

Changes in PUB22 Ubiquitination Modes Triggered by MITOGEN-ACTIVATED PROTEIN KINASE3 Dampen the Immune Response

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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2016-00654-RA1st Editorial decision– *revision requested*

October 10, 2016

The manuscript advances the understanding of E3 ligase regulation and plant immunity. However, many major and minor points of concern are raised on the manuscript that need to be addressed. We ask you to pay attention to the following points in preparing your revision.

1. The quality of your results is high and most of the conclusions are supported by the presented data. However, broadly, there are cases where the quality of your presentations specially details on your data, repeats/replications, quantitation and statistical significance fell short and is a concern and need to be addressed in a revised version. The quality of the immunoblots shown in some of your figures need to be improved. The interpretation of the data presented and the model drawn need to be revisited to make it consistent with the complexity of your data. In some cases, conclusions were drawn without strong supporting evidence.
2. One important concern is that the PUB22 protein level in PUB22 transgenic lines used in your experiments and variants were not determined. It is not clear whether the lines accumulate PUB22 and mutated variants at similar protein levels in the same experiment. Thus, whether the dampening of plant immunity is caused by T62/88 phosphorylation of PUB22 or altered protein levels in unclear. This is a major issue that needs to be addressed.
3. Based on your results, MPK3 is upstream of PUB22 and modulating PUB22 activity. However, it is unclear how loss-of PUB22 function determines the extent to which flg22 stimulation regulates MPK3 and MPK6 signaling. These may require an additional experimentation.
4. Your observation suggests PUB22 is constitutively degraded due to trans-ubiquitination of homodimers and that activation of MAPKs by PRR signaling promotes the accumulation of monomeric PUB22 for substrate degradation. This conclusions need to be better supported by testing whether flg22 stimulation affects PUB22 homodimerization in a MPK3-dependent manner.

5. The cycloheximide experiments conducted using the PUB22-GFP also missing the appropriate controls. To support the conclusion that flg22 peptide stabilizes PUB22, comparison of cycloheximide treated +/- flg22 peptide will be necessary in the same experiment.
6. Discrepancies in the description and the corresponding figures need to be checked and corrected. Please make sure you go through all of the reviewers' comments and address in a revised version.

[Reviewer comments shown below along with author responses]

TPC2016-00654-RAR11st Revision received

January 4, 2017

Reviewing Editor's comments and **author responses**:

Point 1. The quality of your results is high and most of the conclusions are supported by the presented data. However, broadly, there are cases, where the quality of your presentations specially details on your data, i) repeats/replications, ii) quantitation and statistical significance fell short and is a concern and need to be addressed in a revised version. The iii) quality of the immunoblots shown in some of your figures need to be improved. The iv) interpretation of the data presented and the model drawn need to be revisited to make it consistent with the complexity of your data. In some cases, conclusions were drawn without strong supporting evidence.

RESPONSE: We are grateful for the positive comments on our work. We have amended the points as follows:

Repeats/replications: Information has been included as requested.

Quantitation and statistical significance: For protein-stability, we have included quantification (Figures 1B, 1C, 1H, 3A and 3B) and statistical analysis (Figure 1H, 1B Supplementary Figure 7B) of the data. Furthermore, we have modified the labeling in Figure 7B and replaced it by a letter code to indicate statistically significant differences.

Quality of the immunoblots: We have replaced the blots as requested (Figure 2E) and included additional data (Figure 2F) that corroborate our claims.

Interpretation of the data: We elaborated in the Discussion the points addressed by Reviewer #1 and included additional data that strengthen our conclusions.

Point 2. One important concern is that the PUB22 protein levels in the PUB22 transgenic lines used in your experiments and variants were not determined. It is not clear whether the lines accumulate PUB22 and mutated variants at similar protein levels in the same experiment. Thus, whether the dampening of plant immunity is caused by T62/88 phosphorylation of PUB22 or altered protein levels is unclear. This is a major issue that needs to be addressed.

RESPONSE: In our study we show that PUB22 stability, and therefore its accumulation, is linked to its autoubiquitination activity by performing detailed biochemical analyses. Stable transgenic lines expressing PUB22 and mutant variants were characterized in depth and consistently showed the expected differences in protein amounts. In addition, we show the response of PUB22 and its variants after flg22 treatment in Figure 3F. In the revised manuscript we have included additional quantitative data that support our findings and are in agreement with all our prior observations (Supplementary Figure 7B and 7C).

Point 3. Based on your results, MPK3 is upstream of PUB22 and modulating PUB22 activity. However, it is unclear how loss of PUB22 function determines the extent to which flg22 stimulation regulates MPK3 and MPK6 signaling. These may require additional experimentation.

RESPONSE: Our previous work uncovered Exo70B2, a subunit of the exocyst complex, as a target of PUB22 (Stegmann et al. 2012 TPC). Our unpublished data has confirmed that PUB22 and PUB24 target additional components of the vesicle trafficking machinery with direct effect on the signalling output of immune receptors. As a consequence, phenotypes in pub22 pub23 pub24 are, at least in part, the result of missing negative regulation on

components upstream from MPK3, thus placing PUB22, genetically upstream of MPK3. Although, biochemically, PUB22 is downstream of MPK3, as it is phosphorylated by MPK3.

