A MPK3/6-WRKY33-ALD1-Pipecolic acid Regulatory Loop Contributes to Systemic Acquired Resistance

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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.)

TPC2017-00903-RA 1st Editorial decision – *declined* Dec. 28, 2018

Thank you for choosing to send your manuscript entitled "A MPK3/6-WRKY33-ALD1-Pipecolic acid Regulatory Loop Contributes to Systemic Acquired Resistance" for consideration at *The Plant Cell*. Your submission has been evaluated by members of the editorial board as well as expert reviewers in your field, and we regret to inform you that we are not able to recommend publication of this manuscript. We have not made this decision lightly. We have had input from multiple scientists, and have solicited post-review comments as well. Our present policy is to offer streamlined decisions and to not advise on the direction of the work by requesting extensive modifications or substantial additional experiments.

[Additional comments shown below along with author responses]

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2018-00547-RA Submission received July 19, 2018

Reviewer comments on previously declined manuscript and **author responses:**

Editor's comments:

1. Copied below are detailed comments from three external reviewers. All three felt that this work addresses an important gap in our current understanding of systemic acquired resistance. However, all three also felt that the data presented did not adequately support the primary conclusions of the manuscript. In particular, the reviewers expressed concerns about the dexamethasone-inducible MKK4DD construct used in several of the experiments. The primary concern is whether this construct is being expressed at equivalent levels in the different genetic backgrounds employed. To address this concern, the reviewers suggested assessing the levels of MPK3/6 activation at multiple time-points following dexamethasone application in each line.

(Our response 1) Thank you for the critical suggestion. We performed experiments to test phosphorylation of MPK3/MPK6 and protein accumulation of MKK4DD-flag and MPK3/MPK6 in all genetic backgrounds that we used in this study. The result showed that the dexamethasone (DEX)-inducible system is intact and MKK4DD-flag protein is expressed at equivalent levels in all genetic backgrounds (Figure 2B). However, we found that MAPK activation by MKK4DD is compromised in *ald1* and *fmo1* backgrounds, which is not explained by MPK3/6 protein accumulation (Figure 2B). Initially, this result was rather surprising to us because it suggests that MAPK activation triggered by MKK4DD requires the endogenous pipecolic acid (Pip) pathway. However, this result indeed supports our positive feed-back loop model between the MAPKs and the Pip pathway. We did not observe obviously compromised MAPK activation by MKK4DD in the *wrky33* background (Figure 2B). In contrast, *Pto* AvrRpt2-triggered MAPK activation was largely compromised in *wrky33* (Figure 7C). These results are consistent with the observations that MKK4DD-triggered SAR is partially retained (Figure 4C) while *Pto* AvrRpt2-triggered SAR is absent in *wrky33* (Figure 4F).

2. A related concern is that dexamethasone can spread systemically in a plant, thus a dexamethasone induction system is problematic when being used to induce a second systemic signal, as it becomes difficult to assess which signal is moving.

(Our response 2) We regret that we did not sufficiently pay attention to the GUS staining pictures. We agree that in the original GUS staining picture, GUS staining in the tip of leaves was observed only in the DEX-treated plant. However, we sometimes observed this GUS staining regardless of DEX treatment (i.e. even in mock control). Thus, we do not think that this GUS activity in the tip of leaves results from activation of the DEX-inducible system. GUS staining in the tip of Arabidopsis leaves is not uncommon since it was observed in many Arabidopsis transgenic plants (such as Danna et al PNAS 2011 9286-9291; Dhawan et al Plant Cell 2009 1000-1019; Hayes et al Plant Physiol 2010 211-221; Figueroa-Balderas et al Plant Physiol 2006 609-619). We realized that this is caused by using either old plants and/or old GUS staining solution in our hands. We have further repeated experiments with freshly-made GUS staining solution, and we did not observe GUS staining in the tip of leaves in both mock and DEX treatment (Figure S1A). Moreover, we showed that we did not detect MKK4DD transcript by RT-PCR in systemic leaves of MKK4DD plants whose local leaves were treated with DEX (Figure S1B). Therefore, we conclude that DEX does not move to systemic leaves at least to the level that directly activates strong immunity in systemic leaves.

3. More generally, the data regarding the timing of specific signaling steps appear to be inconsistent with your model (e.g. WRKY33 accumulation does not occur until AFTER *ALD1* expression, yet WRKY33 is proposed as an upstream signaling step).

(Our response 3) We apologize for that we used the poor immunoblotting result in which WRKY33-HA protein was not clearly seen at early time points, and we should have paid more attention to this. We observed WRKY33-HA accumulation at early time points in other experiments, but the immunoblotting results did not look pretty due to high backgrounds. We regret that we used the picture in the previous manuscript. To solve this issue, we have additionally repeated this experiment three times independently, and observed that WRKY33-HA consistently accumulates at 6 and 12 hours after infection before *ALD1* gene induction. We have included one representative result in Figure 6B and the other two replicates below in this letter.

4. The reviewers also felt that the large discrepancy in activation levels observed for *Pst* (*avrRpt2*) compared to MKK4DD suggests that the activation loop may be more complex than presented in the model.

(Our response 4) We agree that there is the difference in Pip levels between *Pto AvrRpt2* infection and MKK4DD system. We first hypothesized that temporal dynamics of Pip accumulation between these two systems might be different: i.e. Pip accumulates earlier or later in MKK4DD system than *Pto AvrRpt2* infection. To test this, we have determined Pip levels at five time points in MKK4DD system (Figure 2C), but the result did not support our hypothesis.

