Tissue-specific Ubiquitination by IPA1 INTERACTING PROTEIN 1 Modulates IPA1 Protein Levels to Regulate Plant Architecture in Rice

Jing Wang, Hong Yu, Guosheng Xiong, Zefu Lu, Yongqing Jiao, Xiangbing Meng, Guifu Liu, Xuewei Chen, Yonghong Wang, and Jiayang Li

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Corresponding authors: Jiayang Li, jyli@genetics.ac.cn, and Yonghong Wang, yhwang@genetics.ac.cn.

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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.)

TPC2016-00752-RA 1st Editorial decision – *declined* Sept. 13, 2015

Thank you for choosing to send your manuscript entitled "IPA1 INTERACTING PROTEIN 1 orchestrates IPA1 protein levels to regulate rice plant architecture." for consideration at *The Plant Cell*. …

As you will see from the reviewers comments, there was overall consensus that, while the work is potentially novel, much additional work would be needed to substantiate the most important claims; In particular, the lack of loss of function mutations in *IPI1*, the reliance on overexpression transgenics for molecular analyses, and the need for more thorough investigation (with improved controls) of the differential ubiquitination of IPA1.

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

[Reviewer comments shown below along with author responses]

TPC2016-00879-RA Submission received Nov. 23, 2016

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

Reviewer #1:

The manuscript by Wang *et al*. presents the identification and characterization of a newly identified RING E3 ligase, IPI1, which interacts with IPA1. The authors presented data suggesting that IPI1 could negatively and positively regulates the protein abundance of IPA1 by mediating polyubiquitination with K48-linked and K63-linked polyubiquitin chains, respectively. This is potentially interesting and has added a new concept to what we have known. My major concern is this paper heavily rely on overexpression strategy thus the physiological function of the IPI1 still remains unanswered. We have learned that some proteins cannot be induced by specific stress but when it is overexpressed in transgenic plant do confer resistance to that stress. For example, the HDG11 OE mutant, a homeodomain-START

transcription factor, had higher levels of ABA and Pro than the WT and demonstrated an increased resistance to oxidative/drought stress. However, *HDG11* can't be induced by drought stress and does not endogenously play a role in drought tolerance since the deletion of the factor has no effect on stress responses (Yu et al. 2008 *Plant Cell* 20: 1134-1151). Thus to address the physiological function of IPI1, authors must show the correlation between IPI1 protein and IPA1 protein in wild-type plants. Also characterization of knockout or knock-down mutants of *IPI1* must be presented in order to support what the claimed function.

RESPONSE: To address the reviewer's major concern about the lacking of lose-of-function mutant, we tried our best to generate the IPI1-knockout mutants using the CRISPR/Cas9 technology, as shown in Supplemental Figure 8 in the revised manuscript. In the *IPI1* knockout mutants, the IPA1 protein level was apparently decreased in the shoot apex but increased in the young panicle (Figures 7A and 7D). Furthermore, the *IPI1* knockout mutants also showed altered plant architecture including increased tiller number and grain number per panicle, which is in contrast to *IPI1*-overexpressing transgenic plants (Figures 6).

In addition, the manuscript requires several critical revisions with additional experiments as described below.

Fig 1. (C): GFP control and IPA1-GFP Co-IP results should be presented on the same blot. (D): Marker of nucleus localization should be included.

RESPONSE: As suggested, for Fig 1C we carried out the experiment again on the same blot, and for Fig 1D we co-transformed with a nuclear marker (NLS-RFP) to demonstrate the nuclear localization.

Fig 2. (D): Detailed information of *in vivo* ubiquitination assay in NP and UB:IPI1 should be provided, such as plant growth stage, tissue used, etc.

RESPONSE: Four-week-old seedlings were used in the *in vivo* ubiquitination assay in NP and UB:IPI1. We added detailed information in the method.

