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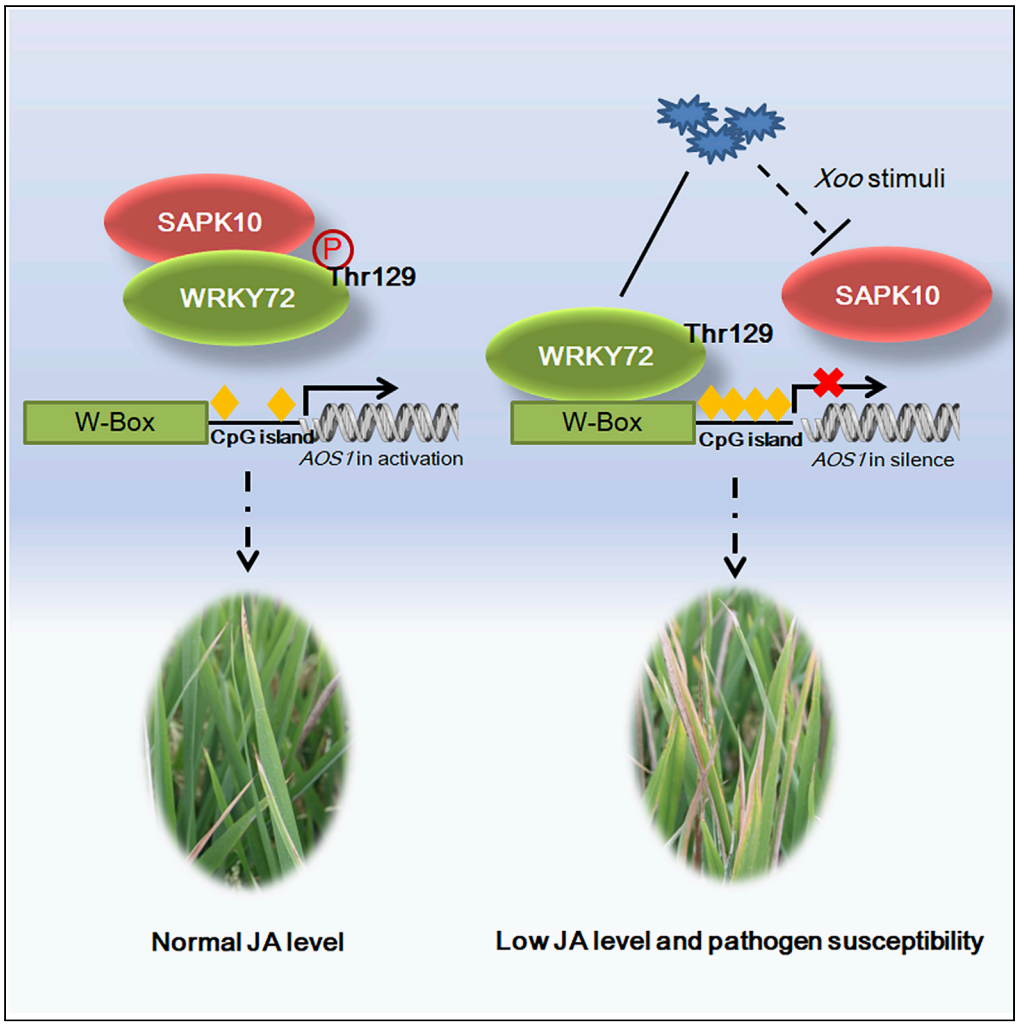

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HIGHLIGHTS

WRKY72 negatively regulates rice resistance to *Xoo* infection and JA synthesis

SAPK10 phosphorylates WRKY72 at Thr 129 to impair its DNA binding on *AOS1*

WRKY72 directly represses *AOS1* transcription to attenuate JA synthesis

WRKY72 recruits hyper DNA methylation on *AOS1* promoter

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SAPK10-Mediated Phosphorylation on WRKY72 Releases Its Suppression on Jasmonic Acid Biosynthesis and Bacterial Blight Resistance

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SUMMARY

Bacterial blight caused by the infection of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a devastating disease that severely challenges the yield of rice. Here, we report the identification of a "SAPK10-WRKY72-AOS1" module, through which *Xoo* infection stimulates the suppression of jasmonic acid (JA) biosynthesis to cause *Xoo* susceptibility. WRKY72 directly binds to the W-box in the promoter of JA biosynthesis gene *AOS1* and represses its transcription by inducing DNA hypermethylation on the target site, which finally led to lower endogenous JA level and higher *Xoo* susceptibility. Abscisic acid (ABA)-inducible SnRK2-type kinase SAPK10 phosphorylates WRKY72 at Thr 129. The SAPK10-mediated phosphorylation impairs the DNA-binding ability of WRKY72 and releases its suppression on *AOS1* and JA biosynthesis. Our work highlights a module of how pathogen stimuli lead to plant susceptibility, as well as a potential pathway for ABA-JA interplay with post-translational modification and epigenetic regulation mechanism involved.

INTRODUCTION

The plant innate immune system is considered to contain two interconnected layers termed PTI (pathogen-associated molecular patterns-triggered immunity) and ETI (effector-triggered immunity) (Jones and Dangl, 2006). Once plant intercepts pathogen-associated molecular patterns (PAMPs) such as chitin and flagellin, it activates downstream defense signaling to provide the first layer (Jones and Dangl, 2006; Saijo et al., 2018). Some virulent pathogens secrete effector proteins to suppress PTI. To fight back, plant resistance (R) proteins trigger ETI that provokes highly efficient defense responses upon effectors (Jones and Dangl, 2006; Peng et al., 2017). PTI and ETI usually result in massive transcriptional reprogramming of defense genes, which indicates the existence of a complex regulatory circuitry composed of transcriptional activators and repressors (Agarwal and Chikara, 2011; Madhunita and Ralf, 2014).

The WRKY family proteins are plant special transcription factors. The WRKY domain contains a conserved WRKYGQK sequence followed by a Cys2His2 or Cys2HisCys zinc-binding motif (Eulgem et al., 2000). WRKY proteins recognize the W-box (T)TGAC(C/T) or W-like box *cis*-regulatory elements, which are often found in many defense gene promoters. In addition to the W-box, it can bind other *cis* elements, such as sugar-responsive element (AA/TAA) in barley and pathogen response element (TACTGCGCTTAGT) in rice (Cai et al., 2008; Cheng et al., 2015; Sun et al., 2003).

WRKY proteins have been reported to play broad and pivotal roles in plant-pathogen interactions and act in a complex signaling network as both positive and negative regulators of disease resistance (Chen and Chen, 2002; Eulgem et al., 2000; Li et al., 2004; Zheng et al., 2006). Till date, a total of 125 WRKY gene family members have been identified and uniquely named by the rice WRKY working group to avoid confusions in nomenclature (hereafter, we follow the nomenclature of rice WRKY working group) (Rice WRKY Working Group, 2012; Ross et al., 2007). Ryu et al. (2006) demonstrated that one-third of the 45 tested WRKY genes in rice were remarkably responsive to the inoculation of bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and the fungal pathogen *Magnaporthe grisea*, which indicated that WRKY genes are extensively involved in plant defense to pathogens (Ryu et al., 2006). Till date, at least 12 WRKY genes have been characterized to be involved in rice disease resistance through diverse mechanisms (Cheng et al., 2015). WRKY12 (originally named as WRKY03), WRKY13, WRKY22, WRKY30, WRKY55 (originally named as WRKY31), WRKY53, WRKY71, and WRKY104 (originally named as WRKY89) are positive regulators of rice

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resistance to pathogens. WRKY12, WRKY55, WRKY53, WRKY71, and WRKY104 could enhance rice resistance to *M. grisea* by up-regulating pathogenesis-related (PR) genes such as *NPR1*, *ZB8*, *POX22.3*, *PR1b*, *PBZ1*, and *Sci2* (Chujo et al., 2014; Liu et al., 2005, 2007; Zhang et al., 2008). Some of the WRKY-positive regulators were implicated in the biosynthesis and signaling of phytohormones such as salicylic acid (SA) and jasmonic acid (JA). For example, WRKY30 activated SA signaling genes in response to *M. grisea* infection, whereas WRKY13 activated a series of SA synthesis and signaling genes and simultaneously suppressed JA synthesis and responsive genes to enhance rice resistance to *M. grisea* and *Xoo* (Cheng et al., 2015; Qiu et al., 2007, 2008). Reported negative regulators include WRKY28, WRKY42, WRKY62, and WRKY76. Most of them increase rice susceptibility to pathogens by suppressing the transcription of defense-related genes, phytoalexin synthesis-related genes, or resistance (*R*) genes (Cheng et al., 2015; Chujo et al., 2013; Peng et al., 2008; Yokotani et al., 2013). Notably, *WRKY45* positively regulates rice resistance to *M. grisea*, but it plays dual roles in rice resistance to bacteria via an alternative splicing model (Shimono et al., 2007, 2012; Tao et al., 2009). Knockout of the variant *WRKY45-1* showed increased resistance to *Xoo* and *Xanthomonas oryzae* pv *oryzicola* (*Xoc*), which was accompanied by a higher level of SA and JA. On the contrary, the variant *WRKY45-2* was found to promote accumulation of JA, but not SA, and eventually play positive functions in response against *Xoo* and *Xoc*. The opposite roles of the two variants in rice-*Xoo* interaction are possibly attributed to their mediation of different defense signaling pathways.

Despite the fact that tremendous progresses on *WRKYs* have been achieved, it is believed that more *WRKY* members are involved in rice immunity, given the extensive involvement of *WRKYs* and complication of rice-pathogen interplays. The current study revealed that *Xoo*-inducible *WRKY72* negatively regulates rice resistance against bacterial blight. WRKY72 directly binds to the promoter of a JA biosynthesis enzyme gene *AOS1* and suppresses its transcription by recruiting DNA methylation on it. SAPK10-mediated phosphorylation on Thr129 of WRKY72 weakens its DNA-binding ability to *AOS1*, promotes the endogenous JA level, and finally enhances *Xoo* resistance.

RESULTS

Transcription of *WRKY72* Is Induced by *Xoo* Infection and Exogenous JA

To find out the rice WRKYs involved in defense against bacterial blight, we performed qRT-PCR analysis of various *WRKYs* in a time line after *Xoo* inoculation and found that *WRKY72* (*LOC_Os11g29870*) is highly induced. The 3,736-bp-long gene encodes a 243-amino acid protein. In its only intron, we identified a SINE (short interspersed nuclear elements)-type transposon (2,812–3,024 bp) (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker). *WRKY72* displayed significantly elevated transcriptional level since 12 HAI (hours after inoculation) and reached highest level (over eight times up-regulation) at 72 HAI, when compared with the 0-HAI samples (Figure 1A). In addition, *WRKY72* also responds to treatment of exogenous phytohormones JA and ABA. *WRKY72* was immediately suppressed by ABA treatment at 4 and 8 HAI, and then increased to a higher level at 12 HAI (Figure 1B). JA treatment showed a similar induction pattern as ABA (Figure 1C). *WRKY72* is constitutively transcribed in various tissues, including leaf, root, panicle, callus, stem, and developing seeds (Figure 1D). To figure out the subcellular localization of WRKY72, we constructed a *pro35S*:WRKY72-GFP vector and co-transformed it with a marker nuclear protein *pro35S*:D53-mKate into rice protoplast. As expected, WRKY72 co-localized with D53 in the nucleus, which supported its functional annotation as a transcription factor (Figure 1E).