Recently, Zeier's group published a paper describing that N-Hydroxypipecolic acid (NHP), converted from Pip by FMO1, is a critical element of SAR (Hartmann et al Cell 2018 456-469). We then hypothesized that NHP accumulates at higher levels in the MKK4DD system than *Pto* AvrRpt2 infection. We detected NHP after MKK4DD activation (Figure 2C). However, we only detected very low accumulation of NHP in wild-type plants infected with *Pto* AvrRpt2 until 24 hpi in a first analysis (data not shown). *FMO1* expression level is higher in MKK4DD system than *Pto AvrRpt2* infection (Figures 3B, 4A, and 4D). Thus, NHP may accumulate at higher levels in the MKK4DD system than *Pto AvrRpt2* infection. Alternatively, the sustained and artificial MAPK activation may strongly activate downstream component(s) of Pip/NHP signaling that would enhance the sensitivity of Pip/NHP and thus lower "the threshold" of signaling metabolites necessary for SAR activation. Besides, the exact amount of Pip/NHP necessary

for SAR induction is not known. With our current method, we feel that the low levels of accumulating NHP upon *Pto AvrRpt2* inoculation cannot be reliably estimated (absolute levels), because the so far used internal standard produces a faint signal close to the NHP peak. We are currently developing methods that can accurately measure low levels of NHP by use of a more suitable internal standard (D_9-labelled NHP which we have chemically synthesized). Once we succeed, we plan to measure NHP at a broader time range with different SAR stimuli, including *Pto AvrRpt2* inoculation. Considering the time required for this development, we think that this is beyond scope of this current manuscript (its main focus on *ALD1* regulation and Pip accumulation). We do not wish to prematurely include these data.

An additional issue that should be addressed in a future manuscript is whether WRKY33 is functioning as a repressor or activator at the *ALD1* promoter.

(Our response 5) In Figures 4 and 6, we showed that *ALD1* expression is compromised in *wrky33* and WRKY33 directly binds to the *ALD1* promoter, suggesting that WRKY33 is a transcriptional activator for *ALD1* expression.

Reviewer #1:

In this report. Wang et al. present an interesting story about SA-independent SAR response mediated by MPK3/MPK6, WRKY33, ALD1, and Pipecolic acid (Pip). It is known that there are MAPK-induced responses that are frequently associated with SAR, but are SA-independent. However, the underlying mechanism(s) are unclear. As a result, the topic of this research is of broad interest.

While the story is interesting, I have major concerns about the key datasets used to support the conclusions in the manuscript.

Specific comments:

1) Most of the data were collected from single time point samples, while time course experiments would be much better to establish the timeline of events described. From the only two datasets with multiple time points (Figure 6B and Figure 7C), I can see clear inconsistency. In Figure 7C, it was shown that *Pto-avrRpt2*-induced MAPK activation is abolished in *ald1* mutants, suggesting the requirement of Pip in *Pto-avrRpt2*-induced MAPK activation. Pathogen-triggered MAPK activation is pretty fast. How fast could Pip be induced in the system? There were only numbers from 24-hour time point in the manuscript.

(Our response 6) Thank you for the critical suggestion. We have determined Pip levels at multiple time points after infection with *Pto AvrRpt2*. The result showed that Pip accumulation significantly increases at 7 hpi (Figure 4E and Figure S2B).

In addition, it was shown that WRKY33 was required for the MAPK activation in the same panel. However, in Figure 6B, WRKY33 protein was shown to accumulate very late, at 24 hours after *Pto-avrRpt2* inoculation, and there was little WRKY33 protein before that. If this is the case, how could WRKY33 be required for an earlier response, MAPK activation?

(Our response 7) Thank you for pointing this out. Please see Our response 3.

2) It was shown in Supplemental Figure 6 that Pip could only induce a very transient activation of MPK3/MPK6, lasted less than 15 min. If *Pto-AvrRpt2*-triggered MPK3/MPK6 activation is fully dependent on Pip (as shown in Figure 7C, *ald1* mutant), how could *Pto-AvrRpt2* triggered an MPK3/MPK6 activation lasted more than several hours?

(Our response 8) This is a very good point. We speculate that sustained MAPK activation in leaf tissues resulted from a mixture of cells which transiently activate MAPKs at different time points. Since no technology is available to investigate MAPK activation at the single cell resolution within a leaf tissue, we think that this issue is beyond our scope. We have added discussion of this point.

3) Is the induction of WRKY33 protein required for other responses triggered by *Pto-AvrRpt2* inoculation? In Figure 6B, WRKY33 accumulation was so late, it is hard to place the activation *ALD1* expression and then Pip biosynthesis as downstream events.

(Our response 9) Thank you for pointing this out. Please see Our response 3.

4) Related to above comments, the authors only pre-treated plants for 24 hours before testing the systemic leaves. Was there enough time for the Pip to be induced and transported to upper leaves if it is the mobile signal?

(Our response 10) We performed time course experiments for Pip accumulation after *Pto AvrRpt2* infection. Pip significantly accumulated after 7 hpi (Figure S2B). Moreover, since the local infected leaves were not removed during systemic bacterial growth assay, SAR mobile signal(s) may be continuously produced and transported to systemic leaves from the local leaves. Nevertheless, we did not claim that Pip or NHP is the SAR mobile signal in this manuscript.

5) Related to comment #4, it was shown in Figure 2B that local leaves at 2 dpi had much higher levels of Pip than at 1 dpi. What is the Pip level in systemic leaves at these time points? Why not test systemic leaves for pathogen resistance at 2 dpi?

(Our response 11) In our experimental system, the local infected leaves were not removed during SAR assay. Thus, the mobile signal(s) would continuously contribute to SAR in systemic leaves. SAR assay after two days after local infection might be better, but our results indicated that SAR assay one day after local infection is sufficient to observe SAR effects in systemic tissues. In this manuscript, our focus is local immune components that contribute to SAR. Therefore, we did not measure Pip in systemic leaves.

