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OsWRKY70 Plays Opposite Roles in Blast Resistance and Cold Stress Tolerance in Rice

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Abstract

The transcription factor WRKYs play pivotal roles in the adapting to adverse environments in plants. Prior research has demonstrated the involvement of *OsWRKY70* in resistance against herbivores and its response to abiotic stress. Here, we reported the functional analysis of *OsWRKY70* in immunity against fungal diseases and cold tolerance. The results revealed that *OsWRKY70* was induced by various *Magnaporthe oryzae* strains. Knock out mutants of *OsWRKY70*, which were generated by the CRISPR/Cas9 system, exhibited enhanced resistance to *M. oryzae*. This was consistent with fortifying the reactive oxygen species (ROS) burst after inoculation in the mutants, elevated transcript levels of defense-responsive genes (*OsPR1b*, *OsPBZ1*, *OsPOX8.1* and *OsPOX22.3*) and the observation of the sluggish growth of invasive hyphae under fluorescence microscope. RNA sequencing (RNA-seq) and quantitative real-time PCR (qRT-PCR) validations demonstrated that differentially expressed genes were related to plant-pathogen interactions, hormone transduction and MAPK cascades. Notably, *OsbHLH6*, a key component of the JA signaling pathway, was down-regulated in the mutants compared to wild type plants. Further investigation confirmed that OsWRKY70 bound to the promoter of *OsbHLH6* by semi-in vivo chromatin immunoprecipitation (ChIP). Additionally, the loss-function of *OsWRKY70* impaired cold tolerance in rice. The enhanced susceptibility in the mutants characterized by excessive ROS production, elevated ion leakage rate and increased malondialdehyde content, as well as decreased activity of catalase (CAT) and peroxidase (POD) under low temperature stress was, which might be attributed to down-regulation of cold-responsive genes (*OsLti6b* and *OslCE1*). In conclusion, our findings indicate that OsWRKY70 negatively contributes to blast resistance but positively regulates cold tolerance in rice, providing a strategy for crop breeding with tolerance to stress.

Keywords Blast resistance, Cold tolerance, Transcriptional regulation, OsWRKY70, Rice

Introduction

Plant growth and development are greatly affected by biotic and abiotic stresses, including pathogen attacks, insect herbivory, extreme temperatures, high salinity and various other factors. To adapt to adverse environments, plants have evolved intricate regulatory mechanisms at the molecular, physiological, biochemical and metabolic levels (Nejat et al. 2017). For example, after perceiving the stimulation, plants promptly and effectively initiate extensive transcriptional reprogramming of gene expression, generating a variety of signaling molecules, including phytohormones, reactive oxygen species (ROS),

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calcium ions (Ca^{2+}) (Buscaill et al. 2014; Ng et al. 2018; Chen et al. 2020). This stress-responsive reprogramming requires the coordinated and precise timing of the involvement of different types of transcription factors (TFs) in both temporal and spatial dimensions (Khan et al. 2018). Genetic and molecular studies have elucidated the functional attributes of TF families such as WRKY, AP2/ERF, NAC, MYB and bHLH in plants (Ng et al. 2018; Kajla et al. 2023).

WRKY TFs are among the largest transcriptional regulatory families in plants. These proteins were divided into three subgroups, namely groups I, II and III, according to the number of WRKY domains and the type of zinc finger structure (Eulgem et al. 2000; Rushton et al. 2010). Up to now, WRKY TFs have been identified in different plant species, including *Arabidopsis thaliana*, *Glycine max*, *Gossypium hirsutum*, *Oryza sativa*, *Solanum tuberosum*, *Triticum aestivum*, *Zea mays* (Khoso et al. 2022; Song et al. 2023; Javed et al. 2023). For instance, there are 90 and 128 WRKYs in *Arabidopsis thaliana* and *Oryza sativa*, respectively (Tian et al. 2020). Some WRKY TFs have been evaluated for their pivotal roles in plant growth and development (Wang et al. 2023a). For example, *AtWRKY10* and *AtWRKY41* are involved in seed development and dormancy (Ding et al. 2014; Xi et al. 2021), while *AtWRKY23* promotes lateral root growth in *Arabidopsis* (Grunewald et al. 2012). The functions of *OsWRKY11*, *OsWRKY36* and *OsWRKY53* have been separately demonstrated in the flowering process, plant height and grain size in rice, respectively (Cai et al. 2014; Lan et al. 2020; Tian et al. 2017).

WRKY TFs serve as critical regulators in plant immune response, specifically binding to W-box cis-element (T)(T)TGAC(C/T) in the promoter region of target genes to modulate transcription (Bakshi et al. 2014; Viana et al. 2018; Saha et al. 2023). In *Arabidopsis*, at least 20 *WRKY* genes have been identified as playing significant roles in diseases or insect resistance, including *AtWRKY28*/*33*/*55*/*70*/*75* (Li et al. 2006; Chen et al. 2013; Wang et al. 2019, 2020; Zhou et al. 2020; Saha et al. 2023). In rice, overexpression of *OsWRKY67* up-regulates defense-related genes (*PR1a*, *PR1b*, *PR4*, *PR10a* and *PR10b*), as well as leads to rapid induction of ROS upon stimulation with chitin and flg22 (Liu et al. 2018). The adaptation results from the interplay between WRKYs and a variety of plant hormones. For example, OsWRKY72 directly binds to the promoter of *AOS1*, which is the jasmonic acid (JA) biosynthesis enzyme gene, negatively regulates JA synthesis and resistance to *Xanthomonas oryzae* pv *oryzae* (*Xoo*) infection (Hou et al. 2019). OsWRKY42 is a negative regulator to *M. oryzae* via repressing JA signaling and OsWRKY45-2 directly activates *OsWRKY13*, whose encoding protein in turn transcriptionally suppresses *OsWRKY42*/*OsWRKY45-2* to regulate blast resistance (Cheng et al. 2015). *OsNPR1*, a key regulator of salicylic acid (SA)-mediated resistance against fungal, bacterial disease and herbivores (Yuan et al. 2007; Feng et al. 2011), is downstream of OsWRKY03 (Liu et al. 2005). Previous research has documented that WRKY TFs play critical roles in plant immune response as one of the downstream substrates of the mitogen-activated protein kinase (MAPK) cascades. For instance, OsMKK10-2-OsMPK3/OsMPK6-OsWRKY31 module participates in the biosynthesis of secondary metabolite camalexin to regulate defense for rice blast pathogen (Wang et al. 2023c). OsWRKY53 is downstream of MAPK cascades, meanwhile, it functions as a negative feedback modulator of *OsMPK3/OsMPK6* in response to striped stem borer (SSB) (Hu et al. 2015). Moreover, OsWRKY53 acts as an early suppressor of induced defenses to mediate the MAPK-regulated *OsWRKY24*/*33*/*70* expression, as well as SSB-induced JA, JA-isoleucine (JA-Ile) and ethylene (ET) biosynthesis (Hu et al. 2015). Collectively, these studies confirm that *WRKY* genes might contribute to multiple biotic stresses through complex signaling cascades such as ROS, plant hormones and MAPKs.

In the past decades, WRKYs have gained extensive attention due to their functions in involving tolerance against abiotic stress (Khoso et al. 2022; Goyal et al. 2023). For example, *AtWRKY25/26/33* positively mediates tolerance to heat stress in *Arabidopsis* (Li et al. 2011). OsWRKY10 negatively regulates thermotolerance in rice by modulating ROS homeostasis and hypersensitive response (Chen et al. 2022). Overexpression of *OsWRKY76* up-regulates peroxidase gene *OsPrx71* and lipid metabolism gene *OsBURP13/OsRAFTIN1*, thus alleviating the damage of low temperatures in rice (Yokotani et al. 2013). Some studies have highlighted the relationship of abiotic stress with *WRKY* genes by regulating the dehydrate-responsive element binding proteins (DREBs) or C-repeat binding factors (CBFs). In particular, the OsWRKY63-OsWRKY76-OsDREB1B signaling cascade module is involved in the regulation of chilling tolerance (Zhang et al. 2022b). OsWRKY28 confers salinity tolerance by directly activating *OsDREB1B* in rice (Zhang et al. 2023). Additionally, overexpression of *OsWRKY55* reduced drought tolerance, is consistent with accelerated water loss and massive accumulation of ROS (Huang et al. 2021).

It has been confirmed that several OsWRKY TFs play multiple roles in the adaptation to both biotic and abiotic stress in rice. For instance, OsWRKY10 is involved in resistance to fungus and thermotolerance (Wang et al. 2023b; Chen et al. 2022). *OsWRKY24* positively regulates fungal disease resistance, which has been identified as a potential candidate gene affecting cold sensitivity (Yokotani et al. 2018; Wu et al. 2023). OsWRKY53 acts as a negative modulator in resistance of bacterial blight, cold

and salt tolerance (Xie et al. 2021; Tang et al. 2022b; Yu et al. 2023), as well as a positive regulator in blast resistance (Chujo et al. 2007). OsWRKY76 plays opposite roles in blast resistance and cold stress tolerance (Yokotani et al. 2013). Empirical evidence suggests that the adaption of a variety of stress is associated with the crosstalk between WRKYs and phytohormones. For example, overexpression of *OsWRKY13* enhanced rice resistance to *M. oryzae* and *Xoo*, which was accompanied by the activation of SA signaling pathways and the suppression of JA signaling pathways (Qiu et al. 2007). OsWRKY45-1 and OsWRKY45-2 are involved in JA and SA signaling, whereas they play opposite roles in bacterial disease resistance (Tao et al. 2009). In addition, OsWRKY45-1 and OsWRKY45-2 act as negative and positive regulators in abscisic acid (ABA) signaling, respectively, while only the latter negatively regulates the tolerance of salt stress (Tao et al. 2011). Furthermore, knock out of *OsWRKY53* mutants confer rice cold tolerance at the booting stage by repressing the anther gibberellin content (Tang et al. 2022b). Therefore, WRKY proteins are potentially important components in plant biotic and abiotic stress responses and are associated with an array of signaling crosstalk.

