nature portfolio

# Peer Review File

# Natural variation of an E3 ubiquitin ligase encoding gene Chalk9 regulates grain chalkiness in rice

Corresponding Author: Professor Changjie Yan

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Grain chalkiness, an undesirable trait that severely compromises rice quality, remains one of the most challenging obstacles in breeding programs aimed at developing high-quality rice varieties. In this study, Hu et al. employed a comprehensive approach combining genome-wide association study (GWAS) with systematic genetic analysis to identify Chalk9 as a crucial locus governing grain chalkiness. Through characterization of diverse genetic materials, the researchers revealed that Chalk9 encodes an E3 ubiquitin ligase that interacts with and mediates the ubiquitination-dependent degradation of OsEBP89, a transcription factor promoting chalkiness formation. They found that a critical 64-bp indel polymorphism in the Chalk9 promoter underlies natural variation in chalkiness among rice germplasms by modulating Chalk9 expression levels. The findings substantially advance our understanding of grain quality regulation and offer practical solutions for overcoming this persistent challenge in rice improvement programs. Overall, this is an impressive reaearch and the results are intriguing to the rice genetic and breeding community, but a few questions remain to be clarified before considering acceptance:
Major：
1. There are three amino acid substitutions between the Chalk9-L and Chalk9-H haplotypes. Do these substitutions affect the function of Chalk9 as an E3 ubiquitin ligase? If this manuscript focuses on Chalk9's role as an E3 ubiquitin ligase, more emphasis should be placed on analysis of the CDS or amino acid changes.
2. In abstract, the authors mentioned "Chalk9-L was strongly selected during japonica rice domestication and gradually incorporated into modern indica breeding programs". The results indicate that the superior chalk9 allele most probably evolved from O. rufipogon through a single lineage, while that of indica probably evolved from O. rufipogon with more complex evolution history. There is no solid evidence to indicate the superior allele of chalk9 in indica variety was introgressed from japonica.
3. The elite haplotype Chalk9-L is highly prevalent in Japonica and O. rufipogon. China has made great progresses in indica rice breeding for high grain quality especial for low chalkiness in recent ten years. What is the proportion of low-chalkiness indica varieties with the chalk9 elite haplotype among the varieties released in recent 10 years in China? We need to know the divergence of the haplotype for chalk9 among indica varieties with low CGR recently released in China? This information is very important for improving rice appearance quality using Chalk9-L in indica rice breeding program.
4. The study reveals high expressed chalk9 decreases the chalkiness by degenerating OsEBP89 (low expression), which interacts with Wx gene. From breeding point of view, the gene OsEBP89, an already cloned gene (Yang et al. Plant Molecular Biology, 2002, 50(3): 379-391) is likely to be more important than chalk9 for regulating chalkiness trait. I concern how about performance of the main agronomic traits including grain yield between mutant and its wildtype? If the elite haplotype of chalk9 has been widely used in modern rice breeding in China, more attentions should be paid for use of the novel haplotype of OsEBP89. Therefore, supplement more information of OsEBP89 is beneficial for rice breeding of high grain quality.
5. It is well known that high yield has trade-off to high grain quality. This study indicates that chalk9 gene can improve grain quality much without compromising productivity. How does the gene realize the synergy between yield and quality by regulating the storage components in endosperm. More experimental data undoubtedly help the readers to understand the potential molecular mechanism.

Minor：
1. By resequencing Chalk9, the authors identified two Indels and four SNPs, which are more strongly associated with grain chalkiness than the top SNP in GWAS. Were any of these SNPs or Indels detected in the GWAS?
2. The authors generated Chalk9-OE and Chalk9-RNAi transgenic plants in the ZH11 background. Which Chalk9 haplotype

does ZH11 carry?
3. Line 130: "Similar expression levels of expression in leaves" may be incorrect. Consider revising for clarity.
4. Line 137: It would be more appropriate to replace "candidate gene" with "functional gene" or "regulatory gene."
5. Line 142: Please correct "RANi" to "RNAi".

Reviewer #2

(Remarks to the Author)
Overall evaluation
The author identified a major gene Chalk9 that controls rice chalkiness through whole genome association studies, explaining 28% of the phenotypic variation. Chalk9 encodes an E3 ubiquitin ligase, which forms the Chalk9-OsEBP89 module with the substrate OsEBP89. The Chalk9-OsEBP89 module maintains the homeostasis of stored substances in the endosperm by regulating Wx and SSP, and the disruption of this homeostasis can lead to chalkiness. The 64bp insertion/deletion in the Chalk9 promoter region is a functional variation that leads to differences in transcription levels and chalkiness. The Chalk9-L excellent allele, which was strongly selected during the domestication process of japonica rice, infiltrated into the high-yield variety Guichao 2, significantly reducing chalkiness but not affecting yield. The author's research has revealed new molecular mechanisms regulating chalkiness and provided new targets for improving rice quality. This research work is meaningful and worthy of recognition, but there is still a lot of room for improvement. I hope the author can address the following concerns of mine properly.

Major concern:
1. Temperature is one of the main environmental factors that contribute to the formation of rice chalkiness. Whether the chalkiness differences caused by natural variations in Chalk9 only manifest at high temperatures or are unrelated to temperature is a highly debated question. Is Chalk9-L more heat-resistant, resulting in better quality of rice under high temperatures?
2. The author mentioned that in the Chalk9 mutant, there was no significant change in the total starch content of the grains, but the amylose content and total protein content increased, followed by chalkiness. So, what is the potential relationship between grain amylose content, protein content, and chalkiness? Does it mean that an increase in protein content necessarily leads to an increase in chalkiness, or an increase in amylose content necessarily leads to an increase in chalkiness? Is there such a pattern?
3. If the author wants to propose the Chalk9-OsEBP89-Wx/SSP module, it is necessary to confirm the interaction between OsEBP89 and SSP in vitro and in vivo through EMSA and ChIP experiments. Otherwise, the description of this module in the text should be modified accordingly.

Minor concern：
1. Line38-40: The author mentioned that rice chalkiness has a negative impact on eating and cooking. So how does it affect eating and cooking? Does rice with high chalkiness necessarily taste bad? Does rice with low chalkiness necessarily taste good?
2. Line991-996: What is the comment about f in Figure 1?
3. In Figure 1c-e, the author needs to add how the p value is obtained. For example, students' two-tailed t test, etc. Other Figures also have similar problems, please modify them together.
4. Line143: Although Yangdao6hao was developed based on the background of 9311, they are not the same thing after all, so it is unreasonable to use 93-11 to represent Yangdao6hao here, and it is suggested to change it to YD6.
5. In Extended Data Fig. 2a, it is suggested to add a note about the longest dotted line.
6. The phrase "of expression" in line 130 is clearly redundant and it is recommended to delete it.
7. Lines 221-222, how did the author find this key amino acid residue site? The text should provide an explanation.
8. In Figure 5e, there may be a problem with the relative protein level of OsEBP89 in NIP. The values of the three duplicate samples are identical, even without standard deviation. The same problem also appears in Figure 5h. I doubt the authenticity of this data.
9. Suggest the author to supplement the plant architecture photos or field photos of Guichao 2 and Chalk9-L near isogenic lines, so that readers can more intuitively observe the results described by the author.

Reviewer #3

(Remarks to the Author)
The study by Hu et al. is about the molecular mechanism of rice endosperm chalkiness via Chalk9-OsEBP89. Through genome-wide association studies in indica rice germplasm, they identified the gene Chalk9 as the key player and revealed two major haplotypes, Chalk-L and Chalk-H, to contribute to the trait. Molecular studies demonstrated the role of Chalk9 as an E3 ubiquitin ligase and OsEBP89 as its direct target for degradation. OsEBP89, a transcription factor, directly regulates amylose as well as various seed storage proteins' synthesis. Further, they emphasized the evolutionary significance of the elite allele Chalk9-L in japonica and indica rice varieties and suggested the importance of the Chalk9-L allele in breeding applications to improve rice appearance quality without yield penalty. It's an interesting and valuable study, and needs the following clarifications:

1. Line 112 incorrectly mentions 15 genes while Supplementary table 2 lists out 18 genes!
2. Suppl fig 2A depicts 6 proteins, though 12 were identified in actuality. A reason for the selection should be mentioned.

3. The reference for a missense SNP not conserved across plant species, unlikely affecting protein function should be mentioned at line 122.
4. IN FIG 1c, P values for all comparisons should be mentioned. Were 8 varieties taken each for high chalk and low chalk data? The samples across which P values were generated should be mentioned in legend (low vs high chalk?)
5. Though the difference in Fig 1D can be visualized, significance should be assigned to the same.
6. Figure 1 legend mentions an 'F' part, which is not visible in the figure.
7. Figure 2 A and E should have independent scale bars for each sub-part of the figure. Similarly, for other figures with sub-parts.
8. The word should be RNAi in line 142 not RANi.
9. The source of expression data for Suppl fig 2C should be mentioned.
10. Nip Chalk-9 mutants should be explained in more details in results.
11. How many varieties were used to re-sequence Chalk9? Did these varieties have any differences in grain chalkiness? Are these from the association panel?
12. Why was Guichao2 used to generate promoter transgenic plants and not Nipponbare or ZH11? What are the differences in their native promoter sequences?
13. Promoter activity was examined in rice protoplasts. How were the protoplasts isolated and transformed (no mention in methods)? Since the gene is expressed in endosperm and not leaf, how were the promoters active in protoplasts?
14. What is the source of data in Suppl fig 4A?
15. A statistical analysis of Fig 4B (RTPCR) should be done. What is the control used?
16. It will be good to mention the expected sizes of proteins in sub-parts of Fig 4.
17. The pull down assay for CHALK9 EBP89 dimer should be performed both ways (GST and MBP pull down). The input lane can have only one protein, not two for confirmation of detection.
18. BiFC should be performed with vector swap (Chalk-cYFP + EBP-nYFP). Similarly, yeast two hybrid assay.
19. The presence of protein bands for both Chalk9 and EBP89 in CoIP input is not correct.
20. Better photographs of Chalk9 promoter activity would be appreciated (Suppl fig 5A).
21. Why was the interaction of EBP89 with Chalk9 tested?
22. Sizes of all proteins should be mentioned at requisite places.
23. The control should also be quantified for Fig 5B, D by ImageJ.
24. Chalky endosperms have lesser amylose content in general. An opposite result here (Fig 6B,C) should be well supported and explained.
25. The correlation coefficient is beyond 0.95 for chalk9 mutants and Nip, which is quite high for them to be compared. This data needs to be supported with other published literature. (Supp fig 7b)
26. Material and methods need to be elaborated, since multiple essential experiments and methods of calculations have been skipped.
27. Grammatical errors here and there need to be rectified.
28. Use of abbreviations are not consistent across the manuscript. For example, even though chalky grain rate is abbreviated as CGR in the manuscript, its mentioned in full length throughout the figures.
29. Extended Data Fig. 1 does not provide data for the statement at lines 99-100. "This locus explained ~28% of the total phenotypic variation".
30. For lines 119-121 "we first evaluated SNPs……. domain (Supplementary Fig. 2a)", data provided in Supplementary Fig. 2a is incomplete
31. From line 991-997 there is a legend starting as "Expression analysis of the candidate genes from GWAS in various tissues…", for which the image is not provided in the Fig. 1 but in Supplementary Fig. 2.
32. Manuscript states association analysis with the identified promoter variants in 149 indica varieties, while the data provided in Fig. 3A-"b,c, The distribution of chalky grain rate (b) and degree of chalkiness (c) in haplotype H (n = 45 accessions) and haplotype L (n = 102 accessions)" totally constitutes only 147 accessions. Also, haplotypes H and L have a wide range of values for CGR and DC from all the accessions while Chalk9 expression has been studied only in selected 24 accessions from both the haplotypes. What was the basis for the selection of these accessions?
33. Supplementary Fig. 5a GUS image for P(Panicle) shows a spikelet instead of a panicle as mentioned.
34. The proteasome inhibitor MG132 usually used at working concentration 10-50uM. The reason for it being used at a higher concentration of 50mM can be mentioned.
35. The pAbAi vector, used in the Y1H assay (Extended Data Fig. 3m), has a selection for uracil and can be used in the dropout medium to ensure stringency in selecting positive interactions.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
You have provided detailed responses and revisions to the review comments, adequately addressing the modifications I suggested. I have no further review comments and recommend acceptance for publication.

Reviewer #2

(Remarks to the Author)
The authors answered my questions, please accept this manuscript to publish on Nature Communications.

