nature portfolio

Peer Review File

TaMPK3 Mediates Synergistic Damage from *Fusarium* Crown Rot and Drought in Wheat

Corresponding Author: Professor Zhao-Shi Xu

Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

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)
Fusarium crown rot (FCR) is emerging as a major disease prevalent in arid and semi-arid regions in wheat production. Thus, it is high valuable to identify genes related to FCR resistance as well the underlying molecular mechanism. In this study, Zheng et al. found that TaMPK3 regulates the expression of downstream sterol synthesis genes by phosphorylating TaWRKY26 to influence FCR resistance and drought tolerance. After review of the manuscript, I have some comments as below:
Major points:
1. Using only the reverse genetic approach to regard TaMPK3 as a targeting gene for FCR is not solid, even though it exhibited the most significant up-regulation in response to FCR, because there are other genes also showed significant up-regulation, how to exclude them?
2. For transcriptome analysis, the cultivars and why use those cultivars as plant materials were not described in the result (L119-L147). Line545-563 give some description of the plant materials: PBZ300 were used for Fusarium crown rot (FCR) inoculated, but Fielder was used for drought treatment. Way not use the same plant materials for transcriptome analysis?
3. It is weird that the mRNA levels were significantly reduced in the gene edited lines (L169). Gene editing in the mutants caused the loss-of-function of the TaMPK3 genes, how can it cause the reduction of gene expression? Is the data reliable?
4. Too many figures in the main text, and English is not good enough.
Minors:
1. Some figures are too small to see clearly, I suggest to enlarge the figures.
2. Line130-147: Only 762 DEGs that were down-regulated under drought treatment and up-regulated under Fpg infection were selected for enrichment analyses. Why not select all DEGs for further analysis?
3. The details of the GO and KEGG enrichment analyses should be described in the Methods section.
4. Line232-234: "… whereas no mycelial development was observed on TaMPK3-OE plants (Figure 4b)". Less mycelial was observed on TaMPK3-OE plants (Figure 4b).
5. Line264-265: "…TaMPK3 regulation were in adjacent regions in chromosomes 5A and 5D.", what does this sentence mean?
6. Figure 7C. No YFP signal was observed in bright field.
7. Line345-346: "…in the promoter regions of sterol synthesis genes." How was the promoter defined? How many bp of DNA sequence upstream of the transcription start site?
8. Line611-619: How many samples were used for co-expression network analysis? It is not clear how this analysis was conducted.

Reviewer #2

(Remarks to the Author)
The manuscript by Zheng et al. presents a well-designed study investigating the synergistic damage caused by Fusarium crown rot (FCR) and drought in wheat, mediated by the TaMPK3 regulatory pathway. The study addresses a critical knowledge gap in understanding the molecular link between FCR susceptibility and drought stress, two major constraints in arid wheat-growing regions. The identification of TaMPK3 as a key mediator of this synergy provides new insights into plant

stress crosstalk. The research integrates multiple approaches, including transcriptome analysis, CRISPR/Cas9-mediated gene editing, overexpression assays, protein-protein interaction studies (Y2H, BiFC, LCI), and physiological phenotyping. This multi-layered validation strengthens the robustness of the conclusions. The manuscript provides valuable insights into the molecular basis of FCR-drought synergy in wheat, with strong potential to inform breeding strategies.

I have a few comments and suggestions for the authors to improve the current manuscript.
1. The introduction briefly mentions limited research on drought-FCR interactions but could more explicitly contrast with prior studies on MAPK pathways in plant stress responses. For example, specify whether MPK3 homologs in other plants (e.g., Arabidopsis, rice) have been implicated in dual stress (pathogen + drought) responses, to highlight the uniqueness of TaMPK3's role in wheat.
2. Explicitly state how this study advances beyond Su et al. (2021, 2024) and Liu et al. (2016), who reported transcriptional overlaps or phenotypic correlations between FCR and drought, by uncovering a molecular regulatory mechanism (TaMPK3-centered) driving the synergy.
3. The manuscript claims TaMPK3 reduces drought tolerance via the ABA pathway (citing Liu et al., 2022) but provides limited new data here. To reinforce this, include key ABA signaling components (e.g., TaPYL4 protein stability, TaSnRK2 activity, or ABA content) in TaMPK3-OE/knockout lines under drought. This would directly connect TaMPK3 to ABA-mediated drought responses.
4. While TaCYP51H37 is validated, the functional relevance of the entire "sterol synthesis gene cluster" (Figure 5d-e) remains underexplored. Do other genes in the cluster (e.g., TaCYP51H35, TaOSC) contribute to FCR resistance? A brief mention of their expression patterns in transgenic lines or preliminary VIGS data would support the "cluster" as a coordinated defense module.
5. For phenotypic data (e.g., disease indices, shoot weight), specify sample sizes and replicate numbers in figure legends. For example, in Figure 2f, how many plants were scored per line? This enhances reproducibility.
6. The study notes that TaMPK3 knockout abolishes synergistic damage without yield penalties under normal conditions. To strengthen relevance, discuss potential breeding strategies (e.g., marker-assisted selection for TaMPK3 variants with reduced expression, or stacking TaCYP51H37 overexpression with TaMPK3 knockout).
7. The research focuses on F. pseudo-graminearum (Fpg). Does the TaMPK3 pathway also mediate synergy with other FCR-causing pathogens (e.g., F. graminearum, F. culmorum, as mentioned in the introduction)? Acknowledging this limitation and suggesting future work would improve contextualization.
8. The discussion mentions TaWRKY26 as a phosphorylation target of TaMPK3, but its role in drought tolerance is unclear. Speculate on whether TaWRKY26 might directly regulate drought-responsive genes (beyond sterol synthesis) to explain its involvement in both stresses.
9. How are about the natural variations of TaMPK3 and TaCYP51H37? Are there TaMPK3 and TaCYP51H37 haplotypic combination effects on FCR and drought tolerance?
10. In the methods, clarify the criteria for DEG calling (e.g., "|log2(FoldChange)| > 1 and p-value < 0.05" is mentioned, but was FDR correction applied?). This is critical for reproducibility.
11. Some figures (e.g., Figure 1g, 5a) use abbreviations ("UP Fpg infected," "DOWN Drought") without explicit definition. Adding brief explanations (e.g., "DEGs upregulated under Fpg infection") would aid readability.
12. For TaCYP51H37 and TaWRKY26 VIGS silencing (Supplemental Figs. 7, 13), include data on off-target effects (e.g., expression of homologous genes) to rule out non-specific silencing.