OsWRKY70 encodes a rice WRKY TF belonging to group I, characterized by the presence of two WRKY domains (Zhang et al. 2015; Li et al. 2023). The resistance to herbivores of OsWRKY70 in rice has been investigated (Li et al. 2015; Ye et al. 2019). However, whether and how OsWRKY70 affects rice immunity to fungal disease and its association with abiotic stress have not been reported. Our previous study has demonstrated that *OsWRKY70* is induced by cold stress (Li et al. 2023). Here, we observed that the transcript level of *OsWRKY70* was up-regulated by different strains of *M. oryzae*. The biological functions of OsWRKY70 were also investigated using knock out mutants of *OsWRKY70*. Our findings revealed that the loss-function of *OsWRKY70* drastically enhanced resistance against *M. oryzae*, while attenuating cold tolerance in rice, demonstrating that OsWRKY70 plays opposite roles in immunity and cold stress response.

Results

OsWRKY70 is a Fungal Pathogen-Induced Gene

The previous finding that OsWRKY70 acts as an early regulator of plant response to herbivores (Li et al. 2015) inspired us to investigate its potential role in immunity against fungal diseases. We first assessed the expression level of *OsWRKY70* by quantitative PCR (qPCR) after spray inoculation with *M. oryzae* strain 318-2. The results showed that the transcript level of *OsWRKY70* was rapidly up-regulated upon infection, achieving a peak of 11.4-fold at 24 h post-inoculation (hpi) (Fig. 1A). To confirm whether the specific fungal pathogens, strains R01-1 and 110-2 were examined. Obviously, the expression of *OsWRKY70* was significantly induced by these strains, resulting in an increase of 9.5-fold and 15.6-fold at 48 hpi, respectively (Fig. 1B, C). Overall, these results indicate that *OsWRKY70* exhibits distinct responses to diverse fungal pathogens.

Generation and Characterization of *OsWRKY70* Knock Out Mutants

To elucidate the function of OsWRKY70, two homozygous mutants, designated *oswrky70-7* and *oswrky70-10*, were generated by CRISPR/Cas9-mediated genome editing. They exhibited a single base A and T insertion in the target sequence of *OsWRKY70*, respectively (Fig. 2A), resulting in the early termination of translation and thus loss of the conserved WRKY domain (Fig. 2B). Under field condition, our observations revealed that there was no significant difference between the mutants and wild

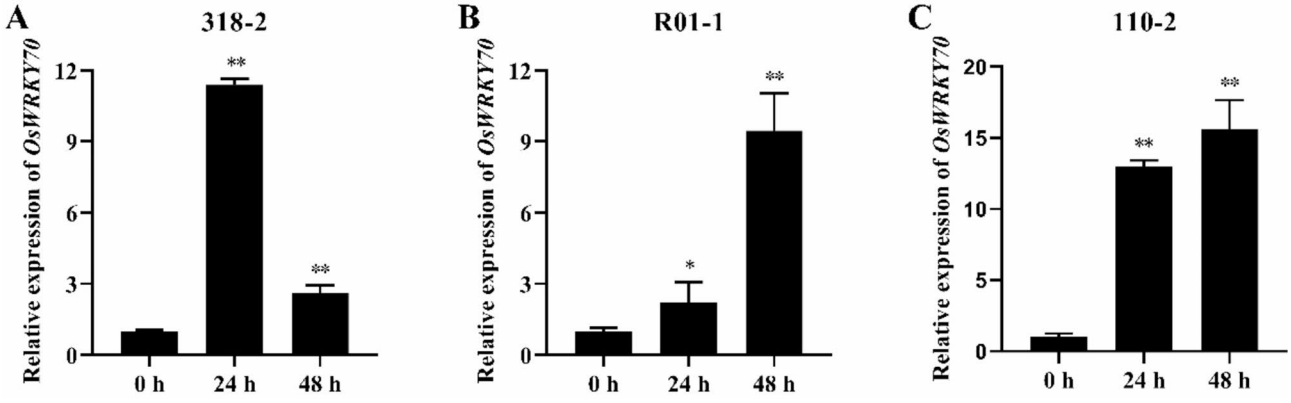

Fig. 1 *M. oryzae* strains infection induced the expression of *OsWRKY70*. (**A-C**) 2-week-old seedlings of NIP plants were inoculated with rice blast by spraying method. The qPCR analysis of *OsWRKY70* expression levels at 0, 24 and 48 h post-inoculation with *M. oryzae* strains 318-2 (**A**), R01-1 (**B**) and 110-2 (**C**). *OsActin* was used as an internal control gene. The expression level of OsWRKY70 in plants under normal condition was set as 1. Data are the means ± SD of three biological replicates. Asterisks indicate significant differences by the Student's t-test (*$P < 0.05$, **$P < 0.01$)

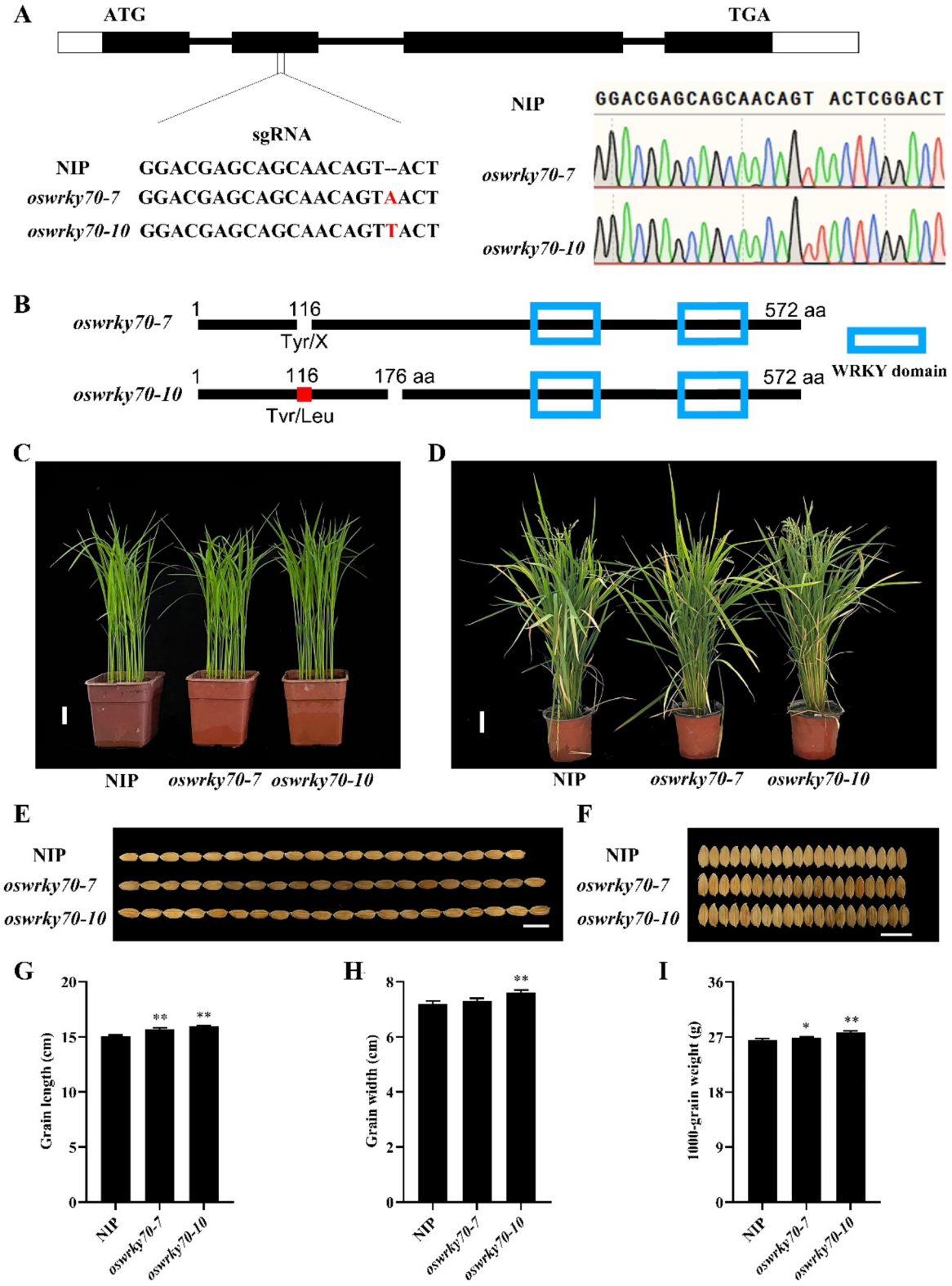

Fig. 2 Generation of mutants and investigations of agronomic traits. (**A**) Knock out of *OsWRKY70* gene by CRISPR/Cas9 technology. Target sequences of single-guide RNA (sgRNA) were listed. The mutation sites were indicated in red. Sequencing results of the *OsWRKY70* in the mutants and wild type. (**B**) Schematic diagrams of OsWRKY70 in knock out mutants. (**C, D**) The plant height at seeding stage (**C**) and heading stage (**D**). Scale bar = 2 cm and 1 dm, respectively. (**E**,**F**) The grain length (**E**) and grain width (**F**). Scale bar = 1 cm, $n = 20$. (**G-I**) The Statistical analysis of grain length (**G**), grain width (**H**) and 1000-grain weight (**I**). Data are the means ± SD of three biological replicates. Asterisks indicate significant differences by the Student's t-test (*$P < 0.05$, **$P < 0.01$)

type in terms of plant height, the tiller count, flag leaf and panicle length (Fig. 2C, D; Supplemental Table 2). Interestingly, the grain length of the mutants was notably greater than that of wild type, while the grain width exhibited a slight increase in *oswrky70-10*, resulting in a promotion in 1000-grain weight of mutants as compared to wild type (Fig. 2E-I). These results suggest that the loss-function of *OsWRKY70* affects rice grain shape rather than its growth and development.

