nature portfolio

# Peer Review File

## Fine-tuning of IPA1 transactivation activity by E3 ligase IPI7-mediated non-proteolytic K29-ubiquitination during Magnaporthe oryzae infection

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The Ideal Plant Architecture 1 (IPA1) transcription factor was well-known by its function to both promote rice yield and immunity. And the regulate role switching of IPA1 to rice immunity or yield based on the phosphorylation and dephosphorylation at its amino acid residue Ser163. However, the mimic phosphorylated IPA1, IPA1(S163D) targets on the promoter of immune response gene WRKY45 but cannot activate its expression which indicates an underlying regulating mechanism.

To solve this problem, the author Shi and the colleagues in this research did the excellent work. They found IPI7, also a RING-finger containing E3 ligase, could interact with and polyubiquitinate both IPA1 and IPA1(S163D) with K29-Ub chain, but without influencing the stability of IPA1. Furthermore, the K29-polyubiquitination on IPA1 with the phosphorylation at Ser163 together co-activate the expression of OsWRKY45. Along with the enhanced phosphorylation of IPA1, the K29-polyubiquitination was also promoted by M. oryzae infection. And the enhanced blast disease resistance caused by higher OsWRKY45 expression in SD-OE transgenic plants reduced to the same level as in the wild type plants in SD-OE/ipi7-ko plants, in accordance with the attenuated OsWRKY45 expression in SD-OE/ipi7-ko plants. In summary, the author revealed that the non-proteolytic K29-polyubiquitinated IPA1 mediated by IPI7 cooperated with the phosphorylated IPA1(S163D) to fine-tune the transactivation activity of IPA1 during the M. oryzae infection in rice.

In general, this research provides comprehensive evidence for the conclusion and the manuscript writing is clear in logic.

Comments:

1.Previous work had shown the IPA1 RNA and protein levels were not significantly affected upon M. oryzae infection. Instead, the phosphorylation of IPA1 was significantly induced by M. oryzae infection, which started to accumulate at 3 hpi, peaks at 6 to 12 hpi, and then subsided to near normal levels within 48 hpi (Wang et al., 2018). Since K29-polyubiquitination of IPA1 is also required for OsWRKY45 activation and rice resistance to rice blast, I think the author should also carry out assays to investigate the IPI7 RNA and protein levels upon M. oryzae infection. Correspondingly, the trend of K29-polyubiquitination of IPA1 upon infection should also be checked together. With these results, it is better to explain the roles of IPI7 in timely fine-tuning the IPA1 transactivation activity.

2.In this work, the author showed IPI7 could interact with and polyubiquitinate both IPA1 and IPA1(S163D), but without influencing the stability of IPA1. Here I want to know whether the stability of IPA(S163D) is influenced by the polyubiquitination? And whether the polyubiquitination level of the IPA1 and IPA(S163D) caused by IPI7 have difference?

3.In Fig. 5c, the middle image indicated the total ubiquitination level of IPA1. It seems like that the total ubiquitination level is weaker than the K29-Ub level, is it because the different exposure time or not?

4.Line 311, the author indicated that they found no disruptions of K sites of IPA1 affected IPI7-mediated ubiquitination, I did not see the results in the manuscript.

5.In figure 1b, the input part of the GST pull-down data to verify IPI7 and IPA1 interaction is incomplete.

6.In figure 3, whether 3a and 3b are repeated clarification that IPI7 has no obvious effects on the stability of IPA1.

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8.In Supplementary Fig. 8b, the In vitro ubiquitination lack the immunoblot detection by ub antibody.

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11.Line 263 – 264, "Only the IPA1 protein……". This sentence is not quite appropriate. Previous studies have found that OsMPK6 phosphorylates WRKY45 at Thr266, Ser294, and Ser299 in vitro. Phosphorylation of Ser294 and/or Ser299 is required for full activation of WRKY45. So, this expression should be reorganized.

12.Supplementary figure 7b, has a strong background.

13.Line 262 "infection" should be changed to "infection".