Reviewer #3

(Remarks to the Author)
The authors have taken outmost care to address all the concerns and have rectified all the points mentioned. The relation between Chalk9-EBP89 in regulating rice grain chalkiness, explored in this article is a considerably important data in understanding this trait.

## Point-by-point response

Reviewer #1 (Remarks to the Author):

Grain chalkiness, an undesirable trait that severely compromises rice quality, remains one of the most challenging obstacles in breeding programs aimed at developing high-quality rice varieties. In this study, Hu et al. employed a comprehensive approach combining genome-wide association study (GWAS) with systematic genetic analysis to identify Chalk9 as a crucial locus governing grain chalkiness. Through characterization of diverse genetic materials, the researchers revealed that Chalk9 encodes an E3 ubiquitin ligase that interacts with and mediates the ubiquitination-dependent degradation of OsEBP89, a transcription factor promoting chalkiness formation. They found that a critical 64-bp indel polymorphism in the Chalk9 promoter underlies natural variation in chalkiness among rice germplasms by modulating Chalk9 expression levels. The findings substantially advance our understanding of grain quality regulation and offer practical solutions for overcoming this persistent challenge in rice improvement programs. Overall, this is an impressive research and the results are intriguing to the rice genetic and breeding community, but a few questions remain to be clarified before considering acceptance:

Response:

We greatly appreciate your positive feedback on our research. In response to your suggestions, we have implemented some improvements, highlighted in blue in the updated manuscript.

Major：

1. There are three amino acid substitutions between the *Chalk9*-L and *Chalk9*-H haplotypes. Do these substitutions affect the function of Chalk9 as an E3 ubiquitin ligase? If this manuscript focuses on Chalk9's role as an E3 ubiquitin ligase, more

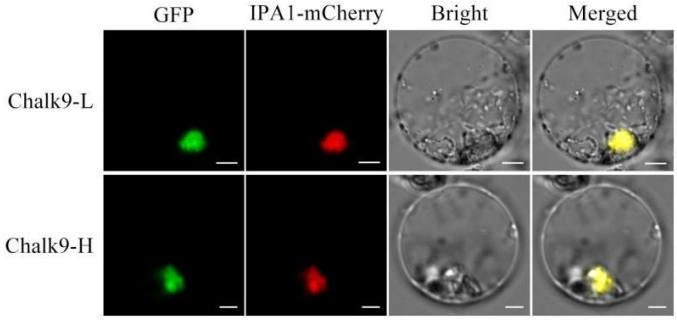
emphasis should be placed on analysis of the CDS or amino acid changes.

Response:

Thank you for your valuable comment. We sincerely appreciate the reviewer's insightful suggestion and fully agree that the functional characterization of the amino acid substitutions between Chalk9-L and Chalk9-H is essential for this study. To determine whether these substitutions impact Chalk9's function as an E3 ubiquitin ligase, we performed two key experiments:

1. Subcellular localization analysis

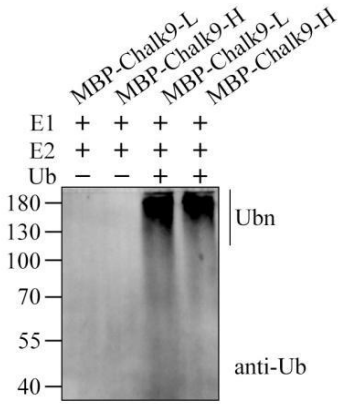
Transient expression assays in rice protoplasts showed identical nuclear localization for both Chalk9-L and Chalk9-H proteins (please see new Supplementary Fig. 8a), suggesting that the amino acid changes do not alter protein localization.




**Supplementary Fig. 8a** Subcellular localization of Chalk9-H-GFP and Chalk9-L-GFP fusion proteins in rice protoplasts. IPA1-mCherry was used as a nuclear marker. Scale bars: 5 μm.

2. *In vitro* ubiquitination assays

Both Chalk9-L and Chalk9-H proteins exhibited comparable autoubiquitination activity in vitro (please see new Supplementary Fig. 8b), indicating similar E3 ligase activity.




**Supplementary Fig. 8b** Ubiquitin ligase activity of Chalk9-L and Chalk9-H. MBP-Chalk9-L and MBP-Chalk9-H were expressed in *E. coli* BL21, and ubiquitinated proteins were detected using an anti-ubiquitin (Ub) antibody.

These results suggest that the amino acid variations between Chalk9-L and Chalk9-H do not affect the Chalk9's molecular function as E3 ubiquitin ligases. The relevant data have been included in the revised manuscript (please see lines 240–245 and new Supplementary Fig. 8).

To further assess the functional consequence of these coding sequence changes in planta, we analyzed transgenic lines expressing either *Chalk9*-L or *Chalk9*-H under the same promoter (*pChalk9*-L). As shown in the results of Fig. 3i–k, both *pChalk9-L::Chalk9-L* and *pChalk9-L::Chalk9-H* transgenic lines significantly reduced chalkiness to a similar extent, indicating that the CDS or amino acid alterations between *Chalk9*-L and *Chalk9*-H do not impair Chalk9's function in regulating chalkiness. In contrast, the *pChalk9-H::Chalk9-H* transgenic line did not significantly reduce chalkiness (see Fig. 3i–k). qRT-PCR analysis indicated that the *Chalk9* expression level in the *pChalk9-H::Chalk9-H* line was lower than in the *pChalk9-L::Chalk9-L* and *pChalk9-L::Chalk9-H* lines (see Fig. 3l), underscoring the importance of promoter-driven expression differences in determining haplotype-specific phenotypic effects. These transgenic results demonstrate that the phenotypic differences between *Chalk9*-L and *Chalk9*-H haplotypes arise from regulatory variations in the promoter region rather than coding sequence polymorphisms. We have accordingly revised the "Results" section to better reflect these findings (please see lines 179–198).

2. In abstract, the authors mentioned "*Chalk9*-L was strongly selected during japonica rice domestication and gradually incorporated into modern indica breeding programs". The results indicate that the superior *Chalk9* allele most probably evolved from *O. rufipogon* through a single lineage, while that of *indica* probably evolved from *O. rufipogon* with more complex evolution history. There is no solid evidence to indicate the superior allele of *Chalk9* in *indica* variety was introgressed from japonica.

Response:

Thank you for your thoughtful comment. We agree that while *Chalk9*-L was strongly selected during *japonica* rice domestication, the available evidence does not conclusively support that this allele was introgressed into *indica* varieties. As our data suggest, the superior *Chalk9* allele in *japonica* likely evolved from *O. rufipogon* through a single lineage, whereas the *indica* allele appears to have a more complex evolutionary origin.

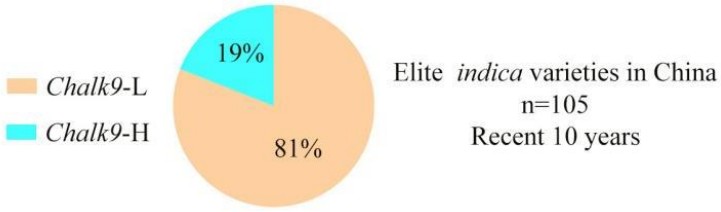
To more accurately reflect this, we have revised the statement in the abstract to: "During rice domestication, *Chalk9*-L was strongly selected in *japonica* but exhibited a complex evolutionary trajectory in *indica*." (please refer to lines 29–30). This modification better aligns with the findings from our evolutionary analysis.

3. The elite haplotype *Chalk9*-L is highly prevalent in *Japonica* and *O. rufipogon*. China has made great progresses in *indica* rice breeding for high grain quality especial for low chalkiness in recent ten years. What is the proportion of low-chalkiness *indica* varieties with the *Chalk9* elite haplotype among the varieties released in recent 10 years in China? We need to know the divergence of the haplotype for *Chalk9* among *indica* varieties with low CGR recently released in China? This information is very important for improving rice appearance quality using *Chalk9*-L in *indica* rice breeding program.

Response:

Thank you very much for your suggestion. To evaluate the prevalence of the elite

haplotype *Chalk9*-L in modern *indica* rice breeding, we genotyped 105 elite *indica* varieties released in China over the past ten years, all of which are recognized for low chalkiness. Using a 64-bp InDel marker in the *Chalk9* promoter, we found that 85 out of 105 varieties (81%) harbor the *Chalk9*-L haplotype, indicating its high frequency and widespread utilization in modern *indica* breeding programs. This observation reflects China's significant advancement in rice quality improvement, particularly in reducing chalkiness, over the past decade. The relevant data have been included in the "Results" section (please refer to lines 392–397 in the revised manuscript) and are presented in the new Supplementary Fig. 14g.



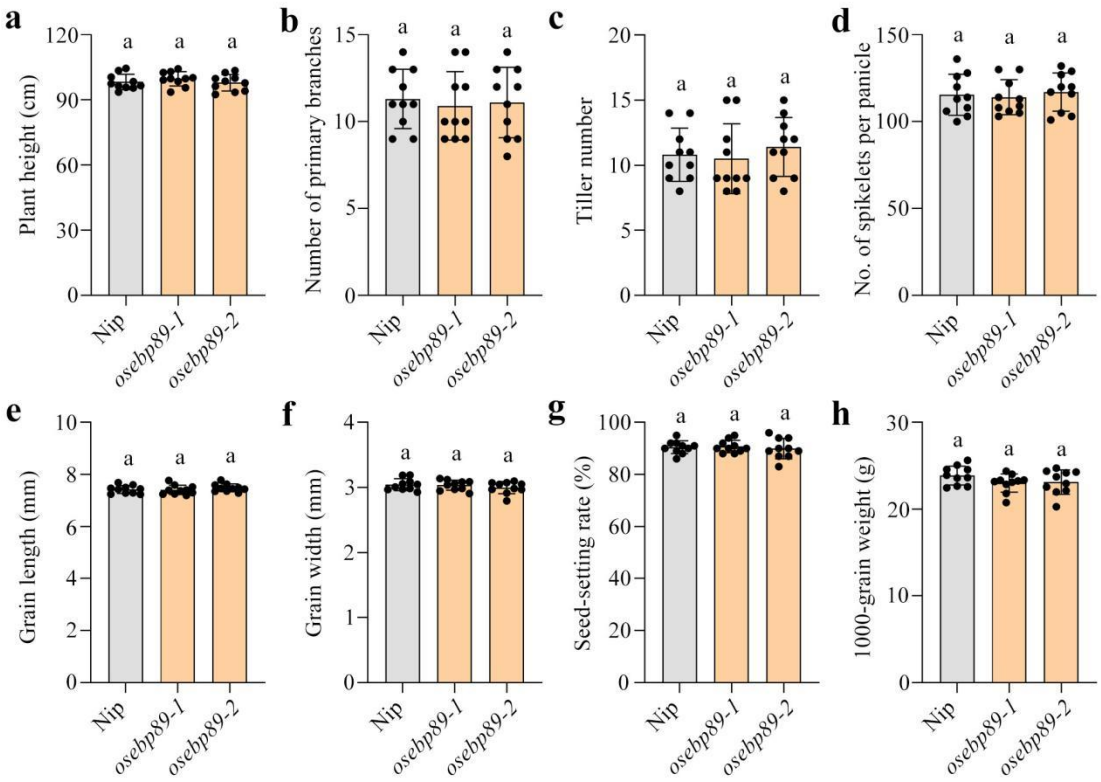

**Supplementary Fig. 14g** The frequency of *Chalk9*-L in the elite *indica* varieties released in China over the past decade.

4. The study reveals high expressed *Chalk9* decreases the chalkiness by degenerating OsEBP89 (low expression), which interacts with *Wx* gene. From breeding point of view, the gene *OsEBP89*, an already cloned gene (Yang et al. Plant Molecular Biology, 2002, 50(3): 379-391) is likely to be more important than *Chalk9* for regulating chalkiness trait. I concern how about performance of the main agronomic traits including grain yield between mutant and its wildtype? If the elite haplotype of *Chalk9* has been widely used in modern rice breeding in China, more attentions should be paid for use of the novel haplotype of *OsEBP89*. Therefore, supplement more information of *OsEBP89* is beneficial for rice breeding of high grain quality.

Response:

We sincerely thank the reviewer for the insightful comment. In response, we conducted a comparative analysis of key agronomic traits between *OsEBP89* knockout mutants and wild-type plants. Traits evaluated included plant height, number of primary

branches, tiller number, number of spikelets per panicle, grain length, grain width, seed-setting rate, and 1000-grain weight. No significant differences were observed in any of these traits under field conditions (please see lines 341–346 in the revised manuscript and new Supplementary Fig. 13). These findings confirm that the loss of function of *OsEBP89* does not compromise key yield-related traits, reinforcing its potential utility in breeding programs aimed at enhancing grain quality without compromising yield.