Loss-Function of *OsWRKY70* Enhances Resistance Against *M. Oryzae*

To ascertain the involvement of *OsWRKY70* in rice blast resistance, we first performed spray inoculation on 2-week-old seedlings with *M. oryzae* 318-2. At 5 days post-inoculation (dpi), the mutants exhibited a reduced overall severity blast compared to wild type (Fig. 3A), as determined by a decrease in the lesion numbers by approximately 47.2% (Fig. 3B). Next, measurement of fungal growth in planta, as revealed by analyzing the genomic DNA level of the *MoPOT2* gene of *M. oryzae*, indicated that *oswrky70-7* and *oswrky70-10* supported less fungal growth, resulting in a reduction of 73.3% and 97.0%, respectively, as compared with that in wild type (Fig. 3C). Additionally, we conducted inoculation to detached leaves and punch-inoculated at 4-week-old seedings with strain 318-2. Consistent with previous findings, the mutants were less susceptible to rice blast than wild type, manifesting as significantly reduced disease lesions and lower fungal biomass (Fig. 3D-F; Figure S1). To further investigate the growth characteristics of *M. oryzae* spores, we infected rice leaf sheath with GFP-tagged strain RB22. At 24 hpi, we observed that almost no appressoria were formed in the leaf sheath cells of mutants, whereas maturing appressoria were found in those of wild type (Fig. 3G). At 48 hpi, only a limited number of invasive hyphae with no branch were present in the mutant cells, while numerous invasive hyphae freely spread to adjacent cells in wild type (Fig. 3H). Collectively, these results demonstrate that OsWRKY70 negatively regulates rice immune response against *M. oryzae*.

ROS Accumulation and Up-Regulated Defense Responsive Genes Enhance Resistance Against *M. Oryzae* in the Mutants

ROS bursts typically trigger plant defense responses (Li et al. 2021). Firstly, we conducted the analysis of ROS in the mutants and wild type after inoculation with *M. oryzae* by histochemical staining. The results showed that the presence of numerous reddish-brown spots on the leaves of mutants upon staining with 3, 3'-diaminobenzidine (DAB) and a significant increase in the number of blue dots were observed on the mutant leaves by nitro-blue tetrazolium (NBT) staining compared with those of wild type (Fig. 4A, B). The findings indicate that enhanced accumulation of H_2O_2 and superoxide anion probably contribute to resistance against rice blast disease. Subsequently, we investigated the expression patterns of defense-related genes. The results revealed that *OsPBZ1*, *OsPOX8.1* and *OsPOX22.3* were dramatically up-regulated in the mutants compared to wild type before inoculation, except for *OsPR1b* (Fig. 4C-F). Upon exposure *M. oryzae* strain 318-2, all four genes were a particularly notable up-regulation in the mutants compared with wild type (Fig. 4C-F). It suggests that loss-function of *OsWRKY70* activate defense response, which might play a pivotal role in response to biotic stress in rice.

Transcriptome Analysis of *OsWRKY70* Knock Out Mutant and qPCR Analysis

To further elucidate mechanisms underlying *OsWRKY70*-mediated immunity response in rice, we examined gene expression differences between *oswrky70-7* (W7) and NIP (N) under normal condition and 48 h post-inoculation with *M. oryzae* using RNA sequencing (RNA-seq). Sample correlation and principal components analysis (PCA) of all genes showed that the three replicates of each treatment clustered together, suggesting good biological replicability (Supplemental Fig. 2). Rice genes whose transcript abundance showed a fold change (FC) ≥ 2 and false discovery rate (FDR) ≤ 0.01 were defined as differentially expressed genes (DEGs). We observed significant clustering differences between W7 and N under normal condition, which identified 2210 DEGs, including 1205 up-regulated and 1005 down-regulated genes. The data revealed that knock out of *OsWRKY70* led to different transcript profiles. Additionally, a comparison of W7 and N at 48 hpi revealed 968 DEGs, containing 482 up-regulated genes, as well as 486 down-regulated genes in the mutant (Supplemental Fig. 2). However, the differences were not significantly enlarged by biotic stress.

KEGG classification illuminated the functional roles of these DEGs were mainly enriched into such as plant-pathogen interaction, plant hormone signal transduction, MAPK signal cascade and other metabolic processes (Fig. 5A, B). Among them, several encoding putative NBS-LRR disease resistance protein genes, *LOC_Os11g44960*, *LOC_Os11g45050* and *LOC_Os11g45180*, as well as the gene *LOC_Os07g44130* in phenylpropanoids metabolism were significantly up-regulated in W7 (Fig. 5C). In addition, *OsbHLH6*, *OsUGT74H4* and *OsWRKY76* were negative regulators of disease resistance involved in JA, SA and MAPK cascade signaling, respectively (Yokotani et al. 2013; Meng et al. 2020; Wu et al. 2022), were also dramatically down-regulated in the mutant after inoculation (Fig. 5C). The findings indicate that complex regulatory networks are activated in