WRKY72 Suppresses Rice Resistance to *Xoo*

To dissect the biological roles of *WRKY72* in rice bacterial blight resistance, we generated both CRISPR/Cas9-mediated mutant lines and over-expression lines of the gene. In T_1 generation, two representative homozygous mutant lines *wrky72-4* and *wrky72-7* and two highly over-expressed lines *OxWRKY72-1* and *OxWRKY72-7* were used for phenotyping. *wrky72-4* and *wrky72-7* harbored a G and a T nucleotide insertion in the coding sequence, respectively, which should have disrupted the WRKY72 translation by shifting the open reading frame, although the transcription of the mutated genes were at the same level as native *WRKY72* (Figures 2A and S1). Both *OxWRKY72-1* and *OxWRKY72-7* showed over 80-fold increase of expression level. The major agronomic traits of the plants were largely the same, except that *OxWRKY72s* had lower yield per plant (Table S1). Upon artificial inoculation of *Xoo*, *OxWRKY72-1* and *OxWRKY72-7* became more susceptible than the wild-type (WT), as the lesion area on *OxWRKY72-1* and *OxWRKY72-7* were 37% and 39%, respectively, whereas 25% of the leaf area of WT displayed necrosis (Figures 2B and 2D). It was also revealed that the *Xoo* growth rates in *OxWRKY72s* were significantly higher than those in the WT at

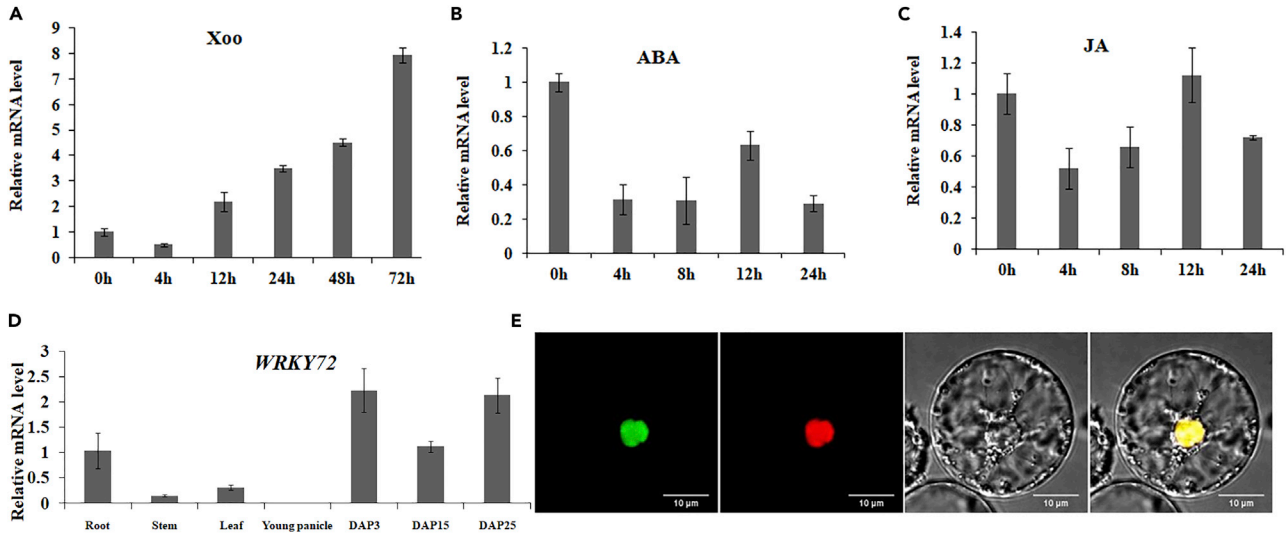

Figure 1. The Temporal-Spatial and Stress Expression Profiles of *WRKY72*

(A–C) The time course expression level of *WRKY72* at *Xoo* inoculation and ABA and JA treatment. (A) *Xoo* and (B) ABA and (C) JA concentrations were ~1 × 10^8 colony-forming unit/mL and 100 μM, respectively.

(D) The expression analysis of *WRKY72* in various tissues and stages. The expression level of leaves was set as 1. DAP: day after pollination.

(E) Subcellular localization of WRKY72. 35S:WRKY72-GFP was co-transformed with a nuclear marker 35S:D53-mKate into protoplast. The fluorescent protein signals from left to right: 35S:WRKY72-GFP, 35S:D53-mKate, bright field, and merged. Scale bars, 10 μm. All data are shown as means ± SD of at least three biological replicates.

3, 7, and 14 DPI (days post inoculation) ($p < 0.05$) (Figure 2C). All these disease index data demonstrated that *WRKY72* negatively regulated rice resistance to *Xoo*. However, *wrky72-4* and *wrky72-7* mutant lines displayed almost identical *Xoo* susceptibility as the WT did, which may be attributed to the functional redundancy of other sibling *WRKY* genes.

WRKY72 Is Phosphorylated by SAPK10 at Thr129

We screened over 1 million colonies from a cDNA library derived from rice young seedlings by using yeast two-hybrid method and detected an interactive kinase SAPK10 (LOC_Os03g41460). SAPK10 may be a negative regulator in response to *Xoo* infection, as its transcription was significantly suppressed by *Xoo* at the first 12 h after inoculation (Figure 3A). We repeated the yeast two-hybrid assay by a reciprocal hybridization and confirmed the SAPK10-WRKY72 interaction in yeast (Figure 3B). The interaction was further verified by pull-down *in vitro* and co-immunoprecipitation *in vivo* assays. The *in vitro* pull-down assay demonstrated that GST-WRKY72 protein could be pulled down by HIS-SAPK10 protein (Figure 3C). Meanwhile, SAPK10-GFP protein, but not the GFP tag control, was specifically co-immunoprecipitated with the WRKY72-FLAG in tobacco (Figure 3D). Thus, we proved the SAPK10-WRKY72 interaction in yeast, *in vitro* and *in vivo*. Subsequently, an *E. coli* kinase assay was performed to examine the kinase-substrate relationship between SAPK10 and WRKY72. When co-transformed with HIS-SAPK10, GST-WRKY72 showed a lagged band as detected by the GST antibody, indicating a phosphorylation has occurred on it (Figure 3E). The phosphorylation was further confirmed by the Phos-tag detection (Figure 3E). Therefore, we concluded that SAPK10 phosphorylates WRKY72 *in vitro*. To specify the SAPK10-dependent phosphosites on WRKY72, we generated the truncated forms of WRKY72 for the kinase assay. The phosphosite was gradually narrowed down to the fragment 1–135 with four potential sites (Figure S2), and finally localized at Thr129 (129^{th} threonine), which is out of, but very close to, the annotated WRKY domain (138–193 amino acid) (Figure 3F).

To test the effect of SAPK10-mediated phosphorylation on the function of WRKY72, we further generated *OxWRKY72*^{T129A} lines, in which the only phosphosite Thr129 was mutated into an alanine (Ala) to block the phosphorylation on it. Two *OxWRKY72*^{T129A} lines with comparable levels of *WRKY72* expression as the *OxWRKY72s* were chosen for *Xoo* susceptibility assay (Figure 2A). Interestingly, *OxWRKY72*^{T129A} lines exhibited higher susceptibility to *Xoo* than the *OxWRKY72* lines (Figure 2D), although the other major

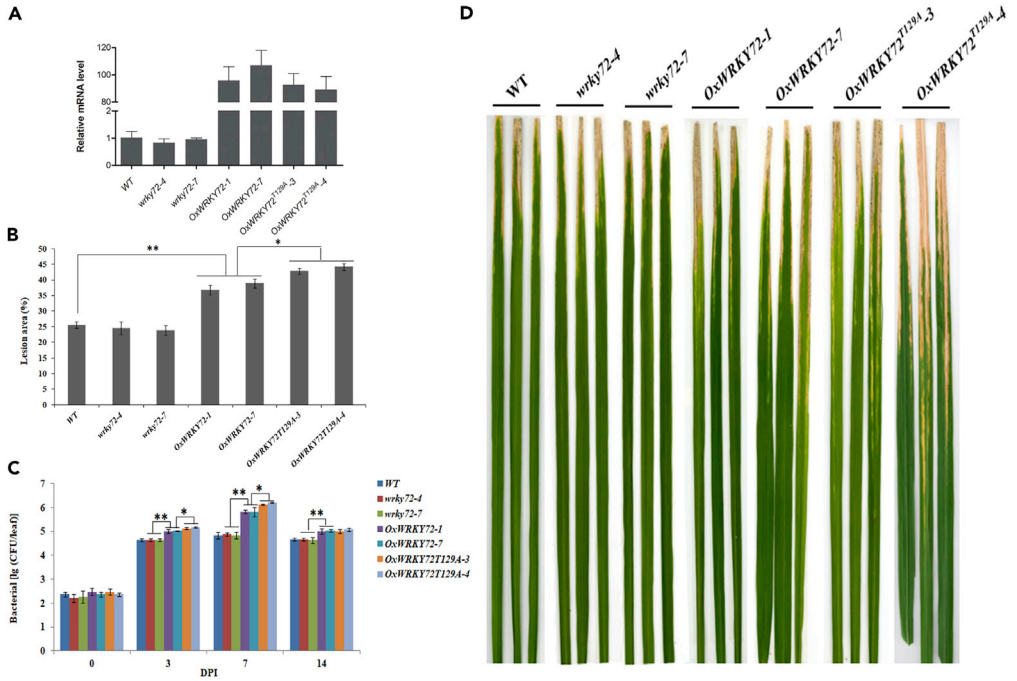

Figure 2. Phenotypical Characterization of *wrky72s*, *OxWRKY72s*, and WT against *Xoo*

(A) The expression analysis of *WRKY72* in *wrky72*, *OxWRKY72*, and WT plant lines.

(B–D) (B) The lesion area (%), (C) bacterial growth rate, and (D) necrosis lesion symposium in *wrky72*, *OxWRKY72*, and WT plant lines. *wrky72-4* and *wrky72-7*: *WRKY72* CRISPR/Cas9 lines; *OxWRKY72-1* and *OxWRKY72-7*: *WRKY72* over-expressing lines; *OxWRKY72*^{T129A}*-3* and *OxWRKY72*^{T129A}*-4*: *WRKY72* over-expressing lines with Thr129 substitution; WT: wild-type. Data are shown as means ± SD of at least three biological replicates. *$p \leq 0.05$, **$p \leq 0.01$ by the Student's t test.

agronomic traits remained largely the same (Table S1), indicating that the phosphorylation on Thr129 might be a key switch to turn off the WRKY72 negative function in disease resistance.

WRKY72 Regulates Defense-Related Genes and JA Biosynthesis Genes

To further elucidate the regulation network, we first performed RT-qCR to examine the transcriptional levels of a couple of JA synthesis genes in *OxWRKY72*, *OxWRKY72*^{T129A}, and WT lines. The results suggested that the all the tested genes including *AOC* (*Allene Oxide Cyclase*, *LOC_Os03g32314*), *AOS1* (*Allene Oxide Synthase 1*, *LOC_Os03g55800*), *AOS2* (*Allene Oxide Synthase 2*, *LOC_Os03g12500*), *LOX1* (*Lipoxygenase 1*, *LOC_Os03g49380*), and *LOX2* (*Lipoxygenase 2*, *LOC_Os03g08220*) were significantly down-regulated in *OxWRKY72*, with the only exception of *OPR7* (*12-Oxophytodienoate reductase*, *LOC_Os08g35740*), which showed an opposite tendency (Figure 4A). More interestingly, the levels of *AOC*, *AOS1*, and *AOS2* were further reduced in *OxWRKY72*^{T129A} when compared with *OxWRKY72* ($p < 0.05$), which is in accordance with the observed higher susceptibility of *OxWRKY72*^{T129A}. We also investigated the levels of PR protein genes *PR1a*, *PR1b*, *PR5*, and *PR10*. *PR1b*, and *PR10* may contribute to the elevated susceptibility of *OxWRKY72* and *OxWRKY72*^{T129A} lines, as both genes were significantly down-regulated (Figure 4B).

Given the obvious down-regulation of the key enzyme genes for JA biosynthesis, we examined the endogenous JA level in *OxWRKY72*, *OxWRKY72*^{T129A}, and WT (Figure 4C). Before the *Xoo* infection, the three types of plants were in a similar and relatively low endogenous JA level, although *OxWRKY72* had a slightly higher level ($p < 0.05$). In the WT, *Xoo* infection significantly stimulated the accumulation of JA to about 16 times higher level than that in the pre-infection condition. However, the *Xoo* infection appeared to have triggered the suppression function of WRKY72 on JA synthesis, as we found that the JA level was significantly reduced in *OxWRKY72* at 72 HAI. Moreover, *OxWRKY72*^{T129A} displayed even lower JA level than *OxWRKY72* at 72 HAI, which is consistent with the observation that *OxWRKY72*^{T129A} lines were more susceptible to *Xoo* than *OxWRKY72s*. The results above suggested that WRKY72 negatively regulates *Xoo* resistance by suppressing JA biosynthesis.

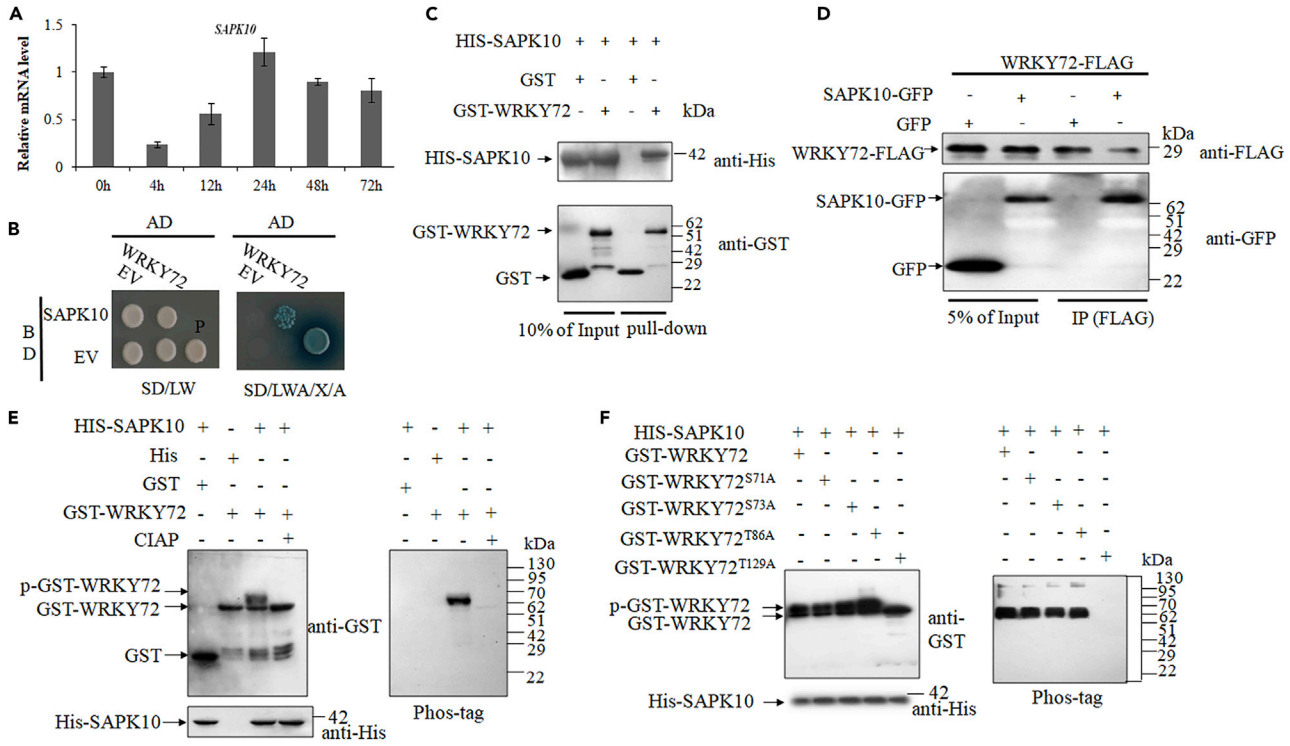

Figure 3. Protein-Protein Interaction and Phosphorylation Site Analysis of WRKY72 and SAPK10

(A) A time course expression level of *SAPK10* at *Xoo* inoculation. Data are shown as means ± SD of at least biological triplicates.

