Peer Review File

Manuscript Title: Activation of TIR signaling boosts pattern-triggered immunity

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

The plant immune system is classically divided into two pathways: pattern- and effector-triggered immunity (PTI and ETI, respectively). While long thought to be distinct, recent work has increasingly revealed overlap/interplay between PTI and ETI. In the current manuscript, Tian, Chen et al report a link between PTI and TIR-NLR (TNLR) signaling machinery. The authors arrived at this connection following observation that SNIPER-OX lines, which have reduced NLR accumulation, are compromised in resistance to Pto hrcC-. From here, the authors characterized PTI responses in TNLR signaling components. The work is generally of high quality, and the conclusions follow from the data for the most part. The work is certainly of broad interest, and thus may be suitable for publication in a journal such as Nature; however, significant issues must be addressed, which are outlined below.

1. While the authors propose a model in which TNLR components regulate PTI signaling, the current data are rather consistent with a model in which TNLR signaling regulate pattern-induced SA accumulation/signaling during PTI. To broaden their conclusions, the authors should test other classical (earlier) PTI outputs, e.g. ROS production, MAPK activation, ethylene production. This is particularly relevant, as a recent bioRxiv paper from the Nurnberger lab (Pruitt et al., 2020) reported that these (earlier) responses depend on TNLR signaling. This would also help resolving the current apparent contradiction between both studies on the generality of these findings for PTI signaling (triggered by both RLKs or RLPs), or specifically downstream of RLPs (as proposed by Pruitt et al., 2020).

2. Also related to this other recent work, the authors there also uncovered the connection between EDS1/PAD4 and PTI, specifically downstream of nlp20 perception. How do the authors explain the conflicting results regarding a direct association between these components and SOBIR1? The authors should address this discrepancy.

3. It is unclear to me why the authors focused exclusively on TNLRs in their analyses. SNIPER-OX lines have reduced levels of both TNLRs and CNLRs. Did the authors ever test whether any PTI outputs also depend on CNLR machinery? Did the authors test ndr1 mutants, for example?

4. Also, it is unclear how the authors decided to focus on the three TIR genes analyses out of the many regulated by flg22 and/or nlp20 treatment.

5. The authors identify the RLCK-VII isoforms PCRK1/2 and PBL19/20 as required for nlp20 responses. The reasoning behind testing just these RLCK-VIIs is unclear, as other members of this family such as BIK1 and PBL1 are extremely-well characterized as functioning downstream of multiple PRRs. Furthermore, PCRK1/2 and PBL19/20 are all members of the RLCK-VII-4 subfamily, which was previously reported to regulate MAPK activation downstream of multiple PRRs (Bi et al 2018 Plant Cell). Did the authors test MAPK activation?

6. Related to the above, Pruitt et al. (bioRxiv) identified a distinct subfamily of RLCK-VIIs, including PBL30/CST as functioning specifically in nlp20 signaling following a screen of many PBL mutant lines. Have the authors directly compared mutants in these different PBLs (all publicly available, cf Rao et al., Plant Physiology 2018) in terms of early (e.g. ROS) and late (e.g. SA accumulation/immunity/gene expression) PTI outputs?

7. The identification of TIR genes as specifically induced (Table S1) seems odd and possibly cherry-picked. What about CNLRs? Are TIR domain genes specifically enriched in induction?

8. It is not clear what the relevance of OX assays with TIR or TNLRs (Fig 3) inducing cell death is to the manuscript, as this is long known and characterized

9. Similarly, what is the specific relevance of the calcium channel blocker expts in Fig 3?

10. It appears that all the experiments with the RLCK mutants were performed with nlp20 only - what about flg22? Did the authors ever test this?

11. It is not always clear how statistics were performed – for example in Fig 1F, it appears that the basal levels of SARD1 expression is reduced in SNIPER-OX lines, but this is not indicated by the statistics. This is a common issue throughout the manuscript (see e.g. Fig 2E), and should be clarified.

Referee #2 (Remarks to the Author):

The authors report that overexpression in Arabidopsis of SNIPER1, an E3 ligase, compromises pattern-triggered immunity (PTI) as judged by the plant response to two pathogens and the elicitors flg22 and nlp20; the latter peptide activates the LRR-RP RLP23. In an earlier paper it was shown that mutation of SNIPER1 (and SNIPER2) in Arabidopsis causes EDS1-dependent lethality and that overexpression of SNIPER1 leads to reduced levels of sensor NLR proteins, including TNLs. They therefore conclude that the reduced accumulation of NLR proteins in SNIPER1 overexpressing lines is the cause of compromised PTI in these plants. I think an equally likely possibility is that overexpression of SNIPER1 causes reduced levels of some key proteins involved in PTI and I don't see any evidence to refute that possibility in the paper. If that is the case, then almost all of their subsequent observations, which they attribute to 'reduced TNL signaling' could simply be due to direct effects of SNIPER1 on one or more PTI components. Note that the observation that certain TNLs cause cell death when overexpressed is not surprising since that has been reported previously. They also report that RLCKs PBL19/20 and PCRK1/2 play important roles in RLP23-mediated PTI, but in fact their evidence is most convincing that PCRK1/2 are the major contributors since they don't actually test a pbl19/20 Arabidopsis mutant, only a pcrk1/2 mutant and other combinatorial mutants with pcrk1/2. Finally, they provide evidence using the TurboID system that SOBIR, a partner of RLP23, interacts with PBL19/20 and PCRK1/2. These experiments, however, require additional controls in the form of some RLCKs that are not expected to interact with SOBIR to show specificity. They do not observe an interaction between SOBIR and EDS1, PAD4, or ADR1 even though these proteins play a role in the compromised RLP23-mediated PTI they observe. This experiment probably should have included RLP23 as was done in the Pruitt et al. paper they cite in which these proteins were shown to interact by co-IP. They end with a model showing that RLP23 signaling proceeds via a calcium influx mechanism to induce expression of TNL genes. However, this model is based on their initial speculation that SNIPER1 suppression of TNLs is the cause of compromised PTI and I do not believe this is unequivocally demonstrated. In general, the paper is based on some unsubstantiated assumptions and also has several instances of overinterpretation of the results.

Referee #3 (Remarks to the Author):

This manuscript makes an intriguing connection between TNL and PTI signaling, primarily through the use of genetic analyses. The genetic analyses and disease assays are well done, but there is a lack of biochemical understanding of these processes. Demonstrating where SNIPER1 acts to

regulate PTI, performing additional PTI-immune related assays (MAPK, callose, etc) for key experiments, and the importance of specific TIR upregulation for PTI responses in Arabidopsis is required.
1. There is no direct evidence to support that reduced sNLR accumulation in SNIPER1 overexpression lines leads to compromised PTI. It is also possible that SNIPER1 overexpression directly regulates the accumulation of PTI receptors, co-receptors, or RLCKs downstream. There are commercial antibodies available against these proteins in Arabidopsis that can be used to demonstrate where SNIPERs act.
2. Do TNL signaling mutants (eds1, pad4, sag1, adr1 triple etc.) show altered expression or accumulation of the immunity-associated genes/proteins? If levels of the immunity-associated protein machinery, such as FLS2, RLP23, BAK1 and RLCKs (BIK1 and SOBIR1), were changed in TNL signaling mutants, the attenuated or lost immune responses upon Pto DC3000 hrcC inoculation or nlp20/flg22 treatment might be due to the affected immunity-associated protein machinery but not the TNL signaling pathway.
3. Do TNL signaling mutants (eds1, pad4, sag1, adr1 triple etc.) show altered expression or accumulation of SA biosynthesis genes/proteins (ICS1, PBS3, EDS5 etc.) besides SARD1 and FMO1? If yes, the attenuated or lost immune responses might be due to altered SA biosynthesis in TNL signaling mutants as opposed to a specific effect on PTI.
4. It is well known that overexpression of some TIRs in N. benthamiana results in cell death and increased SA levels. Also, these phenotypes may not be transferrable to Arabidopsis. The authors need to test what TIRs are able to induce defense in Arabidopsis using either transient expression or protoplast cell death assays. In order to convincingly demonstrate TIR upregulation affects PTI, a genetic approach is needed with higher order TIR mutants.
5. How does flg22-induced immunity change in RLCK mutants (pcrk1/2, pcrk1/2 pbl19/20)? It seems that both FLS2 and RLP23 share the same downstream RLCKs, but this is confusing as a related paper on a pre-print server and previous publications indicate that FLS2 and RLP23 induce different immune responses are require different RLCKs. Will any higher order RLCK mutants affect immune output? These kinases are indicated to act upstream of the early induction of TIR genes in line 276. As shown in Fig. 4E, if they share both RLCKs and TNL signaling pathways, why are flg22- and nlp20-induced immune responses different in TNL signaling mutants?

Author Rebuttals to Initial Comments:

We genuinely thank the three expert reviewers for your constructive and helpful suggestions and comments, which help us to improve the quality and thoroughness of our study. Our responses in blue font can be found beneath each original reviewer's comment below.