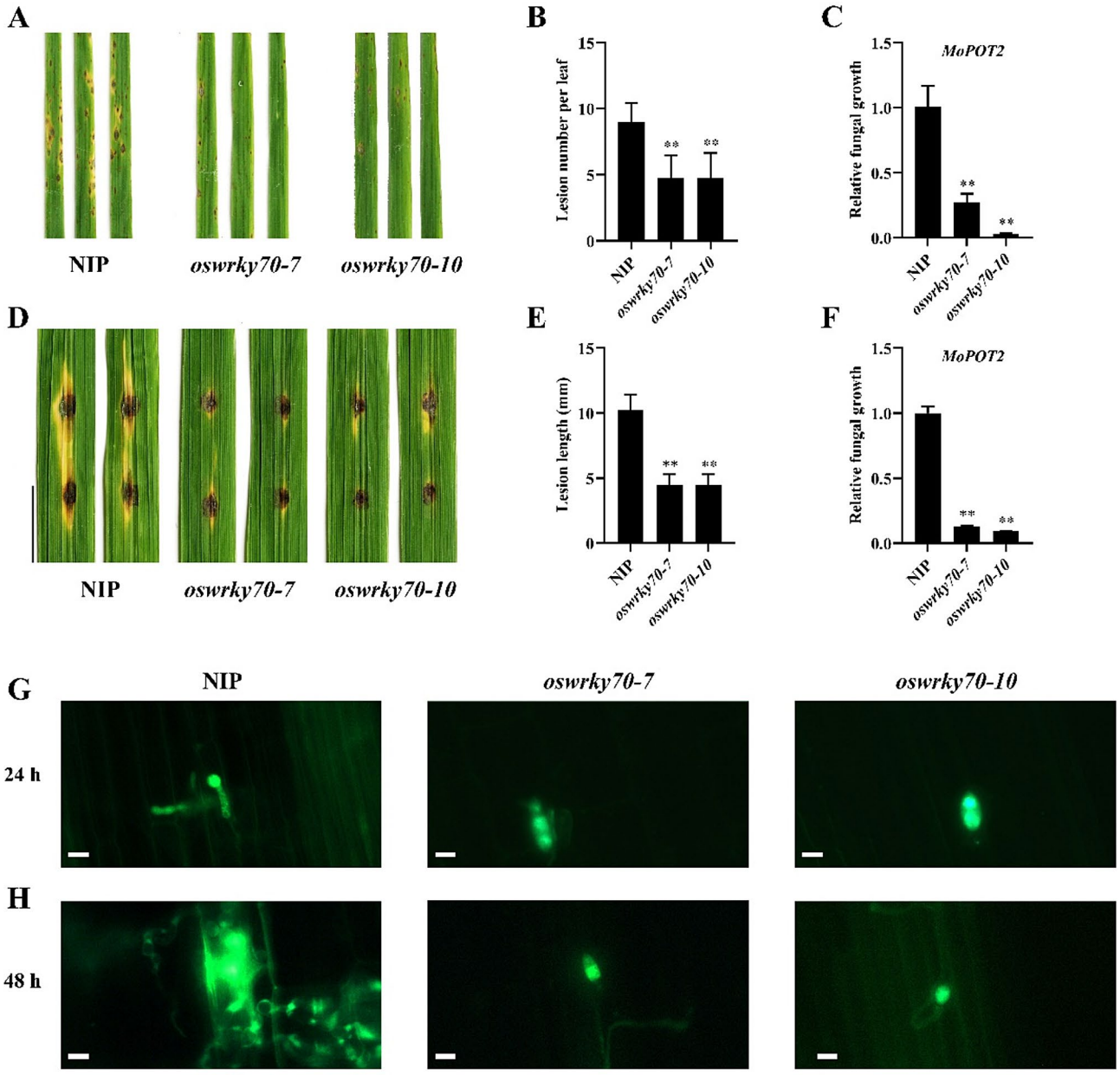

Fig. 3 Loss-function of *OsWRKY70* enhance resistance to *M. oryzae*. (**A**) Spray inoculation with *M. oryzae* spores on seedlings of mutants and wild type. (**B**) Lesion numbers on inoculated leaves at 5 days post-inoculation, $n = 5$. (**C**) Blast fungus biomass was determined by qPCR analysis using the ratio of *M. oryzae* DNA (*MoPOT2*) to rice DNA (*OsAcitn*) in infected leaves. Data are the means ± SD of three biological replicates. Asterisks indicate significant differences by the Student's t-test (*$P < 0.05$, **$P < 0.01$). (**D**) The detached leaves of mutants and wild type were inoculated with the *M. oryzae*. Scale bar = 1 cm. (**E**) Lesion length was determined on leaves at 5 days after inoculation, $n = 5$. (**F**) Relative fungal biomass in the necrotic regions of the detached leaves was assessed by qPCR of the fungal *MoPOT2* and normalized to rice *OsActin*. Data are the means ± SD of three biological replicates. Asterisks indicate significant differences by the Student's t-test (*$P < 0.05$, **$P < 0.01$). (**G**, **H**) Fluorescence microscopic observation of *M. oryzae* strain RB22-GFP infection on leaf sheath at 24 hpi (**G**) and 48 hpi (**H**). Scale bar = 20 μm

timely manners, which is crucial for rice blast resistance. To further screen candidate downstream target genes associated with the immune response of OsWRKY70, the leaves of 2-week-old mutants and wild type were individually collected for qPCR analysis after inoculation. The results revealed that the expression level of hormone, MAPK signaling and metabolic process genes was consistent with the RNA-seq analysis (Fig. 5D-I).

OsWRKY70 Interacts with Promoter of *OsbHLH6* in Vitro

Our finding revealed that the expression level of *OsbHLH6* was down-regulated in the mutants (Fig. 5E). To further explore whether *OsbHLH6* is the candidate target

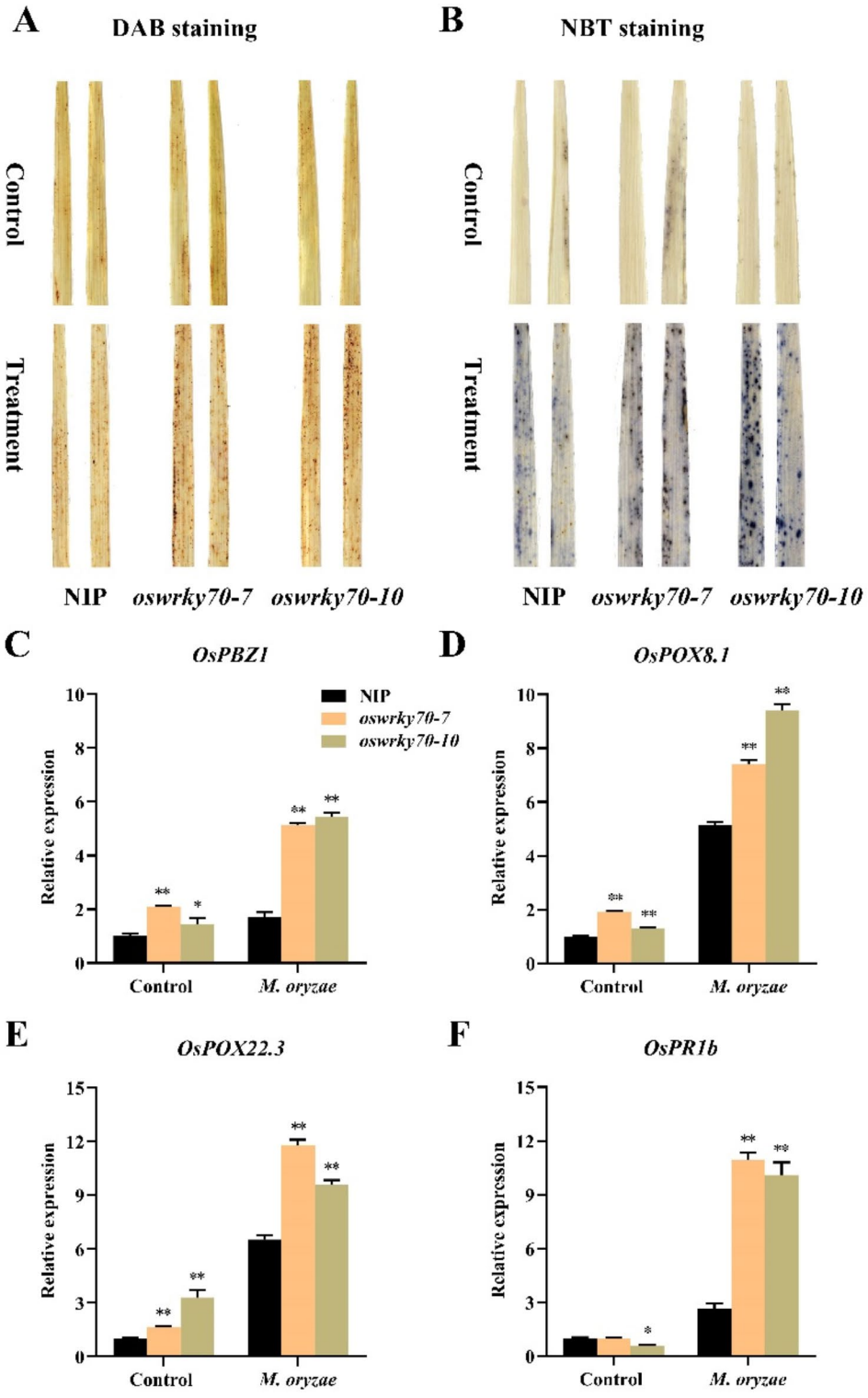

Fig. 4 ROS accumulation and up-regulated defense responsive genes enhance *M. oryzae* resistance in rice. (**A**, **B**) DAB staining (**A**) and NBT staining showed H_2O_2 and superoxide anion accumulation in leaves from 2-week-old seedings cultivated in normal culture and after inoculation with *M. oryzae*. (**C**-**F**) Expression levels of defense-responsive genes *OsPBZ1* (**C**), *OsPOX8.1* (**D**), *OsPOX22.3* (**E**) and *OsPR1b* (**F**) in the leaves at seeding stage without inoculation and post-inoculation with *M. oryzae*. *OsActin* was used as an internal control. The expression level of the tested genes in wild type plants under normal condition was set as 1. Data are the means ± SD of three biological replicates. Asterisks indicate significant differences by the Student's t-test (*$P < 0.05$, **$P < 0.01$)