**Supplementary Fig. 13 Agronomic traits for *OsEBP89* knockout mutants. a–h** Comparisons of plant height (**a**), number of primary branches (**b**), tiller number (**c**), number of spikelets per panicle (**d**), grain length (**e**), grain width (**f**), seed-setting rate (**g**), and 1000-grain weight (**h**) in Nip, *osebp89-1*, and *osebp89-2* plants. Data show means ± SD ($n$ = 16 plants). Identical letters indicate no significant differences ($P \geq 0.05$, one-way ANOVA with Tukey's multiple comparison test); for $P$ values, see Source Data. Source data are provided as a Source Data file.

Additionally, Supplementary Table 13 shows that *OsEBP89* has a single major haplotype in *indica* rice, contrasting with three primary haplotypes in *japonica* rice, indicating a trend of *indica-japonica* differentiation. As the reviewer pointed out, more attention should be paid to the importance of *OsEBP89* in rice. Further investigations are warranted to identify and functionally characterize superior haplotypes of *OsEBP89*.

5. It is well known that high yield has trade-off to high grain quality. This study indicates that *Chalk9* gene can improve grain quality much without compromising productivity. How does the gene realize the synergy between yield and quality by regulating the storage components in endosperm. More experimental data undoubtedly help the readers to understand the potential molecular mechanism.

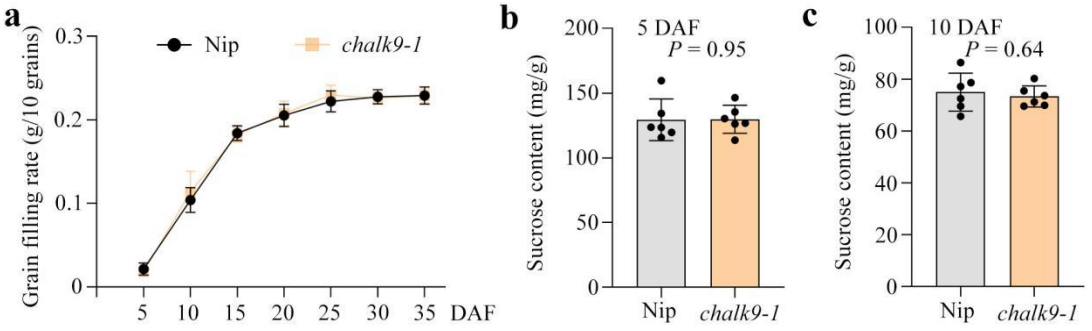
Response:

We appreciate the constructive and insightful comments. Although understanding the trade-off between yield and quality is challenging, we recognize that the underlying mechanisms remain insufficiently elucidated in our original manuscript and agree that further clarification is necessary.

Our study demonstrates that *Chalk9* regulates the expression of genes involved in amylose and SSP biosynthesis, particularly during the late maturation phase, modulating starch granule structure and protein body formation, thereby regulating grain chalkiness. During grain filling, photosynthetic assimilates produced in the leaves are transported to developing grains and converted into storage components through enzymatic reactions. Recent studies by Shi et al. (2024) have shown that the production, transport, and partitioning of these assimilates—primarily sucrose—are crucial in determining the final starch and seed storage protein (SSP) accumulation, ultimately influencing both grain yield and quality.

To investigate this further, we analyzed grain filling rate in wild-type Nip and *Chalk9* knockout mutant plants. No significant differences were observed between Nip and *chalk9-1* mutant from 5 to 35 DAF (please see new Supplementary Fig. 17a), indicating that *Chalk9* does not affect the grain-filling process. We also measured sucrose content in endosperms at 5 and 10 DAF, finding no significant differences between the two genotypes (please see new Supplementary Fig. 17b, c), suggesting that *Chalk9* does not disrupt the supply of photosynthetic assimilates for the synthesis of storage components. These observations are consistent with the unaltered yield in *chalk9* mutants, which

may explain *Chalk9*'s unique ability to avoid yield penalty. Consequently, *Chalk9* may improve grain quality through precise regulation of endosperm structure and storage component composition without compromising yield. These findings have been included in the revised manuscript (please see lines 412–419).




**Supplementary Fig. 17 The effect of *Chalk9* on grain filling and sucrose metabolism in the endosperm. a** Grain filling rate of Nip and *chalk9-1*. DAF, days after flowering. Data show means ± SD ($n$ = 3 biological replicates). **b**, **c** Sucrose content in the endosperm at 5 (**b**) and 10 (**c**) DAF from Nip and *chalk9-1*. Data show means ± SD ($n$ = 6 biological replicates). Statistical analysis was performed by two-tailed Student's $t$-test; for $P$ values, see Source Data. Source data are provided as a Source Data file.

Reference:

Shi, H. et al. Natural variation of *WBR7* confers rice high yield and quality by modulating sucrose supply in sink organs. *Plant Biotechnol. J.* **22**, 2985–2999 (2024).

Minor:

1. By resequencing *Chalk9*, the authors identified two Indels and four SNPs, which are more strongly associated with grain chalkiness than the top SNP in GWAS. Were any of these SNPs or Indels detected in the GWAS?

Response:

Thank you for your insightful question. Among the six variants identified by resequencing *Chalk9* (two Indels and four SNPs), we found that one SNP overlapped with the variants identified in the GWAS results, while the remaining variants, including both the Indels and other SNPs, were not detected in the GWAS. This discrepancy can be attributed to the inherent limitations of low-coverage sequencing in

our GWAS, which may fail to detect certain variants, particularly Indels, due to insufficient sequencing depth or the specific focus of the GWAS on SNP variation. These additional variants, particularly the Indels, exhibited stronger associations with grain chalkiness than the top SNP identified in the GWAS, indicating their potential importance in functional variation at the *Chalk9* locus.

We appreciate your attention to this important distinction and hope this explanation clarifies the relationship between the resequencing and GWAS results.

2. The authors generated *Chalk9*-OE and *Chalk9*-RNAi transgenic plants in the ZH11 background. Which *Chalk9* haplotype does ZH11 carry?

Response:

Thank you for your question. We have carefully examined the *Chalk9* haplotype in the ZH11 background using molecular marker analysis. Our results indicate that ZH11 carries the *Chalk9*-L haplotype. This was determined by analyzing a key 64-bp indel that distinguishes the known *Chalk9* haplotypes. We appreciate your attention to this detail.

3. Line 130: "Similar expression levels of expression in leaves" may be incorrect. Consider revising for clarity.

Response:

Thank you for bringing this to our attention. The original phrase, "expression levels of expression," is incorrect. We have revised the sentence to: "Similar expression levels in leaves." The revised version has been updated in the manuscript (please see line 131).

4. Line 137: It would be more appropriate to replace "candidate gene" with "functional gene" or "regulatory gene."

Response:

Thank you for your insightful suggestion. We agree that the term "functional gene" or "regulatory gene" is indeed more appropriate than "candidate gene" in this context, as it more accurately reflects the gene with demonstrated biological function or regulatory role.

In response, we have revised the manuscript by replacing "candidate gene" with "functional gene" (please see line 138). We appreciate your attention to this detail, which has contributed to improving the clarity of our manuscript.

5. Line 142: Please correct "RANi" to "RNAi".

Response:

Thank you for pointing out this error. We have corrected "RANi" to "RNAi" in the manuscript (please see line 143).

Reviewer #2 (Remarks to the Author):

Overall evaluation

The author identified a major gene Chalk9 that controls rice chalkiness through whole genome association studies, explaining 28% of the phenotypic variation. Chalk9 encodes an E3 ubiquitin ligase, which forms the Chalk9-OsEBP89 module with the substrate OsEBP89. The Chalk9-OsEBP89 module maintains the homeostasis of stored substances in the endosperm by regulating Wx and SSP, and the disruption of this homeostasis can lead to chalkiness. The 64bp insertion/deletion in the Chalk9 promoter region is a functional variation that leads to differences in transcription levels and chalkiness. The Chalk9-L excellent allele, which was strongly selected during the domestication process of japonica rice, infiltrated into the high-yield variety Guichao

2, significantly reducing chalkiness but not affecting yield. The author's research has revealed new molecular mechanisms regulating chalkiness and provided new targets for improving rice quality. This research work is meaningful and worthy of recognition, but there is still a lot of room for improvement. I hope the author can address the following concerns of mine properly.

Response:

We truly value your insightful feedback and greatly appreciate your constructive suggestions.

Major concern:

1. Temperature is one of the main environmental factors that contribute to the formation of rice chalkiness. Whether the chalkiness differences caused by natural variations in Chalk9 only manifest at high temperatures or are unrelated to temperature is a highly debated question. Is Chalk9-L more heat-resistant, resulting in better quality of rice under high temperatures?

Response:

Thank you very much for your insightful comment regarding the role of temperature in chalkiness formation and its potential interaction with the natural variation of *Chalk9*. High temperature (HT) is indeed a critical environmental factor that disrupts endosperm development and promotes the formation of chalkiness during the grain-filling stage (Tabassum et al. 2020; Sreenivasulu et al. 2015).

Although our current study focused on the genetic basis of chalkiness in natural populations, we did not specifically investigate temperature effects. However, we took deliberate measures to minimize temperature-related confounding factors. Notably, we selected experimental materials grown in the Yangzhou region, rather than in Hainan, where temperature conditions differ significantly. Furthermore, due to the prolonged high temperatures experienced in Yangzhou during the 2022 growing season, we

excluded materials from that year from our GWAS analysis. These steps highlight the importance of temperature in chalkiness formation and our efforts to control for its influence in this study.

In the past, we cultivated *Chalk9* mutant materials across various locations (Beijing, Shandong, Yangzhou, and Hainan), encompassing a range of latitudes and temperature regimes. In all four regions, the *Chalk9* mutants consistently exhibited significantly higher chalkiness compared to the wild type, suggesting that *Chalk9* plays a relatively stable role in chalkiness formation irrespective of temperature. However, we cannot rule out some temperature influences. We agree that investigating whether *Chalk9*-L confers improved grain quality under HT conditions is a compelling direction for future research. Such studies would provide valuable insights into the environmental adaptability of *Chalk9* variants and their potential application in breeding heat-tolerant rice varieties.

We have included this explanation in the "Discussion" section (please see lines 457–465 in the revised manuscript).

Reference:

Sreenivasulu, N. et al. Designing climate-resilient rice with ideal grain quality suited for high-temperature stress. *J. Exp. Bot.* **66**, 1737–1748 (2015).

Tabassum, R. et al. *FLOURY ENDOSPERM11-2* encodes plastid HSP70-2 involved with the temperature-dependent chalkiness of rice (*Oryza sativa* L.) grains. *Plant J.* **103**, 604–616 (2020).

2. The author mentioned that in the Chalk9 mutant, there was no significant change in the total starch content of the grains, but the amylose content and total protein content increased, followed by chalkiness. So, what is the potential relationship between grain amylose content, protein content, and chalkiness? Does it mean that an increase in protein content necessarily leads to an increase in chalkiness, or an increase in amylose content necessarily leads to an increase in chalkiness? Is there such a pattern?

Response:

We sincerely appreciate your insightful questions about the potential relationship between amylose content, protein content, and chalkiness. Amylose plays a critical role in starch biosynthesis and granule structure, while seed storage proteins influence the formation and organization of protein bodies. Both components are integral to endosperm development and structure. Alterations in their levels can affect the packing and arrangement of starch granules and protein bodies, potentially disrupting endosperm compactness and generating air spaces, which contribute to chalkiness formation (Chen et al., 2024).

Interestingly, both increases and decreases in these storage components have been associated with chalkiness, suggesting a non-linear relationship. For example, the *opaque3* (*o3*) mutant shows significantly reduced protein and starch content, along with increased chalkiness (Cao et al., 2022). Similarly, two *grains with chalkiness* (*gwc1*) mutants, *gwc1-1* and *gwc1-2*, exhibit lower total starch and amylose contents, coupled with white-belly endosperm phenotypes (Guo et al., 2020). In contrast, *WCR1* knockout mutants show elevated white-core rates with increased total starch and amylose contents but reduced total protein (Wu et al., 2022). Moreover, other studies have shown that enhanced accumulation of storage components can actually reduce chalkiness, as in the case of *WBR7*, which promotes the synthesis of storage compounds and results in lower white-belly rates (Shi et al., 2024).