(B) Yeast two-hybrid assays. BD: pGBKT7; AD: pGADT7; EV: empty vector; SD/LW: -Leu-Trp; SD/LWA/X/A: -Leu-Trp-Ade with the addition of X-α-Gal and aureobasidin A; P: positive control, pGADT7-T/pGBKT7-53.

(C) *In vitro* pull-down of SAPK10 and WRKY72. His-SAPK10 and GST-WRKY72 and GST were expressed and purified in *E. coli* and subjected to GST pull-down assays, then detected by immunoblotting using anti-GST and anti-His antibodies, respectively.

(D) *In vivo* co-immunoprecipitation (IP) assay of SAPK10 and WRKY72. GFP, SAPK10-GFP, and WRKY72-FLAG extracted from infiltrated *Nicotiana benthamiana* leaves were used in a coIP assay. Precipitates were immunoblotted with GFP and FLAG antibodies, respectively.

(E and F) WRKY72 is phosphorylated by SAPK10 at Thr129. The recombinant protein GST-WRKY72 (E) and GST-WRKY72 with potential phosphosites substituted (F) were co-expressed with His-SAPK10 in *E. coli*, respectively. Equal amounts of the GST purified recombinant proteins were detected by immunoblotting using indicated antibodies. CIAP: Calf Intestine Alkaline Phosphatase; p-GST-WRKY72: phosphorylated GST-WRKY72; Phos-tag: biotinylated Phos-tag zinc BTL111 complex.

WRKY72 Directly Suppresses *AOS1* Transcription to Attenuate JA Biosynthesis

The reduced level of endogenous JA and JA biosynthesis rate-limiting genes in *OxWRKY72* lines intrigued us to speculate that WRKY72 directly suppresses the transcription of these genes. To test this hypothesis, we checked the *in vitro* binding of WRKY72 on *AOC*, *AOS1*, and *LOX1* promoter regions by EMSA (electrophoretic mobility shift assay). The results showed that WRKY72 only bound to the promoter of *AOS1*, where a conserved W-box *cis* element exists (Figures 5A, 5B, and S3). The shifted bands were substantially weakened when non-labeled competitive probes were applied, suggesting a highly specific binding of WRKY72 to the promoter of *AOS1 in vitro* (Figure 5B). Meanwhile, the binding was completely impaired when the conserved W-box was mutated; hence this 6-nucleotide sequence (TTGACC) might be the core *cis* element for the binding of WRKY72 (Figure 5C). Moreover, we performed EMSA to investigate the DNA-binding ability of WRKY72 in different phosphorylation status. As shown in Figure 5D, p-GST-WRKY72 (Thr129 site phosphorylated) and WRKY72^{T129D}, in which Thr129 was mutated to mimic constitutive phosphorylation status, both had substantially reduced signal of shifted bands when compared with the non-phosphorylated WRKY72. This observation suggested that SAPK10-mediated phosphorylation on Thr129 turns down WRKY72 function by weakening its DNA-binding ability. Subsequently, we generated *proUbi:WRKY72-FLAG* lines and used the leaves at 72 HAI for chromatin immunoprecipitation-qPCR assay. In total, five fragments representing the promoter, UTR, and coding sequence regions were examined, and we found that WRKY72 was significantly enriched in the W-box region of the *AOS1* promoter when compared with the mock, which proved the binding of WRKY72 to *AOS1* promoter *in vivo* (Figure 5G). Finally, we conducted

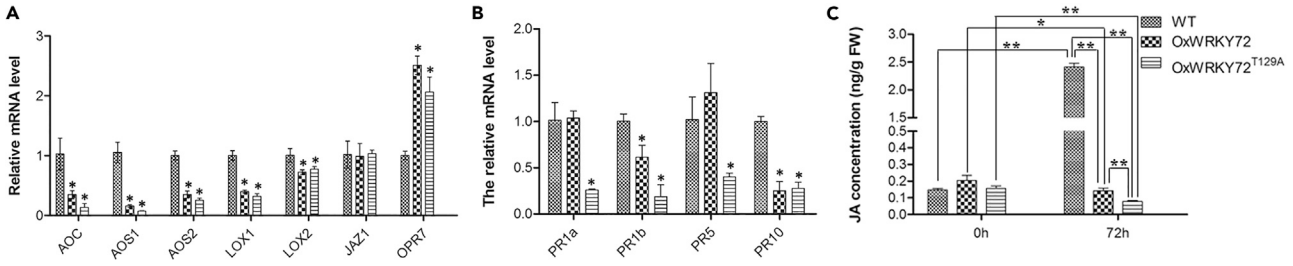

Figure 4. The Transcriptional Abundances of JA Biosynthesis and Pathogenesis-Related Genes and Endogenous JA Level Analysis in WT, *OxWRKY72*, and *OxWRKY72*^{T129A}

(A and B) The transcriptional level of JA biosynthesis and pathogenesis-related genes in WT, *OxWRKY72*, and *OxWRKY72*^{T129A} at 72 h after *Xoo* infection.
(C) The time course endogenous JA level in WT, *OxWRKY72*, and *OxWRKY72*^{T129A}. WT: wild-type; *OxWRKY72*: *WRKY72* over-expression line 1 and line7 sample mixed; *OxWRKY72*^{T129A}: *WRKY72* with Thr129 substitution form's over-expressing line 3 and 4 sample mixed. FW: fresh weight.
Data are shown as means ± SD of three biological replicates. * And ** represent significant difference in the comparison at $p \leq 0.05$ and $p \leq 0.01$, respectively, as determined by the Student's t test.

a dual luciferase (LUC) transient transcriptional activity assay to test the effect of WRKY72 on *AOS1* transcription. When compared with the empty effector, *pro35S*:*WRKY72*:*tNOS* dramatically reduced the firefly LUC reporter level (Figures 5E and 5F), which is in agreement with the observed down-regulation of *AOS1* in *OxWRKY72* lines, indicating that WRKY72 directly suppresses *AOS1* transcription. In support to the observed lower DNA-binding ability of WRKY72^{T129D} in EMSA, we also found that the suppression on *AOS1* was significantly reduced when WRKY72^{T129D} was used as the effector (Figures 5E and 5F).

To figure out the biological functions of *AOS1* in rice disease resistance, we further over-expressed *AOS1* in Nipponbare background. The *OxAOS1* lines had substantially elevated transcription level of *AOS1* and became dwarf, which is a typical phenotype of plants with high endogenous JA levels, given that JA generally represses plant growth (Figures S4A–S4C). After the artificial inoculation of *Xoo*, *OxAOS1* lines exhibited less lesion areas in the leaf and lower bacterial growth rate, suggesting a positive role of *AOS1* in response to *Xoo* infection (Figures 6A–6C).

WRKY72 Induces DNA Hypermethylation on *AOS1* Promoter

DNA methylation has been revealed as a profound epigenetic mechanism involved in gene repression. To address the question that how is *AOS1* suppressed by WRKY72, we checked the DNA methylation pattern and level on the *AOS1* promoter region (minus 251 – minus 73), which is the only potential CpG island as predicted by MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) (Figure 5A). When compared with the WT, the CG and CHH levels of *OxWRKY72* were significantly increased, which led to a significant increase of the total DNA methylation level, although the CHG level was decreased (Figure 7). *WRKY72*^{T129A} exhibited hypermethylation on this region, when compared with WT and *OxWRKY72* (Figure 7). The correlation between the DNA methylation and *AOS1* transcription levels suggested that WRKY72 may induce DNA hypermethylation on *AOS1* promoter to suppress its transcription.

From the results above, a working model of WRKY72 was proposed (Figure 8). Under normal growth conditions, WRKY72 is phosphorylated by SAPK10 at Thr 129, which releases the suppression of WRKY72 to *AOS1* by impairing WRKY72 DNA-binding ability and lowering the DNA methylation level on *AOS1* promoter, thus maintaining a normal endogenous JA level for growth. Under *Xoo* infection, the stimuli represses *SAPK10* transcription and makes the WRKY72 in a non-phosphorylated status, which facilitates the binding of WRKY72 to the W-box *cis* element of *AOS1* promoter to suppress *AOS1* transcription by recruiting hyper DNA methylation on it, and eventually contributes to plant susceptibility by suppressing the endogenous JA biosynthesis (Figure 8). It should be noted that, although our working model proposed a suppressing pathway of JA biosynthesis induced by *Xoo* infection, we did observe that the final endogenous JA level in Nipponbare plants was drastically increased by *Xoo* stimuli at 72 HAI (Figure 4C). Such a phenomenon suggested the existence of a feedback loop comprising suppressing pathways such as SAPK10-WRKY72-AOS1 as well as some unknown activating pathways of JA biosynthesis, whose counterbalance finally decides the endogenous level in rice. In the time point for JA quantification assay, possibly due to the weak level of WRKY72 in WT plants, the WRKY72-mediated suppression might be overcounted

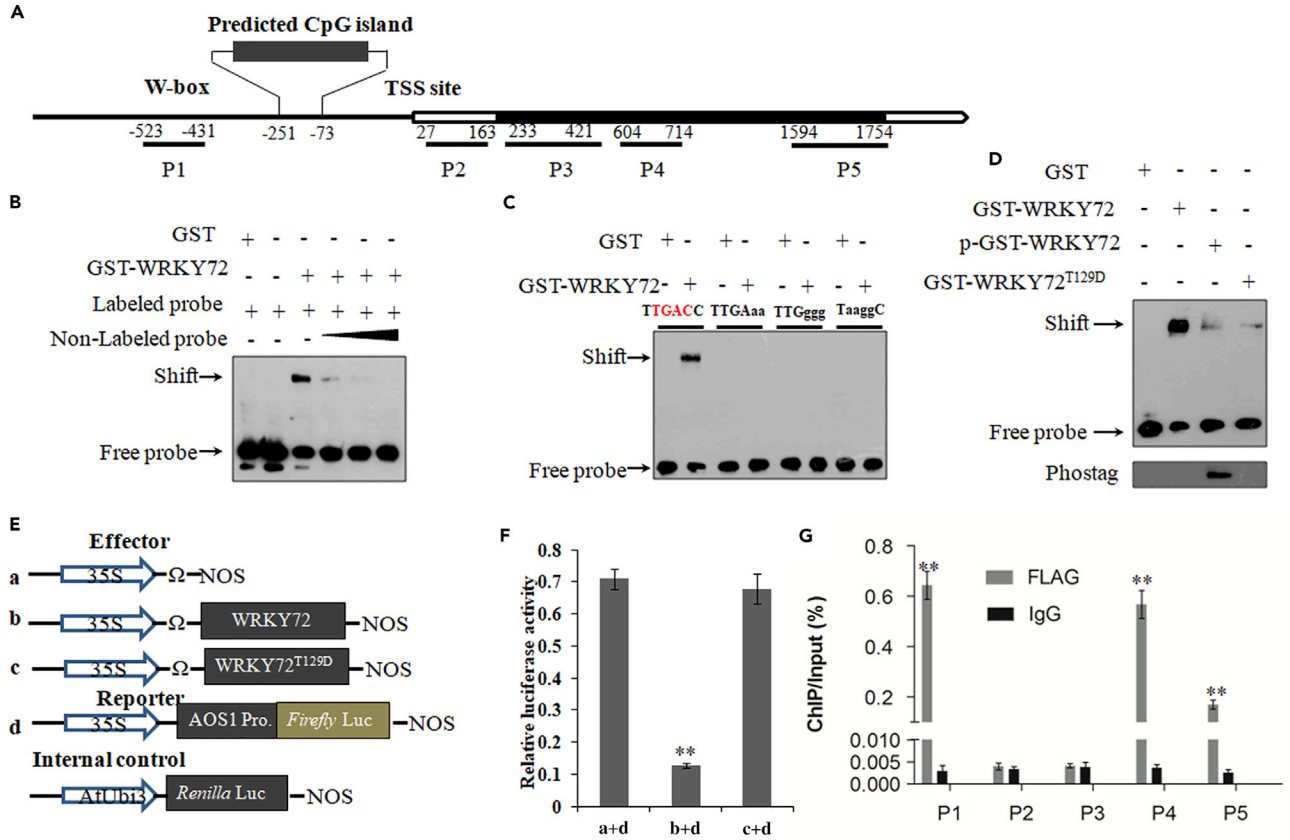

Figure 5. WRKY72 Directly Binds to the W-Box of *AOS1* Promoter and Suppresses Its Transcription, and Phosphorylation of WRKY72 can Reduce its Binding and Suppression Activity

(A) Schematic presentation of the *AOS1* gene structures. Black boxes: exons; blank box: untranslated region; line: promoter. Transcription starting site (TSS) was set as 0. Numbers indicate the distances (bps) to the TSS.