Referees' comments:

Referee #1 (Remarks to the Author):

The plant immune system is classically divided into two pathways: pattern- and effector-triggered immunity (PTI and ETI, respectively). While long thought to be distinct, recent work has increasingly revealed overlap/interplay between PTI and ETI. In the current manuscript, Tian, Chen et al report a link between PTI and TIR-NLR (TNLR) signaling machinery. The authors arrived at this connection following observation that SNIPER-OX lines, which have reduced NLR accumulation, are compromised in resistance to Pto hrcC-. From here, the authors characterized PTI responses in TNLR signaling components. The work is generally of high quality, and the conclusions follow from the data for the most part. The work is certainly of broad interest, and thus may be suitable for

publication in a journal such as Nature; however, significant issues must be addressed, which are outlined below.

Thank you for the positive comments.

1. While the authors propose a model in which TNLR components regulate PTI signaling, the current data are rather consistent with a model in which TNLR signaling regulate pattern-induced SA accumulation/signaling during PTI. To broaden their conclusions, the authors should test other classical (earlier) PTI outputs, e.g. ROS production, MAPK activation, ethylene production. This is particularly relevant, as a recent bioRxiv paper from the Nurnberger lab (Pruitt et al., 2020) reported that these (earlier) responses depend on TNLR signaling. This would also help resolving the current apparent contradiction between both studies on the generality of these findings for PTI signaling (triggered by both RLKs or RLPs), or specifically downstream of RLPs (as proposed by Pruitt et al., 2020).

Thank you for this very important point. As suggested, we have tested other PTI outputs including MAPK activation and ROS production in TIR signaling mutants, please see these data throughout the revised manuscript (Extended Data Figure 5a-c). As we do not have the setup for measuring ethylene production, we did not pursue those assays, especially because they are covered by the related manuscript by Pruitt et al. from Thorsten's group.

Our data indicate that TIR signaling components are required for certain branches, but not all of PTI signaling output, which is reflected in our model. We did not observe any difference in flg22-induced MAPK and ROS production in *eds1* and *adr1* triple, which is consistent with findings from a previous report by Jeff Dangl's group (Bonardi et al. 2011, PNAS). We have included our data below for reviewing purpose. They were not included in the manuscript due to space limitations.

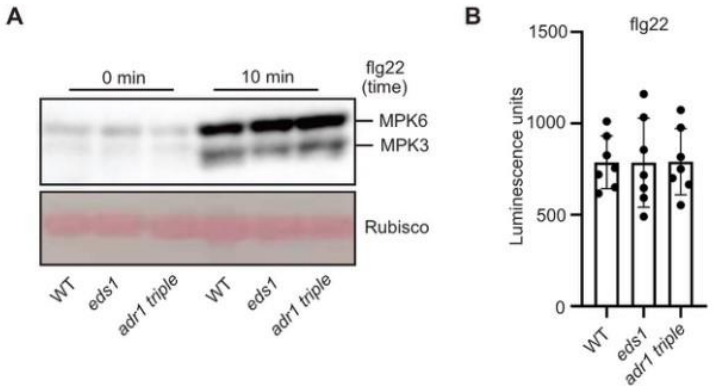

Figure 1. flg22-induced MAPK activation and ROS production in WT, *eds1-24* and *adr1 adr-L1 adr-L2* (*adr1 triple*).
(A) flg22-induced MAPK activation in WT, *eds1-24* and *adr1 adr-L1 adr-L2* (*adr1 triple*) plants.12-day-old seedlings were treated with 0.1μM flg22. MAPK activation was analyzed by immunoblotting using the anti-pERK antibody. Equal loading is confirmed by Ponceau staining of Rubisco.
(B) H_2O_2 production in the indicated genotypes after treatment with 0.1μM flg22 measured as luminescence. The values show the peak of luminescence units from all the time points. Error bars represent SD. The experiment was repeated twice with similar results.

In *eds1*, *pad4* and *adr1* triple mutants, nlp20-induced MAPK activation is also not affected at all (Extended data Fig. 5a and 5b), confirming that TIR signaling components are not required for all signaling events downstream of RLPs. In addition, three TIR genes selected for functional analysis can still be induced by nlp20 and flg22 in the *eds1-24* mutant (Fig. 3b and Extended Data Fig. 8e), indicating that EDS1 acts downstream of up-regulation of the TIR genes. In the *eds1-24* mutant, early induction of several other tested genes including *SOBIR1*, *RLP23*, *BAK1* and *BIK1* by nlp20 is also not affected (Extended data Fig. 6). Furthermore, findings from Thorsten's group also showed that nlp20-induced ethylene production is reduced, but not blocked in TIR signaling mutants (Figure 2a, Figure 3a and Figure S8), confirming that not all signaling outputs in RLP signaling require TIR signaling components. The original model proposed by Pruitt et al., 2020 seems over-simplified. They have revised their model during revision as well.

In a study from Thorsten Nürnberger's group (Wan et al., 2019, New Phytologist), it was shown that nlp20 and flg22 trigger largely overlapping transcriptional reprogramming. 97% percent (1 h treatment with nlp20) and 91% (6 h treatment with nlp20) of transcripts that changed after nlp20 treatment were also affected by flg22 application. A recent study published in Nature Plants by Zipfel's group also showed that transcriptional responses to diverse elicitors including flg22 and nlp20 are remarkably similar. However, flg22-induced defense responses appear to be quicker and stronger than nlp20-induced responses. Both ROS production and MAPK activation are often seen to be induced more dramatically by flg22 than nlp20, and there are many more genes significantly induced by flg22 than nlp20. Thus, it is understandable that *eds1*/*pad4*/*adr1 triple* have

a smaller effect regarding flg22-induced resistance against *Pto* DC3000, since EDS1/PAD4/ADR1s appear to mainly affect one branch of defense signaling downstream of FLS2, which leads to induction of *SARD1* expression and SA biosynthesis, but have almost no effect on ROS production and MAPK activation. Similarly, EDS1/PAD4/ADR1s are also required for induction of *SARD1* expression and SA biosynthesis by nlp20, which play bigger roles in nlp20-induced disease resistance because the other branches of defense signaling downstream of RLP23 is likely weaker and are not sufficient to compensate on the defects caused by loss of TIR signaling.

2. Also related to this other recent work, the authors there also uncovered the connection between EDS1/PAD4 and PTI, specifically downstream of nlp20 perception. How do the authors explain the conflicting results regarding a direct association between these components and SOBIR1? The authors should address this discrepancy.

We repeated the TurboID experiment with RLP23 included in the reactions, as suggested by reviewer #2. We still did not observe labeling of EDS1 or PAD4 by SOBIR1-HATurboID, but we were able to detect some labeling of ADR1 by SOBIR1-HATurboID (Extended data Fig. 10b-d). Thus, we have modified our model to show potential interactions between SOBIR1 and ADR1s on the plasma membrane and contribution of such interaction to defense signaling.

Whether (and how) defense signals from RLPs are directly transduced through the SOBIR1/ADR1s interactions remains to be determined. Nevertheless, direct signaling through ADR1s won't be the whole story, considering that nlp20-induced MAPK activation (Extended data Fig. 5a-b) and expression of a number of genes as mentioned above are not affected in TIR signaling mutants (Fig. 3b, Extended data Fig. 8e and Extended data Fig. 6), and nlp20-induced ethylene production is only partially affected in TIR signaling mutants. In addition, RLCKs such as PCRK1/2/PBL19/20 are also directly involved in early nlp20-induced immune responses.

3. It is unclear to me why the authors focused exclusively on TNLRs in their analyses. SNIPER-OX lines have reduced levels of both TNLRs and CNLRs. Did the authors ever test whether any PTI outputs also depend on CNLR machinery? Did the authors test ndr1 mutants, for example?

We focused on TNLRs for several reasons. First, the autoimmune phenotype of *sniper1/2* double knockout mutant is blocked by *eds1* (Wu et al., 2020, The EMBO journal). We have added a sentence to explain this in the manuscript. Second, several well-defined signaling mutants that specifically block TNLR signaling are available, whereas only one mutant (*ndr1*) is known to affect CNLR-mediated immunity. Disease resistance specified by some CNLRs is affected by *ndr1*, while other CNLs are either not affected or only partially affected by *ndr1*. For example, *ndr1* only has a small effect on autoimmune phenotype caused by activation of the CNLR SUMM2 in *mkk1/2* (Liu et al.,

2020, JIPB). How NDR1 contributes to CNLR-mediated immunity is unknown. In addition to defects in immunity, the *ndr1* mutant was also reported to exhibit defects in plant cell wall-plasma membrane connection (Knepper et al., 2011, Plant Physiology).

We measured nlp20-induced ROS production, MAPK activation, SA accumulation and resistance to *Hpa* Noco2 in the *ndr1* mutant. In *ndr1* mutant plants, nlp20-induced MAPK activation and SA production are not affected, but ROS production is reduced (consistently with a previous report that a small reduction of flg22-induced ROS was observed in *ndr1*). Both basal resistance and nlp20-induced resistance against *Hpa* Noco2 are slightly affected in *ndr1*. This could be due to defects either in cell wall-plasma membrane connection or CNLR signaling. We have included these data here for reviewing purpose, but not in the manuscript due to space limitation.