14.Line 26 "IPA1S(163D)" should be changed to "IPA1(S163D)".

15.Line 27 "fine-tune" should be changed to "fine-tunes".

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors discovered an Ideal Plant Architecture 1 (IPA1)-Interacting protein 7 (IPI7) that functions as a mono-subunit RING-finger E3 ubiquitin (Ub) ligase for adding K29-type poly-Ub chains onto IPA1. IPA1 was discovered by the same group as a transcription factor that can either promote rice growth or enhance resistance to a fungal pathogen, Magnaporthe oryzae. Since it is critical in crop production to optimize the balance between growth and pathogen resistance, the dual activities of IPA1 make it a good model to develop crops with high yield and disease resistance. Through both biochemical and genetic studies, the authors argued that a IPI7-mediated non-proteolytic K29-ubiquitination on phosphorylated IPA1 boosted the binding of IPA1 with WRKY45, thus enhancing pathogen resistance. While this reviewer finds that the research topic is of high impact, there are several puzzles needed to be solved in the current version of the manuscript. The major concerns are summarized below.

1. I am confused by the inconsistent molecular weight of IPA1 presented in Figs. 2, 3, S4, S7, S8, S9, S12. In Figs 2a, S8b, S9, a His-IPA1 with >95 kDa is shown. However, in Figs 2b, 3, S4, and S7, the detected IPA1 band is just around 50 kDa. In Figs 5 and 12, the size of IPA1 is not clear. Why were different sizes of IPA1 detected? How did the authors confirm that the band of IPA1 was correct? Since the authors have generated both overexpression and RNAi-mediated knock-down lines in their previous work (Ref 37), it might be a good idea to use these lines as positive and negative controls to verify the correct bands of IPA1 detected by the anti-IPA1 antibodies, particularly in the in vivo studies.

2. In Fig 2a, the authors detected a heavy smear band by Ub antibodies but only a faint smear band detected by both anti-His and anti-MBP antibodies, which were against to the substrate IPA1 and E3 ligase IPI7, respectively. Although I agree that the heavy band detected by Ub antibodies could result from its high antigenicity, I could not believe that it can make such a large difference with the signal detected by anti-MBP antibodies if we assume that the majority of ubiquitinated proteins in this in vitro ubiquitination system were auto-ubiquitinated IPI7 E3 ligases. Indeed, the signal detected by anti-MBP antibodies in the last lane is only slightly higher than the other lanes.

3. Since the authors have developed an in vitro ubiquitination assay system in Fig 2a, it would be a good idea to test whether His-IPA1(S163D), but not His-IPA1(S163A) (see below), can be ubiquitinated by IPI7 via K29 directly in this system.

4. Since the authors did not find stability changes of IPA1 in Ri22 and IPI7 overexpression lines in Fig 3, it would be expected that MG132 treatment will not enhance the ubiquitinated forms of IPA1. Therefore, in the experiment of Fig 2b, it would be a good idea to include this control. In addition, does the transcription level of IPA1 remain no changes in different samples?

5. In Figs 3 and S7, for quantitative comparisons of protein bands detected by immunoblotting assays, it is necessary to provide a statistical analysis. I am not sure how many replicates that the authors did. Same as Comment 4, does the transcription level of IPA1 remain no changes in different samples? In addition, the authors did not explain how the protein bands were displayed and recorded. If by chemiluminescence on X-ray film, the signal could be out of the linear range easily, thus resulting in misleading conclusions.

6. In Figs 4, S1, S2, what about the effect of a phosphorylation-mull mutant, such as IPA(S163A)?

7. In Figs 5 and 12, I think it is critical to verify the specificity and efficacy of each Ub chain-specific antibody. Neither references nor controls (positive and negative) are provided.

8. In Fig 9, I don't think the data is conclusive. Since the majority of IPA1 proteins are non-ubiquitinated, the lack of difference of mobility shift could simply result from the binding of non-ubiquitinated IPA1 with Biotin-WRKY45P. In addition, a phosphorylation-mull mutant, such as IPA(S163A), would be a good control as well.