6) Induction of Pip by MKK4DD was much lower than that by *Pto-AvrRpt2*. Was there enough Pip translocated to upper leaves to establish SAR?

(Our response 12) We argue that Pip is required for SAR in this manuscript but not that Pip or NHP is the mobile signal. Pip or NHP may be the mobile signal but this requires further experiments, which is beyond the scope of this manuscript.

7) The induction levels of MKK4DD in all crosses after DEX treatment should be examined. Transgenes can get silenced when being crossed to T-DNA insertion mutant background.

(Our response 13) Thank you for the critical suggestion. Please see Our response 1.

8) What is the explanation for an enhanced Pip induction by *Pto-AvrRpt2* in the *sid2* background in Figure 3C? More time points showing the induction dynamics will be very useful.

(Our response 14) Thank you for the thoughtful comment. We have determined Pip levels at multiple time points (Figure 4E). This together with Figure 3C indicates that the *SID2*-mediated SA pathway negatively contributes to Pip accumulation in infected local leaves. We have included this regulation in our revised model in Figure 7E.

9) The speculation that BAK1/BKK1 are involved in perceiving Pip needs further evidence. It could be simply that the lack of BAK1/BKK1 makes the plants less sensitive to Pip because of a change in the overall responsiveness of *bak1 mkk1* mutants.

(Our response 15) BAK1 and BKK1 are involved in perception of multiple MAMPs and DAMPs. We agree that further biochemical evidence is required to conclude that BAK1 and BKK1 are directly involved in perception of Pip, NHP, or further metabolized chemical. We have added new data showing Pip-triggered immunity requires BAK1 and BKK1 (Figure 7D). This further suggests that BAK1 and BKK1 contribute to perception of Pip or Pip-derived chemicals. Nevertheless, we have deleted BAK1 and BKK1 from our revised model to avoid giving the impression to readers that it is demonstrated that Pip or NHP is perceived by BAK1 and BKK1 (Figure 7E).

10) *PR1* induction in MKK4DD after DEX treatment was very low in comparison to Col-0 inoculated with *Pto-AvrRpt2* (Figure 1A and 1C). Is the induction of *PR1* by MKK4DD relevant to SAR response?

(Our response 16) *Pto* AvrRpt2 infection activates multiple signaling pathways, including MAPK and SA. This is likely why *PR1* induction is higher in *Pto AvrRpt2* compared to the MKK4^{DD} system. In this manuscript, we simply used *PR1* expression as a marker of local MAPK activation and do not claim that *PR1* is involved in SAR.

11) Finally, there is a possibility that DEX infiltrated into local leaves can move to upper leaves. DEX is lipid soluble and should be mobile in plant body. In Supplemental Figure 1A (right), there was a clear GUS induction around the hydathodes (tips of each leaf serration). There was no GUS expression around the hydathodes in the mock control

(Supplemental Figure 1A, left).

(Our response 17) Thank you for the critical comment. Please see Our response 2.

In summary, more detailed analyses including time course experiments are needed to establish the timeline of events. Equally important, whether DEX is mobile in Arabidopsis and can move to the upper leaves to induce MKK4DD and then downstream events needs further investigation.

Reviewer #2:

A wide range of factors have been identified for SAR from many prominent research groups worldwide. Unfortunately, it is still unclear how these factors/molecules operate. Findings described in the MS, in that sense, is likely very welcome news to many. The Zeier group has identified/characterized pipecolic acid (Pip), one of the SAR molecules, and in this study further characterizes the molecular mechanism of how Pip functions. The authors analyzed several well-known defense factors (MPK3/6, WRKY33, ADL1, Pip, and BAK1/BKK1) together and concluded that these components are part of a novel SAR regulatory loop. First, the MS started with an intriguing observation that sustained MAPK activation triggered SAR and induced the expression of *ALD1* as well as two other defense marker genes; this activation did not require SID2, thus eliminating SA in the regulatory loop. MPK3/6 was necessary for Pip accumulation and its known substrate WKRY33 was also involved. WRKY33 bound to the *ALD1* promoter, which connects WRKY33 with the production of Pip. Finally, Pip production was shown to be essential for MPK3/6 activation likely through BAK1/BKK1, leading to a model that MPK3/6, WRKY33, ADL1, Pip and BAK1/BKK1 form the SAR regulatory loop. This SAR regulatory loop is very interesting and should be very insightful to a wide range of plant scientists. However, while I am very intrigued by the findings, I have some reservations whether or not this model, the crux of this paper, is fully supported by the data presented. I suggest following experimental supports for the novel SAR regulatory loop.

Figure 1: MKK4-DD in the DEX inducible system was used to activate MPK3/6. This DEX system uses a chimeric transcription factor (GVG) in the 35S promoter. A foreign and constitutive promoter tends to often change its strength via various mechanisms. Therefore, confirmation that MPK3/6 is activated comparably in different backgrounds is important in drawing the conclusion presented. Furthermore, it is also possible that mutations used in the MS may affect the inducible system. For this reason and others, previous studies using a DEX inducible system generally include a confirmatory step for gene induction; for MKK4-DD, one may directly analyze the activation of MPK3/6 as done in Mao et al (2011).

(Our response 18) Thank you for the critical suggestion. Please see Our response 1.

Figures 3, 4 and 5: In addition to *ALD1*, the defense gene markers presented in Figure 1, *PR1* and *FRK1*, should also be presented together. While the amount of Pip is generally well correlated with *ALD1*, it was not with bacterial resistance. Therefore, investigating how defense marker genes are induced in the different backgrounds would help understanding the discrepancy.

(Our response 19) Thank you for the suggestion. We performed additional experiments and have added expression data for three marker genes *PR1*, *ALD1*, and *FMO1* in Figures 3 and 4.