Reviewer #3

(Remarks to the Author)
This manuscript, "Synergistic Damage Caused by Fusarium Crown Rot and Drought in Wheat is Mediated through the TaMPK3 Regulatory Pathway" by Zheng et al. This manuscript investigates the molecular mechanisms underlying the interaction between drought stress and Fusarium crown rot (FCR) in wheat. The authors demonstrate that drought exacerbates FCR severity, while FCR infection further reduces drought tolerance, highlighting a synergistic damage effect. Through transcriptomic analysis and functional validation, they identify TaMPK3 as a key regulator that enhances resistance to FCR but compromises drought tolerance. Mechanistically, TaMPK3 phosphorylates TaWRKY26, which in turn regulates a sterol synthesis gene cluster, with TaCYP51H37 playing a central role in boosting FCR resistance. The study proposes a molecular model of TaMPK3 mediated crosstalk between drought and disease responses, providing valuable insights and potential genetic targets for breeding wheat with improved stress resilience. However, certain aspects require refinement before this can be considered ready for publication:

Comment 1:
In figure 2b, it has only validated the overexpression lines at the transcript level (RT-qPCR), but no direct evidence is provided at the protein level. Since increased mRNA abundance does not necessarily correspond to higher protein accumulation. It is recommended that the authors perform protein-level validation of the overexpression materials, which would greatly strengthen the reliability and persuasiveness of the conclusions.
Comment 2:
In the LCI experiments (Figure 7e), the empty vector control also displayed a strong luminescence signal. Please clarify this phenomenon and improve the reliability of their conclusions.
Comment 3:
In the EMSA experiments (Figure 8e), the free probe bands are not clearly visible in some lanes, with relatively high background, making interpretation less straightforward. And the assay lacks a negative control using an empty protein, which is important to exclude non-specific interactions. It is recommended the authors to include appropriate negative protein controls and improve band clarity to strengthen the reliability of the EMSA results.
Comment 4.

In line 121, “indices of by” should be changed to “indices by conducting”.
Comment 5:
In line 213, “yield trait include spike number” should be changed to “yield traits including or includes spike number”.
Comment 6:
The manuscript presents a clear scientific idea and strong phenotypic evidence, with results that are both novel and relevant. However, beyond the scientific content, the manuscript would benefit from a careful formatting and language revision to ensure full compliance with the journal’s guidelines and to improve the overall clarity, consistency, and readability of the text.
Comment 7: The quality of Figure 8e (EMSA assay) is unsatisfactory. The free probe band is missing, and the binding bands is not linear. An additional competition or mutation assay should be included to enhance experimental reliability.
Comment 8: Figure 5f-p does not have a significance analysis. It is recommended to add one. For the significance analyses in other figures, the standards of * $p<0.05$ and ** $p<0.01$ should be used for marking.
Comment 9: The discussion section focuses on TaMPK3, but it fails to compare its function with other known MAPK genes in wheat that regulate biotic/abiotic stresses. The rationale for why TaMPK3 (rather than other MAPKs) mediates the synergistic effect of FCR resistance and drought tolerance remains unclear. It is recommended to broaden the discussion by incorporating recent studies on wheat MAPK family members in stress responses, clarifying the unique role of TaMPK3 in coordinating FCR resistance and drought tolerance.

Reviewer #4

(Remarks to the Author)
The manuscript by Zheng et al. reports an extensive characterization of a Mitogen-activated protein kinase (TaMPK3) as a key linkage between Fusarium crown rot resistance and drought sensitivity in bread wheat, and TaMPK3 interacts a WRKY transcription factor (TaWRKY26), which further regulates a terpenoid (ellarinacin) biosynthesis pathway to increase resistance to Fusarium crown rot. The authors previously identified that TaMPK3 interacts and destabilizes the ABA receptors to reduce drought tolerance. This work observed that TaMPK3 and TaWRKY26 homoeologue triplets are among the genes with significantly increased expression after Fusarium pseudograminearum inoculation and reduced expression after drought treatment, in line with the phenomenon that drought treatment reduced wheat resistance to F. pseudograminearum-caused crown rot. Using CRISPR/Cas9-derived Tampk3 knockout lines and TaMPK3-overexpressing lines of wheat, the authors showed that TaMPK3 expression level positively correlates with the resistance to F. pseudograminearum, and negatively correlates with drought tolerance. Extracts of TaMPK3-overexpressing lines exhibit greater inhibition to F. pseudograminearum growth in medium than extracts of Tampk3 knockout lines. VIGS silencing of TaWRKY26 genes led to increased FCR symptoms. Transcriptome analysis identifies terpenoid compound ellarinacin biosynthesis pathway genes, including TaCYP51H37, increased expression in the TaMPK3 overexpressing lines and in F. pseudograminearum infected samples. TaCYP51H37-overexpressing lines exhibit increased resistance to F. pseudograminearum and drought tolerance. Noteworthy, TaMPK3-overexpressing lines showed less resistance to crown rot under drought stress, but Tampk3-knockout mutants showed similar resistance to crown rot under drought stress and no drought stress. TaCYP51H37-overexpressing lines also showed similar resistance to crown rot under drought stress and no drought stress. Fusarium Crown Rot is an agriculturally important wheat disease. This work helps in understanding the mechanisms underlying drought enhancement of Fusarium crown rot disease severity. The quantification of disease severity under drought and normal conditions based on two types of F. pseudograminearum inoculations was of high quality. However, a few aspects of data analysis and experimental details require clarification. I have outlined my comments below.

1. Line 405, “In TaCYP51H37-OE lines, the FCR disease indices was higher under drought compared to normal conditions, but the difference was less than that in WT and TaMPK3-OE lines.” It is not clear whether the FCR disease indices were significantly higher under drought than under normal conditions. Statistical analysis should be performed on Figure 10f data.
2. Line 300， “TaCYP51H37-OE lines exhibited enhanced drought tolerance in seedling stage tests”. According to the working model the authors proposed in Figure 10g, it is understandable that TaCYP51H37-overexpression leads to increase in Fusarium crown rot resistance through accumulation of ellarinacin. But how to explain that TaCYP51H37-overexpression leads to higher drought tolerance?
3. The details of the TaMPK3-overexpressing wheat construction are not sufficient in the methods session. It is not clear which one of TaMPK3 homoeologues was driven by the ubiguitin promoter to generate OE-2, OE-6, and OE-11 lines. It is also not clear which one or all the TaMPK3 homoeologues expression levels were examined in Figure 2b. The protein identity percentages among TaMPK3 homoeologues can be provided in the results or labeled in Supplementary Figure 3b.
4. For the convenience of readers, the detailed consequences of protein sequence changes in the three CRISPR-derived lines could be provided, in addition to the consequences of DNA sequence changes shown in Fig. 2a. The authors could clarify the amino acid position of the frame-shift start in the kinase domain in Supplementary Figure 3b.
5. Ref 2 the journal name is incorrect. It should be Australasian Plant Pathology https://doi.org/10.1071/AP09053.
6. Line 283, “TaCYP51H37 catalyzes the final step of ellarinacin synthesis”. TaCYP51H37 should not be italic; it represents an enzyme.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

All my concerns have been addressed in this revised manuscript.

Reviewer #2

(Remarks to the Author)
This revised manuscript made significant revision based on the comments of the four reviewers. The authors performed additional experiments and provided strong new information to support their findings. I was satisfied with the scientific meaning and content of the revision. I have no further comments for the revised manuscript.

