nature portfolio

Peer Review File

A point mutation in VIG1 boosts development and chilling tolerance in rice

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
This manuscript describes a potentially important finding, in the discovery of a genetic locus linked to stress tolerance and productivity in one of the world's most important crops food crops. The figures are largely well produced. The study is detailed and complex but sometimes difficult to follow where the English is a little ambiguous and the clarity could be improved.
The mapping and further genetic characterisation of the locus has been very thoroughly performed and strong evidence presented to show linkage with the gene OsbZIP01. By creating different mutant versions of the gene, the authors present convincing evidence that its different functions (relating to chilling tolerance and yield) are associated with different domains of the protein.
Complete loss of VIG1 does not result in the vig1a point mutant phenotype; evidence is presented to support the conclusion that this is because VIG1 acts redundantly with two similar proteins, ZIP18 and ZIP48 and that the phenotype seen in the vi1a point mutation causes a dominant negative effect that cannot be overcome by the presence of the other two proteins.
1. How does complementation of the vig1a mutant with a wild type bZIP01 sequence rescue the mutant if the mutation is dominant negative? This surely means that the mutant allele will act dominantly over the introduced wild type allele.
2. The authors do not clearly explain how they reconcile the conclusion that the three transcription factors act redundantly (so are to some extent interchangeable) with the observation that they all interact with one another. Are they suggesting they form dimers for instance, and whether it is a homo or heterodimer does not make a difference to the phenotype? Or do they believe the interaction between bZIP01 and bZIP48 is essential for normal function? If it were, you would expect to see a phenotype if either were missing. This might be covered in the manuscript, but it is difficult to find.
3. A major conclusion from the study is that this gene influence chilling tolerance but the chilling work is not as well supported as the other parts of the study. Survival levels are calculated and sensitive plants are shown as wilted but no further assays are performed to show that chilling damage has occurred in some but not other lines. The chilling tolerance/sensitivity phenotype is correlated in the manuscript with altered transcript expression patterns. Whilst this goes some way towards suggesting a mechanism, it would have been helpful to include accepted methods of assessing chilling damage, for instance testing whether the tolerant lines were less prone to reactive oxygen species accumulation.
4. OsbZIP1 has been studied before and is already known to affect plant growth and yield in rice, with effects on auxin metabolism implicated. Although some of these papers are cited in the current manuscript, they are not discussed as fully as they could be, leaving it unclear exactly what is already known about the gene and its effects.
5. The manuscript represents a huge amount of work, much of which is presented as supplemental material. In places this material is essential for understanding the main message and therefore it is sometimes hard to follow the conclusions the authors appear to be drawing from the main figures. As an example, it is not clear how the data presented in main figure 4 shows that interaction between Zip01 and zip18 is necessary for the effect seen. To draw this conclusion from the figure, you would need to see the data showing which mutations destroy the protein interaction.

6. Similarly, in figure 5 the legend states that OsbZIP1 functions synergistically with OSBZIP18. The data clearly show they both have an effect, but I am not sure how one would argue from that figure that they are acting synergistically. Also, the meaning of the word “simultaneously” is not clear on line 368 of the text.
7. The last section of the results describes an important finding, relating to testing the function of these genes in Indica rice. However, the data are presented only as supplementary figure. Data should be presented in the main paper if they are an important part of the story.

Other points.
Some labels are difficult to see on photographs (e.g. figure 4c).

In the first figure, the authors present evidence to show that should growth and particularly root growth is greater in the mutant. Data are presented that show a better survival of chilling in the mutant. However, the legend and text do not state how old the seedlings were when subjected to chilling. The growth data would suggest that the reason seedlings were significantly larger than the wild-type at the time of chilling and therefore chilling tolerance exhibited might simply be a function of a larger less vulnerable plant. It would have been useful to subject plant at a similar stage of development to chilling side-by-side, or to show photographs of the plants at the two-leaf stage going side-by-side before and after stress. In him for all subsequent figures.
Line 248-249. How do these results indicate alternative splicing is occurring? (It is shown to be true later but not clear how you would deduce this beforehand).
Unclear : “vig1a will be converted into vig1b” (line 467). What does this mean?

Reviewer #2:
Remarks to the Author:
This manuscript shows that the vig1a mutation at the VIG1 locus has excellent ST, CT, and GNP, and is very useful for breeding varieties suitable for direct seeding. The authors are also trying to explain the phenotypic differences between vig1a and vig1b by analyzing the presence or absence of important domains of VIG1 protein and its interaction with homologous genes OsbZIP18 and 48. The research content is very interesting and can be highly evaluated. However, there remain ambiguities in how experimental results are presented and their interpretation, and these points need improvement.

Comment 1
Attempts are being made to insert mutations into VIG1, OsbZIP18 and 48 using genome editing technology. However, it is very difficult to understand the relationship between each target site and the presence or absence of each important domain. It is necessary to first explain these carefully and in detail, and then describe the phenotypic results.

Comment 2
I think that VIG1-NK is structurally very similar to vig1b, but unlike vig1b, ST and CT are not good. On the other hand, ST and CT abilities of VIG1-CK is said to be like vig1b. How do you interpret these

results?

Comment 3
It is very difficult to understand the contents of the paragraph starting from line 235. For example, 1) it says that when both OsbZIP01 and 18 are lost, the transgenic line can phenocopy vig1a, but is OsbZIP48 incapable of functioning? Does this mean that it is necessary to form a heterodimer and that a OsbZIP48 homodimer cannot function? 2) VIG1-NK OsbZIP18-NK is the same as the original variety, while vig1b OsbZIP18-CK is similar to vig1a. Also, although vig1b OsbZIP18-NK (1b18-n) is said to be the same as vig1b, it should be explained more clearly and in an easy-to-understand manner. 3) VIG1-NK OsbZIP18-NK (1-n 18-n) and VIG1-NK OsbZIP18-CK (1-n 18-c) are the same as VIG1-NK. In this regard, the discussion mentions OsbZIP18 produce two CDS. This is not very clear, so taking the interaction domain into account, please explain in more detail.

Comment 4
Regarding Figure 6, for example, in vig1a, the target gene has changed from being suppressed to being promoted (indicated by arrows), but isn't it just that the suppressive function is gone? If vig1a promotes it, please explain the basis for this.

Comment 5
I can’t understand the title of line 261. Is “basic region” necessary for the interaction between VIG1 and OsbZIP18? Also, I can’t understand the meaning of the sentence on line 261. Please explain appropriately.

Other points
- Line 100: The causal gene --> The causal mutation?
- Line 127and 155: the casual gene --> the causal gene?
- Line 159: which makes VIG1 a good candidate --> which makes vig1a a good candidate?
- Line 474: the ZIP domain --> the bZIP domain?

Reviewer #3:
Remarks to the Author:
Xiong et al. reported the OsbZIP01 mutants that enhance seedling establishment, chilling tolerance and yield potential are valuable for the application of paddy direct-seeding system in rice. The authors obtained VIG1 gene by map-based cloning, and determined that the phenotypes of vig1a were achieved by editing specific gene coding regions of OsbZIP01 or OsbZIP01 and OsbZIP18. Moreover, they discovered that the leucine zipper region of OsbZIP01 plays an important role for keeping vig1a phenotypes. Since OsbZIP01 and OsbZIP18 as transcription factors, they also identified the downstream target genes with different functions involved in regulating of phenotype seedling vigor, chilling tolerance, and grain number per panicle. The manuscript provided a series of evidences to demonstrate the potential application of OsbZIP01 and OsbZIP18 for enhancing seedling vigor, although these two proteins are known transcription factors, which is helpful for rice breeding, especially paddy-direct seeding. However, the organization of experimental results was

confused, which leads to difficulties for understanding logical relationship. And, some experimental results need to be confirmed.

1. Regarding the map-based cloning, some experimental data should be provided in the manuscript. KD8 (japonica) was used as the parent material to construct the mapping population when mapping VIG1. Therefore, the authors should provide the phenotypes identification of KD8, including SV, CT, and GNP, as well as representative plants with WT and mutant phenotype in F2 population derived from the cross between vig1a and KD8. In addition, it is necessary to clarify the phenotype of 1920 plants was analyzed in Line 127.
2. The reasons and motivations for investigating OsbZIP18 and studying the interaction between VIG1 and OsbZIP18 were not found in the manuscript. More importantly, the previous researches have shown that OsbZIP18 plays the role of a positive regulatory (DOI10.3390/ijms23063215, DOI10.3390/plants13040498, and DOI10.1111/nph.16800), while the results of in this study showed that OsbZIP18 played a negative regulatory role. Therefore, the transcriptional regulatory of OsbZIP18 need to be confirmed.
3. The authors stated that OsbZIP01 that encoding a transcription factor is responsible for vig1a. The authors also mentioned several mutants, including vig1a, vig1b, VIG1-NK, and VIG1-CK, with different types of variations resulting in different mutation forms of OsbZIP01. As a transcription factor, the author should identify the transcriptional activity of these different mutation forms of OsbZIP01 proteins. Explaining the transcriptional activity of proteins with different mutation forms will help to explain the phenotype of vig1a and vig1b.
4. Regarding the regulation mechanism of VIG1, the authors stated that the mutations in the basic region of VIG1 protein, including vig1a, vig1m1, vig1m2, and vig1m3, do not affect the localization of VIG1 and the interaction with OsbZIP18. How to explain the impact of point mutations and basic region deletion on the activity of VIG1a proteins?
5. Based on the results provided by the author, 3.52-kb genomic sequence of OsbZIP01 from WT fully rescued in transgenic plants of vig1a and vig1b; the same 3.52-kb genomic sequence was amplified from vig1a and introduced into vig1b and presented the vig1a phenotype (Supplementary Fig. 5). In the aforementioned different genetic materials, it is uncertain which OsbZIP01 form plays a major role, and the corresponding phenotype is also not very reasonable. For example, vig1a/vig1a (R121H) displayed enhanced seedling vigor, however, VIG1a/VIG1a vig1a/vig1a (Line 133-139) dispayed wild-type phenotype.
6. The author should use ChIP-qPCR to verify the relationship between OsbZIP01 and its targets gene under low temperature conditions.
7. At present, three-leaf seedlings are commonly used to identify cold tolerant phenotypes in rice. As the cold tolerance of two-leaf seedlings may come from seeding vigor, the author should provide more experimental results that can exclude the possibility mentioned above.
8. Three phenotypes were observed in the manuscript. In L145-L147, the author stated that high similarities of vig1a and vig1b in both SV and CT. But, there are significant differences in heading date, plant height, and GNP between vig1a and vig1b (Supplementary Fig. 1d; 3b-c, e-g). Therefore, the cross test that was conducted between vig1a and vig1b is difficult to understand. Additionally, the author should exhibit the phenotype of hybrid F1 plants.
9. It is easily confused that the mutants with different target sites were designed different type name, for example, VIG1-NK and VIG1-CK and VIG1-KO, how to improve it? Based on the two splice

forms of OsbZIP01, the author should display mRNA and protein mutation information as well as mutation site information in appropriate parts in the paper.
10. Fig1d, the left panel may be the pre-treated seedlings, bar? These seedlings really were at “two-leaf-stage” (Line 503)?
11. In Fig. S7c, the observed phenotypes related to chilling tolerance in the manuscript do not consistently match with the provided statistical data.
12. In Fig. 3J, the ticks should be added on horizontal axis.
13. In Fig. S22a-b, the image is not clear, and legend with the same ratio is better.
14. In Fig. S22d-e, the description of experimental conditions should be provided.
15. Line 287, “conversion of vig1a into vig1b”, the phrase should be “phenotypic conversion of vig1a into vig1b”.
16. Line 137, T0 plants?

