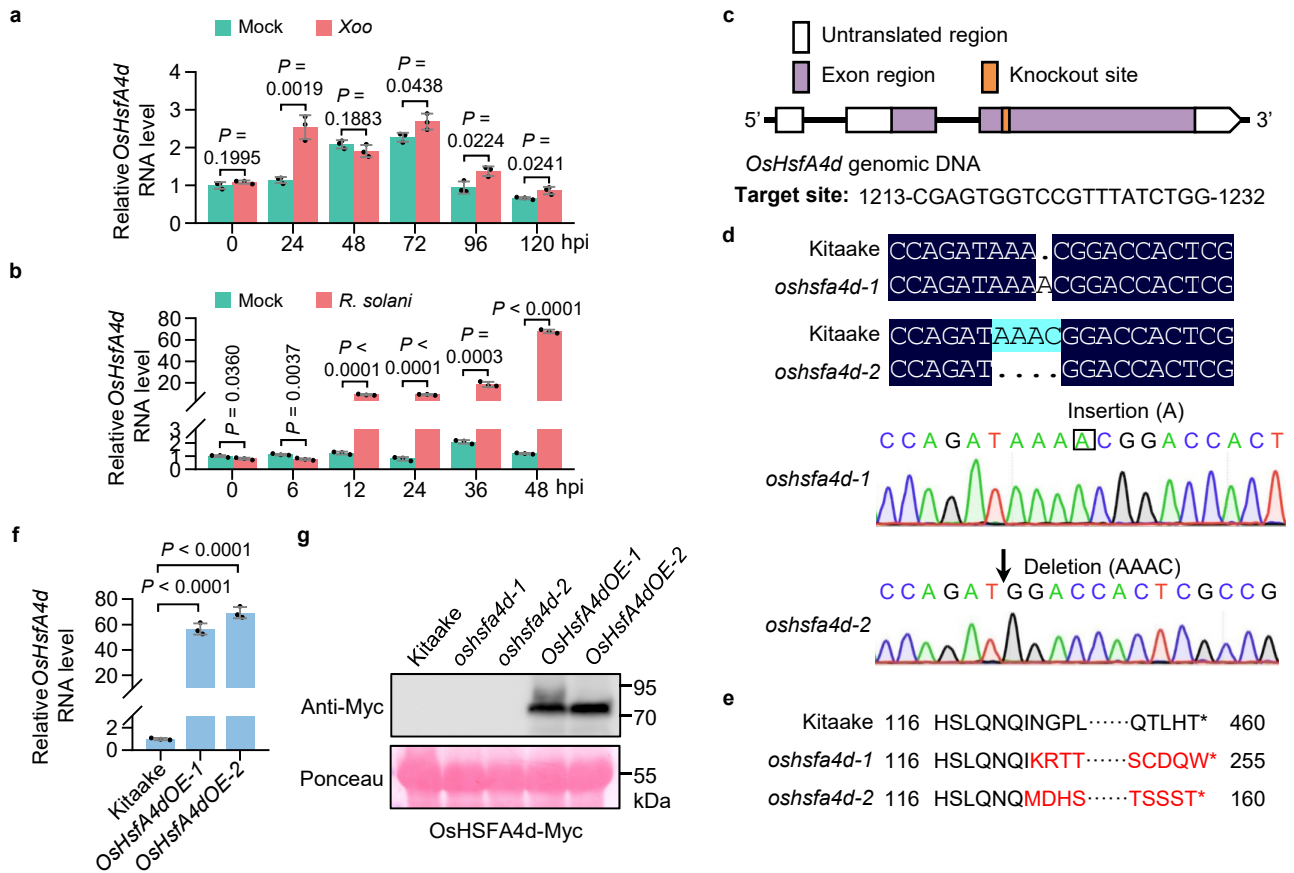


**OsCDPK24 and OsCDPK28 phosphorylate heat shock factor
OsHSFA4d to orchestrate abiotic and biotic stress responses in rice**

Fang et al.