Reviewer #3

(Remarks to the Author)
All the questions raised by the reviewers have been addressed, and no further issues remain

Reviewer #4

(Remarks to the Author)
The revision improved the manuscript and addressed my previous concerns. But several aspects still need to be improved:

1. Figure 2 and Figure 8 show that “catalase (CAT) activity, malondialdehyde (MDA) content, and proline (PRO) content were measured”. But the measurement methods cannot be found in the Methods section. Although their measurements are often considered routine in certain labs, catalase activity and MDA contents are known to change in response to various stresses. Without careful design of sampling details, such as tissue type, plant growth conditions, and tissue amounts, it will be difficult to yield reproducible results.
2. Figure 3C shows wheat germ agglutinin staining. With the presented resolution, it is difficult to see the fungal hyphae. In addition, the method details of wheat germ agglutinin staining cannot be found in the methods section either.
3. Figure 7a, the phosphorylated WRKY26 band can be observed in the lane without ATP. This does not support the statement “The TaWRKY26 transcription factor was verified to be phosphorylation by TaMPK3” (Line 377). By the way, “phosphorylation” here should be “phosphorylated”.
4. Figure 7c, what is the meaning of “competitors”? Line 383 The statement “Electrophoretic mobility shift assay (EMSA) demonstrated that TaWRKY26 binds specifically to the WRKY binding sites in promoters of these sterol synthesis genes (Figure 7 c)” has problems. With competitors, the retarded binding band should be reduced if the binding is specific. However, the Figure 7c binding bands (as pointed to with arrowheads) were similar to those without competitors.
5. Line 803, The listed data PRJNA1139870, PRJNA1390310 can not be found in ncbi database (as of Jan 19). Please make the data accessible.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
The revised manuscript shows that TaMPK3 positively regulated FCR resistance through TaWRKY26, and negatively influencing drought tolerance. The revision has addressed my previous concerns. There is still a minor issue regarding English expressions.
The authors stated that “In this study, the severity of FCR was examined through in vitro infection and in vivo injection under both drought and normal conditions.”, and “we evaluated FCR disease indices by conducting in vitro infection with diseased wheat kernels and in vivo infection by injecting spore suspensions in both drought and normal growth environment.” The term "in vivo" often refers to research conducted on a living organism, while "in vitro" refers to research conducted in a laboratory dish or tube. However, both infection methods were inoculated on wheat plants in this manuscript. It is not appropriate to differentiate these two methods with "in vivo" and "in vitro".

Response to Referees letter

We extend our sincere appreciation to the four reviewers for meticulous and professional review, as well as for insightful and constructive suggestions. These perspectives and suggestions played a pivotal role in refining and enhancing our paper, not only augmenting its structural integrity but also imbuing it with profound scientific significance. Upon careful examination of the aforementioned comments, we comprehended that *TaMPK3* regulatory pathway assume an indispensable function in exploring wheat FCR resistance while providing robust guidance for future endeavors. The letter concludes with an appended comprehensive point-by-point response that addresses all the comments and suggestions made by each reviewer.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Fusarium crown rot (FCR) is emerging as a major disease prevalent in arid and semi-arid regions in wheat production. Thus, it is high valuable to identify genes related to FCR resistance as well the underlying molecular mechanism. In this study, Zheng et al. found that TaMPK3 regulates the expression of downstream sterol synthesis genes by phosphorylating TaWRKY26 to influence FCR resistance and drought tolerance. After review of the manuscript, I have some comments as below:

Major points:

1. Using only the reverse genetic approach to regard TaMPK3 as a targeting gene for FCR is not solid, even though it exhibited the most significant up-regulation in response to FCR, because there are other genes also showed significant up-regulation, how to exclude them?

Thank you for the reviewer's comments. In subsequent experiments, we attempted to identify natural variations of the *TaMPK3* gene; however, natural variations of this gene were found to be extremely limited (Supplementary Figure 26). As a result, the validation process using natural populations was not included. Both natural variation and chemical mutagenesis have their limitations, and mapping a gene with exceedingly scarce variations, such as *TaMPK3*, through forward genetics involves a certain degree of serendipity.

Initially, we aimed to elucidate the reasons behind the aggravated symptoms of FCR under drought conditions through transcriptomic analysis, with the goal of identifying whether certain genes or pathways exhibit significantly different expression patterns under both drought and FCR stress. We discovered that the MAPK signaling pathway involving the *TaMPK3* gene showed completely opposite expression trends under the two stress conditions. We selected *TaMPK3*, the most significantly expressed gene in this pathway, as our research target (Figure 1 g-j). In subsequent experiments, we validated the function of the *TaMPK3* gene using stable overexpression and gene-edited materials. Meanwhile, we analyzed the regulatory pathway of the *TaMPK3* gene and its downstream genes, as well as the FCR resistance function of related genes within the regulatory pathway. These results collectively demonstrate the significance of the *TaMPK3* gene and its regulatory pathway in the modulation of FCR resistance. Of course, there were also many other significantly upregulated genes. We believe there is no need to exclude them, as their expression changes are certainly linked to stress signaling. Should their expression patterns capture our attention, we would also consider them as research targets for further investigation.

Surprisingly, we observed that the expression of *TaCAT2*, a previously reported FCR resistance-related gene identified via GWAS and WES analyses[30], was also markedly upregulated in *TaMPK3*-OE lines (Supplementary Figure 21 b-d). It also indicates that the regulatory pathway involving TaMPK3 holds research value in the study of FCR resistance.

2. For transcriptome analysis, the cultivars and why use those cultivars as plant

materials were not described in the result (L119-L147). Line545-563 give some description of the plant materials: PBZ300 were used for Fusarium crown rot (FCR) inoculated, but Fielder was used for drought treatment. Way not use the same plant materials for transcriptome analysis?

We appreciate the reviewers' comments. The drought-related transcriptome data employed in our initial exploratory phase were derived from pre-existing datasets generated by our laboratory, whereas the transcriptome sequencing for the fusarium crown rot (FCR) experiments was conducted using disease-resistant materials selected during our FCR investigation. Consequently, the same experimental materials were not used across these analyses. In our related experiments, transcriptome sequencing was performed on multiple sets of susceptible and resistant materials, as well as under PEG-simulated drought stress; in all cases, *TaMPK3* gene exhibited a consistent expression pattern.

To address this point, we have supplemented the manuscript with quantitative analyses of *TaMPK3* gene expression in response to FCR and drought stress across Fielder, PBZ300 and five major cultivated varieties (Pinyu8012, Zhengmai1860, Jimai60, Jinhe991, Ningmai58), as well as an analysis of TaMPK3 protein dynamics over the course of *Fpg* infection in the Fielder (Supplementary Figure 3).

3. It is weird that the mRNA levels were significantly reduced in the gene edited lines (L169). Gene editing in the mutants caused the loss-of-function of the TaMPK3 genes, how can it cause the reduction of gene expression? Is the data reliable?

Thank you for your comments. Since our *TaMPK3*-KO lines all introduced premature stop codons at different sites, leading to the production of nonsense mRNA and triggering the nonsense-mediated mRNA decay (NMD) mechanism (Supplementary Figure 5). We designed specific primers for chromosomes A, B, and D, respectively. Detection results showed that the mRNA levels of *TaMPK3-A*, *TaMPK3-B*, and *TaMPK3-D* were extremely low in the *TaMPK3*-KO lines (Figure 2 d). Meanwhile, we detected changes in TaMPK3 protein levels, demonstrating an increase in TaMPK3 protein in TaMPK3-OE lines and a significant reduction in TaMPK3-KO lines

(Supplementary Figure 6).

4. Too many figures in the main text, and English is not good enough.

Thank you for your suggestions. We have made adjustments to the entire text and all the figures.

Minors:

1. Some figures are too small to see clearly, I suggest to enlarge the figures.

Thank you for your suggestions. We have made adjustments to all the figures.

2. Line130-147: Only 762 DEGs that were down-regulated under drought treatment and up-regulated under Fpg infection were selected for enrichment analyses. Why not select all DEGs for further analysis?