These findings underscore the complexity of the relationship between storage components and chalkiness. There appears to be no consistent pattern indicating that increased amylose or protein content necessarily leads to higher chalkiness. Instead, the effect likely depends on how these changes alter the structural organization of the endosperm. The mechanisms underlying chalkiness formation remain incompletely understood. Future studies should explore structural modifications in starch granules and protein bodies, as well as their interactions during endosperm development, to clarify their contributions to chalkiness formation. Thank you for these important questions.

References:

Cao, R. et al. *OPAQUE3*, encoding a transmembrane bZIP transcription factor, regulates endosperm storage protein and starch biosynthesis in rice. *Plant Comm.* **3**, 100463 (2022).

Chen, L. et al. Genes controlling grain chalkiness in rice. *Crop J*. **12** 979–991 (2024).

Guo, L. et al. GWC1 is essential for high grain quality in rice. *Plant Sci*. **290** 110497 (2020).

Shi, H. et al. Natural variation of *WBR7* confers rice high yield and quality by modulating sucrose supply in sink organs. *Plant Biotechnol. J.* **22**, 2985–2999 (2024).

Wu, B. et al. Natural variation in *WHITE-CORE RATE 1* regulates redox homeostasis in rice endosperm to affect grain quality. *Plant Cell* **34**, 1912–1932 (2022).

3. If the author wants to propose the Chalk9-OsEBP89-Wx/SSP module, it is necessary to confirm the interaction between OsEBP89 and SSP in vitro and in vivo through EMSA and ChIP experiments. Otherwise, the description of this module in the text should be modified accordingly.

Response:

Thank you for your insightful and constructive comment. We fully agree that further experimental validation, such as electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP), are necessary to confirm a direct regulatory interaction between OsEBP89 and *SSP* genes.

Although our current study provides preliminary evidence suggesting a potential regulatory link, we acknowledge that the direct regulation of *SSP* genes by OsEBP89 has not been demonstrated. Therefore, we have revised the description of the “Chalk9-OsEBP89-Wx/SSP module” to “Chalk9-OsEBP89 module” throughout the manuscript to more accurately reflect the current level of evidence (please see lines 427 and 502).

Minor concern:

1. Line38-40: The author mentioned that rice chalkiness has a negative impact on eating and cooking. So how does it affect eating and cooking? Does rice with high chalkiness necessarily taste bad? Does rice with low chalkiness necessarily taste good?

Response:

Thank you for your insightful question regarding the impact of rice chalkiness on eating and cooking quality. We appreciate the opportunity to clarify this point.

The endosperm is the main edible portion of rice grains and is predominantly composed of storage substances. The structural composition of the endosperm directly determines the cooking quality of rice (Custodio et al., 2019). Starch, which accounts for approximately 85% of the endosperm, is the most critical component influencing cooking quality. Amylose content (AC) is a major predictor of rice eating and cooking quality, as it has been associated with mechanical textural attributes such as hardness and stickiness. Generally, cooked rice grains with low or intermediate AC exhibit an elastic, sticky, and glossy texture, whereas high AC varieties are characterized by firm and separated grains after cooking (Li et al., 2016). Chalky rice tends to have higher amylose content, contributing to a harder texture after cooling. Additionally, cooking time is influenced by the gelatinization temperature (GT), which is the temperature at which the crystalline structures of starch begin to melt (Custodio et al., 2019). Chalkiness is often associated with lower gelatinization temperature and altered starch properties, which can impact the cooking process and lead to a less uniform texture (Misra et al., 2021). Therefore, chalkiness negatively affects cooking quality primarily by influencing the texture and consistency of the cooked rice.

Notably, consumer preferences for rice eating quality differ by region and culture. For instance, individuals from China, Japan, and Korea tend to prefer rice with low AC and sticky texture upon cooking, whereas individuals from India and Bangladesh prefer high AC rice, which results in firm, separate grains when cooked (Custodio et al., 2019). Regarding taste, rice with high chalkiness does not necessarily taste bad (Xia et al., 2022), as the taste is influenced by multiple factors such as aroma, flavor compounds,

and individual preferences. Conversely, rice with low chalkiness does not always guarantee good taste (Xia et al., 2022), but it generally provides a more appealing texture and consistency, which are important components of eating quality. We acknowledge that the relationship between chalkiness and eating quality is not absolute. Hence, high chalkiness does not necessarily equate to poor eating quality, and low chalkiness does not always indicate superior taste.

To address this, we have revised the relevant "Introduction" section by removing the description of "eating" (please see line 38). Thank you once again for your insightful feedback.

Reference:

Custodio, M. C. et al. Rice quality: how is it defined by consumers, industry, food scientists, and geneticists? *Trends Food Sci. Tech.* **92**, 122–137 (2019).

Li, H., Prakash, S., Nicholson, T. M., Fitzgerald, M. A., & Gilbert, R. G. The importance of amylose and amylopectin fine structure for textural properties of cooked rice grains. *Food Chem*. **196**, 702–711 (2016).

Misra, G. et al. Genome-wide association coupled gene to gene interaction studies unveil novel epistatic targets among major effect loci impacting rice grain chalkiness. *Plant Biotechnol. J.* **19**, 910–925 (2021).

Xia, D. et al. Effects of *Wx* Genotype, Nitrogen Fertilization, and Temperature on Rice Grain Quality. *Front Plant Sci*. **13**, 901541 (2022).

2. Line991-996: What is the comment about f in Figure 1?

Response:

We sincerely apologize for the oversight regarding the mention of panel (f) in the Figure 1 legend. Upon thoroughly reviewing the figure, we confirmed that panel (f) was mistakenly included in the legend. To correct this, we have removed the reference to panel (f) from the Figure 1 legend in the revised manuscript, ensuring that the legend now accurately reflects the content of the figure. We appreciate your careful review and

the opportunity to clarify this issue.

3. In Figure 1c-e, the author needs to add how the *p* value is obtained. For example, students' two-tailed *t* test, etc. Other Figures also have similar problems, please modify them together.

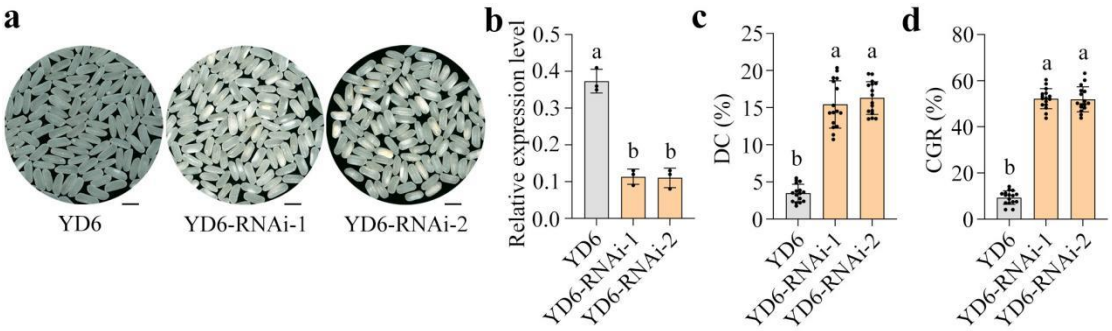
Response:

Thank you for your valuable comment. We appreciate your suggestion to clarify how the *P* values were calculated in Figure 1c–e and other figures. In response, we clarified that two-tailed Student's *t*-test was used to assess statistical significance in Figure 1c–e (please see lines 1174–1176), as well as in the new Supplementary Fig. 14c–f (please see lines 218–219 in the Supplementary Figure file).

4. Line143: Although Yangdao6hao was developed based on the background of 9311, they are not the same thing after all, so it is unreasonable to use 93-11 to represent Yangdao6hao here, and it is suggested to change it to YD6.

Response:

Thank you for highlighting this important distinction. We completely agree with your suggestion and have substituted "93-11" with "YD6" in the relevant sections of the revised manuscript (please refer to lines 144 and 533, and new Supplementary Fig. 4a–d). We appreciate your careful attention to this detail, which has improved the clarity of our work.



**Supplementary Fig. 4 Identification of *Chalk9* RNAi and knockout plants. a** Grain chalkiness in YD6, YD6-RNAi-1, and YD6-RNAi-2 plants. Scale bar: 8 mm. **b** Relative expression levels of *Chalk9* in YD6, YD6-RNAi-1, and YD6-RNAi-2 plants. Data show means ± SD ($n$ = 3 biological replicates). **c**, **d** Degree of chalkiness (**c**) and chalky grain rate (**d**) in YD6, YD6-RNAi-1, and YD6-RNAi-2 plants. Data show means ± SD ($n$ = 16 plants).

5. In Extended Data Fig. 2a, it is suggested to add a note about the longest dotted line.

Response:

We sincerely appreciate your insightful feedback. In response to your suggestion, we have added a note to the relevant figure legend to clarify the meaning of the longest dashed line. Specifically, the note now reads: “The longest dashed line highlights the only non-synonymous SNP, which is not significantly associated with chalkiness.” Please refer to lines 69–70 in the Supplementary Figure file for the updated annotation.

6. The phrase "of expression" in line 130 is clearly redundant and it is recommended to delete it.

Response:

Thank you for your careful review. We agree that the phrase "of expression" is redundant. Accordingly, we have removed it in the revised manuscript to improve clarity and conciseness (please see line 131).

7. Lines 221-222, how did the author find this key amino acid residue site? The text should provide an explanation.

Response:

Thank you for your valuable comment. We appreciate your suggestion to clarify how the key amino acid residue site was identified. In the revised manuscript (please see lines 233–235), we have now added an explanation indicating that the site was

determined through a combination of multiple sequence alignment and structural analysis. Specifically, we compared the RING-finger domain sequences of homologous proteins across diverse plant species to identify conserved and variable regions. Structural modeling was then employed to assess the key site, which was selected based on its location within the functional core of the RING-finger domain. We believe that providing this methodological detail strengthens the transparency and robustness of our analysis.

8. In Figure 5e, there may be a problem with the relative protein level of OsEBP89 in NIP. The values of the three duplicate samples are identical, even without standard deviation. The same problem also appears in Figure 5h. I doubt the authenticity of this data.

Response:

Thank you for your thorough review and valuable comments regarding Fig. 5e and h. We appreciate your attention to the details of our data presentation and statistical analysis. In response to your concern about the relative protein level of OsEBP89 in Nip, we would like to clarify the experimental design and data processing method:

1. Experimental design:

We conducted three independent biological replicates, each including a wild-type Nip sample alongside either *Chalk9* knockout mutants (Fig. 5e) or NIL$^{Chalk9\text{-H}}$ (Fig. 5h). The protein levels of OsEBP89 were detected using Western blot and subsequently analyzed with ImageJ.

2. Data normalization:

In each replicate, the OsEBP89 protein level of Nip was set as the reference (normalized to 1), and the relative protein levels of the corresponding mutant or NIL samples were calculated accordingly. Because this normalization was performed independently for each replicate, the wild-type values appear as “1” across all three replicates. As a result,

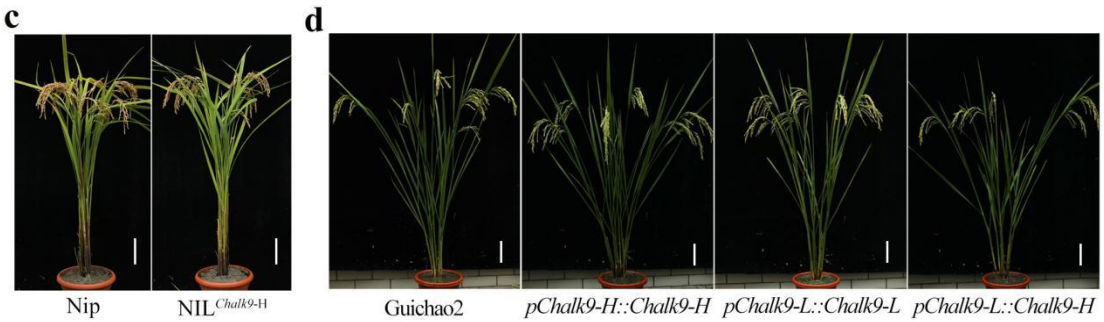
no variation is shown for the Nip samples, and standard deviation is not applicable, as indicated in Fig. 5e and h.

We apologize for any confusion caused by the original presentation and appreciate your scrutiny, which has enabled us to enhance the clarity of our data reporting. To improve transparency, we have revised the legend for Figure 5e and 5h to better elucidate the data analysis process (please see lines 1272–1274 and 1278–1280).