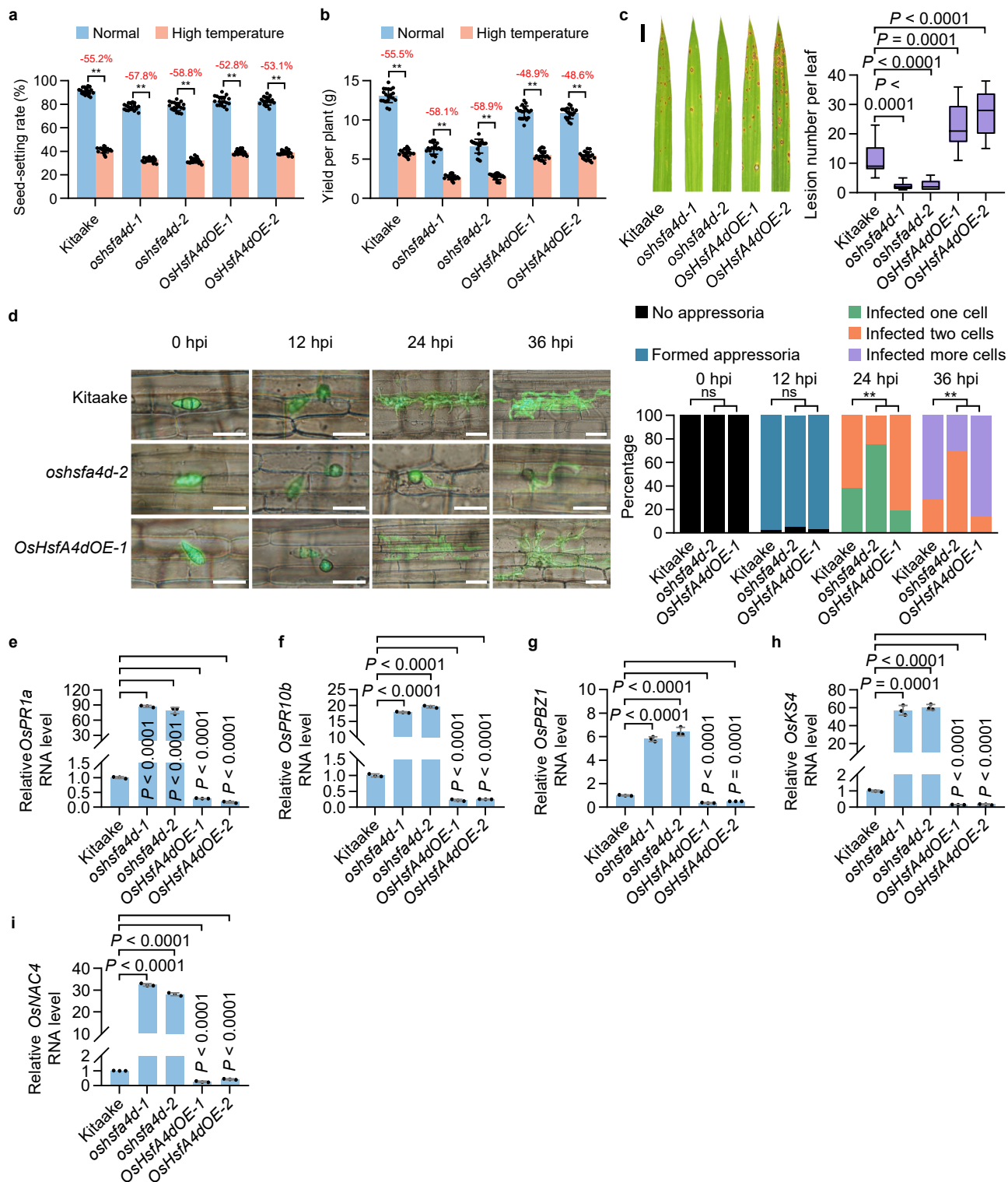
Supplementary Information

Supplementary Figures 1-14



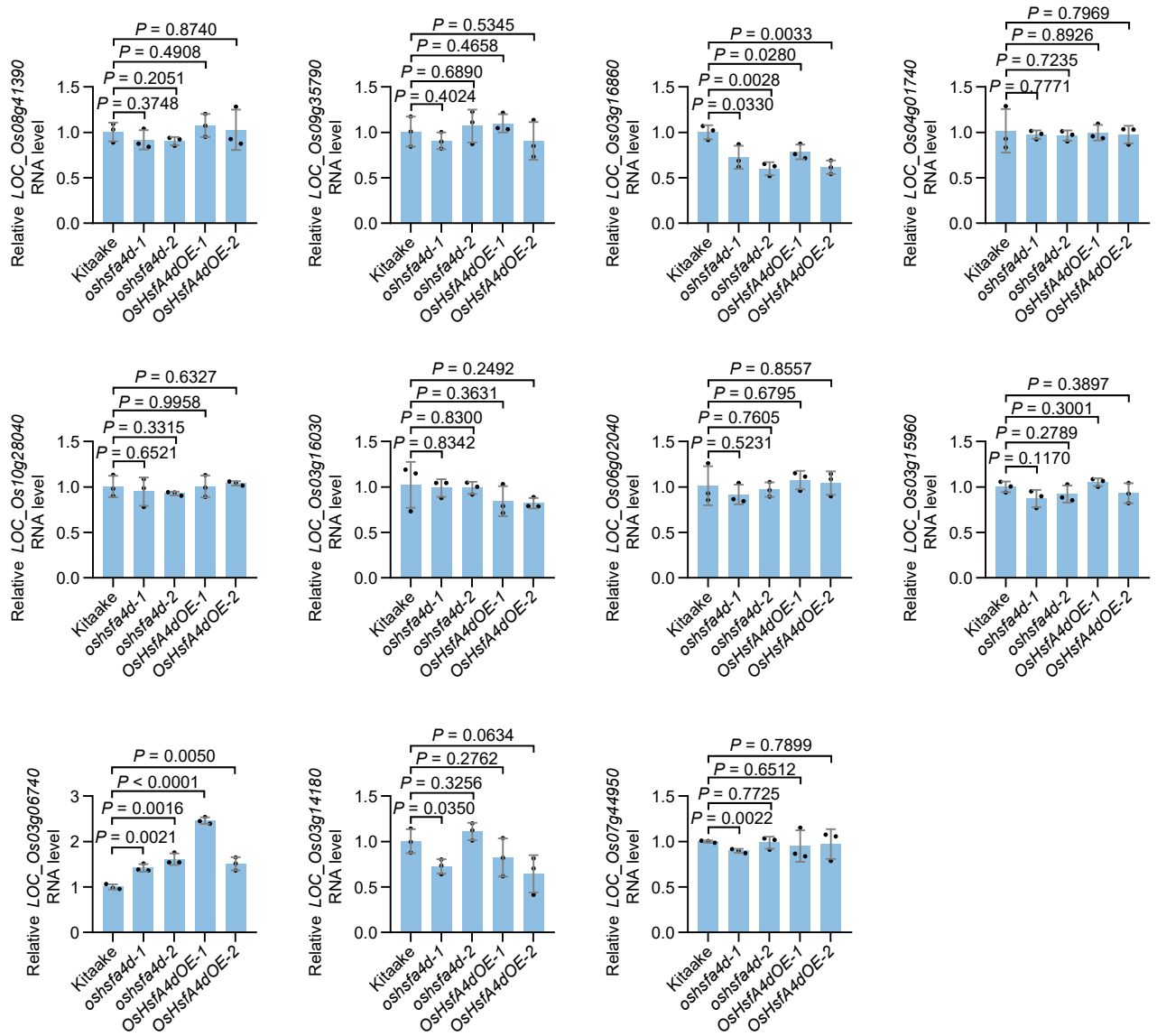
Supplementary Fig. 1 RNA levels of *OsHsfA4d* in response to pathogen infection and the generation of *OsHsfA4d* knockout (KO) and overexpression (OE) rice lines.

a, b RNA levels (mean values $\pm$ SD, $n = 3$ technical replicates) of *OsHsfA4d* in three-week-old Kitaake leaves at 0-120 hpi with *Xoo* (**a**) or at 0-48 hpi with *R. solani* (**b**). **c** Schematic drawing of the *OsHsfA4d* target site for *OsHsfA4d* knockout using CRISPR/Cas9. **d** Alignment of target site sequences of wildtype Kitaake and two independent *OsHsfA4d* knockout lines ($oshhsfa4d-1$ and $oshhsfa4d-2$) based on PCR-based sequencing. **e** Amino acid sequence alignment of OsHsfA4d proteins from Kitaake, $oshhsfa4d-1$ and $oshhsfa4d-2$ lines. Each mutation leads to a frameshift and premature termination in the OsHsfA4d protein. **f** RNA levels of *OsHsfA4d* were measured in three-week-old Kitaake and *OsHsfA4dOE* plants measured by RT-qPCR (mean values $\pm$ SD, $n = 3$ technical replicates). P values were calculated using two-sided Student's t -test (**a, b, f**). **g** Protein levels of OsHsfA4d-Myc were measured in three-week-old Kitaake, $oshhsfa4d$, and *OsHsfA4dOE* plants using anti-Myc in immunoblot analysis. All experiments were repeated three times with consistent results.



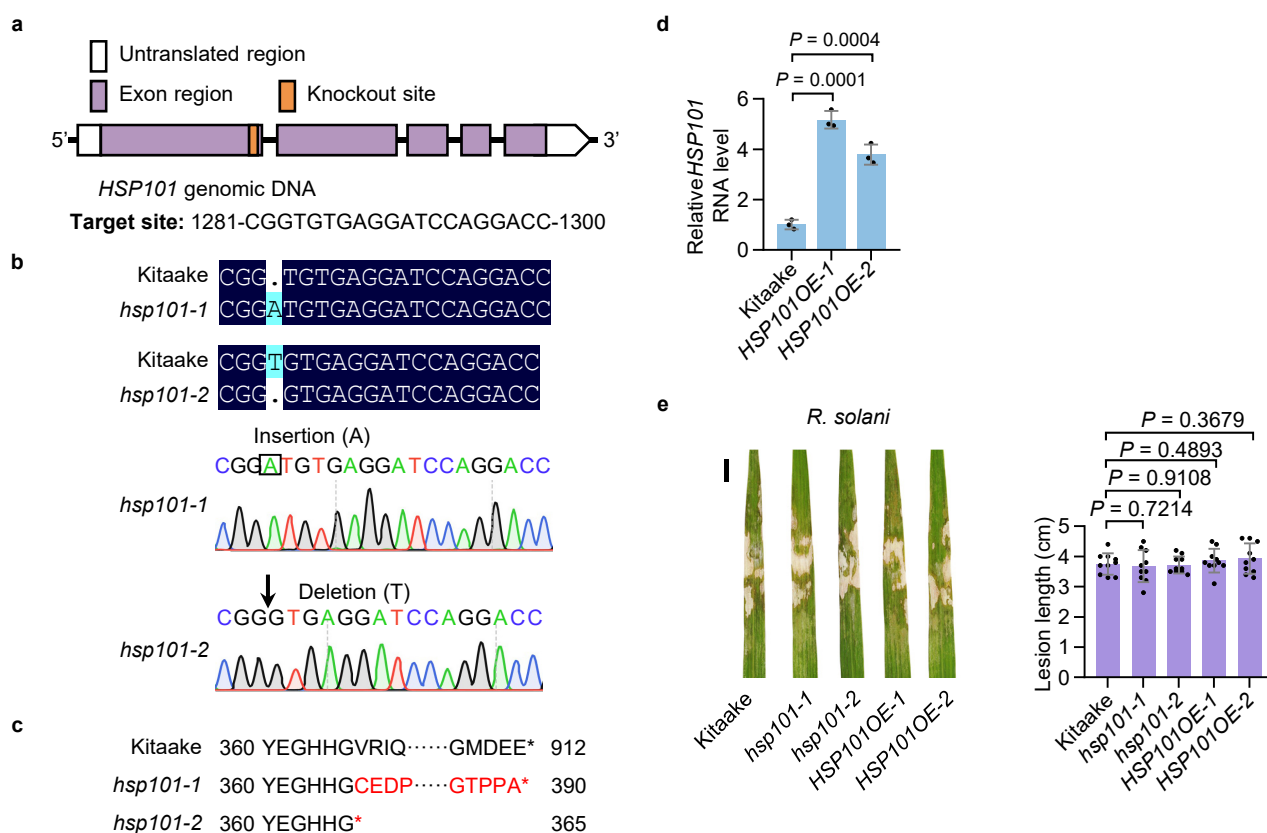
Supplementary Fig. 2 *OshsfA4d* positively regulates thermotolerance at the reproductive stage but negatively modulates immunity in rice.