Dear reviewers:

Thank you for reviewing our manuscript entitled “A point mutation in VIG1 enhances seedling establishment, chilling tolerance and yield potential in rice” (ID: NCOMMS-24-13855A) and your critical comments and suggestions. Those comments are all valuable and very helpful for revising and improving our paper.

We have carefully studied those comments from all reviewers, and have revised the manuscript accordingly. We have shown all changes in the manuscript text file in red letters. A detailed point-by-point response addressing the reviewers’ comments is listed below. We would be grateful if our revised manuscript can be accepted for publication in the *Nature Communications*.

Thank you very much for your efforts in reviewing our manuscript.

Sincerely yours,

Shanguo Yao

Professor, Group leader

Institute of Genetics and Developmental Biology

Chinese Academy of Sciences

Chaoyang District, Beijing 100101, China

Responses to comments by Reviewer #1

This manuscript describes a potentially important finding, in the discovery of a genetic locus linked to stress tolerance and productivity in one of the world's most important crops food crops. The figures are largely well produced. The study is detailed and complex but sometimes difficult to follow where the English is a little ambiguous and the clarity could be improved.

The mapping and further genetic characterization of the locus has been very thoroughly performed and strong evidence presented to show linkage with the gene OsbZIP01. By creating different mutant versions of the gene, the authors present convincing evidence that its different functions (relating to chilling tolerance and yield) are associated with different domains of the protein.

Complete loss of VIG1 does not result in the vig1a point mutant phenotype; evidence is presented to support the conclusion that this is because VIG1 acts redundantly with two similar proteins, ZIP18 and ZIP48 and that the phenotype seen in the vig1a point mutation causes a dominant negative effect that cannot be overcome by the presence of the other two proteins.

Response: Thanks for your positive comments on our work. Please find the detailed point-by-point responses below.

1. How does complementation of the vig1a mutant with a wild type bZIP01 sequence rescue the mutant if the mutation is dominant negative? This surely means that the mutant allele will act dominantly over the introduced wild type allele.

Response: Thank you for the critical concern and sorry for the wrong description. In our manuscript, we would like to show that the *vig1a* allele has a negative activity toward OsbZIP01 and OsbZIP18, which causes dysfunction of both proteins, but not to express that *vig1a* mutant allele is dominant to the wild type allele. Actually, we crossed the *vig1a* with the wide-type KY131, and found that the F_1 plants show the wide-type phenotype both in the seedling (5-day-old) and the heading stages (Response Figure 1-1). Furthermore, a similar result can also be observed in the

complementation test in Supplementary Figure 2. These results indicate that *vig1a* is recessive to the wide-type plants. The genetic phenomenon discovered in this research is similar to the previous reports by Li et al. in *Arabidopsis* (DOI: 10.1101/gad.463608). Thus, to avoid ambiguity, we have changed our description in lines 494-495, 497 of the revised manuscript.

Response Figure 1-1. *vig1a* is a recessive mutant indicated by the genetic test.

2. The authors do not clearly explain how they reconcile the conclusion that the three transcription factors act redundantly (so are to some extent interchangeable) with the observation that they all interact with one another. Are they suggesting they form dimers for instance, and whether it is a homo or heterodimer does not make a difference to the phenotype? Or do they believe the interaction between bZIP01 and bZIP48 is essential for normal function? If it were, you would expect to see a phenotype if either were missing. This might be covered in the manuscript, but it is difficult to find.

Response: Thank you for your concerns and suggestions. From our results, we want to express that OsbZIP18 acts redundantly with OsbZIP01 because when we selected different target sites and knocked out *OsbZIP18* in the KY131, respectively, various homozygous lines (*OsbZIP18*-NK and *OsbZIP18*-CK) presented no phenotypic

alterations (Supplementary Fig. 12; 13), nevertheless *vig1bOsbZIP18*-CK (*1b18*-c) double mutants greatly enhanced the mutant phenotype of *vig1b* (Fig. 3), indicating that *OsbZIP18* functions redundantly with *OsbZIP01*, and the mutant phenotype of *OsbZIP18*-CK can be observed only when *OsbZIP01* fully loses its function in the *vig1b* mutant. Consistently, overexpression of *OsbZIP18* in the *vig1a* mutant could partially rescue the phenotype including SV, CT and GNP of *vig1a* (Supplementary Fig. 19). And the interaction between vig1a and OsbZIP18 is indispensable for the phenotype of *vig1a* (Fig.4 and Supplementary Fig. 23)

For OsbZIP48, it has no redundant function with OsbZIP01 and OsbZIP48 because multiple homozygous knock-out lines of *OsbZIP48* (*OsbZIP48*-NK and *OsbZIP48*-CK) in the background of KY131 showed no phenotypic changes (Supplementary Fig. 14; 15), whereas *vig1bOsbZIP48*-CK (*1b48*-c) double mutants and *vig1bOsbZIP18*-CK*OsbZIP48*-CK (*1b18*-c*48*-c) triple mutants still showed similar phenotype with *vig1b* and *vig1bOsbZIP18*-CK (*1b18*-c) mutants, respectively, indicating that loss function of OsbZIP48 has no or little effect on SV and CT, which is not correlated with whether it forms a homo or heterodimer.

For OsbZIP01, it has no redundant function with OsbZIP18 and OsbZIP48 because When we knocked out *OsbZIP01* at the front end of the second exon (*VIG1*-NK), homozygous lines have no significant changes in SV and CT (Supplementary Fig. 7), whereas after knocking out *OsbZIP01* at the posterior end of the second exon (*VIG1*-CK1), homozygous lines show remarkably enhanced SV and CT (Fig. 2), the functional difference between *VIG1*-NK and *VIG1*-CK1 was proved to be caused by the different alternative splices of *VIG1* but not functional redundancy between these three genes.

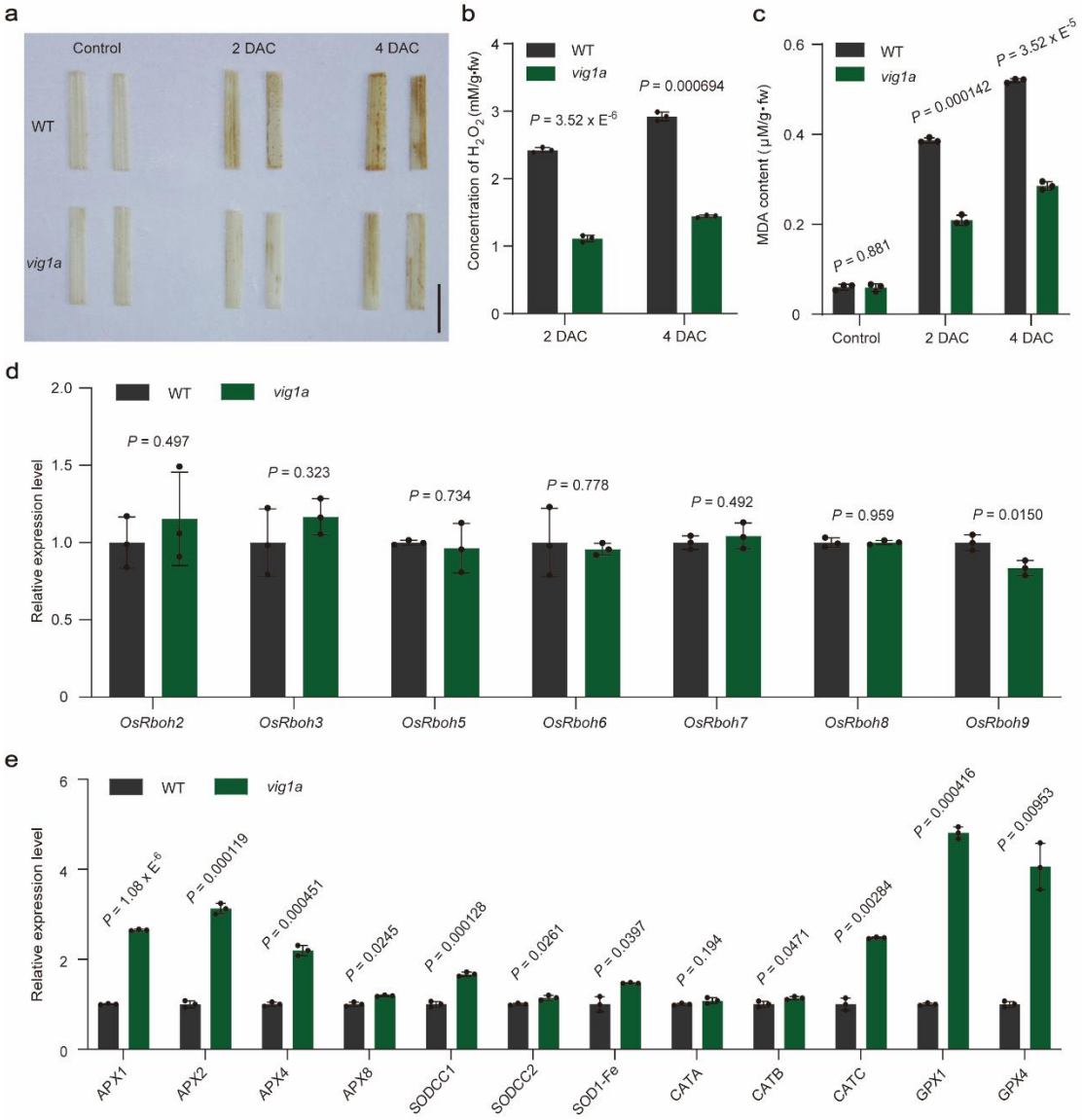
3. A major conclusion from the study is that this gene influence chilling tolerance but the chilling work is not as well supported as the other parts of the study. Survival levels are calculated and sensitive plants are shown as wilted but no further assays are performed to show that chilling damage has occurred in some but not other lines. The

chilling tolerance/sensitivity phenotype is correlated in the manuscript with altered transcript expression patterns. Whilst this goes some way towards suggesting a mechanism, it would have been helpful to include accepted methods of assessing chilling damage, for instance testing whether the tolerant lines were less prone to reactive oxygen species accumulation.

