. The data are the means $\pm$ SDs. *n* = 3 independent biological samples.

Please check all protein expression levels in all LCI assays.

Response: Thank you. We have repeated all the LCI assays and thoroughly checked the protein expression levels (please see Response Fig. 7 below).



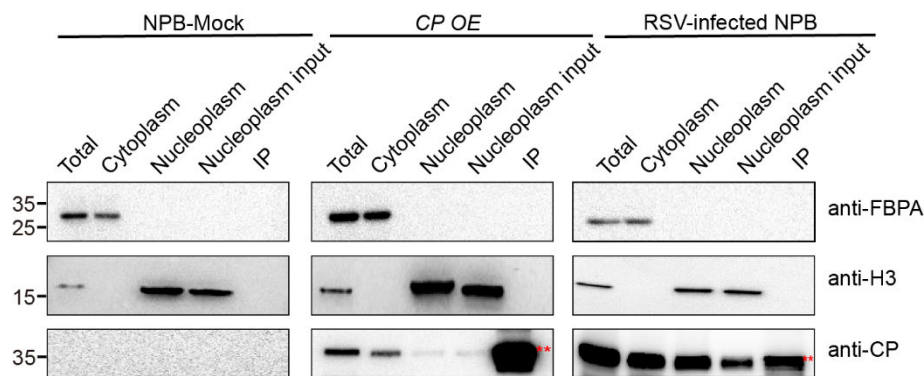
Response Fig. 7. All the proteins in the corresponding LCI assays were correctly expressed. Protein detection of different combinations in LCI assays. The indicated proteins were detected via the corresponding antibodies. The red asterisks indicate the target protein bands.

-Fig. 1b

Lack of molecular size of WB image.

Response: Thank you; we have added the molecular size of the WB image to the revised Fig. 1b.

Fig. 1b



Revised Fig. 1b. IP–MS assays conducted with the nuclear fraction of mock-inoculated NPB plants, *CP OE* plants and RSV-infected NPB plants. FBPA served as a cytoplasmic marker, and histone H3 served as a nuclear marker. IP, immunoprecipitation.

CP band in nucleoplasm input of CP OE looks different.

Uninfected rice as a negative control for CP detection.

Why the CP-IP band in the CP OE plant in Fig. 1b showed a different molecular size?

Why didn't the CP protein show enrichment after IP compared to the input?

Response: Thank you for the detailed observations and questions. We ensured the uniformity of the CP bands' molecular size in the nucleoplasm input of the *CP OE* and the IP samples. Given that we wanted to retain more samples for subsequent mass spectrometry detection, the loading amount of IP samples for western blotting was less than that of the other samples. Therefore, CP was not enriched in the IP samples. We repeated these IP-western blotting experiments and included NPB mock as a negative control for CP detection. During these experiments, we ensured comparable loading amounts of different samples to reflect the enrichment of CP in the IP samples.

The exact time of the RSV treatment for the plants should be indicated in the legend.

Response: Thank you; we have added the exact duration of RSV treatment for the plants to the revised figure legends.

-Fig. 1c

It is not a good way to conduct IP-MS without any proper controls. Using nuclear-localized proteins or different fractions of CP protein as control will be more convincing.

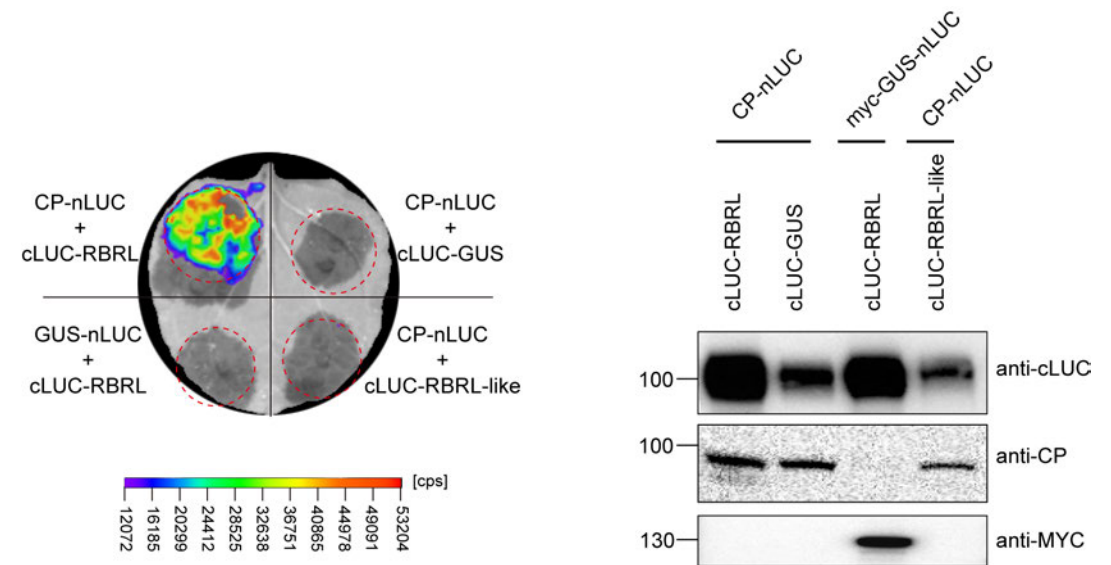
Response: Thank you for the suggestions. We have now used the nuclear fractions of the *CP OE* and NPB-RSV samples to screen for interactors of CP in the nucleus,

and found that RBRL is present in the IP-MS lists of both the *CP OE* and the NPB-RSV samples. We believe this result is more convincing. In addition, we included NPB-mock rice samples as a negative control for CP detection in our IP-MS experiments, as suggested. We also added the raw IP-MS data to Supplementary Table 1.

-Fig. 1e

check the protein expression.

Response: Thank you. We have checked the protein expression levels in Fig. 1e (please see Response Fig. 8 below).

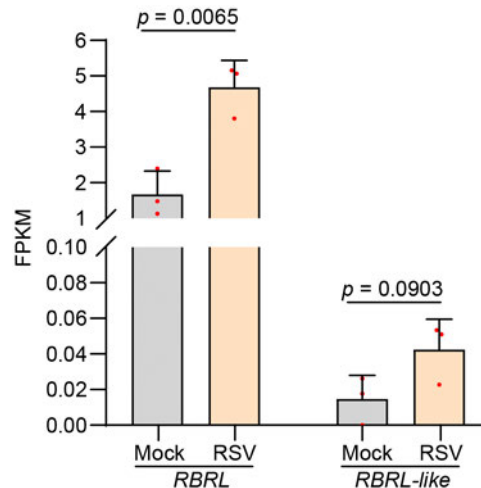


Response Fig. 8. LCI assays demonstrating the interaction between RSV CP and OsRBRL (left, revised Fig. 1e). cLUC-GUS and GUS-nLUC served as negative controls. cps, signal counts per second. Expression analysis of the corresponding proteins in different combinations (right). The proteins were detected via the indicated antibodies.

-Extended data 1

adjust the scale to reflect RBRL-like expression levels, as it appears to be reduced upon RSV infection.

Response: Thank you. We have adjusted the scale as suggested, and we also added the RNA-seq results for *RBRL* and *RBRL-like* generated from this study to the revised Extended Data Fig. 1c.



Revised Extended Data Fig. 1c. Expression analysis of *OsRBRL* and *OsRBRL-like* via transcriptome sequencing (RNA-seq) data. FPKM, fragments per kilobase of exon per million reads mapped. Statistical analysis was performed via two-sided Student's *t* tests, and all *p* values are shown in the figure. The data are the means $\pm$ SDs. *n* = 3 independent biological samples.

-Fig. 2

One-way ANOVA should be used to test the significant difference between different genotypes (d and g).

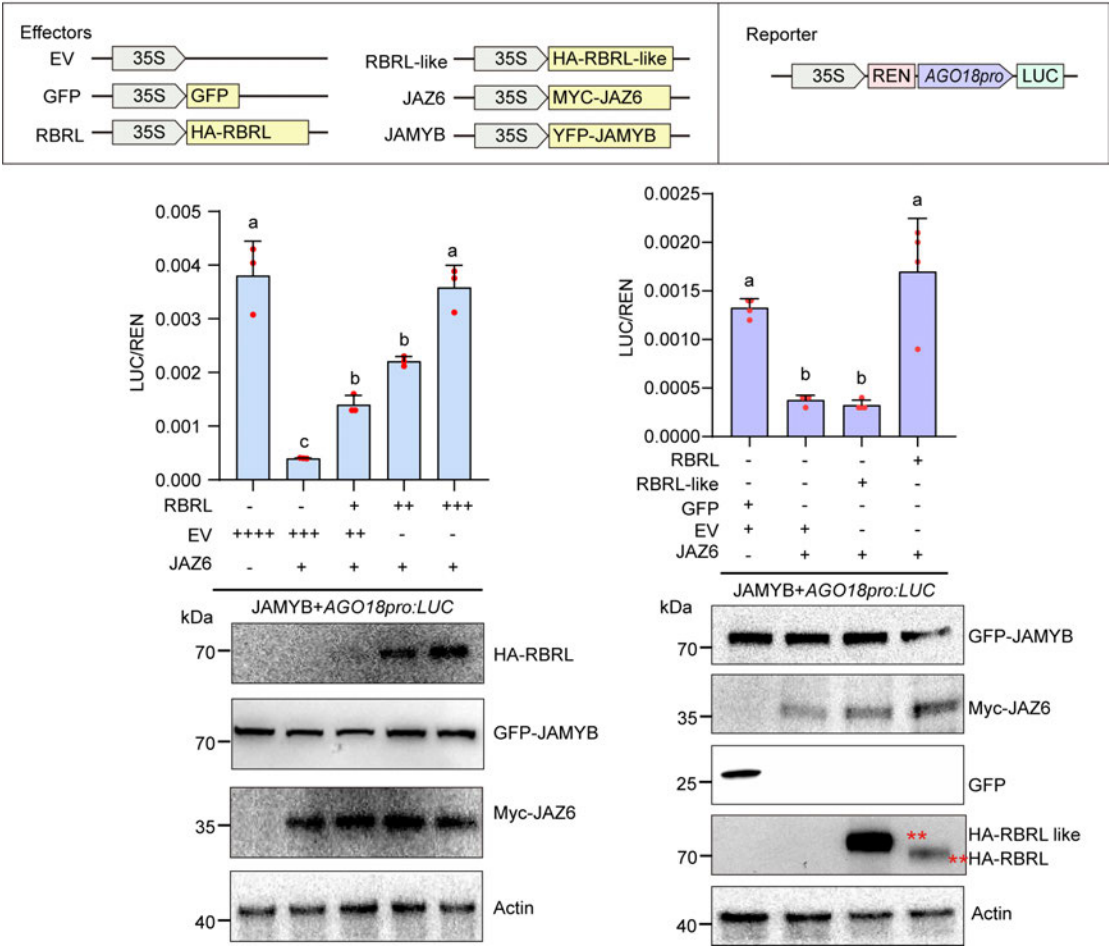
Response: Thank you; we have changed the statistical analysis as suggested in the revised Fig. 2g. For Fig. 2d, we thought it is more appropriate to show the difference between *rb1* mutant or *RBRL* OE and wild-type NPB plants.

-Extended data 3b

check all protein expression levels.

Response: Thank you. We have repeated these experiments and checked the protein expression levels in the revised Extended Data Fig. 4b.

Extended Data Fig. 4b



Revised Extended Data Fig. 4b. OsRBRL promotes the transcription of OsAGO18 in rice protoplasts. A schematic diagram of the constructs is shown in the upper panel. The activities of firefly luciferase (LUC) and Renilla luciferase (REN) were measured sequentially, and the LUC/REN ratio was calculated as the final transcriptional activity. EV: empty vector. Statistical analysis was performed via one-way analysis of variance (ANOVA) with Tukey's multiple comparison test (different letters represent significantly different groups; $p < 0.05$).

Line 204-205

Is there any evidence to show that the E3 ligase activity of RBRL is affected by CP? The degradation assay showing the promotion of RBRL degradation by CP unlikely supports this.

Response: Thank you for your questions. Fig. 3f showed that the addition of CP promotes the instability of RBRL. Additionally, in Extended Data Fig. 9c, we showed

that CP enhances the RBRL-mediated degradation of NINJA3. We have added proper controls for these assays to the revised Fig. 3f and Extended Data Fig. 9c. Furthermore, we conducted *in vitro* ubiquitination assays with the addition of CP and observed a significant increase in the ubiquitination ladder of both RBRL and NINJA3 (please see revised Fig. 3e and Extended Data Fig. 9d). These results suggest that CP enhances the ubiquitination activity of RBRL.

Fig. 3e

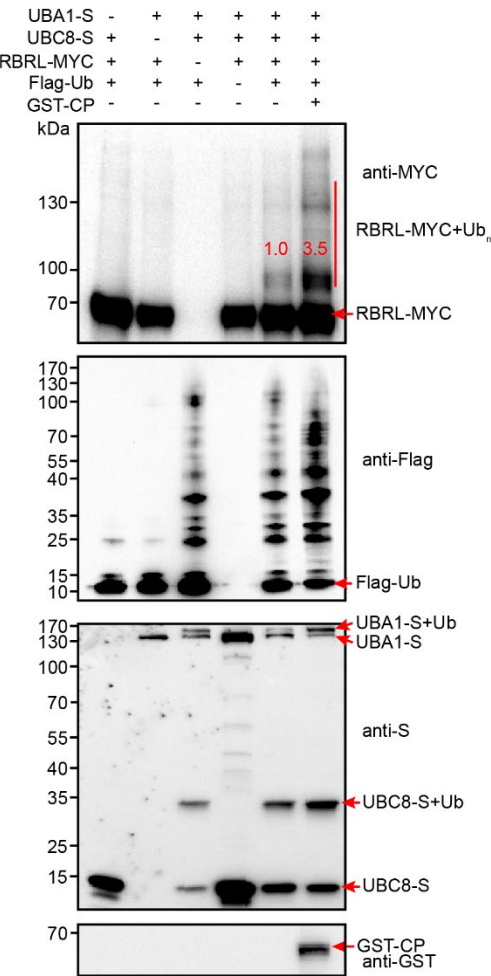
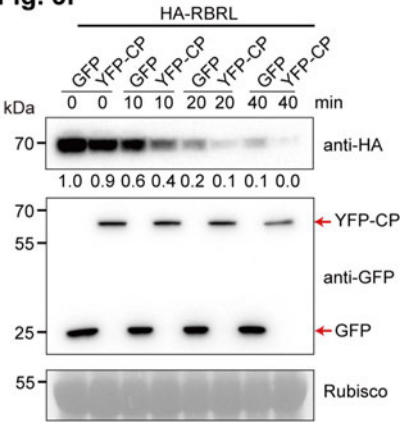
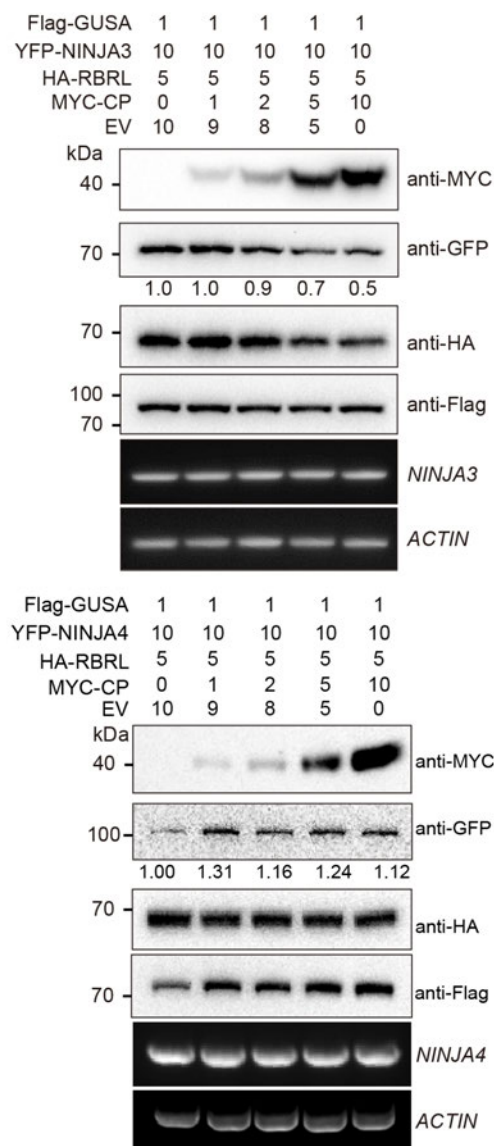