a, b Comparison of thermotolerance among Kitaake, *oshfsfa4d*, and *OsHsfA4dOE* plants at the reproductive stage in growth chambers. These plants were grown at 26°C until heading, then exposed to normal (26°C) or high-temperature (42°C) conditions for 7 days, followed by recovery at 26°C until maturity. Seed-setting rate (**a**, $n = 20$) and grain yield per plant (**b**, $n = 20$) were measured. **c** Photographs of representative lesions and lesion numbers per leaf ($n = 13, 12, 11, 16$, and 16 leaves, respectively) on leaves of three-week-old Kitaake, *oshfsfa4d*, and *OsHsfA4dOE* plants 7 days post spray inoculation with *M. oryzae*. Box plots show the median (50%) and interquartile range (25-75%), and whiskers show minimum and maximum values. **d** Infection stages of *M. oryzae* expressing GFP in sheath cells of four-week-old Kitaake, *oshfsfa4d*, and *OsHsfA4dOE* plants. Representative scanning microscopy images and distribution of fungal infection progressing at 0-36 hpi ($n = 40$ cells). **e-i** RNA levels of *PR* genes in three-week-old Kitaake, *oshfsfa4d*, and *OsHsfA4dOE* plants were measured by RT-qPCR ($n = 3$ technical replicates). Data are presented as mean values $\pm$ SD, and P values were calculated using two-sided Student's *t*-test, and ** indicates $P < 0.01$. Scale bars: 1 cm (**c**) and 20 μ m (**d**). All experiments were repeated three times with consistent results.



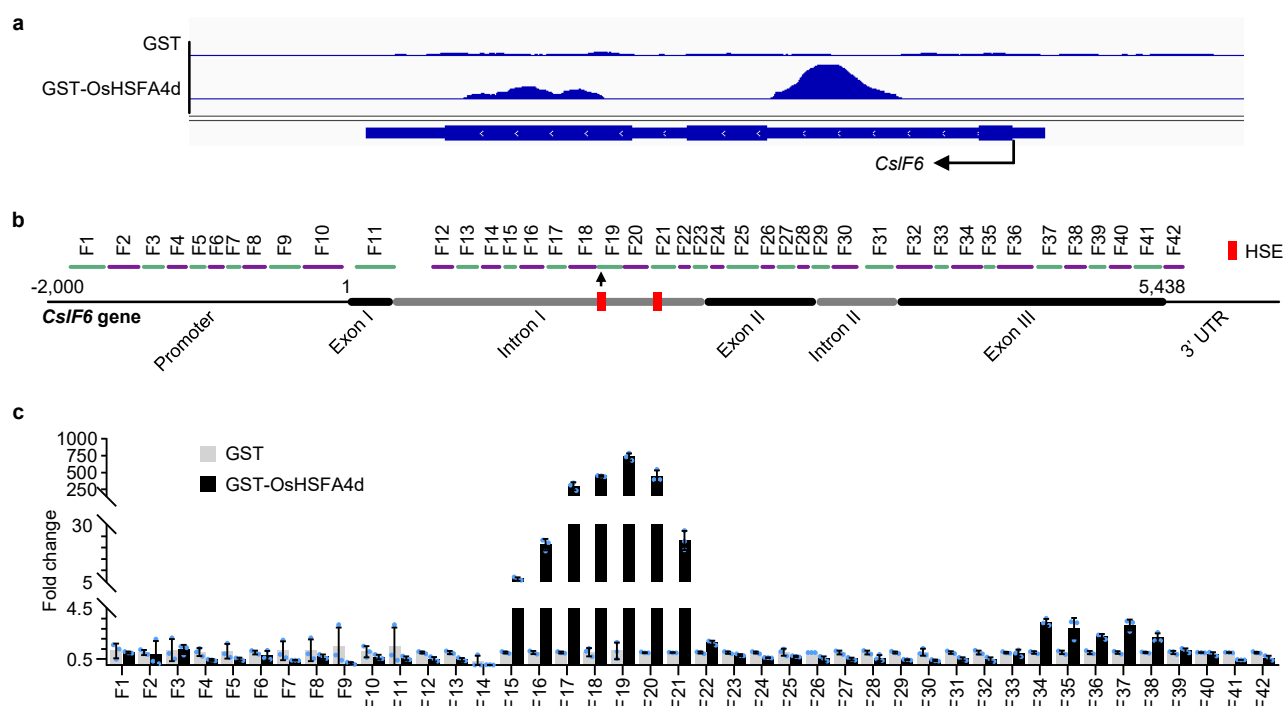
Supplementary Fig. 3 Selection of OsHSFA4d downstream target genes involved in the HS response.

RT-qPCR analysis shows the expression levels of candidate OsHSFA4d downstream genes, which were reported to be involved in the HS response, in three-week-old Kitaake, *oshsfa4d*, and *OsHsfA4dOE* plants (mean values $\pm$ SD, $n = 3$ technical replicates). P values were calculated using two-sided Student's t -test. All experiments were repeated three times with consistent results.



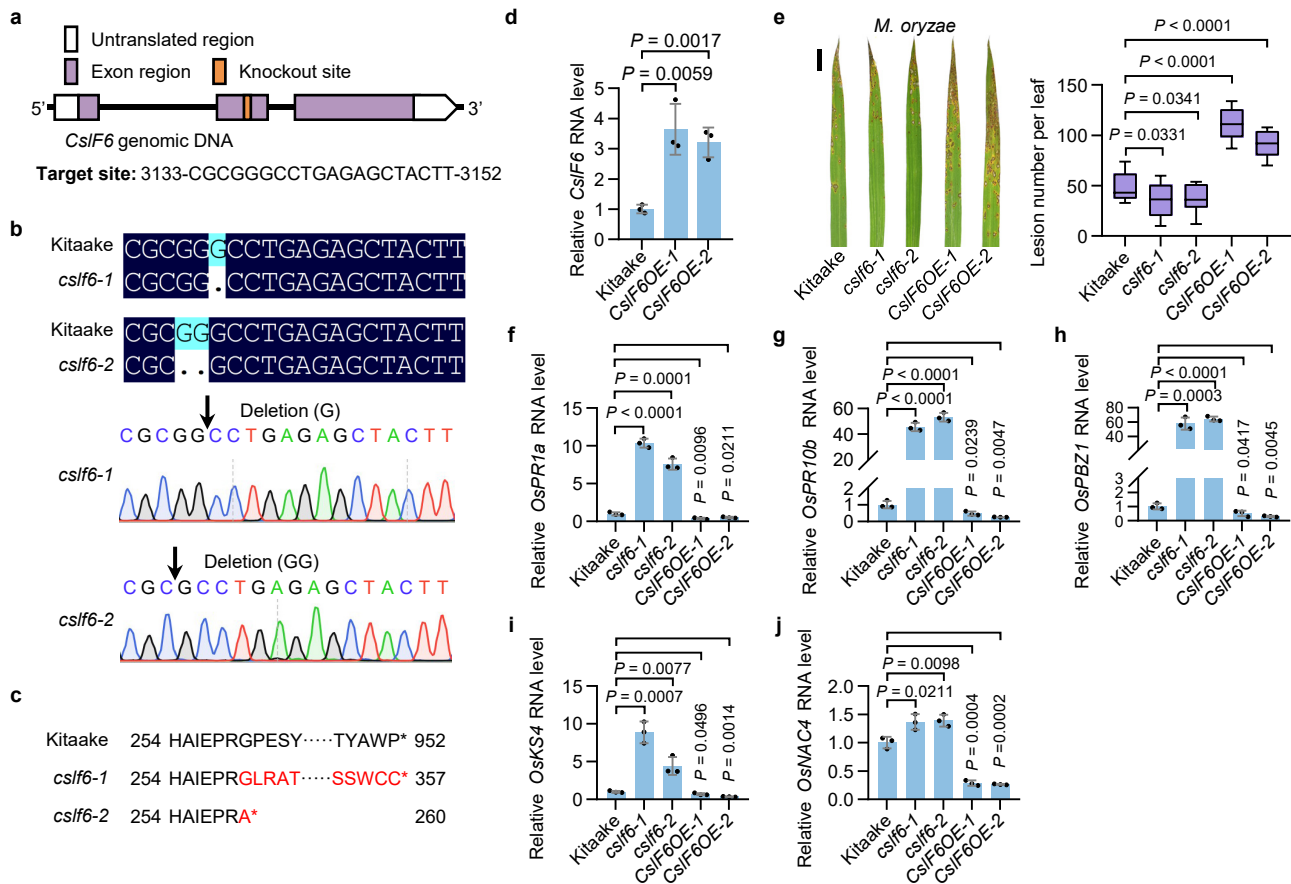
Supplementary Fig. 4 Generation of *HSP101* knockout and overexpression rice lines and *HSP101* has no obvious role in plant disease resistance.