Thank you for your comments. The objective of our experiment is to identify the relationship between differentially expressed genes under two types of stress, in order to explain the more severe symptoms of FCR in drought conditions. We observed a greater overlap among genes that were significantly downregulated under drought stress and significantly upregulated upon FCR infection (Figure 1 g). We aim to explore the connection between the two stresses by focusing on this subset of differentially expressed genes. This stepwise filtering approach is intended to narrow down the scope and ultimately uncover the most relevant gene types. As for the other differentially expressed genes, enrichment analysis was also performed, though the number of genes simultaneously upregulated or downregulated under both drought and FCR conditions was relatively small (Supplementary Figure 1).

3. The details of the GO and KEGG enrichment analyses should be described in the Methods section.

Thank you for your comments. We have incorporated the relevant details into the text as suggested, Line 665-669.

4. Line232-234: "… whereas no mycelial development was observed on TaMPK3-OE plants (Figure 4b)". Less mycelial was observed on TaMPK3-OE plants (Figure 4b).

Thank you for your valuable suggestions. We have made the corresponding changes to the article, Line 252.

5. Line264-265: "…TaMPK3 regulation were in adjacent regions in chromosomes 5A and 5D.", what does this sentence mean?

Thank you for your comments. We have also revised the original text accordingly, Line 286-288. This section aims to convey that the series of genes regulating sterol synthesis form a gene cluster in the genome. Gene clusters with similar expression patterns and related functions often share common regulatory mechanisms, which serves as a key clue for our subsequent research on their regulation.

6. Figure 7C. No YFP signal was observed in bright field.

In the bright field, only images of leaf cells are provided, with YFP signals visible exclusively in the YFP signal channel and the Merge channel (Figure 6 c).

7. Line345-346: "…in the promoter regions of sterol synthesis genes." How was the promoter defined? How many bp of DNA sequence upstream of the transcription start site?

We thank the reviewers for their comments. We have added the relevant information in the text (Line 380) and specified the exact length of the promoter sequence inserted into the LUC vector in Figure 7 d.

8. Line611-619: How many samples were used for co-expression network analysis? It is not clear how this analysis was conducted.

We thank the reviewer for the suggestions and have revised the methods section accordingly, Line 725-728.

Reviewer #2 (Remarks to the Author):

The manuscript by Zheng et al. presents a well-designed study investigating the synergistic damage caused by Fusarium crown rot (FCR) and drought in wheat, mediated by the TaMPK3 regulatory pathway. The study addresses a critical knowledge gap in understanding the molecular link between FCR susceptibility and drought stress, two major constraints in arid wheat-growing regions. The identification of TaMPK3 as a key mediator of this synergy provides new insights into plant stress crosstalk. The research integrates multiple approaches, including transcriptome analysis, CRISPR/Cas9-mediated gene editing, overexpression assays, protein-protein interaction studies (Y2H, BiFC, LCI), and physiological phenotyping. This multi-layered validation strengthens the robustness of the conclusions. The manuscript provides valuable insights into the molecular basis of FCR-drought synergy in wheat, with strong potential to inform breeding strategies.

I have a few comments and suggestions for the authors to improve the current manuscript.

1. The introduction briefly mentions limited research on drought-FCR interactions but could more explicitly contrast with prior studies on MAPK pathways in plant stress responses. For example, specify whether MPK3 homologs in other plants (e.g., Arabidopsis, rice) have been implicated in dual stress (pathogen + drought) responses, to highlight the uniqueness of TaMPK3's role in wheat.

We thank the reviewer for the valuable suggestion and have incorporated this part into the discussion, Line 518-529.

2. Explicitly state how this study advances beyond Su et al. (2021, 2024) and Liu et al. (2016), who reported transcriptional overlaps or phenotypic correlations between FCR and drought, by uncovering a molecular regulatory mechanism (TaMPK3-centered) driving the synergy.

Thank you to the reviewers; this is very helpful for us. We will also incorporate this

part into the discussion, Line 496-504.

3. The manuscript claims TaMPK3 reduces drought tolerance via the ABA pathway (citing Liu et al., 2022) but provides limited new data here. To reinforce this, include key ABA signaling components (e.g., TaPYL4 protein stability, TaSnRK2 activity, or ABA content) in TaMPK3-OE/knockout lines under drought. This would directly connect TaMPK3 to ABA-mediated drought responses.

Thank you very much for your suggestions. Since our previous study has already reported the role of TaMPK3 in regulating the protein stability of TaPYL4 in wheat, we further conducted detection and analysis of the downstream effector genes of TaPYL4. In recent years, the ABA-responsive gene *TaPYL1-1B*[38], the stomatal regulation-associated gene *TaSLAC1*, the ROS scavenging-related gene *TaCAT1*[39], and the stripe rust resistance-related gene *TaASR2L*[40] downstream of TaPYL proteins in the ABA signaling pathway has been identified in wheat. In our assays, these downstream genes were significantly downregulated in *TaMPK3*-OE lines, while they were markedly upregulated in *TaMPK3*-KO lines. This extension of the study further confirms the role of TaMPK3 in the ABA signaling pathway and has been highly beneficial to our research (Supplementary Figure 21 a-d).

4. While TaCYP51H37 is validated, the functional relevance of the entire "sterol synthesis gene cluster" (Figure 5d-e) remains underexplored. Do other genes in the cluster (e.g., TaCYP51H35, TaOSC) contribute to FCR resistance? A brief mention of their expression patterns in transgenic lines or preliminary VIGS data would support the "cluster" as a coordinated defense module.

Thank you very much for your suggestions. We confirmed that the expression of these genes responding to FCR infection and are regulated by TaMPK3 (Figure 4 d-q and Supplementary Figure 9). Furthermore, we have preliminarily explored the effects of other genes in the sterol synthesis gene cluster on FCR resistance and drought tolerance using the VIGS method. The results indicate that these genes also play significant roles in regulating FCR resistance, but they have no significant impact on drought tolerance

(Supplementary Figure 22-24). We greatly appreciate your assistance, and these findings further confirm the critical role of this regulatory pathway in FCR resistance.

5. For phenotypic data (e.g., disease indices, shoot weight), specify sample sizes and replicate numbers in figure legends. For example, in Figure 2f, how many plants were scored per line? This enhances reproducibility.

Thank you for your valuable suggestions. We have added this information to both the figure legends and figure legends.

6. The study notes that TaMPK3 knockout abolishes synergistic damage without yield penalties under normal conditions. To strengthen relevance, discuss potential breeding strategies (e.g., marker-assisted selection for TaMPK3 variants with reduced expression, or stacking TaCYP51H37 overexpression with TaMPK3 knockout).

Thank you very much for the reviewer's comments. We have supplemented the applied value of our study in the Discussion section. Due to the independent pathways through which the *TaMPK3* gene affects drought and FCR resistance, and the fact that knocking out *TaMPK3* significantly improves drought resistance without adversely affecting wheat yield, introducing downstream FCR resistance genes into *TaMPK3*-KO lines represents a feasible breeding strategy. We have included these results in the discussion, Line588-599.

7. The research focuses on F. pseudo-graminearum (Fpg). Does the TaMPK3 pathway also mediate synergy with other FCR-causing pathogens (e.g., F. graminearum, F. culmorum, as mentioned in the introduction)? Acknowledging this limitation and suggesting future work would improve contextualization.

Thank you very much for your suggestions. This is actually a direction we plan to explore in our follow-up research. Since *F. graminearum* is also a major pathogen causing Fusarium head blight, we intend to validate the findings of our study in the context of this disease. Currently, we have compiled six sets of transcriptomic data from *F. graminearum* infected treatments. A comprehensive analysis revealed that the 580

commonly differentially expressed genes include the MAPK signaling pathway we identified. These results will be applied in our subsequent work to further analyze the breeding applications of the TaMPK3 regulatory pathway.