We have included the following sentence in the discussion to highlight this aspect: "Enhanced MAPK activity in the *pub22 pub23 pub24* background may instead be the result of the accumulation of substrates such as Exo70B2 (Stegmann et al., 2012), which potentially control the levels of signalling components upstream of MAPK activation."

Point 4. Your observation suggests PUB22 is constitutively degraded due to trans-ubiquitination of homodimers and that activation of MAPKs by PRR signaling promotes the accumulation of monomeric PUB22 for substrate degradation. This conclusion needs to be better supported by testing whether flg22 stimulation affects PUB22 homodimerization in an MPK3-dependent manner.

RESPONSE: We agree with the reviewer and have included additional results showing that the oligomerization of PUB22 is dependent on MPK3 (please see Figure 5H).

Reviewer #1

This manuscript presents interesting data but there are several missing experiments and missing demonstrations of replication, quantitation and statistical analyses. In some cases I am not convinced by some the data shown. Examples where the ms could be improved by these types of analyses are detailed below.

Point 1. Some of the data don't quite fit together in a clean model and it seems the authors tend to gloss over these complexities/complications. For example, the T62/88A PUB22 mutant does not stabilize (Fig 3E), yet the differences in autoubiquitination compared to WT are not convincing (Fig 4B).

RESPONSE: We agree with the reviewer that this aspect was not explained by our model and not addressed appropriately in the manuscript. We have therefore included the following statement for clarification: "However, the inability of the non-phosphorylatable variant to stabilize after the activation of immune signalling in vivo, although it displays reduced autoubiquitination in vitro, may suggest an additional layer of regulation. We show that PUB22 can heterodimerize with PUB24, opening the possibility that heterodimerization with related PUBs is not significantly affected in the T62/88A variant in vivo and thus its instability is maintained. A second possibility is that the phosphorylated variant is recognized by a ubiquitin-specific protease and contributes to its stabilization as shown for TRIM25 and TRAF6 (Pauli et al., 2014; Lin et al., 2015a)."

We have also included this aspect in the model in Figure 8 by adding a potential additional –unknown– regulatory element.

Point 2. I was confused regarding the relationship between PUB22 and MPK2/3/6. The model shows MAPKs in general, while the work shows MPK3 is the only one of the group phosphorylating PUB22. Then what is the role of enhanced MPK activation in a *pub* mutant background? How does that fit in with the model?

RESPONSE: Because we could not detect ubiquitination of PUB22 by MPK3 in an in vitro assay, we hypothesize that the phenotype observed in the *pub22 pub23 pub24* mutant is a result of accumulated substrates, such as Exo70B2 and others (unpublished), which may affect the signalling by components upstream of MPK3, and thus influence also the activation of MPK6. Subsequently, phenotypes in *pub22 pub23 pub24* are, at least in part, the result of missing negative regulation on components upstream from MPK3, thus placing PUB22 genetically upstream of MPK3, although, biochemically, PUB22 is downstream of MPK3, as it is phosphorylated by MPK3. We have included the following text for clarification: "However, the enhanced MAPK activity in the *pub22 pub23 pub24* background is most likely the result of the accumulation of substrates such as Exo70B2 (Stegmann et al., 2012), which may control the levels of signalling components upstream of MAPK activation." In addition, we elaborated the legend of Figure 8, to include this aspect: "4) Increased stability of the monomeric PUB22 allows it to engage substrates such as Exo70B2 (blue), which are likely to control signalling up-stream of MPK3."

Point 3. Figure 1B. The authors present data that MG132 slows the degradation of PUB22. In the figure it looks like there is significant loss in MG132, which while reasonable because often MG132 does not block 100%. What concerns me is that this loss makes it hard to compare +/- MG132 given that the zero time points are over-exposed. So, I think for the authors to make this claim, the authors should quantitate the data and show the results from several independent experiments.

RESPONSE: We have replaced the blot in Figure 1B by one with lower exposure time and lower band intensity. We confirmed that the band wasn't overexposed using ImageJ and by determining the maximal density values for the samples 1) and 4), which displayed the strongest signal. We have also quantified the percentage of the remaining signal intensities compared to the initial value. PUB22 stabilization after MG132 treatment is also supported by results shown in Figures 1F and 1I.

Point 4. Figure 1B. In the presence of flg22 peptide, there is rapid loss of PUB22-GFP in the presence of cycloheximide. If the authors want to conclude that flg22 peptide stabilizes PUB22, then the experiment should be to compare cycloheximide treated +/- flg22 peptide. PUB22 should be lost even more rapidly in the presence of cycloheximide without flg22 than with flg22.

RESPONSE: We thank the reviewer for pointing this out. Additional data showing the stabilization of PUB22 by flg22 in the presence of CHX was included (Figure 1C).

Point 5. The authors write that this effect is seen within 5 min, precluding a transcriptional effect. But what about a translational effect? That could be rapid.

RESPONSE: We cannot completely rule out an effect on the translational process. However, to the best of our knowledge, mechanisms controlling mRNA translation require the endogenous UTRs; constructs used in this study contained only the coding sequence. Direct regulation of ribosomal translation rates, such as through GCN4 in yeast, are more likely to affect global protein translation rates.