Fig. 3f

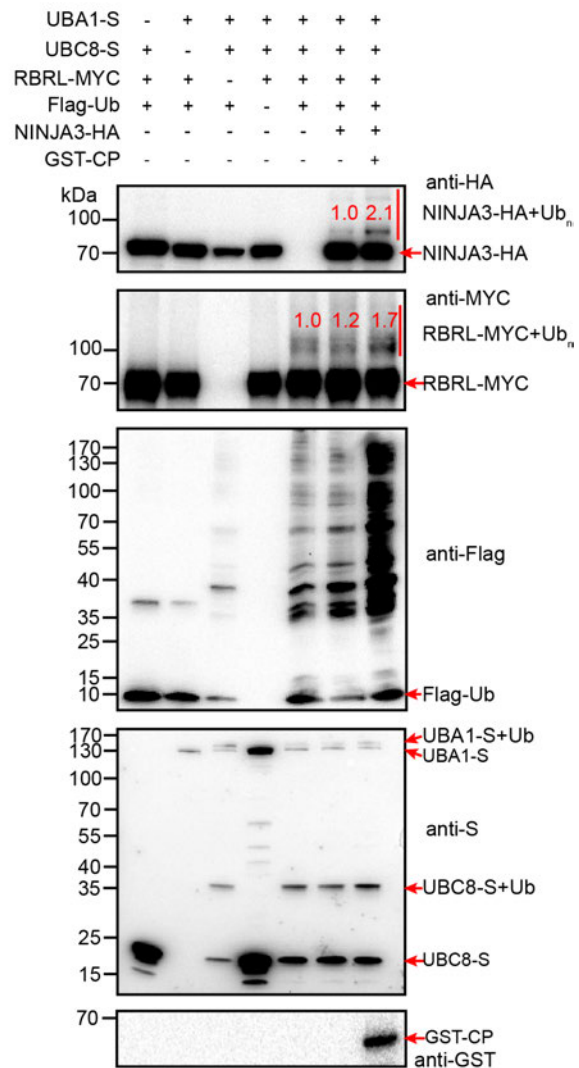


Revised Fig. 3e and 3f. e, CP enhanced the ubiquitin ligase activity of OsRBRL. The bacterial lysates from *E. coli* strains expressing UBA1-S, UBC8-S, Flag-Ub, RBRL-MYC and GST-CP or from strains lacking one of these components were analyzed by immunoblotting with an anti-MYC antibody (upper panel), an anti-Flag antibody (middle panel), an anti-S antibody (lower panel) and an anti-GST antibody (bottom panel). **f**, Time course of CP-promoted OsRBRL self-degradation. The OsRBRL degradation assay was performed by mixing cell extracts from separately infiltrated GFP, YFP-CP and HA-RBRL samples, and GFP served as a negative control. Ponceau S staining (bottom panel) of the Rubisco protein is shown as a loading control.

Extended Data Fig. 9c



Extended Data Fig. 9d



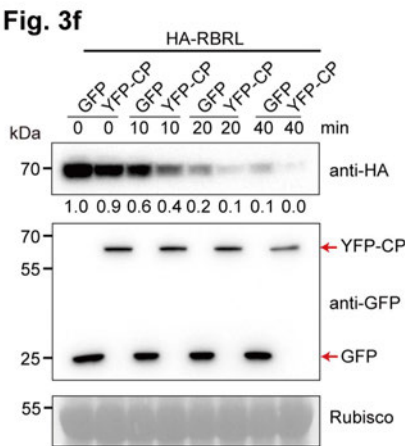
Revised Extended Data Fig. 9c and d. RSV CP promoted the proteolysis of NINJA3 but not that of NINJA4 by increasing the activity of RBRL. c, The *in vivo* degradation of OsNINJA3 was conducted by determining the YFP-NINJA3 protein level via coinfiltration experiments with increasing amounts of MYC-CP. Flag-GUSA was used as the internal control. The mRNA expression levels of the target genes *OsNINJA3* and *ACTIN* were analyzed via RT-PCR. The numbers at the top indicate the ratio of the concentrations of *Agrobacterium* used in coinfiltration. YFP-NINJA4 (bottom panel) served as a negative control. **d,** CP promotes the degradation of NINJA3 by OsRBRL. The bacterial lysates from *E. coli* strains expressing UBA1-S, UBC8-S, Flag-Ub, RBRL-MYC, MBP-NINJA3-HA and GST-CP or from strains lacking one of these components were analyzed by immunoblotting with an anti-HA antibody to detect the ubiquitination of OsNINJA3.

-Fig. 3e

YFP, but not EV, should be used as a negative control.

CP promotes RBRL degradation is not convincing, should include a negative control.

Response: Thank you; we have replaced EV with GFP as the negative control in the revised Fig. 3f (Fig. 3e in the original manuscript).



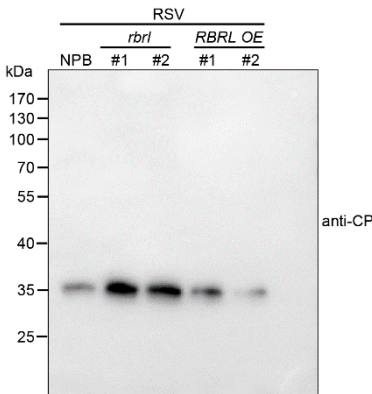
Revised Fig. 3f. Time course of CP-promoted OsRBRL self-degradation. The OsRBRL degradation assay was performed by mixing cell extracts from separately infiltrated GFP, YFP-CP and HA-RBRL samples, and GFP served as a negative control. Ponceau S staining (bottom panel) of the Rubisco protein is shown as a loading control.

Line 210-211

provide the data supporting the claim that “we did not detect ubiquitinated CP in wild-type NPB or RBRL-OE rice plants infected with RSV”.

Response: Thank you. The full gel graph of the western blot analyses revealed that there were no ubiquitinated forms of the CP protein band (please see revised Extended Data Fig. 6a).

Extended Data Fig. 6a

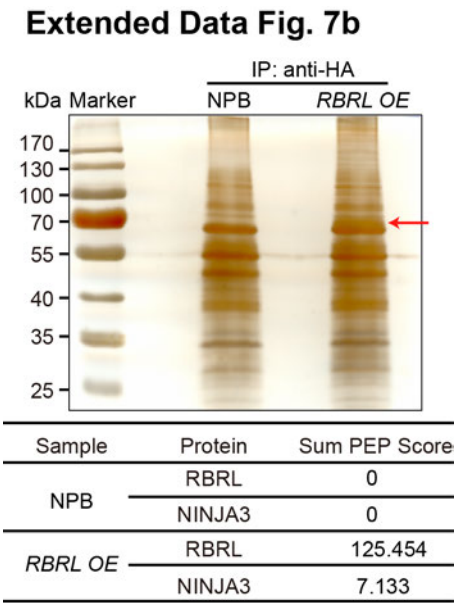


Revised Extended Data Fig. 6a. Full gel graph of the immunoblotting analysis of CP in the indicated plants; no ubiquitinated bands of CP were detected.

-Extended data 4a and b

It is weird that NPB and RBRL OE showed similar protein band signatures with CBB staining after IP. Additionally, there is no specificity band of RBRL in the gel with CBB. Better to present all potential interactors and their unique peptide sequences.

Response: Thank you for the comments. The similar protein band signatures observed in NPB and *RBRL OE* with Coomassie Brilliant Blue staining were due to the insufficient separation of the bands and the lower sensitivity of CBB staining. We have reperformed the IP–silver staining experiments, which are more sensitive, and extended the SDS–PAGE running time. This approach allowed us to detect a specific band (please see the revised Extended Data Fig. 7b), which was confirmed to be the RBRL protein upon mass spectrometry analysis. We also included the raw data (including all potential interactors and their unique peptide sequences) in Supplementary Table 4.



Revised Extended Data Fig. 7b. HA-RBRL and its interaction proteins purified with anti-HA magnetic agarose from proteins extracts of *RBRL OE* rice lines were detected by silver staining. OsRBRL expressed in RBRL OE lines was labeled with a 1× HA tag at the N-terminus. Wild-type NPB plants served as the negative control. Red arrows indicate the specific RBRL band. Scores of the RBRL protein and its interacting protein NINJA3 identified by mass spectrometry in different samples.

-Line 226

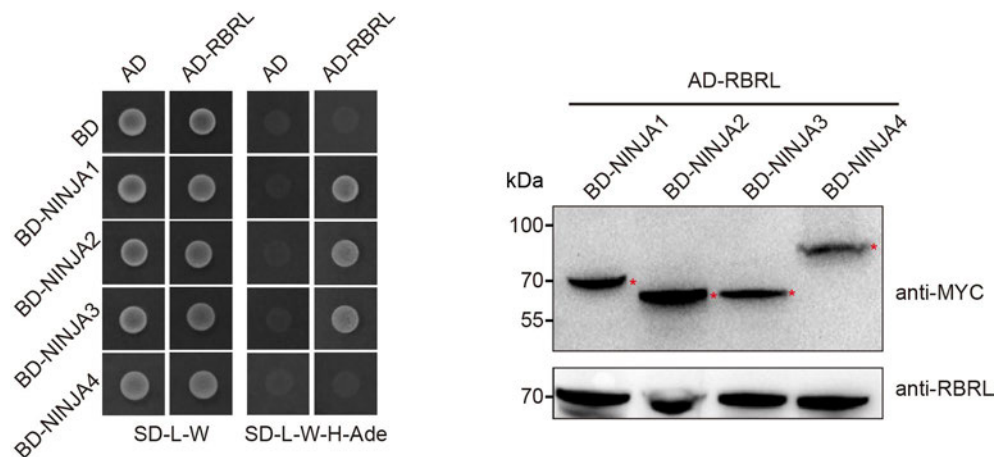
Change “confirmed” to “examined”.

Response: Thank you; we have changed this term in the revised manuscript.

-Fig. 4a

check the protein expression of NINJA4.

Response: Thank you. We have reperformed the yeast two-hybrid assay and checked the expression of these proteins (please see Response Fig. 9 below).



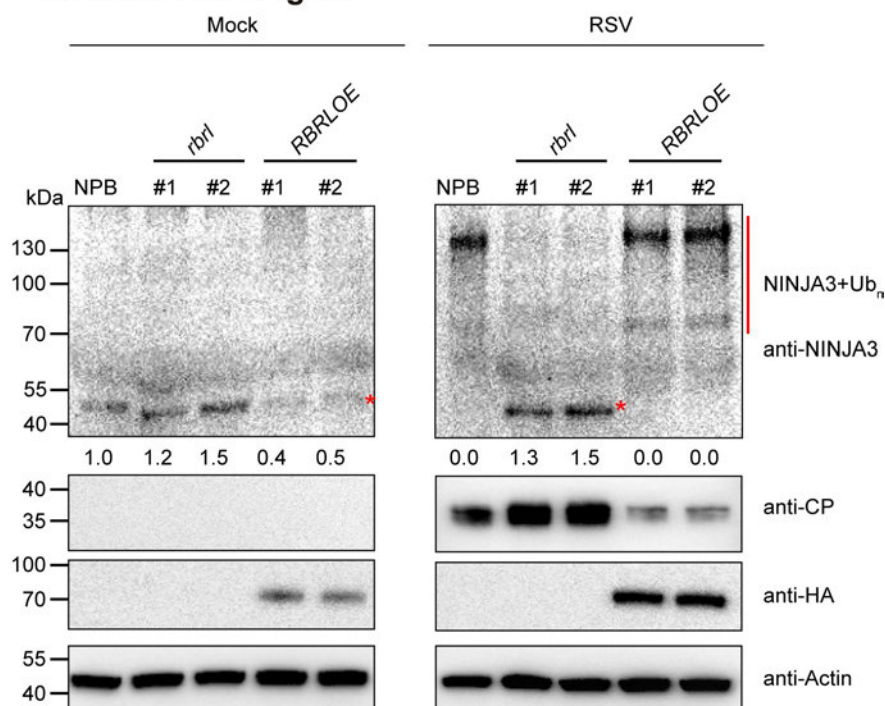
Response Fig. 9. Analysis of interactions between OsNINJAs and OsRBRL via yeast two-hybrid assays (left, revised Fig. 4a). Protein detection of different combinations of the corresponding proteins in the yeast two-hybrid assays (right). The indicated proteins were detected via the corresponding antibodies. The red asterisks indicate the target protein bands.

-Fig. 4d

How about NINJA3 protein levels before and after RSV infection in *rbrl*?

Response: Thank you for your question. We analyzed the NINJA3 protein levels in the NPB, *rbrl*, and *RBRL* OE plants with or without RSV infection. Our results show that under mock conditions, NINJA3 protein levels are significantly greater in *rbrl* mutants than in NPB and *RBRL* OE plants. After RSV infection, NINJA3 in the NPB and *RBRL* OE plants was ubiquitinated and degraded. In contrast, in the *rbrl* mutants, no ubiquitinated NINJA3 bands were detected, and the protein levels remained unchanged after RSV infection (please see the revised Extended Data Fig. 9e). These findings suggest that RBRL is necessary for the RSV-induced ubiquitination and degradation of NINJA3.

Extended Data Fig. 9e



Revised Extended Data Fig. 9e. NINJA3 protein levels are significantly greater in *rbr1* mutants than in NPB and *RBRL* OE plants with or without RSV infection. Immunoblotting analysis of OsNINJA3 levels in the plants indicated by the asterisk. The red line denotes the ubiquitinated form of NINJA3.

-Fig. 4e

NINJA4 would be a more suitable negative control than EV.

Response: Thank you for your suggestion. We have added a new set of experiments using NINJA4 as a negative control as suggested (please see revised Fig. 4e, f).

