

OsCDPK24 and OsCDPK28 phosphorylate heat shock factor OsHSFA4d to orchestrate abiotic and biotic stress responses in rice

Corresponding Author: Professor Xuewei Chen

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)

Global warming has the potential to dramatically reduce agricultural harvests. Heat stress is reported to lead to thermotolerance and suppression of disease resistance in rice. However, the molecular basis underlying this trade-off remains in rice is far from clear. HS factors (HSFs) are important transcription factors that regulate abiotic and biotic stress responses, binding the heat shock element (HSE) in promoters of target genes to regulate their expression. Whether and how HSFs are involved in plant immunity remain to be elucidated. Under abiotic and biotic stresses, the Ca^{2+} signal can be sensed by calcium-dependent protein kinases (CDPKs) and transduced through phosphorylation events, leading to gene expression changes. However, how these events regulate plant thermotolerance and disease resistance under heat stress remains poorly understood. The manuscript by Fang et al discovered that OsCDPK24 and OsCDPK28 are activated by heat stress to phosphorylate OsHSFA4d and enhances its ability to promote HSP101 expression, conferring plant thermotolerance, and CsIF6 expression, suppressing plant disease resistance. The authors provided lots of experiment evidences to support their findings. However, they might need to address and response the following questions before its possible publication.

1. Thermotolerance is a phenotype that organism has the ability to survival under heat stress. Hence it is recommended that replacing 'induce thermotolerance' with 'induce HS responses' in line 20 and line 38.
2. HSF4d binds to CsIF6intron I (instead of promoter) which contains typical HSE to promote CsIF6 expression. It is suggested that the authors discuss the possible underlying mechanism.
3. In line 49-50, 'HSFs bind to the heat shock element (HSE, 5'-nGAAAnnTTCn-3') in promoters of target genes to regulate their expression. In Fig 2g, F8 which contains the HSE is not the most enriched region, while F9 which contains no HSE is the most enriched region. It is suggested that the authors should explain this puzzle.
4. In line 439-442, 'One strategy to optimize OsHsfA4d function is to have OsHsfA4d expressed at low levels under severe disease conditions (mostly at seedling stage) but expressed at high levels when high temperature occurs and thermotolerance is required (at mature stage)'. This optimized expression pattern may be discovered in local varieties and accessions through natural variations analysis which is recommended to perform by the authors.
5. Both OsCDPK24 and OsCDPK28 can phosphorylate OsHSFA4d at S146 to orchestrate plant thermotolerance and disease resistance, what is the functional difference between OsCDPK24 and OsCDPK28? Whether OsCDPK24 or OsCDPK28 could form homodimer?

Reviewer #2

(Remarks to the Author)

This work discovered a regulatory module, consisting of OsCDPK24/OsCDPK28-OsHSFA4d- HSP101/CsIF6, in regulating the trade-off of thermotolerance and disease resistance in rice. Although the role of HSF-HSP in thermotolerance is well known and the role of CsIF6 in defense response has been also reported, the key novel finding of this work is that the OsHSFA4d can be activated by heat stress (HS)-activated CDPKs to positively and negatively regulate thermotolerance and disease resistance, respectively, which provide a mechanistic example to explain the phenomenon that HS induces thermotolerance but often causes decreased immunity to pathogens. The genetic and biochemical evidence are strong and

complete to support the major conclusions. However, I have a few comments for authors as follows.

- 1) Since OsHSFA4d negatively regulates disease resistance, it is logically strange to see that the expression of OsHSFA4d is also strongly induced by pathogen infection. Reasonable explanation is needed.
- 2) What is the biological significance for the activation the expression of CslF6 thus to suppress disease resistance under heat stress? If explained as a trade-off, should the activation of CslF6 be also beneficial for improving thermotolerance? Or the thermotolerance mediated by the module will be compromised or decreased under pathogen infection?
- 3) How CslF6, a cellulose synthase-like protein, suppresses defense response? If the mechanism has been reported, it should be mentioned in the introduction or discussion. If not clear, at least some thoughtful discussion is necessary.
- 4) Does OsHSFA4d also confer thermotolerance at reproductive stage in rice? As heat stress at reproductive stage, rather than seedling stage, is the really big problem for rice production.
- 5) Does OsHSFA4d has any natural variations to be explored for thermotolerance breeding instead of using transgenic approach?
- 6) The oshsfa4d mutant was reported with spotted leaf phenotype, and authors claimed the same in the discussion. But according to the oshsfa4d leaf segment pictures in Figures, the spotted leaf phenotype is hardly visible in the shown leaves even from tillering stage. Please clarify this issue.