Point 6. The authors demonstrate PUB22 autoubiquitination in vitro assays and identify the sites of ubiquitin attachment. They then IP in vivo expressed PUB22 to "confirmed that PUB22 autoubiquitinated in vivo". However, in vivo there are other ligases present, so how does this experiment demonstrate autoubiquitination? Maybe it is ubiquitinated by an unrelated ligase, especially given the result in Fig 3D.

RESPONSE: We agree with the reviewer. Given the fact that PUB22 can heterodimerize with PUB24 and the W40A variant still is degraded, it would be inaccurate to state the above. We have changed the passage to "confirmed that PUB22 is ubiquitinated in vivo".

Point 7. As in Fig 1B, Fig 1F needs quantitation with replication and statistics. The loads are so different between the lines that it is hard to compare the time courses. If these are done by film, the authors should be careful to make sure their exposures are in the linear range of the film for quantitation. The W40A does show detectable loss. Comparison of separate transgenic lines does not prove that the W40A protein is more stable.

RESPONSE: To confirm the differences in stability, we included quantification of relative protein amounts and statistical analysis, which show that the W40A PUB22 variant is more stable (please see Figure 1H).

Point 8. Further demonstration of autoubiquitination - the authors should perform the experiment in Fig 1E with the W40A mutant- show no ubiquitination of mutant PUB22.

RESPONSE: To visualize the ubiquitination of PUB22, high amounts of the protein need to be immunopurified. The very low amounts of PUB22 under basal (non-induced) conditions therefore preclude this experiment. We have previously attempted to carry out the experiment in Figure 1F without pre-treating plants with flg22, but were unable

to obtain sufficient amounts of GFP-PUB22. The use of a proteasome inhibitor is also not possible, as it will bias the analysis.

Point 9. Figure 3. The authors show that stabilization does not occur in the *mpk3* mutant background and when MPK3 is over-expressed, the authors write, "Furthermore, expression of MPK3 resulted in elevated basal levels of PUB22 (Figure 3A), indicating that MPK3 is required and sufficient to induce PUB22 stabilization." To support this claim, the authors need quantitation and replication. The differences in 3A seem small to me and could just represent variation in loading and western processing that can be seen. MPK3 should not be active, so this result, if substantiated, is a bit of a surprise.

RESPONSE: We appreciate the reviewer's point and have included additional quantitative data using a luciferase-PUB22 fusion protein, which corroborates MPK3's function in the stabilization of PUB22 (please see figure 3B). Band intensities in Figure 3A were additionally analysed, and relative amounts calculated to show the difference between the eight different experimental combinations. We agree that MPK3 should theoretically not be active. However, additional expression of MPK3 may lead to the stabilization of PUB22 because of an unfavourable stoichiometry to negative regulators (phosphatases), and/or increased basal activation of signalling due to the protoplastation.

Point 10. Figure 3B. There are higher levels of MKK5DD than RR. Could this confound interpretation of the result?

RESPONSE: We have replaced the figure with results obtained using constructs under the control of a constitutive promoter (please see Figure 3C).

Point 11. Figure 3D. Again, hard to compare lines when the initial load is different. Ones with less protein to start with have less at the end, but does that mean their stability is different? The rates should be quantitated.

RESPONSE: We quantified the degradation of WT and W40A variants of PUB22 and confirmed the faster degradation of WT (please see figure 1G and 1H).

Point 12. Fig 6B. The IB for Ub marks the smear U-box-Ub(n). What is the evidence that these are attached to the Ubox and not also E2-Ubn smear as well as other proteins in the protein preparations? The pattern does not match the top blot. These blots need size markers. In this figure, the lane with W40A alone contains HMW species (top); what are those? They co-migrate with species in the 3rd lane, if they are not ubiquitinated species, what are they? This result is confusing. The protein species without an active ubiquitination reaction should be run in adjacent lanes so we can see what the proteins look like in a (-) reaction.

RESPONSE: The lower panel (IB:Ub) in the lower molecular range (>25kDa) displays three bands. Two UBC8 forms conjugated to ubiquitin can be detected (corresponding to the upper and lower bands; UBC8-Ub1 26kDa and UBC8-Ub2 34,5kDa). Upon re-examination we detected a mislabelling and have corrected it. The identity of the middle band is, however, difficult to determine; it may be a tri-Ub peptide.

The space in between the lower molecular bands (>25kDa) and the higher molecular ones (>45kDa) is a clear indication that the upper species are not polyubiquitinated E2 forms. The higher molecular species start at the expected size of a mono-ubiquitinated form of the U-box (49,3kDa). In addition, the clear laddering pattern indicates that it is not random ubiquitination of impurities. Because the U-box also autoubiquitinates in the presence of the W40A variant, polyubiquitinated U-box can be observed in the 3rd lane.