Fig. 4e

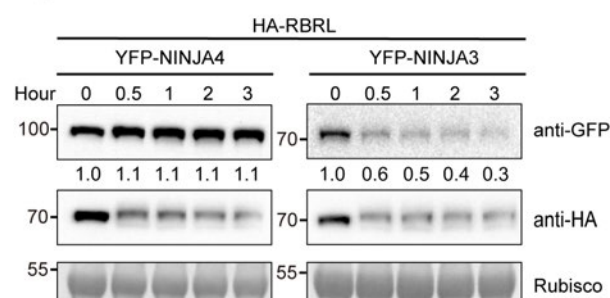
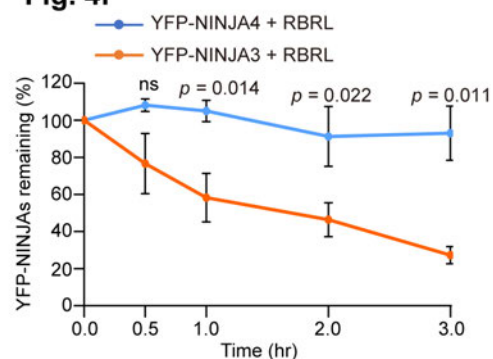


Fig. 4f



Revised Fig. 4e and f. e, Time course of RBRL-promoted NINJA3 degradation. YFP-NINJA4 served as a negative control. Ponceau S staining (bottom panel) of the Rubisco protein served as a loading control. f, The intensities of the bands shown in (e) were

quantified with ImageJ software. Relative values were calculated via comparison with the values at 0 hours (set to 1.0). Statistical analysis was performed via two-sided Student's *t* tests, and all *p* values are shown in the figure. The data are the means $\pm$ SDs. *n* = 3 independent biological samples. ns, not significant.

-Fig. 5b

How about NINJA3 protein levels in CP OE/rb1?

Response: Thank you for your question. Our results show that NINJA3 protein levels are 4.3-fold higher in *CP OE/rb1* plants than that in *CP OE* plants (please see Response Fig. 10).

RESPONSE FIG. 10 REDACTED

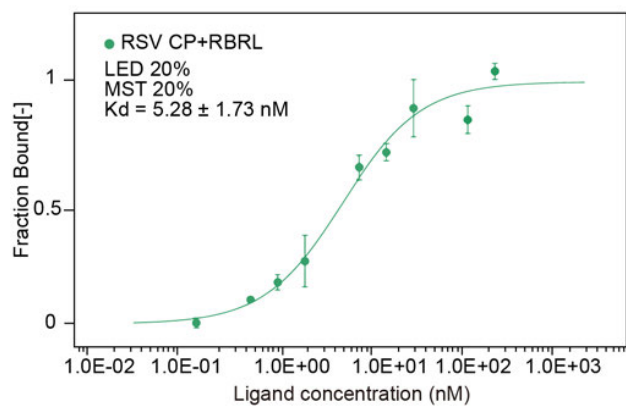
-Extended data Fig 5c

CP-1,-2 did not obviously promote NINJA3 degradation only CP-5 and -10 worked?
Too high threshold?

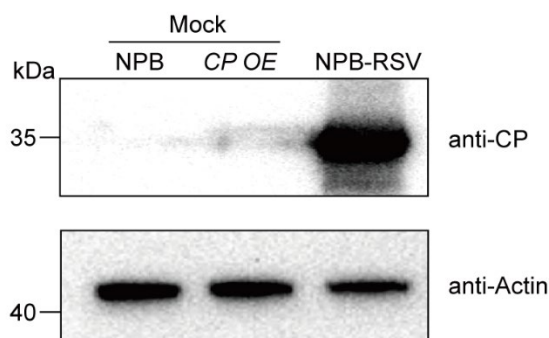
Response: Thank you for bringing this question to our attention. Our appended MST data showed that the dissociation constant (*K_d*) between CP and RBRL was 5.28 nM (please see the revised Extended Data Fig. 2c), indicating that low dose of CP is sufficient to be sensed by RBRL. Besides, the immunoblotting analysis showed that NINJA3 protein levels are 4.3-fold higher in *CP OE/rb1* plants than that in *CP OE* plants, indicating that CP-mediated NINJA3 degradation is dependent on RBRL (please see the above Response Fig. 10). Additionally, the protein level of RSV CP in *CP OE* plants is much lower than that in natural RSV-infected NPB plants, suggesting that low level CP could promote the degradation of NINJA3 in rice plants (Response Fig. 11). Furthermore, we wish to point out that the numbers -1, -2, -5, and -10 in the in the revised Extended Data Fig. 9c (Extended Data Fig. 5c in the original manuscript) represent the ratios of CP, not specific amounts of protein. As shown, even in the -5

and -10 combinations, the proportion of CP is not very high, making up only 5/26 and 10/26 of the total transiently expressed proteins, and the ratio of CP and NINJA3 is 1/2 and 1/1, respectively. Collectively, these results indicated that CP promotes NINJA3 degradation without requiring a high dosage both in rice plants or semi-*in vivo* degradation system.

Extended Data Fig. 2c

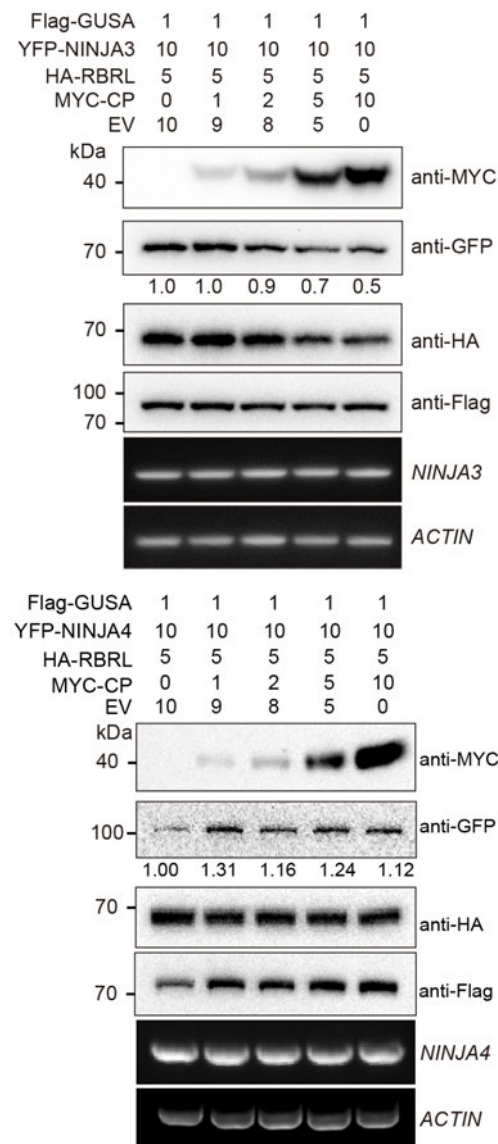


Revised Extended Data Fig. 2c. MST assays showing the binding affinity between RSV CP and OsRBRL. The data are the means $\pm$ SDs. $n = 3$ independent biological samples.



Response Fig. 11. RSV CP accumulated to a much higher level under RSV infection than that in *CP OE* plants. Immunoblotting analysis of the RSV CP levels in the specified plants. All the mock-inoculated rice plants were 6-week-old and all the RSV-infected plants were collected at 4 wpi.

Extended Data Fig. 9c



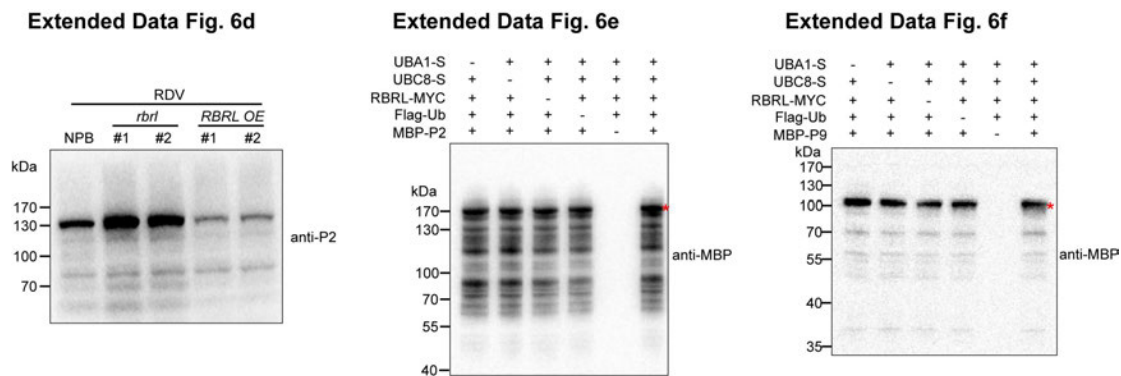
Revised Extended Data Fig. 9c. RSV CP promoted the proteolysis of NINJA3 but not that of NINJA4 by increasing the activity of RBRL. The *in vivo* degradation of OsNINJA3 was conducted by determining the YFP-NINJA3 protein level via coinfiltration experiments with increasing amounts of MYC-CP. Flag-GUSA was used as the internal control. The mRNA expression levels of the target genes *OsNINJA3* and *ACTIN* were analyzed via RT-PCR. The numbers at the top indicate the ratio of the concentrations of *Agrobacterium* used in coinfiltration. YFP-NINJA4 (bottom panel) served as a negative control.

-Extended data Fig 7B

P2 and P9 was degraded by RBRL?

Response: Thank you for your question. Our results indicate that P2 is not degraded by RBRL, as we did not detect ubiquitinated bands of P2 in *RBRL* OE plants.

Additionally, we included P2 and P9 in the ubiquitination assay, and we did not detect any bands corresponding to P2 and P9 ubiquitinated by RBRL (please see Extended Data Fig. 6d-f).



Extended Data Fig. 6d-f. **d**, Immunoblotting analysis of RDV P2 levels in the indicated plants; no ubiquitinated bands of P2 were detected. **e**, Ubiquitination analysis of P2 by RBRL. Bacterial lysates from *E. coli* strains expressing UBA1-S (E1), UBC8-S (E2), Flag-Ub, MBP-P2 and RBRL-MYC or from strains lacking one of these components were analyzed by immunoblotting with an anti-MBP antibody to determine the expression of P2. No ubiquitinated bands of P2 were detected. **f**, Ubiquitination analysis of P9 by RBRL. Bacterial lysates from *E. coli* strains expressing UBA1-S (E1), UBC8-S (E2), Flag-Ub, MBP-P9 and RBRL-MYC or from strains lacking one of these components were analyzed by immunoblotting with an anti-MBP antibody to determine the expression of P9. No ubiquitinated bands of P9 were detected.

Referee #3 (Remarks to the Author): In this paper, the authors present data to provide a mechanistic basis on how RSV CP promotes JA accumulation and induction of AGO18 through MYB transcription factor. Based on the data shown, the authors conclude that RBRL E3 ligase interacts with RSV CP that degrades NINJA3 transcriptional repressor to promote JA responsive genes for antiviral defense. Results presented are interesting but need additional evidence to show that some of the process described occurs during natural RSV infection of rice.

Response: We appreciate the valuable comments and constructive suggestions on our manuscript. We have added additional data to show that some of the processes described here occur during natural RSV infection in rice. Please see our point-by-point responses below.

Specific comments:

1. Authors identified RBRL using CP overexpressing plants through IP-MS. MS methods and data presented lack the standard that is routinely followed. The methods section also only includes description for how they identified RBRL interacting proteins but not CP. Authors state that they identified RBRL and show scores just for CP and RBRL in Fig. 1c table. How did they actually do the MS of IP sample shown in Fig. 1c gel? Is RBRL the only interactor of CP? Similarly for RBRL data in Extended Data Fig. 4B – how were these experiments done? Is NINJA3 the only interactor identified from RBRL IP-MS?

Response: We appreciate the comments and apologize for not providing a detailed description of the IP–MS methods and the raw data in the original manuscript. We have added the detailed protocols for the nuclear–cytoplasmic fractionation IP–MS method used to identify CP-interacting proteins to the “METHODS” section. As shown in Supplementary Tables 1 and 4, RBRL is not the only interactor of CP; similarly, NINJA3 is not the only interactor of RBRL. We focused on RBRL as a potential interactor of CP because RBRL is in the prey lists of both *CP OE* and RSV-infected NPB samples. Moreover, we focused on OsNINJA3 in the prey list of RBRL IP-MS because the NINJA protein has been reported to be an important regulator of JA signaling (Pauwels *et al.*, 2010 Nature).

Reference:

Pauwels, L. *et al.* NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature 464, 788-791 (2010)

METHODS section:

“Nuclear–cytoplasmic fractionation followed by an immunoprecipitation assay

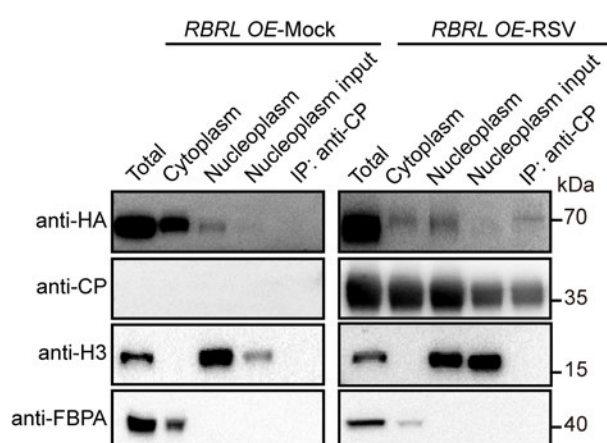
To precisely screen for rice proteins that interact with RSV CP in the nucleus, we performed nuclear–cytoplasmic fractionation followed by immunoprecipitation–mass spectrometry (IP–MS) on RSV-infected NPB and *CP OE* rice, with mock NPB rice used as a control. First, we conducted subcellular fractionation on the rice materials with minor modifications as described previously⁶². The rice materials were harvested and ground into powder in liquid nitrogen at four weeks post RSV infection. The powder (approximately 1 g) was resuspended in Honda buffer (0.4 M sucrose, 2.5% Ficoll, 5% dextran T40, 25 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 0.5% Triton X-100, 0.5 mM PMSF, 10 mM β-mercaptoethanol, RNase inhibitor, and Roche protease inhibitor cocktail) at

a ratio of 2 mL/g. The homogenate was filtered twice through a double-layered Miracloth, and the supernatant was subsequently centrifuged at 1500 *g* for 5 minutes at 4°C. The supernatant was further centrifuged at 10,000 *g* for 10 minutes at 4°C to obtain the cytoplasmic fraction. The pellet containing the nuclear proteins was resuspended in three volumes of nuclear extraction buffer (500 mM KCl, 20 mM Tris HCl pH 8.0, 0.5% Triton X-100, 25% glycerol, 1.5 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, cocktail and 1 mM PMSF) and incubated for 30 minutes at 4°C. Insoluble nuclear residues were then sedimented at 10,000 *g* at 4°C for 10 minutes, and the supernatant was collected for the IP assay. The nuclear extract was diluted with four volumes of dilution buffer (20 mM Tris HCl (pH 8.0), 500 mM KCl, 0.5% Triton X-100, 1 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, cocktail and 1 mM PMSF). The mixture was precleared with protein G for 30 minutes, and then, 1:100 anti-CP antibody was added and incubated for 2 hours, followed by the addition of 1:200 protein G and further incubation for 2 hours. The samples were washed three times with washing buffer (20 mM Tris HCl (pH 8.0), 500 mM KCl, 0.5% Triton X-100, 5% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, cocktail and 1 mM PMSF). Finally, the bead-bound proteins were eluted in 2× Laemmli buffer and subjected to SDS–PAGE. For LC–MS/MS analysis, the gel was subjected to silver staining to identify the candidate CP-interacting proteins.”

2. CP and RBRL interaction: What is the difference between Fig. 1d and Extended Data Fig. 2a? In Extended Data Fig. 2a – the interaction between CP and RBRL seems to be very weak. Since authors have CP and RBRL antibodies; they should use these antibodies to show that this interaction occurs during natural infection of RSV in rice. This is important to put the data in perspective with natural infection in rice.