9. Suggest the author to supplement the plant architecture photos or field photos of Guichao 2 and *Chalk9*-L near isogenic lines, so that readers can more intuitively observe the results described by the author.

Response:

We genuinely appreciate your insightful suggestion. In response to your recommendation, we have included photographs of the plant architecture of Guichao2 and its three transgenic lines under field conditions (please see the new Supplementary Fig. 5d). Concerning the *Chalk9* near-isogenic line, we confirm that the original manuscript already contains plant architecture images of this material (please see the Supplementary Fig. 5c).




**Supplementary Fig. 5 c** Representative photographs of Nip and NIL$^{Chalk9\text{-H}}$ plants at the mature stage in the field. Scale bar: 10 cm. **d** Representative photographs of Guichao2, *pChalk9-H::Chalk9-H*, *pChalk9-L::Chalk9-L*, and *pChalk9-L::Chalk9-H* plants at the mature stage in the field. Scale bar: 10 cm.

Reviewer #3 (Remarks to the Author):

The study by Hu et al. is about the molecular mechanism of rice endosperm chalkiness via Chalk9-OsEBP89. Through genome-wide association studies in indica rice germplasm, they identified the gene Chalk9 as the key player and revealed two major haplotypes, Chalk-L and Chalk-H, to contribute to the trait. Molecular studies demonstrated the role of Chalk9 as an E3 ubiquitin ligase and OsEBP89 as its direct target for degradation. OsEBP89, a transcription factor, directly regulates amylose as well as various seed storage proteins' synthesis. Further, they emphasized the evolutionary significance of the elite allele Chalk9-L in japonica and indica rice varieties and suggested the importance of the Chalk9-L allele in breeding applications to improve rice appearance quality without yield penalty. It's an interesting and valuable study, and needs the following clarifications:

Response:

We are truly grateful for your thoughtful comments and insightful feedback. In response to your suggestions, we have made the necessary revisions, with all changes highlighted in blue in the revised manuscript.

1. Line 112 incorrectly mentions 15 genes while Supplementary table 2 lists out 18 genes!

Response:

Thank you for pointing out the discrepancy regarding 15 genes mentioned in line 112 in the original manuscript. We sincerely appreciate your careful review and apologize for any confusion caused by the lack of clarity in our original text. Below, we provide a detailed explanation.

Using a strict threshold ($P < 1 \times 10^{-6}$), we identified 76 SNPs significantly associated with chalkiness. These 76 SNPs were mapped to a total of 18 genes. To evaluate the potential functional impact of these SNPs, we further classified them based on their

genomic locations:

1. Coding regions: 11 missense SNPs and 1 synonymous SNP.

2. Regulatory regions: 20 SNPs located in promoters or untranslated regions (UTRs).

The 32 SNPs (11 missense + 1 synonymous + 20 regulatory) were assigned to 15 genes. The mention of 15 genes in line 112 of the original manuscript specifically refers to the subset associated with the 32 SNPs located in coding or regulatory regions, which are of particular functional interest. In contrast, the 18 genes are associated with all 76 SNPs, regardless of functional classification. We regret that this distinction was not clearly communicated in the original text, leading to confusion.

To address this issue, we have revised the relevant description in the manuscript to better explain the classifications and gene assignments (please see lines 109–113 and new Supplementary Table 4).

2. Suppl fig 2A depicts 6 proteins, though 12 were identified in actuality. A reason for the selection should be mentioned.

Response:

Thank you for your valuable feedback. We apologize for any confusion regarding the protein selection. Below, we provide a systematic explanation of the rationale behind protein selection. Through SNP classification and protein functional analysis, we initially identified 12 candidate genes. Among these, we prioritized proteins with missense mutations, as these mutations (resulting in amino acid substitutions) are more likely to have a functional impact. Out of the 12 proteins, 6 exhibited missense mutations that resulted in notable amino acid changes in the encoded proteins, making them the primary candidates for further analysis (please see new Supplementary Table 6). Therefore, these six proteins were selected for depiction in the current Supplementary Fig. 3a (previously Supplementary Fig. 2a). We have revised the manuscript to reflect this rationale (please see lines 119–121 and new Supplementary

Table 6). We hope this provides clearer context for the selection process.

3. The reference for a missense SNP not conserved across plant species, unlikely affecting protein function should be mentioned at line 122.

Response:

Thank you for your valuable suggestion. We appreciate your insightful comment regarding the need to substantiate the interpretation of the missense SNP not conserved across plant species. In response, we have added the following reference: "Worth, C., Gong, S., & Blundell, T. Structural and functional constraints in the evolution of protein families. *Nat. Rev. Mol. Cell Biol.* **10**, 709–720 (2009)." This study demonstrates that amino acid substitutions at non-conserved sites often do not compromise protein stability or function, thereby supporting our inference that the identified missense SNP is unlikely to affect protein functionality. The reference has been added at lines 124 and 976–977 in the revised manuscript. We believe that this addition strengthens the scientific rigor and credibility of our interpretation.

4. IN FIG 1c, *P* values for all comparisons should be mentioned. Were 8 varieties taken each for high chalk and low chalk data? The samples across which *P* values were generated should be mentioned in legend (low vs high chalk?)

Response:

Thank you for your thorough review and valuable suggestions. We have carefully addressed your comments regarding Figure 1c. In response, we have now added *P* values for all pairwise comparisons between the high-chalky and low-chalky groups in Figure 1c. Each group comprised 8 rice varieties, and the statistical significance was evaluated using Student's *t*-test to determine the differences between groups. To enhance clarity and reproducibility, we have explicitly stated in the figure legend that the *P* values were calculated based on comparisons between the high- and low-chalky

groups. This additional information has been included in the updated figure legend (please see lines 1167–1169). We believe these revisions further strengthen the transparency and scientific rigor of our study.

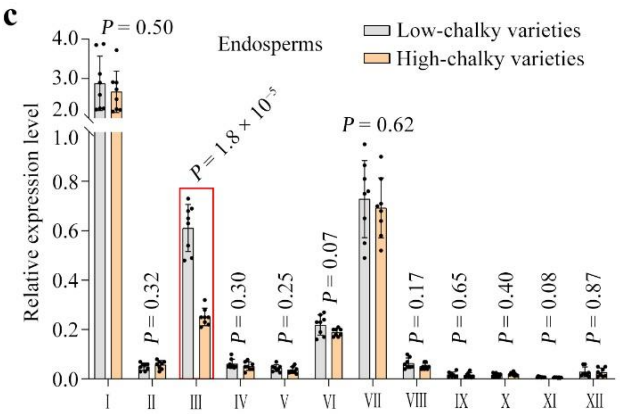



**Fig. 1c** Relative expression levels of the 12 candidate genes in the endosperm of eight high-chalky and eight low-chalky varieties at 20 days after flowering (DAF). The 12 predicted genes in the *Chalk9* locus region are labeled by I to XII. Data show means ± SD ($n$ = 8 varieties). $P$ values were calculated for comparisons between high-chalky and low-chalky groups, with each group comprising 8 varieties.

5. Though the difference in Fig 1D can be visualized, significance should be assigned to the same.

Response:

Thank you for your valuable suggestion. We fully agree that it is important to statistically validate the visual differences observed in Figure 1d. In response, we have performed a statistical analysis using Student's $t$-test to assess the significance of the differences between high-chalky and low-chalky groups. The corresponding $P$ value has been added to Figure 1d to clearly demonstrate the statistical significance. We have also updated the figure legend to include information about the statistical test applied. Please refer to the revised Figure 1d and lines 1170–1172 in the manuscript for these updates.

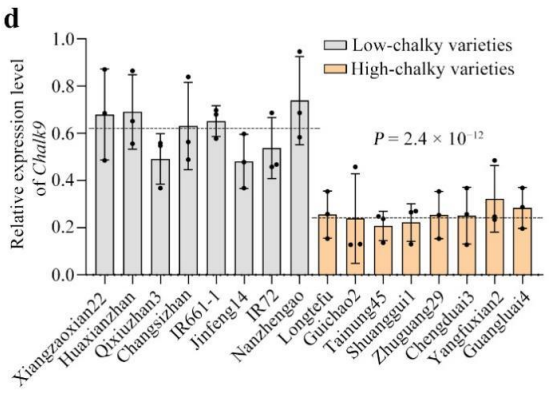



**Fig. 1d** Relative expression level of the candidate gene III (*Chalk9*) in the endosperm from the selected varieties at 20 DAF. The *P* value was calculated for the comparison between high-chalky and low-chalky groups, with each group comprising 8 varieties. Data show means ± SD ($n$ = 3 biological replicates).

6. Figure 1 legend mentions an 'F' part, which is not visible in the figure.

Response:

Thank you for bringing this discrepancy to our attention. We sincerely apologize for this oversight and greatly appreciate your careful and thorough review. After carefully re-examining the figure and its legend, we confirmed that the reference to panel (f) in the legend of Figure 1 was mistakenly retained from an earlier version of the figure layout. In the final version, panel (f) was removed during figure revision, but the legend was not updated accordingly.

We have now corrected this error by removing the reference to panel (f) from the figure legend in the revised manuscript. We have also re-checked all figure legends to ensure consistency with the corresponding figures, in order to prevent similar issues in other parts of the manuscript. Thank you again for your attention to detail, which helped improve the clarity and accuracy of our presentation.

7. Figure 2 A and E should have independent scale bars for each sub-part of the figure. Similarly, for other figures with sub-parts.

Response:

Thank you for your valuable suggestion. We fully agree that providing independent scale bars for each sub-part improves the clarity and precision of data presentation. In response, we have added independent scale bars to all relevant sub-parts, including in Figure 2a, 2e, 3e, 3i, 3n, 6l, Supplementary Figure 4a, 4e, and Supplementary Figure 12c. We believe that these revisions enhance the accuracy and visual consistency of the figures, thereby improving the overall quality of the manuscript.

8. The word should be RNAi in line 142 not RANi.

Response:

Thank you for highlighting the typographical error. We have corrected “RANi” to “RNAi” in the revised manuscript (please refer to line 143).

9. The source of expression data for Suppl fig 2C should be mentioned.

Response:

Thank you for your valuable suggestion. In response, we have clarified that the expression data presented in Supplementary Figure 2c were obtained from the RiceXpro database (http://ricexpro.dna.affrc.go.jp/GGEP/index.html). We have updated the figure legend accordingly to specify the source of the data. Please refer to lines 35–38 in the revised "Supplementary Figure" file.

10. Nip Chalk-9 mutants should be explained in more details in results.

Response:

We appreciate the constructive and insightful comments. In response, we have added a more detailed description of the *Chalk9* mutants in the revised manuscript (please see lines 146–150). Specifically, we state that: “Two mutant alleles, *chalk9-1* and *chalk9-*

*2*, were generated, each with a 1 bp deletion and a 1 bp insertion in the exon of *Chalk9*, resulting in premature stop codons at the 115th and 92nd codons, respectively (Supplementary Fig. 4i–k). These knockout lines exhibited increased chalkiness, with higher CGR and DC values compared to Nip (Fig. 2e–g)." We believe that this addition improves the clarity and completeness of the Results section.

11. How many varieties were used to re-sequence Chalk9? Did these varieties have any differences in grain chalkiness? Are these from the association panel?

Response:

We greatly appreciate your thoughtful questions regarding the re-sequencing of *Chalk9*. In our study, *Chalk9* was re-sequenced in a total of 149 rice varieties, all of which were selected from our association panel. These varieties were specifically chosen to represent a broad range of grain chalkiness phenotypes, encompassing both high and low chalkiness levels. This phenotypic diversity provided an important foundation for exploring the natural genetic variation within the *Chalk9* locus.

The grain chalkiness phenotypic data for these varieties are provided in Supplementary Table 7, alongside the corresponding re-sequencing information. By integrating phenotypic and genotypic data, we were able to assess potential associations between *Chalk9* haplotypes and variation in grain chalkiness. These clarifications have been added to the revised manuscript (please refer to lines 156–157).

12. Why was Guichao2 used to generate promoter transgenic plants and not Nipponbare or ZH11? What are the differences in their native promoter sequences?