Reviewer #3

(Remarks to the Author)

The study presents intriguing insights into the phosphorylation dynamics of OsHSF4d and identifies potential upstream kinases, OsCDPK24 and OsCDPK28, through quantitative IP-MS analysis. While the work addresses a relevant and timely research question, I believe certain methodological aspects and data presentation need improvement to enhance the data quality. Below, I outline several comments and suggestions for the authors to address before the manuscript can be considered for publication in Nature Communications.

1. In Figure 4a, the authors propose OsCDPK24 and OsCDPK28 as candidate upstream kinases of OsHSF4d, based on quantitative IP-MS data comparing kinase abundance between GST-OsHSF4d and GST alone. However, the lack of biological replicates in the two comparisons raises concerns about the reproducibility of the findings. It is strongly recommended to include biological replicates to substantiate the reproducibility of these data.
2. Supplemental Table 4 does not provide quantitative information. The authors should include quantitative data from all biological replicates, as obtained from the Proteome Discoverer (PD) output tables. Additionally, the identification of OsCDPK24 and OsCDPK28 is based on only one unique peptide. Quantitative analysis using a single unique peptide is inadequate due to low protein coverage. A minimum of two unique peptides is required for robust quantitative analysis.
3. In Figure 5f, the labeling of fragment ions (b and y ions) is unclear. For instance, y₂, y₅, y₆, and y₇ ions are not visible in the spectra. The authors should provide a clearer representation of the spectra with explicitly labeled fragment ions for better interpretability.
4. To strengthen the evidence for OsHSF4d phosphorylation by OsCDPK24 and OsCDPK28, it is necessary to perform in vitro kinase reaction of the single-phosphorylation site mutants. These mutants should be subjected to incubate with OsCDPK24 and OsCDPK28, followed by LC-MS/MS analysis. The authors should provide quantitative analysis to confirm that only the S146-containing phosphopeptide shows increased phosphorylation after the kinase reaction, while other Ser/Thr residues do not.
5. Supplemental Table 6 should include quantitative information corresponding to the experiments described above. This data is crucial for validating the conclusions drawn from the kinase activity assays.
6. The manuscript lacks critical details regarding the LC-MS/MS analysis. The authors should include information on the LC-MS/MS setup, database search parameters, and quantitative analysis methods in the Methods section.
7. To comply with data availability standards, the authors should deposit all mass spectrometry raw files, Proteome Discoverer search files, and PD output tables in an online proteomics repository.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The revised manuscript is now substantially improved. Through new experimental data or additional explanation, the authors have responded to all my comments and successfully addressed my concerns. This is an impressive study about thermotolerance and disease resistance regulation in rice. I have no more questions.

Reviewer #2

(Remarks to the Author)

Authors have addressed my concerns and made the revisions accordingly. I have no more comments.

Reviewer #3

(Remarks to the Author)

All my concerns have been appropriately addressed. I would recommend for publication.

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Responses to Reviewer #1:

Global warming has the potential to dramatically reduce agricultural harvests. Heat stress is reported to lead to thermotolerance and suppression of disease resistance in rice. However, the molecular basis underlying this trade-off remains in rice is far from clear. HS factors (HSFs) are important transcription factors that regulate abiotic and biotic stress responses, binding the heat shock element (HSE) in promoters of target genes to regulate their expression. Whether and how HSFs are involved in plant immunity remain to be elucidated. Under abiotic and biotic stresses, the Ca²⁺ signal can be sensed by calcium-dependent protein kinases (CDPKs) and transduced through phosphorylation events, leading to gene expression changes. However, how these events regulate plant thermotolerance and disease resistance under heat stress remains poorly understood. The manuscript by Fang et al discovered that OsCDPK24 and OsCDPK28 are activated by heat stress to phosphorylate OsHSFA4d and enhances its ability to promote HSP101 expression, conferring plant thermotolerance, and CslF6 expression, suppressing plant disease resistance. The authors provided lots of experiment evidences to support their findings. However, they might need to address and response the following questions before its possible publication.