Fig 3. (B): How old were the seedlings? Since IPA1 stability can be much enhanced by MG132 in calli cells and seedling what is actually the major form of IPA1 ubiquitination? is K48-chain of IPI1 a more abundant form? This may answer the physiological function of IPI1. (C): The blot of MYC-IPI1 was too dark and blurry. (D): Was MYC-IPI1 co-expressed in tobacco leaves? Show blot. GFP internal control was too much. The bands are too saturated to show even transfection and expression efficiency.

RESPONSE: For Fig 3B, four-week-old seedlings were used in the assay. As for the major form of IPA1 ubiquitination, we found that IPI1 could promote IPA1 tagged with the K48-ubiquitin chain in leaves and young panicles, but with the K63-ubiquitin chain in shoot apexes (Figure 8A). Taken together with the result from cell-free degradation experiments, it is apparent that K48 is the major form of IPA1 ubiquitination promoted by IPI1. For Fig 3C, we carried out the experiment again as suggested. For Fig 3D, yes, the Myc-IPI1 was co-expressed in tobacco leaves and the blot was showed. As suggested, we carried out the GFP blot experiment again.

Fig 4. (A) The plants must grow in the same conditions and in the same figure. It would be more convincing if the authors combined phenotypes of IPA1 loss-of-function mutants (maybe miR156 OE). In addition to (B) and (C), the direct physiological relationship between tiller number and *IPA1* expression in shoot apex should be presented.

RESPONSE: For Fig 4A, the plants were grown under the same conditions and in the same figure. As suggested, to understand the direct physiological relationship between tiller number and IPA1 abundance in shoot apex, we showed the tiller numbers and protein levels of IPA1 in shoot apexes in NP, *IPA1* RNAi and *IPI1* OE plants in Supplemental Figure 5. (It should be pointed out that the miR156OE transgenic plant has many phenotypes because miRNA156 may target many genes.)

What is the expression pattern of *IPA1*/ *IPI1* gene and protein in different tissues and growth stages? What is young panicle? This must be defined in the M&M.

RESPONSE: As suggested, we showed the expression pattern of *IPI1* in different tissues and growth stages including roots, shoot apexes, leaves and young panicles in Supplemental Figure 6. The expression pattern of *IPA1* had been described in our previous paper (Jiao et al. 2010 *Nature Genetics* 42: 541-544). In addition, we also defined the young panicles (< 3 cm) in the main text.

What are the putative downstream genes of IPA1? Could the authors provide the expression patterns of IPA1 downstream genes in different tissues to prove that the opposite phenotypes were really caused by the differential expression of IPA1 protein?

RESPONSE: Thanks for the suggestion. In our previous study, we identified several important genes as the targets of IPA1, including *OsTB1* and *DEP1*. We therefore detected the expression level of *OsTB1* and *DEP1*, and found as expected that *OsTB1* and *DEP1* were up-regulated in shoot apexes and down-regulated in young panicles in *IPI1*-overexpressing plants (Figure 5) and down-regulated in shoot apexes and up-regulated in young panicles in the *IPI1* knockout mutant (Figure 7).

Fig 5. This part is redundant in my opinion. It could be moved to supplemental data.

(B) Effect of IPI1 levels on the degradation of ipa1. The Myc-IPI1 protein is not proportionally increased.

RESPONSE: As suggested we moved this figure to Supplemental Figure 9. The blot of MYC-IPI1 was too dark and blurry to show the proportional increase of Myc-IPI1. We therefore improved this experiment with a shorter exposure in Supplemental Figure 9B.

Fig. 6. (B) and (D) polyubiquitination of IPA1 was enhanced in both young panicles and shoot apexes in Ub:IPI1/Ri22. The IPA1 protein was mainly modified with the K63-polyubiquitin chain in shoot apexes (Figures 6B), and mainly with the K48-polyubiquitin chain in young panicles (Figures 6D). Where is the evidence that K63-polyubiquitin chain or K48-polyubiquitin chain is really associated with IPA1? It is possible that IPI1 has several target proteins.

RESPONSE: Because specific anti-IPA1 polyclonal antibodies were used in the IP experiment in the *in vivo* ubiquitination assays, most polyubiquitin chain detected should be associated with the IPA1 protein.