(B) EMSA assay showing WRKY72 could directly bind to the promoter of *AOS1*. The 5-, 10-, and 30-fold excess non-labeled probes were used for competition.

(C) EMSA showing TTGACC is required by WRKY-72 binding to the promoter of *AOS1*. Probe sequence (60 bp) containing W-box (TTGACC). W-box region is shown in (A). TTGACC was substituted by TaaggC, TTGggg, and TTGAaa in the mutant probe, and the substitution nucleotide acids were marked with lowercases.

(D) EMSA assay showing the binding between WRKY72 and *AOS1* is suppressed by phosphorylation of Thr129 in WRKY72 protein. GST-WRKY72, purified protein; p-GST-WRKY72: phosphorylated proteins; GST-WRKY72^{T129D}: mimic phosphorylated proteins. The phosphorylated proteins were chemiluminescence detected by biotinylated Phos-tag zinc complex.

(E) Scheme of the constructs used in LUC transient transcriptional activity assay.

(F) LUC transient transcriptional activity assay in rice protoplast. Reporter: proAOS1:LUC; effectors: pro35S:WRKY72:tNOS and WRKY72^{T129D}. The fLUC/rLUC ratio represents the relative activity of 35S promoter.

(G) Validation of the direct binding of WRKY72 to the promoter and coding sequence (CDS) of *AOS1* by chromatin immunoprecipitation (ChIP)-qPCR. P1–P5 indicates the regions detected by ChIP-qPCR shown in (A).The enrichment values were normalized to Input. IgG-immunoprecipitated DNA was used as a control check (CK).

All values are mean ± SD with biological triplicates. **$p < 0.01$ by the Student's t test.

by the JA biosynthesis activation pathways, which eventually gave rise to the elevated JA level in the WT plant. Nevertheless, in the OxWRKY72 lines with magnified WRKY72 effects, the suppression pathway overrode the activating pathways and finally led to lower JA level and pathogen susceptibility after *Xoo* infection, which perfectly matched the JA quantification assay result in Figure 4C.

DISCUSSION

WRKY72 Suppresses Endogenous JA Level in Defense

It has been well known that JA, as an activating signal molecule, triggers immunity to confer broad-spectrum resistance for plants (Okada et al., 2015). Pathogen infection or other forms of biotic attack stimulate

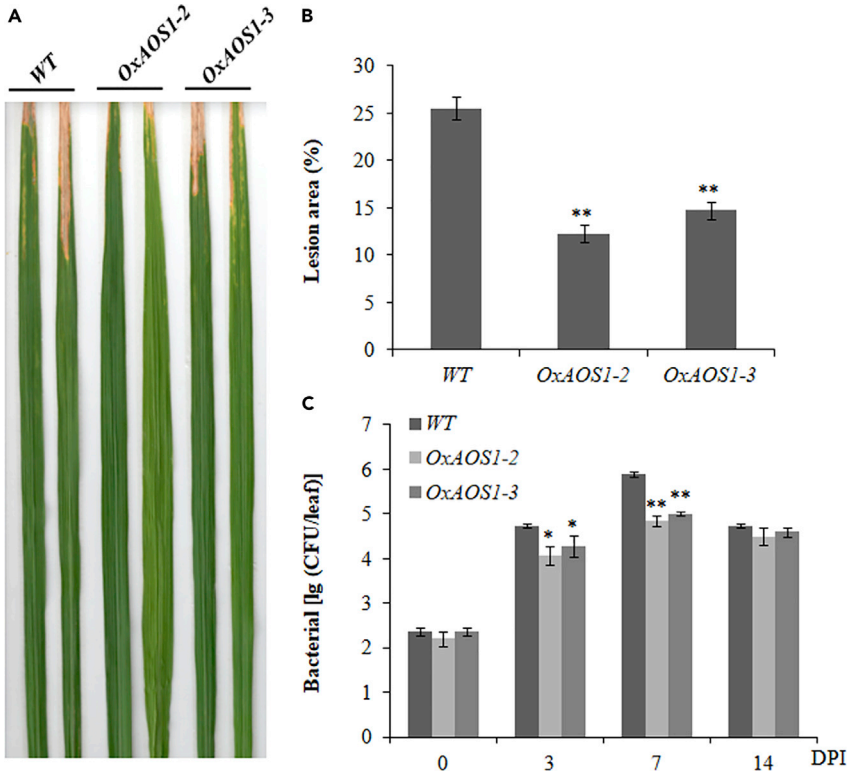

Figure 6. Phenotypical Characterization of *OxAOS1s* and WT against *Xoo*

(A–C) Necrosis lesion symposium (A), the lesion area (%) (B), and bacterial growth rate (C) in *OxAOS1* and WT plant lines. *OxAOS1-2* and *OxAOS1-3*: *AOS1* over-expressing lines; WT: wild-type. Data are shown as means ± SD of at least three biological replicates. *$p \leq 0.05$, **$p \leq 0.01$ by the Student's t test.

rapid biosynthesis of JA and its derivatives, which would promote the expression of defense-related proteins and secondary metabolites such as alkaloids, terpenoids, and PR proteins (Campos et al., 2014). Meanwhile, genetically knocking out or knocking down JA biosynthesis or signaling genes led to higher susceptibility of the plants to various pathogens. In *Arabidopsis*, disruption of JA receptor gene *Coi1* or JA-Ile synthesis gene *JAR1* makes the plants susceptible to necrotrophic pathogens or soil fungus, respectively (Staswick et al., 2002, 2010). Likewise, rice jasmonate-deficient plants *cpm2* and *hebiba* were found to lose their resistance to an originally incompatible avirulent strain of *M. grease*, whereas ectopic expression of *AOS2* encoding a JA production enzyme enhanced the plant resistance to pathogenic fungi (Mei et al., 2006; Riemann et al., 2013). JA may also promote plant resistance to hemi-necrotrophic *Xoo*. For example, JA signaling genes *JAZ8* and *MYC2* both are involved in rice resistance to bacterial blight (Uji et al., 2016; Yamada et al., 2012).

WRKYs are very important transcription factors in plants. The majority of over 100 members in rice are found to be involved in plant defense response in either a negative or a positive manner. The negative regulator members include WRKY28, WRKY42, WRKY62, and WRKY76, but their regulatory mechanism may vary from each other. WRKY62 and WRKY76 cause pathogen susceptibility by regulating a list of defense-related genes or interacting with the intracellular kinase domain of Xa21 to affect its protein cleavage and nuclear localization (Park and Ronald, 2012; Peng et al., 2008; Yokotani et al., 2013). Recently, emerging evidences linked the WRKY-regulated pathogen response to JA accumulation or signaling. For example, WRKY42-knockdown and WRKY42-over-expressing plants showed increased resistance and susceptibility to *M. oryzae*, which are accompanied by increased and reduced JA content, respectively (Cheng et al., 2015). In this research, we found that WRKY72 negatively regulates rice response to *Xoo* infection by suppressing JA biosynthesis, as the WRKY72 over-expression lines became more susceptible upon *Xoo* inoculation. A couple of JA biosynthesis genes were significantly down-regulated in *OxWRKY72* lines. Moreover, we provided several layers of *in vivo* and *in vitro* evidences to show that WRKY72 directly binds to the conserved

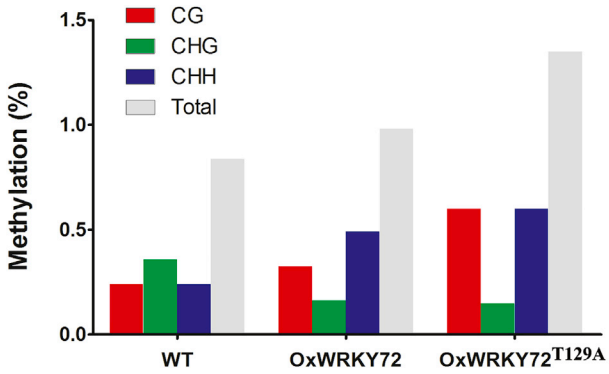

Figure 7. DNA Methylation Patterns and Level in WT, *OxWRKY72*, and *OxWRKY72*^{T129A}

CG, CHG and CHH: three methylation patterns; WT: wild-type; *OxWRKY72*: *WRKY72* over-expression line 1 and line7 sample mixed; *OxWRKY72*^{T129A}: *WRKY72* with Thr129 substitution form's over-expressing line 3 and 4 sample mixed. DNA methylation region detected is shown in Figure 5A.

W-box *cis* element of JA biosynthesis rate-limiting enzyme gene *AOS1* and suppresses *AOS1* transcription, which eventually reduced endogenous JA level and rice resistance to bacterial blight. AOS enzymes catalyze the conversion of 13-HPOT (13-hydroperoxy-9,11,15-octadecatrienoic acid) to 12,13-EOT ((9Z,11E,15Z,13S,12R)-12,13-epoxy-9,11,15-octadecatrienoic), which is the first step toward JA biosynthesis (Schaller, 2001). Among the four *AOS* genes in rice (*AOS1-AOS4*), *AOS2* has been characterized as a pathogen-inducible gene, and its over-expression lines had higher levels of JA and stronger resistance to *M. grisea* (Mei et al., 2006). Likewise, the current study revealed that over-expression of *AOS1* also enhanced plant resistance to *Xoo* infection, when compared with the WT. In another study, *AOS1* was isolated by positional cloning as *Pre* (*precocious*) controlling juvenile-to-adult phase transition in rice. *Pre* exhibited long leaf with precociously acquired adult features in midrib formation, shoot meristem size and plastochron, and more importantly, lower endogenous JA level (Hibara et al., 2016). Although the authors did not explore the potential roles of *AOS1* in disease resistance, it is rational to expect a higher susceptibility of *aos1* to *Xoo* infection.

SAPK10-Mediated Phosphorylation Turns Down WRKY72 Function as a Repressor

Post-translational modifications, particularly protein phosphorylation, have been long recognized as a significant regulatory mechanism controlling transcription factor activity (Meng et al., 2013; Yang et al., 2017). In plant defense, MAPK (mitogen-activated protein kinase) is a major type of kinase that can phosphorylate disease resistance-related transcription factors such as WRKYs. Phosphorylation within the SP cluster of WRKY proteins by MAPKs is thought to exert a booster function in the expression of downstream genes (Asai et al., 2002; Ishihama and Yoshioka, 2012; Pitzschke et al., 2009). In *Arabidopsis*, AtMPK3 and AtMPK6 directly phosphorylated AtWRKY33 to enhance the production of phytoalexin camalexin and phytohormone ethylene, whereas non-phosphorylated AtWRKY33 was not able to fully rescue *wrky33* mutant, implying that MAPK-dependent phosphorylation activates AtWRKY33 function (Li et al., 2012; Mao et al., 2011; Wang et al., 2018). Similarly, Chujo et al. (2014) found that WRKY53-mediated resistance to rice blast fungus strain Ina86-137 relied on the phosphorylation on its serine-proline residues by MPK3/MPK6. Over-expressing a phosphomimic mutated version of WRKY53 (WRKY53SD) rice plants elevated the expression level of defense-related genes and enhanced disease resistance to *M. oryzae* compared with native WRKY53-over-expressing rice plants (Chujo et al., 2014). It was suggested that the positive effect of MAPK-dependent phosphorylation on WRKYs might be achieved by increasing its DNA-binding activities on target genes (Ishihama and Yoshioka, 2012; Koo et al., 2009; Menke et al., 2005).