For Figure 5, the observation that *wrky33* became resistant to *Pma* is different from an earlier report (Zheng et al, 2006). Thus, expression analysis of the defense marker genes is likely helpful to clarify the difference between this and earlier report. Also, this contrasting outcome needs to be discussed as WRKY33 has been considered to be a negative regulator for resistance against Pseudomonas. For instance, overexpression of *WRKY33* was shown to repress defense responses against *Pst* in Zheng et al (2006). How does this negative regulator work in a positive defense regulatory loop? Detailed discussion of the WRKY33 role in defense signaling seems necessary to explain outcomes in the MS.

(Our response 20) In Zheng et al 2006, the authors showed that *wrky33* mutants are as susceptible as wild type plants against *Pto* DC3000. Consistently, in this study, we did not see a big difference in *Pto* DC3000 growth between *wrky33* and wild-type plants (Figure 4). However, as the reviewer pointed out, *wrky33* mutant plants were resistant against *Pma* ES4326 compared with wild-type plants. This is consistent with the notion that *WRKY33* is a

negative regulator of basal immunity against bacterial pathogens as described in Zheng et al 2006 (Zheng et al Plant J 2006 592-605). In addition, *WRKY33* is a negative regulator of the SA pathway (Birkenbihl et al Plant Physiol 2012 266-285). In this study, we showed that WRKY33 is a positive regulator of ALD1-mediated Pip accumulation, which is a critical component for SAR. Thus, we think that WRKY33 is a negative regulator of local defense via SA suppression and is a positive regulator of SAR via Pip accumulation. We have added a discussion of this point and revised our model in Figure 7E.

Figure 7: It is interesting that BAK1/BKK1 is needed for this regulatory loop. Unfortunately, bacterial resistance analysis against Pseudomonas was not presented to confirm the possibility of BAK1/BKK1 being in the SAR loop. Without this analysis, the step between Pip and MPK in the SAR loop is not fully connected. Also, to further strengthen the loop model, the gene expression analysis is likely necessary with the higher order mutants.

(Our response 21) Thank you for the suggestion. We have performed additional bacterial growth assay to determine Pip-triggered immunity against the bacterial pathogen in *bak1 bkk1*. The result showed that *BAK1* and *BKK1* are required for this (Figure 7D). Please also see Our response 15.

A minor feedback: Lines 103 and 104: There seems to be an unintended paragraph change.

(Our response 22) Thank you for pointing this out. We have corrected this.

Reviewer #3:

Systematic acquired resistance (SAR) is an induced system-level immunity following local tissue exposure to compatible or incompatible pathogens, microbe- or damage-associated molecular patterns, and stresses. Although salicylic acid (SA) signaling is critical for the manifestation of SAR, the local generation of multiple small metabolites has been suggested to act as mobile signals to promote SAR at the distal secondary sites. The expression of two SAR regulatory genes, *AGD2-LIKE DEFENSE RESPONSE PROTEIN1* (*ALD1*) and *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1*) is locally and systematically enhanced in SAR-induced plants. Complementary metabolite and genetic analyses suggested that ALD1 is responsible for the production of a Lys catabolite, pipecolic acid (Pip), and essential for SAR, as exogenous Pip can complement the defect in *ald1*. This manuscript provided interesting new evidence that the sustained (4-10 h) MAPK3/6 activation stimulated by *Pto AvrRpt2*, but not *Pto* or *Psm*, inoculation led to WRKY33-ALD1 activation and Pip accumulation. Significantly, Pip rapidly activated *MPK3/6*, and WRKY33 and ALD1 appeared to be critical for the positive feedback loop in the sustained MAPK3/6 activation by *Pto AvrRpt2*, which contributed to SAR.

The experimental data provided much correlated evidence to support the new findings and conclusions. However, the results are quite complex relying on DEX-inducible MKK4DD and MKK4DD *sid2* or *mpk3 sid2*, *mpk6 sid2* and *wrky33 sid2*. For example, Pip accumulation was very low (1.3 µg/g FW in Fig. 2B and 0.9 µg/g FW in Fig. 4B) 24 h after strong and long-term (24 h) MAPK3/6 activation by DEX-induced MKK4DD, but was highly induced (>15 µg/g FW in Fig. 3C and 4E) by *Pto AvrRpt2* inoculation, which activated MAPK3/6 for shorter period (Fig. 7C. Up from 3 h to 12 h, but not 20 h in Fig. 4B by Tsuda et al. PLOS genetics 2013), despite similar *ALD1* gene induction. Although *mpk3* or *mpk6* reduced *ALD1* expression and Pip levels induced by *Pto AvrRpt2* inoculation (Fig. 3C), the level of Pip is still significantly higher than what was induced by DEX-induced MKK4DD. Moreover, in the absence of SID2, the loss of SAR induced by *Pto AvrRpt2* inoculation in *mpk3 sid2* and *mpk6 sid2* seemed to be independent of Pip accumulation (Fig. 3C, all > 15 µg/gFW). Thus, the correlation between MPK3/6-Pip and SAR remained unclear and did not support the proposed model on the regulatory loop in Fig. 7E.

(Our response 23) Thank you for the critical comment. Please see Our response 4.

Similar issues also need careful attention and explanations to link WRKY33-ALD1-Pip to SAR based on the data obtained with MKK4DD *wrky33* (low Pip accumulation) and *wrky33 sid2* (high Pip accumulation but complete loss of SAR) (Fig. 4). To support the specificity of AvrRpt2 signaling, it is important to test whether *Pms*-triggered SAR might be independent of ALD1 and Pip (e.g., no MAPK3/6 activation by ALD1-Pip) despite high Pip accumulation (Fig. 5).

(Our response 24) It has been shown that Pip accumulation and SAR triggered by *Pma* ES4326 infection are dependent on *ALD1* (Návarová et al Plant Cell 2012 5123-5141).