a Schematic drawing of the *HSP101* target site for *HSP101* knockout using CRISPR/Cas9. **b** Alignment of target site sequences of wildtype Kitaake and two independent *HSP101* knockout lines (*hsp101-1* and *hsp101-2*) based on PCR-based sequencing. **c** Amino acid sequence alignment of *HSP101* proteins of Kitaake, *hsp101-1* and *hsp101-2* plants. Each mutation leads to a frameshift and premature termination in the *HSP101* protein. **d** RNA levels of *HSP101* in three-week-old Kitaake and *HSP101OE* plants were measured by RT-qPCR (mean values $\pm$ SD, $n = 3$ technical replicates). **e** Photographs of representative lesions and lesion lengths (mean values $\pm$ SD, $n = 10$ lesions) on leaves of three-week-old Kitaake, *hsp101*, and *HSP101OE* plants 2 dpi with *R. solani*. Scale bar, 1 cm. P values were calculated using two-sided Student's t -test (**d**, **e**). All experiments were repeated three times with consistent results.



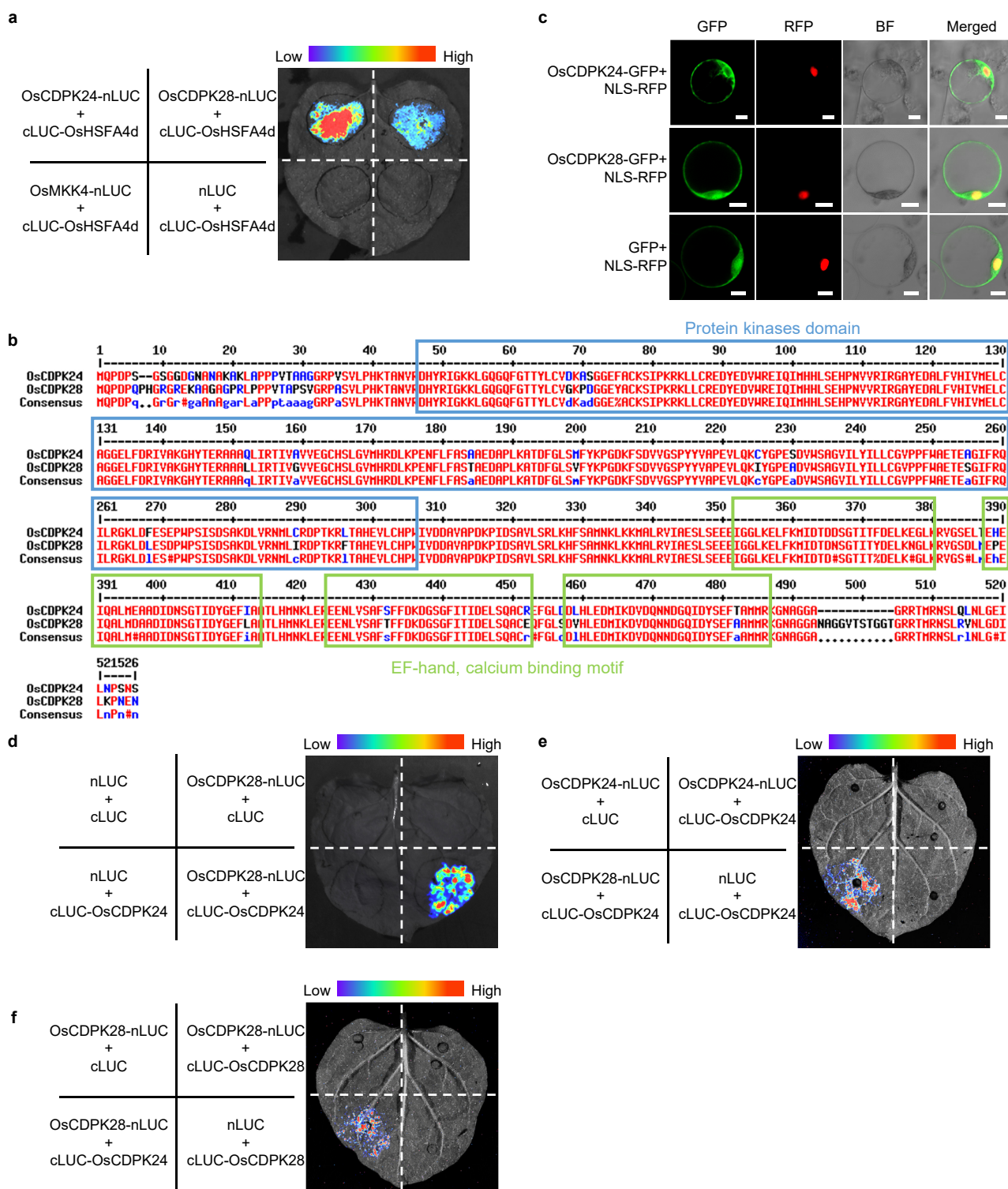
Supplementary Fig. 6 OsHSFA4d binds to the HSE in *CsIF6*_{intron I}

a OsHSFA4d binds to the first intron of *CsIf6* in DAP-seq. **b, c** DAP-qPCR assay to detect OsHSFA4d binding to the first intron of *CsIf6*. Schematic drawing of the locations of 42 fragments (F1-F42) of the promoter, exons, introns, and 3' UTR of *CsIf6*. The heat shock elements (HSEs) were shown in red boxes (**b**). Fold enrichment of sonicated rice DNA pulled down by GST-OsHSFA4d (**c**, mean values $\pm$ SD, $n = 3$ technical replicates). GST was used as a negative control. All experiments were repeated three times with consistent results.