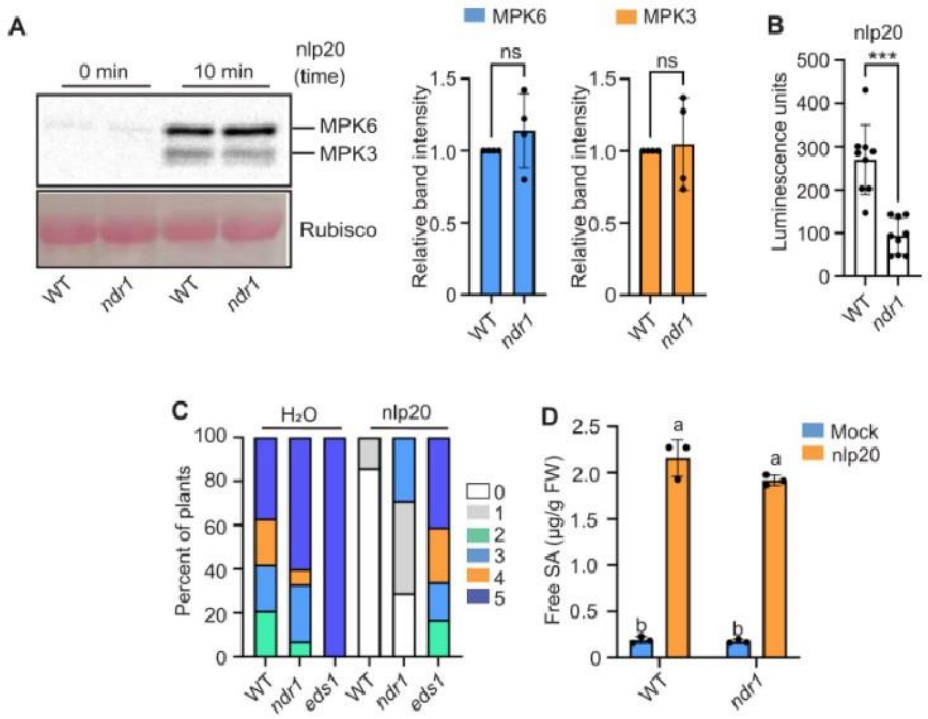

Figure 2. nlp20-induced immune responses in *ndr1-1* mutant plants.
(A) nlp20-induced MAPK activation in *ndr1-1* mutant plants. 12-d-old seedlings were treated with 0.1µM nlp20. MAPK activation was analyzed by immunoblotting using the anti-pERK antibody. Equal loading is confirmed by Ponceau staining of Rubisco. Quantification of the phosphorylated MPK6 and MPK3 10 min after treatment with 0.1µM nlp20 is shown in the right. Error bars represent SD from four different experiments. ($p < 0.05$, two-tailed student's *t*-test; n=4).
(B) H_2O_2 production in the indicated genotypes after treatment with 1µM nlp20 measured as luminescence. The values show the peak of luminescence units from all the time points. Error bars represent SD from nine biological replicates. (***, $p < 0.01$, two-tailed student's *t*-test; n=9). The experiment was repeated three times with similar results.
(C) Growth of *Hpa* Noco2 on the local leaves of the indicated plants. Three-week-old soil-grown plants were pretreated with H_2O or 1 µM nlp20 and sprayed with *Hpa* Noco2 spores (50,000 spores/ml) 24 hours later. Disease ratings are as described in Methods. The experiment was repeated twice with similar results.
(D) Levels of free SA in four-week-old soil-grown plants of the indicated genotypes after treatment with H_2O (mock) or 1 µM nlp20 for 24h. Different letters indicate genotypes with statistical differences ($p < 0.05$, one-way ANOVA test; n=3). The experiment was repeated three times with similar results.

4. Also, it is unclear how the authors decided to focus on the three TIR genes analyses out of the many regulated by flg22 and/or nlp20 treatment.

The three TIR genes were selected as examples for further study because they are commonly induced by flg22 and nlp20. We have added a sentence to explain this in the manuscript.

5. The authors identify the RLCK-VII isoforms PCRK1/2 and PBL19/20 as required for nlp20 responses. The reasoning behind testing just these RLCK-VIIs is unclear, as other members of this family such as BIK1 and PBL1 are extremely-well characterized as functioning downstream of multiple PRRs. Furthermore, PCRK1/2 and PBL19/20 are all members of the RLCK-VII-4 subfamily,

which was previously reported to regulate MAPK activation downstream of multiple PRRs (Bi et al 2018 Plant Cell). Did the authors test MAPK activation?

Our analysis of PCRK1/2 and PBL19/20 was initially aimed to identify RCLKs involved in promoting SA production in nlp20 responses. We tested mutants of *PCRK1/2* (and the closely related *PBL19/20*) because of our previously established connection of PCRK1/2 to SA biosynthesis in PTI (Kong et al., 2016 Plant Physiology). We have included a sentence in the text to explain this.

We have measured MAPKs activity in *pcrk1/2 pbl19/20* mutants upon nlp20 treatment (Extended data Fig. 9d and 9e). There are significant reductions of MPK3/6 phosphorylation induced by nlp20. Previously it was reported that chitin-induced MAPK activation was blocked, but flg22-induced MAPK activation was not affected in the *rlck-vii-4* mutant (Rao et al., Plant Physiology, Jian-Min Zhou's group). However, under our experimental condition, we consistently observed a small but significant reduction of MPK3/6 phosphorylation induced by flg22 in the *pcrk1/2 pbl19/20* mutants (Extended data Fig. 9j and 9k).

6. Related to the above, Pruitt et al. (bioRxiv) identified a distinct subfamily of RLCK-VIIs, including PBL30/CST as functioning specifically in nlp20 signaling following a screen of many PBL mutant lines. Have the authors directly compared mutants in these different PBLs (all publicly available, cf Rao et al., Plant Physiology 2018) in terms of early (e.g. ROS) and late (e.g. SA accumulation/immunity/gene expression) PTI outputs?

We have measured nlp20-induced ROS production, MAPK activation, SA production and resistance to *Hpa* Noco2 in the *rlckvii-7* mutant which were identified to affect nlp20-induced ethylene production. There is a dramatic reduction of nlp20-induced ROS production, marginally decreased MAPK activation, and partially compromised resistance against *Hpa* Noco2, but SA production is not affected. We have included these data below for reviewing purpose. They were not included in the manuscript because Pruitt et al. originally identified and characterized this group of RLCKs as regulators in nlp20-induced immunity and we would not want to steal their thunder and undermine their story.