[FIGURE REDACTED]

8. The discussion mentions TaWRKY26 as a phosphorylation target of TaMPK3, but its role in drought tolerance is unclear. Speculate on whether TaWRKY26 might directly regulate drought-responsive genes (beyond sterol synthesis) to explain its involvement in both stresses.

We thank the reviewer for the suggestion. Using the VIGS approach, we validated whether TaWRKY26 plays a role in drought tolerance in wheat. The results showed that silencing the *TaWRKY26* gene did not significantly alter the drought resistance of wheat (Supplementary Figure 20 i-m). Additionally, we examined the downstream effector genes of the ABA signaling pathway influenced by TaMPK3 and found that these genes are not affected by TaWRKY26 (Supplementary Figure 21 g). These findings suggest that the TaMPK3-mediated ABA signaling pathway operates independently of the FCR resistance pathway.

9. How are about the natural variations of TaMPK3 and TaCYP51H37? Are there TaMPK3 and TaCYP51H37 haplotypic combination effects on FCR and drought tolerance?

Thank you very much for your suggestions. We examined the variation in the TaMPK3 and TaCYP51H37 genes and found that these genes exhibit limited variation, with no haplotypes identified that are applicable to breeding (Supplementary Figure 26, 29 and 30). However, we observed the presence of multiple non-expressed copies of sterol biosynthesis-related gene clusters in the genome (Supplementary Figure 27), which contain abundant variations (Supplementary Figure 31-33). We anticipate that expressible haplotypes could be identified from these clusters for use in breeding. The relevant descriptions were added to the discussion, Line 568-587.

10. In the methods, clarify the criteria for DEG calling (e.g., "|log2(FoldChange)| > 1 and p-value < 0.05" is mentioned, but was FDR correction applied?). This is critical for reproducibility.

Thank you for your suggestions. We have reviewed the data and made corresponding revisions in the methods, Line 665-666.

11. Some figures (e.g., Figure 1g, 5a) use abbreviations ("UP Fpg infected," "DOWN Drought") without explicit definition. Adding brief explanations (e.g., "DEGs upregulated under Fpg infection") would aid readability.

Thank you for your valuable suggestions. We have added detailed annotations to the images.

12. For TaCYP51H37 and TaWRKY26 VIGS silencing (Supplemental Figs. 7, 13), include data on off-target effects (e.g., expression of homologous genes) to rule out non-specific silencing.

We thank the reviewers for their comments. We screened genes that may have potential off-target effects and analyzed their expression levels in the transcriptome. The results showed that most of these genes were not expressed under normal, drought, or FCR-

induced conditions. Meanwhile, we examined other expressed off-target genes (Supplemental Fig 34).

Reviewer #3 (Remarks to the Author):

This manuscript, "Synergistic Damage Caused by Fusarium Crown Rot and Drought in Wheat is Mediated through the TaMPK3 Regulatory Pathway" by Zheng et al. This manuscript investigates the molecular mechanisms underlying the interaction between drought stress and Fusarium crown rot (FCR) in wheat. The authors demonstrate that drought exacerbates FCR severity, while FCR infection further reduces drought tolerance, highlighting a synergistic damage effect. Through transcriptomic analysis and functional validation, they identify TaMPK3 as a key regulator that enhances resistance to FCR but compromises drought tolerance. Mechanistically, TaMPK3 phosphorylates TaWRKY26, which in turn regulates a sterol synthesis gene cluster, with TaCYP51H37 playing a central role in boosting FCR resistance. The study proposes a molecular model of TaMPK3 mediated crosstalk between drought and disease responses, providing valuable insights and potential genetic targets for breeding wheat with improved stress resilience. However, certain aspects require refinement before this can be considered ready for publication:

Comment 1:

In figure 2b, it has only validated the overexpression lines at the transcript level (RT-qPCR), but no direct evidence is provided at the protein level. Since increased mRNA abundance does not necessarily correspond to higher protein accumulation. It is recommended that the authors perform protein-level validation of the overexpression materials, which would greatly strengthen the reliability and persuasiveness of the conclusions.

Thank you for your valuable suggestions. We have incorporated protein abundance-related detection of TaMPK3 and TaCYP51H37. (Supplementary Figure 3, 6 and 8).

Comment 2:

In the LCI experiments (Figure 7e), the empty vector control also displayed a strong luminescence signal. Please clarify this phenomenon and improve the reliability of their conclusions.

Thank you for the comments. We checked the original images and found that the signal occurs at the pinhole location, and after reducing the signal strength, the signal at the pinhole location can be eliminated. This may be due to the weak interaction signals in this experiment. To validate the experimental results, we repeated the experiment with ensured signal strength, and replaced the images. (Figure 6 d).

Comment 3:

In the EMSA experiments (Figure 8e), the free probe bands are not clearly visible in some lanes, with relatively high background, making interpretation less straightforward. And the assay lacks a negative control using an empty protein, which is important to exclude non-specific interactions. It is recommended the authors to include appropriate negative protein controls and improve band clarity to strengthen the reliability of the EMSA results.

Thank you for your valuable suggestions. We have re-verified the relevant experiments in the article and replaced the corresponding images (Figure 7 c).

Comment 4.

In line 121, “indices of by” should be changed to “indices by conducting”.

Thank you for the comments; we have made the corresponding changes in the article, Line 124.

Comment 5:

In line 213, “yield trait include spike number” should be changed to “yield traits including or includes spike number”.

Thank you for your valuable suggestions. The relevant content in the article has been

revised, Line 218.

Comment 6:

The manuscript presents a clear scientific idea and strong phenotypic evidence, with results that are both novel and relevant. However, beyond the scientific content, the manuscript would benefit from a careful formatting and language revision to ensure full compliance with the journal's guidelines and to improve the overall clarity, consistency, and readability of the text.

Thank you for your valuable suggestions. We have made improvements to the entire text accordingly.

Comment 7:

The quality of Figure 8e (EMSA assay) is unsatisfactory. The free probe band is missing, and the binding bands is not linear. An additional competition or mutation assay should be included to enhance experimental reliability.

Thank you for your valuable suggestions. Your repeated emphasis on the EMSA results made us realize the importance of the issue. We have since repeated the experiment and replaced the images (Figure 7 c). This has been very helpful to us.

Comment 8:

Figure 5f-p does not have a significance analysis. It is recommended to add one. For the significance analyses in other figures, the standards of * $p<0.05$ and ** $p<0.01$ should be used for marking.

Thank you for your valuable suggestions. Statistical analysis results have been added to the images (Figure 4 g-q).

Comment 9:

The discussion section focuses on TaMPK3, but it fails to compare its function with other known MAPK genes in wheat that regulate biotic/abiotic stresses. The rationale for why TaMPK3 (rather than other MAPKs) mediates the synergistic effect of FCR

resistance and drought tolerance remains unclear. It is recommended to broaden the discussion by incorporating recent studies on wheat MAPK family members in stress responses, clarifying the unique role of TaMPK3 in coordinating FCR resistance and drought tolerance.

Thank you for the reviewer's suggestion. We have incorporated relevant reports on other members of the MAPK family into the discussion to highlight the significance of the association between TaMPK3 and FCR. The relevant content was added to the discussion, Line 511-529.