In this study, we demonstrated that SAPK10 kinase cloud physically binds to and phosphorylates WRKY72 at Thr129. In contrast to the above-mentioned cases that phosphorylation activated WRKYs, the phosphorylation on WRKY72 weakened its DNA-binding ability to *AOS1* promoter, to thus release the inhibition on JA accumulation. In support of this finding, the *OxWRKY72*^{T129A} lines, which had blocked SAPK10 target phosphosite, showed drastically reduced transcription of downstream JA biosynthesis genes, endogenous JA level, and resistance to *Xoo* infection than the native *OxWRKY72* lines. Hence, the indication is that the SAPK10-dependent phosphorylation on WRKY72 turns down its function as a transcription repressor in plant defense, representing

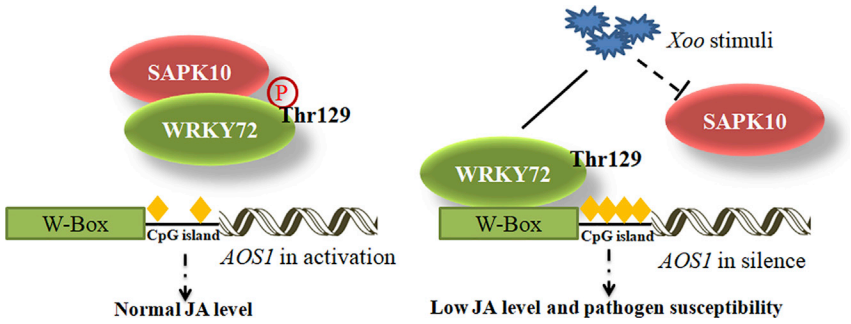

Figure 8. Working Model of WRKY72-Mediated Regulation against *Xoo* Infection in Rice

Line and dotted line indicate positive and negative regulation in transcription, respectively; ⓟ indicates the phosphorylation status of the protein; and yellow rhombus indicates DNA methylation on the region.

a diverse mechanism to the previously reported MAPK-WRKY module. SAPK10 is an ABA-inducible SnRK2-type kinase involved in ABA signaling (Kobayashi et al., 2010). So far, the effects of ABA on plant disease resistance remain elusive. ABA likely plays negative roles in plant defense; however, the interplay of ABA with other phytohormones often produces complicated network and possibly promotes defense in plants (Ton et al., 2009). Notably, a couple of rice SnRK2s have been implicated in response to *Xoo* infection (Xu et al., 2013). Our results hint a pathway "SAPK10-WRKY72-AOS1" in the cross talk of ABA-JA as well as in the ABA-mediated plant defense response, which will be further explored in our future study.

WRKY72 Recruits DNA Hypermethylation to Repress *AOS1*

DNA cytosine methylation is usually connected with transcriptional silence of the target genes in numerous biological processes, including plant defense (Chen and Zhou, 2013; Wang et al., 2018). RdDM (RNA-directed DNA methylation) is a major mechanism to recruit DNA methyltransferases to the target site to execute DNA methylation. In such a case, DNA methylation is guided by a series of 21- to 24-nt small interfering RNA (siRNAs) with high homology with the target sites. The siRNAs could be derived from viral replication intermediates, products of endogenous RNA-directed RNA polymerase, transcribed inverted repeats, or TEs (transposable elements) (Wassenegger et al., 1994). One of the well-documented RdDM cases in rice defense is TE-siR815 (Zhang et al., 2016). TE-siR815 is an siRNA that originates from a MITE (miniature inverted repeat transposable elements). TE existed in the first intron of *WRKY45*, whose two variants *WRKY45-1* and *WRKY45-2* play opposite roles in response to *Xoo* and *Xoc* infection (Tao et al., 2009). Only the negative player *WRKY45-1* produces TE-siR815, which imposes DNA hypermethylation on *ST1* via an RdDM mechanism to abolish WRKY45-mediated pathogen resistance (Zhang et al., 2016). In our study, the total DNA cytosine methylation level on *AOS1* promoter was negatively correlated with the *AOS1* transcription level and endogenous JA level in WT, *OxWRKY72*, and *OxWRKY72*^{T129A} lines, implying that the repressor role of WRKY72 may be achieved by inducing DNA methylations on the promoter region of its direct target *AOS1*. Nevertheless, the question that how was the DNA methyltransferase recruited to the target sites remain to be addressed. The identified SINE TE in *WRKY72* intron might be a good clue that WRKY72 suppresses target genes through an RdDM mechanism, given the reported example of its homolog WRKY45 and many SINE-directed DNA methylation cases in mouse and human beings (Estécio et al., 2012; Yates et al., 2003).

Limitations of the Study

In this study, we revealed a "SAPK10-WRKY72-AOS1" module, through which *Xoo* infection suppresses JA biosynthesis to cause *Xoo* susceptibility. However, as we have discussed in the article, the final endogenous JA level in plant is determined by the counterbalance of both activation and suppression pathways. Therefore, figuring out the *Xoo*-activated JA biosynthesis regulatory pathways would be of great interest for us to elucidate the comprehensive reaction of rice in response to *Xoo* infection. In addition, although we provided clues that WRKY72 represses *AOS1* transcription via RdDM, the detailed mechanism needs to be explored in future studies.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.06.009.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

J.Z. and S.H. planned and designed the research; Y.H., Y.W., L.T., X.T., L.W., and L.L. performed experiments; Y.H., Y.W., and J.Z. analyzed data; Y.H. and J.Z. wrote the manuscript. Y.H. and Y.W. contributed equally.

DECLARATION OF INTERESTS

All the authors declare no conflicts of interests in this paper.

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REFERENCES

Agarwal, P., and Chikara, J. (2011). WRKY: its structure, evolutionary relationship, DNA-binding selectivity, role in stress tolerance and development of plants. Mol. Biol. Rep. *38*, 3883–3896.

Asai, T., Tena, G., Plotnikova, J., Willmann, M., Chiu, W.-L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. Nature *415*, 977–983.

Cai, M., Qiu, D., Yuan, T., Ding, X., Li, H., Duan, L., Xu, C., Li, X., and Wang, S. (2008). Identification of novel pathogen-responsive cis-elements and their binding proteins in the promoter of OsWRKY13, a gene regulating rice disease resistance. Plant Cell Environ. *31*, 86–96.

Campos, M.L., Kang, J.H., and Howe, G.A. (2014). Jasmonate-triggered plant immunity. J. Chem. Ecol. *40*, 657–675.

Chen, C., and Chen, Z. (2002). Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced Arabidopsis transcription factor. Plant Physiol. *129*, 706–716.

Chen, X., and Zhou, D.X. (2013). Rice epigenomics and epigenetics: challenges and opportunities. Curr. Opin. Plant Biol. *16*, 164–169.

Cheng, H., Liu, H., Deng, Y., Xiao, J., Li, X., and Wang, S. (2015). The WRKY45-2 WRKY13 WRKY42 transcriptional regulatory cascade is required for rice resistance to fungal pathogen. Plant Physiol. *167*, 1087–1099.

Chujo, T., Miyamoto, K., Ogawa, S., Masuda, Y., Shimizu, T., Kishi-Kaboshi, M., Takahashi, A., Nishizawa, Y., Minami, E., Nojiri, H., et al. (2014). Overexpression of phosphomimic mutated OsWRKY53 leads to enhanced blast resistance in rice. PLoS One *9*, e98737.

Chujo, T., Miyamoto, K., Shimogawa, T., Shimizu, T., Otake, Y., Yokotani, N., Nishizawa, Y., Shibuya, N., Nojiri, H., Yamane, H., et al. (2013). OsWRKY28, a PAMP-responsive transrepressor, negatively regulates innate immune responses in rice against rice blast fungus. Plant Mol. Biol. *82*, 23–37.

Estécio, M.R.H., Gallegos, J., Dekmezian, M., Yue, L., Liang, S., and Issa, J.P.J. (2012). SINE retrotransposons cause epigenetic reprogramming of adjacent gene promoters. Mol. Cancer Res. *10*, 1332.

Eulgem, T., Rushton, P.J., Robatzek, S., and Somssich, I.E. (2000). The WRKY superfamily of plant transcription factors. Trends Plant Sci. *5*, 199–206.

Hibara, K.I., Isono, M., Mimura, M., Sentoku, N., Kojima, M., Sakakibara, H., Kitomi, Y., Yoshikawa, T., Itoh, J.I., and Nagato, Y. (2016). Jasmonate regulates juvenile-adult phase transition in rice. Development *143*, 3407.

Ishihama, N., and Yoshioka, H. (2012). Post-translational regulation of WRKY transcription factors in plant immunity. Curr. Opin. Plant Biol. *15*, 431–437.

Jones, J.D.G., and Dangl, J.L. (2006). The plant immune system. Nature *444*, 323–329.

Kobayashi, Y., Murata, M., Minami, H., Yamamoto, S., Kagaya, Y., Hobo, T., Yamamoto, A., and Hattori, T. (2010). Abscisic acid-activated SNRK2 protein kinases function in the gene-regulation pathway of ABA signal transduction by phosphorylating ABA response element-binding factors. Plant J. *44*, 939–949.

Koo, S.C., Moon, B.C., Kim, J.K., Kim, C.Y., Sung, S.J., Kim, M.C., Cho, M.J., and Cheong, Y.H. (2009). OsBWMK1 mediates SA-dependent defense responses by activating the transcription factor OsWRKY33. Biochem. Biophys. Res. Commun. *387*, 365–370.

Li, G., Meng, X., Wang, R., Mao, G., Han, L., Liu, Y., and Zhang, S. (2012). Dual-level regulation of ACC synthase activity by MPK3/MPK6 cascade and its downstream WRKY transcription factor during ethylene induction in Arabidopsis. PLoS Genet. *8*, e1002767.

Li, J., Brader, G., and Palva, E.T. (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell *16*, 319–331.

Liu, X., Bai, X., Wang, X., and Chu, C. (2007). OsWRKY71, a rice transcription factor, is involved in rice defense response. J. Plant Physiol. *164*, 969–979.

Liu, X.Q., Bai, X.Q., Qian, Q., Wang, X.J., Chen, M.S., and Chu, C.C. (2005). OsWRKY03, a rice transcriptional activator that functions in defense signaling pathway upstream of OsNPR1. Cell Res. *15*, 593–603.

Madhunita, B., and Ralf, O. (2014). WRKY transcription factors: jack of many trades in plants. Plant Signal. Behav. *9*, e27700.

Mao, G., Meng, X., Liu, Y., Zheng, Z., Chen, Z., and Zhang, S. (2011). Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in Arabidopsis. Plant Cell *23*, 1639–1653.

Mei, C., Qi, M., Sheng, G., and Yang, Y. (2006). Inducible overexpression of a rice allene oxide synthase gene increases the endogenous jasmonic acid level, PR gene expression, and host resistance to fungal infection. Mol. Plant Microbe Interact. *19*, 1127–1137.

Meng, X., Xu, J., He, Y., Yang, K.Y., Mordorski, B., Liu, Y., and Zhang, S. (2013). Phosphorylation of an ERF transcription factor by Arabidopsis MPK3/MPK6 regulates plant defense gene induction and fungal resistance. Plant Cell *25*, 1126–1142.

Menke, F.L., Kang, H.Z., Park, J.M., Kumar, D., and Klessig, D.F. (2005). Tobacco transcription factor WRKY1 is phosphorylated by the MAP kinase SIPK and mediates HR-like cell death in tobacco. Mol. Plant Microbe Interact. *18*, 1027–1034.

Okada, K., Abe, H., and Arimura, G. (2015). Jasmonates induce both defense responses and communication in monocotyledonous and dicotyledonous plants. Plant Cell Physiol. *56*, 16–27.

Park, C.J., and Ronald, P.C. (2012). Cleavage and nuclear localization of the rice XA21 immune receptor. Nat. Commun. *3*, 920.

Peng, Y., Bartley, L.E., Chen, X., Dardick, C., Chern, M., Ruan, R., Canlas, P.E., and Ronald, P.C. (2008). OsWRKY62 is a negative regulator of basal and Xa21-mediated defense against Xanthomonas oryzae pv. oryzae in rice. Mol. Plant *1*, 446–458.

Peng, Y., Wersch, R.V., and Zhang, Y. (2017). Convergent and divergent signaling in PAMP-triggered immunity and Effector-triggered immunity. Mol. Plant Microbe Interact. *31*, 403–409.

Pitzschke, A., Schikora, A., and Hirt, H. (2009). MAPK cascade signalling networks in plant defence. Curr. Opin. Plant Biol. *12*, 421–426.

Qiu, D., Xiao, J., Ding, X., Xiong, M., Cai, M., Cao, Y., Li, X., Xu, C., and Wang, S. (2007). OsWRKY13 mediates rice disease resistance by regulating defense-related genes in salicylate- and jasmonate-dependent signaling. Mol. Plant Microbe Interact. *20*, 492–499.

Qiu, D., Xiao, J., Xie, W., Liu, H., Li, X., Xiong, L., and Wang, S. (2008). Rice gene network inferred from expression profiling of plants overexpressing OsWRKY13, a positive regulator of disease resistance. Mol. Plant *1*, 538–551.

Rice WRKY Working Group (2012). Nomenclature report on rice WRKY's - conflict regarding gene names and its solution. Rice *5*, 3.

Riemann, M., Haga, K., Shimizu, T., Okada, K., Ando, S., Mochizuki, S., Nishizawa, Y., Yamanouchi, U., Nick, P., and Yano, M. (2013). Identification of rice Allene Oxide Cyclase mutants and the function of jasmonate for defence against Magnaporthe oryzae. Plant J. *74*, 226–238.

Ross, C., Liu, Y., and Shen, Q. (2007). The WRKY gene family in rice (Oryza sativa). J. Integr. Plant Biol. *49*, 827–842.

Ryu, H.S., Han, M., Lee, S.K., Cho, J.I., Ryoo, N., Heu, S., Lee, Y.H., Bhoo, S.H., Wang, G.L., Hahn, T.R., et al. (2006). A comprehensive expression analysis of the WRKY gene superfamily in rice plants during defense response. Plant Cell Rep. *25*, 836–847.