Response: Thank you for your high evaluation and constructive suggestions for our manuscript. Following your kind suggestions, we have thoroughly revised the manuscript by adding new data and refining the result descriptions as follows.

1. Thermotolerance is a phenotype that organism has the ability to survival under heat stress. Hence it is recommended that replacing ‘induce thermotolerance’ with ‘induce HS responses’ in line 20 and line 38.

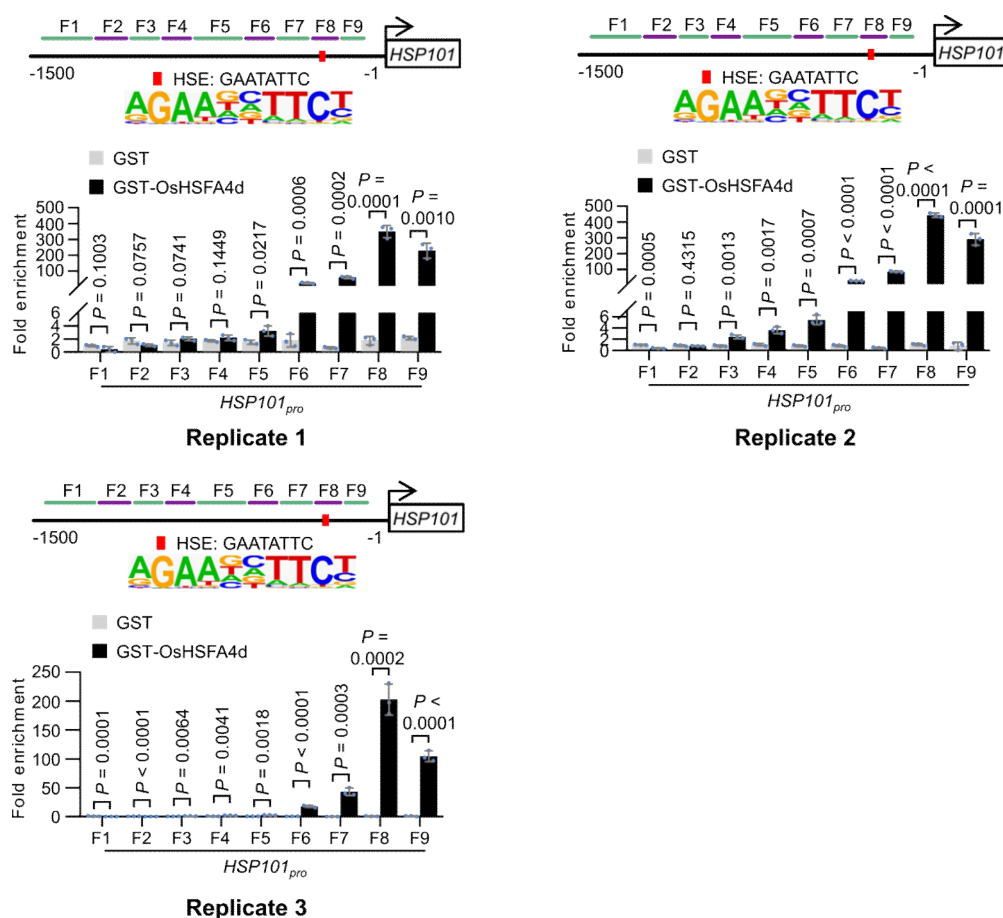
Response: Following your suggestion, we have replaced ‘induce thermotolerance’ with ‘induce HS responses’ in line 20 and line 39 on page 2.

2. HSFA4d binds to CslF6intron I (instead of promoter) which contains typical HSE to promote CslF6 expression. It is suggested that the authors discuss the possible underlying mechanism.

Response: Following your suggestion, we have added the discussion of the underlying mechanism by which OsHSFA4d binds to the first intron of *CslF6* to promote its expression (please see lines 222-227 on page 8 in the revised manuscript). We presume that the binding of OsHSFA4d to *CslF6* intron may either directly enhance transcription or facilitate mRNA processing, thereby increasing *CslF6* mRNA accumulation. Given that previous studies in animals and plants have shown that transcription factors can bind to internal introns or exons containing typical *cis*-elements to regulate target gene expression (Wei et al., *Science*, 2022, 377: eabi8455; Yang et al., *Nat. Commun.*, 2020, 11: 5118; Shetty et al., *Mol. Cell. Biol.*, 2005, 25: 5404-5416; Xia et al., *Plant Biotechnol. J.*, 2021, 19: 897-909), this potential mechanism may also apply to these similar scenarios.