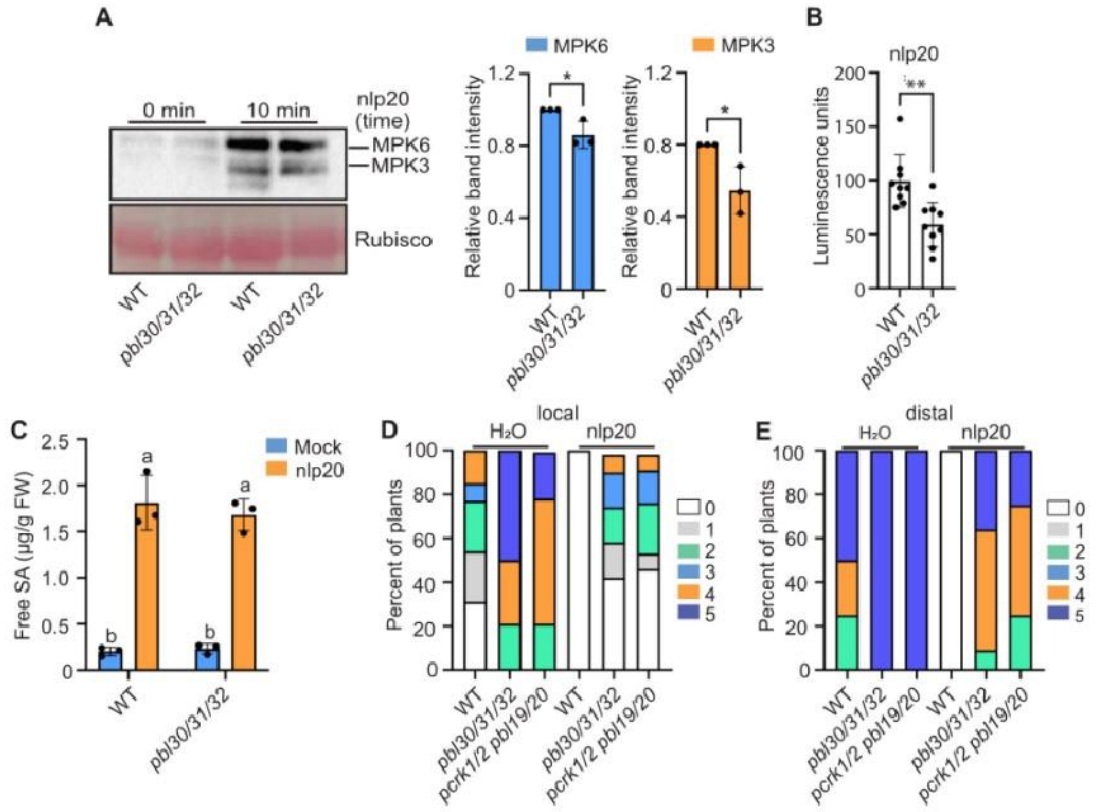

Figure 3. nlp20-induced PTI response in *pbl30/31/32* mutant plants.
(A) nlp20-induced MAPK activation in *pbl30/31/32* mutant plants. 12-day-old seedlings were treated with 0.1µM nlp20. MAPK activation was analyzed by immunoblotting with the anti-pERK antibody. Equal loading is confirmed by Ponceau staining of Rubisco. Quantification of the phosphorylated MPK6 and MPK3 10 min after treatment with 0.1µM nlp20 in the indicated genotypes is shown on the right. Error bars represent SD from three different experiments. (*, $p < 0.05$, two-tailed student's *t*-test; n=3).
(B) H_2O_2 production in the indicated genotypes after treatment with 1µM nlp20 measured as luminescence. The values show the peak of luminescence units from all the time points. Error bars represent SD from nine biological replicates. (**, $p < 0.01$, two-tailed student's *t*-test; n=9). The experiment was repeated three times with similar results.
(C) Levels of free SA in four-week-old soil-grown plants of the indicated genotypes after treatment with H_2O (mock) or 1 µM nlp20 for 24h. Different letters indicate genotypes with statistical differences ($p < 0.05$, one-way ANOVA test; n=3). The experiment was repeated three times with similar results.
(D) Growth of *Hpa* Noco2 on the local leaves of the indicated plants. Three-week-old soil-grown plants were pretreated with H_2O or 1 µM nlp20 and sprayed with *Hpa* Noco2 spores (50,000 spores/ml) 24 hours later. Disease ratings are as described in Methods. The experiment was repeated twice with similar results.
(E) Growth of *Hpa* Noco2 on the distal leaves of the indicated plants. Three-week-old soil-grown plants were pretreated with H_2O or 1 µM nlp20 and sprayed with *Hpa* Noco2 spores (50,000 spores/ml) 24 hours later. Disease ratings are as described in Methods. The experiment was repeated twice with similar results.

7. The identification of TIR genes as specifically induced (Table S1) seems odd and possibly cherry-picked. What about CNLRs? Are TIR domain genes specifically enriched in induction?

Thanks a lot for pointing this out. We initially only looked at the expression of TIR genes because we were focusing on the contribution of TIR signaling to PTI. We have now expanded the analysis on the CNLs. Interestingly, only a few of them are induced by nlp20 or flg22 treatment. TIR domain genes

but not CNL genes are found to be significantly enriched in induction. This has now been added to the revised manuscript.

8. It is not clear what the relevance of OX assays with TIR or TNLRs (Fig 3) inducing cell death is to the manuscript, as this is long known and characterized

We have removed the figure of cell death caused by the overexpression of the *TIR/TNLRs* genes. We have included additional data to show that OX of the three *TIR* genes can activated *pSARD1-Luc* expression in *Arabidopsis* protoplasts (Extended Data Fig. 8c), as suggested by reviewer #3.

9. Similarly, what is the specific relevance of the calcium channel blocker expts in Fig 3?

The experiment was carried out to determine whether there is a potential connection between calcium influx and induction of TIR gene expression. In the revised manuscript, we also included data showing up-regulation of the three *TIR* genes in the gain-of-function mutants of CNGCs (*crp22* and *cngc20-4*) to support the potential connection between calcium influx and induction of *TIR* gene expression.

10. It appears that all the experiments with the RLCK mutants were performed with nlp20 only - what about flg22? Did the authors ever test this?

As suggested, we have tested the RLCK mutants with flg22. The data are now included in Extended Data Fig. 9j-p. flg22-induced defense responses appear to be modestly affected in the mutants.

11. It is not always clear how statistics were performed – for example in Fig 1F, it appears that the basal levels of SARD1 expression is reduced in SNIPER-OX lines, but this is not indicated by the statistics. This is a common issue throughout the manuscript (see e.g. Fig 2E), and should be clarified.

We have included additional statistical analysis (See in the Figures and legends) and showed the P values in the figures following the journal guidelines. The main text is also modified accordingly.

Referee #2 (Remarks to the Author):

The authors report that overexpression in Arabidopsis of SNIPER1, an E3 ligase, compromises pattern-triggered immunity (PTI) as judged by the plant response to two pathogens and the elicitors flg22 and nlp20; the latter peptide activates the LRR-RP RLP23. In an earlier paper it was shown that mutation of SNIPER1 (and SNIPER2) in Arabidopsis causes EDS1-dependent lethality and that

overexpression of SNIPER1 leads to reduced levels of sensor NLR proteins, including TNLs. They therefore conclude that the reduced accumulation of NLR proteins in SNIPER1 overexpressing lines is the cause of compromised PTI in these plants. I think an equally likely possibility is that overexpression of SNIPER1 causes reduced levels of some key proteins involved in PTI and I don't see any evidence to refute that possibility in the paper. If that is the case, then almost all of their subsequent observations, which they attribute to 'reduced TNL signaling' could simply be due to direct effects of SNIPER1 on one or more PTI components.

This is a very good point. We have measured the endogenous protein levels of FLS2 and BAK1 in the *SNIPER1* ox lines and found no significant difference between WT and two *SNIPER1* ox lines used in the ms (Extended data Fig. 2a and 2b).

Since we don't have good antibodies against other major PTI signaling components in flg22 and nlp20-induced immune responses, we overexpressed SNIPER1 together with epitope-tagged RLP23, SOBIR1 and several RLCKs including BIK1, PCRK2 and PBL19 in *N. benthamiana*. While overexpression of *SNIPER1* resulted in reduced accumulation of the NLR protein SNC1, it did not have any effect on the accumulation of RLP23, SOBIR1, or the tested RLCKs (Extended data Fig. 2c-2h). We also used TurboID to test whether SNIPER1 interacts with RLP23, SOBIR1 or the RLCKs in *N. benthamiana* and no interactions were observed (Extended data Fig. 3). Although we are not absolutely certain that SNIPER1 does not directly affect any PTI signaling components, it is unlikely that the reduced TNL signaling is due to direct effects of SNIPER1 on these main PTI components. More importantly, our genetic analysis on the TIR signaling mutants *eds1*, *pad4* and *adr1 triple* mutant provided additional support that TNL signaling is required for PTI.

Note that the observation that certain TNLs cause cell death when overexpressed is not surprising since that has been reported previously.

As suggested, we have removed the figure of cell death caused by the overexpression of the *TIR/TNL* genes. We added new data to show that OX of the three *TIR/TNL* genes can activated *pSARD1-Luc* expression in *Arabidopsis* protoplasts (Extended Data Fig. 8c), as suggested by reviewer #3.

They also report that RLCKs PBL19/20 and PCRK1/2 play important roles in RLP23-mediated PTI, but in fact their evidence is most convincing that PCRK1/2 are the major contributors since they don't actually test a pbl19/20 Arabidopsis mutant, only a pcrk1/2 mutant and other combinatorial mutants with pcrk1/2.

This is a very good point. Since we do not have a *pbl19/20* double mutant and most of our analysis was done with the *pcrk1/2 pbl19/20* quadruple mutant, we have revised our conclusion as PCRK1/2 are the major contributors and PBL19/20 have some additive effects on RLP23-mediated immunity, considering that *pcrk1/2 pbl19/20* quadruple mutant displayed more severely compromised nlp20-induced resistance against *Hpa* Noco2 in both local and distal tissue (Fig. 4a and Extended Data Fig. 9a).

Finally, they provide evidence using the TurboID system that SOBIR, a partner of RLP23, interacts with PBL19/20 and PCRK1/2. These experiments, however, require additional controls in the form of some RLCKs that are not expected to interact with SOBIR to show specificity.

We have repeated the Turbo-ID experiment using the non-signaling RLCK RKS1 as a negative control (Fig. 4c).

They do not observe an interaction between SOBIR and EDS1, PAD4, or ADR1 even though these proteins play a role in the compromised RLP23-mediated PTI they observe. This experiment probably should have included RLP23 as was done in
the Pruitt et al. paper they cite in which these proteins were shown to interact by co-IP.

Thanks a lot for the good suggestion. We repeated the TurboID experiment with RLP23 included in the reactions. Although we still did not see labeling of EDS1 or PAD4 by SOBIR1-HATurboID, but we were able to detect some labeling of ADR1 by SOBIR1-HATurboID (Extended Data Fig. 10b-d). Thus, we modified our model to show potential interactions between SOBIR1 and ADR1s on the plasma membrane.