P11 "Mutation in *ipa1* Has No Effect on Polyubiquitination Mediated by IPI1". This subheading must indicate which mutation.

RESPONSE: Thanks for the suggestion. We have revised the subheading as "Mutation in *ipa1* Disturbing the miR156 Target Sites Has No Effect on Polyubiquitination Mediated by IPI1".

Is IPI1 ubiquitously abundant in WT? Since the function of IPA1 is to maintain ideal architecture of rice, why is IPA1 so unstable (clear band only appear after MG132 treatment)?

RESPONSE: IPI1 is ubiquitously expressed, but its expression levels in different tissues are varied in WT (Supplemental Figure 6). The IPA1 abundance is regulated by IPI1, an E3 ligase in the 26S protein degradation system. Therefore, as a inhibitor MG132 inhibits the IPA1 degradation in the assay and the Western blot showed a stronger band. In fact, the IPA1 band is still there without adding MG132 though it is much weaker. In addition, in this cell-free degradation assay, CHX was added to inhibit the protein synthesis, which might be the reason why a much weaker IPA1 band was detected without MG132 treatment.

Reviewer #2:

This manuscript reports a protein interactor for IPA, a SPL-type transcription factor in rice regulating shoot architecture. The interactor is a ubiquitin E3 ligase, previously uncharacterized, called IPI1. The authors show that this protein is an active E3 and can ubiquitinate recombinant IPA1 *in vitro*. The curious result is that OE of *IPI1* leads to a reduction in IPA1 protein in panicles as expected, but does not reduce IPA1 in shoot apices. The authors present intriguing data that the form of IPA1 is different between the two and IPI is responsible for differential chain formation. However, there are some missing pieces that weaken the argument and these are explained below.

1. The protein differences in 4J are not convincing and need better normalization controls. Such as actin western blot with appropriate exposure. It is possible that there is no effect in the shoot apex- possibly because there is little expression of the ligase (or maybe it is destabilized in this organ??). Rather than an increase in the shoot apex, perhaps there is no effect. The authors should careful compare protein levels with hopefully multiple controls to clearly establish a difference. I agree that the difference appears to be larger in the *ipa* background, but why would that be the case?

RESPONSE: As suggested, we performed the assay again with Actin as internal controls and obtained consistent results in both *IPI1*OE plants (Figure 5A and D) and newly generated *IPI1* knockout mutants (Figure 7A and D). In the *ipa1* background, the protein level of IPA1 is so great abundant to be tagged by IPI1, which might be the reason for the case.

2. Figure 6A. *In vitro* ubiquitination. The nature of the ub-ub chains made, depends to some extent on the species of E2 used. Some E2s show strong preference for one type of chain, and others can make different chains and others show specific chain formation with a specific E3. Therefore, the presence of K48 and K63 chains from *in vitro* assays does not prove the nature of chains present *in vivo*. The E2 used in the assays has to be specified - the specific one used and the source. Similarly, the sources of E1, creatine kinase and ubiquitin should be listed. Independent of which E2, the authors may not be using the physiologically relevant E2. I suggest that this experiment be removed.

RESPONSE: As suggested, we removed this experiment in the revised manuscript.

3. The molecular explanation of how OE of *IPI1* can lead to hyper-accumulation of IPA1 in the shoot apex, but decreased level in panicles, is that the enzyme functions differently, attaching K63 chains in the former, stabilizing the protein, but attaching K48 chains in the former, targeting it for degradation. This explanation stems from the use of chain-specific antibodies. These have been reported and used in the literature. Since this experiment is the central, and most novel data of the paper, it seems reasonable to request control experiments. One would be to show that the commercial antibodies do indeed show the specificity as indicated. How many times have we tried a reagent that did not perform as claimed? The authors could purchase K48 and K63 chains and test them with the Ab preparations used. Second, the authors should perform an IP with pre-immune sera to show that chains, or other proteins with chains co-IP with antibodies. Maybe the same experiment using an *IPA*-null plant if that exists, to show that the results are not obtained when IPA is not present? Certainly the first two experiments are easily performed.