Response: Thanks for the comments and critical suggestions. Given that *vig1a* presents extremely higher chilling tolerance than the wide type KY131 (Figure 1d, g), we have checked the reactive oxygen species accumulation in both 14-day-old KY131 and *vig1a* seedling leaves after two- or four-days chilling stress (2 DAC or 4 DAC) treatment via 3,3'-diaminobenzidine (DAB) staining according the previous report (DOI: 10.1111/pce.13717). Our results showed that at the normal growth condition, the KY131 and *vig1a* have no accumulation of hydrogen peroxide (H_2O_2) (Response Figure 1-2a). However, KY131 accumulated more H_2O_2 after two- or four-days chilling stress treatment (Response Figure 1-2a). In addition, the H_2O_2 concentrations were quantified using 0.1-gram leaves of both materials after chilling stress by the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Catalog no. A22188) according to the manufacturer's instructions (Response Figure 1-2b). Overproduction of H_2O_2 often results in the peroxidation of membrane lipids and subsequent malondialdehyde (MDA) accumulation (doi: 10.1155/2014/360438). Thus, we have examined the MDA in both leaves of KY131 and *vig1a* after two- or four-days chilling stress (2 DAC or 4 DAC) treatment. Consistently, the MDA content in the KY131 was significantly higher than that of in *vig1a* after chilling stress, whereas it remained the same in both the KY131 and *vig1a* under the normal growth condition (Response Figure 1-2c).

Given that the ROS accumulation in plant cells under stress conditions is dynamically controlled by the regulatory networks of ROS-generating system, mainly those *Rboh* genes and ROS-scavenging system, such as SOD, ascorbate peroxidase, catalase isozyme, and glutathione peroxidase (DOI: 10.1111/tpj.13299; DOI: 10.3390/antiox7110169; DOI: 10.1111/pce.13717), thus we have detected the expression of all nine rice *Rboh* genes (DOI: 10.1007/s00726-017-2491-5) in both

leaves of KY131 and *vig1a* after four-days chilling stress treatment. Our results showed that only the expression of genes *OsRboh2*, *OsRboh3*, *OsRboh5*, *OsRboh6*, *OsRboh7*, *OsRboh8* and *OsRboh9* can be detected in both the WT and *vig1a* leaves (Response Figure 1-2d). And except for *OsRboh9*, which had significantly lower expression level in *vig1a* than the WT, other genes showed comparable expression in both the WT and *vig1a* (Response Figure 1-2d), indicating that the expression of those *OsRboh* genes is not responsible for the higher ROS accumulation in the WT. Thus, we have further detected those ROS-scavenging genes including *APX1*, *APX2*, *APX4*, *APX8*, *SODCC1*, *SODCC2*, *SOD1-Fe*, *CATA*, *CATB*, *CATC*, *GPX1*, GPX4 because of their relatively higher expression levels in seedling leaves (Cy3 signal intensity > 5000) indicated by the Rice Expression Profile Database RiceXPro (https://ricexpro.dna.affrc.go.jp/category-select.php), and found that except for *CATA*, other genes showed remarkably higher expression levels in *vig1a* than that of in the WT (Response Figure 1-2e), suggesting that less accumulation of ROS in *vig1a* can be attributed to the enhanced expression of those ROS-scavenging genes.



Response Figure 1-2. *vig1a* showed lower ROS accumulation and enhanced ROS scavenging.

4. OsbZIP1 has been studied before and is already known to affect plant growth and yield in rice, with effects on auxin metabolism implicated. Although some of these papers are cited in the current manuscript, they are not discussed as fully as they could be, leaving it unclear exactly what is already known about the gene and its effects.

Response: Thank you for your suggestions. We have fully discussed the function of OsbZIP1 in rice different biological processes in lines 411-425 of our revised manuscript.

5. The manuscript represents a huge amount of work, much of which is presented as supplemental material. In places this material is essential for understanding the main message and therefore it is sometimes hard to follow the conclusions the authors appear to be drawing from the main figures. As an example, it is not clear how the data presented in main figure 4 shows that interaction between Zip01 and zip18 is necessary for the effect seen. To draw this conclusion from the figure, you would need to see the data showing which mutations destroy the protein interaction.

Response: We are sorry for the lack of the protein sequence comparisons among different knock-out lines. According to your helpful suggestions, we have conducted multiple sequence alignments among vig1a, VIG1-CK1^{vig1a} and VIG1-CK2^{vig1a} proteins, and have supplied this result as the Supplementary Figure 23 in our revised manuscript. For other knock-out lines under different material backgrounds, the protein sequence comparisons among different knock-out mutants and the corresponding wide-type have been provided as Supplementary Data 1 to Supplementary Data 8.

6. Similarly, in figure 5 the legend states that OsbZIP1 functions synergistically with OsbZIP18. The data clearly show they both have an effect, but I am not sure how one would argue from that figure that they are acting synergistically. Also, the meaning of the word “simultaneously” is not clear on line 368 of the text.

Response: We are sorry for these imprecise descriptions. We have revised the ‘VIG1 functions synergistically with OsbZIP18’ to be ‘VIG1 functions with OsbZIP18’ in the legend of Figure 5 (line 1433) in the revised manuscript. And we have also changed the description of ‘OsbZIP18 bound simultaneously to genes’ to be ‘OsbZIP18 bound to genes’ in the line 387 of our revised manuscript.

7. The last section of the results describes an important finding, relating to testing the function of these genes in Indica rice. However, the data are presented only as supplementary figure. Data should be presented in the main paper if they are an important part of the story.

Response: Thanks for your constructive suggestions. We have presented the data of

NIL-*vig1a* in the main text and set as Figure 6 in our revised manuscript.

Other points.

Some labels are difficult to see on photographs (e.g. figure 4c).

Response: We are sorry for the unclear images. To make the photographs easier to distinguish, we have replaced more clearer figures for Figure 4, Supplementary Figure 3, Supplementary Figure 5, Supplementary Figure 6, Supplementary Figure 20, Supplementary Figure 22 and Supplementary Figure 25 in the text.

In the first figure, the authors present evidence to show that should growth and particularly root growth is greater in the mutant. Data are presented that show a better survival of chilling in the mutant. However, the legend and text do not state how old the seedlings were when subjected to chilling. The growth data would suggest that the reason seedlings were significantly larger than the wild-type at the time of chilling and therefore chilling tolerance exhibited might simply be a function of a larger less vulnerable plant. It would have been useful to subject plant at a similar stage of development to chilling side-by-side, or to show photographs of the plants at the two-leaf stage going side-by-side before and after stress. In him for all subsequent figures.

Response: We are sorry for the missing information of the age of seedlings when they were subjected to chilling. In fact, in this research, all seedlings were cultivated for 14 days when we conducted chilling stress treatment, and we have supplied this information in the line 536 of our revised manuscript and also indicated it in the legend of Figure 1 (line 924) in the main text.

To the best of our knowledge, there are no corresponding reports supporting that if the seedling is large, it is cold-tolerant, if all treated seedlings are at the same growth stage, such as the cold tolerance of *d14* and *d14m57-1* with its wide-type plants Shi (DOI: 10.1111/nph.14977). Compared with the wide-type plants Shi, though *d14* and *d14m57-1* mutants showed smaller seedling size, they still exhibited higher chilling tolerance. The similar results can also be observed in *OsGA2ox1*-OE plants with its

wide-type material ZH11 (DOI: 10.1016/j.jplph.2021.153406), and *OsCAF1B*-OE (BO) lines with its wide-type cultivar TNG67 (DOI: 10.1007/s11103-020-01079-8), indicating that the seedling size has no direct correlation with its chilling tolerance if the treated seedlings are at the same growth stage.

In this research, we have presented photographs of the plants at the third-leaf stage side-by-side before and after chilling stress treatment to make our results easier to understand.

Line 248-249. How do these results indicate alternative splicing is occurring? (It is shown to be true later but not clear how you would deduce this beforehand).

Response: Thanks for the critical concern. To reveal genetically the generation of the *vig1a* mutant, we have created a series of double and triple mutants for genes *OsbZIP01*, *OsbZIP18* and *OsbZIP48* by crossing the *VIG1*-NK or *vig1b* mutant with knock-out lines (NK and CK) of genes *OsbZIP18* and *OsbZIP48*. After analyzing their phenotypes, we have found that the phenotype of *vig1bOsbZIP18*-CK (*1b18*-c) double mutants resembled that of the *vig1a* (Fig. 3), whereas *vig1bOsbZIP18*-NK (*1b18*-n) double mutants still phenocopied *vig1b* (Supplementary Figure 17). Actually, if *OsbZIP18* doesn't have different splices, the *1b18*-n double mutant should show similar phenotype with the *1b18*-c double mutant. Given that the huge differences between these two different double mutants, we deduced that *OsbZIP18* probably has different splices and conducted 5' RACE to confirm our speculation (Supplementary Figure 18). And we have indicated it in lines 263-264 of the revised manuscript.

Unclear: "vig1a will be converted into vig1b" (line 467). What does this mean?

Response: We are sorry for this writing mistake. We have revised '*vig1a* will be converted into *vig1b*' to be 'the phenotype of *vig1a* will be converted into that of the *vig1b*' in lines 498-499 of the text.

Reviewer #2 (Remarks to the Author):

This manuscript shows that the vig1a mutation at the VIG1 locus has excellent ST, CT, and GNP, and is very useful for breeding varieties suitable for direct seeding. The authors are also trying to explain the phenotypic differences between vig1a and vig1b by analyzing the presence or absence of important domains of VIG1 protein and its interaction with homologous genes OsbZIP18 and 48. The research content is very interesting and can be highly evaluated. However, there remain ambiguities in how experimental results are presented and their interpretation, and these points need improvement.

Response: Thanks for your positive comments on our work. Please find the detailed point-by-point responses below.

Comment 1

Attempts are being made to insert mutations into VIG1, OsbZIP18 and 48 using genome editing technology. However, it is very difficult to understand the relationship between each target site and the presence or absence of each important domain. It is necessary to first explain these carefully and in detail, and then describe the phenotypic results.