9. Line 294. If we assume the authors' data presented in Figure 5 is correct, the data only suggest that IPA1 is K29-ubiquitinated. However, it does not support that this ubiquitination is necessary for the IPA1-mediated response to M. oryzae. IPI7 could also lead to another type of ubiquitination that is necessary for this response.

10. Lines 311-312, I don't understand what the authors meant. There is no data provided to support this conclusion. If K0 IPA1 has been tested, please show the data. I feel the entire paragraph has too much speculation.

Minor Comments.

1. Line 57, "location" is not a good word. "site" might be better.

2. Line 70, "CULLIN" should be "cullin 1" or "CUL1". "SCF-box" is not a conventional term. It should be "SCF". However, if the authors meant SCF, the sentence does not include many other CUL-RING E3 ligases.

3. Line 81, "MAMPs" ?

4. Line 83, "RLCKs"?

5. Line 123, "IPA1" could be "IPA1's".

6. Line 161, "Fig. 6a-c" could be simply "Fig. 6".

**REVIEWER COMMENTS**

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The Ideal Plant Architecture 1 (IPA1) transcription factor was well-known by its function to both promote rice yield and immunity. And the regulate role switching of IPA1 to rice immunity or yield based on the phosphorylation and dephosphorylation at its amino acid residue Ser163. However, the mimic phosphorylated IPA1, IPA1(S163D) targets on the promoter of immune response gene WRKY45 but cannot activate its expression which indicates an underlying regulating mechanism.. In summary, the author revealed that the non-proteolytic K29-polyubiquitinated IPA1 mediated by IPI7 cooperated with the phosphorylated IPA1(S163D) to fine-tune the transactivation activity of IPA1 during the M. oryzae infection in rice.

In general, this research provides comprehensive evidence for the conclusion and the manuscript writing is clear in logic.

1. Previous work had shown the IPA1 RNA and protein levels were not significantly affected upon *M. oryzae* infection. Instead, the phosphorylation of IPA1 was significantly induced by *M. oryzae* infection, which started to accumulate at 3 hpi, peaks at 6 to 12 hpi, and then subsided to near normal levels within 48 hpi (Wang et al., 2018). Since K29-polyubiquitination of IPA1 is also required for OsWRKY45 activation and rice resistance to rice blast, I think the author should also carry out assays to investigate the IPI7 RNA and protein levels upon *M. oryzae* infection. Correspondingly, the trend of K29-polyubiquitination of IPA1 upon infection should also be checked together. With these results, it is better to explain the roles of IPI7 in timely fine-tuning the IPA1 transactivation activity.

Response: Thanks for your suggestion. To analyze whether the RNA levels of *IPI7* are altered upon *M. oryzae* infection, we have measured the transcripts levels of *IPI7* and found that *IPI7* is not induced by *M. oryzae* infection compared to mock treatment (Supplementary Fig. 14) (Line271-272). To analyze whether the protein levels of IPI7 are altered by *M. oryzae* infection, we generated the IPI7-GUS and IPI7-GFP transgenic plants. To our disappointment, although the transcript levels of *IPI7* were clearly increased, we could not detect IPI7-GUS or IPI7-GFP protein using GUS and GFP antibodies, respectively. Moreover, we also failed to generate a specific antibody to detect native IPI7 protein in rice plants.

To better explain the roles of IPI7 in the temporal fine-tuning of IPA1 transactivation activity, we performed an *in vivo* ubiquitination assay at different hours post-inoculation (hpi) with *M. oryzae*. We found that the K29-polyubiquitination of IPA1 started to accumulate at 3 hpi, peaked at 6 hpi, then returned to basal levels at 12 hpi (Supplementary Fig. 11b). (Line223-225)

2. In this work, the author showed IPI7 could interact with and polyubiquitinate both IPA1 and IPA1(S163D), but without influencing the stability of IPA1. Here I want to know whether the stability of IPA(S163D) is influenced by the polyubiquitination? And whether the polyubiquitination level of the IPA1 and IPA(S163D) caused by IPI7 have difference?