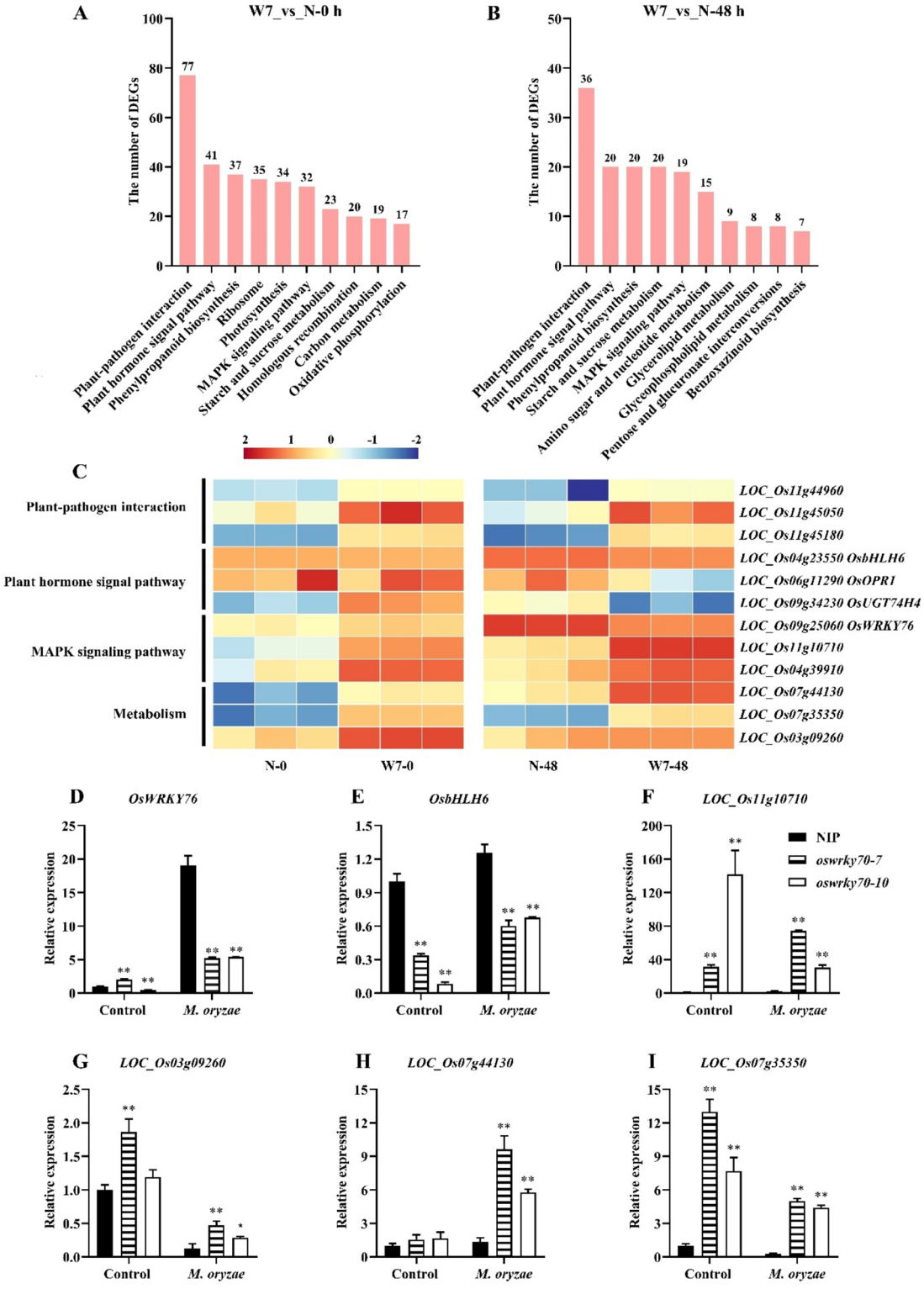

Fig. 5 Transcriptomic analysis of *oswrky70-7* before and after blast inoculation, as well as qPCR analysis to verify some DEGs. (**A**, **B**) KEGG pathway analysis of the DEGs between the mutant and wild type without inoculation (**A**) and 48 h post-inoculation (**B**) with *M. oryzae* 318-2. (**C**) Heatmaps showing the part of DEGs related to plant-pathogen interaction, plant hormone signal pathway, MAPK signaling pathway and metabolism. (**D-I)** Quantitative PCR analysis of *OsWRKY76* (**D**), *OsbHLH6* (**E**), *LOC_Os11g101710* (**F**), *LOC_Os03g09260* (**G**), *LOC_Os07g44130* (**H**) and *LOC_Os07g35350* (**I**). 2-week-old wild-type mutants and wild type plants were grown in soil under 16 h light/8 h dark conditions and were treated under inoculation for 0 and 48 h. The leaves were collected for RNA extraction. Three biological replicates were performed. Relative expression levels were normalized by the transcript level of the *OsActin* gene as an internal control and the expression level of each gene of interest in wild type plants under normal condition was set as 1. Data are the means ± SD of three biological replicates. Asterisks indicate significant differences by the Student's t-test (*$P < 0.05$, **$P < 0.01$)

gene of OsWRKY70 in immune response, we initially analyzed the promoter region of *OsbHLH6* and found ten W-box elements (Supplemental Table 3; Fig. 6A). Notably, the candidate fragments encompassed one or two core sequences (TGAC) characteristic of W-box. Then, we conducted a semi-in vivo chromosome immunoprecipitation qPCR (ChIP-qPCR) assay to verify the interaction, which employed the purified recombinant His-OsWRKY70 protein (Fig. 6B; Supplemental Fig. 3) and DNA fragments of rice genomic. The ChIP-qPCR results confirmed a significant approximately 4.0-fold enrichment of the P1 fragment in His-OsWRKY70 compared to the His control, while no enrichment was observed for the P2 fragment (Fig. 6C). These findings suggest that OsbHLH6 might serve as a downstream target gene for OsWRKY70.

Loss-Function of *OsWRKY70* Impairs Cold Tolerance in Rice

Our previous study that *OsWRKY70* was induced by cold stress (Li et al. 2023) prompted us to investigate whether OsWRKY70 is involved in cold tolerance. Consequently, 2-week-old seedlings were subjected to 4 °C for 3 days and returned to normal growth conditions for a recovery period of 7 days. The mutants exhibited pronounced leaf curling symptoms after cold stress and a significant proportion of them could not be recovered compared with wild type (Fig. 7A), demonstrating that OsWRKY70 is a positive regulator of cold tolerance in rice. This observation is consistent with the survival rate of *oswrky70-7* and *oswrky70-10*, which were 25.3% and 3.3%, compared to 42.7% of wild type (Fig. 7B). Moreover, higher levels of electrolyte leakage and MDA content were in the mutants than that in wild type (Fig. 7C, D). We further performed histochemical staining to detect ROS bursts in rice plants. After cold treatment, deeper reddish brown and darker bluish-purple spots in the mutant leaves than that in wild type by DAB and NBT staining (Fig. 8A, B), reflecting excessive H_2O_2 and superoxide anion in the mutants, respectively. Subsequently, the activity of two antioxidant enzymes crucial for scavenging ROS was examined. A significant decrease in CAT activity was observed in the *oswrky70-7* compared to wild type at 24 h after cold treatment (Fig. 8C). Especially, *oswrky70-10* exhibited approximately 30% decline after 12 h of cold treatment compared to wild type (Fig. 8C). Similarly, POD activity significantly reduced after exposure to cold for 12 h in the mutants. These results indicate that an imbalance of ROS homeostasis contributes to cellular oxidative membrane damage in OsWRKY70-regulated cold tolerance in rice. To assess the potential downstream genes of OsWRKY70 in response to cold, we detected the expression level of *OsLti6b*, *OsICE1* and *OsCOLD1*, which are cold-related genes (Kim et al. 2007; Ma et al. 2015; Zhang et al. 2017). The results revealed that the mutants showed down-regulation of *OsLti6b* and *OsICE1*, while no significant difference in the expression of *OsCOLD1* was observed (Fig. 8E-G), implying that knock out of *OsWRKY70* attenuates cold tolerance in rice presumably due to the suppressed expression of *OsLti6b* and *OsICE1*.

Discussion

Transcription factor OsWRKY70, a member of WRKY group I, has been established to function as a transcriptional activator (Li et al. 2015; Zhang et al. 2015). Phylogenetic analysis revealed that OsWRKY70 shares up to 52.43% and 62.87% amino acids identities with OsWRKY24 and OsWRKY53, respectively (Li et al. 2023). Repression of *OsWRKY24* (Yokotani et al. 2018) and overexpression of *OsWRKY53* (Chujo et al. 2007)

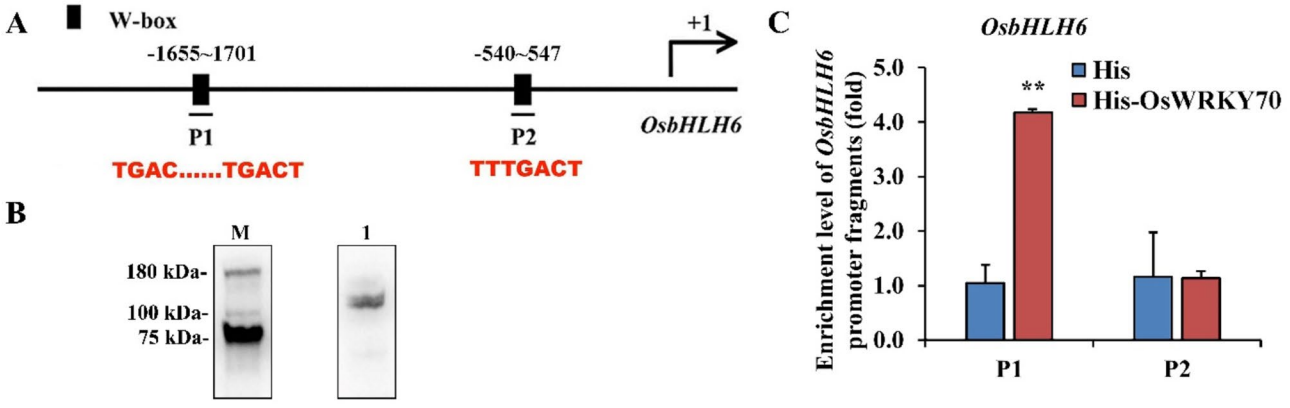

Fig. 6 OsWRKY70 interacts the promoter of *OsbHLH6* in vitro. (**A**) Schematic of the *OsbHLH6* promoter. Black rectangles indicate W-box (TGAC core sequences) cis-elements in the promoter of *OsbHLH6*. (**B**) The Western blot with purification of His-OsWRKY70 obtained from recombination of *E. coli* BL21. (Line 1, eluted His-OsWRKY70; M, protein marker). (**C**) The semi-in vivo ChIP-qPCR assay showed that His-OsWRKY70 enriched the P1 fragment of *OsbHLH6* promoter. DNA fragments co-incubated with His was used as a negative control. Relative enrichment is represented as the normalized ratio of the ChIP DNA to the input genomic DNA at the site. P1 and P2 are the fragments of the promoter. Data are the means ± SD of three biological replicates. Asterisks indicate significant differences by the Student's t-test (*$P < 0.05$, **$P < 0.01$)