It remains to be determined whether (and how) defense signals from RLPs are directly transduced through the SOBIR1/ADR1s interactions. Nevertheless, direct signaling through ADR1s/EDS1/PAD4 won't be the whole story, considering that nlp20-induced MAPK activation (Extended data Fig. 5a-b) and nlp20-induced ethylene production is only partially affected in TIR signaling mutants. In addition, three *TIR* genes selected for functional analysis can still be induced by nlp20 in the *eds1-24* mutant (Fig. 3b), indicating that EDS1 acts downstream of up-regulation of the *TIR* genes. In the *eds1-24* mutant, early induction of several other tested genes including *SOBIR1*, *RLP23*, *BAK1* and *BIK1* by nlp20 is also not affected (Extended data Fig. 6). Moreover, RLCKs such as PCRK1/2/PBL19/20 are also directly involved in early nlp20-induced immune responses.

They end with a model showing that RLP23 signaling proceeds via a calcium influx mechanism to induce expression of TNL genes. However, this model is based on their initial speculation that

SNIPER1 suppression of TNLs is the cause of compromised PTI and I do not believe this is unequivocally demonstrated.

We have addressed this in our response to the first comment. With the additional experimental evidence that SNIPER1 does not affect the protein levels of all major RLP23 signaling components we tested, we think it is unlikely that the reduced PTI signaling is due to direct effects of SNIPER1 on main PTI components. In addition, we showed that TNL genes are rapidly induced in a calcium-dependent, but EDS1-independent manner following treatment with nlp20 (Fig. 3a and 3b), and overexpression of three selected *TIR/TNL* genes can activate the expression of the *pSARD1-Luc* reporter expression in *Arabidopsis* protoplasts and SA accumulation in *N. benthamiana*. We also included new data (Fig. 3C and Extended Data Fig. 8f) showing that the three *TIR/TNL* genes we tested are up-regulated in the CNGC gain-of-function mutants *cpr22* and *cngc20-4*, which exhibit constitutive defense responses. We believe these new experimental data enhances our modified model.

In general, the paper is based on some unsubstantiated assumptions and also has several instances of overinterpretation of the results.

We have toned down our claims in the main text and also performed additional experiments as suggested to address the concerns raised.

Referee #3 (Remarks to the Author):

This manuscript makes an intriguing connection between TNL and PTI signaling, primarily through the use of genetic analyses. The genetic analyses and disease assays are well done, but there is a lack of biochemical understanding of these processes. Demonstrating where SNIPER1 acts to regulate PTI, performing additional PTI-immune related assays (MAPK, callose, etc) for key experiments, and the importance of specific TIR upregulation for PTI responses in Arabidopsis is required.

Thank you for the constructive and very helpful suggestions. We have performed additional experiments as listed below to address each of them. Several preprints recently uploaded to bioRxiv addressed the biochemical mechanisms of how EDS1/PAD4/SAG101 and ADR1s/NRG1s contribute to activation of defense responses in TIR signaling (bioRxiv 2021.02.25.431980; bioRxiv 2020.12.21.423810; bioRxiv 2021.05.23.445317). Because these findings in these preprints have not been peer-reviewed and there is strict space limitation for our revision, we did not discuss them in our manuscript.

1. There is no direct evidence to support that reduced sNLR accumulation in SNIPER1 overexpression lines leads to compromised PTI. It is also possible that SNIPER1 overexpression directly regulates the

accumulation of PTI receptors, co-receptors, or RLCKs downstream. There are commercial antibodies available against these proteins in Arabidopsis that can be used to demonstrate where SNIPERs act.

This is a very good point. We have measured the protein levels of FLS2 and BAK1 using endogenous antibodies and found no significant difference between WT and two *SNIPER1* ox lines (Extended data Fig. 2a and 2b). (Extended data Fig. 2a and 2b). Because we don't have good antibodies against other major PTI signaling components in flg22 and nlp20-induced immune responses, we overexpressed SNIPER1 together with epitope-tagged RLP23, SOBIR1 and several RLCKs including BIK1, PCRK2 and PBL19 in *N. benthamiana*. While overexpression of SNIPER1 resulted in reduced accumulation of the NLR protein SNC1, it did not show any effect on RLP23, SOBIR1 and the tested RLCKs (Extended data Fig. 2c-2h). We also used Turbo-ID to test whether SNIPER1 interacts with RLP23, SOBIR1 and the RLCKs in *N. benthamiana*, and no interactions were observed (Extended data Fig. 3). Although we cannot be absolutely certain that SNIPER1 does not directly affect any PTI signaling components, it is unlikely that the reduced TNL signaling is due to direct effects of SNIPER1 on these main PTI components.

2. Do TNL signaling mutants (eds1, pad4, sag1, adr1 triple etc.) show altered expression or accumulation of the immunity-associated genes/proteins? If levels of the immunity-associated protein machinery, such as FLS2, RLP23, BAK1 and RLCKs (BIK1 and SOBIR1), were changed in TNL signaling mutants, the attenuated or lost immune responses upon Pto DC3000 hrcC inoculation or nlp20/flg22 treatment might be due to the affected immunity-associated protein machinery but not the TNL signaling pathway.

This is a great point. We have compared the transcript levels of *FLS2*, *BAK1*, and *BIK1* with or without flg22 treatment (Extended data Fig. 4i-4k), and *RLP23*, *SOBIR1* and *BIK1* with or without nlp20 treatment (Extended data Fig. 6), in wild type and *eds1* mutant plants. We did not observe any difference of the transcript levels of these PTI components in wild type and *eds1* mutant plants treated with the respective elicitors.

3. Do TNL signaling mutants (eds1, pad4, sag1, adr1 triple etc.) show altered expression or accumulation of SA biosynthesis genes/proteins (ICS1, PBS3, EDS5 etc.) besides SARD1 and FMO1? If yes, the attenuated or lost immune responses might be due to altered SA biosynthesis in TNL signaling mutants as opposed to a specific effect on PTI.

SARD1 is known to regulate the transcription of the SA biosynthesis genes *ICS1*, *EDS5* and *PBS3*. Considering that *eds1*, *pad4* and *adr1 triple* mutants have reduced *SARD1* expression levels, we would expect the transcript levels of *ICS1*, *EDS5* and *PBS3* are affected by blocking TIR signaling. When we measured the transcript levels of these three genes in flg22 or nlp20-treated Col-0 and *eds1* mutant plants, the expression levels of these three genes were indeed significantly lower in *eds1* compared to wild type. This is consistent with the reduced SA levels in *eds1* mutant upon flg22

and nlp20 treatment. We have included these data here (Fig. 4 for reviewing purpose) and but not in the manuscript due to space limitation. In our model, the reduced induction of SA production in the TIR signaling mutants is a main contributing factor to the attenuated or lost immune responses. Although it is known that SA can induce the expression of a large number of defense-related genes, it takes some time to increase SA biosynthesis and accumulate high levels of SA after elicitor treatment, and early PTI responses such as rapid induction of *TIR/TNL* genes and *SARD1* usually happen before there is significant increase in SA levels (> 4h in our hands).

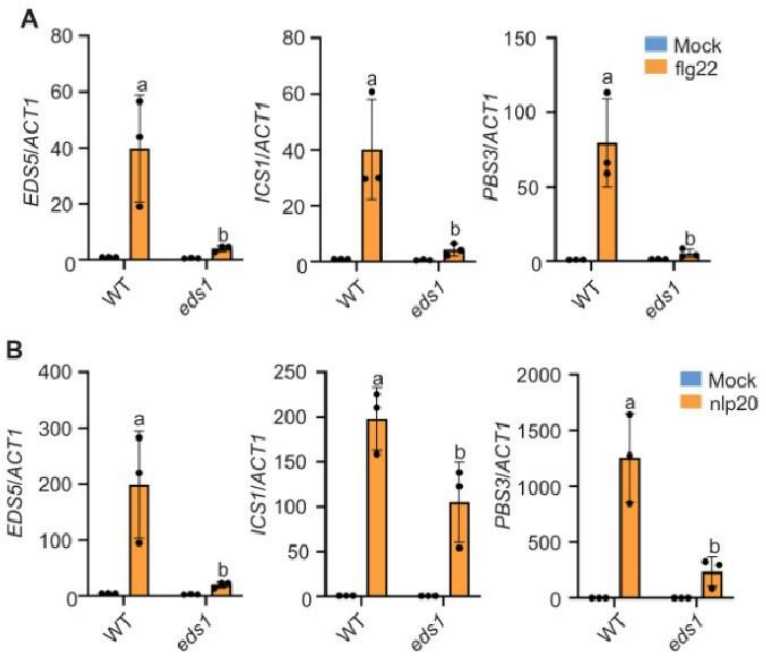

Figure 4. flg22- or nlp20-induced expression of *ICS1*, *EDS5* and *PBS3* in *eds1-24* mutant plants. (A, B) Relative expression level of *ICS1*, *EDS5* and *PBS3* in the WT and *eds1-24* mutant plants upon 1 μM flg22(A) or 1 μM nlp20(B). Total RNA was isolated from 4-week-old soil-grown plants. *ACTIN1* was used as the reference gene, and the expression of each gene in the WT plants was set as 1. Error bars represent SD from three biological replicates. Different letters indicate genotypes with statistical differences ($p < 0.05$, one-way ANOVA test; n=3).The experiment was repeated twice with similar results.