Response: Thank you for the comments. The difference between Fig. 1d and Extended Data Fig. 2a lies in the beads used for the IP assays. Owing to differences in the affinity of the beads, the strength of the interactions observed in the IP results may vary. Following your suggestion, we used CP antibodies to perform co-IP experiments on *RBRL OE* rice plants naturally infected with RSV. To confirm the specificity of the interactions in the nucleus, we used the nuclear fraction to perform a co-IP assay (please see the revised Extended Data Fig. 5a). RBRL was detected in IP samples via anti-CP antibodies, which was consistent with our previous findings, confirming that CP and RBRL stably interact in the nuclear fraction.

Extended Data Fig. 5a



Extended Data Fig. 5a. CP interacts with RBRL during natural infection of RSV in rice. IP assays were conducted with the nuclear fraction of mock-inoculated and RSV-infected *RBRL OE* plants. FBPA served as a cytoplasmic marker, and histone H3 served as a nuclear marker. Mock-inoculated *RBRL OE* plants served as a negative control.

3. Fig. 2: CP OE lines: How do authors rule out classical CP-mediated resistance (DOI: 10.1073/pnas.89.20.9865) in these experiments?

Response: Thank you for your question. We believe that our study provides a molecular mechanism for elucidating classical CP-mediated resistance. Hayakawa *et al.* (1992) reported that overexpressing the RSV coat protein (CP) in rice increased antiviral immunity (Hayakawa *et al.*, PNAS). However, the underlying mechanism remains unclear. Our studies in 2015 and 2020 further confirmed that CP enhances rice antiviral immunity (Wu *et al.*, eLife; Yang *et al.*, Cell Host & Microbe). Additionally, we revealed that CP mediates antiviral defense by activating the JA signaling pathway, which subsequently induces the expression of the broad-spectrum antiviral component *OsAGO18* (Yang *et al.*, Cell Host & Microbe). In this study, we primarily showed that RSV CP is sensed by an RBR-type E3 ligase, OsRBRL, which subsequently activates the JA pathway. Therefore, this study highlights a molecular mechanism of CP-mediated resistance. Moreover, we believe that additional factors may be required for the CP-mediated antiviral immunity pathway.

References:

- Hayakawa, T. *et al.* Genetically engineered rice resistant to rice stripe virus, an insect-transmitted virus. *Proc. Natl. Acad. Sci. U. S. A.* 89, 9865-9869 (1992).
- Wu, J. *et al.* Viral-inducible argonaute 18 confers broad-spectrum virus resistance in rice by sequestering a host microRNA. *eLife* 4, e05733 (2015).
- Yang, Z. *et al.* Jasmonate signaling enhances RNA silencing and antiviral defense in rice. *Cell Host Microbe* 28, 89-103 (2020).

4. Authors state in lines 188-189: “To understand the molecular function of RBRL, we performed in vivo self-ubiquitination assays (utilizing the plant ubiquitination cascade reconstituted in bacteria)”. If it is done in bacteria – how can this be in vivo? In vivo – in bacteria or in plants? The reference should be provided and methods should clearly described in the methods section on this.

Response: Thank you for the insightful comments and suggestions. We apologize for not describing the methods of the detailed procedures in the original manuscript, and we have added this information to the revised “METHODS” section. Ubiquitination assays were performed according to a previous study (Han *et al.*, 2017 Plant J.). The components required for the ubiquitin cascade reaction were reconstituted in a prokaryotic system (*Escherichia coli*). This reconstitution provides the necessary conditions and environment for the ubiquitination reaction; therefore, this system is referred to as an *in vivo* assay in bacteria but not in plants. We have revised the manuscript to avoid confusing readers.

Reference:

Han, Y. *et al.* Reconstitution of the plant ubiquitination cascade in bacteria using a synthetic biology approach. Plant J. 91, 766-776 (2017).

METHODS section:

Protein expression and ubiquitination assay in *E. coli*

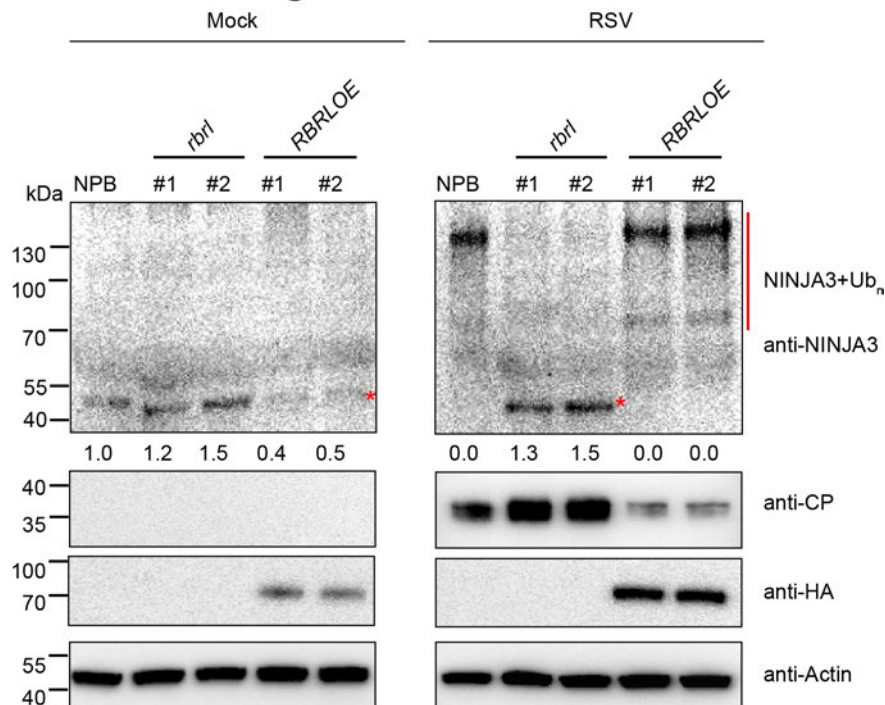
The protein expression and ubiquitination assays conducted in the *Escherichia coli* strain BL21 (DE3) were performed according to previous studies⁴³. Briefly, different combinations of the indicated expression vectors were transformed into the *E. coli* strain BL21 (DE3), and the strains were cultured in Luria–Bertani (LB) liquid medium at 37°C. Then, 0.5 mM IPTG was added to the medium when the absorbance at 600 nm reached 0.4–0.6 to induce the expression of the target proteins. The bacteria were further cultured at 28°C for 10–12 hours and then stored at 4°C overnight. The crude proteins were extracted and analyzed via western blotting with the corresponding antibodies.

5. Fig. 2d-h data: The data presented is hard to interpret. Authors state that RSV CP promotes RBRL degradation. At the same time RSV infection induces RBRL at the RNA and protein level. If this is the case why there is increased RBRL in Fig. 2g when CP is high during virus infection? If CP promotes RBRL activation and degradation, then one would expect to see less RBRL in Fig 2g. Therefore, it is important to show that the ubiquitination of RBRL occurs in vivo in rice and CP promotes its activation

RBRL. Since authors have RBRL antibodies, can they show RBRL is indeed ubiquitinated in rice? Then what is the consequence of this ubiquitination in RSV infected samples? This will provide direct evidence that RBRL targets RSV CP, and this promotes RBRL E3 activity in rice during natural RSV infection.

Response: Thank you for the comments and questions. We found that CP enhanced the E3 ligase activity of RBRL through release of autoinhibition. Therefore, coexpressing RBRL with CP in tobacco or the ubiquitination system induced the ubiquitination of the E3 ligase itself and promoted its degradation. In rice plants, NINJA3 was identified as a substrate of RBRL, and increased E3 ligase activity of RBRL promoted the degradation of its substrate, NINJA3. Similarly, in the *RBRL*-overexpressing lines, the NINJA3 protein had a greater level of ubiquitination upon RSV infection than did the NPB or *rbrl* mutants (please see the revised Extended Data Fig. 9e). We also detected the ubiquitination of RBRL *in vivo* in rice plants through an IP assay using RBRL antibodies and determined the ubiquitination levels of the IP products with ub antibodies (please see **Response Fig. 12**). Our results indicated that RBRL undergoes low-level ubiquitination in rice during RSV infection.

Extended Data Fig. 9e



Extended Data Fig. 9e. Immunoblotting analysis of OsNINJA3 levels in the plants indicated by the asterisk. The red line denotes the ubiquitinated form of NINJA3.

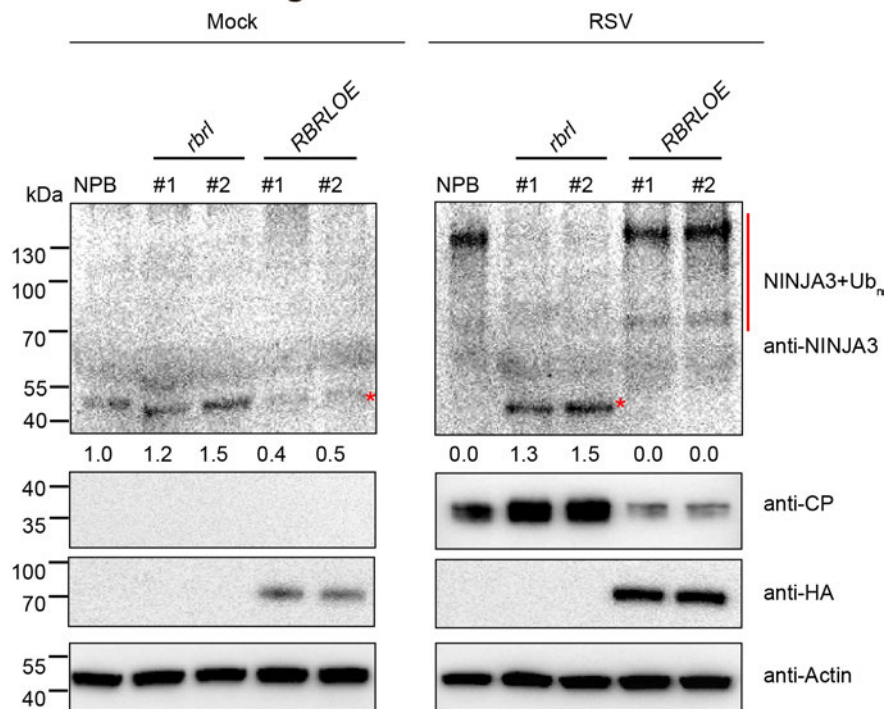
RESPONSE FIG. 12 REDACTED

6. Fig. 4: Authors nicely show that NINJA3 is ubiquitinated during RSV infection using NINJA3 antibodies. Authors could use the same antibodies to show in rice during RSV interaction, NINJA3 indeed interacts with RBRL and this interaction promotes NINJA3 degradation during RSV infection in rice. Similar experiments could be done using *rbrl* and RBRL OE lines. These experiments will demonstrate that in natural infection, NINJA3 is degraded through RBRL.

Response: Thank you for your valuable suggestion. We appreciate your recognition of our findings on NINJA3 ubiquitination during RSV infection. We attempted to perform IP experiments using NINJA3 antibodies, but unfortunately, the NINJA3 antibodies we prepared were not suitable for IP experiments. However, following your suggestion, we used HA-tag antibodies for IP in the NPB and *HA-RBRL OE* lines, which confirmed the interaction between RBRL and NINJA3 in rice during natural infection (please see **Response Fig. 13**). To determine the levels of NINJA3 in the *RBRL OE* and *rbrl* lines, we used NINJA3 antibodies for direct detection. These results were consistent with our previous conclusions: NINJA3 protein levels were significantly increased in the *rbrl* lines under both mock and RSV conditions, whereas NINJA3 levels were lower in the *RBRL OE* lines than in the NPB and *rbrl* lines (please see **Extended Data Fig. 9e**).

RESPONSE FIG. 13 REDACTED

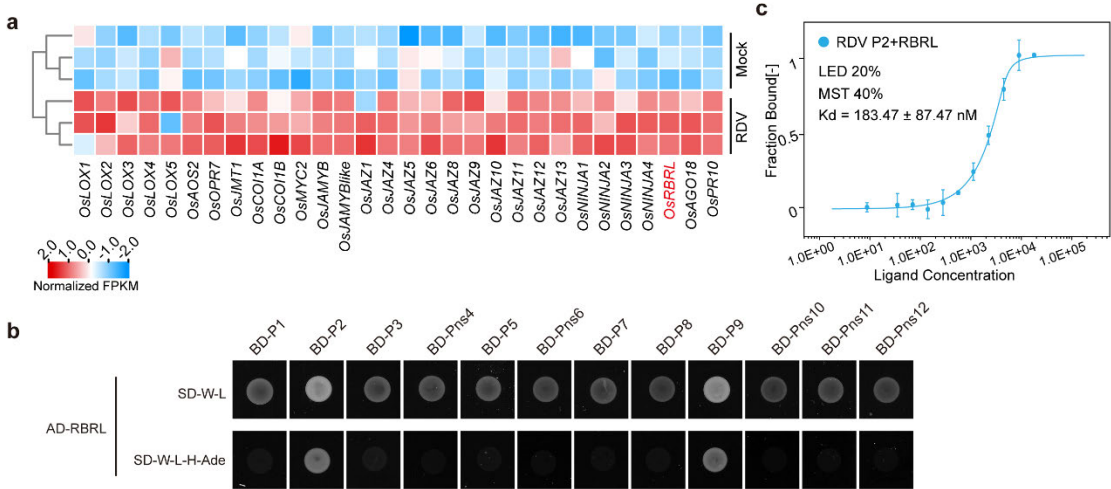
Extended Data Fig. 9e



Extended Data Fig. 9e. Immunoblotting analysis of OsNINJA3 levels in the plants indicated by the asterisk. The red line denotes the ubiquitinated form of NINJA3.

7. Fig. 6: The data shown indicate that RBRL OE lines show enhanced resistance to RDV. Based on this the authors conclude that “RBRL mediates a broad spectrum of natural antiviral defenses”. Difficult to make this conclusion. Is the enhanced resistance due to indirect effect because of increased JA and JA responsive genes in RBRL OE lines? Do RDV P2 and P9 localize to the nucleus? RDV P2 has been shown to hijack auxin signaling and GA, how does this intertwine with JA data shown here?

Response: Thank you for the insightful comments. RDV and RSV are two distinct viruses. RDV belongs to the *Phytoreovirus* genus (Reoviridae), whereas RSV belongs to the *Tenuivirus* genus (Phenuiviridae). The *RBRL OE* lines were resistant to both RDV and RSV, suggesting a broad-spectrum antiviral role of RBRL. As shown in Extended Data Fig. 14a, RBRL and JA signaling genes were also induced by RDV infection. Additionally, metabolomic analysis following RDV infection revealed that RDV infection increases the levels of JA-related metabolites (see Response Fig. 14 below, unpublished data), which suggests that RDV infection can activate the JA signaling pathway. RBRL also interacted with RDV capsid proteins P2 and P9 (Extended Data Fig. 14b and 14c). These results suggest that RBRL might also be involved in regulating rice resistance to RDV by perceiving RDV CPs and triggering JA signaling. We also analyzed the localization of RDV P2 via nuclear–cytoplasmic fractionation experiments and found that RDV P2 and RBRL were colocalized to both the nucleus and the cytoplasm (please see Response Fig. 15). Our previous research demonstrated that RDV inhibits IAA10 in the auxin signaling pathway and hijacks *ent*-kaurene oxidases in the gibberellin biosynthesis pathway. Owing to the complexity of the hormone interaction network during plant host–virus interactions, RDV P2 facilitates viral infection through the inhibition of auxin signaling by hijacking *ent*-kaurene oxidases and IAA10. To counter defense, crop plants employ RBRL to recognize P2, thus triggering the JA signaling pathway to inhibit viral infection. This intricate interplay exemplifies the ongoing evolutionary arms race between viral infection strategies and plant immune responses. As mentioned, we cannot exclude the possibility that indirect effects mediate antiviral defense against RDV because of increased JA- and JA-responsive genes in *RBRL OE* lines.