Response: Thanks for your suggestion. To test whether the stability of IPA1(S163D) is influenced by IPI7-mediated ubiquitination, we expressed IPI7-GFP and IPA1(S163D)-HA *in vivo* and found that the stability of IPA1(S163D) was not influenced by IPI7 (Supplementary Fig. 8d) (Line 184-185). Next, we performed *in vitro* and *in vivo* ubiquitination assays to compare the polyubiquitination levels of IPA1 and IPA1(S163D) proteins caused by IPI7. The results showed that there were no significant differences in the polyubiquitination of IPA1 and IPA1(S163D) mediated by IPI7 (Supplementary Fig. 8b-c, Line181-184)

3. In Fig. 5c, the middle image indicated the total ubiquitination level of IPA1. It seems like that the total ubiquitination level is weaker than the K29-Ub level, is it because the different exposure time or not?

Response: As you speculated, the exposure times for these two images were different. We cleared up this misunderstanding by using images with similar exposure time lengths (Fig. 5c).

4. Line 311, the author indicated that they found no disruptions of K sites of IPA1 affected IPI7-mediated ubiquitination, I did not see the results in the manuscript.

Response: Thanks for reminding us. We have added these results in the revised manuscript (Supplementary Fig. 16, Line 326). All K-site mutants of IPA1 showed similar IPI7-mediated ubiquitination levels.

5. In figure 1b, the input part of the GST pull-down data to verify IPI7 and IPA1

interaction is incomplete.

Response: Thanks for your reminding. We repeated the GST pull-down experiment for IPI7 and IPA1, and the new intact result is shown in the revised manuscript in Fig. 1b.

6. In figure 3, whether 3a and 3b are repeated clarification that IPI7 has no obvious effects on the stability of IPA1.

Response: Thanks for your suggestion. We have rearranged this figure as you suggested. Now, Fig 3a is the result showing that the stability of IPA1 is not affected by IPI7 in tobacco leaves and Fig 3d shows a similar result using rice protoplast cells. These results indicate that IPI7 does not affect the stability of IPA1 *in vivo* (Fig 3f, g).

7. In Supplementary Fig. 2, I think there should set a non-phosphorylated IPA1 control which might be more clearly shown IPI7 mediated IPA1(S163D) rather than IPA1 is essential for WRKY45 expression activation.

Response: Thanks for your suggestion. We have added IPA1(S163A) as a non-phosphorylated IPA1 control to show that IPI7-mediated ubiqutination of IPA1(S163D), but not IPA1, is essential for activation of *WRKY45* expression (Supplementary Fig. 2b).

8. In Supplementary Fig. 8b, the In vitro ubiquitination lack the immunoblot detection by ub antibody.

Response: Thanks for your suggestion. We repeated the *in vitro* ubiquitination experiment and detected ubiquitination using both Ub and His antibodies (Supplementary Fig. 8b).

9. In figure 4, I think there also should set a non-phosphorylated IPA1 control to clearly present polyubiquitination and phosphorylation co-activate the expression of WRKY45.

Response: Thanks for your suggestion. We have added IPA1(S163A) as a non-phosphorylated IPA1 control to show that polyubiquitination and phosphorylation co-activate the expression of *WRKY45* (Supplementary Fig. 9b).

10. For rice gene's expression and protein stability, I think the experiments should be better performed in rice (eg., rice protoplasts or transgenic plants) instead of in tobacco system. For example, Fig. 3a, IPA1 protein stable assay. At the same time, Fig. 3b, the abundance of GFP level is different, how did you normalized and calculated the IPA1 protein level?

Response: Thanks for your suggestion. We have performed the IPA1 protein stability assay in rice protoplasts and the results are shown in the revised Fig. 3d. To avoid misunderstanding of the previous result in Fig. 3b, we have removed that data (Fig 3b).

11. Line 263 – 264, "Only the IPA1 protein……". This sentence is not quite appropriate. Previous studies have found that OsMPK6 phosphorylates WRKY45 at Thr266, Ser294, and Ser299 in vitro. Phosphorylation of Ser294 and/or Ser299 is required for full activation of WRKY45. So, this expression should be reorganized.