4. It is well known that overexpression of some TIRs in N. benthamiana results in cell death and increased SA levels. Also, these phenotypes may not be transferrable to Arabidopsis. The authors need to test what TIRs are able to induce defense in Arabidopsis using either transient expression or protoplast cell death assays. In order to convincingly demonstrate TIR upregulation affects PTI, a genetic approach is needed with higher order TIR mutants.

Thanks a lot for this great suggestion. We performed transient expression analysis as suggested and found that overexpression of the three selected *TIR/TNL* genes can indeed activate the expression of the *pSARD1-Luc* reporter gene in *Arabidopsis* protoplasts (Extended data Fig. 8c). In addition,

overexpression of several elicitor-induced *TIR/TNL* genes including *SNC1*, *LAZ5* and *At2g32140* was known to result in constitutively activated defense responses in Arabidopsis plants.

Because a large number of TIR/TNL genes (>20) are induced at early time point of PTI, it is likely that they have additive effects on promoting PTI. While it would be very nice to have higher order mutants knocking out most of these *TIR/TNL* genes and use such mutants to measure PTI responses, it could take years to combine enough TIR/TNL mutants in order to see an effect on PTI because the loss of a few TIR/TNLs most likely can be compensated by other TIR/TNLs. We think that the data from overexpression of *TIR/TNL* genes from our study and previous literature have already provided solid support that induction of *TIR* genes could contribute to PTI, which is consistent with the observed PTI defects in TIR signaling mutants and *SNIPER1* ox lines.

5. How does flg22-induced immunity change in RLCK mutants (pcrk1/2, pcrk1/2 pbl19/20)? It seems that both FLS2 and RLP23 share the same downstream RLCKs, but this is confusing as a related paper on a pre-print server and previous publications indicate that FLS2 and RLP23 induce different immune responses are require different RLCKs. Will any higher order RLCK mutants affect immune output? These kinases are indicated to act upstream of the early induction of TIR genes in line 276. As shown in Fig. 4E, if they share both RLCKs and TNL signaling pathways, why are flg22- and nlp20-induced immune responses different in TNL signaling mutants?

We have tested flg22-induced immune responses in the *pcrk1/2 pbl19/20* mutants. The data are now included in Extended data Fig. 9. flg22-induced defense responses appear to be modestly reduced in the mutants.

FLS2 and RLP23 induce very similar immune responses such as ROS production, MAPK activation and SA production. In a study from Thorsten Nürnberger's group (Wan et al., 2019, New Phytologist), it was shown that nlp20 and flg22 trigger largely overlapping transcriptional reprogramming. 97% percent (1 h treatment with nlp20) and 91% (6 h treatment with nlp20) of transcripts that changed after nlp20 treatment were also affected by flg22 application. A recent study published in Nature Plants by Zipfel's group also showed that transcriptional responses to diverse elicitors including flg22 and nlp20 are remarkably similar. We think the main differences between immune responses activated by FLS2 and RLP23 are the magnitude and dynamics of the responses. In our hands, flg22-induced defense responses are usually stronger than nlp20-induced responses. Both ROS production and MAPK activation are induced more dramatically by flg22 than nlp20. While it is not surprising that similar RLCKs are used to transduce defense signals from FLS2 and RLP23, the relative contributions of each RLCK may differ in flg22 and nlp20-induced immune responses, as FLS2 and SOBIR1 (signaling kinase partner for RLP23) may have different affinity toward these RLCKs. Unfortunately, there are so many RLCKs that have been shown to be potentially involved in the process and there are extensive overlaps in the functions of these RLCKs. It is challenging to completely block all the immune output downstream of FLS2 or RLP23 by combining a limited number of rlck mutants.

We have analyzed flg22 and nlp20-induced MAPK activation and ROS production in the TIR signaling mutants as suggested. We did not show any data on callose deposition. There are big variations in callose deposition among plants of the same genotype under our growth condition, probably because callose deposition is very sensitive to environment conditions as reported by Ton, et al. (2011, MPMI -07-10-0149, Callose Deposition: A Multifaceted Plant Defense Response).

In *eds1*, *pad4* and *adr1 triple* mutants, MAPK activation induced by flg22 as well as nlp20 is also not affected at all (Extended data Fig. 5a and 5b; Fig.1 for reviewing purpose; previous report by Bonardi et al. 2011, PNAS), indicating that TIR signaling components are not required for all signaling events downstream of both FLS2 and PLP23. Findings from Thorsten's group also showed that nlp20-induced ethylene production is reduced but not blocked in TIR signaling mutants, confirming that not all signaling output in RLP23 signaling requires TIR signaling components (The model proposed by Pruitt et al., 2020 is a bit over-simplified in this regard). As flg22-induced defense responses are stronger than nlp20-induced responses, it is understandable that *eds1/pad4/adr1*s have a smaller effect regarding flg22-induced resistance against *Pto* DC3000, since EDS1/PAD4/ADR1 appear to mainly affect one branch of defense signaling downstream of FLS2, which leads to induction of *SARD1* expression and SA biosynthesis, but have almost no effect on ROS production and MAPK activation. The reduced flg22-induced defense signaling in *eds1/pad4/adr1*s could be compensated by the other signaling branches. Similarly, EDS1/PAD4/ADR1 are also required for induction of *SARD1* expression and SA biosynthesis by nlp20, but they may play a bigger role in nlp20-induced disease resistance because the other branches of defense signaling downstream of RLP23 is weaker and are not sufficient to compensate on the defects caused by loss of TIR signaling.

Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

Summary:
I thank the reviewers for having performed experiments to address comments previously raised. In sum, the major findings of this revised manuscript are however that:
1. PTI induces TNL gene expression.
2. TNL over-expression induces defense.
3. Blocking TNL signaling affect SA-related PTI.
4. PRCK1/2 and PBL19/20 play a role in nlp20-induced SA responses.
The major issues remaining are that Points 1 and 2 are ultimately not that novel; point 3 is not sufficiently substantiated as there is so far no direct link demonstrated between PTI and the function of specific TNLs as the model remains based on correlations, and point 4 somehow seems unrelated to the major focus of the manuscript. In addition, the discrepancy with the data presented in the bioRxiv manuscript from the Nurnberger's group are still insufficiently discussed, and the data generated to try addressing them should definitely be presented as part of the manuscript and not as for the rebuttal only, as these are very important for the community.

Specific comments:
• In the rebuttal and manuscript, the authors indicate that TIR machinery is required for "some" but "not all" branches of PTI. In fact, it is clearer in the revision that TNL signalling is specific to SA

biosynthesis/signalling and not PTI signalling per se - a more accurate title would be "PAMP-induced SA accumulation requires TNLRs/TIR signalling" or something similar.
• There is still no direct genetic evidence that this is specific to TNLs without contribution from CNLs. The authors point out that fewer CNLs than TNLs are induced transcriptionally, but this alone is insufficient for such a conclusion. The NDR1 data should be added to the manuscript.
• The authors still do not satisfactorily address the discrepancies between their manuscript and Pruitt et al, which posits a more direct involvement of TIR signalling components in (early) PTI responses. At the very least, the ROS and MAPK data included in the response should be included in the manuscript proper.
• The authors repeatedly claim that calcium-dependent induction of TNLs is a distinct "branch" of PTI signalling, separate from MAPK, ROS, etc. However, they have not tested this and thus the claim is misleading. While MAPK activation and ROS are not affected by their mutants, they only tested the calcium dependence of TNL gene induction, and have not tested whether this also depends on ROS and/or MAPKs. While technically challenging for MAPKs, this could easily be done for ROS using either rbohd mutants or pharmacological inhibition/scavenging (e.g. DPI).
• Lines 247-254: I am not convinced by this reasoning. flg22 induced far more TNLs than nlp20 yet nlp20-mediated PTI is more dependent on TNLs?
• Line 26: I would replace 'conserved' by 'extracellular', as not all apoplastic elicitors are necessarily conserved, and some of them are also effectors.
• Line 28: for consistency with point above, I would write "(NLRs) recognize intracellular effector proteins.
• Line 47-49: misleading, as BAK1 is a co-receptor, not an adaptor. To simplify, RLP+SOBIR=RK, which then recruit BAK1 as co-receptor upon ligand-binding.
• Line 50: FLS2 = FLAGELLIN-SENSING 2.
• Line 72-74: transition here is a bit odd, and I would thus suggest to refer to recent publications showing an interplay between PTI and ETI.
• Line 85: should rather be "compromises SA accumulation triggered by Pto DC3000 hrcC".
• Line 89: what are "specific PAMP elicitors"?
• Line 100: the logic of testing the accumulation of PRRs or PTI components can be questioned by the fact that MAPK activation or ROS production is not affected in SNIPER-ox plants, thus arguing against an effect on upstream components. Same comment for Line 145.
• Line 109-111: and consistent with the lack of effect on ROS or MAPKs.
• Line 113: should be "nlp20- and flg22-induced SA responses".
• Line 115: the impact of SNIPER-OX could also be due to yet unknown SNIPER substrates.
• Line 134: should be "SA-linked PTI responses". Same comment for line 157.
• The selection of the three TIR genes tested upon transient over-expression in N. benthamiana and Arabidopsis mesophyll protoplasts still seems arbitrary, as over-expression of any NLR is likely to activate immune responses. Would the authors see a different response if they were to test TNLs not induced transcriptionally during PTI?
• Line 200: the results with the cngc mutants could also be simply interpreted that auto-immunity in general induce the expression of these TIR genes; they do not necessarily show an upstream role for calcium.
• Fig 1a, 2b, 2d, S9b, S9p: show full y-axis scale.
• Fig 4b (and others similarly) – were statistics performed with the control (water) samples? It seems clear that the rlck mutants have reduced basal SA – is this bourn out by statistical analyses?
• Fig 4c: RKS1 is not mentioned in the text.
• All protein blots require MW size markers

Referee #2 (Remarks to the Author):

I am satisfied with the authors' revisions and believe this is an important contribution to our understanding of pattern-triggered immunity.