3. In line 49-50, ‘HSFs bind to the heat shock element (HSE, 5’-nGAAnnTTCn-3’) in promoters of target genes to regulate their expression. In Fig 2g, F8 which contains the HSE is not the most enriched region, while F9 which contains no HSE is the most enriched region. It is suggested that the authors should explain this puzzle.

Response: Thanks for your careful reading and pointing out this puzzle. We speculate that this puzzle may be caused by the procedure of preparing fragmented DNA for DAP-qPCR. As described in the Methods section (see lines 650-651 on page 21 in the revised manuscript), the fragmented DNA was approximately 500 bp in length. Given that the F8 and F9 fragments are adjacent and both smaller than 150 bp, it is likely that the qPCR template contained large amounts of fragments that overlap between F8 and F9, leading to our observation that F8 and F9 were both enriched in the similar magnitude order. To resolve this, we optimized the experimental conditions by purifying the GST-OsHSFA4d protein with higher purity, and repeated the experiment with three biologically independent replicates, to obtain similar results with better quality. The results showed that the F8 fragment containing HSE is the region with the highest enrichment of OsHSFA4d in the *HSP101* promoter, instead of F9 fragment (see Figures of three biological replicates below the response, and Fig. 2g and lines 170-172 on page 6 in the revised manuscript), further suggesting that OsHSFA4d mainly binds to the HSE in the *HSP101* promoter.



4. In line 439-442, ‘One strategy to optimize OsHsfA4d function is to have OsHsfA4d expressed at low levels under severe disease conditions (mostly at seedling stage) but expressed at high levels when high temperature occurs and thermotolerance is required (at mature stage)’. This optimized expression pattern may be discovered in local varieties and accessions through natural variations analysis which is recommended to perform by the authors.

Response: Following your suggestion, we analyzed natural variation in the 2.1-kb promoter and coding region of *OsHsfA4d* across 4,045 rice accessions using RiceVarMap2 (Yu et al., *Rice*, 2021, 14: 16). We identified 36 variants in promoter and one 6-bp InDel in coding region, and classified them into four haplotypes (Hap1-Hap4) (see Supplementary Fig. 14b and lines 482-485 on page 15 in the revised manuscript). Among 33 high-quality sequenced varieties (Qin et al., *Cell*, 2021, 184: 3542-3558), we selected one representative variety per haplotype and measured *OsHsfA4d* expression levels in leaves at seedling, tillering, and reproductive stages (see Supplementary Table 10 and lines 485-487 on page 15 in the revised manuscript). The results showed that Hap2 in rice 93-11, as an elite haplotype, showed relatively low *OsHsfA4d* expression level compared with other haplotypes during seedling stage; whereas Hap1 in rice R527, as another elite haplotype, exhibited higher *OsHsfA4d* expression during reproductive stage (see Supplementary Fig. 14c and lines 487-490 on page 15 in the revised manuscript). These results indicate that introducing Hap2 into other rice varieties using molecular marker technology may enhance seedling disease resistance, while introducing Hap1 may improve thermotolerance at the reproductive stage.

5. Both OsCDPK24 and OsCDPK28 can phosphorylate OsHSFA4d at S146 to orchestrate plant thermotolerance and disease resistance, what is the functional difference between OsCDPK24 and OsCDPK28? Whether OsCDPK24 or OsCDPK28 could form homodimer?