Extended Data Fig. 14a-c. **a**, RDV infection induces the expression of *RBRL* and activation of the JA pathway. Expression analysis of genes related to JA synthesis and JA

signaling in mock-inoculated and RDV-infected wild-type Zhonghua11 rice plants via transcriptome sequencing data from a previous study. The numbers are the Z scores of the FPKM values for each gene. FPKM, fragments per kilobase of transcript per million. **b**, Analysis of the interactions between RDV proteins and OsRBRL via Y2H assays. OsRBRL interacts with the capsid proteins of RDV. **c**, MST assays showing the binding affinity between RDV P2 N-terminus and OsRBRL. The data are the means $\pm$ SDs. $n = 3$ independent biological samples.

RESPONSE FIG. 14
REDACTED

RESPONSE FIG. 15
REDACTED

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have conducted a substantial effort in an attempt to address all of the comments that I had raised during my first review. This involved a considerable number of additional experimentation, for which I acknowledge the authors. In most cases, the novel experimentation and data were satisfactory in the sense that they filled the caveats spotted and mitigated the inconsistencies put forward. That did not only regard my comments but, as far as I can judge, also most of the comments of the other reviewers. I have a few concerns/comments left, raised by the novel data, which I'd like the authors to address.

Response: We are most grateful to thank you for carefully assessing our newly added data and the detailed suggestions on how to further improve our manuscript. We have thoroughly deliberated your new comments and suggestions, and have done our best to address them. We have provided point-by-point responses to each comment below, along with relevant excerpts for easy review.

1/ The authors have provided many valuable control data, yet in several cases only in 'Response Figures', not included in the manuscript. Particularly for Response Figures #1 and 2, I would like to see these data incorporated in the final manuscript (as ED or SI data).

Response: Thank you for your valuable advice. We have now incorporated Response Figures #1 and #2 into the revised manuscript – please see the revised Extended Data Figures 2, 5 and 8.

2/ Figures ED3 and ED10. It is not clear on which gene sets the GO analysis is performed on. That is the case for 3e, 3j and 10d, which respectively refer to the DEGs shown in 3c, 3h and 10b. In each case it is not clear however whether the GO analysis is for instance done only on the DEG sets in the overlap of the Venn diagrams, or of all DEGs combined or on DEGS of either mock or RSV transcriptomes. This should be indicated in the legend.

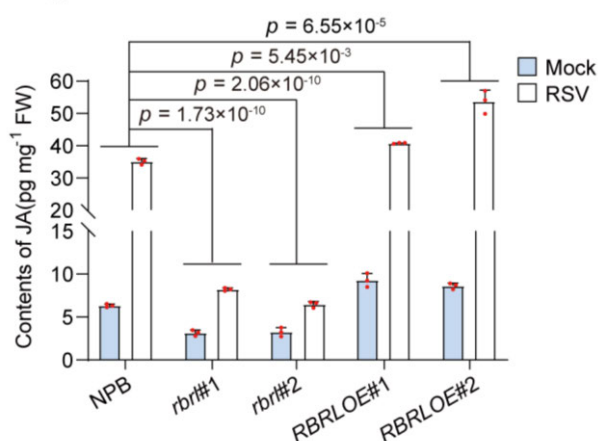
Response: Thank you for the important suggestions. As advised, we consolidated all the DEGs presented in Extended Data Figures 3c, 3h and 10b (which becomes Extended Data 2i, 2m and 7b in the present revision) to conduct the GO analysis shown in Extended Data Figures 3e, 3j and 10d (which becomes Extended Data 2k,

2p and 7d in the present revision), respectively, clarifying the transcriptional effects of *OsRBRL* or *OsNINJA3*. We have revised the legends of the revised Extended Data Figures 2k, 2p and 7d as follows: Extended Data Figures **2k** “Gene Ontology (GO) analysis showing the top 20 enrichment terms of **all** the DEGs shown in **(i)**”, Extended Data Figures **2p** “Gene Ontology (GO) analysis showing the top 20 enrichment terms of **all** the DEGs shown in **(m)**”, and Extended Data Figures **7d** “Gene Ontology (GO) analysis showing the top 20 enrichment terms of **all** the DEGs shown in **(b)**”.

3/ Fig 1m. I agree with the general impressions that increases in JA levels seem less in the *rbrl* backgrounds but proper statistics need to be conducted to verify the significance of the genotype X treatment effect and really support the robustness of the conclusion. Furthermore, on line 123, the term ‘is essential’ should be replaced by ‘contributes’ as there is clearly still a 2-fold increase in JA levels in the *rbrl* background, indicating that other factors/processes contribute as well and RBRL is not the only player.

Response: Thank you for the important suggestions on improving our presentation and description. We have revamped the statistical analysis of Fig. 1m data by performing a two-way analysis of variance (ANOVA). The *p* values for the interactions between genotype and treatment are shown in the revised Fig. 1m. The results confirm the significance of the genotype × treatment effect and firmly support the robustness of the conclusion. We have also changed the term ‘is essential’ to ‘contributes’ in the manuscript, as suggested.

Fig. 1m



Revised Fig. 1m. RBRL contributes to RSV-induced JA accumulation. Quantification of the JA concentrations in the NPB, *rbrl*, and *RBRL* OE plants with or without RSV infection. Rice plants were collected at 3 wpi. Statistical analysis was performed by two-way analysis

of variance (ANOVA). The p values for the interactions between genotype and treatment are shown in the figure.

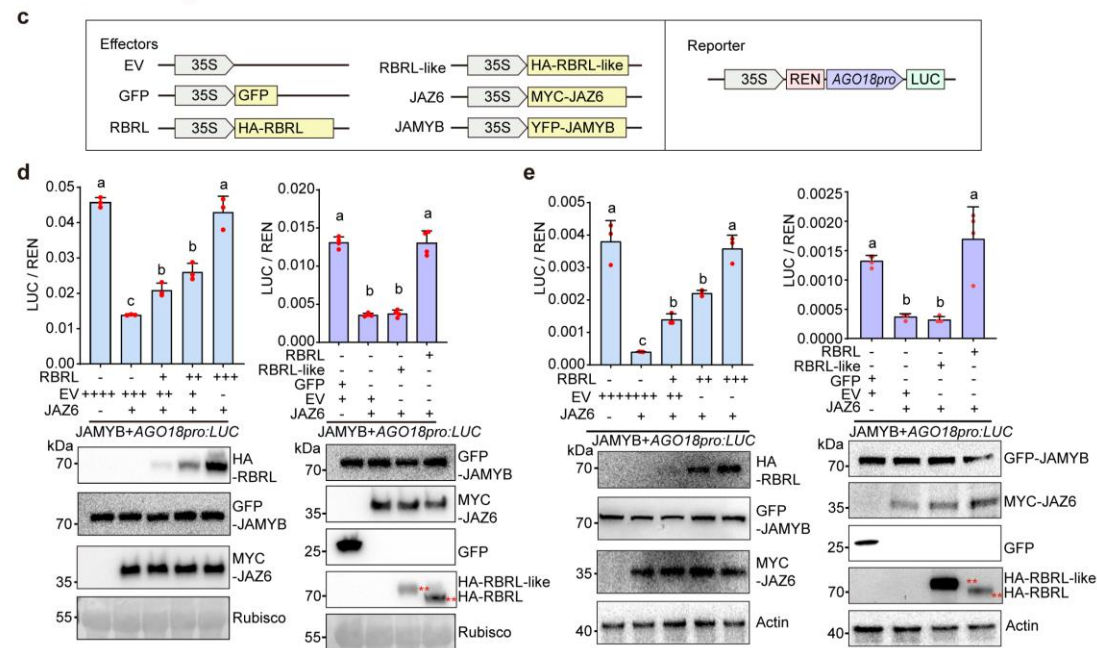
4/ ED Fig 4. I agree the data in the rice protoplast provide results consistent with the hypotheses. Yet, I firmly disapprove the practice of omission of puzzling data. That equals inappropriate scientific conduct in my opinion. The authors thereby also avoid to respond to my concern. Nonetheless, given the substantial efforts done by the authors to address my comments, I tend to agree that the analogous, yet puzzling, findings obtained should perhaps not be seen anymore as something conflicting. Yet, keeping proper scientific conduct in mind, I would suggest to include both the old (bentha leaves) and new (rice protoplast) data in a new ED Fig 4. That will provide future readers a full view on the experimentation conducted by the authors and potential inspiration for (other) researchers, interested in following-up of the findings presented in this manuscript.

Response: We sincerely apologize for the omission referred to, and thank you very much for your understanding. We have now revised the ED Fig. 4b (which becomes Extended Data Fig. 3c-e in the current revision). During the last revision, we reconstructed the phylogenetic tree of NINJAs from rice and other species according to your suggestion. As shown in the Extended Data Fig. 5c, AFP3 from *Arabidopsis* (*Arabidopsis thaliana*) and A0A1S3Z6S5 from tobacco (*Nicotiana tabacum*) belong to a subclade, which is very closely related to the OsNINJA1/2/3 subclade. Hence, it is possible that AFP orthologs of *N. benthamiana* could be targeted by OsRBRL, as you speculated. In addition, a previous study found that the *Arabidopsis afp2* loss-of-function mutant phenotypically resembled that of the *jaz-D* decuple mutant, possibly implicating AFPs involved in regulating JA signaling pathway (Hong *et al.*, 2020, PNAS). These likely explain why the results from *N. benthamiana* leaves and rice protoplasts agreed with each other. As you suggested, we have included the results from both *N. benthamiana* leaves and rice protoplasts in the revised Extended Data Fig. 3c-e to provide readers with a full view on the experimentation.

Reference:

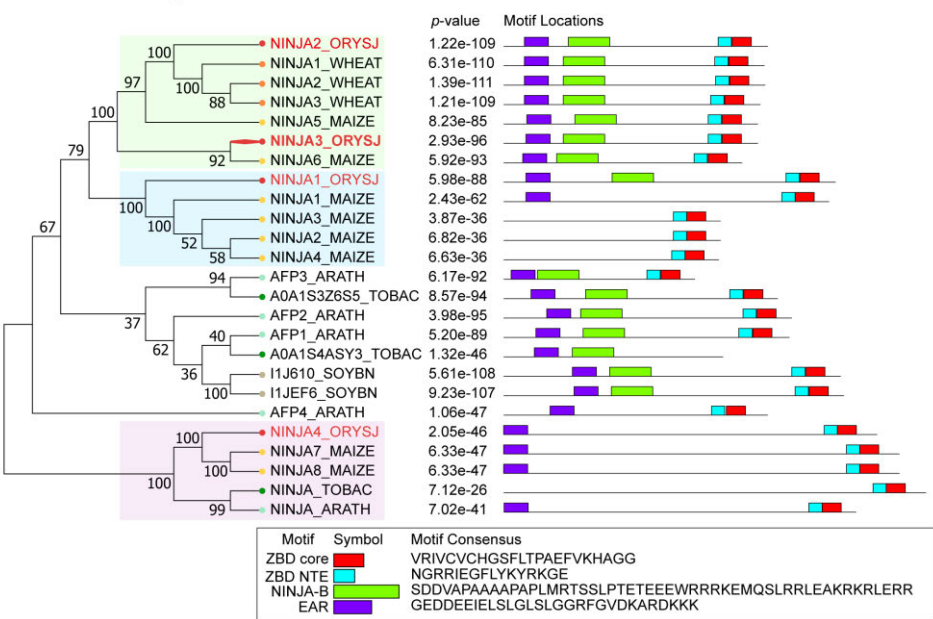
Hong, S. A.-O., *et al.* Heterologous microProtein expression identifies LITTLE NINJA, a dominant regulator of jasmonic acid signaling. *Proc Natl Acad Sci U S A* 117, 26197-26205 (2020).

Extended Data Fig. 3c-e



Revised Extended Data Fig. 3c-e. OsRBRL promotes the transcription of OsAGO18. Dual-luciferase assays in *N. benthamiana* leaves (**d**) and rice protoplasts (**e**). A schematic diagram of the constructs is shown in (**c**). The activities of firefly luciferase (LUC) and Renilla luciferase (REN) were measured sequentially, and the LUC/REN ratio was calculated as the final transcriptional activity. EV: empty vector. Statistical analysis was performed via one-way analysis of variance (ANOVA) with Tukey's multiple comparison test (different letters represent significantly different groups; $p < 0.05$). Immunoblotting analysis of all the proteins in the corresponding dual-luciferase assays were shown in the lower panels.

Extended Data Fig. 5c



Extended Data Fig. 5c. Phylogenetic analysis of NINJA proteins in rice and other

plant species. Rice NINJA proteins are highlighted in red. Conserved motif analysis was performed using the online software program MEME (<http://meme-suite.org/tools/meme>).

5/ ED fig 9e. The findings presented in this panel are puzzling. Why would one actually be able to see poly-ubiquitinated NINJA in RSV-infected rice plants? That is quite unusual, as far as I know, given that poly-ubiquitinated proteins are typical proteasomal targets? Based on the immunoblots shown, it appears even that there is no degradation of NINJA by RBRL in rice whatsoever, but actually full conversion and even stabilization to poly-ubiquitinated NINJA? That would implicate a need for a revisit of the model proposed by the authors? Or is this experiment conducted in the presence of MG132? This is not indicated in the legend and I also do not think it is the case, given one can see degradation of NINJA in mock conditions in the RBRL-OE line. Along those lines, do the authors then observe poly-ubiquitinated NINJA in the experiment shown in ED Fig 9b?

Response: Thank you for your comments. To the best of our understanding, ubiquitination and degradation are two distinct processes, and the rate of ubiquitination does not necessarily correlate to the rate of proteasomal degradation. For instance, the phytochrome interacting factor 1 (PIF1) undergoes rapid ubiquitination and degradation under red light (Shen *et al.*, 2008 Plant Cell). Nonetheless, ubiquitinated PIF1 remains detectable through immunoblotting, even in the absence of proteasomal inhibitor treatment (please see Reference Fig. 1 and 2 excerpted from Shen *et al.*, 2008 Plant Cell and Zhu *et al.*, 2015 Nat. Commun., respectively). Besides, the degradation rate of certain proteins varies under different plant growth conditions (Li *et al.*, 2017 Plant Cell). In our case, viral infections significantly perturbed the normal growth of rice (Fig. 2a, below), which may have influenced the degradation rate of certain proteins.

Proteasome core particle assembly begins with the formation of an α ring, followed by the addition of β subunits (Rousseau and Bertolotti, 2018 Nat. Rev. Mol. Cell Biol.). The antibodies against the α -ring subunit PAG1(PSMA3) have been proven to be an effective tool in dissecting proteasome assembly in both mammals and Arabidopsis (Nandi *et al.*, 1997 EMBO J.; Han *et al.*, 2019 New Phytol.).

To address the concern, we attempted to detect proteasome assembly in mock-inoculated and RSV-infected rice plants using a PAG1 antibody (Han *et al.*, 2019 New Phytol.). For this experiment, we used Arabidopsis proteasomes as the marker (Han *et al.*, 2019 New Phytol.). We observed that protein extracts of the RSV-infected wild-

type NPB plants contained clearly detectable incomplete proteasomes in the native PAGE gel (please see Response Fig. 1 below). This result suggests that the assembly of the proteasomes was compromised by RSV infection. Additionally, RSV CP was found to enhance RBRL activity and promote the RBRL-mediated ubiquitination of NINJA3 (please see Extended Data Fig. 6d below). Finally, we repeated the experiment in Extended Data Fig. 9b (which is Extended Data Fig. 6b in the present revision) and observed the accumulation of ubiquitinated NINJA3 after treatment with MG132 (please see Response Fig. 2 below). This also suggests that inhibiting proteasome activity indeed suppressed the degradation of ubiquitinated NINJA3. Taken together, the difference between ubiquitination and degradation rates may explain why we detected ubiquitinated NINJA3 in RSV-infected NPB but not in mock-treated plants.