Reviewer #2

This manuscript presents findings that suggest a possible MPK3-mediated negative feedback loop in the plant U-box type E3 ligase, PUB22-mediated immune signaling. Earlier publications by the authors suggested that PUB22/23/24 act as a negative regulator of PTI by ubiquitinating the exocyst complex subunit Exo70B2. In this report, the authors

present data to show that PUB22 can undergo autoubiquitination and subsequent degradation in the cell. As a negative feedback to prevent unchecked immune responses, the MPK3 phosphorylates PUB22 at two sites (one is localized within the U-box domain, the other one is localized at a disordered region), which stabilizes PUB22 from autoubiquitination and subsequent degradation and thus promotes the negative effect of PUB22 on immune signaling. The authors have presented a large volume of data in this manuscript, and a MAK3-mediated negative feedback loop would be a novel finding if it is supported by convincing data. That said, I have a few concerns regarding the data presented.

Point 1. In the earlier publication by the authors (Curr. Biol 2008), PUB22/23/24 were shown to be highly induced 1h after PAMP treatment, which raise the question that the increased accumulation of PUB22 is due to PAMP triggered induction of the PUB22 gene expression, or due to stabilization by MAPK3 phosphorylation, or both? The author should present a time course of qPCR results of the expression of the *PUB22* gene upon flg22 treatment.

RESPONSE: We and others have previously shown that PUB22, PUB23 and PUB24 and other related E3s are rapidly induced by a wide array of PAMPs and also biotic stresses (Navarro et al. 2004, Plant Physiology; Trujillo et al. 2008, Current Biology; Yee and Goring 2009, Journal of Experimental Biology). Our results indicate that both stabilization and gene induction are required for PUB22 to accumulate. Notably, we show that stabilization is a prerequisite, even when expressed under the control of a constitutive promoter (Figure 3F). The same holds true for the complementation of the *pub22 pub23 pub24* phenotypes by the non-phosphorylatable form of PUB22 (Figures 7A and 7B). These results, therefore, uncouple protein accumulation driven by gene expression from protein stabilization resulting from reduced degradation.

Point 2. Additionally, it is likely that phosphorylation of PUB22 by MPK3 promotes its E3 activity towards the substrate Exo70B2 (Figure 4D upper panel), which attenuates the immune signaling. And the accumulation of PUB22 might be due to transcriptional induction but not stabilization.

RESPONSE: We agree with the reviewer in that by inhibition of autoubiquitination through phosphorylation, PUB22 is able to dedicate its activity towards a substrates, such as Exo70B2. This dedicated activity may be reflected by an increase in ubiquitination of Exo70B2 in vitro, as seen in Figure 4D. Moreover, the addition of ubiquitin chains onto the E3 may also result in a steric hindrance towards substrates in vitro, and therefore, reduced autoubiquitination may also contribute to enhanced interaction. As described above (1.), our data indicate that stabilization is a key event that allows PUB22 to accumulate.

Point 3. Finally, in several figures (such as Fig 2E), the difference between controls and the construct tested is marginal in the western blots, which makes it hard to draw a clear conclusion. This is especially worrisome if the experiment was performed only once without extra repeats. Furthermore, as it is common sense that different exposure times can change the signal strength when performing western blotting, the authors should run samples to be compared in the same gel and check the western blot signal on the same blot for the same experiment, such as the blots in Figures 3E and 7A. The signal strength cannot reflect the protein level to be compared in Figure 7A, as they are on separate blots thus likely have different exposure times.

RESPONSE: We have replaced this figure and show a clear MPK3-dependent mobility shift of PUB22 (please see Figure 2E and 2F). We agree with the reviewer and apologize for the misunderstanding. For comparability, we always run PAGEs in parallel and blot the gel sections containing the protein of interest simultaneously onto the same membrane. Hence, exposure time is identical. We have amended the figure legend accordingly and included additional information in the materials and methods section.

Reviewer #3

Furlan et al. describe the mechanism by which PUB22 accumulates upon PAMP stimulation. Using in vitro and in planta interaction studies, they nicely demonstrated the specific interaction and phosphorylation of PUB22 by MPK3 at T62 and T88. The authors then showed that accumulation of PUB22 depended on MPK3 and the phosphorylation

sites T62 and T88. In vitro studies further revealed that auto-ubiquitination of PUB22 was reduced by active MPK3 and mutation of T62, and that the latter had only little effect on PUB22 ubiquitination of Exo70B2, a substrate of PUB22. Using in vitro and in planta interaction studies, the authors then addressed PUB22 homodimer formation and identified, among others, that mutation of T62 altered its ability to self-associate. They then showed that PUB22 trans-ubiquitinated itself and that the auto-ubiquitination levels of PUB22 correlated with the extent to which PUB22 self-associated. Expression of PUB22 and mutated PUB22 in *pub22 pub23 pub24* plants showed partial reduction of flg22-triggered MPK3/6 activation, which appeared stronger in plants expressing mutated PUB22. The authors then performed bacterial infections and showed that *pub22 pub23 pub24* mutants expressing PUB22 and T62/T88E but not T62/T88A mutated PUB22 supported higher bacterial titers, suggesting the physiological importance of PUB22 phosphorylation by MPK3 in anti-bacterial immunity.

I agree that this manuscript significantly advances the understanding of modes of E3 ligase regulation and beyond, including the regulation of plant immunity. The experiments were well-designed and carefully conducted, including the appropriate controls. The data were properly analyzed and quantified. Overall, the quality of this study is high and most of the authors' conclusions are supported by the presented data.