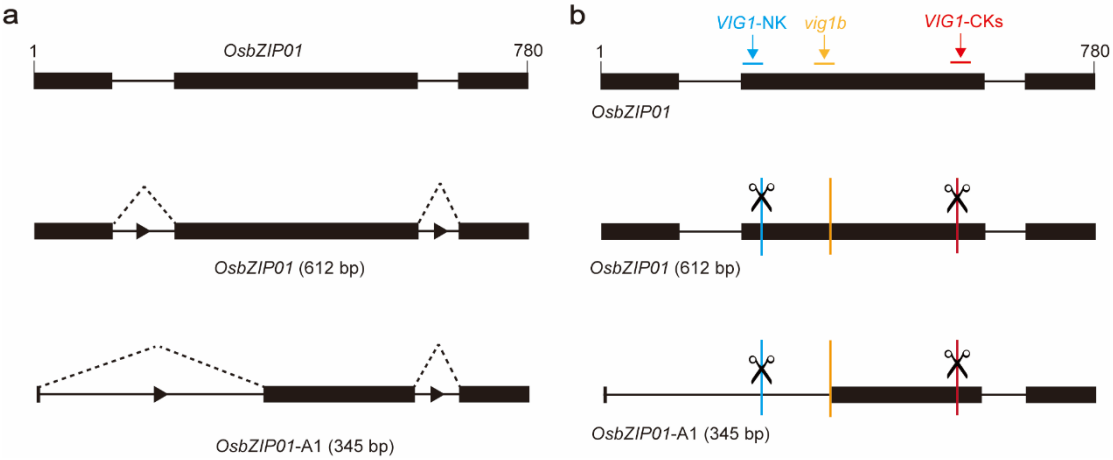
Response: Thanks for your comments and critical suggestions. To make the relationship between each target site and the presence or absence of each important domain easier to understand, we have conducted multiple sequence comparison for different mutant proteins encoded by those knock-out lines with their corresponding wide-type proteins (Supplementary Data 1 to Supplementary Data 8). Additionally, according to your helpful suggestions, we have explained the resulting mutant proteins caused by mutations in different knockout lines before describing their phenotypic results in the revised manuscript.

Comment 2

I think that VIG1-NK is structurally very similar to vig1b, but unlike vig1b, SV and CT

are not good. On the other hand, SV and CT abilities of VIG1-CK is said to be like vig1b. How do you interpret these results?

Response: Thanks for your critical concerns. In the process of functional analysis of *VIG1*, we found that *VIG1* has alternative splicing and generates two different splices (Supplementary Data 9d-e and Response Figure 1-3a), which is consistent to the previous report (DOI: 10.1093/plphys/kiad334). For *VIG1*-NK, the knockout target was designed at the front end of the second exon of the 612 bp splice of *OsbZIP01*, whereas it locates at the first intron of the 345 bp splice of *OsbZIP01*, which resulted in the destruction of the 612 bp splice but the reservation of the 345 bp splice (Response Figure 1-3b), making the SV and CT unchanged. For *vig1b*, the 14 bp deletion occurred in the second exon of the 612 bp splice of *OsbZIP01*, which also locates at the splicing site of the 345 bp splice of *OsbZIP01* (Response Figure 1-3b), leading to dysfunction of both splices and therefore enhanced SV and CT. For *VIG1*-CKs, the knockout target was designed at the second exon of both the 612 bp and 345 bp splices of *OsbZIP01*, which caused the impairment of both the 612 bp and 345 bp splices (Response Figure 1-3b), resulting in enhanced SV and CT similar to that of the *vig1b* mutant. Therefore, although *VIG1*-NK is structurally very similar to *vig1b*, it doesn't show elevated SV and CT.



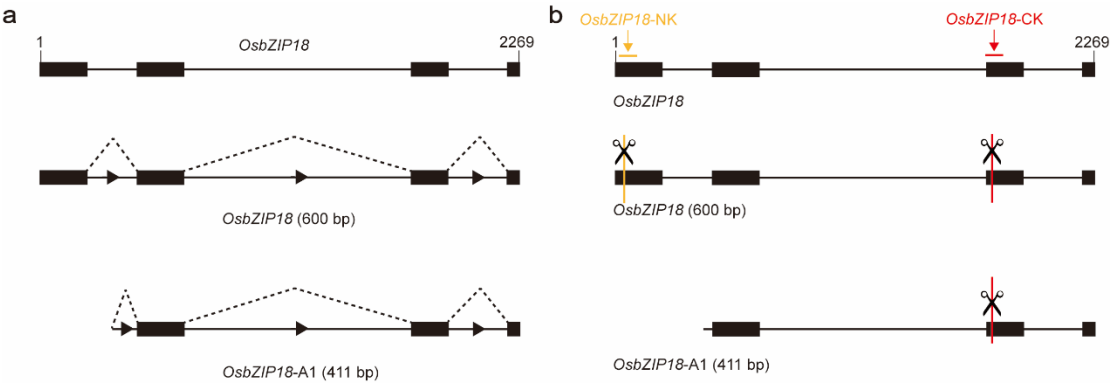
Response Figure 1-3. The phenotypic differences between *VIG1*-NK and *vig1b* are caused by different splices of *OsbZIP01*.

Comment 3

It is very difficult to understand the contents of the paragraph starting from line 235. For example, 1) it says that when both OsbZIP01 and 18 are lost, the transgenic line can phenocopy vig1a, but is OsbZIP48 incapable of functioning? Does this mean that it is necessary to form a heterodimer and that a OsbZIP48 homodimer cannot function? 2) VIG1-NK OsbZIP18-NK is the same as the original variety, while vig1b OsbZIP18-CK is similar to vig1a. Also, although vig1b OsbZIP18-NK (1b18-n) is said to be the same as vig1b, it should be explained more clearly and in an easy-to-understand manner. 3) VIG1-NK OsbZIP18-NK (1-n 18-n) and VIG1-NK OsbZIP18-CK (1-n 18-c) are the same as VIG1-NK. In this regard, the discussion mentions OsbZIP18 produce two CDS. This is not very clear, so taking the interaction domain into account, please explain in more detail.

Response: Thank you very much for pointing out these issues. 1) Based on our phenotypic results, we discovered that the phenotype of single mutation of gene *OsbZIP48* resembles that of the wide-type (Supplementary Figure 14 and 15). In addition, the phenotype of *vig1bOsbZIP48*-CK (*1b48*-c) double mutant resembles that of the *vig1b* mutant (Figure 3d-g, j). Moreover, the phenotype of *vig1bOsbZIP18*-CK*OsbZIP48*-CK (*1b18*-c*48*-c) triple mutant resembles that of the *vig1bOsbZIP18*-CK (*1b18*-c) double mutant (Figure 3d-g, j). These results indicate that OsbZIP48 has little or no effect on rice SV, CT and GNP, which is independent of whether it forms a heterodimer or homodimer. 2) According to your helpful suggestions, we have changed the description of the phenotype of *VIG1*-NK *OsbZIP18*-NK, *vig1b OsbZIP18*-CK and *vig1b OsbZIP18*-NK double mutants to make it easier to understand, which can be observed in lines 255-262 of our revised manuscript. 3) In our research, we found that the phenotype of single mutation of *OsbZIP18* resembles that of the wide-type (Supplementary Figure 12 and 13). And *OsbZIP18* has alternative splicing and generates a 600 bp splice and a 411 bp splice, respectively. For *OsbZIP18*-NK, only the 600 bp splice was destroyed without impairing the 411 bp splice, whereas for *OsbZIP18*-CK, both splices were impaired (Response Figure 1-4). Taking the interaction domain into account, we could find that mutation in *OsbZIP18*-CK1 results in the impairment of both splices and the interaction domain of their encoding proteins

(Supplementary Data 5 and Response Figure 1-4). However, the phenotype of *OsbZIP18*-CK1 resembled that of the wide-type, indicating that single mutation of OsbZIP18, no matter the interaction domain is impaired or not, doesn't alter rice SV, CT and GNP (Supplementary Figure 13). Whereas the phenotype of *vig1bOsbZIP18*-CK resembled that of the *vig1a* mutant, indicating the predominant role of *VIG1* in regulating SV, CT and GNP. Thus, the phenotype of *VIG1*-NK *OsbZIP18*-NK (*1*-n*18*-n) and *VIG1*-NK *OsbZIP18*-CK (*1*-n*18*-c) resembled that of the original variety *VIG1*-NK.



Response Figure 1-4. The effects of different types of *OsbZIP18* knockout lines on its two splices.

Comment 4

Regarding Figure 6, for example, in vig1a, the target gene has changed from being suppressed to being promoted (indicated by arrows), but isn't it just that the suppressive function is gone? If vig1a promotes it, please explain the basis for this.

Response: We are sorry for this wrong description. As you state, the suppressive function of VIG1 and OsbZIP18 are actually blocked in the *vig1a* mutant, rather than showing a promoting function. And we have corrected it in the Figure 7 and indicated it in lines 1028-1029 of our revised manuscript.

Comment 5

I can't understand the title of line 261. Is "basic region" necessary for the interaction between VIG1 and OsbZIP18? Also, I can't understand the meaning of the sentence

on line 261. Please explain appropriately.

Response: We are sorry for the unclear description of this sentence. To make the title easier to understand, we have revised it in lines 277-279 of the text. And in this title, we want to express that the mutant phenotype of *vig1a* also can be obtained by other mutations in the basic region of OsbZIP01, and it relies on the interaction between VIG1 basic region mutated proteins and OsbZIP18. From our results, the basic region is not necessary for the interaction between VIG1 and OsbZIP18 (Supplementary Figure 20a, c, d).

Other points

- Line 100: The causal gene --> The causal mutation?
- Line 127 and 155: the casual gene --> the causal gene?
- Line 159: which makes VIG1 a good candidate --> which makes vig1a a good candidate?
- Line 474: the ZIP domain --> the bZIP domain?

Response: We are sorry for these writing mistakes. We have revised 'The causal gene' to 'The causal mutation' in line 100, 'the casual gene' to 'the causal gene' in line 129 and 157, 'which makes VIG1 a good candidate' to 'which makes *vig1a* a good candidate' in line 162, 'the ZIP domain' to 'the leucine zipper region' in lines 507-508 of our revised manuscript.