RESPONSE: Thanks. As suggested, we have validated the specificity of K48- and K63- antibodies using purchased K48 and K63 chains (Supplemental Figure 12A). Then, we performed an IP with pre-immune sera to detect the special of K48- and K63-Ab (Supplemental Figure 12B). No *IPA*-null plant is available for now.

4. If K63 chains stabilize IPA1, then one might expect that most IPA1 is modified with chains to protect it, this may be another important piece of data. If one looks at a western of total protein (shoot apex), is IPA1 predominantly one band or can a smear of higher MW forms be detected? It is difficult to explain the differences in total protein accumulation observed if only a small percentage of the protein is modified. For K48, these species are transient, so one would expect only a small percentage to be modified.

RESPONSE: Thanks. We now showed a bigger area around the band in Figure 8A, and a very weak smear of higher MW forms of IPA1 could be seen in the shoot apex. Although K63 chains stabilize IPA1, it appears that K63-labled IPA1 might quickly associate with its targets and de-ubiquitination quickly, which is a dynamic process. Similar events were also reported in animals, such as TRAF6, a E3 ligase, which activates its substrates dependent on the K63-chain system (Cao et.al 1996 *Nature* 383:(443-446), Yamashita et al. 2009 *Molecular Cell* 31(6): 918–924. Deng et al. 2000 *Cell* 103(2): 351-361).

5. The other point is that if IPA1 is differently modified, the experiments do not show that IPI1 is responsible. They have shown that IPI1 can catalyze the two different chains (but that could be a function of the E2 used) *in vitro*, and that IP'ed IPA1 has different chains- but the link between the two is not direct. Are these chains lost in the *ipi* mutant?? This result would strengthen the relationship between the two.

RESPONSE: Thanks for the suggestion. Compared with Ri22, in *Ub:IPI1/Ri22* transgenic plants, the K63-polyubiquitination of IPA1 was enhanced in shoot apexes, while its K48-polyubiquitination was enhanced in young panicles and leaves (Figure 8B to 8G), indicating that IPI1 is responsible. At mean time, in *Ri22* plants the IPA1 protein was tagged with both K48- and K63-polyubiquitin chains in shoot apexes and leaves, and mainly with the K48-polyubiquitin chain in young panicles, suggesting that IPA1 might be tagged by multiple E3. We therefore think that these chains might not disappear in the *IPI1*-knockout mutant.

The "*in vivo*" ubiquitination assay is a misleading description of this experiment. The experiment is to determine the state of ubiquitination of IPA1 *in vivo*. It is not analogous to *in vitro* ubiquitination assays where ubiquitination occurs

during the assay. I suggest another name. How about calling it detecting IPA ubiquitination *in vivo*? [The cited reference does not call it an *in vivo* ubiquitination assay.]

RESPONSE: Thanks, we have changed the name as suggested.

Reviewer #3:

The manuscript written by Wang *et al*. described the identification and characterization of *IPI1*, encoding a RING-finger E3 ligase that could interact with IPA1, which is an important gene regulating plant architecture and grain yield in rice. The authors found that IPI1 could promote IPA1 ubiquitination with two different types of polyubiquitin chains (k63 and k48) in shoot apexes and panicles, respectively, fine-tune the protein level of IPA1, and then affect plant architecture in rice. These findings revealed the regulation mechanism of plant architecture involving the post-transcriptional modification of IPA1, and provided new insights into the complex molecular mechanism of plant type in rice.

(1) In this manuscript, the authors just provided the *IPI1*-overexpressing transformation evidences in both Nipponbare and Ri22 genetic backgrounds, and these results showed that the transgenic plants had shorter and thinner culm and smaller panicle size than those of the controls. Hence, if the authors can provide the *IPI1*-downregulation or knockout transformation evidence, showing a positive effect on grain yield, it would further confirm the finding about the genetic regulation of plant architecture in rice.