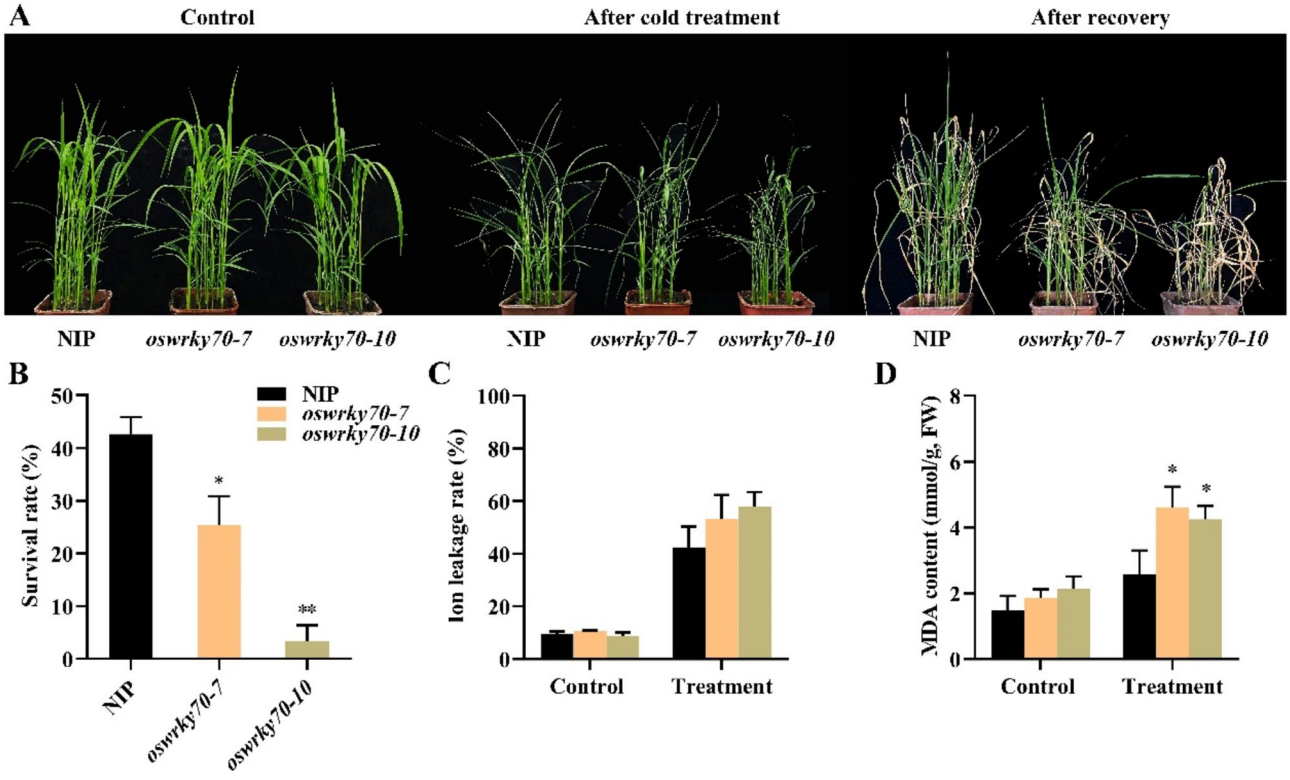

Fig. 7 Knock out of *OsWRKY70* reduced cold tolerance in rice. (**A**) 2-week-old seedlings of the mutants and wild type were subjected to 4 ℃ for 3 days in a growth chamber and then cold stressed recovered under 25–28 ℃ for 7 days. (**B**) The survival rate of seedlings after recovery were calculated. (**C**) Ion leakage rate in leaves of the cold-stressed mutants and wild type. (**D**) Content of MDA between mutants and wild type under normal condition and cold treatment. Data are the means ± SD of three biological replicates and each replicate includes at least 15 independent seedlings. Asterisks indicate significant differences by the Student's t-test (*$P < 0.05$, **$P < 0.01$)

confers blast resistance in rice. Our findings demonstrated that knock out of *OsWRKY70* mutants enhanced resistance against *M. oryzae* (Fig. 3; Figure S1), implying that *OsWRKY70* negatively regulates the fungal immunity in rice. However, it seems paradoxical that the increased transcriptional expression level of *OsWRKY70* upon infection (Fig. 1). Similarly, this feedback-like has been reported in some rice TFs. For instance, *OsbHLH6* and *ONAC083*, which are induced by inoculation with *M. oryzae*, act as negative regulators of disease resistance (Bi et al. 2023; Meng et al. 2020). Additionally, OsWRKY24, OsWRKY53 and OsWRKY70 may be functionally redundant in grain regulation (Tang et al. 2022a). Considering that predicted interactions of these proteins (data have no shown), we presume that up-regulated *OsWRKY70* might be involved in adjusting the intensity of defense response by cooperating with OsWRKY24 or OsWRKY53 to protect the plant from biotic stress. Therefore, further generation of double and triple mutant plants for these genes will contribute to elucidating the molecular mechanism of blast resistance. Our KEGG analysis revealed that the altered genes are involved in plant-pathogen interactions, hormone signaling transduction, MAPK cascades and so on, indicating that OsWRKY70 is a key component of immunity response (Fig. 5). Plant NBS-LRR genes, a class of the resistance (R) genes, encode immune receptors that help defend against pathogens infection (Wang et al. 2023d). For instance, rice NBS-LRR protein Pit interacts with OsRac1 and induces the generation of ROS and hypersensitive response to resist the invasion of *M. oryzae* (Kawano et al. 2014). *Pi63* encodes a typical NBS-LRR protein, whose expression level is closely related to disease resistance (Xu et al. 2014). In our study, *LOC_Os11g44960*, *LOC_Os11g45050* and *LOC_Os11g45180*, which are assumed to encode NBS-LRR proteins, were up-regulated expression in the mutant (Fig. 5C). It is suggested that these genes might be conferred to the resistance to blast. The activation of the JA signaling pathway improved resistance against disease in rice (Okada et al. 2015; Wang et al. 2021; Qiu et al. 2022). *OsbHLH6*, a transcription activator, negatively regulates rice blast resistance, which has been shown to play a pivotal role in modulating the JA and SA signaling pathways (Kiribuchi et al. 2004; Meng et al. 2020). Knock out of the *OsbHLH6* mutant downregulates the expression of *OsJAZ* family genes, which exhibits severe damage upon exposure to herbivores (Valea et al. 2022). In our study, the *OsbHLH6* gene transcript significantly decreased in *oswrky70-7* and

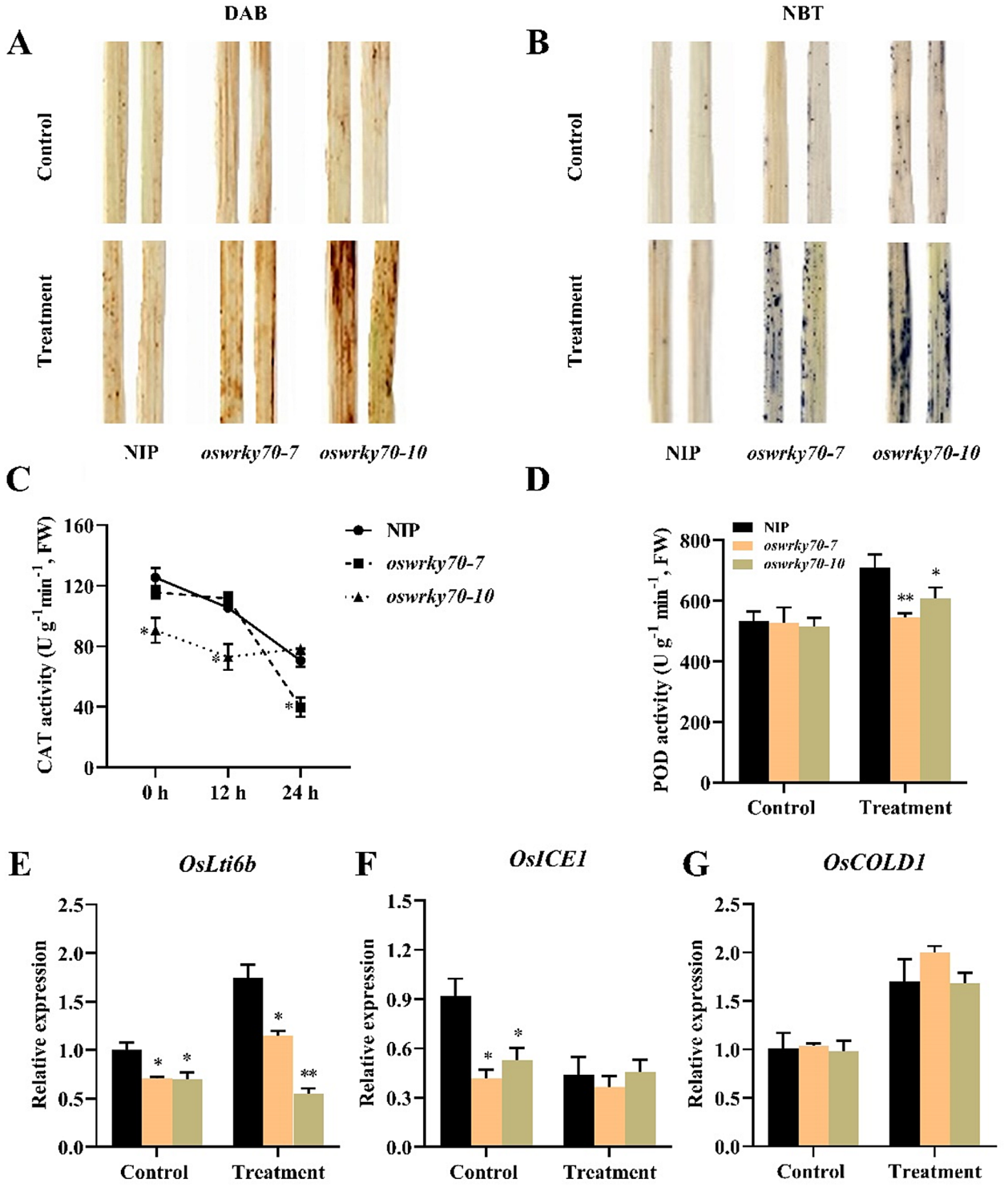

Fig. 8 ROS burst and the expression levels of cold-related genes in *oswrky70* mutants in response to cold. (**A**) H_2O_2 accumulation checked by DAB staining. (**B**) The accumulation of superoxide anion in leaves detected by NBT staining. (**C**, **D**) Enzyme Activity of CAT (**C**) and POD (**D**) from seedings of 2-week-old wild type and *oswrky70* mutants before and after cold stress treatment. Data are the means ± SD of three biological replicates. Asterisks indicate significant differences by the Student's t-test (*$P < 0.05$, **$P < 0.01$). (**E-G**) The expression levels of cold-related genes *OsLti6b* (**E**), *OsICE1* (**F**) and *OsCOLD1* (**G**) from the seedings of 2-week-old wild type mutants and mutants without and with cold treatment. Relative expression levels were normalized by the transcript level of the *OsActin* gene as an internal control and the expression level of each gene of interest in wild type plants under normal condition was set as 1. Data are the means ± SD of three biological replicates. Asterisks indicate significant differences by the Student's t-test (*$P < 0.05$, **$P < 0.01$)