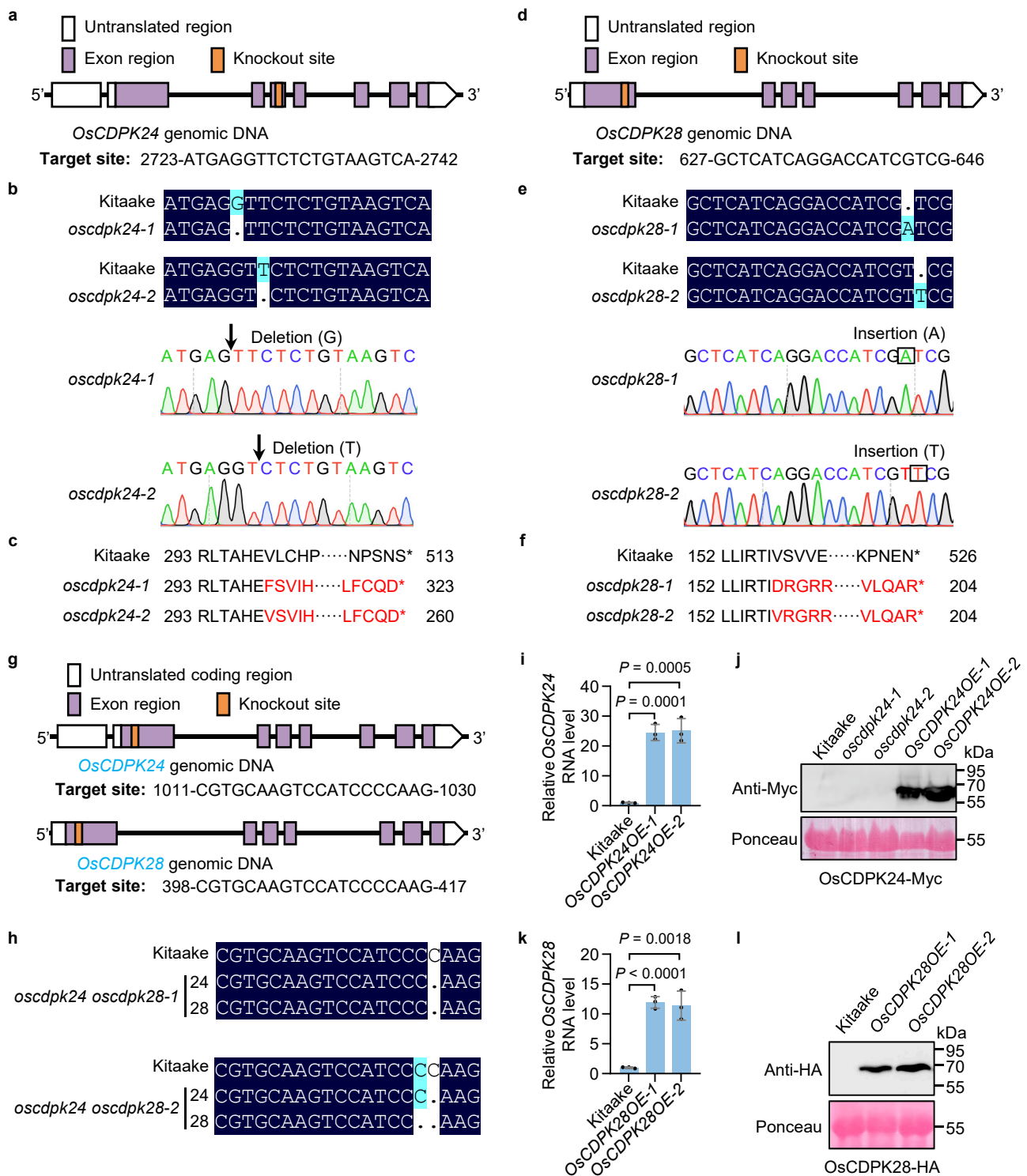
Supplementary Fig. 7 *CsIF6* suppresses plant disease resistance.

a Schematic drawing of the *CsIF6* target site for *CsIF6* knockout using CRISPR/Cas9. **b** Alignment of target site sequences of wildtype Kitaake and two independent *CsIF6* knockout lines (*csIf6-1* and *csIf6-2*) based on PCR-based sequencing. **c** Alignment of amino acid sequences of CSLF6 proteins of Kitaake, *csIf6-1*, and *csIf6-2* plants. Each mutation leads to a frameshift and premature termination in the CSLF6 protein. **d** RNA levels of *CsIF6* in three-week-old Kitaake and *CsIF6OE* plants were measured by RT-qPCR (mean values $\pm$ SD, $n = 3$ technical replicates). **e** Representative lesions and lesion numbers per leaf (mean values $\pm$ SD, $n = 15, 14, 12, 15$, and 12 leaves, respectively) on leaves of three-week-old Kitaake, *csIf6*, and *CsIF6OE* plants 7 days post spray inoculation with *M. oryzae*. Box plots show the median (50%) and interquartile range (25-75%), and whiskers show minimum and maximum values. Scale bar, 1 cm. **f-j** RNA levels of *PR* genes in three-week-old Kitaake, *csIf6*, and *CsIF6OE* plants measured by RT-qPCR (mean values $\pm$ SD, $n = 3$ technical replicates). P values were calculated using two-sided Student's *t*-test (**d-j**). All experiments were repeated three times with consistent results.



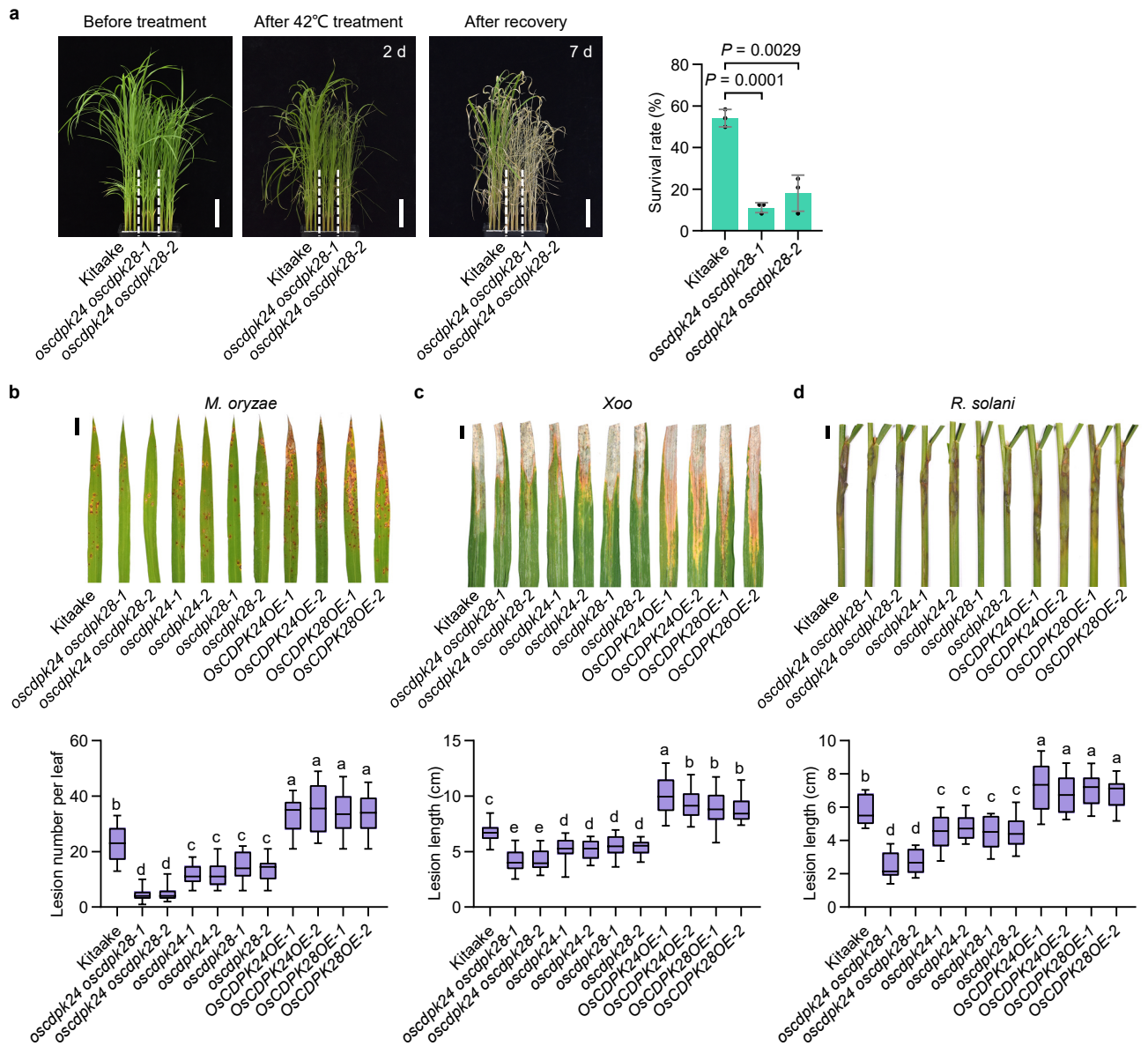
Supplementary Fig. 8 OsCDPK24, OsCDPK28, and OsHSFA4d form a complex.

a Luciferase complementation imaging (LCI) was performed to test interaction between OsHSFA4d/OsCDPK24, OsHSFA4d/OsCDPK28 and OsHSFA4d/OsMKK4 in transiently transfected *N. benthamiana* leaves. OsMKK4 was used as a negative control. **b** Amino acid sequence alignment for OsCDPK24 and OsCDPK28. The protein kinase domain is highlighted in blue boxes. The Ca^{2+} binding motif is indicated in green boxes. **c** Subcellular localization of OsCDPK24 and OsCDPK28 in rice protoplasts. NLS-RFP marks the nucleus. Scale bar, 10 μm . **d** Interaction between OsCDPK24 and OsCDPK28 detected by LCI in transiently transfected *N. benthamiana* leaves. **e** Self interaction of OsCDPK24 was detected by LCI in transiently transfected *N. benthamiana* leaves. **f** Self interaction of OsCDPK28 was detected by LCI in transiently transfected *N. benthamiana* leaves. Empty vectors expressing nLUC and cLUC were used as negative controls (**a**, **d-f**). All experiments were repeated three times with consistent results.