Response:

Thank you for your insightful questions regarding our choice of Guichao2 for generating promoter transgenic plants. Since *Chalk9* acts as a negative regulator of chalkiness (Fig. 2), we expect that transgene-induced upregulation will reduce

chalkiness. Nipponbare and ZH11 possess the *Chalk9*-L haplotype, which is linked to low grain chalkiness and higher *Chalk9* expression. In these low-chalky varieties, the added expression from the transgene may not result in a noticeable phenotypic change. In contrast, Guichao2 carries the *Chalk9*-H haplotype, which is associated with high chalkiness and lower *Chalk9* expression. This background is more suitable for detecting transgene effects, as the resulting reduction in chalkiness is more pronounced. Therefore, we selected Guichao2 as the transgenic background to generate promoter transgenic plants. We have included this information in the revised manuscript (please see lines 182–184).

In addition, based on the haplotype analysis, Nipponbare and ZH11 harbor the *Chalk9*-L haplotype, while Guichao2 harbors the *Chalk9*-H haplotype. Thus, the differences in native promoter sequences between Nipponbare/ZH11 and Guichao2 primarily correspond to the distinction between the *Chalk9*-L and *Chalk9*-H haplotypes.

13. Promoter activity was examined in rice protoplasts. How were the protoplasts isolated and transformed (no mention in methods)? Since the gene is expressed in endosperm and not leaf, how were the promoters active in protoplasts?

Response:

Thank you very much for raising these important points. We sincerely appreciate your thoughtful and constructive questions. In response, we have now added a detailed description of the procedures used for rice protoplast isolation and transformation in the revised Methods section (please see lines 641–659). These additions provide clarity on the technical steps involved in evaluating promoter activity using this system.

Although *Chalk9* is predominantly expressed in rice endosperm, our RT-PCR results (please see Fig. 4b) and the newly added Supplementary Fig. 7a indicate that it is also expressed at low but detectable levels in leaf tissues. This suggests that the *Chalk9* promoter retains basal transcriptional activity in leaf cells, which enables us to monitor its activity using leaf protoplasts.

We fully acknowledge the importance of tissue specificity in gene expression studies. However, due to the inherent biological characteristics of rice endosperm development, isolating viable protoplasts from endosperm tissue is technically challenging. The coenocytic phase lasts only about two days, followed by a brief three-day cellularization phase. Shortly thereafter, cells undergo rapid programmed cell death between 12 and 20 days after fertilization (DAF). By the time the endosperm matures, the starchy endosperm cells are largely non-viable and function primarily in storage, making them unsuitable for transient expression assays. Therefore, as a practical and widely accepted alternative, we employed rice leaf protoplasts to evaluate promoter activity. While this system may not fully replicate the cellular environment of the endosperm, it provides a useful and reproducible platform for functional analysis of promoter constructs. This approach is commonly used in rice molecular biology research when tissue-specific protoplasts are difficult to obtain.

We hope this explanation addresses your concerns and clarifies the rationale behind our experimental design.

14. What is the source of data in Suppl fig 4A?

Response:

Thank you very much for your suggestion. The expression data were obtained from RiceXPro (http://ricexpro.dna.affrc.go.jp/GGEP/index.html). We have revised the figure legend in the updated Supplementary Fig. 6a (please refer to lines 88–89 in the "Supplementary Figure" file).

15. A statistical analysis of Fig 4B (RTPCR) should be done. What is the control used?

Response:

We sincerely appreciate your valuable suggestion. In response, we have now performed statistical analysis of the RT-PCR data presented in Fig. 4b. Specifically, we quantified

gene expression based on three biological replicates and applied multiple comparison analysis (using one-way ANOVA followed by Tukey's post-hoc test) to assess statistical significance among different groups. The significance levels have been indicated in the updated figure using standard annotations, as shown in the revised version of Fig. 4b.

Furthermore, *OsActin* was employed as the internal reference gene to normalize expression levels, due to its stable expression profile in rice under a wide range of conditions. We have updated the figure legend accordingly in the revised manuscript to include both the statistical method and the internal control gene used (please see lines 1232–1234). We believe these improvements enhance the robustness and interpretability of the data.

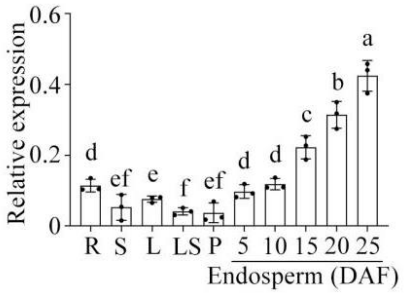



**Fig. 4b** Quantitative PCR with reverse transcription (qRT–PCR)-based transcript abundance analysis of *Chalk9* in various tissues. R, root; S, stem; L, leaf; LS, leaf sheath; P, panicle; DAF, days after flowering. Data show means ± SD ($n$ = 3 biological replicates). *OsActin* was used as a control. Different letters indicate significant differences ($P < 0.05$, one-way ANOVA with Tukey's multiple comparison test); for $P$ values, see Source Data.

16. It will be good to mention the expected sizes of proteins in sub-parts of Fig 4.

Response:

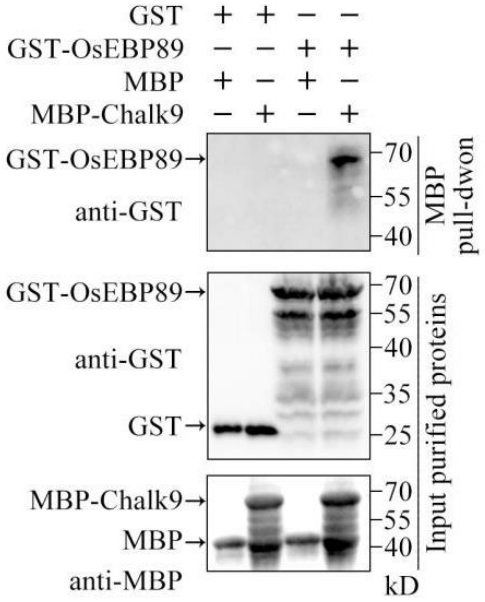
We sincerely appreciate your valuable suggestion. In response to your comment, we have added the expected molecular weights of all target proteins in the corresponding sub-parts of the Figure 4 legend to improve clarity and help readers better interpret the immunoblotting results. Specifically, these molecular weights are now indicated in the revised figure legend at lines 1236–1237, 1242–1244, and 1247–1249 of the manuscript.

We believe this addition enhances the informational value of the figure and facilitates a clearer understanding of the protein bands observed in each panel.

17. The pull down assay for CHALK9 EBP89 dimer should be performed both ways (GST and MBP pull down). The input lane can have only one protein, not two for confirmation of detection.

Response:

Thank you very much for your insightful comments and suggestions. In response to your recommendation, we have performed an additional MBP pull-down assay to complement the original GST pull-down experiment and confirm the interaction between Chalk9 and OsEBP89 from both directions (please see the new Supplementary Fig. 9c).




**Supplementary Fig. 9c** Pull-down assay. MBP-Chalk9 was used as bait, and the pull-down GST-OsEBP89 was detected by the anti-GST antibody. The expected molecular weights of the target proteins are as follows: MBP-Chalk9 (64 kD), GST-OsEBP89 (62 kD), MBP (42 kD), and GST (26 kD).

To address your concerns regarding the validation of input detection in our pull-down assays, we present a detailed description of the experimental procedures. The coding regions of *OsEBP89* and *Chalk9* were amplified and cloned into the pGEX-5X-1 and

pMAL-c5X vectors, respectively. The resulting constructs, along with the empty vectors, were individually transformed into *E. coli* BL21 for protein expression induced by IPTG. The expressed proteins (GST, GST-OsEBP89, MBP, and MBP-Chalk9) were then purified using affinity chromatography. Before conducting the pull-down assays, we validated the presence of the target proteins in the input samples using specific antibodies: anti-MBP for MBP and MBP-Chalk9 and anti-GST for GST and GST-OsEBP89, ensuring that the input samples contained the intended proteins. Consequently, in the input validation experiments, both GST- and MBP-tagged proteins were detected for confirmation.

For the GST pull-down assay, the target proteins were incubated with glutathione-sepharose resins for 3 hours at 4°C, followed by five washes with wash buffer to eliminate non-specific interactions. The bound proteins were eluted by boiling in a loading buffer for 5 minutes and analyzed by SDS-PAGE, with detection using an anti-MBP antibody. Similarly, for the MBP pull-down assay, the target proteins were incubated with amylose resins, washed, eluted, and analyzed by SDS-PAGE, followed by detection using an anti-GST antibody. This reciprocal pull-down strategy provides stronger evidence for the specific interaction between Chalk9 and OsEBP89.

All corresponding experimental details and methodological updates have been added to the revised "Methods" section (please refer to lines 724–736). We thank you again for your valuable suggestions, which have helped us strengthen the rigor and completeness of our protein interaction analysis.

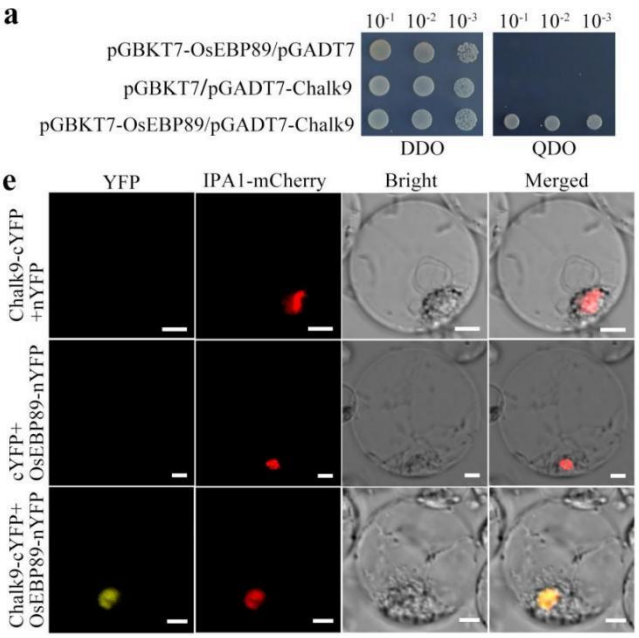
18. BiFC should be performed with vector swap (Chalk-cYFP + EBP-nYFP). Similarly, yeast two hybrid assay.

Response:

Thank you very much for your valuable comments and suggestions. In response to your recommendation, we have performed vector swap experiments for both the yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays, as

suggested. Specifically, we swapped the vectors to test the interaction in both orientations: Chalk9-cYFP with OsEBP89-nYFP for BiFC, and pGBKT7-OsEBP89 with pGADT7-Chalk9 for Y2H.

The updated results, including the additional data from these vector swap experiments, have been incorporated into the revised manuscript (please refer to the new Supplementary Fig. 9a and 9e). These experiments help validate the robustness of the interaction between Chalk9 and OsEBP89 by demonstrating that the interaction is independent of the orientation of the tagged proteins.




**Supplementary Fig. 9 a** The interaction between OsEBP89 and Chalk9 in the yeast two-hybrid assay. Strains carrying two indicated constructs were grown on a synthetic dropout medium. **e** The interaction between Chalk9 and OsEBP89 is demonstrated by bimolecular fluorescence complementation (BiFC) assays in rice protoplasts. The N-terminal fragment of YFP (nYFP) fused with OsEBP89 and the C-terminal fragment of YFP (cYFP) fused with Chalk9 were co-expressed in rice protoplasts. IPA1-mCherry was used as a nuclear control.

19. The presence of protein bands for both Chalk9 and EBP89 in CoIP input is not correct.

Response:

Thank you for your comment regarding the protein bands for Chalk9 and OsEBP89 in the Co-IP input. We appreciate your attention to detail and would like to clarify the experimental design and results to address your concern.

We performed three sets of protoplast transformation experiments: (1) transfection with OsEBP89-GFP alone, (2) transfection with Chalk9-HA alone, and (3) co-transfection with OsEBP89-GFP and Chalk9-HA. The total protein extracts from the transformed protoplasts were immunoprecipitated using anti-HA beads at 4 °C for 2 hours. The eluted proteins and Co-IP input samples were then analyzed by immunoblotting with anti-HA (1:3000, ab9110, Abcam) and anti-GFP (1:3000, ab290, Abcam) antibodies. The results are as follows:

(1) For the OsEBP89-GFP alone transfection: In the input, only the OsEBP89-GFP band should be detected, while no Chalk9-HA band should be observed, because only OsEBP89-GFP was expressed in the transformed protoplasts. In the HA-IP sample, OsEBP89-GFP nor Chalk9-HA bands should be detected, confirming that OsEBP89-GFP does not non-specifically bind to anti-HA beads.