References:

- Shen, H., *et al.* Light-induced phosphorylation and degradation of the negative regulator PHYTOCHROME-INTERACTING FACTOR1 from *Arabidopsis* depend upon its direct physical interactions with photoactivated phytochromes. *Plant Cell* 20(6), 1586—1602 (2008).
- Zhu, L., *et al.* CUL4 forms an E3 ligase with COP1 and SPA to promote light-induced degradation of PIF1. *Nat. Commun.* 6, 7245 (2015).
- Li, L., *et al.* Protein degradation rate in *Arabidopsis thaliana* leaf growth and development. *Plant Cell* 29(2), 207—228 (2017).
- Rousseau, A., and Bertolotti, A. Regulation of proteasome assembly and activity in health and disease. *Nat. Rev. Mol. Cell biol.*, 19(11), 697—712 (2018).
- Nandi, D., *et al.* Intermediates in the formation of mouse 20S proteasomes: implications for the assembly of precursor beta subunits. *The EMBO J.* 16(17), 5363—5375 (1997).
- Han, J., *et al.* The $\beta 5$ subunit is essential for intact 26S proteasome assembly to specifically promote plant autotrophic growth under salt stress. *The New Phytol.* 221(3), 1359—1368 (2019).

REFERENCE FIG. 1
REDACTED

REFERENCE FIG. 1
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REFERENCE FIG. 2
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Fig. 2a

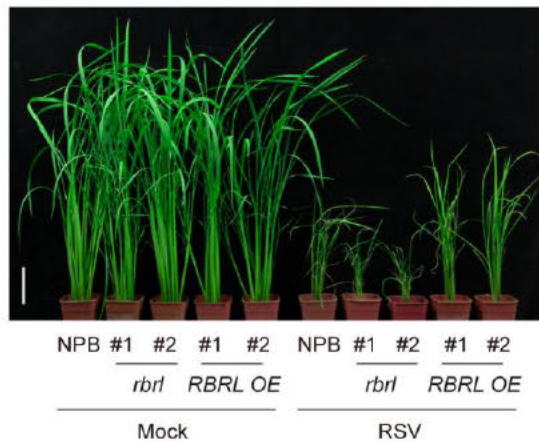
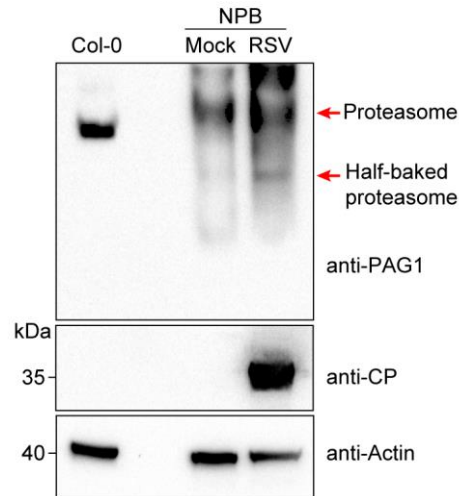


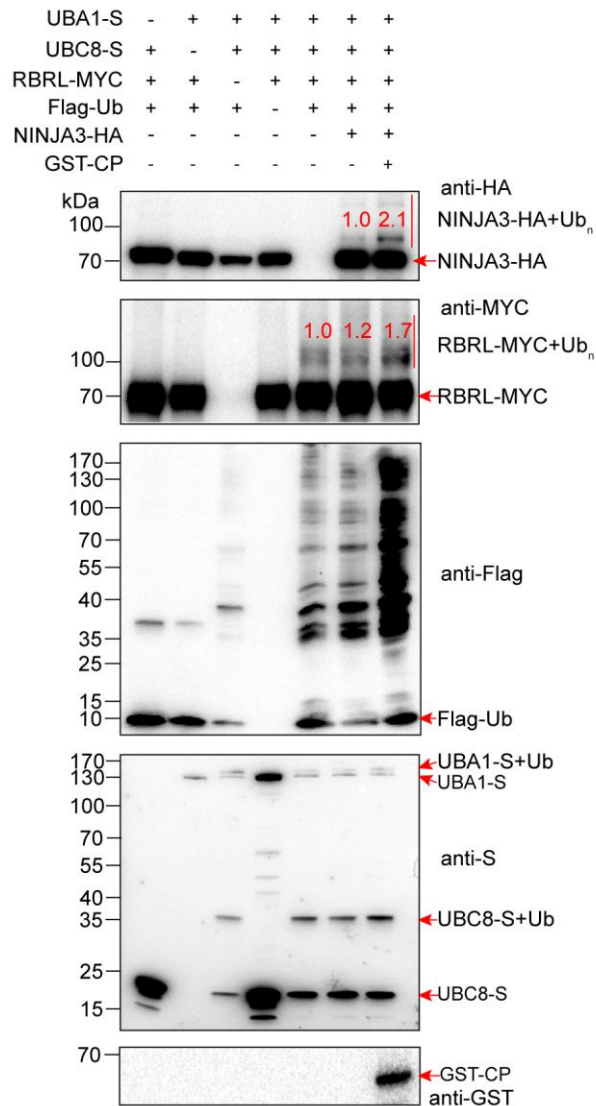
Fig. 2a. *OsRBRL* plays a positive role in rice antiviral defense. Images showing mock-inoculated or RSV-infected NPB, *rbt1*, and *RBRL OE* plants. Scale bar, 10 cm. Images were taken at 4wpi.

Response Fig. 1



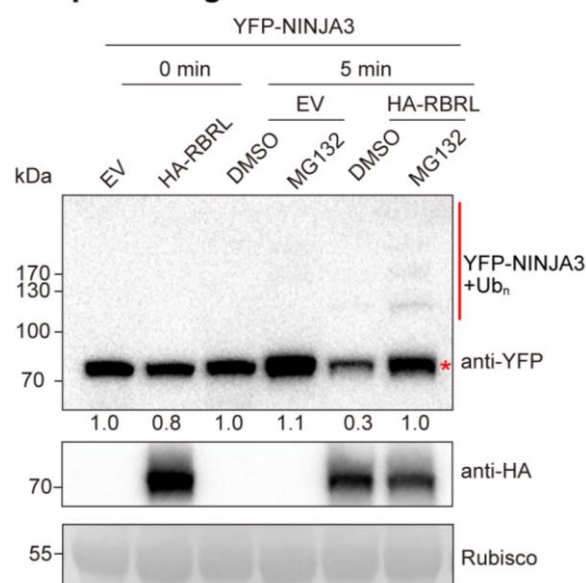
Response Fig. 1. The assembly of proteasome was affected in RSV-infected NPB plants. Proteasome assembly in the *Arabidopsis thaliana* Col-0 ecotype, mock-treated wild-type NPB rice plants and RSV-infected NPB plants were detected with anti-PAG1 antibody via native polyacrylamide gel electrophoresis (PAGE). Proteasome and half-baked proteasome are indicated. Actin was used as the loading control.

Extended Data Fig. 6d



Extended Data Fig. 6d. CP promotes the ubiquitination of NINJA3 by OsRBRL. The bacterial lysates from *E. coli* strains expressing UBA1-S, UBC8-S, Flag-Ub, RBRL-MYC, MBP-NINJA3-HA and GST-CP or from strains lacking one of these components were analyzed by immunoblotting with an anti-HA antibody to detect the ubiquitination of OsNINJA3.

Response Fig. 2



Response Fig. 2. OsRBRL mediates the ubiquitination and degradation of OsNINJA3 through the 26S proteasome. Proteasome inhibitor MG132 was added to the corresponding protein mixture samples at a final concentration of 50 μ M to prevent protein degradation through the 26S proteasome. Dimethyl sulfoxide (DMSO) was used as a control. Asterisk indicated YFP-NINJA3. The red line denotes the ubiquitinated form of NINJA3.

6/ I find the rationales/speculations raised by the authors on my inquiry about the possible molecular mechanism by which CP would stimulate the RBR E3 Ub-ligase activity worthwhile to be included in the discussion in the manuscript. Particularly, because a similar inquiry was raised by another referee as well.

Response: Thank you for your valuable advice. We have added these rationales/speculations to the discussion section in the revised manuscript as follows:

“Results from HHARI and TRIAD proteins, orthologs of OsRBRL, indicate that binding by their interactors could release their autoinhibition by opening the mask of the Ariadne domain on the active site^{39,43}. RSV CP-RBRL interaction promotes RBRL self-ubiquitination and RBRL-mediated ubiquitination of NINJA3 in bacterial systems. Therefore, CP appears to release the autoinhibition of OsRBRL, possibly through interaction, thereby exposing the active site”.

Referee #2 (Remarks to the Author):

The authors have largely addressed my comments with additional experiments, which provide essential support of their conclusions in the revised version.

Response: Thank you for the valuable comments and constructive suggestions on improving our manuscript. After thoroughly considering the two new comments, we have provided point-by-point responses to each comment below, along with relevant excerpts for your convenience.

I have only two comments:

1. CP degrades and also promotes RBRL activation. How the two opposite functions work or are coordinated mechanistically? They should at least discuss this. In addition, the Figure 6 model is not straight and informative.

Response: We sincerely appreciate your comment for providing new perspectives that have enriched our discussion. When reconstituting the ubiquitination cascade in *E. coli*, CP activates RBRL, promotes RBRL self-ubiquitination and RBRL-mediated ubiquitination of NINJA3 (please see Fig 3e and Extended Data Fig. 6d). When co-expressing RBRL and CP in tobacco leaves, CP interacts with RBRL and promotes the degradation of RBRL (please see Fig. 3f below). However, in RSV-infected rice or during co-expression of RBRL with CP and OsNINJA3 in tobacco leaves, CP facilitates the RBRL-mediated degradation of NINJA3 (please see Extended Data Fig. 6c, e below). These results indicated that the extent of CP-mediated degradation and activation of RBRL varies with context. Similar mechanisms have been observed in other E3 ubiquitin ligases, for example, the Keep on Going (KEG) ubiquitin ligase. Under normal conditions, KEG ubiquitinates ABI5, ABF1, ABF3, and other substrates, promoting their degradation, while its self-ubiquitination is relatively weak. When ABA levels increase, KEG's self-ubiquitination also rises, leading to its degradation and a subsequent increase in ABI5 accumulation (Yu *et al.*, 2016 Mol. Plant; Liu *et al.*, 2010 Plant Cell). According to your suggestion, we have added this feature of RBRL in the discussion section in the revised manuscript as follows: "Interestingly, we found that CP-mediated degradation and activation of RBRL varies with context, similar to Keep on Going, a previously reported E3 ubiquitin ligase⁵⁸. When co-expressing RBRL and CP in tobacco leaves, CP primarily promotes the degradation of RBRL. Meanwhile, in RSV-infected rice or co-expressing RBRL with CP and OsNINJA3 in tobacco leaves, CP facilitates the degradation of NINJA3 by RBRL".

In addition, we have simplified the model in revised Extended Data Fig. 10m (see

also below) to make it more precise and concise.

References:

Yu, F., *et al.* Ubiquitin-proteasome system in ABA signaling: from perception to action. *Mol. Plant* 9(1), 21-33 (2016).

Liu, H., and Stone, S. L. Absciscic acid increases Arabidopsis ABI5 transcription factor levels by promoting KEG E3 ligase self-ubiquitination and proteasomal degradation. *Plant Cell* 22(8), 2630-2641(2010).

Fig. 3e

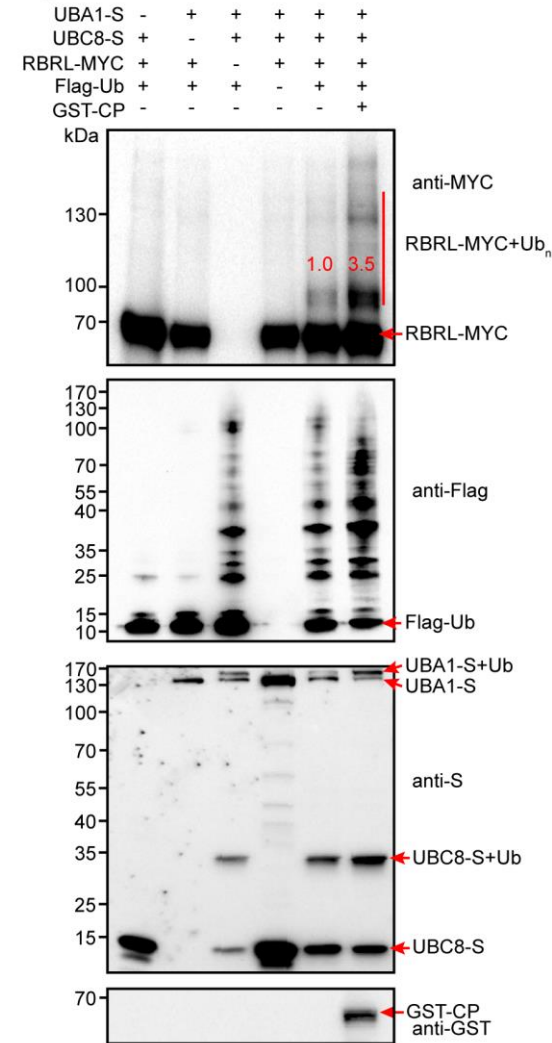


Fig. 3f

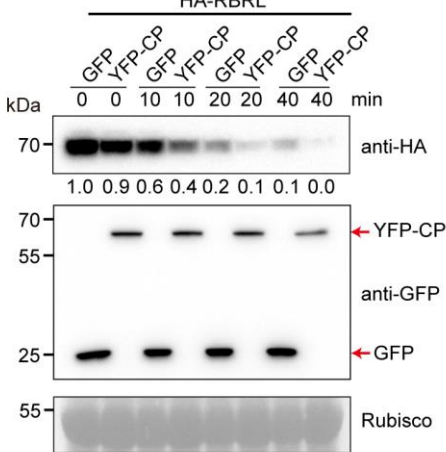


Fig. 3e-f. CP enhances the ubiquitin ligase activity of RBRL. **e**, The bacterial lysates from *E. coli* strains expressing UBA1-S, UBC8-S, Flag-Ub, RBRL-MYC and GST-CP or from strains lacking one of these components were analyzed by immunoblotting with an anti-MYC antibody (upper panel), an anti-Flag antibody (middle panel), an anti-S antibody (lower panel) and an anti-GST antibody (bottom panel). **f**, Time course of CP-promoted OsRBRL self-degradation. The OsRBRL degradation assay was performed by mixing cell extracts from separately infiltrated GFP, YFP-CP and HA-RBRL samples, and GFP served as a negative control. Ponceau S staining (bottom panel) of the Rubisco protein is shown as a loading control.