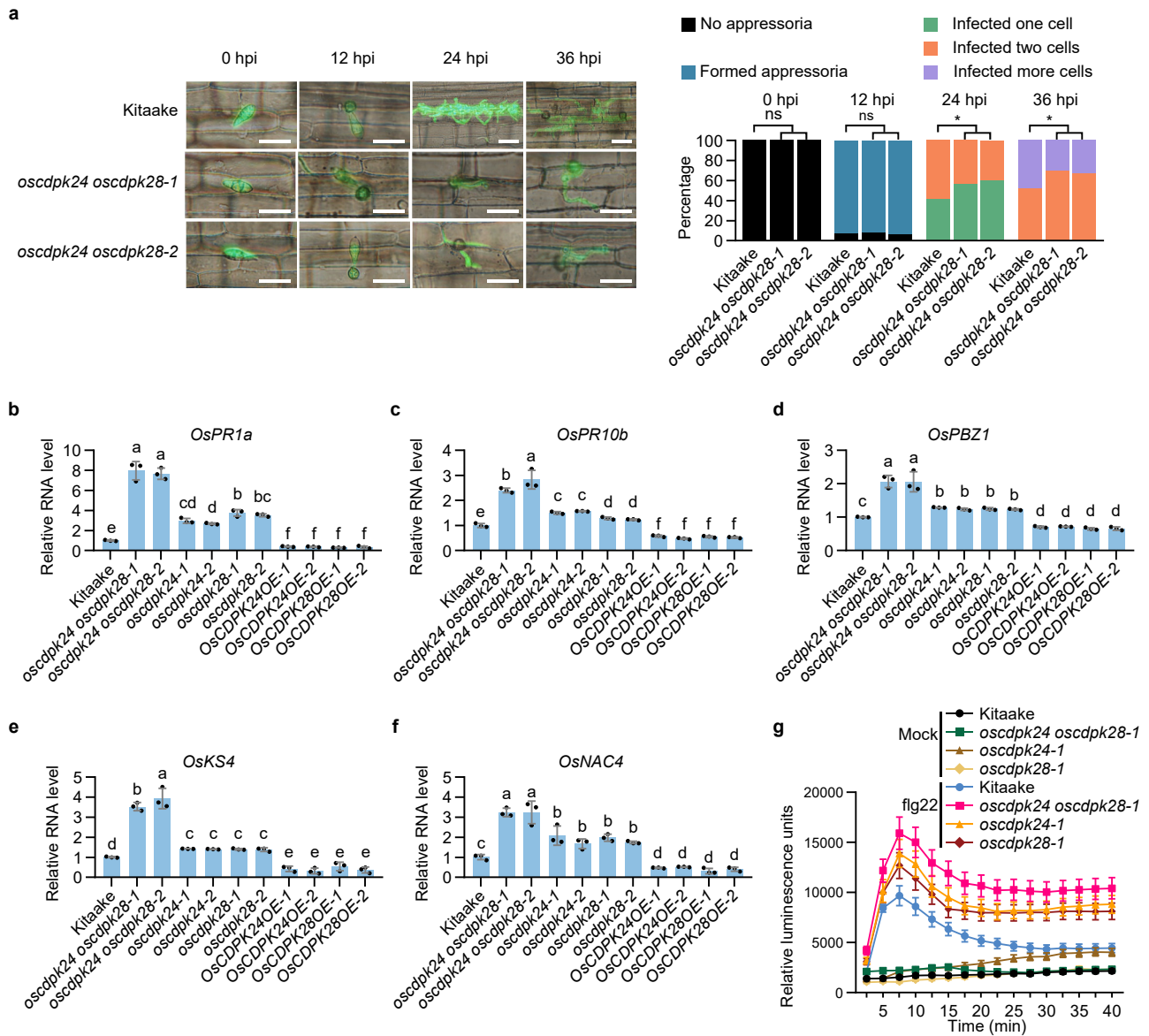
Supplementary Fig. 9 Generation of *OsCDPK24/28* knockout and overexpression rice lines.

a Schematic drawing of the *OsCDPK24* target site for *OsCDPK24* knockout using CRISPR/Cas9. **b** Alignment of target site sequences of wildtype Kitaake and two independent *OsCDPK24* knockout lines (*oscdpk24-1* and *oscdpk24-2*) analyzed based on PCR-based sequencing. **c** Amino acid sequence alignment of *OsCDPK24* proteins of Kitaake, *oscdpk24-1*, and *oscdpk24-2* lines. Each mutation leads to a frameshift and premature termination in the *OsCDPK24* protein. **d** Schematic drawing of the *OsCDPK28* target site for *OsCDPK28* knockout. **e** Alignment of target site sequences of Kitaake and two independent *OsCDPK28* knockout lines (*oscdpk28-1* and *oscdpk28-2*). **f** Amino acid sequence alignment of *OsCDPK28* proteins of Kitaake, *oscdpk28-1*, and *oscdpk28-2* plants. Each mutation leads to a frameshift and premature termination in the *OsCDPK28* protein. **g** Schematic drawing of the *OsCDPK24* and *OsCDPK28* target site sequences for *OsCDPK24* and *OsCDPK28* double knockout. **h** Alignment of target site sequences of Kitaake and two independent lines with *OsCDPK24* and *OsCDPK28* double knockout (*oscdpk24 oscdpk28-1* and *oscdpk24 oscdpk28-2*). **i** RNA levels of *OsCDPK24* were measured in three-week-old Kitaake and *OsCDPK24OE* plants (mean values $\pm$ SD, $n = 3$ technical replicates). **j** Protein levels of *OsCDPK24*-Myc were detected in three-week-old Kitaake, *oscdpk24*, and *OsCDPK24OE* plants by anti-Myc in immunoblot analysis. **k** RNA levels of *OsCDPK28* in three-week-old Kitaake and *OsCDPK28OE* plants (mean values $\pm$ SD, $n = 3$ technical replicates). **l** Protein levels of *OsCDPK28*-HA were detected in three-week-old Kitaake and *OsCDPK28OE* plants by anti-HA in immunoblot analysis. P values were calculated using two-sided Student's t -test (**i**, **k**). All experiments were repeated three times with consistent results.