The statement that "the different signaling pathways leading to SAR induction all converge at the level of *ALD1* activation (Fig. 2A, Sup Fig. 2. P. 8, line 241-243)" was not fully supported by the data presented in this manuscript.

(Our response 25) Thank you for pointing this out. We agree that this description was exaggerated. We have now carefully stated as "these different signaling pathways leading to SAR induction converge at *ALD1*".

To further support the proposed connection between MAPK3/6-WRKY33-ALD1, it is a good idea to explain why the WRKY33-HA protein accumulation (at 24 h but not 12 h, Fig. 6C) was "uncoupled" from the peak of MPK3/6 activation at 4-10 h after AvrRpt2 induction (Fig. 7C and Fig. 4B by Tsuda et al. PLOS genetics 2013).

(Our response 26) Thank you for the suggestion. Please see Our response 3.

Since the effectiveness of ALD1-Pip signaling is dependent on FMO1 and Pip only weakly and transiently activates MAPK3/6 (15 min), it is important to analyze MPK3/6 activation by *Pto AvrRpt2* in *fmo1* and *sard4*, and test the possibility to rescue the MAPK response in *ald1* by adding Pip after AvrRpt2 inoculation (Fig. 7C), which will help understand the nature and site of Pip synthesis, signaling and propagation in vivo.

(Our response 27) Thank you for the suggestion. We have measured MAPK activation in *fmo1* after *Pto AvrRpt2* infection. The result showed that MAPK activation is compromised in *fmo1* (Figure S6). We did not include *sard4* since Pip accumulation is only partially compromised in this mutant (Hartmann et al Plant Physiol 2017 124-153). We also performed experiments to test if Pip supplementation rescues MAPK activation in *ald1* after *Pto AvrRpt2* infection. However, we unfortunately did not detect MAPK activation in wild-type plants as well as in *ald1* with Pip supplementation. We detected MAPK activation in wild-type plants after *Pto AvrRpt2* infection with mock control but not in *ald1* as in Figure 7C. Pretreatment of plant roots with Pip may not work as endogenous Pip. Alternatively, Pip pretreatment might have de-sensitized plants for MAPK activation at the time of bacterial infection as observed for the MAMP flg22-triggered MAPK activation (Tsuda PLoS Genet 2013 e1004015). As this requires future investigation, we think that this is beyond our scope of the current manuscript.

There could be more clear and informative discussions on the different preference for MPK3 and MPK6 by AvrRpto2 and elevated Pip, respective, in SAR induction (Fig. 3A vs. Fig. 7D), and why *mpk3* or *mpk6* completely blocked Pto AvrRpt2-mediated SAR only in the *sid2* background but not in Col-0, if the function of MPK3/6 is, as proposed, to activate ALD1-Pip when the level of Pip was much higher in *mpk3 sid2* and *mpk6 sid2* than in *mpk3* and *mpk6* (Fig. 3A). The SA and Pip redundancy cannot explain the SAR results because Pip production was hardly compromised in *mpk3 sid2* and *mpk6 sid2*. I also found the following statement confusing: "SAR predominantly depends on SA when it is activated by local infection with *Pto* and *Pma*" (p. 11, lines 315-317) and "ALD1 is commonly required for SAR triggered by local infection with *Pma*" (p. 11, line 321). It is also unclear why *wrky33* abolished Pto-induced SAR without a known link between WRKY33 and SA (Sup. Fig. 4B).

(Our response 28) Thank you for the suggestion. This confusion was probably caused by our intention not to include systemic tissues in our model. SA acts as a negative regulator of Pip accumulation in leaves locally infected with *Pto AvrRpt2* (Figure 3C and Figure 4E). However, SA is required to amplify SAR signals in systemic leaves (Bernsdorff et al Plant Cell 2016 102-129). *mpk3* or *mpk6* plants have reduced Pip but the intact SA pathway, which is sufficient to activate SAR (Figure 3). We think that wild-type level Pip accumulation with compromised in the SA pathway in the double mutants results in compromised SAR (Figure 3). In *sid2* plants, even though SA pathway is compromised, Pip accumulation is elevated (Figures 3 and 4E), which might compensate for the loss of the SA pathway for SAR induction (Figure 3). We have modified discussion accordingly. Please also see Our response 20.

A minor note: Perhaps replacing *Pma* with *Pseudomonas syringae pv. maculicola* (*Psm*) ES4326 for consistency in the literature.

(Our response 29) Both *Psm* and *Pma* have been used in various papers. We prefer *Pma* for *Pseudomonas syringae* pv. *maculicola* since we use *Pto* for *Pseudomonas syringae* pv. *Tomato*.

TPC2018-00547-RA 1st Editorial decision – *accept with minor revision* Aug, 17, 2018

We have received reviews of your manuscript entitled "A MPK3/6-WRKY33-ALD1-Pipecolic acid Regulatory Loop Contributes to Systemic Acquired Resistance." On the basis of the advice received, the board of reviewing editors

would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on revision based on the comments of our reviewers. In particular, please consider the following:

All three reviewers felt some of your conclusions needed to be qualified based on the data included in the paper. In particular, reviewer 3 pointed out several aspects of your data that indicate your model shown in Figure 7E is oversimplified and ignores some of your own results. In revising your manuscript, please specifically address the following issues:

[Provided below along with author responses]

---------- Reviewer comments:

[Provided below along with author responses]

TPC2018-00547-RAR1 1st Revision received Aug. 21, 2018

Reviewer comments on previous submission and **author responses**:

Editor's comments:

1) The observation that MKK4(DD)-MAPK3/6 activation is not affected in by mutations in WRKY33 (Fig. 2B) and *Pto-AvrRpt2*-MPK3/6 activation is only partially reduced by *wkry33* mutation (Fig. 7C).