Referee #3 (Remarks to the Author):

In this revision, the authors have effectively addressed most of my previous concerns. There has been intense interest in overlap between PTI and ETI signaling in the past year. Thus, the work should be of broad interest. It is still not clear at what level these SNIPER acts and the reason for slightly different outputs from RLKs and RLPs. However, I think the MS is suitable without these details of SNIPER mechanics. I am also not convinced of the interpretation that RLKs simply have a stronger response – as there are differential immune outputs between RLPs (strong ethylene production and no seedling growth inhibition) and RLKs even if transcriptional reprogramming is similar.

Author Rebuttals to First Revision:

Responses to reviewers:

Referee #1 (Remarks to the Author):

Summary:

I thank the reviewers for having performed experiments to address comments previously raised. In sum, the major findings of this revised manuscript are however that:

1. PTI induces TNL gene expression.

2. TNL over-expression induces defense.

3. Blocking TNL signaling affect SA-related PTI.

4. PRCK1/2 and PBL19/20 play a role in nlp20-induced SA responses.

The major issues remaining are that Points 1 and 2 are ultimately not that novel; point 3 is not sufficiently substantiated as there is so far no direct link demonstrated between PTI and the function of specific TNLs as the model remains based on correlations, and point 4 somehow seems unrelated to the major focus of the manuscript. In addition, the discrepancy with the data presented in the bioRxiv manuscript from the Nurnberger's group are still insufficiently discussed, and the data generated to try addressing them should definitely be presented as part of the manuscript and not as for the rebuttal only, as these are very important for the community.

We really appreciate this reviewer's constructive and very helpful suggestions.

Point 1 and 2: Although the RNA-seq dataset we used for analyzing the expression of TNL/TIR genes during PTI were from public resource, the dramatic over-representation of TNL/TIR genes (but not CNL genes) during early PTI response was a surprise to us. This leads to the theory that induction of TNL/TIR genes is used to boost plant defense during PTI, which we regard as an important conceptual advance in the understanding of PTI signaling.

Point 2 is not a main point of our study. Although it is known that overexpression of some TNLs induces defense responses (reference 18 and 19) as mentioned in our manuscript (Line 136-137), the overexpression analysis of selected PTI-induced TNL/TIR genes (shown as Extended data) is necessary to confirm that at least some of the early induced TNL/TIR genes during PTI can indeed activate defense responses when overexpressed. Together, data related to Point 1 and 2 support the new concept that induction of TNL/TIR genes is used in PTI to promote plant defense, which we believe is novel.

Point 3: Our conclusion "blocking TNL signaling affects PTI" is mainly drawn from the strong genetic evidences obtained from the analysis of TIR signaling mutants *eds1*, *pad4* and *adr1 triple*, similar to the one from the manuscript by Pruitt et al. submitted to Nature together with ours. These genetic data are consistent with the observed PTI defects observed in *SNIPER1* overexpression lines, which were known to have reduced sensor NLR levels. Although it would be nice to link PTI to the functions of specific TNLs, this would require the generation of higher order mutants knocking out *TIR/TNL* genes which are rapidly induced during PTI (>20 gene) and using such mutants to measure PTI responses. It could take years to

combine enough TIR/TNL mutants in order to see an effect on PTI, as the loss of a few TIR/TNLs most likely can be compensated by other TIR/TNLs. We believe such experiment is out of the scope for the current study and is not essential for our main conclusion.

Point 4: Although it seems that Point 4 is not well connected to the major focus of the manuscript, identification of the role of PCRK1/2 and PBL19/20 in nlp20-induced immunity is crucial to place these RLCKs between the RLP23/SOBIR1 receptor complex and TIR/TNLs in the signaling pathway, as induction of the TIR genes is dependent on PCRK1/2 and PBL19/20. This distinguishes our model from the one by Nurnberger's group where they suggested that SOBIR1 directly activates TIR signaling through association with EDS1/PAD4/ADR1s. To better connect Point 4 and the main point of the manuscript, we have moved the data showing the requirement of PCRK1/2 and PBL19/20 for the induction of TIR genes by nlp20 to the Main Figure (Fig. 3C) and brought up the requirement of RLCKs for nlp20-induced immunity in the discussion (Line 216-221) of the revised manuscript.

Specific comments:

• In the rebuttal and manuscript, the authors indicate that TIR machinery is required for "some" but "not all" branches of PTI. In fact, it is clearer in the revision that TNL signalling is specific to SA biosynthesis/signalling and not PTI signalling per se - a more accurate title would be "PAMP-induced SA accumulation requires TNLRs/TIR signalling" or something similar.

Although SA accumulation is dramatically affected in the TIR signaling mutants *eds1*, *pad4* and *adr1 triple*, it is not the only defense response that is affected. The induction of SARD1 and FMO1 during PTI is also dependent on EDS1/PAD4/ADR1s. Background information of SARD1 and FMO1 has been added to the manuscript for clarification (Line 64-65). SARD1 encodes a master transcription factor that regulates the expression of a large number of immune regulators in addition to SA biosynthetic genes (Sun et al. 2015). FMO1 encodes a key enzyme for the biosynthesis of N-hydroxylpipecolic acid, which is another important plant defense signal (Hartmann et al., 2018). In addition, nlp20-induced ROS is also significantly reduced in *eds1*, *pad4* and the *adr1 triple* mutant. Despite that TIR machinery is not required for some branches of PTI, such as MPK activation, flg22/nlp20-induced resistance against *Pto* DC3000 or *Hpa* Noco2 is compromised and growth of *Pto* DC3000 hrcC is enhanced in TIR signaling mutants. Although the current title "Activation of TIR signaling is required for pattern-triggered immunity" seems justified to us, it might be a bit too strong. So we changed the title to "Activation of TIR signaling boosts pattern-triggered immunity".

• There is still no direct genetic evidence that this is specific to TNLs without contribution from CNLs. The authors point out that fewer CNLs than TNLs are induced transcriptionally, but this alone is insufficient for such a conclusion. The NDR1 data should be added to the manuscript.

We have added the ndr1-1 data, shown in our previous "responses to the reviewers", to the manuscript as Extended Data Fig. 7.

• The authors still do not satisfactorily address the discrepancies between their manuscript and Pruitt et al, which posits a more direct involvement of TIR signalling components in (early) PTI responses. At the very least, the ROS and MAPK data included in the response should be included in the manuscript proper.

The data on ROS production and MAPK activation induced by flg22 and nlp20 in the TIR signaling mutants are now included in Extended Data Fig. 5 of the manuscript. Pruitt et al showed that SOBIR1 interacts with EDS1/PAD4/ADR1s and proposed that defense signals are transduced directly through the SOBIR1 and EDS1/PAD4/ADR1s interactions, which does not explain the requirement of various RLCKs in nlp20-induced immune responses and no effect on MAPK activation when TIR signaling is blocked. This has been discussed in the revision (Line 216-221).

• The authors repeatedly claim that calcium-dependent induction of TNLs is a distinct "branch" of PTI signalling, separate from MAPK, ROS, etc. However, they have not tested this and thus the claim is misleading. While MAPK activation and ROS are not affected by their mutants, they only tested the calcium dependence of TNL gene induction, and have not tested whether this also depends on ROS and/or MAPKs. While technically challenging for MAPKs, this could easily be done for ROS using either rbohd mutants or pharmacological inhibition/scavenging (e.g. DPI).

The induction of TIR genes by flg22 and nlp20 was blocked by GdCl3 (Fig. 3a and Extended Data Fig. 8d), suggesting that activation of Ca^{2+} signaling is required for the induction of TIR genes. In the manuscript, we suggested that elevation in cytosolic Ca^{2+} levels during PTI may play a crucial role in the induction of TIR genes. We have toned down our conclusion in the text, and did not claim calcium-dependent induction of TNLs is a distinct "branch" of PTI signaling, separate from MAPK and ROS.