Point 1. A key finding of this study is presented in Figure 7. One major criticism is that studies carried out in stable transgenic lines were done without showing whether the lines accumulate PUB22 and mutated variants at similar protein levels in the same experiment. The authors examined PUB22 transcript levels in these lines (Figure S5), stating that transcript accumulation was similar across the lines. However, Figures 3 and S5 suggest differences in steady state transcript and protein levels. It therefore remains elusive whether the dampening of PTI is caused by T62/88 phosphorylation of PUB22 or altered protein levels.

RESPONSE: In our revised manuscript, we included additional quantitative data regarding the accumulation of PUB22 and mutant variants. This data substantiates previous observations and confirms that minor differences in transcript levels are not reflected by protein accumulation. For instance, the variants W40A and T62E display lower levels of transcript compared to WT, but accumulate higher levels of protein (please see Supplemental Figure 6). Therefore, our in depth characterization of stable transgenic lines consistently show throughout, the expected differences in protein amounts.

These results are further substantiated by detailed biochemical analyses showing that PUB22 stability, and therefore its accumulation, is linked to its autoubiquitination activity. In the revised manuscript, we have included additional data further supporting the proposed mechanism as suggested by the reviewer.

Point 2. Given that MPK3 is upstream of PUB22 and the specific MAPK modulating PUB22 activity, it remains unclear how then loss-of PUB22 function determines the extent to which flg22 stimulation activates (and prolongs?) MPK3 and MPK6 signaling. Have the authors tested for MPK3 and MPK6 protein levels in these lines?

RESPONSE: We have indeed considered this possibility earlier on. However, already in our previous work we did not observe any changes in MPK3, MPK4 or MPK6 levels (Trujillo et al. 2008). However, additional studies from our group uncovered Exo70B2, a subunit of the exocyst complex, as a target of PUB22 (Stegmann et al. 2012 TPC). Our unpublished data has confirmed that PUB22 and PUB24 target additional components of the vesicle trafficking machinery with direct effect on the signalling output from immune receptors. As a consequence, phenotypes in *pub22 pub23 pub24* are, at least in part, the result of missing negative regulation of components upstream from MPK3, thus placing PUB22 upstream of MPK3 genetically. However, biochemically, PUB22 is downstream of MPK3, as it is phosphorylated by MPK3. Higher amounts of PUB22, as seen for the T62E phosphomimic, most likely result in the targeting of PUB22 substrates that are upstream of MPK3, resulting in a dampened immune response.

We have included the following sentence in the Discussion to better explain this aspect: "Enhanced MAPK activity in the *pub22 pub23 pub24* background may instead be the result of the accumulation of substrates such as Exo70B2 (Stegmann et al., 2012), which potentially control the levels of signalling components upstream of MAPK activation."

In addition, we elaborated the legend of Figure 8 to include this aspect: "4) Increased stability of the monomeric PUB22 allows it to engage substrates such as Exo70B2 (blue), which are likely to control signalling up-stream of MPK3."

Point 3. It also remains unclear why PtoDC3000 devoid of the AvrPto and AvrPtoB effectors was used for the infection experiments. As these effectors target the PRR complex, do the authors suspect that loss-of PUB22 function alters PRR complexes e.g. abundance?

RESPONSE: From our previous and ongoing work, resistance phenotypes in PUB E3 mutant lines are easier to detect using the *P. syringae* DC3000 Δ AvrPto/AvrPtoB. We have shown that PUB22, PUB23 and PUB24 negatively regulate PAMP-triggered signalling. In addition, as mentioned above, PUB22 and related E3s may be linked to receptor-mediated signalling via regulating the vesicular traffic machinery. As pointed out by the reviewer, AvrPtoB has been shown to target receptor-like kinases (FLS2), as well as kinases (Fen), thus hampering signalling. One possible explanation for the suitability of the Δ AvrPto/AvrPtoB strain in our hands is that its reduced capability to hamper receptor-triggered signalling improves the traceability of PAMP-triggered signalling dependent phenotypes in PUB mutants.

We have included the following sentence to explain our choice: "Reduced virulence of this strain improves the detection of PAMP related phenotypes."

Point 4. Furthermore, the authors present a model in which PUB22 is constitutively degraded due to trans-ubiquitination of homodimers and then the activation of MAPKs by PRR signaling promotes the accumulation of monomeric PUB22 for substrate degradation. I think it is possible to better support this model by testing whether flg22 stimulation affects PUB22 homodimerization in an MPK3-dependent manner. Current data presented in Figure 5 show constitutive homodimer formation, which is reduced by T62A and T62E mutations (both phosphonull and phosphomimic) as well as enhanced by T62I mutation. Likewise, both phosphonull and phosphomimic T62A and T62E mutations reduce PUB22 auto-ubiquitination activity (Figure 4C), while T62I mutation enhances auto-ubiquitination of PUB22 (Figure 6C). However, phosphomimic T62/88E but not phosphonull T62/88A complements the increased bacterial resistance in *pub22 pub23 pub24* mutants (Figure 7B).

RESPONSE: In our revised manuscript, we have included data showing that the dimerization of PUB22 U-box is MPK3-dependent (please see Figure 5H). For this, we expressed the PUB22- U-box split luciferase constructs with MKK5 DD in Col-0 WT or *mpk3* background. Results corroborate our model in which MPK3 controls PUB22 oligomerization.