(Our response 1) As we discussed in Discussion, other transcription factors in addition to WRKY33 also regulate *ALD1* expression and thereby Pip accumulation. We have included "other TFs" in our model to avoid over simplification of our findings (now Figure 7F).

2) The observation that the *bak bkk1* double mutation blocks PIP-induced resistance (Fig. 7D) and MPK activation (Fig 7C) need to be incorporated in your model (Fig. 7E) (note: you need to correct line 312, which states that this double mutation did not affect resistance).

(Our response 2) We have incorporated BAK1/BKK1 in our model (now Figure 7F) and have corrected the error. Now the description reads (line 307-313):

"We observed significant Pip-induced resistance against *Pma* in Col-0, as well as *mpk3*, *mpk6*, and *wrky33* (Figure 7E), indicating that the MPK3/MPK6- and WRKY33-based regulatory loop can be bypassed by high amounts of Pip. However, consistent with our observation that Pip-induced root growth inhibition and MAPK activation were compromised in *bak1 bkk1* (Figure 7A and 7B), Pip-induced immunity against *Pma* required *BAK1 BKK1* (Figure 7E)."

3) There should be more emphasis on the observation that FMO is required for MKK-DD activation of MPKs and disease resistance (Fig. 2), which strongly indicates that NHP is the key signaling molecule, not PIP.

(Our response 3) We have added a sentence (line 185-187) in Results to make it clear that NHP is the key signaling molecule in MAPK-triggered SAR. Please also see Our response 18.

"Considering that Pip is metabolized to NHP by FMO1 and that Pip-induced responses requires *FMO1* (Chen et al., 2018; Hartmann et al., 2018), NHP is the key signaling molecule in MAPK-mediated SAR."

We have further indicated the involvement of FMO1 and NHP in the discussion of the SAR regulatory loop (lines 300 and 325).

4) Please provide your thoughts on why MKK4-DD activation of downstream MPK3/MPK6 requires ALD1 and FMO1 (Fig. 2B). MPK3/MPK6 are known to be direct targets/substrates of MKK4DD, thus it is difficult to understand the requirement for ALD1 and FMO1.

(Our response 4) This observation was also surprising to us. We have added a paragraph in Discussion in which we have speculated why MKK4DD-triggered activation of MPK3 and MPK6 requires ALD1 and FMO1 (line 426-439). Please also see Our response 10.

"The observation that MAPK activation triggered by MKK4^{DD} (Figure 2B) requires *ALD1* and *FMO1* was rather surprising to us because MKK4^{DD} would be able to directly phosphorylate MPK3 and MPK6 without other components. However, this suggests that MKK4^{DD} requires additional components whose activity depends on Pip/NHP to achieve sustained activation of MPK3 and MPK6 in plants. We speculate that Pip/NHP may condition the proper formation of MKK4DD-MPK3/MPK6 complex through, for instance, affecting the subcellular localization of MKK4^{DD}, MPK3, and MPK6. Alternatively, MKK4^{DD} triggers initial phosphorylation of MPK3 and MPK6, which then triggers sustained activation of MPK3 and MPK6 dependently on Pip/NHP. Recently, it has been shown that MPK6 phosphorylates the upstream MAPK kinase kinase MAPKKK5 to enhance activation of MPK3 and MPK6 (Bi et al Plant Cell 2018). Thus, Pip/NHP signaling may ensure, for instance, expression of *MAPKKK5* and this positive feedback mechanism may be required for MKK4^{DD}-triggered sustained activation of MPK3 and MPK6. Nevertheless, these speculations need to be experimentally tested."

5) Please modify Figure 2 so that immunoblots from separate gels are bounded by their own boxes. Do NOT splice together independent gels.

(Our response 5) We have separated gels by boxes based on individual immunoblot membranes according to the suggestion.

Please note the following:
-Supplemental materials should be restricted to large datasets and tables, presentation of replicates, and validation of reagents, methods, or genotypes. Any data that are used to support claims must be in the main manuscript. Supplemental figure legends must indicate what figure in the main manuscript is supported by the supplemental data presented.
-You have a lot of supplemental figures. Please make sure they meet these criteria, otherwise they need to be in the main paper.

(Our response 6) We have moved the previous supplementary Figure 6 to the new Figure 7D. Accordingly, the previous Figure 7D and 7E are now Figure 7E and 7F.

-Please see the attached file for comments on the figures, which you can correct in the revision. You can download the attachment, open in acrobat and view in comment mode. please apply the comments to all figures in the paper.

(Our response 7) Thank you very much for the comments. We have revised those figures accordingly.

-Sampling methods and nature of "biological replicates" should be described precisely (i.e. different plants, parts of plants, pooled tissue, independent pools of tissue, sampled at different times, etc), along with a clear description of and rationale for any statistical analyses conducted. The reader should know exactly what was sampled; what forms the basis of the calculation of any means and statistical parameters reported. This is also necessary to ensure that proper statistical analysis was conducted.

(Our response 8) We think that our figure legends contain sufficient information for the nature of replicates (biological replicates, independent trials) and the detail of statistical analysis.

Reviewer #1:

I reviewed a previous submission of this manuscript. This version is much improved and the authors have addressed all my concerns very well.

1) I still have one question about Figure 2B. It is hard to believe that MKK4DD activation of downstream MPK3/MPK6 requires ALD1 and FMO1. MPK3/MPK6 are direct targets/substrates of MKK4DD!

(Our response 9) This observation was also surprising to us. We have added a paragraph in the Discussion in which we have speculated why MKK4DD-triggered activation of MPK3 and MPK6 requires ALD1 and FMO1. Please see Our response 4.