Response: We appreciate your suggestion. We have reorganized this sentence as follows: These two post-translational modifications (ubiquitination and phosphorylation) of IPA1 protein play crucial roles in activating plant immunity (Line 275-276).

12. Supplementary figure 7b, has a strong background.

Response: Thanks for your suggestion. We have repeated this assay and showed a new result (Supplementary Fig. 7e).

13. Line 262 "infection" should be changed to "infection".

Response: We are sorry for this mistake and have changed this word (Line 272).

14. Line 26 "IPA1S(163D)" should be changed to "IPA1(S163D)".

Response: We are sorry for the typo error and have corrected this word (Line 26).

15. Line 27 "fine-tune" should be changed to "fine-tunes".

Response: We are sorry for this mistake and have changed this word (Line 27).

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors discovered an Ideal Plant Architecture 1(IPA1)-Interacting protein 7 (IPI7) that functions as a mono-subunit RING-finger E3 ubiquitin (Ub) ligase for adding K29-type poly-Ub chains onto IPA1. IPA1 was discovered by the same group as a transcription factor that can either promote rice growth or enhance resistance to a fungal pathogen, Magnaporthe oryzae. Since it is critical in crop production to optimize the balance between growth and pathogen resistance, the dual activities of IPA1 make it a good model to develop crops with high yield and disease resistance. Through both biochemical and genetic studies, the authors argued that a IPI7-mediated non-proteolytic K29-ubiquitination on phosphorylated IPA1 boosted the binding of IPA1 with WRKY45, thus enhancing pathogen resistance. While this reviewer finds that the research topic is of high impact, there are several puzzles needed to be solved in the current version of the manuscript. The major concerns are summarized below.

1. I am confused by the inconsistent molecular weight of IPA1 presented in Figs. 2, 3, S4, S7, S8, S9, S12. In Figs 2a, S8b, S9, a His-IPA1 with >95 kDa is shown. However, in Figs 2b, 3, S4, and S7, the detected IPA1 band is just around 50 kDa. In Figs 5 and 12, the size of IPA1 is not clear. Why were different sizes of IPA1 detected? How did the authors confirm that the band of IPA1 was correct? Since the authors have generated both overexpression and RNAi-mediated knock-down lines in their previous work (Ref 37), it might be a good idea to use these lines as positive and negative controls to verify the correct bands of IPA1 detected by the anti-IPA1 antibodies, particularly in the in vivo studies.

Response: We are sorry for this confusion. We expressed His-IPA1 using the pCold TF vector (TaKaRa, Cat# 3365), which carries a cold shock carrier that contains the soluble label "Trigger Factor (TF) companion". The "trigger factor" (48 kDa) is a prokaryotic ribosome related molecular chaperone protein (48 kDa), which is beneficial to the co-translational folding of newly expressed peptides. Therefore, the His-IPA1protein in Fig2a, S8b, and S16 is actually the His-TF-IPA1 protein. The IPA1 antibody has been validated in previous study, which can detect IPA1 protein *in vivo* as a band around 50 kDa (Jiao, Y. et al., *Nat. Genet.* 42, 541-544, 2010). Thus,

the size of His-TF-IPA1 is > 95 kDa. To avoid the misunderstanding, we have replaced His-IPA1 with His-TF-IPA1.

2. In Fig 2a, the authors detected a heavy smear band by Ub antibodies but only a faint smear band detected by both anti-His and anti-MBP antibodies, which were against to the substrate IPA1 and E3 ligase IPI7, respectively. Although I agree that the heavy band detected by Ub antibodies could result from its high antigenicity, I could not believe that it can make such a large difference with the signal detected by anti-MBP antibodies if we assume that the majority of ubiquitinated proteins in this in vitro ubiquitination system were auto-ubiquitinated IPI7 E3 ligases. Indeed, the signal detected by anti-MBP antibodies in the last lane is only slightly higher than the other lanes.