(2) For the Chalk9-HA alone transfection: In the input, only the Chalk9-HA band should be detected, and no OsEBP89-GFP band should be observed, because only Chalk9-HA was expressed in the transformed protoplasts. In the HA-IP sample, only the Chalk9-HA band was detected, and no OsEBP89-GFP band was observed, demonstrating the specificity of the anti-HA beads.

These two sets of experiments served as negative controls, ensuring the specificity of the Co-IP assay.

(3) For the co-transfection of OsEBP89-GFP and Chalk9-HA: In the input, both Chalk9-HA and OsEBP89-GFP bands were detected, because OsEBP89-GFP and Chalk9-HA were expressed in the transformed protoplasts. In the HA-IP sample, both Chalk9-HA and OsEBP89-GFP bands were detected, indicating the physical interaction between the two proteins.

Our Co-IP results (Fig. 4f) are consistent with the above description. We have included

this detailed methodology of the Co-IP assay in the “Methods” section of the revised manuscript (please see lines 704–715). We hope this clarification resolves your concern.

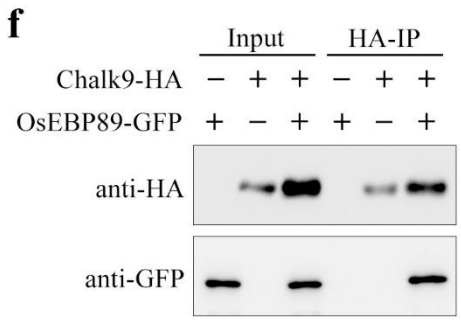


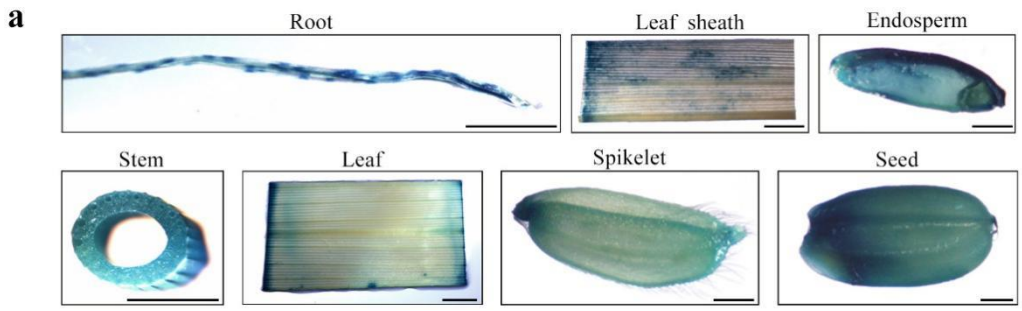

**Fig. 4f** Co-immunoprecipitation (Co-IP) assay of rice protoplasts co-expressing Chalk9-HA and OsEBP89-GFP. Total protein extracts were incubated with magnetic agarose beads conjugated to HA-tag antibody. The immunoprecipitants were probed with antibodies against HA and GFP. IP, immunoprecipitation.

20. Better photographs of *Chalk9* promoter activity would be appreciated (Suppl fig 5A).

Response:

Thank you very much for your helpful suggestion. We fully agree that clear and high-quality images are essential for accurately interpreting promoter activity. In response to your comment, we have replaced the original GUS staining images with higher-resolution photographs that provide better visualization of staining patterns. The updated images have been included in the revised manuscript as new Supplementary Fig. 7a (previously Supplementary Fig. 5a).

We hope that these improved photographs more clearly demonstrate the spatial expression driven by the *Chalk9* promoter and enhance the overall clarity and presentation of the data.



**Supplementary Fig. 7a** *Chalk9* promoter activity was monitored using *Chalk9pro::GUS* transgenic plants. Histochemical GUS staining was performed on the root, stem, leaf, leaf sheath, spikelet, endosperm, and seed. Scale bar: 1 mm.

21. Why was the interaction of EBP89 with Chalk9 tested?

Response:

Thank you for your question. In our study, *Chalk9* was identified as a key regulatory gene controlling grain chalkiness in rice through GWAS analysis. *Chalk9* is predicted to encode an E3 ubiquitin ligase. To explore its molecular function in regulating chalkiness, we performed yeast two-hybrid assays to screen for potential interacting proteins. Through this screening, we identified OsEBP89, a transcription factor previously reported to be involved in amylose biosynthesis (Zhu et al., 2003), as an interactor of Chalk9.

Given that alterations in amylose content have been linked to chalkiness formation in rice grains (Cao et al., 2022), we hypothesized that OsEBP89 could be a degradation substrate of Chalk9, and that Chalk9 may regulate amylose synthesis through modulating OsEBP89 stability. Investigating the interaction between Chalk9 and OsEBP89 was therefore a critical step toward elucidating the molecular mechanism underlying Chalk9-mediated regulation of chalkiness.

Reference:

Cao, R. et al. *OPAQUE3*, encoding a transmembrane bZIP transcription factor, regulates endosperm storage protein and starch biosynthesis in rice. *Plant Comm.* **3**, 100463 (2022).

Zhu, Y., Cai, X. L., Wang, Z. Y. & Hong, M. M. An interaction between a MYC protein and an EREBP protein is involved in transcriptional regulation of the rice *Wx* gene. *J. Biol. Chem*. **278**, 47803–47811 (2003).

22. Sizes of all proteins should be mentioned at requisite places.

Response:

Thank you for your valuable feedback. We completely agree that explicitly reporting protein molecular weights is crucial. In response to your suggestion, we have systematically included protein size information in the relevant figure legends for Figures 4 and 5, as well as Supplementary Figure 9. Please refer to lines 1236–1237, 1242–1244, 1247–1249, 1260–1261, 1265–1267, and 1285–1286 in the revised main manuscript, along with lines 137–139 and 148–149 in the updated "Supplementary Figure" file.

23. The control should also be quantified for Fig 5B, D by ImageJ.

Response:

Thank you for your valuable suggestion regarding the quantification of the control signals. In response, we have performed densitometric analysis of the Actin control bands in Fig. 5b and 5d using ImageJ software, following standard procedures. The quantified values have now been included in the updated versions of these panels in the revised manuscript. This added analysis enhances the rigor of our data presentation. We appreciate your suggestion, which has helped us further strengthen the quality of our results.

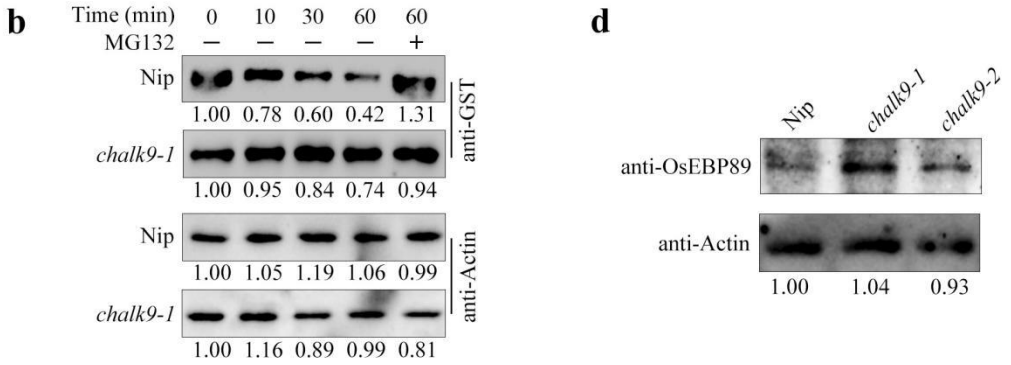



**Fig. 5 b** Cell-free degradation of GST-OsEBP89 in the protein extracts from Nip and *chalk9-1* seedlings. Protein levels of GST-OsEBP89 were detected using anti-GST antibody, and Actin was used as a loading control for total protein extraction. Relative fold changes of GST-OsEBP89 to Actin were quantified by ImageJ and marked on the bottom of the lanes. The protein level at time point 0 min was marked as 1. The expected molecular weights of the target proteins are as follows: GST-OsEBP89 (62 kD) and

Actin (42 kD). **d** Detection of OsEBP89 protein abundance in Nip and *chalk9-1* plants. Total proteins were extracted from seeds at 18 DAF. OsEBP89 protein abundance was determined by immunoblotting.

24. Chalky endosperms have lesser amylose content in general. An opposite result here (Fig 6B,C) should be well supported and explained.

Response:

Thank you for your insightful comment. We appreciate your concern and have provided a detailed explanation to address this point. Our findings can be supported and explained as follows:

1. Recent research by Li et al. (2023) has demonstrated that different *Wx* alleles (e.g., $Wx^a$ and $Wx^b$) significantly influence amylose content (AC) and chalkiness formation. Specifically, $Wx^a$ is associated with high AC (>24%) and increased chalkiness, whereas $Wx^b$ corresponds to moderate AC (~16%) and reduces chalkiness. Using CRISPR/Cas9 to edit the $Wx^a$ promoter region, downregulating *Wx* expression was found to reduce AC and effectively alleviate chalkiness formation.

2. A recent study by Tu et al. (2025) proposed a model in which *LCG1* expression during the grain-filling phase plays a key role in regulating amylose content and chalkiness. In *indica* rice varieties, the expression of *LCG1* in the endosperm is relatively low, leading to weaker inhibition of the transcriptional activity of the OsBP5/OsEBP89 complex. This results in higher *Wx* expression, increased amylose content, and greater chalkiness. In contrast, in *japonica* rice varieties, *LCG1* is more highly expressed, effectively inhibiting the OsBP5/OsEBP89 complex. This results in reduced *Wx* expression, lower amylose content, and preventing chalkiness formation.

Our observation of increased amylose content in chalky endosperms (Figure 6b, c) is consistent with these recent findings. We have incorporated this explanation into the "Discussion" section (please see lines 444–455 in the revised manuscript). Thank you again for your insightful comment.

Reference:

Li, J. et al. Fine mapping of the grain chalkiness quantitative trait locus *qCGP6* reveals the involvement of *Wx* in grain chalkiness formation. *J. Exp. Bot*. **74**, 3544–3559 (2023).

Tu, B. et al. The LCG1-OsBP5/OsEBP89-*Wx* module regulates the grain chalkiness and taste quality in rice. *Plant Biotechnol. J*. **23**, 36–50 (2025).

25. The correlation coefficient is beyond 0.95 for chalk9 mutants and Nip, which is quite high for them to be compared. This data needs to be supported with other published literature. (Supp fig 7b).

Response:

Thank you very much for your comment. We recognize that a correlation coefficient greater than 0.95 may initially appear quite high. However, similar patterns have been observed in rice grain transcriptome studies: (1) Zha et al. (2019) reported a correlation of 0.96 between chalky and translucent endosperm regions, despite identifying 986 upregulated and 916 downregulated genes. (2) Xu et al. (2021) noted that there were 282 downregulated and 242 upregulated genes in wild type and chalky mutants based on RNA sequencing.

Additionally, the *Chalk9* mutants were developed in the Nip genetic background. While the disruption of *Chalk9* significantly alters grain chalkiness, many other key agronomic traits remain largely unchanged, indicating that numerous conserved genetic and biochemical networks in rice are unaffected. This explains the high overall correlation between Nip and *Chalk9* knockout mutant. Furthermore, the mutation targets a single gene (*Chalk9*), and such knockouts typically do not drastically impact the expression of most genes in the genome. Instead, *Chalk9* may primarily influence downstream genes within specific pathways. Thank you again for your thorough evaluation.

Reference:

Xu, X. et al. *OsYUC11*-mediated auxin biosynthesis is essential for endosperm

development of rice. *Plant Physiol***. 3**, 934–950 (2021).

Zha, K. Expression of Maize MADS Transcription Factor *ZmES22* Negatively Modulates Starch Accumulation in Rice Endosperm. *Int. J. Mol. Sci*. **20**, 483 (2019).

26. Material and methods need to be elaborated, since multiple essential experiments and methods of calculations have been skipped.

Response:

Thank you very much for your valuable feedback regarding the "Methods" section. We fully agree that clear and comprehensive methodological descriptions are essential for the reproducibility and transparency of scientific research. In response to your comments, we have thoroughly revised and significantly expanded this section to include detailed information on all major experimental procedures, including those previously omitted.