Meanwhile, we have compared the induction of TIR gene expression in WT and *rbohd* mutant plants by flg22 and nlp20 as suggested by the reviewer. We found that the induction of the three TIR genes tested was not affected in the rbohd mutant, suggesting that ROS is not required for the induction of these TIR genes. We have included this new data in Extended Data Fig. 8f and 8g. In our model (Fig. 3e), we did not draw the relationship between MAPKs and TIR gene induction, because of the lack of data on (and no easy way to test) whether MAPKs play any role in TIR gene induction or not. The evidence we have so far support that calcium influx is required and sufficient to induce the expression of the three tested TIR genes.

• Lines 247-254: I am not convinced by this reasoning. flg22 induced far more TNLs than nlp20 yet nlp20-mediated PTI is more dependent on TNLs?

Not only more TNL genes, flg22 also induces about 1600 more other genes, which is twice as many as the number of genes induced by nlp20. In the study by Wan et al. (New Phytologist, 2019), it was shown that 97% percent of transcripts that changed 1 h after nlp20 treatment were also affected by flg22 application. However, flg22 additionally induced 1638 gene that were not induced by nlp20, which may also contribute to flg22-induced defense against pathogens and could compensate for the loss of TIR signaling. We have included this reference to our discussion to strengthen the reasoning (line 223-227).

• Line 26: I would replace 'conserved' by 'extracellular', as not all apoplastic elicitors are necessarily conserved, and some of them are also effectors.

We have replaced 'conserved' with 'extracellular' as suggested.

• Line 28: for consistency with point above, I would write "(NLRs) recognize intracellular effector proteins.

We have made the suggested change.

• Line 47-49: misleading, as BAK1 is a co-receptor, not an adaptor. To simplify, RLP+SOBIR=RK, which then recruit BAK1 as co-receptor upon ligand-binding.

"adaptor kinase" have been changed to "kinase".

• Line 50: FLS2 = FLAGELLIN-SENSING 2.

We have made the suggested change.

• Line 72-74: transition here is a bit odd, and I would thus suggest to refer to recent publications showing an interplay between PTI and ETI.

We have revised the text according to the suggestion.

• Line 85: should rather be "compromises SA accumulation triggered by Pto DC3000 hrcC".

We have added additional background information on SARD1 and FMO1 to the related paragraph (Line 64-65). Since the expression of SARD1 and FMO1 as well as the growth of Pto DC3000 hrcC are all affected (in addition to SA), we used "compromised PTI" instead of "compromised SA accumulation" in the summary sentence of the paragraph (line 72). As mentioned above, SARD1 encodes a master transcription factor that regulates the expression of a large number of immune regulators in addition to SA biosynthetic genes. FMO1 encodes a key enzyme for the biosynthesis of N-hydroxylpipecolic acid, which is another important plant defense signal.

• Line 89: what are "specific PAMP elicitors"?

"specific PAMP elicitors" has been changed to "PAMP elicitors".

• Line 100: the logic of testing the accumulation of PRRs or PTI components can be questioned by the fact that MAPK activation or ROS production is not affected in SNIPER-ox plants, thus arguing against an effect on upstream components. Same comment for Line 145.

We agree. The fact that MAPK activation or ROS production is not affected in SNIPER-ox plants argues against an effect on upstream components. However, the experiments were performed to confirm this per request by the other two reviewers (Same for Line 145). We have reorganized the paragraph (line 84-97) to make it read better.

• Line 109-111: and consistent with the lack of effect on ROS or MAPKs.

We have made the change as suggested.

• Line 113: should be "nlp20- and flg22-induced SA responses".

As mentioned above, in addition to nlp20/flg22-induced SA accumulation, nlp20/flg22-induced SARD1 and FMO1 expression as well as nlp20/flg22-induced protection against pathogens are affected in the SNIPER1 overexpression lines, so we used "compromised nlp20 and flg22-induced defense responses" instead of "compromised SA responses" here. As mentioned above, SARD1 encodes a master transcription factor that regulates the expression of a large number of immune regulators in addition to SA biosynthetic genes. FMO1 encodes a key enzyme for the biosynthesis of N-hydroxylpipecolic acid,

which is another important plant defense signal. Although SA is linked to many aspects of plants defense, SARD1 and FMO1 are involved in more than just SA responses.

• Line 115: the impact of SNIPER-OX could also be due to yet unknown SNIPER substrates.

We have toned down by changing the impact of SNIPER-OX from "most likely due to the general reduction of sNLRs levels" to "likely due to the general reduction of sNLRs levels", and also noted the possibility of SNIPER1 targeting other unknown immune regulators other than sNLRs (Line 96-97).

• Line 134: should be "SA-linked PTI responses". Same comment for line 157.

Same argument as our responses to Line 85 and Line 113. Although SA is linked to many aspects of plants defense, SARD1 and FMO1 are involved in more than just SA responses.

• The selection of the three TIR genes tested upon transient over-expression in N. benthamiana and Arabidopsis mesophyll protoplasts still seems arbitrary, as over-expression of any NLR is likely to activate immune responses. Would the authors see a different response if they were to test TNLs not induced transcriptionally during PTI?

Theoretically we could have picked any TIR genes induced by flg22 or nlp20 for the overexpression analysis. But logistically it is simpler to pick the genes that are induced by both flg22 and nlp20, which was why we selected the three TIR genes. To us, testing TNLs that are not induced transcriptionally during PTI would not address the question whether up-regulation of such TIR genes contributes to induction of defense responses. We think it is unlikely that the predicted NLRs are all functional. Overexpression of those unfunctional NLRs would not activate immune responses, which makes it worthwhile testing some of the selected NLR genes induced during PTI.

• Line 200: the results with the cngc mutants could also be simply interpreted that auto-immunity in general induce the expression of these TIR genes; they do not necessarily show an upstream role for calcium.

The results with the cngc mutants show that influx of calcium caused by the gain-of-function cngc mutants is sufficient to induce the TIR genes. We have added a sentence on this to the text (Line 176). This was done during revision to strengthen the other data that calcium influx is required for the induction of TIR genes by flg22 and nlp20.

• Fig 1a, 2b, 2d, S9b, S9p: show full y-axis scale.

The figures have been modified to show the full y-axis scale.

• Fig 4b (and others similarly) – were statistics performed with the control (water) samples? It seems clear that the rlck mutants have reduced basal SA – is this bourn out by statistical analyses?

Statistical analysis has been performed with the control samples. P values have been added to the control (water) samples in Fig 4b as well as others.

• Fig 4c: RKS1 is not mentioned in the text.

RKS1 is now mentioned in the text.

• All protein blots require MW size markers

We have added MW size markers in the gel pictures.

Referee #2 (Remarks to the Author):

I am satisfied with the authors' revisions and believe this is an important contribution to our understanding of pattern-triggered immunity.

Referee #3 (Remarks to the Author):

In this revision, the authors have effectively addressed most of my previous concerns. There has been intense interest in overlap between PTI and ETI signaling in the past year. Thus, the work should be of broad interest. It is still not clear at what level these SNIPER acts and the reason for slightly different outputs from RLKs and RLPs. However, I think the MS is suitable without these details of SNIPER mechanics. I am also not convinced of the interpretation that RLKs simply have a stronger response – as there are differential immune outputs between RLPs (strong ethylene production and no seedling growth inhibition) and RLKs even if transcriptional reprogramming is similar.

We fully agree with the reviewer that RLKs may not simply have a stronger response. We will revise the statement in our discussion section accordingly.

Reviewer Reports on the Second Revision:

Referee #1 (Remarks to the Author):

The authors have mostly satisfactorily addressed my comments.

As the authors postulate that non-induced TNLs are likely "unfonctional", it would be have been nice to test this experimentally by testing if their over-expression induces cell death or not.

The authors should also state in the text that the data with the cngc mutants do not necessarily mean that calcium influx is "sufficient" to induce TIR gene expression since the mutants used are actually auto-immune, and thus the cause cannot be necessarily distinguished from the consequence here. I would not be surprised that other auto-immune mutants (independent of being related to calcium signaling) show similar constitutive expression of these TIR genes.

Finally, as a minor point, in line 89, "PAMP elicitors" should be "PAMPs" as these are by definition elicitors.

Author Rebuttals to Second Revision:

Responses to referees' comments:

Referee #1 (Remarks to the Author):

The authors have mostly satisfactorily addressed my comments.

As the authors postulate that non-induced TNLs are likely "unfonctional", it would be have been nice to test this experimentally by testing if their over-expression induces cell death or not.

>>> Whether non-induced TNLs contribute to PTI will be tested in the future.

The authors should also state in the text that the data with the cngc mutants do not necessarily mean that calcium influx is "sufficient" to induce TIR gene expression since the mutants used are actually auto-immune, and thus the cause cannot be necessarily distinguished from the consequence here. I would not be surprised that other auto-immune mutants (independent of being related to calcium signaling) show similar constitutive expression of these TIR genes.

>>> We have modified the text as suggested (Line 173-174: Whether the expression of these *TIR* genes are induced directly by the elevated Ca^{2+} levels or indirectly by the constitutive defense responses in *cpr22* and *cngc20-4* remains to be determined).

Finally, as a minor point, in line 89, "PAMP elicitors" should be "PAMPs" as these are by definition elicitors.

>>> This has been corrected.