oswrky70-10 mutants before and after inoculation compared to the wild type (Fig. 5C, E). Given the finding that OsWRKY70 enhances rice herbivore resistance to SSB mediated by JA signaling (Li et al. 2015), we hypothesized that *OsbHLH6* might be a potential downstream target of OsWRKY70. Then, we analyzed the promoter of *OsbHLH6* and further confirmed the binding of OsWRKY70 using a semi-in vivo ChIP assay (Fig. 6). Therefore, it would be interesting in our future work to verify the interactions in vivo by ChIP and to generate their double mutant plants for exploring the regulatory relationships. Advanced thinking is that JA and SA signaling crosstalk commonly manifests as a reciprocal antagonism or adaptation (Thaler et al. 2012). In our work, the transcript level of *OsUGT74H4* was decreased in mutant after infection by RNA-seq analysis (Fig. 5C). OsUGT74H4 may inactivate SA through glycosylation modification, negatively regulating the resistance of rice to bacterial diseases (Wu et al. 2022). Both positive and negative transcriptional regulations of SA biosynthesis are required to fine-tune the SA levels for optimal defense without causing unnecessary fitness costs (Ding et al. 2020). Therefore, we suspect that knock out of *OsWRKY70* might affect SA content in response to fungal pathogen infection. Phenylpropanoids are considered to be secondary metabolites involved in plant defense responses (Kishi-Kaboshi et al. 2010). Previous research has revealed that *CYP72A1*, the cytochrome P450 gene, positively regulates the production of ROS and the accumulation of defense-related secondary metabolites in basic immune response (Zhang et al. 2022a). The Cinnamate-4-hydroxylase (C4H) belongs to the cytochrome monooxygenase, which is the second key enzyme in the phenylpropane metabolic pathway (Yang et al. 2005). According to our transcriptome analysis, there were more than 20 DEGs in Phenylpropanoids biosynthesis without and after inoculation (Fig. 5A). Among them, *LOC_Os07g44130*, which encodes putative cytochrome P450, was up-regulated more than 5.5-fold in mutants after infection (Fig. 5H). Thus, it is also worthy of further study and exploration of issues that OsWRKY70 participates in phenylpropanoids biosynthesis to adapt to biotic stress.

The loss-function of *OsWRKY70* mutants reduced tolerance to cold stress (Fig. 7), suggesting that OsWRKY70 is a positive regulator of cold tolerance. It has been demonstrated that chilling usually causes excessive ROS accumulation in rice (Marchi et al. 2012; Zhang et al. 2022b; Zhai et al. 2024). *OsWRKY63* and *OsWRKY76* might affect ROS homeostasis in the regulation of cold tolerance (Yokotani et al. 2013; Zhang et al. 2022b). We observed that knock out of *OsWRKY70* mutants accumulated a large amount of H_2O_2 and superoxide anion under cold stress condition (Fig. 8A, B). This is consistent with the measurement of reduced activities of CAT and POD, as well as increased electrolyte leakage levels and MDA content (Figs. 7C and D and 8C and D). Cold tolerance is primarily regulated by cold-responsive (COR) regulon (Wani et al. 2021). Our findings revealed that the expression level of *OsLti6b* and *OsICE1* was significantly down-regulated in mutants (Fig. 8E, F), which has been documented to positively regulate cold tolerance in rice (Kim et al. 2007; Nakamura et al. 2011; Xia et al. 2021). What is more, COR regulon also mediates the expression of *AtWRKY6/22/30/40/32/187* (Banerjee et al. 2015; Wani et al. 2021), hinting that there may be feedback or cooperation between OsWRKY70 and COR regulon in chilling stress. Unlike cold-responsive genes, *OsCOLD1* is involved in sensing cold to trigger Ca^{2+} signaling for chilling tolerance (Ma et al. 2015). There were no differences in the transcript level of *OsCOLD1* between the mutants and wild type before and after cold treatment (Fig. 8G), suggesting that *OsCOLD1* may be the upstream of *OsWRKY70*-mediated signaling. Additionally, the changes in OsCOLD1 protein structure and membrane fluidity in response to low temperature might initiate signaling (Ma et al. 2015). Thus, another possibility is that the mutation of *OsWRKY70* affects the structure and dynamics of OsCOLD1 to regulate cold tolerance.

We demonstrated that OsWRKY70 functions as a negative regulator of rice against *M. oryzae* while a positive regulator of cold tolerance in rice (Figs. 3 and 7). Previous findings showed that temperature is an important factor affecting the occurrence of rice blast. For instance, cold summers increase the frequency and severity of fungal pathogen disease and bring heavy yield losses in the northern regions of Japan (Hironori et al. 2004). The blast lesion area and fungal growth in the inoculated seedlings at a warm temperature, 22 °C, is greater than those at 28 °C (Qiu et al. 2022). Additionally, 22 °C compromises basal resistance in rice by reducing JA biosynthesis and signaling (Qiu et al. 2022), suggesting that JA are involved in temperature-modulated plant resistance. We found that knock out of *OsWRKY70* mutants down-regulated *OsbHLH6* expression in response to rice blast (Fig. 5E). However, the regulatory relationship between OsWRKY70 and *OsbHLH6* whether involving temperature-mediated fungal pathogen resistance is yet to be clarified. In addition, more and more evidences imply that WRKY TFs are involved in the MAPK signaling pathway to adapt to biotic stress. For example, OsMKK10-20-OsMPK6 pathway is required for *OsWRKY45*-mediated resistance against *M. oryzae* (Ueno et al. 2013). OsWRKY31, which is phosphorylated by OsMPKs, elevates DNA-binding activity and confers enhanced blast resistance in rice (Wang et al. 2023c). Previously, OsMPK3 and OsMPK6 regulates OsWRKY70 in herbivore resistance (Li et al. 2015). OsMPK3, phosphorylated and activated by the calcium-dependent protein

kinase CPK18, negatively regulates rice blast resistance (Xie et al. 2014). In addition to biotic stress, MAPK cascades have also been confirmed to confer abiotic stress responses, including chilling, salt and drought. For instance, OsICE1 is phosphorylated by OsMPK3, resulting in inhibition of OsICE1 ubiquitination and enhanced resistance to chilling damage (Zhang et al. 2017). Furthermore, OsMPK3 has positively modulated salt tolerance by attenuating the accumulation of ROS (Zhang et al. 2018). Consequently, further validation of the interaction with OsMPK3 and OsWRKY70 will contribute to aid in elucidating the molecular mechanism in response to fungal pathogen infection and cold stress.

Materials and methods

Plants and Pathogens

The wild type rice *Oryza sativa L. japonica* 'Nipponbare' (NIP) was used to generate knock out of *OsWRKY70* mutants in this study. All rice seeds were soaked in culture dishes with water at 37℃ for one day. On the second day, the seeds were rinse for 2–3 times and added a small amount water to promote germination at 37 °C. Then, the seeds with same germination state were planted to the substrate soil. The plants were grown under normal conditions (25–28℃, 14 h light/10 h dark photoperiod, 50–75% relative humidity). For agronomic trait analysis, the tiller count, flag leaf, panicle length, grain length, grain width and 1000-grain weight of these plants were grown in the paddy fields of Hunan Agricultural University, Changsha, China.

Magnaporthe oryzae strains 318-2 from College of Agronomy in Hunan Agricultural University, as well as *M. oryzae* strains 110-2, R01-1 and GFP-tagged RB22 from Institute of Plant Protection in Chinese Academy of Agricultural Sciences, were cultured on oatmeal agar for 2 weeks at 28 °C and the spores were collected with sterile water containing 0.02% Tween-20. For *M. oryzae* strains treatment, 2-week-old seedlings of NIP were inoculated using *M. strains* 318-2, 110-2 and R01-1 for gene expression analysis of *OsWRKY70*. The plants were sprayed with spores of *M. oryzae* strains (2×10^5 spores/mL), covered with a plastic box in the dark for 24 h (25–28 °C, approximately 100% relative humidity) and transferred to normal alternation of light and dark (25–28 °C, approximately 100% relative humidity). Leaf samples were collected at 0, 24 and 48 h post-inoculation, frozen in liquid nitrogen and stored at -80 °C until use. Each treatment was performed with three biological replicates. The control plants (0 h) were left blast-free.