Specifically, we have added detailed descriptions of the experimental protocols, including the calculation of phenotypic variance explained, RNA-seq analysis, protoplast isolation and transformation, yeast one-hybrid assay, immunoblotting, protein–protein interaction assays (yeast two-hybrid, co-immunoprecipitation, and pull-down assays), as well as population genetic and evolutionary analyses. All additions and revisions have been clearly marked in blue font in the revised manuscript. For your convenience, the relevant updates can be found in the following sections: lines 562–565, 615–622, 641–659, 673–686, 704–715, 719–736, 779–790, 820–827, 842–843, 848–852, and 859–871.

We sincerely appreciate your suggestion, which has helped improve the clarity and completeness of our methodological documentation.

27. Grammatical errors here and there need to be rectified.

Response:

Thank you very much for your careful reading and valuable feedback regarding the grammatical issues in our manuscript. We sincerely appreciate your attention to language accuracy, which is essential for ensuring clarity and effective communication of our findings.

In response, we thoroughly proofread the entire manuscript and corrected all identified grammatical and typographical errors. All grammar-related revisions have been clearly marked in blue in the revised manuscript.

We believe these improvements have enhanced the overall readability and presentation of our work. Thank you once again for your constructive suggestions.

28. Use of abbreviations are not consistent across the manuscript. For example, even though chalky grain rate is abbreviated as CGR in the manuscript, its mentioned in full length throughout the figures.

Response:

Thank you very much for your valuable feedback regarding the inconsistent use of abbreviations in our manuscript. We fully agree that maintaining consistency in abbreviation usage is essential for clarity and professional presentation. In response to your comment, we have carefully reviewed and revised the manuscript to ensure that the abbreviations for “chalky grain rate” (CGR) and “degree of chalkiness” (DC) are used consistently across all figures, figure legends, and relevant sections of the main text.

Specifically, these revisions have been applied to Fig. 2c, d, f, g; Fig. 3b, c, f, g, j, k, o, p; Fig. 6m, n; Supplementary Fig. 1a–d; Supplementary Fig. 2; Supplementary Fig. 4c, d, g, h; Supplementary Fig. 12d, e; and Supplementary Fig. 14c–f. These changes help ensure a clearer and more uniform presentation of data throughout the manuscript.

We sincerely appreciate your careful observation and helpful suggestion.

29. Extended Data Fig. 1 does not provide data for the statement at lines 99-100. “This locus explained ~28% of the total phenotypic variation”.

Response:

Thank you for your thoughtful evaluation of our manuscript and for pointing out the discrepancy between the statement in lines 99–100 and the corresponding figure. We appreciate your attention to the accuracy of data reporting.

The ~28% phenotypic variance explained (PVE) by the locus was calculated using the standard formula implemented in GEMMA, which takes into account the effect size (β), standard error (SE), minor allele frequency (MAF), and sample size (N) obtained from our GWAS results, as described in Teslovich et al. (2010). We apologize for not explicitly stating the basis for this calculation in the original version of the manuscript.

To clarify this, we have now revised the “Methods” section to include a detailed explanation of the PVE calculation procedure (see lines 562–565). In addition, we have included the corresponding PVE data in Supplementary Table 2, ensuring transparency and traceability of the reported value.

We thank you again for highlighting this issue, which has allowed us to improve the clarity and completeness of our manuscript.

Reference:

Teslovich, T. et al. Biological, clinical and population relevance of 95 loci for blood lipids. *Nature* **466**, 707-713 (2010).

30. For lines 119-121 “we first evaluated SNPs……. domain (Supplementary Fig. 2a)”, data provided in Supplementary Fig. 2a is incomplete.

Response:

Thank you for bringing this concern to our attention. Your suggestion aligns with the earlier comment (Comment #2: Suppl Fig 2A shows 6 proteins, whereas 12 were

identified in reality. The reasoning behind the selection should be mentioned.). We apologize for any confusion resulting from the lack of clarity in the original text. Allow us to clarify our rationale for protein selection through the following explanation. We first assessed the potential functional impact of missense SNPs among 12 candidate genes and found that missense mutations occurred in only 6 of the 12 genes. These 6 proteins were selected for depiction because they represent the subset of proteins directly impacted by missense mutations. To eliminate any ambiguity, we have revised the text accordingly (please see lines 119–121).

31. From line 991-997 there is a legend starting as “Expression analysis of the candidate genes from GWAS in various tissues…”, for which the image is not provided in the Fig. 1 but in Supplementary Fig. 2.

Response:

Thank you for your careful attention and for pointing out this inconsistency. As you correctly noted, the legend segment beginning with “Expression analysis of the candidate genes from GWAS in various tissues…” (original lines 991–997) does not correspond to any panel in Figure 1 but instead refers to data presented in Supplementary Fig. 2 in the original manuscript.

This issue is related to a previous comment (Comment #6), which highlighted the mention of a non-existent panel “f” in Figure 1. We sincerely apologize for this oversight and confirm that the redundant legend segment was mistakenly retained and is not relevant to the main figure.

To address this, we have now removed the redundant portion from the Figure 1 legend to ensure consistency between the text and the corresponding data. We appreciate your attention to this detail, which helped us improve the clarity and accuracy of the manuscript.

32. Manuscript states association analysis with the identified promoter variants in 149 indica varieties, while the data provided in Fig. 3A-"b,c, The distribution of chalky grain rate (b) and degree of chalkiness (c) in haplotype H (n = 45 accessions) and haplotype L (n = 102 accessions)" totally constitutes only 147 accessions. Also, haplotypes H and L have a wide range of values for CGR and DC from all the accessions while Chalk9 expression has been studied only in selected 24 accessions from both the haplotypes. What was the basis for the selection of these accessions?

Response:

Thank you for your meticulous attention to data consistency. We sincerely apologize for the oversight in the original manuscript. Upon re-examining the raw data in Fig. 3b and 3c, we found that haplotype L includes 104 accessions, and haplotype H includes 45 accessions (H:45 + L:104 = 149), which aligns with the manuscript's statement of "149 *indica* varieties." We have corrected the error in the legend of Fig. 3 (please see line 1199).

Regarding *Chalk9* expression analysis, RNA was extracted from endosperm samples at 15 DAF to measure *Chalk9* mRNA levels. Ideally, it would have been optimal to include all accessions in the expression analysis. However, due to the short duration of midday flowering, it was challenging to precisely label the flowering spikelets of all accessions at the anthesis stage. Furthermore, these accessions exhibited a wide range of heading dates, which posed additional challenges for synchronized sampling under field conditions. To minimize environmental effects, we randomly selected accessions with similar heading dates within each haplotype. Although these accessions displayed a broad spectrum of grain chalkiness, we did not deliberately select accessions based on chalkiness phenotypes. Instead, the selection of accessions was based solely on practical considerations related to field operability. Accordingly, 24 accessions from each haplotype were randomly selected for *Chalk9* expression analysis. We have added this clarification to the revised manuscript (please see lines 168–170).

33. Supplementary Fig. 5a GUS image for P(Panicle) shows a spikelet instead of a panicle as mentioned.

Response:

Thank you for your careful observation and for bringing this to our attention. We sincerely apologize for the mislabeling in the original version of the manuscript. You are absolutely correct that the image labeled as “P (Panicle)” in Supplementary Fig. 5a actually shows a spikelet rather than a full panicle.

To rectify this issue and avoid any confusion for readers, we have revised the figure and corrected the label to “Spikelet” in the updated version of the supplementary figure (please see new Supplementary Fig. 7a). We appreciate your attention to detail, which has helped us improve the accuracy and clarity of our data presentation.

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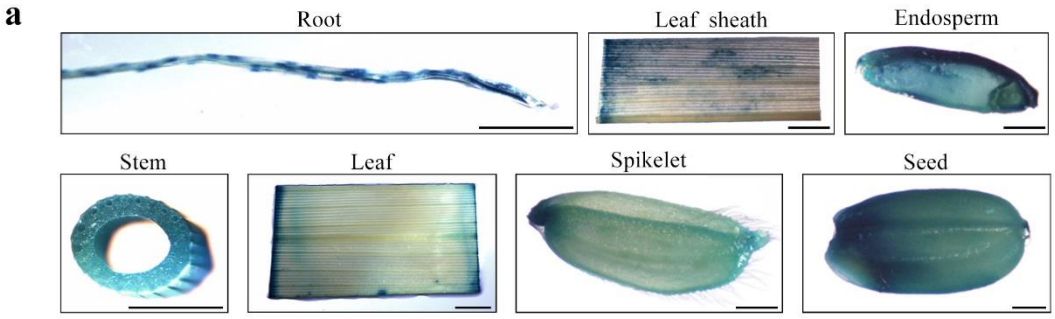


**Supplementary Fig. 7a** *Chalk9* promoter activity was monitored using *Chalk9pro::GUS* transgenic plants. Histochemical GUS staining was performed on the root, stem, leaf, leaf sheath, spikelet, endosperm, and seed. Scale bar: 1 mm.

34. The proteasome inhibitor MG132 usually used at working concentration 10-50uM. The reason for it being used at a higher concentration of 50mM can be mentioned.

Response:

Thank you for your careful attention to the experimental details regarding the use of the proteasome inhibitor MG132. We sincerely apologize for the typographical error in the original manuscript. The reported concentration of 50 mM was incorrect and should

have been 50 μM, which falls within the commonly used and effective range (10–50 μM) for proteasome inhibition in plant systems.

We have corrected this mistake in the "Methods" section of the revised manuscript (please see line 767) to accurately reflect the concentration used in our experiments. We are grateful for your thorough review, which helped us improve the accuracy and reliability of our methodological reporting.

35. The pAbAi vector, used in the Y1H assay (Extended Data Fig. 3m), has a selection for uracil and can be used in the dropout medium to ensure stringency in selecting positive interactions.

Response:

Thank you for your insightful comment regarding the yeast one-hybrid (Y1H) assay. We apologize for not providing sufficient detail about the experimental procedure in the original manuscript. Below is a detailed explanation of the procedure:

1. The pBait-AbAi vectors (containing *GluB1a*, *GluB2*, *GluB4*, *PROLM20*, *PROLM22*, or *PROLM23*) were first transformed into the Y1HGold yeast strain. The transformants were then plated on SD/-Ura plates to select for positive colonies.

2. Next, the optimal AbA concentration for each bait strain was determined. The bait strains were plated on SD/-Ura medium supplemented with varying concentrations of AbA (100, 200, 300, or 500 ng/mL). Growth was monitored to identify the minimal inhibitory concentration of AbA.

3. Successful pBait-AbAi transformants were streaked onto SD/-Ura plates for further selection and used for competent cell preparation. The prey construct pGADT7-OsEBP89 was then introduced into these competent pBait-AbAi yeast cells via transformation.

4. After pre-selection on SD/-Ura medium, positive protein-DNA interactions were

verified by plating the transformants on SD/-Leu agar plates supplemented with AbA at the optimal inhibitory concentration.

This methodology adheres to the standardized procedures outlined in the Yeast One-Hybrid System User Manual (Clontech Laboratories). We regret not clearly describing the role of uracil selection in the dropout medium in the original manuscript and have now included this detailed procedure in the revised manuscript (please see lines 779–790).

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response:

We sincerely appreciate the reviewer's time and effort in co-reviewing our manuscript as part of the *Nature Communications* initiative. We are grateful for your valuable feedback, thoughtful comments, and constructive suggestions. We have carefully considered all the points raised and made the revisions accordingly. All modifications are highlighted in blue in the revised manuscript.

## Point-by-point response

Reviewer #1 (Remarks to the Author):

You have provided detailed responses and revisions to the review comments, adequately addressing the modifications I suggested. I have no further review comments and recommend acceptance for publication.

Response:

We sincerely appreciate your time and constructive feedback on our manuscript. We are grateful for your acknowledgment of our revisions and your recommendation for acceptance. Your insightful comments have significantly improved the quality of our work. Thank you again for your valuable contributions.

Reviewer #2 (Remarks to the Author):

The authors answered my questions, please accept this manuscript to publish on Nature Communications.

Response:

We sincerely appreciate your time and positive feedback. Thank you for your valuable comments and for supporting our manuscript's publication in Nature Communications.

Reviewer #3 (Remarks to the Author):

The authors have taken outmost care to address all the concerns and have rectified all the points mentioned. The relation between Chalk9-EBP89 in regulating rice grain chalkiness, explored in this article is a considerably important data in understanding this trait.

Response:

We sincerely appreciate your thoughtful evaluation and kind acknowledgment of our efforts in addressing all the raised concerns. We are particularly grateful for your

recognition of the significance of our findings regarding the Chalk9-EBP89 regulatory mechanism in rice grain chalkiness. Your insightful feedback has greatly strengthened our manuscript. Thank you for your time and valuable contribution to this work.