Saijo, Y., Loo, E.P., and Yasuda, S. (2018). Pattern recognition receptors and signaling in plant-microbe interactions. Plant J. *93*, 592–613.

Schaller, F. (2001). Enzymes of the biosynthesis of octadecanoid-derived signalling molecules. J. Exp. Bot. *52*, 11–23.

Shimono, M., Koga, H., Akagi, A., Hayashi, N., Goto, S., Sawada, M., Kurihara, T., Matsushita, A., Sugano, S., and Jiang, C.J. (2012). Rice WRKY45 plays important roles in fungal and bacterial disease resistance. Mol. Plant Pathol. *13*, 83–94.

Shimono, M., Sugano, S., Nakayama, A., Jiang, C.J., Ono, K., Toki, S., and Takatsuji, H. (2007). Rice WRKY45 plays a crucial role in Benzothiadiazole-inducible blast resistance. Plant Cell *19*, 2064–2076.

Staswick, P.E., Tiryaki, I., and Rowe, M.L. (2002). Jasmonate response locus JAR1 and several related Arabidopsis genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. Plant Cell *14*, 1405–1415.

Staswick, P.E., Yuen, G.Y., and Lehman, C.C. (2010). Jasmonate signaling mutants of Arabidopsis are susceptible to the soil fungus Pythium irregulare. Plant J. *15*, 747–754.

Sun, C., Palmqvist, S., Olsson, H., Borén, M., Ahlandsberg, S., and Jansson, C. (2003). A Novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the iso1 promoter. Plant Cell *15*, 2076.

Tao, Z., Liu, H., Qiu, D., Zhou, Y., Li, X., Xu, C., and Wang, S. (2009). A pair of allelic WRKY genes play opposite roles in rice-bacteria interactions. Plant Physiol. *151*, 936–948.

Ton, J., Flors, V., and Mauchmani, B. (2009). The multifaceted role of ABA in disease resistance. Trends Plant Sci. *14*, 310–317.

Uji, Y., Taniguchi, S., Tamaoki, D., Shishido, H., Akimitsu, K., and Gomi, K. (2016). Overexpression of OsMYC2 results in the up-regulation of early JA-rresponsive genes and bacterial blight resistance in rice. Plant Cell Physiol. *57*, 1814–1827.

Wang, Y., Schuck, S., Wu, J., Yang, P., Doering, A.C., Zeier, J., and Tsuda, K. (2018). A MPK3/6-WRKY33-ALD1-pipecolic acid regulatory loop contributes to systemic acquired resistance. Plant Cell. *30*, 2480–2494.

Wassenegger, M., Heimes, S., Riedel, L., and Sänger, H.L. (1994). RNA-directed de novo methylation of genomic sequences in plants. Cell *76*, 567–576.

Xu, M., Huang, L., Zhang, F., Zhu, L., Zhou, Y., and Li, Z. (2013). Genome-wide phylogenetic analysis of stress-activated protein kinase genes in rice (OsSAPKs) and expression profiling in response to xanthomonas oryzae pv. oryzicola infection. Plant Mol. Biol. Rep. *31*, 877–885.

Yamada, S., Kano, A., Tamaoki, D., Miyamoto, A., Shishido, H., Miyoshi, S., Taniguchi, S., Akimitsu, K., and Gomi, K. (2012). Involvement of OsJAZ8 in jasmonate-induced resistance to bacterial blight in rice. Plant Cell Physiol. *53*, 2060–2072.

Yang, W., Zhang, W., and Wang, X. (2017). Post-translational control of ABA signalling: the roles of protein phosphorylation and ubiquitination. Plant Biotechnol. J. *15*, 4–14.

Yates, P.A., Burman, R., Simpson, J., Ponomoreva, O.N., Thayer, M.J., and Turker, M.S. (2003). Silencing of mouse Aprt is a gradual process in differentiated cells. Mol. Cell. Biol. *23*, 4461–4470.

Yokotani, N., Sato, Y., Tanabe, S., Chujo, T., Shimizu, T., Okada, K., Yamane, H., Shimono, M., Sugano, S., Takatsuji, H., et al. (2013). WRKY76 is a rice transcriptional repressor playing opposite roles in blast disease resistance and cold stress tolerance. J. Exp. Bot. *64*, 5085–5097.

Zhang, H., Zeng, T., Hong, H., Chen, Z., Wu, C., Li, X., Xiao, J., and Wang, S. (2016). Transposon-derived small RNA is responsible for modified function of WRKY45 locus. Nat. Plants *2*, 16016.

Zhang, J., Peng, Y., and Guo, Z. (2008). Constitutive expression of pathogen-inducible OsWRKY31 enhances disease resistance and affects root growth and auxin response in transgenic rice plants. Cell Res. *18*, 508–521.

Zheng, Z., Qamar, S.A., Chen, Z., and Mengiste, T. (2006). Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J. *48*, 592–605.

ISCI, Volume *16*

Supplemental Information

SAPK10-Mediated Phosphorylation on WRKY72 Releases Its Suppression on Jasmonic Acid Biosynthesis and Bacterial Blight Resistance

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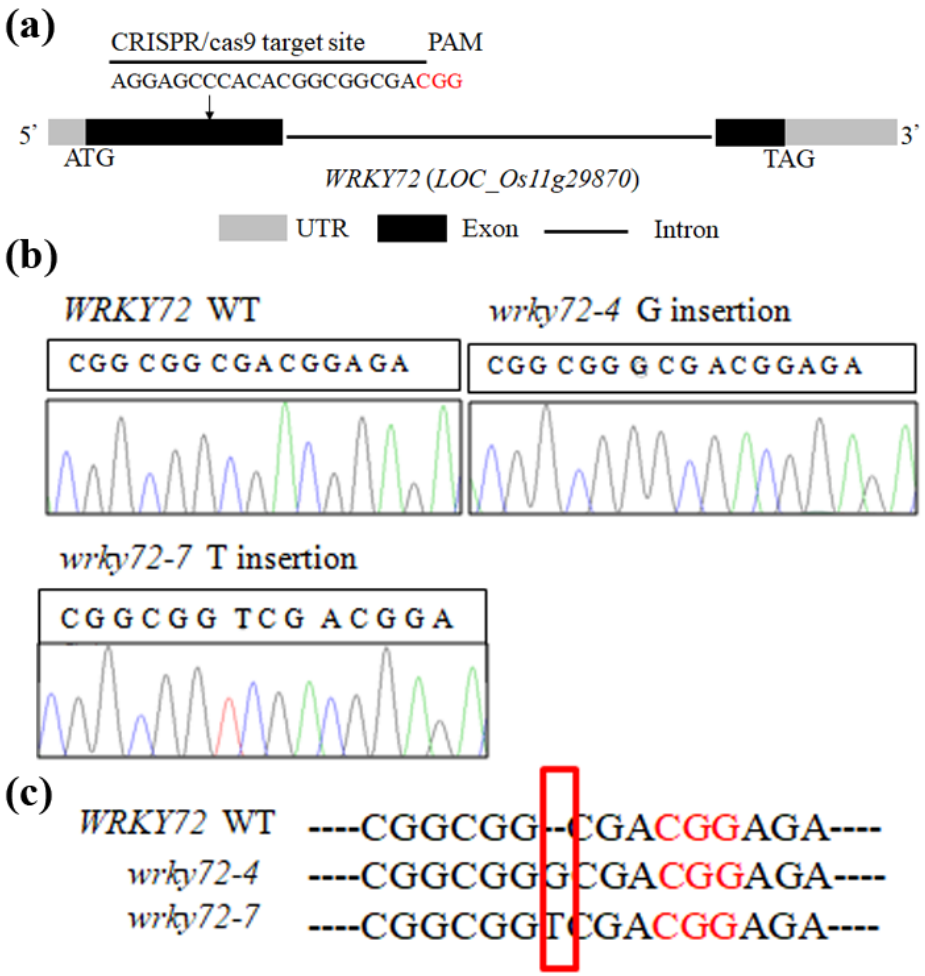
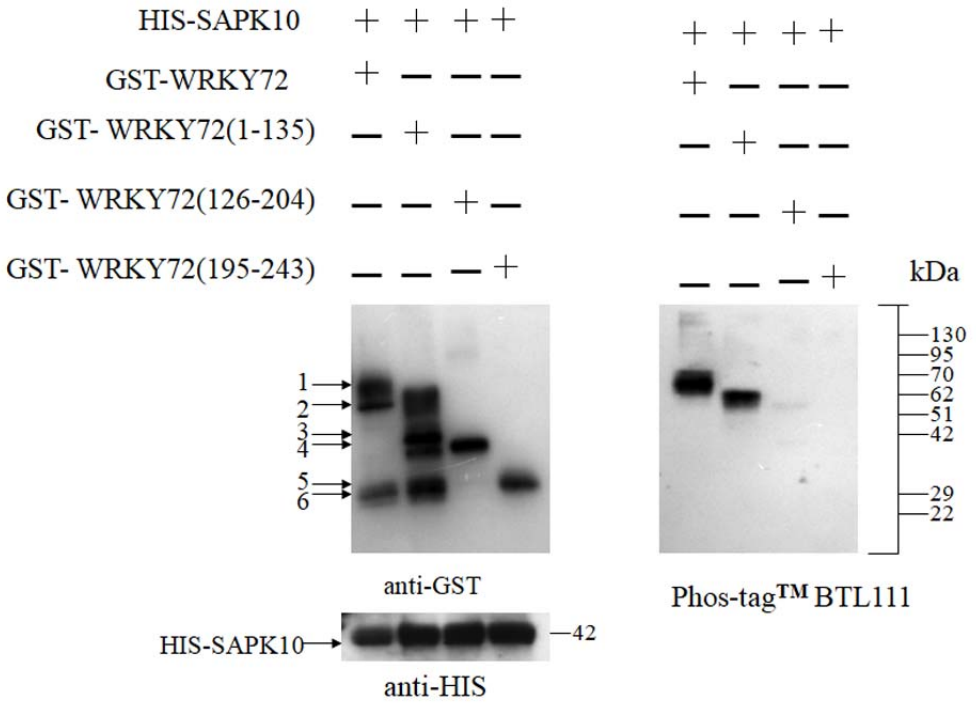
Supporting information

Figure S1. Sanger Sequencing of the Mutated Sites in Homozygous Mutants of *wrky72-4 and wrky72-7*, Related to Figure 2.



(a) Schematic presentation of the *WRKY72* structure and gene editing site. (b) Sanger sequencing chromatograph of the target site on *WRKY72*. (c) Summary of the mutations in the gene edited lines.

Figure S2. *In vitro* Phosphorylation of the Gradually Narrowed Fragment of WRKY72, Related to Figure 3.



1:p- WRKY72; 2:GST-WRKY72;3. GST- WRKY72(1-135);4:GST- WRKY72(126-204);5:GST-WRKY72(195-243); 6.GST.

Figure S3. EMSA of WRKY72 on *AOC* and *LOX1* Promoter Regions, Related to Figure 5.

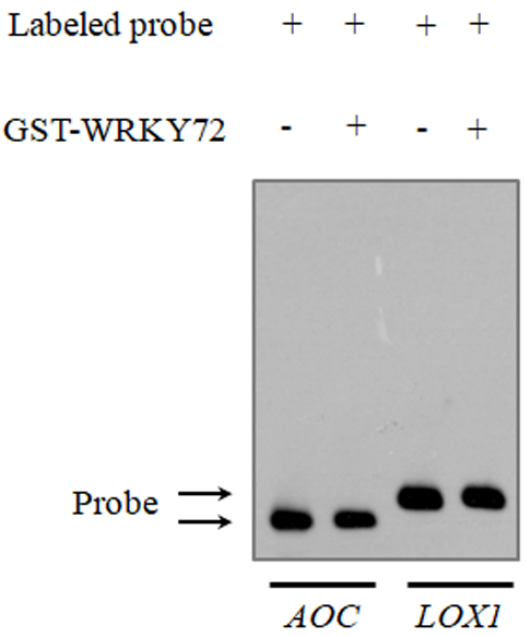
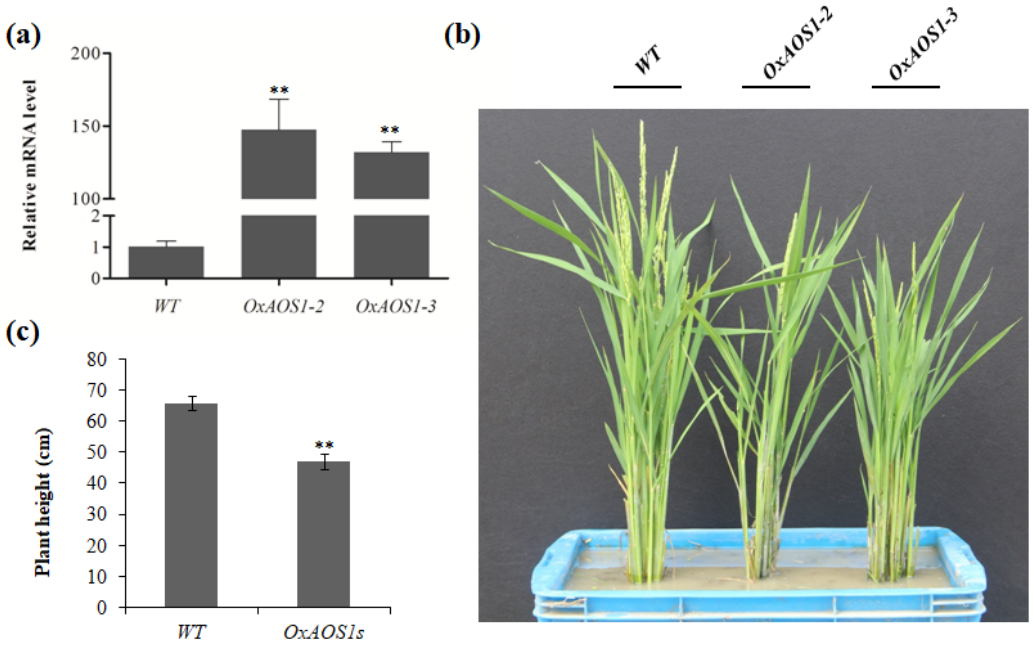


Figure S4. Major Agronomic Traits of *OxAOS1s* and WT, Related to Figure 6.