Flag-GUSA	1	1	1	1	1
YFP-NINJA3	10	10	10	10	10
HA-RBRL	5	5	5	5	5
MYC-CP	0	1	2	5	10
EV	10	9	8	5	0

kDa						
40						anti-MYC
70						anti-GFP
70						anti-HA
100						anti-Flag
70						NINJA3
						ACTIN

Flag-GUSA	1	1	1	1	1
YFP-NINJA4	10	10	10	10	10
HA-RBRL	5	5	5	5	5
MYC-CP	0	1	2	5	10
EV	10	9	8	5	0

kDa						
40						anti-MYC
100						anti-GFP
70						anti-HA
70						anti-Flag
						NINJA4
						ACTIN

	1	2	3	4	5	6	7	8
UBA1-S	-	+	+	+	+	+	+	+
UBC8-S	+	-	+	+	+	+	+	+
RBRL-MYC	+	+	-	+	+	+	+	+
Flag-Ub	+	+	+	-	+	+	+	+
NINJA3-HA	-	-	-	-	-	+	+	+
GST-CP	-	-	-	-	-	-	-	+

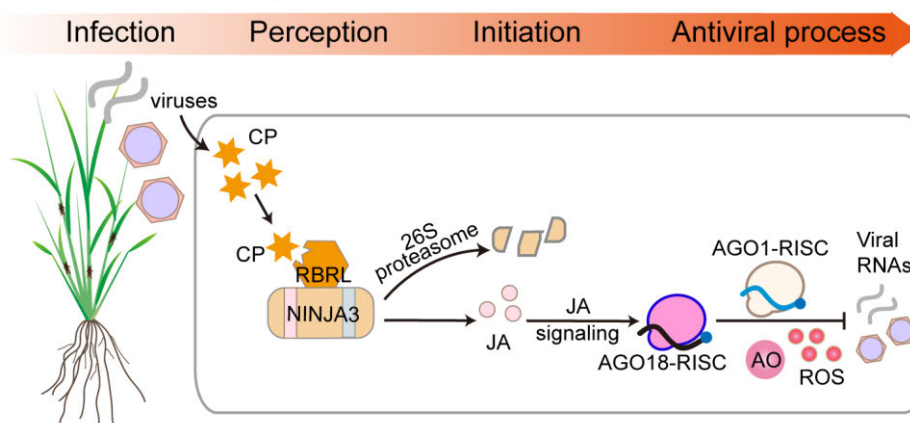
Western blot analysis of NINJA3 ubiquitination. The figure consists of five panels. Panel 1: anti-HA blot showing NINJA3-HA ubiquitination (Ub_n) in lanes 7 and 8, with molecular weight markers at 100 and 70 kDa. Red arrows point to bands at 1.0, 2.1, and 2.1 kDa. Panel 2: anti-MYC blot showing RBRL-MYC ubiquitination (Ub_n) in lanes 7 and 8, with molecular weight markers at 100 and 70 kDa. Red arrows point to bands at 1.0, 1.2, and 1.7 kDa. Panel 3: anti-Flag blot showing Flag-Ub ubiquitination in lanes 7 and 8, with molecular weight markers from 10 to 170 kDa. Red arrows point to bands at 15, 35, 40, 55, 70, 100, 130, and 170 kDa. Panel 4: anti-S blot showing UBA1-S and UBC8-S ubiquitination in lanes 7 and 8, with molecular weight markers from 15 to 170 kDa. Red arrows point to bands at 15, 25, 35, 40, 55, 70, 100, 130, and 170 kDa. Panel 5: GST-CP and anti-GST blot showing GST-CP ubiquitination in lane 8, with a molecular weight marker at 70 kDa. Red arrows point to bands at 70 and 170 kDa.

[illegible]

13/23

OsNINJA3 was conducted by determining the YFP-NINJA3 protein level via co-infiltration experiments with increasing amounts of MYC-CP. Flag-GUSA was used as the internal control. The mRNA expression levels of the target genes *OsNINJA3* and *ACTIN* were analyzed via RT-PCR. The numbers at the top indicate the ratio of the concentrations of *Agrobacterium* used in co-infiltration. YFP-NINJA4 (bottom panel) served as a negative control. **d**, CP promotes the ubiquitination of NINJA3 by OsRBRL. The bacterial lysates from *E. coli* strains expressing UBA1-S, UBC8-S, Flag-Ub, RBRL-MYC, MBP-NINJA3-HA and GST-CP or from strains lacking one of these components were analyzed by immunoblotting with an anti-HA antibody to detect the ubiquitination of OsNINJA3. **e**, Immunoblotting analysis of OsNINJA3 levels in the plants indicated by the asterisk. The red line denotes the ubiquitinated form of NINJA3.

Extended Data Fig. 10m



Extended Data Fig. 10m. A proposed working model for initiation of the antiviral defense via RBRL. Upon RSV infection, CP is perceived by E3 ligase OsRBRL to promote the degradation of OsNINJA3 orchestrated by the ubiquitination activity of OsRBRL. The degradation of OsNINJA3, a negative regulator of the JA signaling pathway, activates a COI1-independent JA signaling pathway, leading to increased JA biosynthesis and upregulation of OsAGO18 expression. The elevated JA level subsequently induces COI1-dependent JA signaling cascades further promoting the expression of OsAGO18. The increased expression of OsAGO18 enhances the host antiviral defense both by alleviating the repression of AGO1-mediated antiviral RNA silencing and promoting the upregulation of ROS levels by AO, as previously reported.

2. I noticed that the master JA signaling suppressors, JAZs, were also strongly induced in Extended Figure 14a, I am wondering whether JAZs are targeted by CP-RBRL and/or how JAZ are actively involved in the pathway.

Response: Thank you for your comments. We were also curious whether JAZs are the targets of RBRL since JA signaling is activated in *RBRL* overexpression rice lines.

However, JAZs were not detected in the IP-MS analysis with OsRBRL as the bait (refer to Supplementary Table 4). To further determine whether JAZs are targeted by RBRL, we have conducted semi-*in vivo* protein degradation assays using JAZ6, which is known to be involved in the JA-AGO18 antiviral signaling pathway (Yang et al., 2020 Cell Host Microbe) and its expression level was strongly induced in Extended Data Fig. 10a. *YFP-JAZ6*, *HA-RBRL* and *GFP* were separately expressed in tobacco leaves via agroinfiltration, and the samples were subsequently mixed for degradation assays. The results showed that the degradation rate of YFP-JAZ6 remained consistent whether it was incubated with HA-RBRL or GFP (please see Response Fig. 3 below). These results suggest that RBRL does not influence the stability of JAZ6. This is probably because JAZ is primarily degraded by the COI1 pathway as previously reported (Thines *et al.*, 2007 Nature), and the increased expression of JAZs occurs due to elevated levels of JA. This rise in JA levels triggers a negative feedback regulation within the JA signaling pathway (Hickman *et al.*, 2017 Plant Cell).

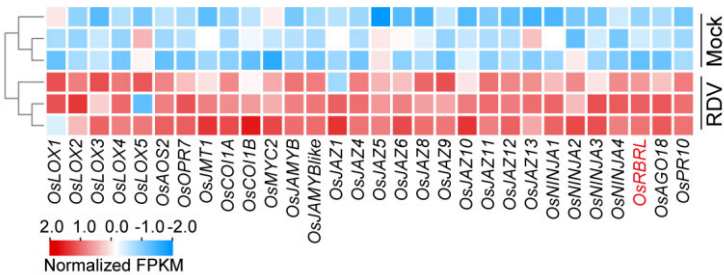
References:

Yang, Z., *et al.* Jasmonate signaling enhances RNA silencing and antiviral defense in rice. *Cell Host Microbe*, 28(1), 89–103 (2020).

Thines, B., *et al.* JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature*, 448(7154), 661–665 (2007).

Hickman, R., *et al.* Architecture and dynamics of the jasmonic acid gene regulatory network. *Plant Cell*, 29(9), 2086–2105 (2017).

Extended Data Fig. 10a



Extended Data Fig. 10a. The expressions of JA-responsive genes were upregulated upon RDV infection. Expression analysis of genes related to JA synthesis and JA signaling in mock-inoculated and RDV-infected wild-type Zhonghua11 rice plants via transcriptome sequencing data from a previous study. The numbers are the Z scores of the FPKM values for each gene. FPKM, fragments per kilobase of transcript per million.

RESPONSE FIG. 3 REDACTED

Referee #3 (Remarks to the Author):

In the revised version, the authors have performed additional experiments and addressed many of the issues raised in the previous review cycle.

Response: We appreciate your valuable comments and constructive suggestions for improving our manuscript. After carefully considering the two new comments, we have provided point-by-point responses to each below, along with relevant excerpts for your convenience.

One issue that still has some issue is the ubiquitination of OsNINJA3 by OsRBRL in vivo during virus infection that the authors should provide explanation. The extended Data Fig. 9e: Overall the top blots are grainy and overexposed compared to other blots in 9e. In the mock-treated RBRL OE, NINJA3 is very low. One could argue that this is due to RBRL-mediated degradation. However, the ubiquitinated form of NINJA3 is not present in these lanes. Also, a high molecular weight protein band present in RSV infected samples is absent in mock with RBRL OE. In RSV infected NPB and RBRL OE – typically ubiquitination should have some laddering but authors detected a high

molecular weight band. Furthermore, the lower bands are absent in the NPB lane with RSV. Authors should explain what these results imply in terms of NINJA3 degradation by OsRBRL.

Response: Thank you for your comments. The OsNINJA3 in the top blots of Extended Data Fig. 9e (which is Extended Data Fig. 6e in the present revision) was detected using a polyclonal antibody against NINJA3 generated in this study. In contrast, other blots were detected with commercialized monoclonal antibodies (anti-HA and anti-Actin) or an anti-CP monoclonal antibody provided by other research groups (Fu *et al.*, 2018 Mol. Plant). The differences in antibody titers and expression levels of various proteins may account for the grainy and overexposed appearance of the top blots compared to the others.

Ubiquitination assay in Extended Data Fig. 6d suggested that RSV CP enhances RBRL activity and promotes the RBRL-mediated ubiquitination of NINJA3. This may explain why detecting ubiquitinated NINJA3 is easier in RSV-infected plants than that in mock-treated plants. Moreover, to the best of our understanding, ubiquitination and degradation are two distinct processes, and the rate of ubiquitination does not necessarily correspond with the rate of proteasomal degradation. The degradation rate of certain proteins correlates with the growth conditions of plants (Li *et al.*, 2017 Plant Cell). In our case, RSV infections significantly disturb the normal growth of rice (please see Fig. 2a) and thus may affect the 26S proteasome assembly and degradation of proteins.

To further test our hypothesis, we detected proteasome assembly in both mock-treated and RSV-infected NPB plants using the antibody against the α -ring subunit PAG1 (PSMA3), which has been proven to be an effective tool in examining proteasome assembly in both mammals and Arabidopsis (Nandi *et al.*, 1997 EMBO J.; Han *et al.*, 2019 New Phytol.). Arabidopsis proteasome was also detected as a positive control. The result showed that the RSV-infected wild-type NPB plant displayed a clear half-baked proteasome band in the native PAGE gel (please see Response Fig. 4 below). This result suggests that proteasome assembly in RSV-infected NPB plants was affected compared to mock-treated NPB plants.

Taken together, the discrepancy between ubiquitination and degradation rates may explain why we detected ubiquitinated NINJA3 in RSV-infected NPB but not in mock-treated plants. The absence of the lower bands in the NPB-RSV and *RBRL OE*-RSV lanes in Extended Data Fig. 9e (which is Extended Data Fig. 6e in the present revision) suggested that almost all NINJA3 proteins in RSV-infected NPB and *RBRL*

OE plants were converted into ubiquitinated NINJA3. This is consistent with the enhanced ubiquitination of NINJA3 mediated by RBRL upon RSV infection and irregular proteasome assembly in RSV-infected NPB plants.

References:

Fu, S., *et al.* Rice stripe virus interferes with S-acylation of remorin and induces its autophagic degradation to facilitate virus infection. *Mol. Plant* 11, 269—287 (2018).

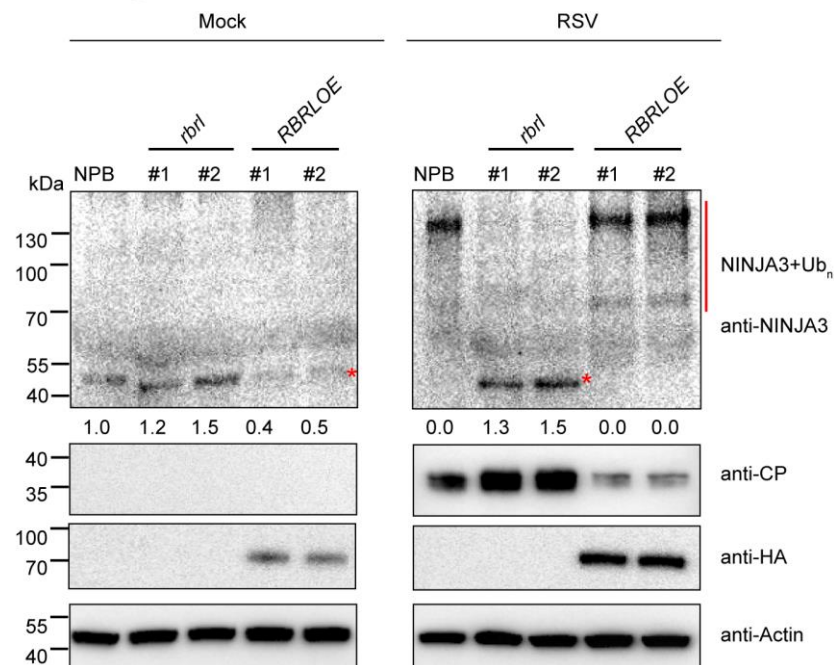
Li, L., *et al.* Protein degradation rate in *Arabidopsis thaliana* leaf growth and development. *Plant Cell* 29(2), 207—228 (2017).

Rousseau, A., and Bertolotti, A. Regulation of proteasome assembly and activity in health and disease. *Nat. Rev. Mol. Cell biol.*, 19(11), 697—712 (2018).

Nandi, D., *et al.* Intermediates in the formation of mouse 20S proteasomes: implications for the assembly of precursor beta subunits. *The EMBO J.* 16(17), 5363—5375 (1997).

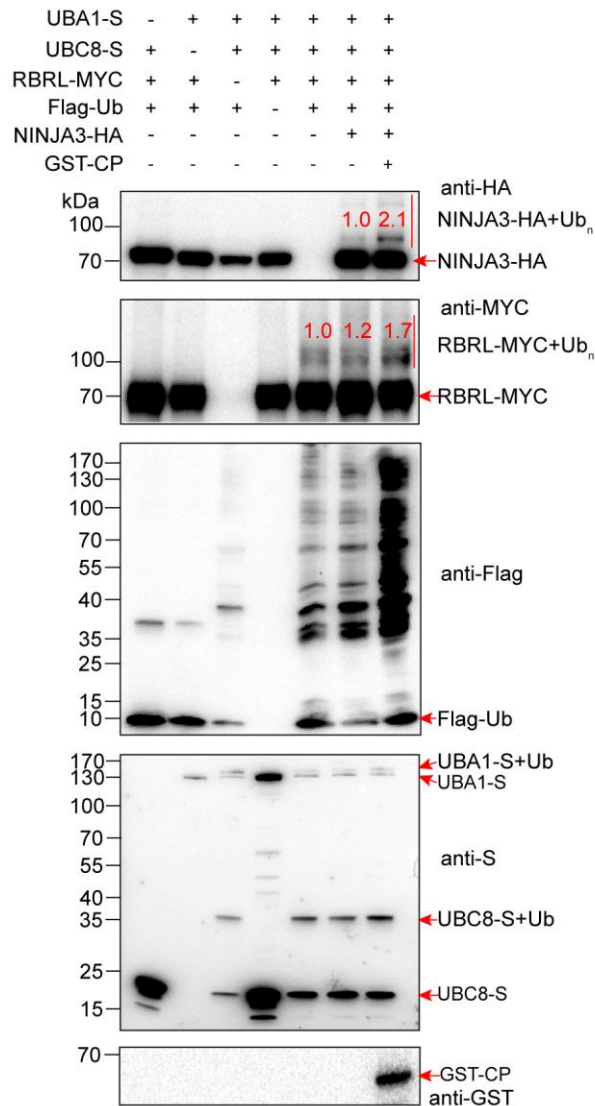
Han, J., *et al.* The $\beta 5$ subunit is essential for intact 26S proteasome assembly to specifically promote plant autotrophic growth under salt stress. *The New Phytol.* 221(3), 1359—1368 (2019).