Point 5. Another major criticism is the quality of the immunoblot analysis shown in Figure 2E.

RESPONSE: We have replaced this figure by a result showing a clearer difference with improved quality (please see figure 2E) and included additional data showing the lack of phosphorylation of PUB22 in the *mpk3* background (please see figure 2F). We also provide new mass-spectrometric data that confirm the phosphorylation of T62 and T88 in vivo (please see Supplementary Figure 4).

TPC2016-00654-RAR12ndEditorial decision – accept with minor revision

February 13, 2017

The manuscript was improved and the comments to the previous version were addressed to a large extent. As you will see from the comments by the reviewers, there are still issues that need to be resolved. Reviewer #1 has the most concern with discrepancies in the data shown in the different Figures as well as the significance of differences which led to some of your conclusions. A serious concern was raised by reviewer #1 (point 5) that you have to respond to. You may be able to address some of these with quantitation of the data and providing explanations for the discrepancies and inconsistencies pointed out or present additional experimental results. Reviewer#2 also has specific questions regarding the use of E2 that at the minimum require a thorough discussion.

[Reviewer comments shown below along with author responses]

TPC2016-00654-RAR22ndRevision received

February 17, 2017

Reviewer comments and **author responses**:

Reviewer #1:

The story is interesting and there are several experiments that are convincing. However, the study still lacks required quantitation that once obtained could affect interpretation of the data (see points 2 and 5), and there is an internal inconsistency that needs to be dealt with (see points 3 and 6).

Point 1. Figure 1B. These data are very confusing to me. In Figure 1A, it was shown that flg22 lead to increase in GFP-PUB22. The hypothesis is that flg22 treatment results in less degradation of PUB22. But then the text says, lets test if PUB22 is unstable using CHX and using the data in Figure 1B with a CHX time course, the text says that "PUB22 protein levels were markedly reduced at 30 min and almost depleted 60 min after cycloheximide (CHX) treatment, indicating active turn-over (Figure 1B)." But at the top of the lane is indicated that flg22 is present. Flg22 should lead to stabilization of the protein (according to the hypothesis). Treatment with CHX + flg22 should lead to slowed degradation. I don't understand this experiment. Why treat with flg22?

RESPONSE: We apologize for the confusion and are grateful to you for pointing this out. The labelling of the figure refers to the induction of the plants which carry GFP-PUB22 under control of its native promoter. The treatment with flg22 is required to induce GFP-PUB22 expression. Seedlings were rinsed before adding CHX or CHX/MG132. We have removed the flg22 treatment labelling and elaborated the description of the experiment in the legend (please see Figure 1B and line #916).

Point 2. It seems that the right part of Fig 1C is the same as the left time course in 1B. CHX chase with flg22 present. There is a bit of spread in the data. 1B: 30 min =34% left 1C: 30 min=49% left.

RESPONSE: Figure 1B was carried out using native promoter lines, while Figure 1C shows lines with a ubiquitin promoter. Differences between the experiments can be explained by, i) different promoters driving expression endogenous vs ubiquitin10, and ii) residual flg22 in the tissue of seedlings after rinsing (Figure 1B).

Point 3. I don't have any problems with there being some differences, but this is why we replicate experiments, generate curves from multiple experiments and ask if the curves are different using statistics. I would like to see such an analysis here. Curves generated from replicated experiments comparing the degradation rate of PUB22 in CHX chase assays in plants either treated or not with flg22. It is clear that flg22 does not completely stabilize PUB22, so there is a slowing of the degradation rate, what is the difference? What is the effect? Is it a 2-fold slowing? This rate difference could be of great biological interest.

RESPONSE: In principal, we agree with the reviewer and have included the quantification of the degradation as previously requested (Figure 1H). We also agree that calculating the degradation rate may be informative. However, we ask the reviewer to consider that the degradation of PUB22 is very fast, leading to a rapid decrease in protein levels, already within 15 min. In addition, basal levels of the protein are very low. Taking this into account, we have described the degradation of PUB22 in the best possible way, within our technical possibilities, and shown that it significantly differs between PUB22 versions. Having said this, I disagree regarding the relevance and significance of determining the exact degradation rate, because the main point remains that there is a difference between the PUB variants and it has a biological impact (see Figure 7).

Point 4. By writing (line 171), "agonist-triggered stabilization" one imagines that the protein is stable, but its not, its degradation has been slowed. Maybe the text here could more accurately describe the effect.

RESPONSE: We respectfully disagree. We employ the term "stabilization" to refer to the process and not to a state and therefore believe that it is appropriate.

Point 5. It seems appropriate to test the ubiquitination ability of the W40A, such as the experiment in 1F, using the W40A form, showing that ubiquitinated forms cannot be seen for W40A when one can see them for WT in cells.

RESPONSE: As pointed out in our previous response, in order to compare the levels of ubiquitination relative to the inactive W40A mutant, high amounts of the WT protein must first be immunopurified to visualize its ubiquitination. The amount of PUB22 under basal (non-induced) conditions is very low, precluding this experiment. The use of a proteasome inhibitor is also not possible, as it will bias the analysis.