2) If there is a positive feed forward regulation as depicted in Figure 7E, then why WRKY33 is not required? Based on the authors' model, the activation of MPK3/MPK6 in this system has two different routes, one through the gain-of-function MKK4DD, which does not require other signaling components; and the other through the activation of

endogenous MKK4/MKK5 through WRKY33-ALD1/FMO1 pathway. If this is the case, the authors should see some different in the *mkk4 mkk5* mutant background. As a result, I suggest that the authors test *mkk4 mkk5* double mutant to see whether there is a requirement of endogenous MKK4/MKK5 after MKK4DD induction or pathogen infiltration.

(Our point 10) As we discussed in Discussion, other transcription factors in addition to WRKY33 also regulate *ALD1* expression thereby Pip accumulation. We have included "other TFs" in our model (now Figure 7F).

We have speculated that MKK4DD-triggered MAPK activation requires the positive feedback mechanism involving MAPKKK5 and that Pip/NHP signaling ensures activity of MAPKKK5. If this is the case, investigating mutants of the intermediate kinases *MKK4* and *MKK5* is important as suggested by Reviewer 3. However, we think that this is beyond our scope of this manuscript. We would address this issue in the future.

BTW: The western blots in this figure can be improved. In the first panel, the SDS-PAGE gels could be run for longer time (another 15 min after the tracking dye runs out) to achieve better separation of MPK6 and MPK3. In the second panel, the authors could use the affinity-purified anti-flag M2 antibody from Sigma. The smaller non-specific band will disappear and image will be clear.

(Our response 11) Thank you very much for the excellent guidance. We will perform such immunoblotting experiments in the future as suggested.

Reviewer #2:

This version of MS has been significantly improved, thanks to several added experiments and revised discussion. There are several places, however, where tone-down in their interpretation and minor revision would improve the MS.

1) Figure 1B. MKK4-DD triggered SAR is diminished but not abolished in *sid2*. Perhaps, soften the subtitle in line 146 and its associated discussion. The whole MS tends to dismiss the role of SA. However, many figures support the role of SA, if they are carefully examined.

(Our response 12) We have deleted "independently of SA" from the subtitle (line 143) and modified the relevant text (line 155-158). It now reads:

"Indeed, we observed that SAR is triggered in MKK4^{DD} and to a lesser extent, MKK4^{DD} *sid2* after DEX treatment in local leaves (Figure 1B), whereas no SAR was observed after DEX treatment in Col-0, *sid2*, and transgenic plants harboring DEX-inducible GUS (GVG::GUS) (Figure 1B)."

We have also modified the title for Figure 1:

"MAPK-mediated SAR does not require SA" was changed to "MAPK-mediated SAR is largely independent of SA".

Bacterial titer at 0.001 of A600 is relatively high (5 times higher than what is used in general). If additional experiments have been performed at a lower titer, please mention.

(Our response 13) Since this bacterial dose robustly triggered SAR in our hands, we did not test different doses of bacterial titer for SAR assays. In addition, we showed that plants induce distinct transcriptome responses to *Pto* and *Pto* AvrRpt2 upon infection with this bacterial dose (Mine et al 2018 Plant Cell 1199-1219). Thus, we think that this bacterial dose is appropriate.

Figure 3 - *sid2* displayed genetic interaction with *mpk3* and *mpk6* when the gene transcription was tested. Yet, in line 204, it was noted that there is no detectable role of SA. This statement is not justified.

(Our response 14) We have deleted "no detectable role of SA" from the sentence.

Figure 4A. *PR1*, *ALD1*, and *FMO1* were still induced in *wkry33*. It is arguable that *PR1* and *FMO1* were more induced in terms of fold change. Soften the interpretation.

(Our response 15) We have rephrased the sentence (line 219-222). It now reads:

"Consistent with our hypothesis, levels of local *ALD1* expression as well as *PR1* and *FMO1* and Pip accumulation after MAPK activation were reduced in *wrky33* (Figure 4A and 4B) while MKK4^{DD} protein is induced after DEX treatment similarly to wild type background (Figure 2B).

In Line 311, it was indicated that there was significant Pip-induced resistance. Figure 7D appears to indicate otherwise. Please rephrase

(Our response 16) Thank you very much for pointing this out. We have corrected this error. Please see Our response 2.

Figure 7E missed BAK/BIK. Please place this component in the model.

(Our response 17) We have incorporated BAK1/BKK1 into the model. Please see Our response 2.

Reviewer #3:

This manuscript addresses a key question on the molecular link between the sustained MPK3/6 signaling and the establishment of systemic acquired resistance (SAR). Five local stimuli that have been shown to trigger SAR were investigated and compared, including DEX-inducible MKK4(DD) expression or infection by *Pto-AvrRpt2* that can induce sustained MPK3/6 activation, *Pto* or *Pma* infection that does not induce sustained MPK3/6 activation, and pipecolic acid (Pip) treatment that triggers transient and weak MPK3/6 activation. The comprehensive analyses suggested the existence of distinct branches of SAR signaling and proposed that these pathways converge at ALD1 and Pip induction in local leaves. Based on the assumption that MPK3/6 activate WRKY33 and a set of correlated findings using various mutants, it was concluded that the MPK3/6-WRKY33-ALD1-Pip regulatory loop contributes to SAR.

The authors have added time course information on the MKK4(DD)-MAPK3/6 activation in various mutants, *Pto-AvrRpt2*-MPK3/6 activation in *fmo1*, and measurements of Pip and NHP accumulation, as well as more marker gene and WRKY33-HA expression analyses to support their conclusions on the importance of the MPK3/6-WRKY33-ALD1-Pip regulatory loop in SAR. Thoughtful discussions for the complex results and the revised model have significantly improved this new manuscript.