Response: We are sorry for this confusion. Fig. 2a shows a heavy smear band detected by Ub antibodies but only a faint smear band detected by both anti-His and anti-MBP antibodies, which is mainly due to their different exposure times. We have replaced them with another set of images with similar exposure times in the revised Fig. 2a.

3. Since the authors have developed an in vitro ubiquitination assay system in Fig 2a, it would be a good idea to test whether His-IPA1(S163D), but not His-IPA1(S163A) (see below), can be ubiquitinated by IPI7 via K29 directly in this system.

Response: Thanks for your suggestions. We performed the *in vitro* assay and found that His-TF-IPA1(S163A) can be ubiquitinated by adding IPI7 (Supplementary Fig. 8b). However, IPI7 cannot promote the transactivation activity of IPA1(S163A) (Supplementary Fig. 9b and 10), which might be due to the differences in the 3D structures of plain IPA1, phosphorylated IPA1, ubiquitinated IPA1, phosphorylated and ubiquitinated IPA1 (We have discussed the possible reason in the discussion section Line 311-315).

Moreover, we detected the *in vitro* ubiquitination of IPA1 mediated by IPI7 using the K29 antibody and obtained a plain image (shown below). During the ubiquitination process, E3 ligases play a crucial role in governing substrate specificity, while the E2 ubiquitin–conjugating enzyme is often considered as a “carrier of ubiquitin” determining the length and topology of ubiquitin chains and the

processivity of the chain assembly reaction. E3 selects the right E2 to generate the appropriate Ub signal on the target protein, thus controlling the fate of a given substrate. The lysine residue in the substrate that accepts the next ubiquitin is usually determined by E2, and E2 can also determine the linkage specificity and length of the ubiquitin chains, thereby transferring the activated ubiquitin to the substrate (Yau, R. & Rape, M. *Nat. Cell Biol.*18, 579-586 2016; Nakamura, N. Ubiquitin System. *Int J Mol Sci.* 19, 2018. ) There are 48 members of E2s in rice plants (Bae & Kim, *Biochem Bioph Res*. 444, 575–580, 2014). Which one is the right E2 helping IPI7 to form K29-ubiquitin chain on IPA1 is an interesing story that remains to be investigated. We have disscussed it in our manuscript (Line 316-321).

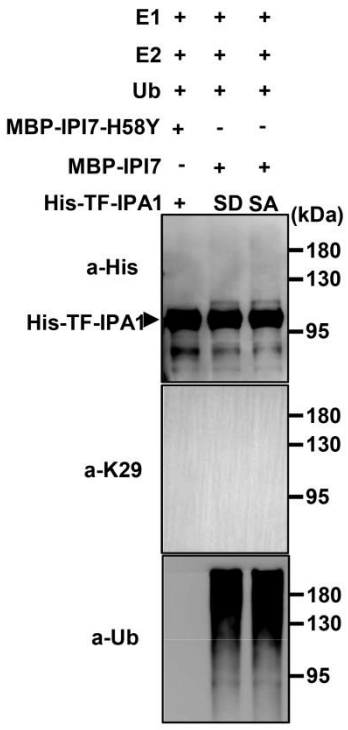


Figure legend: *In vitro ubiquitination* of His-TF-IPA1(S163D) and His-TF-IPA1(S163A) by MBP-IPI7. MBP-IPI7(H58Y) was used as negative control. Immunoblotting was performed with antibodies against His, Ub or K29-polyubiquitin chain (K29). The presence (+) or absence (-) of components in the reaction mixture is indicated.

4. Since the authors did not find stability changes of IPA1 in Ri22 and IPI7 overexpression lines in Fig 3, it would be expected that MG132 treatment will not enhance the ubiquitinated forms of IPA1. Therefore, in the experiment of Fig 2b, it

would be a good idea to include this control. In addition, does the transcription level of IPA1 remain no changes in different samples?