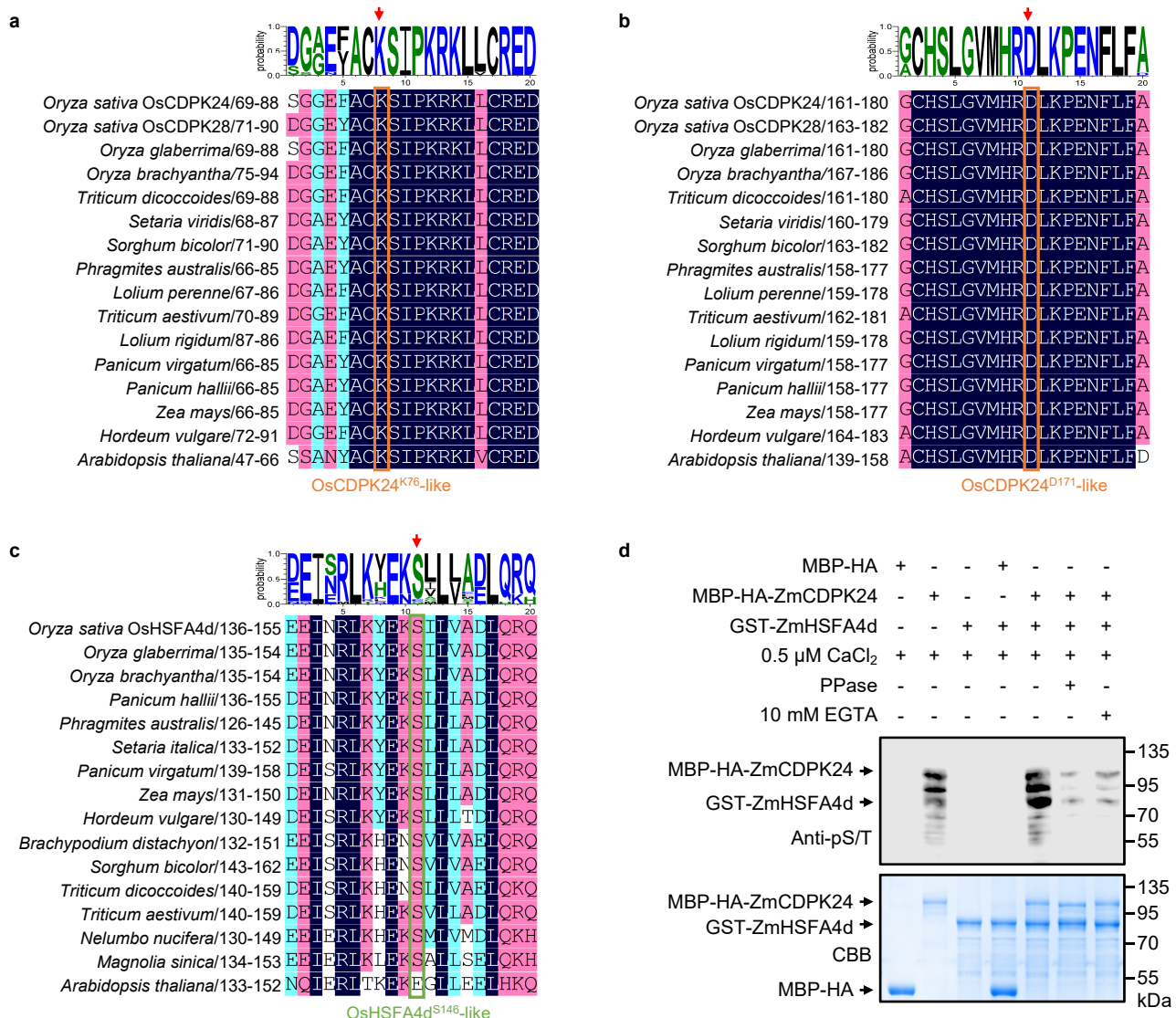
Supplementary Fig. 10 OsCDPK24 and OsCDPK28 promote plant thermotolerance but suppress disease resistance.

a Representative rice plants and survival rates (mean values $\pm$ SD, $n = 3$ biological replicates) of three-week-old Kitaaake and *oscdpk24 oscdpk28* lines before and after 42°C high temperature treatment for three days, followed by recovery at 26°C for seven days. P values were calculated using two-sided Student's t -test. **b** Photographs of representative lesions and lesion lengths (mean values $\pm$ SD, $n = 23, 25, 25, 20, 21, 15, 20, 15, 12, 10$, and 12 lesions, respectively) on leaves of three-week-old Kitaaake, *oscdpk24 oscdpk28*, *oscdpk24*, *oscdpk28*, *OsCDPK24OE*, and *OsCDPK28OE* plants 7 days post punch inoculation with *M. oryzae*. **c** Photographs of representative lesions and lesion lengths (mean values $\pm$ SD, $n = 14$ lesions) on leaves of tillering-stage Kitaaake, *oscdpk24 oscdpk28*, *oscdpk24*, *oscdpk28*, *OsCDPK24OE*, and *OsCDPK28OE* plants 14 dpi with *Xoo*. **d** Photographs of representative lesions and lesion lengths (mean values $\pm$ SD, $n = 9$ lesions) on leaf sheaths of tillering-stage Kitaaake, *oscdpk24 oscdpk28*, *oscdpk24*, *oscdpk28*, *OsCDPK24OE*, and *OsCDPK28OE* plants 5 dpi with *R. solani*. Different letters above bars indicate differences at $P < 0.05$ by one-way ANOVA with LSD test (**b-d**). Box plots show the median (50%) and interquartile range (25-75%), and whiskers show minimum and maximum values (**b-d**). Scale bars: 5 cm (**a**) and 1 cm (**b-d**). All experiments were repeated three times with consistent results.



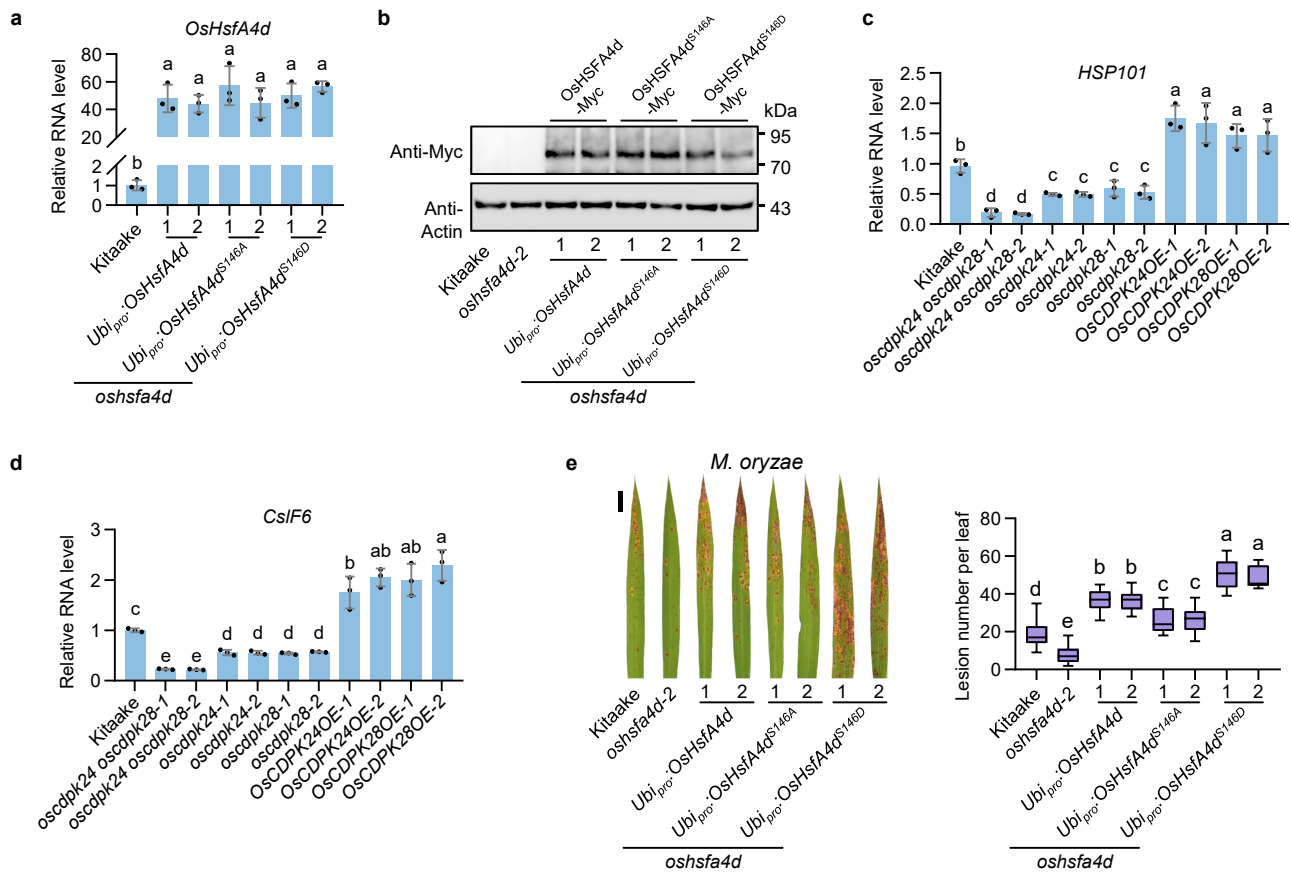
Supplementary Fig. 11 OsCDPK24 and OsCDPK28 suppress plant immunity.

a Infection stages of *M. oryzae* expressing GFP in leaf sheaths of four-week-old Kitaake and *oscdtk24 oscdtk28* plants. Scanning microscopy images and distribution of fungal infection progressing at 0-36 hpi (mean values $\pm$ SD, $n = 40$ cells). Scale bar, 20 μ m. P values were calculated using two-sided Student's t -test, and * indicates $0.01 < P < 0.05$. **b-f** RNA levels of *PR* genes were measured in three-week-old Kitaake, *oscdtk24 oscdtk28*, *oscdtk24*, *oscdtk28*, *OsCDPK24OE*, and *OsCDPK28OE* plants (mean values $\pm$ SD, $n = 3$ technical replicates). Different letters above bars indicate differences at $P < 0.05$ by one-way ANOVA with LSD test. **g** ROS bursts were detected by the luminol-horseradish peroxidase method in small pieces of leaves from three-week-old Kitaake, *oscdtk24 oscdtk28*, *oscdtk24* and *oscdtk28* treated with or without flg22 (mean values $\pm$ SD, $n = 3$ biological replicates). All experiments were repeated three times with consistent results.