Some comments and suggestions are provided as follows:

1. Although discussed by the authors, the ALD1-Pip "centric" model may still need to be further updated based on the recent Pip-FMO1-NHP paper (Hartmann et al., 2018). "Although exogenous Pip application activates the transcription of key regulatory genes in plant immunity and SAR, the prominent Pip-inducible transcriptional response observed in the wild-type was absent in the *fmo1* mutant." Importantly, Pip does not activate SAR in *fmo1*. Moreover, data presented in this manuscript showed that both MKK4(DD)-MAPK3/6 activation and *Pto-AvrRpt2*-MPK3/6 activation were blocked in the *fmo1* mutant. Even though L-Pip transiently activated MPK3/6, NHP (or further metabolites), the product of FMO1 instead of Pip, could be the "actual" signaling molecule for SAR. The emphasis on ALD1 and Pip, although important to lead to NHP or other signals, may need to be modified soon to be more accurate, especially on the sustained MPK3/6 activation induced by *Pto-AvrRpt2* infection.

(Our response 18) We agree with Reviewer 3 that we and/other researchers need to update how Pip-derived metabolites such as NHP induces SAR. As shown in Chen et al 2018 PNAS and Hartmann et al 2018 Cell, NHP is the FMO1-generated downstream metabolite of Pip. As we discussed, NHP or NHP-derived metabolites such as NHP-hexose (Hartmann and Zeier, 2018; information now introduced into the discussion section, line 400)) may be the actual mobile signal for SAR (discussion, line 405). We have shown that Pip-derived chemical(s) trigger MAPK activation dependently on *BAK1 BKK1*. This implies that such metabolite(s) are sensed by pattern recognition receptor(s) on the plasma membrane as for DAMPs. For this, key updates should include the identification of receptor(s) for the mobile metabolite, which facilitates dissection of SAR induction mechanisms. Please also see Our response 3.

2. The importance of WRKY33 in the MPK3/6-WRKY regulatory loop may be overemphasized without the actual data to show that the phosphorylated WRKY33 by MPK3/6 or the WRKY33 phosphorylation mutant controls the *ALD1* promoter. Furthermore, MKK4(DD)-MAPK3/6 activation was not affected in the *wrky33* mutant (Fig. 2B) and *Pto-AvrRpt2*-MPK3/6 activation was only partially reduced in *wkry33* (Fig. 7C), which differentiate the two MPK3/6 activation pathways and suggest that other TFs could play important roles.

(Our response 19) Thank you for the critical suggestion. We have revised our model in which other transcription factors "other TFs" are also involved in the SAR regulatory loop (now Figure 7F). Please also see Our response 1.

3. Despite the complete lack of SAR in *wrky33* induced by *Pto-AvrRpt2*, the *ALD1* and *FMO1* expression or Pip accumulation was still highly induced (Fig. 4D, E, F). It remains possible that WRKY33 may play more important roles in SAR, but only a minor role in the local leaves focused in this study.

(Our response 20) Thank you for the critical suggestion. We have revised our model in which other transcription factors "other TFs" are also involved in the SAR regulatory loop (now Figure 7F). Please also see Our response 1.

4. The expression of *FMO1* and *ALD1* is not always co-regulated and the Pip levels may not be directly linked to NHP and SAR, e.g., the basal level of *FMO1* was lower in *mpk3*, *mpk6* or MKK4DD plants before the induction (Fig. 3A, 4A), thus the induction levels appeared to be similar, and the significance of the Pip levels associated with different induction for SAR remained a tricky issue (Fig. 3C, 4B, 4E and 5C).

(Our response 21) As described in the previous response letter, we were unable to reliably detect NHP, which is Pip-derived and the key metabolite for SAR induction, upon *Pto AvrRpt2* infection due to very low accumulation in the investigated time period. With our current method, we feel that the low levels of accumulating NHP upon *Pto AvrRpt2* inoculation cannot be reliably estimated (absolute levels), because the so far used internal standard produces a faint signal close to the NHP peak. We are currently developing methods that can accurately measure low levels of NHP by use of a more suitable internal standard (D_9-labelled NHP which we have chemically synthesized). Once succeeded, we plan to measure NHP at a broader time range with different SAR stimuli, including *Pto AvrRpt2* inoculation. This would allow us to precisely correlate gene expression, Pip, and NHP levels, which then might solve this issue. Considering the time required for this development, we think that this is beyond scope of this current manuscript (its main focus on *ALD1* regulation and Pip accumulation).

5. The "positive" roles of WRKY33 in SAR induced by *Pto-AvrRpt2*, *Pto* or *Pma* infection may not be explained by MPK3/6, and yet the important "positive" role of SA in these types of SAR is confounded by the "negative" role of WRKY33 on SA.

(Our response 22) We agree to that we need to incorporate SA suppression by WRKY33 into our model. We have added SA suppression by WRKY33 in systemic leaves to the model (now Figure 7F).

6. In the model for the MPK3/6-WRKY33-ALD1-Pip regulatory loop, the L-Pip-activated root inhibition or SAR may still require FMO1, and the "negative" role of WRKY33 in SAR after Pip treatment also needs some explanation (Fig. 7D).

(Our response 23) We have revised our model (now Figure 7F) in which the arrow to "X" mobile signal comes from only NHP (but not Pip) and WRKY33 suppresses SA in systemic tissues. This SA suppression by WRKY33 may explain why *wrky33* showed stronger Pip-induced immunity compared to wild type plants.

TPC2018-00547-RAR1 2nd Editorial decision – *acceptance pending* **Aug. 27, 2018**

We are pleased to inform you that your paper entitled "A MPK3/6-WRKY33-ALD1-Pipecolic acid Regulatory Loop Contributes to Systemic Acquired Resistance" has been accepted for publication in *The Plant Cell*, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor **Sept. 12, 2018**