Reviewer #3 (Remarks to the Author):

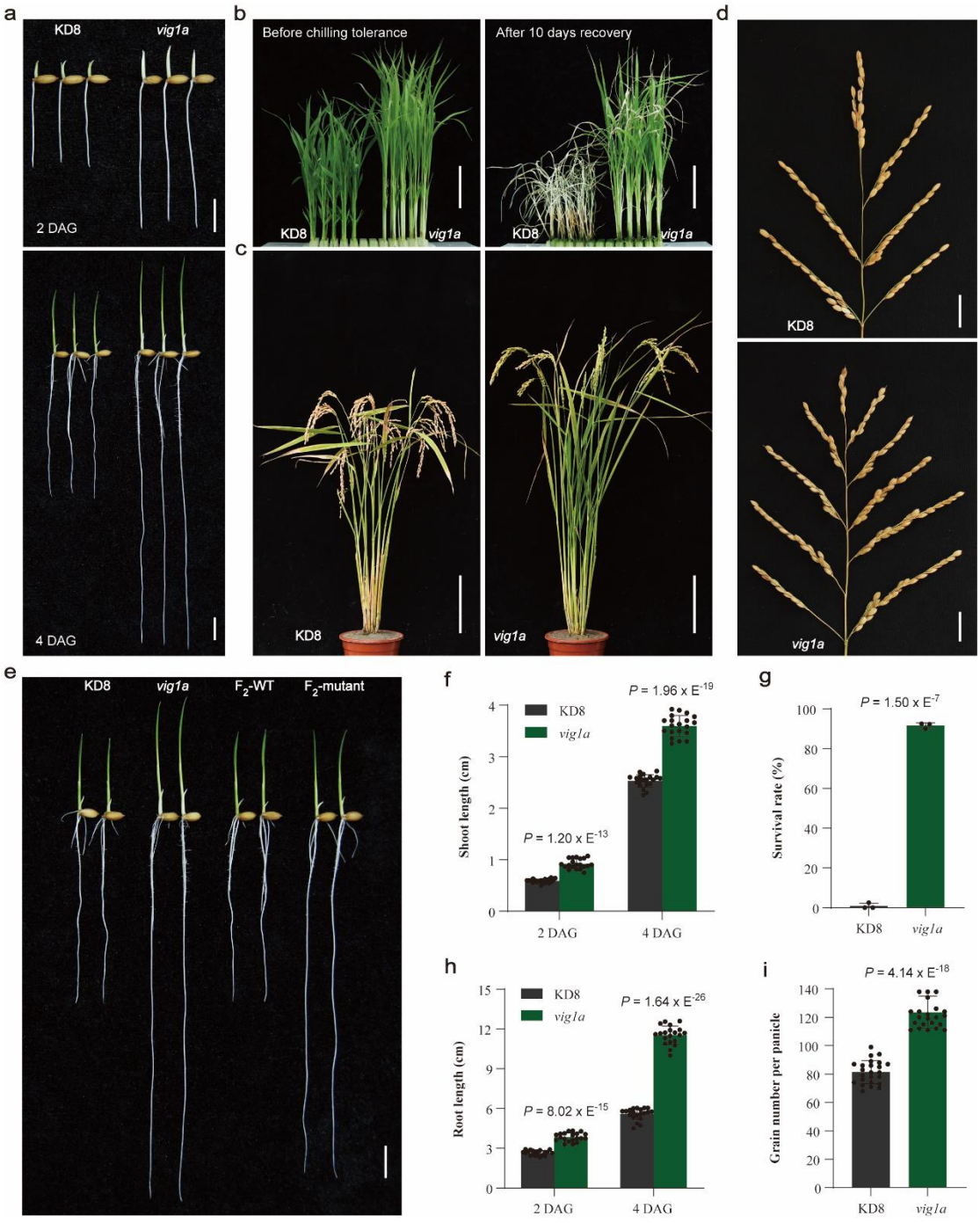
Xiong et al. reported the OsbZIP01 mutants that enhance seedling establishment, chilling tolerance and yield potential are valuable for the application of paddy direct-seeding system in rice. The authors obtained VIG1 gene by map-based cloning, and determined that the phenotypes of vig1a were achieved by editing specific gene coding regions of OsbZIP01 or OsbZIP01 and OsbZIP18. Moreover, they discovered that the leucine zipper region of OsbZIP01 plays an important role for keeping vig1a

phenotypes. Since OsbZIP01 and OsbZIP18 as transcription factors, they also identified the downstream target genes with different functions involved in regulating of phenotype seedling vigor, chilling tolerance, and grain number per panicle. The manuscript provided a series of evidences to demonstrate the potential application of OsbZIP01 and OsbZIP18 for enhancing seedling vigor, although these two proteins are known transcription factors, which is helpful for rice breeding, especially paddy-direct seeding. However, the organization of experimental results was confused, which leads to difficulties for understanding logical relationship. And, some experimental results need to be confirmed.

Response: Thank you for the positive feedback and helpful suggestions on how to improve our manuscript. Please find the detailed point-by-point responses below.

1. Regarding the map-based cloning, some experimental data should be provided in the manuscript. KD8 (japonica) was used as the parent material to construct the mapping population when mapping VIG1. Therefore, the authors should provide the phenotypes identification of KD8, including SV, CT, and GNP, as well as representative plants with WT and mutant phenotype in F2 population derived from the cross between vig1a and KD8. In addition, it is necessary to clarify the phenotype of 1920 plants was analyzed in Line 127.

Response: Thanks for your constructive suggestions. Compared with *vig1a*, KD8 shows extremely lower SV, CT and GNP (Response Figure 1-5). Given that the great differences in seminal root length between these two materials at the seedling stage (Response Figure 1-5a-d, f-i), *vig1a* was crossed with KD8 to generate the F_2 mapping population and we took the seminal root length as the phenotype to locate gene *VIG1* (Response Figure 1-5e). These results were supplied as Supplementary Figure 2 in the text.



Response Figure 1-5. Phenotypic comparison between KD8 and *vig1a*.

2. The reasons and motivations for investigating OsbZIP18 and studying the interaction between VIG1 and OsbZIP18 were not found in the manuscript. More importantly, the previous researches have shown that OsbZIP18 plays the role of a positive regulatory (DOI10.3390/ijms23063215, DOI10.3390/plants13040498, and DOI10.1111/nph.16800), while the results of in this study showed that OsbZIP18

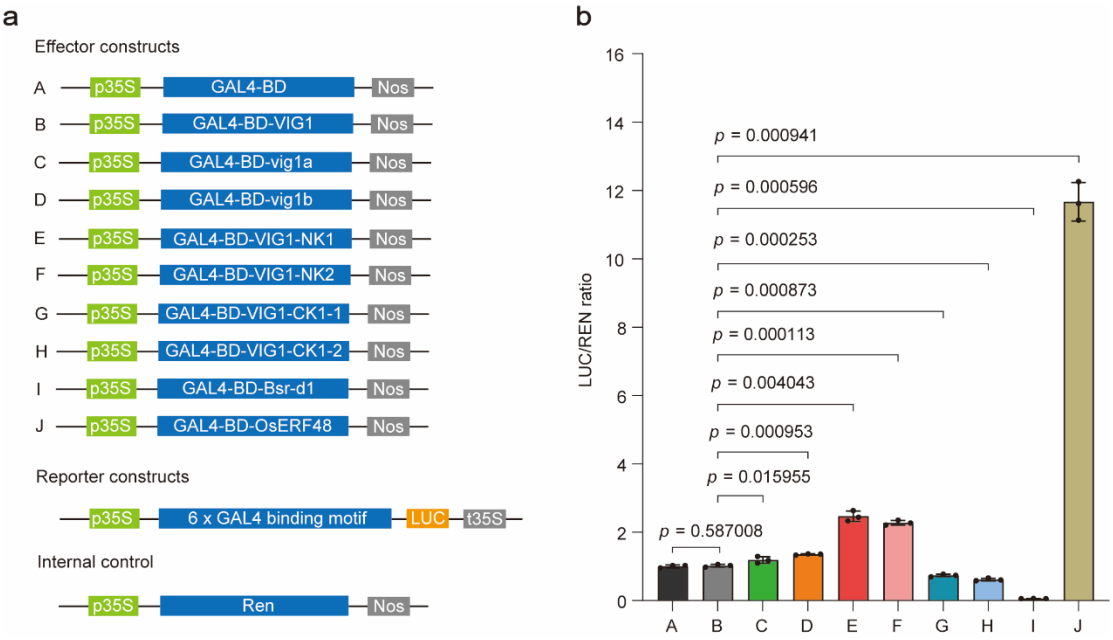
played a negative regulatory role. Therefore, the transcriptional regulatory of OsbZIP18 need to be confirmed.

Response: Thank you for the critical concerns. During our functional analysis of the *VIG1* gene, we found that *VIG1* is the homolog of *AtHY5* and *AtHYH*, two paralogs in *Arabidopsis*. As the crucial regulators of photomorphogenesis, AtHY5 and AtHYH can form heterodimers and homodimers, respectively (DOI: 10.1101/gad.969702). Phylogenetic analysis showed that OsbZIP18 and OsbZIP48 are the closest paralogs of VIG1 in the rice genome, these three bZIP TFs are homologous proteins of both AtHY5 and AtHYH, which motivated us to investigate the function of both genes *OsbZIP18* and *OsbZIP48*, and studying the interaction among VIG1, OsbZIP18 and OsbZIP48. And we have pointed out the reasons and motivations for investigating OsbZIP18 and studying the interaction between VIG1 and OsbZIP18 in lines 207-208 of our revised manuscript.

In addition, as a central transcription factor, AtHY5 acts as an activator in primary root elongation (DOI: 10.1111/jipb.13099), flavonoid and pigment accumulation (DOI: 10.1016/j.molp.2016.07.002), nutrient uptake and utilization (DOI: 10.1111/nph.19516), and also as a repressor in hypocotyl elongation (DOI: 10.1038/35013076). These reports showed that AtHY5 acts both as an activator and a repressor according to the different traits in *Arabidopsis*. Therefore, we tend to consider that OsbZIP18, similar to *Arabidopsis* AtHY5, might also act both as an activator and a repressor, which depends on the traits it's involved in.

3. The authors stated that OsbZIP01 that encoding a transcription factor is responsible for vig1a. The authors also mentioned several mutants, including vig1a, vig1b, VIG1-NK, and VIG1-CK, with different types of variations resulting in different mutation forms of OsbZIP01. As a transcription factor, the author should identify the transcriptional activity of these different mutation forms of OsbZIP01 proteins. Explaining the transcriptional activity of proteins with different mutation forms will help to explain the phenotype of vig1a and vig1b.

Response: Thank you for the helpful suggestion. According to your suggestion, we have conducted a LUC assay to examine the transcriptional activity of different mutant forms of OsbZIP01 proteins (Response Figure 1-6). In this assay, OsbZIP01 mutant proteins including vig1a, vig1b, VIG1-NK, and VIG1-CK1 were fused to yeast GAL4-BD and used as the effectors with the yeast GAL4-BD as the empty control, the transcriptional repressor Bsr-d1 as the negative control (DOI: 10.1016/j.cell.2017.06.008) and the transcriptional activator OsERF48 as the positive control (DOI: 10.1111/pbi.12716), respectively (Response Figure 1-6a). In addition, the LUC vector, which contains six copies of GAL4 binding motif and luciferase coding region, was used as the reporter, and the vector that expressed Renilla luciferase (Ren) was employed as the internal control (Response Figure 1-6a). The effectors were then co-transformed with the reporter and the internal control into rice protoplasts in via the PEG (polyethylene glycol) mediated method, and the relative LUC/REN ratio was measured. The relative LUC/REN ratios of OsbZIP01 mutant proteins were normalized against WT, which was set to 1 in Response Figure 1-6b. Our results showed that VIG1 showed no significant transcriptional activation or repression activity compared with the GAL4-BD empty vector. Compared with VIG1 wide-type protein, vig1a, vig1b and VIG1-NKs proteins showed significantly enhanced transcriptional activation activity while VIG1-CK1 presented remarkably elevated transcriptional repression activity. However, we found that *VIG1*-CK1 showed similar SV, CT and GNP with *vig1b* (Figure 1l-n, Figure 2 and Supplementary Figure 4a-g), indicating that the changes of transcriptional activity in different mutant forms of OsbZIP01 proteins are not responsible for the phenotypic divergence of different mutants.