RESPONSE: Thanks. We have tried our best to generate the *IPI1*-knockout mutants using the CRISPR/Cas9 technology, as shown in Supplemental Figure 8 in the revised manuscript. In the *IPI1* knockout mutants, the IPA1 protein level was apparently decreased in the shoot apex but increased in the young panicle (Figures 7A and 7D). Furthermore, the *IPI1* knockout mutants indeed showed positive effect in increasing tiller number and grain number per panicle, which is in contrast to *IPI1*-overexpressing transgenic plants (Figures 6).

(2) The authors found that over expression of *IPI1* in the japonica variety Nipponbare background could reduce the tiller number, which was similar to the phenomenon of *IPA1*-overexpressing transgenic plants. While other traits in *IPI1*-overexpressing transgenic plants were opposite to those of *IPA1*-overexpressing transgenic plants. In other words, the result in the ectopic expression of *IPI1* gene under a strong constitutive promoter cannot explain the regulation mechanism of plant architecture involving IPA1 in native conditions. Therefore, my concern is whether and how IPI1 affects tiller number through regulating IPA1 abundance in native conditions, for example, in the Nipponbare or Ri22 background.

RESPONSE: Using the newly created *IPI1* knockout mutant, we showed that IPA1 abundance is regulated by IPI1 in native conditions. Furthermore we also carried out an additional three experiments to understand how IPI1 affects tiller number through regulating IPA1 abundance in native conditions. 1. We performed a cell-free degradation assay in shoot apexes and young panicles to detect the stability of IPA1. As shown in Supplemental Figure 11, the degradation of IPA1 is slower in shoot apexes than in young panicles. 2. To avoid the effects of strong promoter on IPI1's *in vivo* function, we created *IPI1* native promoter-driven *IPI1*-overexpressing transgenic plants (*IPI1:IPI1-GUS*). Similar phenotypes were also observed as Ub:IPI1, including reduced plant height and tiller numbers (Supplemental Figure 7). 3.

In our previous study, we identified several important genes as the targets of IPA1, including *OsTB1* and *DEP1*. We therefore detected the expression levels of *OsTB1* and *DEP1*. As expected, *OsTB1* and *DEP1* are up-regulated in shoot apexes and down-regulated in young panicles in *IPI1*-overexpressing plants (Figure 5) and down-regulated in shoot apexes and up-regulated in young panicles in *IPI1* knockout mutant (Figure 7). All these results confirmed that IPA1 abundance is regulated by IPI1 in native conditions.

TPC2016-00879-RA 1st Editorial decision – *revision requested* Dec. 19, 2016

We have received reviews of your manuscript entitled "IPA1 INTERACTING PROTEIN 1 orchestrates IPA1 protein levels to regulate rice plant architecture." Thank you for submitting your best work to *The Plant Cell*.

The two reviewers that commented on your initial submission were able to review the revised manuscript and we have had a post-review consultation to clarify several points and highlight the most significant issues to address in a revision, which we are offering as an option. Indeed, as you will see below, although the two reviewers and this reviewing editor agree that the work is substantive and may become acceptable for publication pending revision and re-review, there remained several important questions on the data and, in particular, details on replication, quantification, and reproducibility. In this letter, we ask that you address the reviewers' comments listed below, including additional experimentation where appropriate, for example, in quantifying western blots. To provide better guidance on the most pressing issues, we ask you to pay attention to the following points in preparing your revision.

For all experiments and data involving transgenic lines, details on whether more than one line was evaluated would be expected. For overexpression experiments, typically multiple independent lines are generated and compared quantitatively to each other and wild-type plants. At least two independent lines would be ideal, and with multiple replicate plants evaluated for each line, with appropriate statistics. If multiple independent lines could not be generated and evaluated, please explain why.