Response: Our results showed that (1) OsCDPK24 and OsCDPK28 show similar subcellular localization (see Supplementary Fig. 8c and line 278 on page 9) (2) in rice, individual knockout of OsCDPK24 or OsCDPK28 similarly inhibits thermotolerance but enhances disease resistance (see Fig. 4i, j, Supplementary Fig. 10 and lines 296-303 on page 10 in the revised manuscript); (3) both OsCDPK24 and OsCDPK28 are able to phosphorylate the S146 amino acid residue of OsHSFA4d, thereby enhancing its transcriptional activation function (see Fig. 5h, i and lines 352-366 on pages 11-12; Fig. 6a-e and lines 382-394 on pages 12-13 in the revised manuscript). These findings suggest that OsCDPK24 and OsCDPK28 exhibit function similarities. We have added the relevant description in the main text (see lines 366-368 on page 12 in the revised manuscript). Our new LCI experiments in *N. benthamiana* revealed that neither OsCDPK24 nor OsCDPK28 could interact with themselves (see Supplementary Fig. 8e, f and lines 281-283 on page 9 in the revised manuscript), suggesting that OsCDPK24 or OsCDPK28 cannot form their respective homodimers. However, LCI and co-IP results showed that OsCDPK24 and OsCDPK28 interact with each other, and these two kinases also interact

with OsHSFA4d respectively (see Fig. 4b-g, Supplementary Fig. 8a, d and lines 258-287 on page 9 in the revised manuscript). Moreover, compared with single knockout, the double knockout of *OsCDPK24* and *OsCDPK28* has a more significant effect on weakening the thermotolerance while enhancing disease resistance in plants (see Fig. 4i, j, Supplementary Fig. 10 and lines 296-303 on page 10 in the revised manuscript), suggesting that combined activity of *OsCDPK24* and *OsCDPK28* exerts a stronger effect than either kinase individually. Taken together, these results suggest that *OsCDPK24* and *OsCDPK28* function similarly but also coordinate to certain extent with each other to regulate OsHSFA4d phosphorylation to regulate plant thermotolerance and disease resistance.

Responses to Reviewer #2:

This work discovered a regulatory module, consisting of OsCDPK24/OsCDPK28-OsHSFA4d-HSP101/CsIF6, in regulating the trade-off of thermotolerance and disease resistance in rice. Although the role of HSF-HSP in thermotolerance is well known and the role of CsIF6 in defense response has been also reported, the key novel finding of this work is that the OsHSFA4d can be activated by heat stress (HS)-activated CDPKs to positively and negatively regulate thermotolerance and disease resistance, respectively, which provide a mechanistic example to explain the phenomenon that HS induces thermotolerance but often causes decreased immunity to pathogens. The genetic and biochemical evidence are strong and complete to support the major conclusions. However, I have a few comments for authors as follows.

Response: Thank you for your high evaluation and constructive suggestions for our manuscript. Following your kind suggestions, we have thoroughly revised the manuscript by adding new data and refining the result descriptions as follows.

1. Since OsHSFA4d negatively regulates disease resistance, it is logically strange to see that the expression of OsHSFA4d is also strongly induced by pathogen infection. Reasonable explanation is needed.

Response: Following your suggestion, we have added relevant discussion regarding this phenomenon (please see lines 132-135 on page 5 in the revised manuscript). Not all genes induced by pathogen infection in the host positively regulate disease resistance. In fact, certain susceptibility genes, such as the wildtype gene *Bsr-d1* of *broad-spectrum resistance in Digu 1 (bsr-d1)* and *OsWRKY28*, are induced by pathogen infection and subsequently facilitate pathogen infection, (Zhu et al., *Nat. Commun.*, 2023, 14: 8399; Zhu et al., *Dev. Cell*, 2024, 59: 1–14). Our results showed that pathogen infection induces *OsHsfA4d* expression to promote virulence (see Fig. 1b, Supplementary Fig. 1a, b and lines 95-97 on page 4; Fig. 1e-g, and lines 117-125 on pages 4-5 in the revised manuscript), suggesting that *OsHsfA4d* functions as a susceptibility gene, similar to the wildtype gene *Bsr-d1* (Li et al., *Cell*, 2017, 170: 114-126) and *OsWRKY28* (Zhu et al., *Dev. Cell*, 2024, 59: 1–14).

2. What is the biological significance for the activation the expression of CsIF6 thus to suppress disease resistance under heat stress? If explained as a trade-off, should the activation of CsIF6 be also beneficial for improving thermotolerance? Or the thermotolerance mediated by the module will be compromised or decreased under pathogen infection?