Response: Thank you for your suggestions. Although our study did not find stability changes of IPA1 in Ri22 and *IPI7* overexpression lines, it does not mean that MG132 treatment would not enhance the ubiquitinated forms of IPA1 because a previous study has indicated that the IPA1 protein can be degraded via the ubiquitin-26S proteasome degradation system, which can be inhibited by MG132 (Wang, J. *et al. Plant Cell.* 29, 697-707, 2017). Actually, it is a commmon event in cells that one protein is ubiquitinated at different sites promoted by different E3 ligases and then enters different fates. For example, The p53 protein in animals is a transcription factor that initiates cell cycle arrest and apoptosis and by this counteracts tumorigenesis. More than ten E3 ligases have been identified to promote ubiquitination of p53. These E3 ligases help to decorate p53 with ubiquitin and small ubiquitin-like proteins to control the fate of p53 protein through degradation, stability or subcellular localization of p53 (Pan, M. and Blattner, C. *Cancers.* 13(4): 745, 2021). Therefore, in the experiment of Fig 2b, MG132 treatment might also enhance the ubiquitination of IPA1 *in vivo*, which may not be associated with IPI7 function.

We have detected the transcript levels of IPA1 in these samples and found no obvious changes (Supplementary Fig. 5b and 7d).

5. In Figs 3 and S7, for quantitative comparisons of protein bands detected by immunoblotting assays, it is necessary to provide a statistical analysis. I am not sure how many replicates that the authors did. Same as Comment 4, does the transcription level of IPA1 remain no changes in different samples? In addition, the authors did not explain how the protein bands were displayed and recorded. If by chemiluminescence on X-ray film, the signal could be out of the linear range easily, thus resulting in misleading conclusions.

Response: Thanks for your suggestions. We performed statistical analysis for these assays as you suggested (Fig 3 and Supplementary Fig. 7). All of these assays have been carried out for more than three times. We now show the results of another repeat for Supplementary Fig. 7.

In addtion, we have assessed the transcript levels of IPA1 in these samples and found no clear changes (Supplementary Fig.5b and 7d).

We have explained how the protein bands were displayed and recorded for quantitation in the Material and Methods section (Line 494-506).

6. In Fig. S4, S1, S2, what about the effect of a phosphorylation-mull mutant, such as IPA(S163A)?

Response: Thanks for your suggestions. Firstly, we carried out LUC-reporter experiments for IPA1 phosphorylation-null mutant, IPA1(S163A). IPI7 has no effects on the transactivation activity of IPA1(S163A), as shown in Supplementary Figs. 2b, 9b, and 10.

Next, we performed *in vitro* ubiquitination assay to detect whether IPI7 could ubiquitinate the phosphorylation-null mutant, IPA1(S163A). IPI7 can ubiquitinate IPA1(S163A) similarly as IPA1(S163D), as shown in Supplementary Fig. 8b,

7. In Figs 5 and 12, I think it is critical to verify the specificity and efficacy of each Ub chain-specific antibody. Neither references nor controls (positive and negative) are provided.

Response: Thank you for the suggestions. However, these Ub chain-specific antibodies were ordered from the ABclonal Technology company and the Bio-swamp company. The specificities of all of these antibodies have been verified by different researchers. Their detailed information can be found on the company websites. We have added the information of these antibodies in the Methods section (Line 453-459). Unfortunately, it is beyond the scope of this report to verify each individual antibody.

8. In Fig 9, I don't think the data is conclusive. Since the majority of IPA1 proteins are non-ubiquitinated, the lack of difference of mobility shift could simply result from the binding of non-ubiquitinated IPA1 with Biotin-WRKY45P. In addition, a phosphorylation-mull mutant, such as IPA(S163A), would be a good control as well.

Response: Thanks for your suggestions. We used IPA1(S163A) as a control in this experiment as you suggested. The results showed that the IPA1(S163A) could not bind to the biotin-labeled *WRKY45* promoter, which is consistent with our previous study (Wang, J. *et al. Science* 361, 1026-1028, 2018), and that IPI7 has no effects on the interaction between IPA(S163A) and the *WRKY45* promoter (Supplementary Fig.15b).

To avoid possible confusions, we have removed the conclusion of the data you deemed inconclusive, but rather discussed it in the discussion section as a guide for future research (Line 293-294).