Reviewer #4 (Remarks to the Author):

The manuscript by Zheng et al. reports an extensive characterization of a Mitogen-activated protein kinase (TaMPK3) as a key linkage between Fusarium crown rot resistance and drought sensitivity in bread wheat, and TaMPK3 interacts a WRKY transcription factor (TaWRKY26), which further regulates a terpenoid (ellarinacin) biosynthesis pathway to increase resistance to Fusarium crown rot. The authors previously identified that TaMPK3 interacts and destabilizes the ABA receptors to reduce drought tolerance. This work observed that TaMPK3 and TaWRKY26 homoeologue triplets are among the genes with significantly increased expression after Fusarium pseudograminearum inoculation and reduced expression after drought treatment, in line with the phenomenon that drought treatment reduced wheat resistance to F. pseudograminearum-caused crown rot. Using CRISPR/Cas9-derived Tampk3 knockout lines and TaMPK3-overexpressing lines of wheat, the authors showed that TaMPK3 expression level positively correlates with the resistance to F. pseudograminearum, and negatively correlates with drought tolerance. Extracts of TaMPK3-overexpressing lines exhibit greater inhibition to F. pseudograminearum growth in medium than extracts of Tampk3 knockout lines. VIGS silencing of TaWRKY26 genes led to increased FCR symptoms. Transcriptome analysis identifies terpenoid compound ellarinacin biosynthesis pathway genes, including TaCYP51H37,

increased expression in the TaMPK3 overexpressing lines and in F. pseudograminearum infected samples. TaCYP51H37-overexpressing lines exhibit increased resistance to F. pseudograminearum and drought tolerance. Noteworthy, TaMPK3-overexpressing lines showed less resistance to crown rot under drought stress, but Tampk3-knockout mutants showed similar resistance to crown rot under drought stress and no drought stress. TaCYP51H37-overexpressing lines also showed similar resistance to crown rot under drought stress and no drought stress. Fusarium Crown Rot is an agriculturally important wheat disease. This work helps in understanding the mechanisms underlying drought enhancement of Fusarium crown rot disease severity. The quantification of disease severity under drought and normal conditions based on two types of F. pseudograminearum inoculations was of high quality. However, a few aspects of data analysis and experimental details require clarification. I have outlined my comments below.

1. Line 405, “In TaCYP51H37-OE lines, the FCR disease indices was higher under drought compared to normal conditions, but the difference was less than that in WT and TaMPK3-OE lines.” It is not clear whether the FCR disease indices were significantly higher under drought than under normal conditions. Statistical analysis should be performed on Figure 10f data.

Thank you for the comments. We have made the corresponding revisions in the article. Additionally, we repeated this part of the experiment and included data on the disease index. The updated statistical analysis results are presented in Figure 8 m-n.

2. Line 300, “TaCYP51H37-OE lines exhibited enhanced drought tolerance in seedling stage tests”. According to the working model the authors proposed in Figure 10g, it is understandable that TaCYP51H37-overexpression leads to increase in Fusarium crown rot resistance through accumulation of ellarinacin. But how to explain that TaCYP51H37-overexpression leads to higher drought tolerance?

We appreciate the reviewer's comments. We believe that the enhanced drought resistance of the *TaCYP51H37*-OE line is also related to the production of sterol

compounds. Ellarinacin is a compound structurally similar to plant cholesterol compound. Previous studies have shown that the external application of plant cholesterol can significantly improve the drought resistance of rice[47]. In soybean, enhancing sterol biosynthesis or the exogenous application of these compounds can improve crop tolerance to drought stress through their antioxidant effects[46]. The relevant content was added to the discussion, Line 544-562.

3. The details of the TaMPK3-overexpressing wheat construction are not sufficient in the methods session. It is not clear which one of TaMPK3 homoeologues was driven by the ubiguitin promoter to generate OE-2, OE-6, and OE-11 lines. It is also not clear which one or all the TaMPK3 homoeologues expression levels were examined in Figure 2b. The protein identity percentages among TaMPK3 homoeologues can be provided in the results or labeled in Supplementary Figure 3b.

Thank you for your comments. We have included the specific accession numbers of the overexpressed genes in the Methods section, Line 613-615. In Figure 2, we have added separate detection results of the *TaMPK3* homoeologous genes from chromosomes A, B, and D. Additionally, in Supplementary Figure 4, we have included the protein identity percentages among TaMPK3 homoeologous genes and marked the divergent amino acid sites.

4. For the convenience of readers, the detailed consequences of protein sequence changes in the three CRISPR-derived lines could be provided, in addition to the consequences of DNA sequence changes shown in Fig. 2a. The authors could clarify the amino acid position of the frame-shift start in the kinase domain in Supplementary Figure 3b.

Thank you for your suggestion. We have added this section to illustrate the frameshift mutations and premature termination codons introduced in three *TaMPK3*-KO lines (Supplementary Figure 5).

5. Ref 2 the journal name is incorrect. It should be Australasian Plant Pathology https://doi.org/10.1071/AP09053.

Thank you very much. We have made the revisions in the article, Line 833-834.

6. Line 283, “TaCYP51H37 catalyzes the final step of ellarinacin synthesis”. TaCYP51H37 should not be italic; it represents an enzyme.

Thanks for the comments. We have already made the changes in the article, Line 306.

Response to Referees letter

We sincerely appreciate the insightful and constructive feedback from the four reviewers. These meticulous efforts and professional suggestions have been invaluable to our work. The letter concludes with an appended comprehensive point-by-point response that addresses all the comments and suggestions made by each reviewer.

REVIEWER COMMENTS

Reviewer #4 (Remarks to the Author):

The revision improved the manuscript and addressed my previous concerns. But several aspects still need to be improved:

1. Figure 2 and Figure 8 show that “catalase (CAT) activity, malondialdehyde (MDA) content, and proline (PRO) content were measured”. But the measurement methods cannot be found in the Methods section. Although their measurements are often considered routine in certain labs, catalase activity and MDA contents are known to change in response to various stresses. Without careful design of sampling details, such as tissue type, plant growth conditions, and tissue amounts, it will be difficult to yield reproducible results.

We sincerely thank the reviewers for valuable comments, which are highly meaningful for improving our manuscript. The detailed experimental protocols and sampling procedures have been incorporated into the Methods section, line 653-671.

Determination of Malondialdehyde (MDA) content, Proline (PRO) content, and Catalase (CAT) activity

Physiological indicators (MDA content, PRO content, and CAT activity) were quantified using commercial assay kits from Suzhou Comin Biotechnology Co., Ltd. (Suzhou, China). Measurements were performed in 96-well plates according to the manufacturer's protocols for tissue sample extraction and detection, with absorbance readings obtained via a microplate reader. Take 0.05 g of treated plant leaves, grind them in liquid nitrogen, and proceed with physiological index analysis. The results were calculated based on sample mass according to the specified formula in the corresponding manuals (MDA: https://www.cominbio.com/uploads/soft/190325/2-1Z325154356.pdf; PRO content: https://www.cominbio.com/uploads/soft/180727/1-1PHGF414.pdf; CAT activity: https://www.cominbio.com/uploads/soft/210330/1-210330161952.pdf).