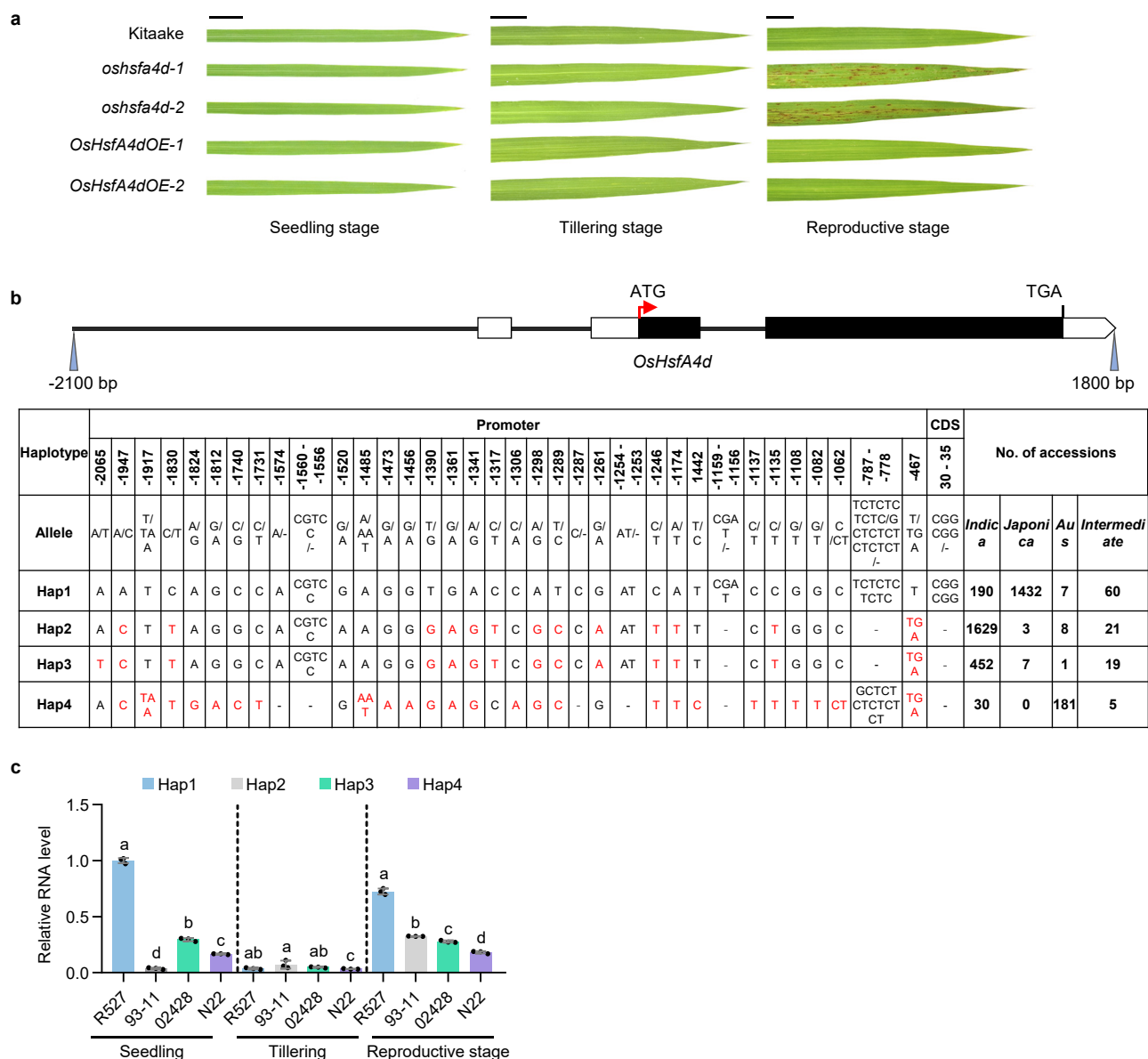
Supplementary Fig. 12 Conservation of the OsCDPK24/28-mediated OsHSFA4d phosphorylation mechanism in the plant kingdom.

a, b K76 (**a**), D171(**b**), and their neighboring residues of OsCDPK24 are conserved among orthologous proteins from different plant species. The red arrows and orange boxes highlight the conserved K76-like (**a**) and D171-like (**b**) residues in OsCDPK24 orthologues. **c** S146 and neighboring residues of OsHSFA4d are relatively conserved among different plant species. The red arrow and green box highlight the conserved S146-like residues in OsHSFA4d orthologues. **d** In vitro phosphorylation of GST-ZmHSFA4d by MBP-HA-ZmCDPK24 or mock (MBP-HA) after a 30-min kinase reaction. Phosphorylation was detected by anti-pS/T on membrane after separation in a PAGE gel. EGTA was used as a Ca²⁺-chelating agent; PPase, phosphatase. This experiment was performed in three independent replicates with similar results obtained.



Supplementary Fig. 13 S146 is essential for OsHsfA4d function.

a RNA levels of *OsHsfA4d* in leaves of three-week-old Kitaake, *Ubi_{pro}:OsHsfA4d/oshfsfa4d*, *Ubi_{pro}:OsHsfA4d^{S146A}/oshfsfa4d* and *Ubi_{pro}:OsHsfA4d^{S146D}/oshfsfa4d* (n = 3 technical replicates). **b** Protein levels of OsHsfA4d-Myc in leaves of three-week-old Kitaake, *oshfsfa4d-2*, *Ubi_{pro}:OsHsfA4d/oshfsfa4d*, *Ubi_{pro}:OsHsfA4d^{S146A}/oshfsfa4d*, and *Ubi_{pro}:OsHsfA4d^{S146D}/oshfsfa4d* were detected by anti-Myc in immunoblot analysis. **c, d** RNA levels of *HSP101* and *CsIF6* genes in three-week-old *oscdpk24 oscdpk28*, *oscdpk24*, *oscdpk28*, *OsCDPK24OE*, and *OsCDPK28OE* plants were measured by RT-qPCR (n = 3 technical replicates). **e** Photographs of representative lesions and lesion numbers per leaf (n = 23, 22, 10, 11, 17, 21, 10, and 8 lesions, respectively) on leaves of three-week-old Kitaake, *Ubi_{pro}:OsHsfA4d/oshfsfa4d*, *Ubi_{pro}:OsHsfA4d^{S146A}/oshfsfa4d* and *Ubi_{pro}:OsHsfA4d^{S146D}/oshfsfa4d* plants 7 days post spray inoculation with *M. oryzae*. Different letters above bars indicate differences at P < 0.05 by one-way ANOVA with LSD test (**a, c-e**). Box plots show the median (50%) and interquartile range (25-75%), and whiskers show minimum and maximum values (**e**). Scale bars: 1 cm (**e**). All experiments were repeated three times with consistent results.



Supplementary Fig. 14 *OsHsfA4d* suppress cell death, and the analysis of its natural variations sites.

a Representative leaves of Kitaake, *oshsfa4d*, and *OsHsfA4dOE* plants phenotypes during seedling, tillering, and reproductive stages. Scale bars: 1 cm. **b** Genetic diversity analysis of *OsHsfA4d* across 4,045 rice accessions. Schematic representation of sequence variations and haplotype distribution in both promoter and coding sequence (CDS) regions. **c** RNA levels of *OsHsfA4d* were measured in leaves of seedling, tillering, and reproductive plants of R527, 93-11, 02428, and N22 (mean values $\pm$ SD, $n = 3$ technical replicates). Different letters above bars indicate differences at $P < 0.05$ by one-way ANOVA with LSD test. All experiments were repeated three times with consistent results.