(a) The expression analysis of *AOS1* in *OxAOS1* and WT plant lines. (b-c) The height analysis of *OxAOS1* and WT plant lines. Data are shown as means ± SD of at least three biological replicates. *: P≤0.05, **≤0.01 by the Student's *t* test.

Table S1. Major Agronomic Traits of *WRKY72* CRISPR and Over-expressing Plants, Related to Figure 2.

	Plant height (cm)	Flowering date (days)	Seed length (cm)	Seed width (cm)	Yield /plant (g)
WT	69.7±1.06	72.0±2.00	7.7±0.10	3.4±0.10	30.57±0.53
wrky72-4	69.3±2.17	70.0±1.00	7.8±0.31	3.4±0.21	29.97±1.49
wrky72-7	69.9±1.07	70.7±0.58	7.6±0.15	3.3±0.15	31.82±1.39
OxWRKY72-1	68.7±1.20	70.3±1.53	7.4±0.40	3.5±0.06	27.93±0.46*
OxWRKY72-7	68.6±1.15	71.3±1.53	7.6±0.25	3.4±0.15	26.63±1.27*
OxWRKY72^{T129A}-3	69.9±2.18	72.0±1.73	7.7±0.21	3.4±0.10	26.58±0.72**
OxWRKY72^{T129A}-4	68.6±1.18	71.7±2.08	7.5±0.32	3.3±0.23	26.22±0.95**

The data is presented as the means ± SD of at least three biological replicates. The significant difference between the WT and *WRKY72* CRISPR and over-expressing plants is determined by the Student's *t* test, the single asterisk indicates P≤0.05, and double asterisks indicate P≤0.01. WT: wild-type; *wrky72-4* and *wrky72-7*: *WRKY72* CRISPR plants; *OxWRKY72-1* and *OxWRKY72-7*: *WRKY72* over-expressing plants; *OxWRKY72^{T129A}-3* and *OxWRKY72^{T129A}-4*: *WRKY72* over-expressing plants with Thr129 substituted by Alanine.

Table S2. Sequences of Primers Used in This Study, Related to Figure 1, 2, 3, 4, 5, 6 and 7.

Name	Gene ID	Primer sequence (5' to 3')
Primers for over-expression constructs		
WRKY72-KpnI-F	LOC_Os11g29870	GGGGTACCATGGAGAACTTCCCGATACTCTTTG
WRKY72-BamHI-R		CGGGATCCCTACTGGAACATGTGGGAAGCAGCA
WRKY72-FLAG-KpnI-F		AAAGGTACCATGGAGAACTTCCCGATACTCTTTG
WRKY72-FLAG-BamHI-R		CGGGATCCCTAGGGCCCCCCCTCGACTTTATCGTCA
OxAOS1F	LOC_Os03g55800	TACGAACGATAGCCGATGGCCACGGCGGCGGCT
OxAOS1R		GGACTCTAGAGGATCTCAGAAGGTGGCCTTCTTG
Primers for subcellular localization construct		
ScWRKY72F		CAGTGGTCTCACAACATGGAGAACTTCCCGATACT
ScWRKY72R		CAGTGGTCTCATACACTGGAACATGTGGGAAGCAG
Primers for qRT-PCR		
qWRKY72F	LOC_Os11g29870	CAAGGGTGCAACGTGAAGAA
qWRKY72R		ATTTCTCGATGGGGTGCGTG
qAOCF	LOC_Os03g32314	GCCAAGGTGCAGGAGATGTTCG
qAOCR		AGCCGCTTGTCCAGGCTTCC
qAOS1F	LOC_Os03g55800	CGGGACATGGTGGTGGAGA
qAOS1R		GGAGTCGTATCGGAGGAAGAGC
qAOS2F	LOC_Os03g12500	TCGTCGGAAGGCTGTTGC
qAOS2R		ACGATTGACGGCGGAGGT
qLOX1F	LOC_Os03g49380	CCAACCAGACAAAGGCAGTA
qLOX1R		GGGAGAACACCCTCAACAATA
qLOX2F	LOC_Os08g39840	CGACGACCGTGTCTACGACTA
qLOX2R		CGTCTCCGACTTAGGGTCTTTT
qOPR7F	LOC_Os08g35740	GAAGGTGGTGGATGCTGTT
qOPR7R		TTTAGGATACTTGCCATAGGAG
qPR1aF	LOC_Os07g03710	CGTGTCGGCGTGGGTGT
qPR1aR		GGCGAGTAGTTGCAGGTGATG
qPR1bF	LOC_Os01g28450	TACGCCAGCCAGAGGAGC
qPR1bR		GCCGAACCCCAGAAGAGG
qPR5F	LOC_Os12g43430	TACAACGTCGCCATGAGCTTC
qPR5R		ACTTGGTAGTTGCTGTTGCCG
qPR10F	LOC_Os12g36830	CCATGAAGCTTAACCCCGATG
qPR10R		AGCTTGCCCACCTTGCTTT
Primers for yeast two-hybrid		
SAPK10-EcoRI-F	LOC_Os03g41460	CGGAATTCATGGACCGGGCGGCGCTGACGGTGG

SAPK10-PstI-R		AACTGCAGTCACATAGCGTATACTATCTCCCCA
WRKY72-EcoRI-F		CGGAATTCATGGAGAACTTCCCGATACTCTTTG
WRKY72-BamHI-R		AAAGGATCCCTACTGGAACATGTGGGAAGCAGC A
Primers for pull-down		
WRKY72-BamHI-F		AAAGGATCCATGGAGAACTTCCCGATACTCTTTG
WRKY72-SmaI-R		AAACCCGGGAACTACTGGAACATGTGGGAAGC AGCA
SAPK10-EcoRI-F		CGGAATTCATGGACCGGGCGGCGCTGACGGTGG
SAPK10-XhoI-R		CCGCTCGAGTCACATAGCGTATACTATCTCCCCA
Primers for co-IP		
WRKY72-XbaI-F		GCTCTAGAATGGAGAACTTCCCGATACTCTTTG
WRKY72-SmaI-R		TCCCCCGGGCTGGAACATGTGGGAAGCAGCAG CA
SAPK10-KpnI-F		GGGGTACCATGGACCGGGCGGCGCTGACGGTG G
SAPK10-XbaI-R		GCTCTAGACATAGCGTATACTATCTCCCCA
Primers for WRKY72 mutation (overlap-PCR)		
WRKY72-S71A-mutation-F		GGAGGAGCTCGCCAACTCCAAGCAG
WRKY72-S71A-mutation-R		GCTTGGAGTTGGCGAGCTCCTCCAT
WRKY72-S73A-mutation-F		GCTCTCCAACGCCAAGCAGGCCGGC
WRKY72-S73A-mutation-R		CGGCCTGCTTGGCGTTGGAGAGCTC
WRKY72-T86A-mutation-F		CGGTGGTGCAGCCAGGAGCCCACAC
WRKY72-T86A-mutation-R		GTGGGCTCCTGGCTGCACCACCGTC
WRKY72-T129A-mutation-F		CGCGTTCCAGGCCCGCAGCCAGGTC
WRKY72-T129A-mutation-R		CCTGGCTGCGGGCCTGGAACGCGAA
WRKY72-T129D-mutation-F		CGCGTTCCAGGACCGCAGCCAGGTC
WRKY72-T129D-mutation-R		CCTGGCTGCGGTCCTGGAACGCGAA
Primers for EMSA		
AOS1probeF1		AAGGTGGTGAGCCCAATCACCACCTTACTGGTT GACCTGCCGAAGACCGCTCCGCTGGCC
AOS1probeR1		GGCCAGCGGAGCGGTCTTCGGCAGGTCAACCA GTAAGGTGGTGATTGGGCTCACCACCTT
AOS1probeF2		AAGGTGGTGAGCCCAATCACCACCTTACTGGTT GAAATGCCGAAGACCGCTCCGCTGGCC
AOS1probeR2		GGCCAGCGGAGCGGTCTTCGGCATTTCAACCAG TAAGGTGGTGATTGGGCTCACCACCTT
AOS1probeF3		AAGGTGGTGAGCCCAATCACCACCTTACTGGTT GGGGTGCCGAAGACCGCTCCGCTGGCC
AOS1probeR3		GGCCAGCGGAGCGGTCTTCGGCACCCCAACCA GTAAGGTGGTGATTGGGCTCACCACCTT
AOS1probeF4		AAGGTGGTGAGCCCAATCACCACCTTACTGGTA AGGCTGCCGAAGACCGCTCCGCTGGCC

AOS1probeR4		GGCCAGCGGAGCGGTCTTCGGCAGCCTTACCAG TAAGGTGGTGATTGGGCTCACCACCTT
AOCprobeF		TTGGAAAGGTACGATGTCAAAAAAATAAATTTG ACCATTATTTTCTATTATAATATGTAT
AOCprobeR		ATACATATTATAATAGAAAATAATGGTCAAATTTA TTTTTTTGACATCGTACCTTTCCAA
LOX1probeF		TCGGTCCGATCGATCGAGTCCACGGCCATGAGC TTAGCTTGTCATGCACGTAGCTTAATTAGGCCCG GAACTTCCAGTCT
LOX1probeR		AGACTGGAAGTTCCGGGCCTAATTAAGCTACGT GCATGACAAGCTAAGCTCATGGCCGTGGACTCG ATCGATCGGACCGA
Primers for dual luciferase transcriptional activity assay		
AOS1-XhoI-F		AATCTCGAGGGAGTACTAGCAGCTAGCAG
AOS1-SalI-R		TAAGTCGACTTCATGTCCATCTCGTGCCC
WRKY72-XbaI-F		GCTCTAGAATGGAGAACTTCCCGATACTCTTTG
WRKY72-KpnI-R		GGGGTACCCTACTGGAACATGTGGGAAGCAGCA
Primers for ChIP-qPCR		
cAOS1F1		ACGCACTGGGCGTAAAAG
cAOS1R1		GAGGAGCCCTAAGACACC
cAOS1F2		TCTCCTCCACTTTTAAAA
cAOS1R2		CTAGCTCCAACTGCAAGT
cAOS1F3		TTGCATCTCGTTCGCGTC
cAOS1R3		AAGTACTCGTACCTGTCC
cAOS1F4		TCTTCACCGGCACCTTCA
cAOS1R4		AGAAGAGGAGGGTCTTGA
cAOS1F5		TGCACGACAAGCAGTGCG
cAOS1R5		TCAGAAGGTGGCCTTCTTGA
Primers for DNA bisulfite conversion		
mAOS1F		TAGGTGTAGGTGTGTAGATGGTGTTA
mAOS1R		CTCACCTACTAACTACTAATACTCC

Transparent Methods

Plant materials

Rice cultivar Nipponbare (*Oryza sativa* ssp *japonica*) and all transgenic plants used in this study were planted in the experimental field and greenhouse in China National Rice Research Institute. Plants in booting-stage were used for artificial *Xoo* inoculation assay.

Vector construction and rice transformation

The coding sequence (CDS) of *WRKY72* was PCR amplified from Nipponbare leaf cDNAs. Mutations of the phosphosite on WRKY72 were introduced by PCR using synthesized oligos. The CDS fragments were ligated into vector pU1301 under the driving of a maize ubiquitin promoter (Zhang et al., 2010). For *OxWRKY72-FLAG*, the CDS without stop codon was in frame fused with 3X FLAG tag at the end with *Kpn*I and *Bam*HI sites and cloned into pU1301. CRISPR/Cas9 system for *WRKY72* knock-out construct was adopted from a previous report (Ma et al., 2015). Annealed double strand oligos of the gDNA sequences were cloned into the pYLgRNA-OsU3 using *Bsa*I site (Thermo, Waltham, U.S.A.). All the constructs were introduced into *Agrobacterium* strain EHA105 and then transformed into Nipponbare embryonic calli. The sequences of the primes used are presented in Table S2.