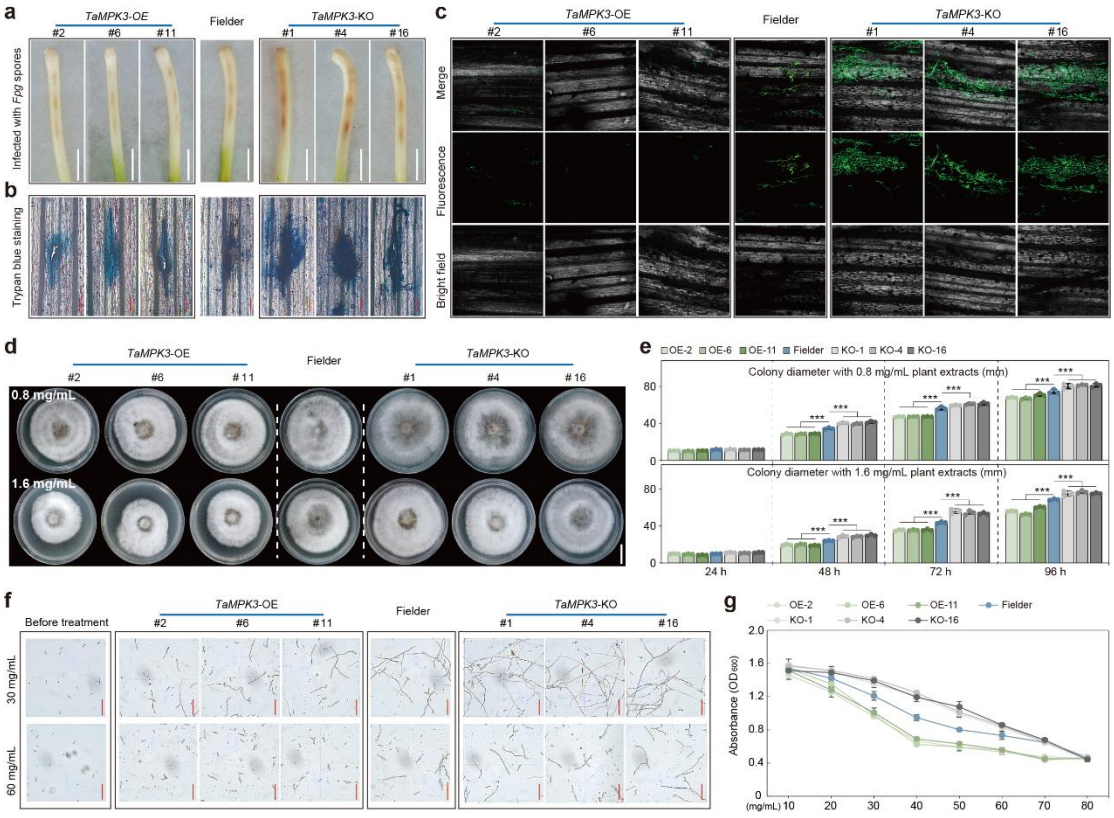
For the drought treatment experiment, physiological parameters were assessed five days after rewatering. In the experiment examining the impact of FCR on drought resistance, the samples were collected five days after *Fpg* infection. To ensure the wheat stem base was sufficiently thick for inoculation with *Fpg* spore suspension at the onset of drought symptoms, an additional 100 ml of water was applied to each pot during the seedling stage, in accordance with the initial soil moisture requirements of the drought treatment.

2. Figure 3C shows wheat germ agglutinin staining. With the presented resolution, it is difficult to see the fungal hyphae. In addition, the method details of wheat germ agglutinin staining cannot be found in the methods section either.

We are grateful to the reviewer for insightful comments. In response, we have adjusted the mycelium signal intensity and improved the WGA staining protocol in the Methods section accordingly, line 726-732.

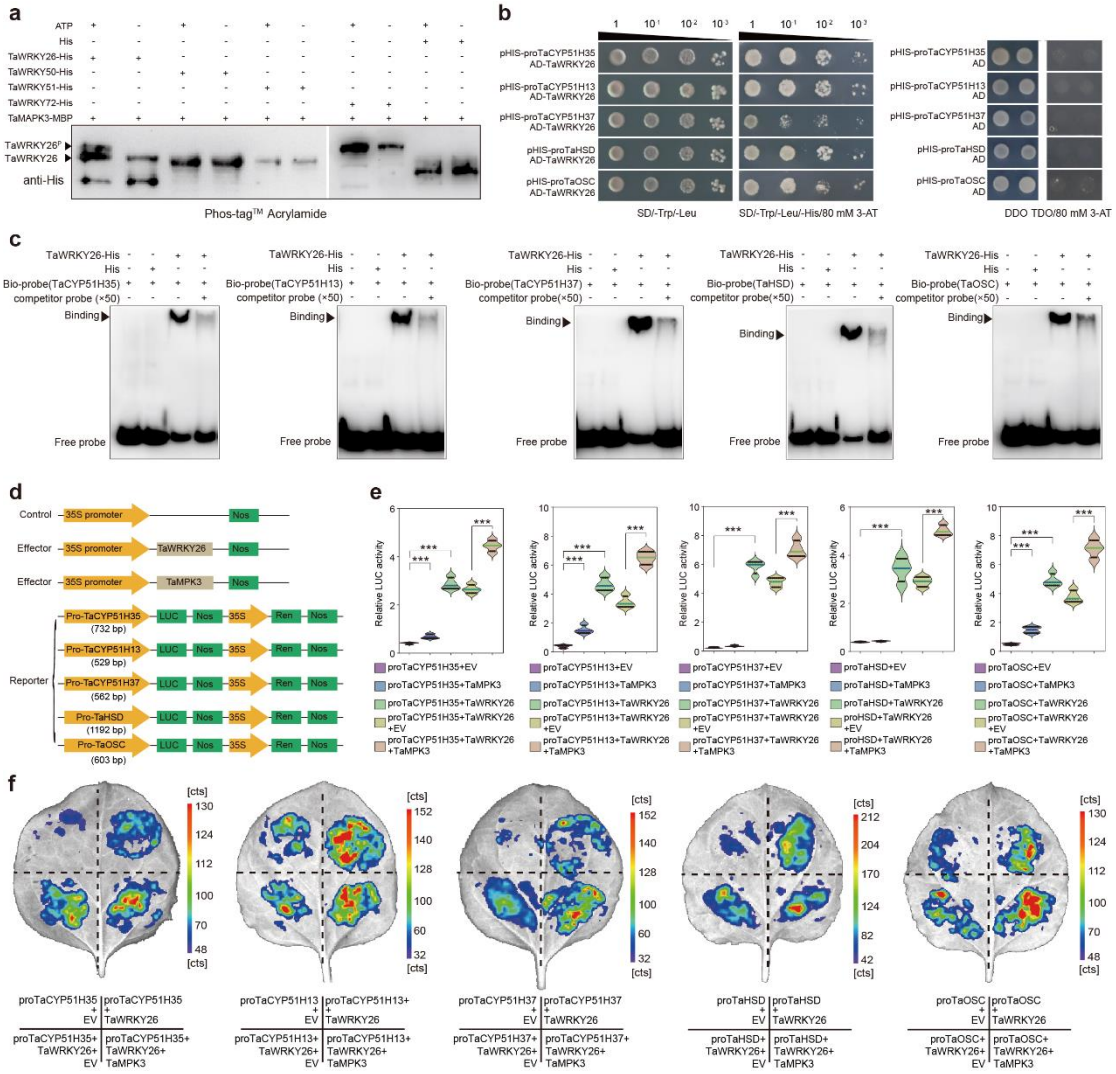
For Wheat Germ Agglutinin (WGA) staining**,** the basal tissue from healthy wheat stems was immersed in spore suspensions for 6 h and subsequently cultured on moist filter paper for three days. The outermost layer of the stem tissue was peeled off and incubated in 1 M KOH at 37°C for 24 h in darkness. The stems were rinsed three times

with 50 mM Tris–HCl (pH 7.4) and stained with WGA at a concentration of 20 μg/mL in the Tris–HCl buffer under dark condition at 4°C for 24 h. The samples were then observed with a Zeiss LSM700 microscope.