Response: Our study reveals how rice utilizes *OsHsfA4d* to balance the trade-off between thermotolerance and disease resistance, providing significant insights into the biological significance of why plants are more susceptible to diseases under heat stress. Since both disease resistance and thermotolerance are energy-cost processes, we presume that rice

likely activates *CsIF6* expression under heat stress to inhibit disease resistance, thereby conserving sufficient energy for thermotolerance. We have added the relevant discussion into the Discussion section (see lines 436-441 on page 14 in the revised manuscript).

Interestingly, OsHSFA4d negatively regulates disease resistance via *CsIF6* but positively regulates thermotolerance via *HSP101* (see Fig. 2j and lines 190-194 on page 7; Fig. 3e-g and lines 235-237 on page 8 in the revised manuscript). Whereas, *CsIF6* does not regulate thermotolerance, and *HSP101* does not regulate disease resistance (see Fig. 2k, l and lines 194-197 on page 7; Fig. 3d and lines 233-235 on page 8 in the revised manuscript). These facts suggest that the activation of *CsIF6* is not beneficial for improving thermotolerance. Given that OsHSFA4d suppresses disease resistance through *CsIF6* while enhancing thermotolerance through *HSP101*, it is possible that the thermotolerance mediated by the module of OsHSFA4d-*CsIF6*/*HSP101* would be compromised or decreased under pathogen infection. We have added relevant discussion into the Discussion section (see lines 441-448 on page 14 in the revised manuscript).

3. How *CsIF6*, a cellulose synthase-like protein, suppresses defense response? If the mechanism has been reported, it should be mentioned in the introduction or discussion. If not clear, at least some thoughtful discussion is necessary.

Response: Following your suggestion, we have added relevant discussion on how *CsIF6* suppresses plant defense responses into the Discussion section (see lines 449-457 on page 14 in the revised manuscript). The Mixed-Linkage Glucan synthesized by *CsIF6* has been reported as a signaling molecule to negatively regulate plant defense responses and cell death (Vega-Sánchez et al., *Plant Physiol.*, 2012, 159: 59-69). Similarly, the hemicellulose xyloglucan synthesis gene *MURUS3* in *Arabidopsis thaliana* negatively regulates defense gene expression and plant resistance to downy mildew pathogen (Tedman-Jones et al., *Plant J.*, 2008, 56: 691-703). The cyclic beta-(1,2)-glucan produced by bacteria inhibit plant defense responses (Rigano et al., *Plant Cell*, 2007, 19: 2077-2089). In addition, the barley β -1,3-endoglucanase *HvBGLUII* releases a conserved β -1,3;1,6-glucan decasaccharide from the soluble extracellular polysaccharide matrix of fungi, which subverts host immunity (Chandrasekar et al., *Plant Cell*, 2022, 34: 2765-2784). Thus, glucan may suppress plant immune responses through complex mechanisms, including regulating enzyme activity, evading immune recognition, interfering with signaling pathways, and altering microbial community structure.

4. Does OsHSFA4d also confer thermotolerance at reproductive stage in rice? As heat stress at reproductive stage, rather than seedling stage, is the really big problem for rice production.

Response: Thank you for raising such a valuable question. We have added data to show that OsHSFA4d confers rice thermotolerance at the reproductive stage similarly as it does at seedling stage (see Supplementary Fig. 2a, b and lines 109-116 on page 4 in the revised

manuscript). We found that under normal conditions, *oshsfa4d* mutants showed significantly reduced seed-setting rates and yield compared to the wildtype Kitaake, likely due to the fitness cost caused by enhanced disease resistance. *OsHsfA4dOE* plants exhibited intermediate yield reductions between Kitaake and *oshsfa4d*, likely due to the fitness cost caused by improved thermotolerance. Following high-temperature treatment, *oshsfa4d* plants displayed greater yield losses than Kitaake, while *OsHsfA4dOE* plants showed better yield maintenance. These results suggest that OsHSFA4d confers thermotolerance at both seedling and reproductive stage in rice.

5. Does OsHSFA4d has any natural variations to be explored for thermotolerance breeding instead of using transgenic approach?