There was discussion on the valuable new results from the single CRISPR-generated loss-of-function *IPI* mutant. In particular, the reviewers in post-review consultation asked why only one mutant was generated by the company when typically two independent mutants would need to be presented to validate the loss-of-function phenotypes. Related to this, is it clear that the truncated protein is indeed resulting in a loss-of-function plant, and not a dominant negative or less likely, a neomorph? This reviewing editor feels that the data on the single-base insertion allele is likely creating a loss-of-function null mutant, but this should be addressed in the response to reviewers. As well, it was requested that the methods section on CRISPR be elaborated to indicate how the company generated the allele, and whether homozygous Cas9 negative seed were provided, how many gRNA target sets were selected, etc. Of course, the most conclusive evidence of recessive loss-of-function would be to evaluate segregating families from the original first generation transgenics, or from outcrosses to wild-type plants. Was this done with this single allele or with a second allele?

The previous point relates to the general comment that the methods section deserves greater attention, including more details on experimentation, such as number of replicates performed for Western blots and quantitation. In particular, a major comment was made on Supplemental Figure 11, which presents differential degradation of IPA1 in the shoot versus young panicles. Reviewer 2 asked why this important result was not in the main figures, and why replication and statistical analyses of the data from multiple independent experiments was not performed. Again, the methods do not provide sufficient details on how this experiment was performed, with replicates or not, etc.

Finally, we ask that you address Reviewer 2's concern on Figure 8, that the evidence for the observed enrichment of K63 is linked to IPA1. Several controls are recommended to address this point, including better presentation of loading controls and the recommendation that this experiment also be performed under denaturating conditions. This would involve needing to reduce SDS so antibody can be added, and then IP and Western blot. Reviewer 2 has suggested removing these data if the above cannot be done. Differential effects on IPA accumulation by altered expression of the ligase is interesting by itself.

Please contact us if there are ambiguous comments or if you wish to discuss the revision.

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

[Provided below along with author responses]

TPC2016-00879-RAR1 1st Revision received Feb. 21, 2017

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

Reviewer #1:

This manuscript described the identification and characterization of *IPI1*, encoding a RING-finger E3 ligase, which

could interact with IPA1, a transcription factor regulating plant architecture and grain yield in rice. Interestingly, the authors found that IPI1 could promote ubiquitination of IPA1in shoot apexes and panicles, which resulted in enhancing the stability of IPA1 in shoot apexes and promoting the degradation of IPA1 in young panicle. The phenotypes of both *IPI1* over-expressing transgenic plants and knock-out mutants were consistent with the protein levels of IPA1. Especially, *IPI1* knock-out mutants had more tillers and larger panicles, ultimately leading to a dramatically increase of grain yield per plant (+48.2%) compared with the wild type. These results demonstrated that IPI1 regulated the development of tiller and panicle through the post-transcriptional modification of IPA1, and also provided new insights into the complex regulation mechanism of plant architecture in rice. The authors presented several data supporting their finding, which is very interesting. Both experimental design and data analyses were performed very well. Altogether, this manuscript was well written, reported good analyses and advanced the rice biology field, the authors need to check the data of yield-related traits in comparison of *IPI1* knock-out mutants and wild type (in Figure 6). Because the increase ratio of grain number was not consistent with that of yield per plant. Was there a significant difference in grain weight between the *ipi1-1* mutant and wild type?

RESPONSE: Thanks. We have checked the grain weight of the *ipi1-1* mutant and wild type. As shown in Figure 6, although the grain weight of the *ipi1-1* mutant is lower ($p = 0.03$), the yield per plant is still higher because of the increased tiller number and grains per plant.

Reviewer 1 provided the following comment in post-review consultation: I agree with Reviewer #2's comments. The authors need to provide more information, including E2 isozymes, the number of independent transgenic plants, and statistical analyses of the data from multiple independent experiments.

RESPONSE: Many thanks. We have added the detailed information about the assays in Methods. We also performed statistical analyses of the data for the experiments and their results can be found in the revised figures.