Extended Data Fig. 6e



Extended Data Fig. 6e. NINJA3 protein levels are significantly greater in *rbrl* mutants than in NPB and RBRL OE plants with or without RSV infection. Immunoblotting analysis of OsNINJA3 levels in the plants indicated by the asterisk. The red line denotes the ubiquitinated form of NINJA3.

Extended Data Fig. 6d



Extended Data Fig. 6d. CP promotes the ubiquitination of NINJA3 by OsRBRL. The bacterial lysates from *E. coli* strains expressing UBA1-S, UBC8-S, Flag-Ub, RBRL-MYC, MBP-NINJA3-HA and GST-CP or from strains lacking one of these components were analyzed by immunoblotting with an anti-HA antibody to detect the ubiquitination of OsNINJA3.

Fig. 2a

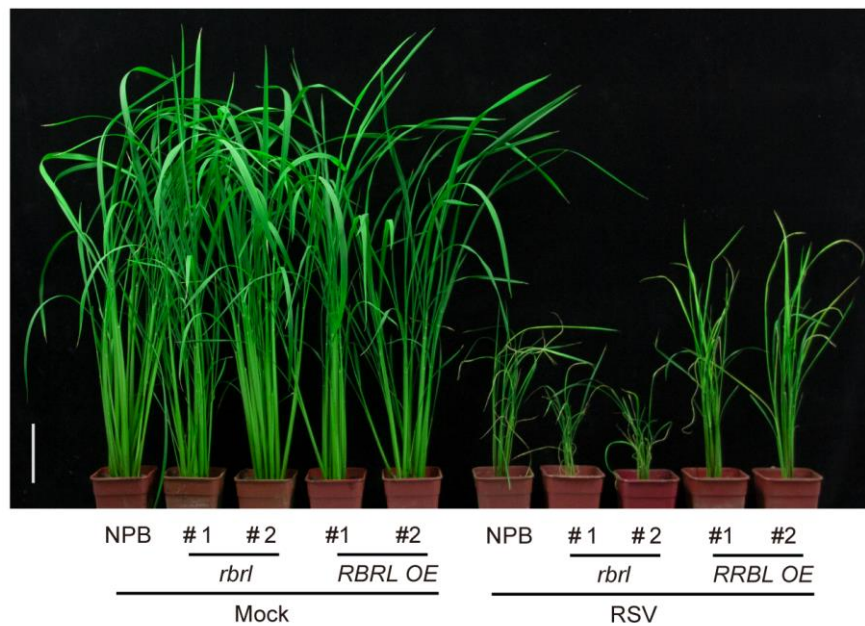
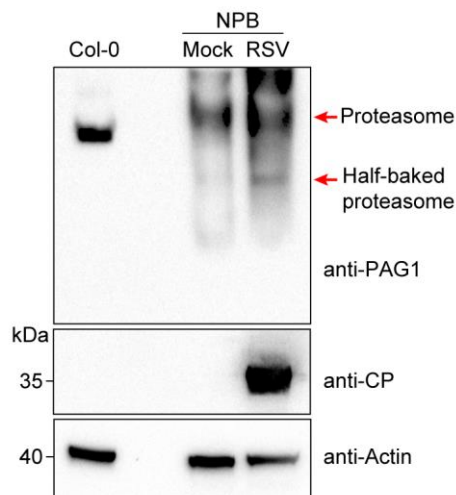


Fig. 2a. *OsRBRL* plays a positive role in rice antiviral defense. Images showing mock-inoculated or RSV-infected NPB, *rbtl*, and *RBRL OE* plants. Scale bar, 10 cm. Images were taken at 4wpi.

Response Fig. 4



Response Fig. 4. The assembly of proteasome was affected in RSV-infected NPB plants. Proteasome assembly in the *Arabidopsis thaliana* Col-0 ecotype, mock-treated wild-type NPB rice plants and RSV-infected NPB plants were detected with anti-PAG1 antibody via native polyacrylamide gel electrophoresis (PAGE). Proteasome and half-baked proteasome are indicated. Actin was used as the loading control.

Response Fig. 12: Not sure how authors conclude that RBRL is self-ubiquitinated in NPB infected with RSV. There is increased accumulation in RSV treated but there is no ubiquitinated laddering one should observe if it is ubiquitinated.

Response: Thank you for your comments. The experiments showing that CP promotes the self-ubiquitination of RBRL were conducted when over-expressing RBRL in *E. coli* (please Fig. 3e). As the expression levels of RBRL in rice plants are relatively low, we suspect that the extent of RBRL ubiquitination is weak in NPB wild-type. Additionally, RBRL is more stable than the mutant form without the NTD and Ariadne domains, and the conserved cysteine residue (C305) in the RING2 domain is essential for the activity of RBRL (please see Fig. 3c, d). These results indicated that the natural state of RBRL is partially autoinhibited, as reported for homologs (Duda *et al.*, 2013 Structure; Spratt *et al.*, 2014 Biochem. J.). Therefore, detecting the ubiquitination of RBRL in mock-inoculated NPB plants is challenging. Although RSV CP activates RBRL and promotes RBRL self-ubiquitination in bacterial systems, in RSV-infected rice, CP facilitates the RBRL-mediated ubiquitination and degradation of NINJA3 (please see Fig. 3e and Extended Data Fig. 6e). These results indicated that RBRL ubiquitinates itself or its substrate OsNINJA3 varies with context, similar to Keep on Going (KEG), a previously reported E3 ubiquitin ligase (Yu *et al.*, 2016 Mol. Plant; Liu *et al.*, 2010 Plant Cell). Therefore, we suspect that the extent of RBRL ubiquitination is weak even in RSV-infected plants, which would explain why it was undetectable in Response Fig. 12. However, we repeated Response Fig. 12 using a new antibody specifically recognizes poly-ubiquitin provided by other research groups (Yu *et al.*, 2016 Mol. Plant). The results suggested that RBRL is ubiquitinated in NPB plants, and RSV infection enhanced its ubiquitination (please see Response Fig. 5 below).

References:

- Duda, David M. *et al.* Structure of HHARI, a RING-IBR-RING ubiquitin ligase: autoinhibition of an Ariadne-family E3 and insights into ligation mechanism. Structure 21, 1030-1041 (2013).
- Spratt, Donald E., Walden, H. & Shaw, Gary S. RBR E3 ubiquitin ligases: new structures, new insights, new questions. Biochem. J. 458, 421-437 (2014).
- Yu, F., *et al.* Ubiquitin-proteasome system in ABA signaling: from perception to action. Mol. Plant 9(1), 21-33 (2016).
- Liu, H., and Stone, S. L. Abscisic acid increases Arabidopsis ABI5 transcription factor levels by promoting KEG E3 ligase self-ubiquitination and proteasomal degradation. Plant Cell 22(8), 2630-2641(2010).
- Yu, F., *et al.* ESCRT-I component VPS23A affects ABA signaling by recognizing ABA receptors for endosomal degradation. Mol. Plant, 9(12), 1570–1582 (2016).

Fig. 3c

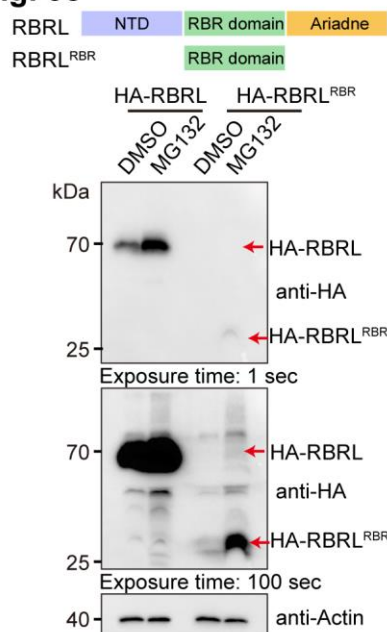


Fig. 3d

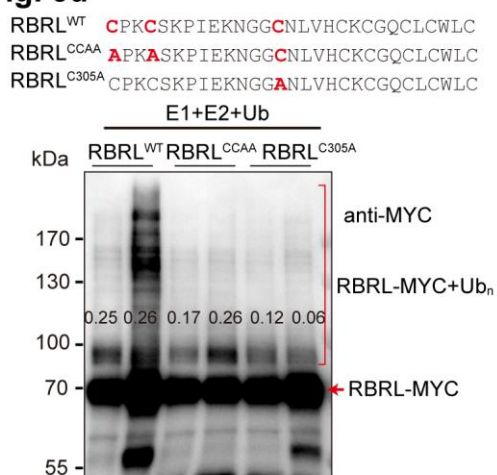


Fig. 3e

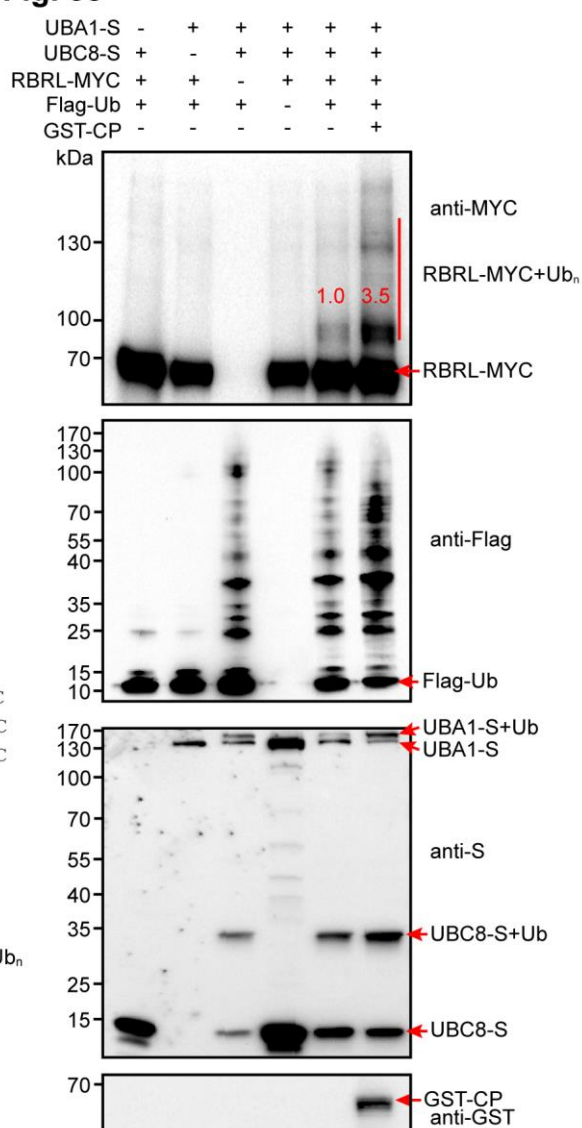
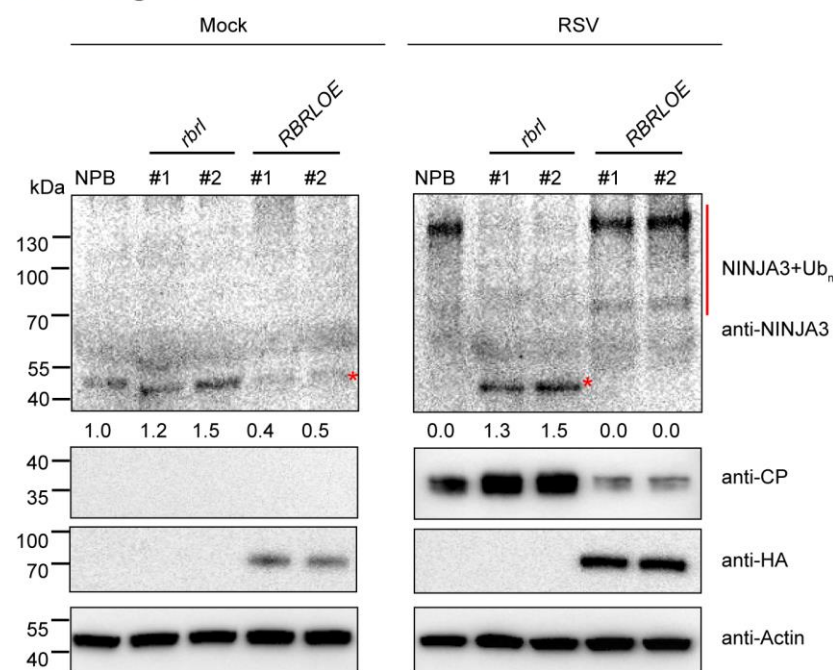


Fig. 3 c-e. Natural state of RBRL is partially autoinhibited and CP enhances the ubiquitin ligase activity of RBRL. **c**, Effect of MG132 on the stability of the full-length OsRBRL and the RBR domain of OsRBRL. Actin served as sample processing controls. **d**, Autoubiquitination of OsRBRL and its corresponding point mutants. The RBRL-MYC+Ub_n/RBRL-MYC ratios are listed. **e**, The bacterial lysates from *E. coli* strains expressing UBA1-S, UBC8-S, Flag-Ub, RBRL-MYC and GST-CP or from strains lacking one of these components were analyzed by immunoblotting with an anti-MYC antibody (upper panel), an anti-Flag antibody (middle panel), an anti-S antibody (lower panel) and an anti-GST antibody (bottom panel).

Extended Data Fig. 6e



Extended Data Fig. 6e. NINJA3 protein levels are significantly greater in *rbr1* mutants than in NPB and *RBRLOE* OE plants with or without RSV infection. Immunoblotting analysis of OsNINJA3 levels in the plants indicated by the asterisk. The red line denotes the ubiquitinated form of NINJA3.

RESPONSE FIG. 5 REDACTED

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have addressed all of my remaining concerns adequately. I particularly like to acknowledge them for their answers and efforts to respond to my comments #4 and #5.

As in the previous review round, I find the response figures #1 and #2 very informative (and convincing), so I would encourage to include these as well in the final manuscript.

Response: We are very grateful for your careful assessment of our newly added data and for your valuable advice on further improving our manuscript. We wish to participate in transparent peer review with redacted information mentioned in the cover letter since those results are related to our other unpublished studies. We have now incorporated Response Figures #1 and #2 into the revised manuscript; please see the revised Extended Data Figures 6b and 6f.

Referee #2 (Remarks to the Author):

I appreciate the efforts of the authors to address my concerns raised during the second round of review. I now agree that their conclusions are well supported. I have no more concern.

Response: We greatly appreciate your thorough evaluation of our newly added data and your valuable suggestions for enhancing our manuscript. We wish to participate in transparent peer review with redacted information mentioned in the cover letter since those results are related to our other unpublished studies.

Referee #3 (Remarks to the Author):

The authors have addressed the issues raised in the last review.

Response: We sincerely appreciate your meticulous evaluation of our newly incorporated data and your insightful advice on improving our manuscript. We wish to participate in transparent peer review with redacted information mentioned in the cover letter since those results are related to our other unpublished studies.