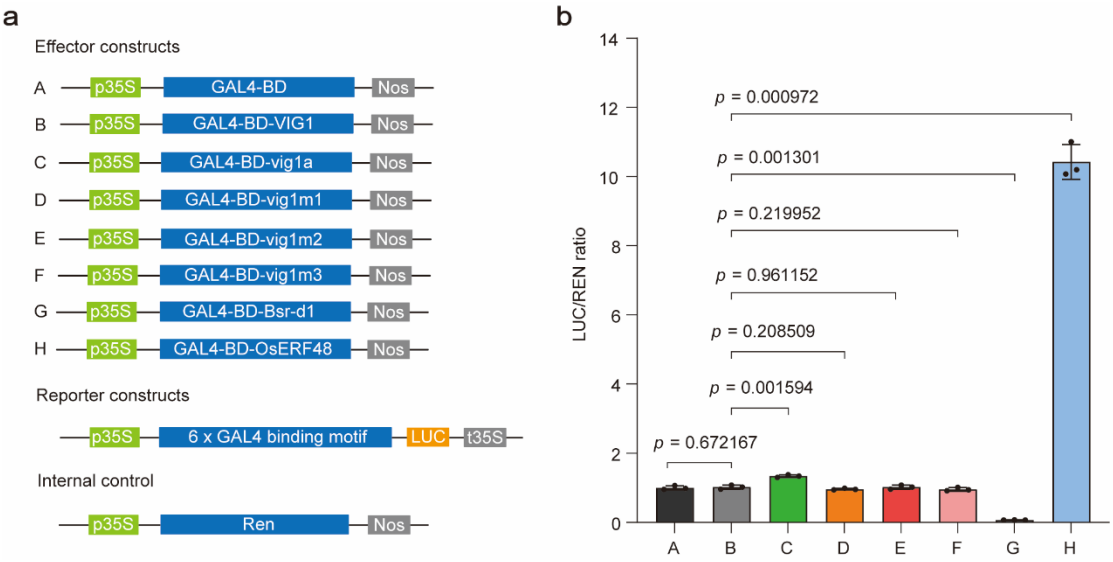


Response Figure 1-6. The transcriptional activity of different mutant forms of OsbZIP01 proteins.

4. Regarding the regulation mechanism of VIG1, the authors stated that the mutations in the basic region of VIG1 protein, including vig1a, vig1m1, vig1m2, and vig1m3, do not affect the localization of VIG1 and the interaction with OsbZIP18. How to explain the impact of point mutations and basic region deletion on the activity of VIG1 proteins?

Response: Thanks for your critical concern. This is a very interesting point that we still don't fully understand. One our hypothesis is that mutations in the basic region of VIG1 alter the transcriptional activity of the protein itself. To explore the effect of different mutations in the basic region of VIG1, we have performed a LUC assay to confirm the transcriptional activity of these VIG1 mutant proteins (Response Figure 1-7). Our results showed that except for vig1a which presented significantly elevated transcriptional activation activity compared with VIG1, other VIG1 basic region mutated proteins including vig1m1, vig1m2, and vig1m3 showed no remarkable alterations on the transcriptional activity, suggesting that the mutant phenotype of VIG1 basic region mutated proteins are not caused by the changes of its transcriptional activity. Another possible explanation lies on that mutations in the basic region of VIG1 alter the spatial structure of the protein，change its interaction with OsbZIP18, and the solution to this

issue will largely rely on the dissection of the crystal structures of these mutant proteins in the future research.

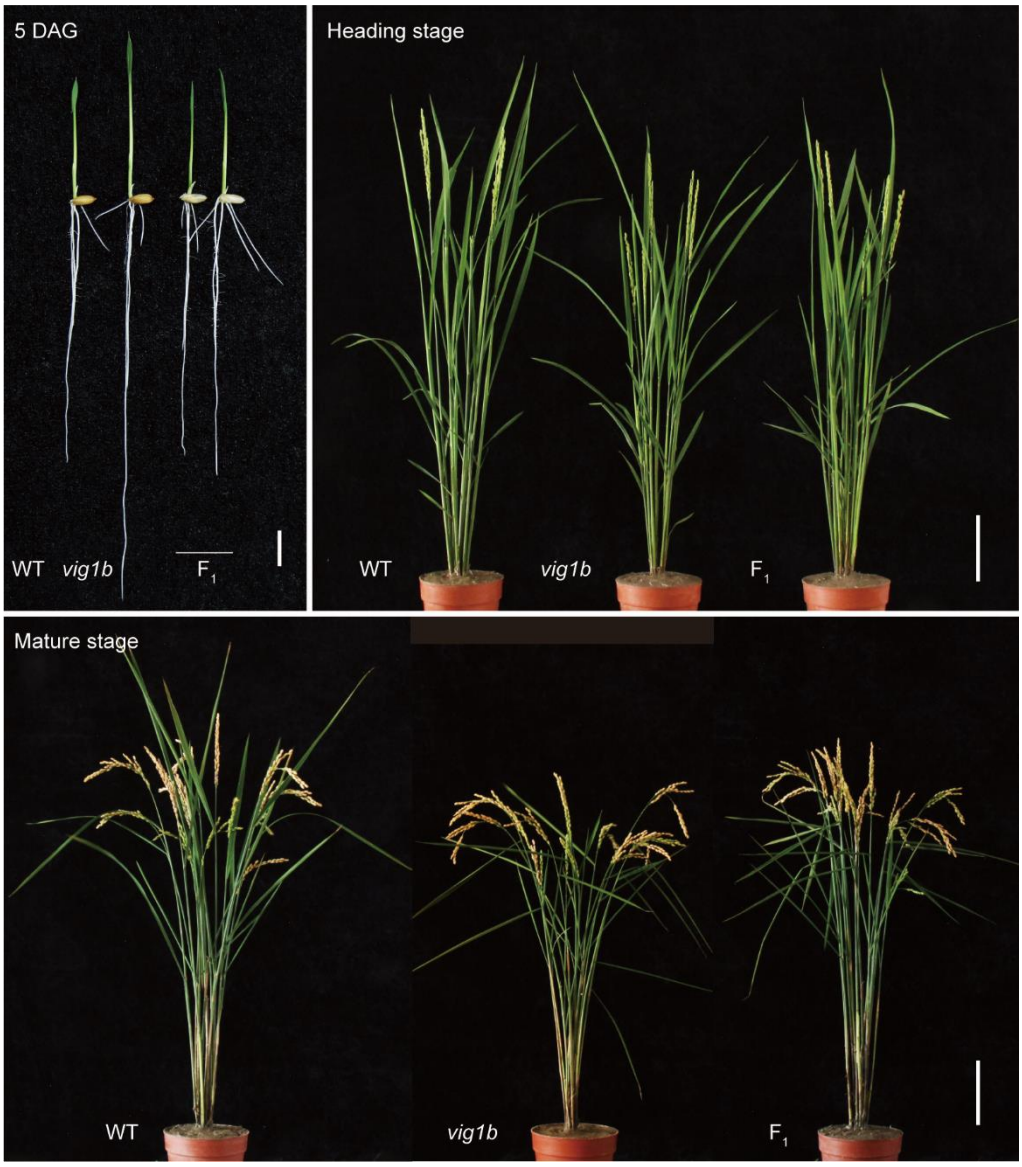


Response Figure 1-7. The transcriptional activity of different basic region mutated proteins of OsbZIP01.

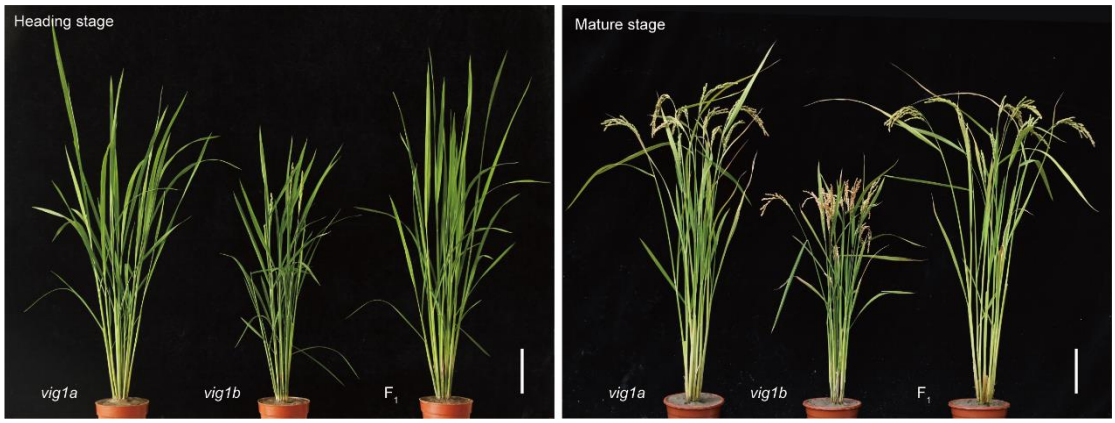
5. Based on the results provided by the author, 3.52-kb genomic sequence of OsbZIP01 from WT fully rescued in transgenic plants of vig1a and vig1b; the same 3.52-kb genomic sequence was amplified from vig1a and introduced into vig1b and presented the vig1a phenotype (Supplementary Fig. 5). In the aforementioned different genetic materials, it is uncertain which OsbZIP01 form plays a major role, and the corresponding phenotype is also not very reasonable. For example, vig1a/vig1a (R121H) displayed enhanced seedling vigor, however, VIG1a/VIG1a vig1a/vig1a (Line 133-139) displayed wild-type phenotype.

Response: Thank you for the critical concern. In the process of functional analysis of *VIG1*, we have found that *vig1a* and *vig1b* are both fully recessive mutants of *VIG1* (Response Figure 1-1, Response Figure 1-8), and *vig1a* is fully dominant to *vig1b* (Response Figure 1-9), which is indicated not only by the cross tests but also through the complementation results (Supplementary Figure 3, Supplementary Figure 5 and Supplementary Figure 6).