Point 6. At the time of Figure 1F, one cannot conclude that the observed ubiquitination is from self ubiquitination. Going from ubiquitination in a purified in vitro system and showing it is ubiquitinated when extracted from plants does not prove that the ubiquitination seen in vivo comes from self ubiquitination. This statement at this time in the story should be modified. "To confirm the function of autoubiquitination in the self-catalysed degradation of PUB22," It's a hypothesis that needs a test. The data shown in 1G and H is a good test of that hypothesis to show E2 interaction needed for instability. But there are issues.

RESPONSE: The text has been modified to "test" instead of "confirm" (please see line #164).

Point 7. Figure 3 present data on the relative stabilities of phosphomimic version. As mentioned in 2 above, the fig22 effect seems be ~2-fold. So, one would expect the phosphomimics to still be degraded, but only 2-fold more slowly because they are stabilized 2-fold relative to the form that cannot be phosphorylated. So, this is where quantitation of the degradation rates would strengthen the data and lead to more robust conclusions. For example, line 270-271, "Notably, mutant variants are still degraded, including the inactive W40 mutant, suggesting that E3 turn-over is mediated by additional factors." But I would argue that we expect the effect to be modest since the effect of fig22 is modest (figure 1-see #2 above).

RESPONSE: We respectfully disagree, as can be seen in Figures 1A, 1C, 1F, together with the effect of MPK3 function shown in 3A, 3B and 3C, and the results with the PUB22 variants, all of which support the significance of the described mechanism. We would also like to argue that a reduction of the degradation rate by half is significant, and must be viewed in combination with the induction of PUB22 transcription during the immune response, which in combination leads to marked accumulation of the protein.

However, we show that mutant variants accumulate different levels of protein, while having comparable levels of transcript, in agreement with our proposed mechanism. As mentioned above, we have described the degradation of PUB22 WT and variants in the best possible way, within our technical possibilities. However, the high degradation level combined with low protein amounts limit our options. One of the reasons that we use the Ubiquitin10 promoter was to avoid massive overexpression in order to obtain lines expressing physiological concentrations. On the downside, these lines do not provide a large window of detection.

Furthermore, our study addresses these issues from many angles and experimental approaches, and even though a degradation rate may be informative, based on the ample data provided, it is highly unlikely to result in an alternative interpretation.

Point 8. The differences between the data presented in Fig 1G and 3E are troubling and led to differing conclusions. Why is the W40A mutant stable in Fig 1G but not in figure 3E? Again, maybe there is a quantitative difference that is not seen in 1G with just one short time point? Anyway, the instability in 3E argues against the conclusion that degradation is cis-mediated because a form that cannot be self-ubiquitinated is unstable as presented in 3E. These data must be reconciled better than presented and discussed here.

RESPONSE: We respectfully disagree. The results shown in figure 1G indicate that 17% of PUB22 W40A is degraded within the first 15 min. We acknowledge that the reduction is not statistically significant at this early time point. However, it is incorrect to extrapolate this observation to argue that at a later time point (30 min, Figure 3E), this reduction will not have increased. The reason for analysing protein levels at 15 min is because of the rapid degradation and low levels of the WT protein.

In regard to the reviewer's second point, which states that a continued degradation of the variants argues against the proposed mechanism relying on ubiquitination in trans, two things need to be considered. First, regulatory switches, especially when they involve changes in an interaction, do not necessarily result in all or nothing responses; they can involve subtle shifts in affinity. We confirm these changes in the interaction of PUB22 (Figure 5F, 5G and 5H). Notably, all variants still interacted, and hence, will still be able to trans-autoubiquitinate. Secondly, we also show that PUB22 can form heterodimers (Figure 5E). This opens the possibility that the stability of PUB22 is

interconnected with other E3 ligases which can contribute to the instability of the mutant variants. This possibility is included in the Discussion (please see lines #584 - 594).

Reviewer #2:

The authors answered most of the concerns by the reviewers and editor in this revised manuscript. However, I still have a few concerns.

Point 1. As the authors mentioned in the Discussion and the data presented in Fig 3E and 3F suggests, there are additional factors that regulate the accumulation/degradation of PUB22, which raises the question of how significant is the mechanism the authors are trying to prove in this manuscript to the PUB22-mediated immune signaling and other U-box type E3s-mediated signaling in general; is it a major or just minor contribution? Moreover, the authors' data could not rule out the impact of PUB22/23/24 on the MPK3 (see the following paragraph).

RESPONSE: We would argue that a protein which is subject to many levels of regulation, functioning in an interdependent network, is not an argument against, but rather a confirmation of its relevance, and thus, of the mechanisms that control its activity. In addition, we contend that the relevance of a function lies not within the "amplitude" of single observations, but in the overall impact. Our work uncovers a direct route of crosstalk between different types of PTMs, which, as we show in Figure 7, is relevant for the dampening of the immune response.

Point 2. There are two possible biochemical consequences of interaction of MPK3 and PUB22, MPK3 phosphorylates PUB22 or PUB22 ubiquitinates MPK3. The authors ruled out the latter by an in vitro ubiquitination assay (Suppl Fig 3B). However, the failure to ubiquitinate MPK3 and MPK6 by PUB22 in that experiment might be due to the E2 they used. As we know, E3 is the major governing factor for substrate specificity. But E2 also contributes to the process. In other words, they may have not used the right E2 for the reaction. The authors thus need compare in vivo the ubiquitination status of MPK3 in WT and *pub22/23/24* in the presence of inhibitors for deubiquitination and proteasome.