Generation of *OsWRKY70* Knock Out Mutants

For the construction of knock out of *OsWRKY70* vector, the sequence (5'-GGACGAGCAGCAACAGTAC T-3') of *OsWRKY70* genomic locus was conducted as the guide RNA through CRISPR Primer Designer v1.1.2. We designed two primers sequences (LP: 5'-TGGCGG GACGAGCAGCAACAGTACT-3'; RP: 5'-AAACAG TACTGTTGCTGCTCGTCCC-3') to synthesized the sgRNA expression cassette. This sequence was inserted into the pHUN4c12 plasmid, which was linearized using *Bsa* I enzyme (NEB, USA). The vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 through electroporation, which were further used to transform the rice callus. After screening with hygromycin and sequence confirmation, two homozygous knockouts *oswrky70-7* and *oswrky70-10* were obtained and the T3 generation seedlings were used for further analysis in this work. The primers used in the plant vector construction and identification are listed in Table S1.

Fungal Pathogen Resistance Experiment

For spraying with *M. oryzae* spores (2×10^5 spores/mL), 2-week-old transgenic seedlings were used for pathogen infection. Leaves were collected at 0 and 24 h post-inoculation for expression analysis of defense-related genes. After inoculation for 5 d, the lesions were scanned and leaves were collected for DNA extraction to evaluate the relative fungal growth. The qRT-PCR was measured to analyze and compare the genomic level of *M. oryzae POT2* in wild type and the mutant leaves with that of the rice *OsActin* as an internal control. All of primers were listed in Table S1.

For detached leaf inoculation, leaves of 4-week-old rice seedlings were cut into pieces (about 5 cm × 1 cm) and float on the distilled water in culture dishes. Apply a drop of the spore suspension (10 μL of 4×10^5 spores/mL) to the wound of each leaf and keep the dishes at 25–28℃ for 5 days. (Darkness is not required). The lesions were photographed and measured at 5 d post-inoculation and leaves were collected for detecting relative biomass.

For punch inoculation, 4-week-old seedlings grown in the field were made a wound using a hole punch. 10 μL spore suspension (4×10^5 spores/mL) was dropped onto wound. The inoculated region was wrapped with tape to maintain humidity. At 5 d post-inoculation, the lesions were pictured and surveyed. Leaf samples were conducted for measuring relative growth of *M. oryzae*. Blast inoculation was performed as previously described (Gu et al. 2023). *M. oryzae* strain 318-2 was used for rice blast inoculation. All experiments were independently repeated at least three times.

For leaf sheaths infection, detached sheaths of 4-week-old rice plants were inoculated with GFP-tagged *M. oryzae* strain RB22 spores (4×10^5 spores/mL) and kept on dished with approximately 100% humidity for 24 h and 48 h in the dark. Images of conidial germination, appressorium development and invasive hyphal growth

were recorded using a fluorescence microscope (Mshot, China).

Evaluation of Cold Tolerance

For cold stress tolerance assay, 2-week-old knock out of *OsWRKY70* mutants and wild type plants were grown under normal condition and then transferred into a growth chamber with the temperature set at 4 °C with a cycle of 14 h light/10 h dark for 3 days, followed by transferring to the growth room with the normal condition for recovery. Plants with green leaves and healthy young leaves after transferring to the normal growth condition were considered as survivals and surviving plants were evaluated at 7 days after recovery from cold treatment. Survival rate was calculated as the ratio of the number of survived plants over the total number of treated plants. Leaf samples at 0 and 24 h were used for determination of malondialdehyde (MDA) content and electric conductivity using the colorimetric method and electrical conductivity meter, respectively (Guan et al. 2012). Leaves at 0, 12 and 24 h were measured the enzyme activity catalase (CAT) and Leaves at 0 and 12 h were detected the peroxidase (POD) activity, as previously described (Wang et al. 2022). Leaves were collected at 0 and 24 h for expression analysis of cold-related genes. The specific primers were exhibited in Table S1. Cold treatment in each of the experiments included three biological replicates with at least 15 plants and the experiments were independently repeated three times.

Histochemical Staining Analysis

For fungal pathogen infection, 2-week-old seedlings of wild type and *oswrky70* mutants were sprayed with *M. oryzae* 318-2 spores (2×10^5 spores/mL) for detection of ROS burst. Leaf samples were collected at 0 and 24 h post-inoculation to carry out Histochemical Staining. For cold stress treatment, 2-week-old transgenic plants were transferred to a growth chamber with temperature set at 4℃. Leaf samples were collected at 0 and 6 h after treatment. 3, 3'-diaminobenzidine (DAB) and nitro-blue tetrazolium (NBT) staining for H_2O_2 and superoxide anion accumulation in plants, respectively, were conducted as previously described (Jambunathan 2010).

DNA Extraction

Rice leaf genomic DNA was extracted, according to the CTAB method (Semagn et al. 2014).

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted using Ultrapure RNA Kit (Cwbio, China). RNA was reverse transcribed to cDNA using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, China). Quantitative Real-Time PCR (qRT-PCR) was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China). The reaction was carried out in the CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). Rice *OsActin* gene was used as an internal standard to normalize. All of primers used for mRNA detection of target genes are shown in Table S1. Three replicate experiments were performed for each sample. The relative quantitation method ($2^{-\Delta\Delta CT}$) was used to evaluate quantitative variation among replicates.

Expression and Purification of Recombinant Protein

The full-length cDNA of *OsWRKY70* was PCR-amplified and cloned into the pCold-TF vector (Takara, Japan). Specific primers used for PCR amplification for this gene are listed in Table S1. The constructs were transformed into *Escherichia coli* BL21 (DE3) (Vazyme, China). OsWRKY70 recombinant protein was induced by adding 0.4 mM isopropyl-β-thiogalactopyranoside (IPTG) at 15˚C for 24 h. Cells were collected and the recombinant protein was purified using *ProteinIso*® Ni-NTA Resin for His (TransGen, China) according to the manufacturer's instructions.

Transcriptome Analysis

Libraries for RNA-seq were constructed and sequenced by Biomarker Technologies Co., Ltd (Beijing, China). Briefly, RNAs were quantified by NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific), agarose gel electrophoresis and Agient2100/LabChip GX. mRNAs were isolated from total RNAs by poly (A) selection, fragmented into short fragments and converted to cDNAs. cDNAs were ligated to adapters and the suitable fragments were selected for PCR amplification as templates. All the RNA-seq libraries were pair-end sequenced on an Illumina NovaSeq6000 platform. mRNA sequencing data analysis were performed as reported (Love et al. 2014). Low-quality reads were removed and adapters were trimmed to obtain clean reads, which were mapped to the reference genome (Oryza_sativa.MSU_v7.0.genome.fa). Transcriptome analysis was performed using BMKCloud (www.biocloud.net). The expression level of each gene was calculated as the FPKM value (fragments per kilobase of transcript per million mapped reads). For differential gene expression analysis, fold change (FC) ≥ 2 and false discovery rate (FDR) ≤ 0.01 as screening criteria. Fold Change represents the ratio of expression between two samples (groups). False Discovery Rate (FDR) is obtained by correcting for the difference significance p-value (p-value), indicating the significance of the difference.

Semi-Vivo Chromatin Immunoprecipitation Assay

The analysis of ChIP was conducted as previously described (Li et al. 2017). Total DNA of Nipponbare and purified His-OsWRKY70 were used for a semi-vivo

chromatin immunoprecipitation (semi-vivo ChIP) assay. 2-week-old seedlings were used for total DNA extraction. The total DNA was sheared into 200–800 bp fragments using ultrasonic crusher. The His fusion protein was affinity-purified on Ni-NTA Resin. His-OsWRKY70 and DNA fragments were co-incubated for 2 h. The incubation buffer includes: 50 mM Tris, 1mM EDTA, 100 mM KCl, adjust pH to 7.0 by HCl, 5% Glycerol, 0.1% Triton X-100; add freshly-made 100 mM DTT to reaction solution to make final concentration of DTT at 1 mM. After co-incubation, Ni-NTA Resin was washed three times using incubation buffer. Then 4 µL 5 M NaCl was added into the sample for each 100 µL volume and was incubated for 4 h to break down cross-linked His-OsWRKY70 and DNA fragments. The prepared DNA in ChIP was applied for qRT-PCR using respective primer pairs (Table S1) in a ChamQ Universal SYBR qPCR Master Mix with a CFX Connect Real-Time PCR Detection System. PCR reactions were performed in triplicate for each sample and the expression levels were normalized to the input sample for enrichment detection. The fold enrichment was calculated against *OsActin* No addition of antibodies (NoAbs) served as a negative control.

Data Analysis

For qRT-PCR analyzes, disease assays, agronomic traits assessment and cold tolerance assays significant differences between samples/lines and the corresponding controls were analyzed using two-tailed Student's t test for pairwise comparisons.

Abbreviations

JA	Jasmonic acid
SA	Salicylic acid
ABA	Abscisic acid
ROS	Reactive oxygen species
MAPK	Mitogen-activated protein kinase
DAB	3, 3'-diaminobenzidine
NBT	Nitro-blue tetrazolium
POD	Peroxidase
CAT	Catalase
MDA	Malondialdehyde
DEG	Differentially expressed gene
KEGG	Kyoto encyclopedia of genes and genomes
PCA	Principal component analysis
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time PCR
ChIP	Chromatin immunoprecipitation

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12284-024-00741-9.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author Contributions

JL, GX, and HZ considered the project and designed the study. JL experimented and measured the data. YC, RZ, RW and BW reviewed and edited the manuscript.

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Data Availability

No datasets were generated or analysed during the current study.

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare no competing interests.

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