3. Figure 7a, the phosphorylated WRKY26 band can be observed in the lane without ATP. This does not support the statement "The TaWRKY26 transcription factor was verified to be phosphorylation by TaMPK3" (Line 377). By the way, "phosphorylation" here should be "phosphorylated".

We are grateful to the reviewers for valuable feedback and have updated Figure 7 a and the corresponding text as suggested, line 379. In Figure 7a, we incorrectly labeled the lanes where ATP was added. ATP was actually added to lanes 1, 3, 5, 7, and 9, and this has been corrected in the figure. We thank the reviewer for their careful review and assistance, and we have also checked the labeling in other figures to avoid similar errors.



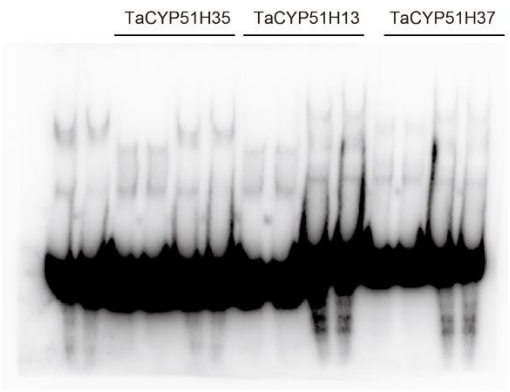
4. Figure 7c, what is the meaning of "competitors"? Line 383 The statement "Electrophoretic mobility shift assay (EMSA) demonstrated that TaWRKY26 binds specifically to the WRKY binding sites in promoters of these sterol synthesis genes (Figure 7 c)" has problems. With competitors, the retarded binding band should be reduced if the binding is specific. However, the Figure 7c binding bands (as pointed to with arrowheads) were similar to those without competitors.

We sincerely appreciate the reviewer's insightful suggestions, which have greatly enhanced the quality of the manuscript. The corresponding revisions have been incorporated into Figure 7 c and the main text line 384-386.

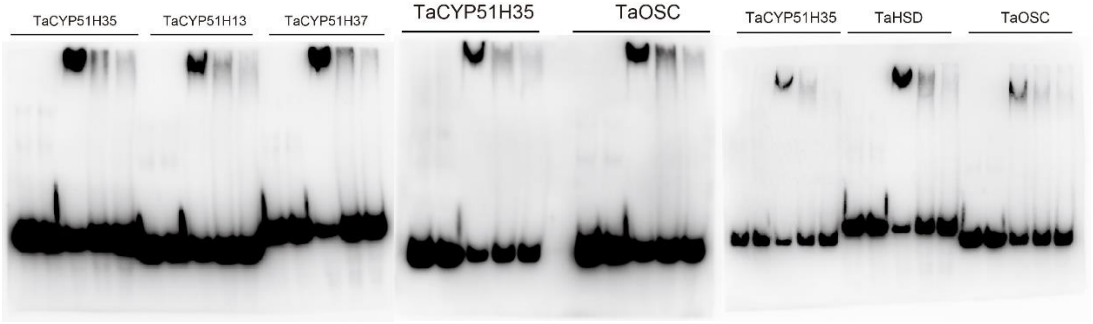
Electrophoretic mobility shift assays (EMSA) confirmed that TaWRKY26 binds to WRKY binding site segments within the promoters of these sterol synthesis genes.

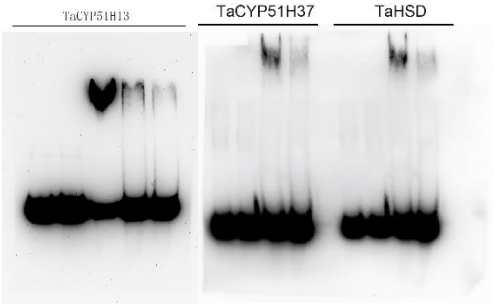
In this experiment, to enhance the competitive effect of the 50× competitive probe, we modified the reagent addition sequence: the unlabeled probe was added first, followed by a 5 minutes reaction period, before introducing the biotin-labeled probe. After a 30 minutes reaction at 25°C, the products were analyzed by gel electrophoresis. On the other hand, we found that the previously obtained TaWRKY26 protein had undergone significant degradation. Therefore, we re-induced and purified a new batch of the protein, which eliminated the non-specific bands in the image.

Original protein:



New protein:





5. Line 803, The listed data PRJNA1139870, PRJNA1390310 can not be found in ncbi database (as of Jan 19). Please make the data accessible.

Thank you for the reminder from the reviewer. The data for PRJNA1139870 and

PRJNA1390310 have not yet reached their release date and will be made available upon acceptance of the manuscript. The reviewer can access the data through the reviewer channel for evaluation.

PRJNA1139870:

https://dataview.ncbi.nlm.nih.gov/object/PRJNA1139870?reviewer=nqtoo26r1fj5hkkbiqt7ln10hl

PRJNA1390310:

https://dataview.ncbi.nlm.nih.gov/object/PRJNA1390310?reviewer=46q27n44es1jik2buqmh981s1k

Response to Referees letter

We sincerely thank the reviewers for their insightful comments and constructive suggestions, which have significantly improved our manuscript. We have carefully addressed each point raised in their final review.

REVIEWER COMMENTS

Reviewer #4 (Remarks to the Author):

The revised manuscript shows that TaMPK3 positively regulated FCR resistance through TaWRKY26, and negatively influencing drought tolerance. The revision has addressed my previous concerns. There is still a minor issue regarding English expressions.

The authors stated that “In this study, the severity of FCR was examined through in vitro infection and in vivo injection under both drought and normal conditions.”, and “we evaluated FCR disease indices by conducting in vitro infection with diseased wheat kernels and in vivo infection by injecting spore suspensions in both drought and normal growth environment.” The term “in vivo” often refers to research conducted on a living organism, while "in vitro" refers to research conducted in a laboratory dish or tube. However, both infection methods were inoculated on wheat plants in this manuscript. It is not appropriate to differentiate these two methods with “in vivo” and “in vitro”.

We sincerely thank the reviewers for their valuable suggestions, which have greatly strengthened our manuscript. In response, we have revised the terminology throughout the text: “in vitro infection” has been replaced with “stem surface inoculation”, and “in vivo injection” with “stem internal inoculation”. The revised paragraphs are provided below:

“In this study, the severity of FCR was examined through stem surface inoculation and

stem internal injection under both drought and normal conditions."

"To identify the cause of the high incidence of FCR in wheat under drought conditions, we evaluated FCR disease indices by conducting stem surface inoculation with diseased wheat kernels and stem internal infection by injecting spore suspensions in both drought and normal growth environments"

"(a) Illustration of stem surface infection using *Fpg* infected wheat kernels. (b, c) Disease symptoms and indices following stem surface infection under drought and normal conditions; n = 36 biologically independent plants. (d) Illustration of stem internal injection using *Fpg* spore suspensions. (e, f) Disease symptoms and indices following stem internal injection; n = 36 biologically independent plants."