RESPONSE: We agree with the reviewer in that the modification of MPK3 by PUB22 cannot fully be ruled out. However, three points argue against this: i) We have previously analysed MPK3 levels and have not detected any changes in the protein levels, nor shifts in size that would be indicative of ubiquitination (Trujillo et al. 2008 and manuscript submitted elsewhere). Also, in spite of a large community of people working on MAPKs, I am not aware of any reports suggesting the ubiquitination or altered proteins levels due to activation of the immune response ii) The E2 used for the in vitro ubiquitination assays was UBC8, which is highly processive and was employed successfully to detect the ubiquitination of Exo70B2 as shown in this and previous work (Stegmann et al., 2012). In addition, the same E2 was also used to determine the in vitro ubiquitination of the FLS2 kinase domain by PUB12 and PUB13. iii) Finally, although the interaction and phosphorylation of PUB ligases by kinases is a common theme (Gu et al., 1998; Samuel et al., 2008; Mbengue et al., 2010), there is only one reported exception to date, which is the ubiquitination of FLS2 by PUB12 and PUB13 (Lu et al., 2011). It is therefore conceivable that the PUB-Kinase association results, at least some cases, in a one-way modification.

Point 3. Furthermore, to rule out effect of PUB22 on the MPK3, the authors need examine if there is similar prolonged MPK3 activity in the UBQ10::GFP-PUB22 variants/*pub22/23/24* lines (those lines used in Fig 7B)? It is also possible other U-box E3 such as PUB23 and PUB24 form heterodimer/oligomer with PUB22 to modify MPK3 (there have been several reports that RING/U-box E3 forms homo- or heterodimer to catalyze ubiquitination).

RESPONSE: We believe that the time points chosen for the analyses suffice for our conclusions. To analyse the duration of MPK activity may be interesting, but would not give us additional information.

Point 4. According to the data presented and the authors proposed in Fig 8, phosphorylation of PUB22 helps stabilize PUB22 monomer that ubiquitinates Exo7B2 for degradation. A key piece of data is missing to support the working model: the author need to show the level/accumulation of Exo7B2 in the mutant lines they used in 7B by transient expression. The working model cannot explain the prolonged MPK3 activity in the *pub22/23/24* mutant.

RESPONSE: We would like to point out that we have previously shown that PUB22 targets Exo70B2 for degradation and that *exo70B2-3* plants display reduced MPK3 activity (Stegmann et al. 2012). It seems therefore, reasonable to propose in a model that Exo70B2 degradation takes place after stabilization of the E3. The immune phenotypes

resulting from the expression of PUB22 and the mutant variants in the *pub22 pub23 pub24* background (Figure 7) also support this.

Point 5. The authors proposed that phosphorylation of PUB22 by MPK3 inhibits the autoubiquitination of PUB22 and hence degradation by the 26 proteasome. However, in Fig 4B the T62/88E has slight higher autoubiquitination than T62/88A at 2 h (upper panel, IB:PUB22). It appears to me the T62/88E mutation reduced the processivity of the ubiquitination reaction but not the activity of PUB22.

RESPONSE: We respectfully disagree; changes in the processivity regarding the autoubiquitination or substrate ubiquitination cannot be assessed within the frame of our experiments. This would require a different type of assay (e.g. ubiquitin discharge). We can only conclude that there are differences in the autoubiquitination, and not major differences in substrate ubiquitination. In addition, the sites of the identified phosphorylation also argue against changes in the processivity because they are unlikely to affect the binding of the E2. The same holds true for the conserved basic residue, which may contribute to the conformational restriction of the E2-Ub conjugate (please see lines #524-534).

We also disagree with the observation that the autoubiquitination of T62/88E is higher than T62/88A at the later time point. The levels are comparable at the later time point, while, as pointed out, reduced at 1h in Figure 4B.

Reviewer #3:

Point 1. I appreciate that the authors included additional data and text that addressed my concerns and significantly improved the manuscript. However, the new data reveal some potential differences: Comparing lines A, T62/88A shows higher accumulation than WT in Fig3E as compared to T62/88A showing lower levels than WT. Lines B were not re-analyzed but showed lower T62/88A levels compared to WT in Fig3E. This raises the question of which lines were used in Fig. 7A and B. Ideally the authors would provide a side-by-side comparison of MPK activation and PUB22 transcript and protein accumulation from the same materials.

RESPONSE: For results in Figure 7 we used lines B, which show comparable levels of transcript. We would like to stress that the overall accumulation and general behaviour of the proteins, as also seen when expressed transiently, are all in agreement with our model, despite minor variation. We used lines B for assays and have included this information in the Materials and Methods section (please see line #619).

TPC2016-00654-RAR23rdEditorial decision – *acceptance pending*

February 19, 2017

We are pleased to inform you that your paper entitled "Dampening of the Immune Response by Changes in PUB22 Ubiquitination Modes Triggered by MPK3" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor

March 3, 2017