The reason why the wide type (KY131) is dominant to *vig1a* is mainly because of the defective photomorphogenesis of *vig1a* without altering the protein size and major domains of OsbZIP01, whereas the wild type allele is able to perceive light better. The reason why *vig1a* is dominant to *vig1b* is because that the 14-bp deletion in *vig1b* disrupts the protein structure and integrity of OsbZIP01, whereas it is more completely preserved in *vig1a*.



Response Figure 1-8. *vig1b* is a recessive mutant of *OsbZIP01*.



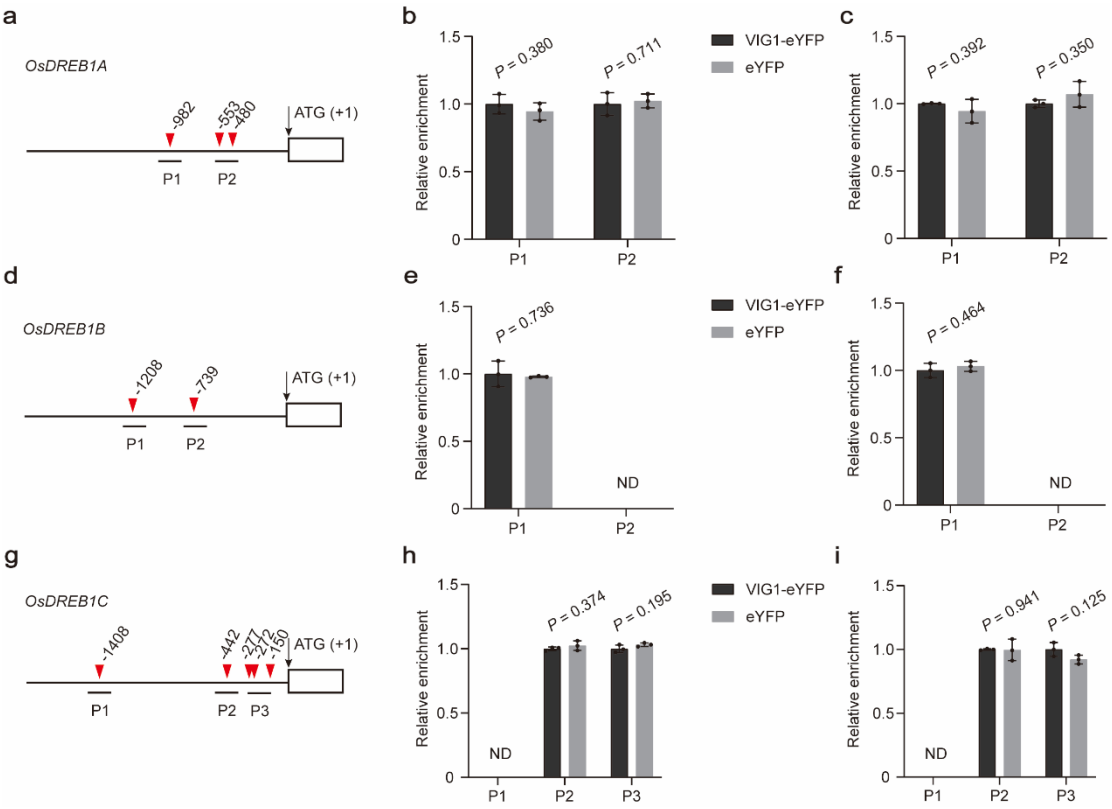
Response Figure 1-9. *vig1a* is dominant to *vig1b* both in the heading stage and the mature stage.

6. The author should use ChIP-qPCR to verify the relationship between OsbZIP01 and its targets gene under low temperature conditions.

Response: Thank you for the constructive suggestion. We have conducted ChIP-qPCR by transforming the VIG1 fused eYFP (VIG1-pSAT6) and its corresponding empty vector pSAT6 into the 14-day-old rice leaf protoplasts via PEG mediated method, the subsequent protoplasts were cultivated under 28°C and the dark condition for 16 h, and then transferred into 4°C for 1 h or 6 h treatment. The resulting protoplasts were collected and used to perform subsequent ChIP assays according to the previous report (DOI: 10.1186/s13007-017-0192-4) with slight modifications. The modifications are that the sonication is conducted via Covaris™ S220 sonicator and the DNA precipitation is performed via the ChIP DNA Clean & Concentrator (ZYMO RESEARCH, Cat. No.: D5205). The beads used in this assay is GFP-Agarose (LABLEAD, Cat. No.: PGA025) which has high affinity and specificity to bind to the eYFP protein.

From the RNA transcriptome data in the text (Supplementary Figure 24), we know that genes *OsDREB1A*, *OsDREB1B*, *OsDREB1C*, *OsDREB1E*, *OsDREB1H*, *OsDREB1G* are the potential downstream target of OsbZIP01 (VIG1) and are correlated with chilling tolerance. Thus, we have examined putative binding sites of VIG1 in the 2.0 kb promoters just upstream of the start codon of those genes and identified single or

multiple A-, G- or C-box *cis*-elements only in genes *OsDREB1A*, *OsDREB1B* and *OsDREB1C* promoters (Response Figure 1-10a, d, g), and we have designed qPCR probes according these target positions (P1 to P3) (Response Figure 1-10a, d, g). Compared with the eYFP control, ChIP-qPCR data of VIG1-eYFP show no significant enrichment of chromatin DNA fragments at the indicated promoter region of genes *OsDREB1A*, *OsDREB1B* and *OsDREB1C* both after 1 h (Response Figure 1-10b, e, h) and 6 h (Response Figure 1-10c, f, i) chilling stress treatment. The relative enrichment was normalized with total input. Values are means ± SD of three independent experiments. ND means no CT values detected in the qPCR assays. These results indicate that VIG1 has no obvious enrichment on those gene promoters under low temperature conditions.



Response Figure 1-10. VIG1 shows no obvious enrichment on *OsDREB* genes under low temperature conditions.

7. At present, three-leaf seedlings are commonly used to identify cold tolerant phenotypes in rice. As the cold tolerance of two-leaf seedlings may come from seeding

vigor, the author should provide more experimental results that can exclude the possibility mentioned above.

Response: We are sorry for this writing mistake. Actually, all seedlings were cultivated for 14 days when we conducted chilling stress treatment, and it already reached three-leaf stage. Thus, we have revised it in lines 536 of the text.

8. Three phenotypes were observed in the manuscript. In L145-L147, the author stated that high similarities of vig1a and vig1b in both SV and CT. But there are significant differences in heading date, plant height, and GNP between vig1a and vig1b (Supplementary Fig. 1d; 3b-c, e-g). Therefore, the cross test that was conducted between vig1a and vig1b is difficult to understand. Additionally, the author should exhibit the phenotype of hybrid F1 plants.

Response: Thank you for the concern. When we obtained the *vig1a* and *vig1b* mutant at different times after screening a large NaN_3-treated population, we were attracted by the extremely longer roots of both *vig1a* and *vig1b* because of the great potential of longer roots in cultivating high yielding rice. Therefore, we were curious about that these two mutants are caused by mutation of one gene or different genes, which triggered us to conduct the cross between *vig1a* and *vig1b*, and finally found these two mutants are allelic. The phenotype of hybrid F_1 plants is presented in (Response Figure 1-9).

9. It is easily confused that the mutants with different target sites were designed different type name, for example, VIG1-NK and VIG1-CK and VIG1-KO, how to improve it? Based on the two splice forms of OsbZIP01, the author should display mRNA and protein mutation information as well as mutation site information in appropriate parts in the paper.

Response: Thank you for your constructive suggestions. To avoid confusion caused by the name of *VIG1*-CK and *VIG1*-KO, we have renamed the '*VIG1*-CK' under the KY131 background to be '*VIG1*-CK1' and the '*VIG1*-KO' under the KD8 background to be '*VIG1*-CK2' in the revised manuscript. According to your suggestion, we have added

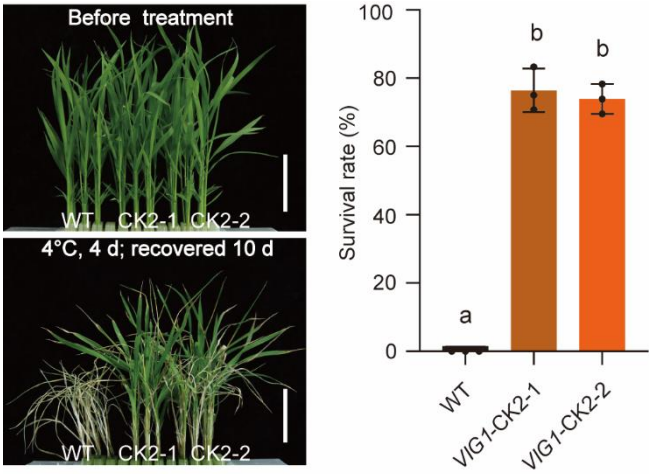
the information about mRNA and protein mutation as well as mutation site based on the two splice forms of OsbZIP01 as the Supplementary Data 1 to Supplementary Data 3, and Supplementary Data 9 in the text.

10. Fig1d, the left panel may be the pre-treated seedlings, bar? These seedlings really were at “two-leaf-stage” (Line 503)?

Response: Thank you for your kind reminder. 1) The left panel is the pre-treated seedlings and we have added bars in all chilling stress-related figures in the revised manuscript. 2) We are sorry for this writing mistake. All seedlings were cultivated for 14 days when we conducted chilling stress treatment, and it already reached three-leaf stage. We have corrected it in lines 536 of the revised manuscript.

11. In Fig. S7c, the observed phenotypes related to chilling tolerance in the manuscript do not consistently match with the provided statistical data.

Response: We are sorry for this inconsistency. We have checked our raw data and corrected it in the revised manuscript. Additionally, we have also reconducted this experiment to confirm the result, and have found that different replications obtain similar phenotypes for the chilling tolerance of *VIG1*-CK2 lines (Response Figure 1-11).



Response Figure 1-11. Knock-out lines of *VIG1* in the KD8 background showed significantly enhanced chilling tolerance.

12. In Fig. 3J, the ticks should be added on horizontal axis.

Response: Thanks for pointing this out. We have added the ticks on horizontal axis in Fig. 3J of our revised manuscript.

13. In Fig. S22a-b, the image is not clear, and legend with the same ratio is better.

Response: Thanks for your kind reminder and constructive suggestion. We have replaced the Fig. S24a-b in the revised manuscript with clearer images, and have changed the legend with the same ratio.

14. In Fig. S22d-e, the description of experimental conditions should be provided.

Response: Thank you for your critical suggestion. We have provided the detail description of experimental conditions for Fig. S24d-e in lines 1356-1358 of our revised manuscript.