Response: Following your suggestion, we analyzed natural variation in the 2.1-kb promoter and coding region of *OsHsfA4d* across 4,045 rice accessions using RiceVarMap2 (Yu et al., *Rice*, 2021, 14: 16). We identified 36 variants in promoter and one 6-bp InDel in coding region, and classified them into four haplotypes (Hap1-Hap4) (see Supplementary Fig. 14b and lines 482-485 on page 15 in the revised manuscript). Among 33 high-quality sequenced varieties (Qin et al., *Cell*, 2021, 184: 3542-3558), we selected one representative variety per haplotype and measured *OsHsfA4d* expression levels in leaves at seedling, tillering, and reproductive stages (see Supplementary Table 10 and lines 485-487 on page 15 in the revised manuscript). The results showed that Hap2 in rice 93-11, as an elite haplotype, showed relatively low *OsHsfA4d* expression level compared with other haplotypes during seedling stage; whereas Hap1 in rice R527, as another elite haplotype, exhibited higher *OsHsfA4d* expression during reproductive stage (see Supplementary Fig. 14c and lines 487-490 on page 15 in the revised manuscript). These results indicate that introducing Hap2 into other rice varieties using molecular marker technology may enhance seedling disease resistance, while introducing Hap1 may improve thermotolerance at the reproductive stage.

6. The *oshsfa4d* mutant was reported with spotted leaf phenotype, and authors claimed the same in the discussion. But according to the *oshsfa4d* leaf segment pictures in Figures, the spotted leaf phenotype is hardly visible in the shown leaves even from tillering stage. Please clarify this issue.

Response: Thank you for raising such a valuable question. Our study revealed that the *oshsfa4d* mutant leaves did not exhibit cell-death-induced spotted leaf phenotypes during the seedling and tillering stages, with visible cell death phenotypes appearing at the reproductive stage (Supplementary Fig. 14a and lines 467-469 on page 15 in the revised manuscript). Consistently, as previously reported, the leaves of the *oshsfa4d* mutant did not exhibit obvious spotted leaf phenotypes until the reproductive stage (Yamanouchi et al., *Proc. Natl. Acad. Sci.*, 2002, 99: 7530-7535). Since the leaf segments of the *oshsfa4d*

mutant used for thermotolerance and disease resistance assays were collected in the seedling or tillering stages, the spotted leaf phenotype was hardly visible at that time.

Responses to Reviewer #3:

The study presents intriguing insights into the phosphorylation dynamics of OsHSF4d and identifies potential upstream kinases, OsCDPK24 and OsCDPK28, through quantitative IP-MS analysis. While the work addresses a relevant and timely research question, I believe certain methodological aspects and data presentation need improvement to enhance the data quality. Below, I outline several comments and suggestions for the authors to address before the manuscript can be considered for publication in Nature Communications.

Response: Thank you for your high evaluation and constructive suggestions for our manuscript. Following your suggestions, we have added biological replicates of the data, improved the presentation of the data, and refined the description of the methods to enhance the data quality of mass spectrum in the revised manuscript. For the detailed revision, please refer to the responses below.

1. In Figure 4a, the authors propose OsCDPK24 and OsCDPK28 as candidate upstream kinases of OsHSF4d, based on quantitative IP-MS data comparing kinase abundance between GST-OsHSF4d and GST alone. However, the lack of biological replicates in the two comparisons raises concerns about the reproducibility of the findings. It is strongly recommended to include biological replicates to substantiate the reproducibility of these data.

Response: Following your suggestion, we have added the results of three biological replicates for the mass spectrum experiments related to Fig. 4a. The results of added three biological replicates are consistent with the results in our previous manuscript, all showing that OsHSFA4d could specifically pull-down multiple peptides of the OsCDPK24 and OsCDPK28 proteins (please see Fig. 4a, Supplementary Table 4, and lines 253-257 on pages 8-9 in the revised manuscript). These biological replicates enhance the reproducibility of our findings, and further support that OsHSFA4d interacts with OsCDPK24 and OsCDPK28.

2. Supplemental Table 4 does not provide quantitative information. The authors should include quantitative data from all biological replicates, as obtained from the Proteome Discoverer (PD) output tables. Additionally, the identification of OsCDPK24 and OsCDPK28 is based on only one unique peptide. Quantitative analysis using a single unique peptide is inadequate due to low protein coverage. A minimum of two unique peptides is required for robust quantitative analysis.