Reviewer #2:

The authors present an interesting story. Convincing data are presented that an E3 (IPI1) interacts with IPA1 and ubiquitinates it *in vitro* and when expression is altered, IPA1 levels are affected *in vivo*. An interesting observation was made that the effect of IPI1 on IPA1 is different in the shoot versus the panicle and propose that IPI makes different chains on IPA in the two different organs. The latter data are not strong, based on one type of experiment that has some flaws (see #9 below). The analysis of a CRISPR allele also has problems. In general, information on the number of independent plants analysed, the number of times the experiment was repeated and quantitation of data are lacking- all these weaken the conclusions.

1. Figure 2A. The activity of IPI is demonstrated with an *in vitro* ubiquitination assay. A key component in this reaction is the E2 because the type of E2 used could determine whether or not active and what kind of chain that is formed. The identity of the E2 must be given in the material and methods and figure legend. Which of the E2 isozymes was used? Information on whether purchased, purified in lab should be indicated. The source of E1 should be reported as well, although E1's role is more generic. Others can't repeat the work if they don't now the materials used.

RESPONSE: Thanks. The enzyme components used *in vitro* ubiquitination assay were obtained from Boston Biochem: E1 (Cat#E-305) and E2 (Cat#E2-607), which have been described in Methods in the revised manuscript.

2. Figure 3A. The conclusion from this panel is that "degradation of IPA1 in cells could be strongly inhibited by MG132..." First, this is a lysate, so the assay is not "in cells", the authors call it a "cell-free degradation assay". Second, a time zero or input would improve this experiment. Is there any degradation is this system over time in the presence of MG132. Third, what was the incubation time? The description of this assay in the material and methods is confusing. It is not clear how the experiment is performed. DMSO treatment not mentioned. If the chemicals were added to intact plants, ground up and then western blot performed, that is not in vitro test. That is a test of the inhibitor on in vivo expression.

RESPONSE: Many thanks. 1, We have changed the sentence to "degradation of IPA1 could be strongly inhibited by MG132". 2, As suggested, we have added the time zero in Figure 3A to improve this experiment. 3, Sorry for missing the experimental details and we have provided the complete information in "Cell free degradation assay"

in the Methods in the revised version.

3. Figure 3B. The effect of MG132 on intact cells is very weak, not very convincing. Only one blot is shown, this experiment has to have replication, quantitation and statistics.

RESPONSE: Thanks. To make this result convincing, we first adjusted the image and performed quantitation. As shown in Figure 3B, the effect of MG132 on intact cells is significant. In addition, we have performed the assay twice and similar results were obtained (replicates are given here as follows).

4. Figure 3D. The image is too dark, the quality is not up to publication standards.

RESPONSE: We have adjusted the image as suggested.

5. Figure 4. The results from over-expressing *IPI1* are presented. However, it is not clear how many independent transgenic lines were analyzed. One than one line should be analyzed. And the number of lines should be reported in the material and methods and in the figure legend. The *n* in the Figure legend is 5. Is this from one experiment and one plant? or is this from several plants in the same experiment or 5 independent experiments? Similarly, in Sup. Fig 2, is this expression of one transgenic line?

RESPONSE: Thanks. In Figure 4, ten independent plants from one transgenic line were analyzed (sorry for the mistake by labeling *n* = 5, which is actually 10). To confirm this result, we also analyzed another independent *IPI1* over-expression transgenic lines (Ub:IPI1-2) in Sup. Fig 4. We have added the detailed information in Methods and the figure legends. The *n* in the related figures indicates the number of plants in one experiment and we have described it in the revised manuscript. In Sup. Fig 3, the expression level is represented from one transgenic line. We have added this information in the legend.

6. Fig 6 and Supplemental Figure 8. The CRISPR allele is not well described. The authors write that "Sequence analysis revealed that a G insertion happened in the fourth exon of *IPI1*, which results in a truncated IPI1 lacking 283-aa at the C-terminal (Supplemental Figure 8A). We found this insertion at the N-terminal of *IPI1* could abolish its nucleus localization and interaction with IPA1 (Supplemental Figure 8B and 8C)."