9. Line 294. If we assume the authors' data presented in Figure 5 is correct, the data only suggest that IPA1 is K29-ubiquitinated. However, it does not support that this ubiquitination is necessary for the IPA1-mediated response to M. oryzae. IPI7 could also lead to another type of ubiquitination that is necessary for this response.

Response: We have reorganized this sentence: the IPI7-mediated enhancement of K29-polyubiquitination of IPA1 is crucial for the IPA1-mediated response to *M. oryzae* (Line 308).

In IPA1 protein, we only detected a reduction in K29-type ubiquitination of IPA1 in *ipi7-ko* plants (Fig. 5a and Supplementary 11a), indicating that only K29-type ubiquitination of IPA1 is associated with IPI7. Therefore, we rule out other types of ubiquitination mediated by IPI7. Moreover, in transactivation assays, we detected significant light signals derived from *proWRKY45:*LUC only when the reporter was co-expressed with both IPA1(S163D)-HA and IPI7-MYC, but not with IPA1(S163A) and IPI7-MYC (Fig. 4a and Supplementary 9). Therefore, IPI7 must promote the ubiquitination of IPA7(S163D) to enhance *proWRKY45*:LUC expression, because if IPI7 ubiquitinates other proteins to enhance *proWRKY45*:LUC expression, the IPA1(S163A)/IPI7-MYC control should also enhance *proWRKY45*:LUC expression, but it did not. These results argue against involvement of other types of ubiquitination.

10. Lines 311-312, I don't understand what the authors meant. There is no data provided to support this conclusion. If K0 IPA1 has been tested, please show the data. I feel the entire paragraph has too much speculation.

Response: Thank you for the suggestions. We have performed *in vitro* ubiquitination experiments using IPA1 lysine mutant proteins, including IPA1(K37R), IPA1(K117R), IPA1(K124R), IPA1(K86R), IPA1(K88R), IPA1(K131R), and IPA1(K161R). As the results shown in Supplementary Fig. 16, none of seven single-K-site disruptions in IPA1 affected IPI7-mediated ubiquitination (Supplementary Fig. 16). Thus,

IPI7-mediated ubiquitination likely occurs on other amino acids of IPA1.

Minor Comments.

1. Line 57, “location” is not a good word. “site” might be better.

Response: We have replaced "location" with "site".

2. Line 70, “CULLIN” should be “cullin 1” or “CUL1”. “SCF-box” is not a conventional term. It should be “SCF”. However, if the authors meant SCF, the sentence does not include many other CUL-RING E3 ligases.

Response: Thanks for your suggestion, we have revised Line 70 as follows: "SKP1-CUL1-F-box (SCF), CUL3-BTB, and CUL4-DDB1-DWD." (Vierstra, R. D. *Nat. Rev. Mol. Cell Biol.* 10, 385-387 2009.)."

3. Line 81, “MAMPs” ?

Response: MAMPs is an abbreviation for microbe-associated molecular patterns. We have given the full name in the revised manuscript (Line 81).

4. Line 83, “RLCKs”?

Response: We have removed this word (Line 84).

5. Line 123, “IPA1” could be “IPA1’s”.

Response: Thanks for your suggestion, we have revised this word (Line 125).

6. Line 161, “Fig. 6a-c” could be simply “Fig. 6”.

Response: Thanks for your suggestion, we have revised it as you suggested (Line 166).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

In the revised manuscript, the authors have fully addressed all of my concerns. I believe the data to be convincing and sufficient to demonstrate the role of plants such as rice, utilize non-proteolytic K29-ubiquitination as a response to pathogen infection to fine-tune the Ideal Plant Architecture 1 （IPA1）transactivation activity for promoting immunity. Clearly, many interesting avenues for follow up will emerge from this work from the authors as well as other groups in the field of plant-microbe interaction. I am looking forward to seeing this work published.

Reviewer #2 (Remarks to the Author):

I appreciate the authors' effort in addressing my comments. I am happy with the current version and don't have any more comments.