Rice bacterial blight inoculation

Virulent *Xoo* strain (ZJ173) was used for the inoculation assay. Briefly, booting stage plants were inoculated with ZJ173 (3×10^8/mL) by a leaf clipping method (Chen et al., 2002). Disease was scored as the percent lesion area (lesion length/leaf length) at 14 days after inoculation. The bacterial growth rate for ZJ173 strain was also determined by counting colony forming units (CFU).

Quantitative RT-PCR (qRT-PCR)

Total RNA of various tissues were isolated using Trizol (Invitrogen, Carlsbad, U.S.A.), and then reverse transcribed using first strand cDNA synthesis Kit (Toyobo,

Shanghai, China). qPCR was conducted using gene-specific primers and THUNDERBIRD SYBR® qPCR Mix (Toyobo, Shanghai, China) on a BioRad real-time PCR CFX96 system. An ubiquitin gene was used as an internal control. The data was analyzed by evaluating threshold cycle (CT) values. The relative mRNA level of tested genes was normalized to ubiquitin gene and calculated by the $2^{-\Delta\Delta CT}$ method. The experiment was performed with three biological replicates.

Subcellular localization analysis

The full CDS of *WRKY72* was ligated into vector pBWA(V)-HS fused to generate the *pro35S*:WRKY72-GFP construct. The rice protoplast was prepared as previously described (Qiu et al., 2016). Around 5 grams rice leaf was stripped into 0.5 mm size, which were digested in 10 mL enzyme solution (1.5% cellulose R10, 0.75% macerozyme R10, 0.6 M mannitol, 10 mM MES pH=7.5) for 6 hours in dark with gentle shaking at 28℃. The filtered protoplasts were washed with 10 mL ice cold W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 2 mM KH_2PO_4, 2 mM MES, 5 mM glucose, pH 5.7) two times, and finally suspended in 500 μL MMG solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH=5.8). Then, the plasmid was transformed into the prepared protoplast by incubating in PEG (0.6 M mannitol, 100 mM $CaCl_2$, 40% PEG4000) for 30 minutes at room temperature. *pro35S*:D53-mKate was used as a nuclear marker. Lastly, the fluorescent protein signals were observed under a confocal microscope (Leica, Wetzlar, Germany).

Yeast two-hybrid assays

The matchmaker GAL4 two-hybrid system (Clontech, CA, U.S.A.) was used for Y2H assays. Full-length CDS of *WRKY72* was cloned into the pGADT7 vector, and the CDS of *SAPK10* was cloned into the pGBKT7 vector. Primers used are listed in Table S2. Constructs were co-transformed into the yeast strain Y2H Gold. SD plates lacking Trp and Leu were used to select the co-transformed colonies. The protein interactions were detected by the visualization of blue colonies on the SD plates with X-α-Gal (0.04 mg/mL) and Aureobasidin A (100 ng/mL), lacking Trp, Leu, and Ade.

Pull-down assays

Full-length CDS of *WRKY72* and *SAPK10* were cloned into pGEX-4T-1 (GE Healthcare, Chicago, U.S.A.) and pET28a (Thermo, Waltham, U.S.A.) vectors, respectively. Primers used are listed in Table S2. The recombinant protein GST-WRKY72 and HIS-SAPK10 were produced in *E.coli* DE3 (Transgen, Beijing, China), and purified using the GST-Sefinose ^{TM} Kit (Sangon Biotech, Shanghai, China) and 6× HIS-Tagged Protein Purification Kit (CWBIO, Beijing, China) according to the manuals, respectively. The tested interactive proteins were incubated with glutathione high capacity magnetic agarose beads (Sigma-Aldrich) in pull-down buffer (50 mM Tris-HCl, pH 7.5, 5% glycerol, 1 mM EDTA, 1 mM DDT, 1 mM PMSF, 0.01% Nonidet P-40, and 150 mM KCl) at 4 °C for 2 hours. After washing five times with pull-down buffer, the beads were suspended in 50 μL 1 × PBS and 10 μL 6 × SDS protein loading buffer for 10% SDS-polyacrylamide (PAGE) gel electrophoresis and immunoblotting analysis. Proteins were detected using Supersignal West Pico Chemiluminescent Substrate (Thermo, Waltham, U.S.A.) and the ChemDoc^{TM} Touch Imaging system (Bio-Rad). The dilution for anti-HIS (Cat: CW0083, CWBIO, Beijing, China) and anti-GST (Cat: CW0085, CWBIO, Beijing, China) was 1: 5000.

Co-IP assays

The full-length CDS of *WRKY72* and *SAPK10* were cloned into pF3PZPY122 (Menon et al., 2005) and pCAMBIA1300-GFP vectors, respectively. Primers used are listed in Table S2. WRKY72-FLAG was transiently co-expressed with empty GFP or SAPK10-GFP in tobacco leaves by *Agrobacterium* infiltration. Two grams of the transformed tissues were ground into fine powders and resuspended in protein extraction buffer (25 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, 1% NonidetP-40, 5 % glycerol, 1 mM PMSF, 20 μM MG132, and 1x Roche protease inhibitor cocktail (Roche, Basel, Switzerland). After a brief centrifugation (20,000g for 10 minutes), the resulting supernatant was incubated with anti-FLAG M2

magnetic beads (Sigma-Aldrich, St Louis, U.S.A.) at 4 °C for 2 hours. Subsequently, the beads were washed five times with washing buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.2% Triton X-100, 1 mM PMSF, and 1x Roche protease inhibitor cocktail), eluted with 50 μL FLAG elution buffer (25 mM Tris-HCl, pH 7.5, and 0.2 mg/mL 3x Flag peptide) (Sigma-Aldrich, St Louis, U.S.A.) at 25 °C for 30 minutes. Proteins were detected using Supersignal West Pico Chemiluminescent Substrate (Thermo, Waltham, U.S.A.) and the ChemDoc™ Touch Imaging system (Bio-Rad, CA, U.S.A.). The dilution for anti-FLAG (Sigma-Aldrich, St Louis, U.S.A.) and anti-GFP (Cat: CW0086, CWBIO, Beijing, China) antibodies was 1:5000.

In *E.coli* phosphorylation assays

Different forms of *WRKY72* (*WRKY72*, *WRKY72*^{S71A}, *WRKY72*^{S73A}, *WRKY72*^{T86A}, and *WRKY72*^{T129A}) were cloned into the pGEX-4T-1 vector, respectively. The different truncated version of *GST-WRKY72* constructs was generated by overlap-PCR. Full-length CDS of *SAPK10* were cloned into pET28a vector. Primers used are listed in Table S2. GST-WRKY72 or GST tag alone were co-expressed with HIS-SAPK10 in *E. coli* strain DE3 (Transgen, Beijing, China), and purified using the GST-Sefinose ™ Kit (Sangon Biotech, Shanghai, China). The purified proteins (100 ng) were incubated with CIAP (Takara, Dalian, China) at 37 °C for 30 minutes, and then subjected to immunoblotting analysis of phosphorylated proteins using biotinylated Phos-tag™ zinc complex BTL111 purchased from Wako (http://www.Phos-tag.com).

EMSA assays

p-GST-WRKY72 (phosphorylated GST-WRKY72) were purified from the *E.coli* co-expressing GST-WRKY72 and HIS-SAPK10. Biotin labeled oligonucleotides were synthesized by TsingKe Company (seen in Table S2). Equal amount of the probe oligos was mixed, heated to 95°C for 2 minutes, and annealed by gradually cooling down to 25°C. Then, the purified proteins were pre-incubated with the binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/μL poly(dI-dC), and 0.05% NP-40) at room temperature for 20

minutes, followed by incubating with 20 fmol labeled probes with or without non-labeled competitive probes for another 20 minutes. Subsequently, the incubated samples were electrophoresed on 6% PAGE gels running with 0.5X Tris-borate-EDTA buffer (TBE) and transferred to a Nylon membrane at 100 V for 30 minutes and cross-linked on a transilluminator equipped with 312 nm bulbs. Lastly, the fluorescence signaling was detected by Chemiluminescence according to LightShift® Chemiluminescent EMSA Kit (Cat: 20148, Thermo, Waltham, U.S.A.).

Dual Luciferase transcriptional activity assay in rice protoplasts

The Dual Luciferase Reporter Gene Assay Kit (Beyotime, Shanghai, China) was used to measure the luciferase activity. Firstly, the transformed protoplasts were re-suspended in 50 μL Lysis buffer, and 30-50 μL of lysate was used to measure the luciferase activity in one well of 96-well plate. 100 μL of firefly luciferase assay substrate buffer was added into the lysate and the firefly luciferase (fLUC) activity was measured with the Infinite® 200 Pro (Tecan, Mannedorf, Switzerland). After that, 100 μL of Stop & renilla luciferase substrate buffer was added to the reaction and the renilla luciferase (rLUC) activity was measured. Relative luciferase activity was calculated as the ratio between fLUC and rLUC (fLUC/rLUC). 35S, 35S:GAL4-fLUC, and AtUbi:rLUC were used as an effector, a reporter and an internal control, respectively. Triple biological repeats were performed for each sample. Primers used for the vector construction are listed in Table S2. All of the plasmids used in this assay were purified with the Plasmid Midi Kit (Qiagen, Dusseldorf, Germany).

ChIP-quantitative PCR (ChIP-qPCR)

ChIP was performed as described previously (Hou et al., 2015). Briefly, chromatin was isolated from 2 g cross-linked leaves of *proUbi:WRKY72-FLAG* plant. Isolated chromatin was sheared to approximately 100 to 500 bp by sonication. Then, the DNA/protein complex was immune-precipitated with ChIP-grade antibody against FLAG (Cat: F1804, Sigma-Aldrich, St Louis, U.S.A.). After reverse cross-linking and proteinase K treatment, the immunoprecipitated DNA was extracted with

phenol/chloroform. The immunoprecipitated and input DNA was used for quantitative PCR using gene specific primers (seen in Table S2). The quantitative PCR results were analyzed by following a method reported in the manual of Magna ChIP™ HiSens kit (Millipore, MA, U.S.A.). All the quantitative ChIP-PCR was performed in three biological replicates. The enrichment values were normalized to the input sample.

Quantification of hormones

Free JA quantification was conducted and the methods were modified form a previous report using the Waters ACQUITY UPLC Xevo TQ HPLC-MS/MS system (You et al., 2016). Approximately 0.5 g leaf samples were finely ground in liquid nitrogen and extracted with 10 mL buffer (isopropanol: water: hydrochloric acid, 2: 1: 0.002 v/v). The extracts were shaken at 4°C for 30 minutes and 20 mL dichloromethane was then added. After that, the samples were shaken at 4°C for 30 minutes and centrifuged at 13,000 rpm for 5 minutes. The organic phase was extracted and dried under liquid nitrogen. The pellets were dissolved in 150 μL methanol (0.1% methane acid) and filtered with a 0.22 μm filter membrane. Lastly, the purified product (2 μL each injection) was subjected to HPLC-MS/MS analysis. The quantitative data was obtained using the peaks of the precursor ions 209.2 and the product ions 58.9. MS conditions were as follows: the spray voltage was 4,500 V; the pressure of the air curtain, nebulizer, and aux gas were 15, 65, and 70 psi, respectively; and the atomizing temperature was 400°C. The experiment was performed with three biological replicates.

DNA bisulfite conversion for Sanger sequencing and methylation analysis

Total genomic DNA was extracted using a DNeasy Plant Maxi Kit (Qiagen, Dusseldorf, Germany) and modified using an EpiTect Bisulfite kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. Then, the modified DNA was amplified, purified and cloned into pMD18-T vector (Takara, Dalian, China). The amplified primers were designed online

(http://www.urogene.org/methprimer/) and seen in Table S2. Lastly, At least 30 clones were sequenced for each sample and the bisulfite sequencing results were analyzed by the Kismeth website (http://katahdin.mssm.edu/kismeth). The methylation levels of the three types of cytosines (CG, CHG, CHH) were calculated by dividing the number of non-converted (methylated) cytosines by the total number of cytosines.

References

Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B., Yang, Z., Li, H., and Liu, Y. (2015). A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Mol Plant ***8***, 1274-1284.

Menon, S., Rubio, V., Wang, X., Deng, X.W., and Wei, N. (2005). Purification of the COP9 signalosome from porcine spleen, human cell lines, and Arabidopsis thaliana plants. Methods Enzymol ***398***, 468-481.

Qiu, J., Hou, Y., Tong, X., Wang, Y., Lin, H., Liu, Q., Zhang, W., Li, Z., Nallamilli, B.R. and Zhang, J. (2016) Quantitative phosphoproteomic analysis of early seed development in rice (Oryza sativa L.). *Plant Mol Biol* ***90***, 249-265.

You, C., Zhu, H., Xu, B., Huang, W., Wang, S., Ding, Y., Liu, Z., Li, G., Chen, L. and Ding, C. (2016) Effect of Removing Superior Spikelets on Grain Filling of Inferior Spikelets in Rice. *Frontiers in Plant Science* ***7***, 1161.

Zhang, J., Nallamilli, B.R., Mujahid, H., and Peng, Z. (2010). OsMADS6 plays an essential role in endosperm nutrient accumulation and is subject to epigenetic regulation in rice (Oryza sativa). Plant Journal ***64***, 604–617.