In yeast, when results are negative, one needs to show that the protein is there, but doesn't interact. I also don't understand why the insertion is referred to as "at the N-terminal of IPI1." Doesn't seem to be at the N-terminus.

These plants are referred to as *IPI1*-knockout plants. These are NOT knock-out plants unless the authors can show no RNA expression or no protein expression. They could show that it is recessive to the wild type allele, suggesting it is loss of function, but I don't see how it is shown that this is a knock-out allele.

RESPONSE: Many thanks for the comments. As suggested, we have changed the description of the CRISPR allele and added more information of the *ipi1* mutant in Methods and in Supplemental Figures 9 and 10.

To confirm the negative results of yeast two-hybrid, we detected the protein levels of IPA1 and IPI1 in yeast cells, and the result is shown in Supplemental Fig 9E in the revised manuscript.

We agree with the reviewer's comments that *ipi1-1* is not a knockout mutant. We showed that the mutation on the cDNA sequence of *IPI1* in the *ipi1-1* mutant generates a new stop codon, which results in a truncated ipi1 lacking 283-aa at the C-terminal (just four amino acids are new to IPI1). In our results, we also indicated that the C-terminal of IPI1 is essential for its nucleus localization and interaction of IPA1. Therefore, we believe that, at least to the regulation of IPA1, *ipi1-1* is a loss-of-function mutant and we therefore have revised it as suggested. To further confirm this result, we also analyzed another independent *ipi1-2* mutant generated by CRISPR/Cas9 and similar phenotypes were obtained and the result is shown in Supplemental Figure 10 in revised manuscript.

7. The protein differences in Fig 7 are small. How many times were these comparisons made?

RESPONSE: The assays were repeated twice and they are consistent and the replicate is given below. The quantitation data have also been given in the revised manuscript.

8. Supplemental Figure 11 is key, since it tries to show differential degradation of IPA1 in the shoot vs young panicles. This needs replication, statistical analyses of the data from multiple independent experiments. It is not convincing as is. It also should be in the main body of the paper. A description of how this experiment was performed is needed. I did not find it in the material and methods.

RESPONSE: Many thanks. We isolated the total protein from different tissues including shoot apex and young panicles using native buffer, and performed a cell-free degradation assay as described in Methods. As suggested, we have changed our description. It is an *in vitro* degradation assay. We have added it as Figure 3 with quantitation data in the revised manuscript.

9. The conclusion that IPA1 is linked with K63 chains comes from Figure 8, an IP with anti-IPA ab then western blotting with K-63 and K-48 specific Ab. They nicely show the specificity of the ab in Supp Fig 12. However, a concern is that there is no proof that the enrichment of K63 seen is linked to IPA1. K63-linked proteins could be associated with IPA1, with the K63 chains not directly attached to IPA1. The IP conditions were under native conditions, retaining protein-protein interactions. For more convincing data, the IPs need to be performed after complete denaturation of the proteins, then reduction of the SDS to allow an IP, then SDS-PAGE and a western.

RESPONSE: Many thanks for the comment. We have tried our best perform the experiment after denaturation of the proteins as suggested, but we could not detect the ubiquitination after co-IP assay (the result is given below). It is known that to detect the protein ubiquination *in vivo*, one must enrich abundant target proteins in the IP assay. It is very likely that the failed detection under the denatured conditions might be due to very low abundance of IPA1 proteins. We understand the reviewer's caution. Due to this technical barrier, we could not confirm whether K63 is directly linked to or interacts with IPA1. We therefore have changed the conclusion to "IPI1 Promotes Differential Ubiquitination of IPA1-mediated Complex in Shoot Apexes and Panicles" and Fig. 9 as well in the revised manuscript.

TPC2016-00879-RAR1 2nd Editorial decision – *acceptance pending* **March 1, 2017**

We are pleased to inform you that your paper entitled "Tissue-specific Ubiquitination by IPA1 INTERACTING PROTEIN 1 Modulates IPA1 Protein Levels to Regulate Plant Architecture in Rice" has been accepted for publication in *The Plant Cell*, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor **March 9, 2017**