Response: Following your suggestions, we have added quantitative analysis of the added three biological replicates in Supplementary Table 4 using MaxQuant software (version 2.4.14.0) and presented the corresponding quantitative data (see Supplementary Table 4 and lines 253-257 on pages 8-9 in the revised manuscript). The results showed that at least two peptides from OsCDPK24 or OsCDPK28 were detected in the pull-down products

and used for quantification, which significantly enhanced the robustness of our GST pull-down results. Moreover, the quantitative analysis showed that the protein abundance of OsCDPK24 and OsCDPK28 pulled down by GST-OsHSFA4d was significantly higher than that pulled down by the control GST, suggesting that OsHSFA4d interacts with OsCDPK24 and OsCDPK28.

3. In Figure 5f, the labeling of fragment ions (b and y ions) is unclear. For instance, y2, y5, y6, and y7 ions are not visible in the spectra. The authors should provide a clearer representation of the spectra with explicitly labeled fragment ions for better interpretability.

Response: Following your suggestion, we have replaced the spectra of Figure 5f in the previous manuscript by the spectra with more visible labels of b and y ions (especially y2, y5, y6, and y7) in the revised manuscript (see Fig. 5f and lines 345-347 on page 11 in the revised manuscript).

4. To strengthen the evidence for OsHSFA4d phosphorylation by OsCDPK24 and OsCDPK28, it is necessary to perform in vitro kinase reaction of the single-phosphorylation site mutants. These mutants should be subjected to incubate with OsCDPK24 and OsCDPK28, followed by LC-MS/MS analysis. The authors should provide quantitative analysis to confirm that only the S146-containing phosphopeptide shows increased phosphorylation after the kinase reaction, while other Ser/Thr residues do not.

Response: Thank you for offering us such valuable advice. We previously analyzed single-phosphorylation site mutants for OsHSFA4d residues phosphorylated by OsCDPK24 and OsCDPK28 using immunoblotting, and found that OsCDPK24 and OsCDPK28 phosphorylate OsHSFA4d mainly at S146 (Fig. 5g, h and lines 347-355 on page 11 in the revised manuscript). Following your suggestions, we have conducted in vitro kinase reaction experiments, incubating the single-phosphorylation site mutant OsHSFA4d^{S146A} with OsCDPK24 and subsequently analyzing the samples by phospho-mass spectrometry (phos-MS). The quantitative analyses of phos-MS from three biological replicates showed that, after co-incubation with OsCDPK24, phosphopeptides containing other serine and threonine residues in OsHSFA4d^{S146A} were detected, whereas the phosphopeptide containing the 146th amino acid residue was not able to be detected (see Supplementary Table 7 and lines 356-361 on pages 11-12 in the revised manuscript). Moreover, our previous immunoblotting using an antibody recognizing phosphorylated S146 showed that the S146A mutation significantly reduced the phosphorylation of OsHSFA4d by OsCDPK24 (see Fig. 5i and lines 363-366 on page 12 in the revised manuscript). Taken together, these results further support that S146 of OsHSFA4d is a key amino acid residue phosphorylated by OsCDPK24.

5. Supplemental Table 6 should include quantitative information corresponding to the experiments described above. This data is crucial for validating the conclusions drawn from the kinase activity assays.

Response: Following your suggestion, we have added quantitative analysis on the mass spectrometry data in Supplementary Table 6. The quantitative analysis showed that OsCDPK24 elevates the phosphorylation levels of multiple serine and threonine residues in OsHSFA4d (see Supplementary Table 6 and lines 344-345 on page 11 in the revised manuscript). These results suggest that OsCDPK24 directly phosphorylates OsHSFA4d, and is consistent with the results in the previous manuscript.

6. The manuscript lacks critical details regarding the LC-MS/MS analysis. The authors should include information on the LC-MS/MS setup, database search parameters, and quantitative analysis methods in the Methods section.

Response: Following your suggestions, we have added detailed information on the LC-MS/MS setup, database search parameters, and quantitative analysis methods in the Methods section (see lines 677-693 on page 22; lines 737-753 on page 24 in Methods of the revised manuscript).

7. To comply with data availability standards, the authors should deposit all mass spectrometry raw files, Proteome Discoverer search files, and PD output tables in an online proteomics repository.

Response: Following your suggestion, we have deposited all the mass spectrometry raw files, MaxQuant search files, and MaxQuant output tables in the online proteomics repository (see lines 793-796 on page 25 in the revised manuscript).