15. Line 287, “conversion of vig1a into vig1b”, the phrase should be “phenotypic conversion of vig1a into vig1b”.

Response: Thanks for pointing this out. We have revised “conversion of *vig1a* into *vig1b*” to be “phenotypic conversion of *vig1a* into *vig1b*” in lines 304-305 of the manuscript.

16. Line 137, T0 plants?

Response: We are sorry for this writing mistake. Actually, all complementary strains used for phenotypic investigations are T_3 plants. And we have corrected this mistake in line 139 of our revised manuscript.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
I thank the authors for carefully addressing my concerns and for the useful extra experimental data supplied and the improved figures. The writing and explanations still need some work to attain full clarity.

Reviewer #2:
Remarks to the Author:
The authors responded to reviewer comments properly. This revised manuscript significantly improves upon ambiguities in the presentation and interpretation of experimental results. As I judged previously, in this manuscript, the authors show that the vig1a mutation at the VIG1 locus is highly useful for rice improvement for direct seeding due to its superior ST, CT, and GNP, and they adequately explain the phenotypic differences between vig1a and vig1b by analyzing the protein structures and interactions with the homologous genes 18 and 48. Thus, I think this manuscript is at the level of being accepted.

Reviewer #3:
Remarks to the Author:
As mentioned in my first review, I believe this is a significant study that sheds light on the regulatory mechanism of three individual traits, including seedling vigor, cold sensitivity, and grain yield. The authors illustrated that the vig1a, which confers enhanced SV, CT and GNP, can be mimicked by mutations in the basic region of VIG1. These results can be applied to rice breeding through genome editing approaches. Authors have conducted additional analyses and experiments and added the appropriate explanations in addressing most of the concerns raised in my previous review. The revised manuscript was improved. However, there are still a few concerns which need to be addressed.

1. Regarding the functional redundancy. The substitution of R121H in the basic region of OsbZIP01 resulted in the vig1a phenotype, and this result indicated that there are not redundant effects among OsbZIP01, OsbZIP18, and OsbZIP48. However, the all knock-out lines (including OsbZIP18-NK, OsbZIP18-CK, OsbZIP48-NK, and OsbZIP48-CK) showed no obvious phenotypic alterations, which suggested a functional redundancy among them (Line 245). These two conclussions are contradictory to each other. Why?
2. Regarding the functional redundancy. If these three transcription factors (OsZIP01/VIG1, OsbZIP18, and OsbZIP48) are functional redundancy, vig1a with R121H substitution and vig1b with 14-bp deletion in OsbZIP01 leads to the phenotype.
3. The four mutation forms (vig1a, vig1m1, vig1m2, and vig1m3) showed the same interactions with bZIP18, the same localizations, the same phenotypes, however, the transcriptional activity of vig1a

was enhanced significantly and of other mutated forms (vig1m1, vig1m2, and vig1m3) were no significant alterations. It is difficult to understand the same phenotype based on the activity and interaction of vig1a, vig1m1, vig1m2, vig1m3.

4. In addition, many figures lacked the correct legends, such as, S6f, s9b, s9d, s16e, s16f, s17e, s17g, s19g, s19f, s25e-j.

Dear reviewers:

Thank you very much for reviewing our manuscript entitled “A point mutation in VIG1 enhances seedling establishment, chilling tolerance, and yield potential in rice” (ID: NCOMMS-24-13855A) and your insightful feedback and constructive comments. Those comments are all valuable for revising and improving our paper.

We have carefully studied those comments from all reviewers, and have revised the manuscript accordingly. We have sought assistance from a professional scientific writing service to refine the manuscript and response to reviewers accordingly, to improve the clarity and precision of the language. All changes in the manuscript text file and response to reviewers are indicated in red letters. A detailed point-by-point response addressing the reviewers’ comments is listed below.

We deeply appreciate the time and expertise you have invested in evaluating our work.

Sincerely yours,

Shanguo Yao

Professor, Group leader

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Reviewer #1 (Remarks to the Author):

I thank the authors for carefully addressing my concerns and for the useful extra experimental data supplied and the improved figures. The writing and explanations still need some work to attain full clarity.

Response: Thank you very much for your constructive and valuable feedback on our revised manuscript. To ensure that the full text meets clear and unambiguous publication standards, we have sought assistance from a professional scientific writing service to refine the manuscript and explanations accordingly, and have improved the clarity and precision of the language.

Reviewer #2 (Remarks to the Author):

The authors responded to reviewer comments properly. This revised manuscript significantly improves upon ambiguities in the presentation and interpretation of experimental results. As I judged previously, in this manuscript, the authors show that the vig1a mutation at the VIG1 locus is highly useful for rice improvement for direct seeding due to its superior ST, CT, and GNP, and they adequately explain the phenotypic differences between vig1a and vig1b by analyzing the protein structures and interactions with the homologous genes 18 and 48. Thus, I think this manuscript is at the level of being accepted.

Response: Thank you for providing your thoughtful review and the positive assessment of our manuscript. We are pleased to hear that the revisions have effectively addressed the points raised by the reviewer and that you find the paper to be of a suitable level for acceptance. Your feedback has been invaluable in guiding our revisions and we are grateful for your support.

Reviewer #3 (Remarks to the Author):

As mentioned in my first review, I believe this is a significant study that sheds light on the regulatory mechanism of three individual traits, including seedling vigor, cold sensitivity, and grain yield. The authors illustrated that the vig1a, which confers enhanced SV, CT and GNP, can be mimicked by mutations in the basic region of VIG1. These results can be applied to rice breeding through genome editing approaches. Authors have conducted additional analyses and experiments and added the appropriate explanations in addressing most of the concerns raised in my previous review. The revised manuscript was improved. However, there are still a few concerns which need to be addressed.

Response: We appreciate the reviewer's positive feedback and helpful suggestions on how to improve our manuscript. Please find the detailed point-by-point response below.

1. Regarding the functional redundancy. The substitution of R121H in the basic region of OsbZIP01 resulted in the vig1a phenotype, and this result indicated that there are not redundant effects among OsbZIP01, OsbZIP18, and OsbZIP48. However, the all knock-out lines (including OsbZIP18-NK, OsbZIP18-CK, OsbZIP48-NK, and OsbZIP48-CK) showed no obvious phenotypic alterations, which suggested a functional redundancy among them (Line 245). These two conclusions are contradictory to each other. Why?

Response: Thank you for your valuable concern. The substitution of R121H in the basic region of OsbZIP01 resulted in the *vig1a* phenotype, which indicated that the OsbZIP01 has no redundant functions with OsbZIP18 and OsbZIP48. Nevertheless, no obvious phenotypic alterations were observed in the knock-out lines of *OsbZIP18*, which is possibly due to the overlapped functions between OsbZIP18 and OsbZIP01 in regulating rice SV, CT, and GNP. If OsbZIP01 functions normally, the mutated effects of OsbZIP18 on SV, CT, and GNP are unlikely to exhibit, in the *OsbZIP18*-NK and *OsbZIP18*-CK plants. These results indicate that OsbZIP01 plays a major role in SV, CT and GNP, and that the role of OsbZIP18 is also important, but visible only in

the absence of OsbZIP01.

In this study, we discovered that *OsbZIP48* has little to no effects on SV, CT, and GNP (Supplementary Figure 14, Supplementary Figure 15, Supplementary Figure 16, and Figure 3), which is independent on the redundant effects. The results suggested that OsbZIP01 and OsbZIP18 have distinct and cooperative functions in regulating SV, CT, and GNP, which is similar to the effect of the two rice phytochromes (phyA and phyB) on the control of de-etiolation (DOI: 10.1105/tpc.105.035899). Compared to the wild-type, the *phyB* single mutant exhibited clear defects in seedling de-etiolation under continuous red-light conditions (Rc). On the other hand, the *phyA* single mutant appeared normal, while the *phyAphyB* double mutant presented more pronounced defects in seedling de-etiolation than that of in the *phyB* single mutant (Figure 2B in DOI: 10.1105/tpc.105.035899).

Therefore, to avoid ambiguity, we have changed all descriptions concerning the functional redundancy between OsbZIP01 and OsbZIP18 in the text.

2. Regarding the functional redundancy. If these three transcription factors (OsZIP01/VIG1, OsbZIP18, and OsbZIP48) are functional redundancy, vig1a with R121H substitution and vig1b with 14-bp deletion in OsbZIP01 leads to the phenotype.
Response: We apologize for the incorrect description. As you have mentioned, OsbZIP01 plays distinct roles in modulating SV, CT, and GNP in both *vig1a* and *vig1b* mutants. The result indicates that OsbZIP01 doesn't have redundant functions with OsbZIP18 and OsbZIP48. Indeed, OsbZIP01 and OsbZIP18 exhibit overlapping and cooperative functions, and both play crucial roles in regulating SV, CT, and GNP. Therefore, we have revised all descriptions concerning the functional redundancy in lines 32, 203-204, 254, 256-257, 283, 490, and 515 in the latest revision of the manuscript.

3. The four mutation forms (vig1a, vig1m1, vig1m2, and vig1m3) showed the same

interactions with bZIP18, the same localizations, the same phenotypes, however, the transcriptional activity of vig1a was enhanced significantly and of other mutated forms (vig1m1, vig1m2, and vig1m3) were no significant alterations. It is difficult to understand the same phenotype based on the activity and interaction of vig1a, vig1m1, vig1m2, vig1m3.

Response: This is a very interesting point that we still don't fully understand. In this study, though vig1a exhibited significantly enhanced transcriptional activity, compared to the other mutated forms of VIG1, it still interacted with OsbZIP18 and showed similar phenotypes in SV, CT, and GNP. One interpretation is that the elevated transcriptional activity in vig1a is caused by the specific site-specific amino acid mutation (R121-H121), but the elevation in transcriptional activity has little to no impacts on the phenotype. That is, the mutant site itself, rather than the increase in transcriptional activity, has an effect on SV, CT, and GNP. Another lies on that the elevated transcriptional activity in vig1a does produce noticeable phenotypic differences, compared to other VIG1 mutant proteins (vig1m1, vig1m2, and vig1m3), but we have not paid attention to these differences.

4. In addition, many figures lacked the correct legends, such as, S6f, s9b, s9d, s16e, s16f, s17e, s17g, s19g, s19f, s25e-j.

Response: We apologize for the absence of the correct legends. Based on your kindly reminder, we have added appropriate legends to the corresponding images at the respective positions.

Reviewers' Comments:

Reviewer #3:
Remarks to the Author:
All my concerns were addressed in the revised version or explained in the response.

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

All my concerns were addressed in the revised version or explained in the response.

Response: We would like to express our gratitude to the reviewer for the careful review and insightful comments, which have been of great help to us in revising and improving the manuscript. Thank you once again for taking the time to review our work and provide such constructive feedback.