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

HIGHLIGHTS

- WRKY72 negatively regulates rice resistance to Xoo infection and JA synthesis
- SAPK10 phosphorylates WRKY72 at Thr129 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 (TACTGCCTTAGT) 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
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*, *Z8*, *POX22.3*, *PR1b*, *PBZ1*, and *Sc12* (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_Os11g29970) 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: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 homologous mutant lines and over-expression lines of the gene. In T1 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
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 (129th 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 OxWRKY72T129A lines, in which the only phosphosite Thr129 was mutated into an alanine (Ala) to block the phosphorylation on it. Two OxWRKY72T129A lines with comparable levels of WRKY72 expression as the OxWRKY72s were chosen for Xoo susceptibility assay (Figure 2A). Interestingly, OxWRKY72T129A lines exhibited higher susceptibility to Xoo than the OxWRKY72 lines (Figure 2D), although the other major
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-qPCR to examine the transcriptional levels of a couple of JA synthesis genes in OxWRKY72, OxWRKY72T129A, 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 OxWRKY72T129A when compared with OxWRKY72 (p < 0.05), which is in accordance with the observed higher susceptibility of OxWRKY72T129A. 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 OxWRKY72T129A 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, OxWRKY72T129A, 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, OxWRKY72T129A displayed even lower JA level than OxWRKY72 at 72 HAI, which is consistent with the observation that OxWRKY72T129A lines were more susceptible to Xoo than OxWRKY72s. The results above suggested that WRKY72 negatively regulates Xoo resistance by suppressing JA biosynthesis.
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 WRKY72T129D, 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
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 WRKY72T129D in EMSA, we also found that the suppression on AOS1 was significantly reduced when WRKY72T129D 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). WRKY72T129A 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 counter-balance 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.
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
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-lle 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. oryzae*, 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 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 ≤ 0.05, **p ≤ 0.01 by the Student’s t test.
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, Chuo et al. (2014) found that WRKYS3-mediated resistance to rice blast fungus strain InaB6-137 relied on the phosphorylation on its serine-proline residues by MPK3/MPK6. Over-expressing a phosphomimic mutated version of WRKYS3 (WRKYS3SD) rice plants elevated the expression level of defense-related genes and enhanced disease resistance to M. oryzae compared with native WRKYS3-over-expressing rice plants (Chuo 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\textsuperscript{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...
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, OwxWRKY72, and OwxWRKY72T129A 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 (Estético 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.

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AUTHOR CONTRIBUTIONS
J.Z. and S.H. planned and designed the research; Y.H., Y.W., L.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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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.

| Protein                | Phosphorylation |
|------------------------|-----------------|
| HIS-SAPK10             | ++              |
| GST-WRKY72             | + + +           |
| GST-WRKY72(1-135)      | + + +           |
| GST-WRKY72(126-204)    | + + +           |
| GST-WRKY72(195-243)    | + + +           |

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.

| Component | Phosphorylation |
|-----------|-----------------|
| Labeled probe | ++ ++ ++ |
| GST-WRKY72   | - + - + |

Probe

*Figure S2.* In vitro phosphorylation of the gradually narrowed fragment of WRKY72, related to Figure 3.

*Figure S3.* EMSA of WRKY72 on AOC and LOX1 promoter regions, related to Figure 5.
Figure S4. Major Agronomic Traits of OxAOSIs and WT, Related to Figure 6.

(a) The expression analysis of AOSI in OxAOSI and WT plant lines. (b-c) The height analysis of OxAOSI and WT plant lines. Data are shown as means ± SD of at least three biological replicates. *: P ≤ 0.05, **: P ≤ 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*      |
| *OxWRKY72T129A-3*    | 69.9±2.18         | 72.0±1.73             | 7.7±0.21         | 3.4±0.10        | 26.58±0.72**     |
| *OxWRKY72T129A-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; *OxWRKY72T129A-3* and *OxWRKY72T129A-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| GGGGTACCATGGAGAACTTCCCGGACTCTCTTTTGG    |
| WRKY72-BamHI-R            |               | CGGGATCCCTACTGGAACATGTTGGAACAGCAGCA     |
| WRKY72-FLAG-KpnI-F        |               | AAAAGTACCATGGAGAACTTCCCCGATACCTCTTTTG   |
| WRKY72-FLAG-BamHI-R       |               | CGGGATCCCTAGGCCCCCCTCGAATTTTCGTA        |
| OxAOS1F                   | LOC_Os03g55800| TACGAACGATAGGCCGATGCCACGGCGCCGCTG      |
| OxAOS1R                   |               | GACCTCTAGAGGATCTCAGAAGGTGCGGCTTCTTG    |
| Primers for subcellular localization construct |               |                                          |
| ScWRKY72F                 |               | CAGTGGTCTCTACAACATGGAGAACCTCCCCGATACT  |
| ScWRKY72R                 |               | CAGTGGTCTCTACACATGGAGAACCTCCCCGATACT  |
| Primers for qRT-PCR       |               |                                          |
| qWRKY72F                  | LOC_Os11g29870| CAAGGGTGCAACGTGAAGAA                  |
| qWRKY72R                  |               | ATTTCTCGATGGGGTGCGTG                   |
| qAOCF                     | LOC_Os03g32314| GCCAAGGTGCAGGAGATGTTGCG               |
| qAOCR                     |               | AGCCGCTTCCTCGCAAGCCTTC                |
| qAOS1F                    | LOC_Os03g55800| CGGGAACATGTTGCGGAGAAGA               |
| qAOS1R                    |               | GAGTGGTCTTCGAGGAGAAGAAGA              |
| qAOS2F                    | LOC_Os03g12500| TCGTCGGAAGGCTGTTCG                    |
| qAOS2R                    |               | AGCATGGCAACGGCGAGGT                   |
| qLOX1F                    | LOC_Os03g49380| CCAACCAGACAAAGGACATG                 |
| qLOX1R                    |               | GGGAAGACACCCCTCAAAATA                 |
| qLOX2F                    | LOC_Os08g39840| CGACGAGCGCTGCTACTGACTA                |
| qLOX2R                    |               | CTCGTTCCGACTTTAGGGTTCTTTT             |
| qOPR7F                    | LOC_Os08g35740| GAAGGTGTTGATGCTGT                     |
| qOPR7R                    |               | TTAAGGATACCTTGCCATAGGA                |
| qPR1aF                    | LOC_Os07g03710| CGTTGCCAGGCGGTTG                      |
| qPR1aR                    |               | GGCAGATCGTGTACGAGGTGT                 |
| qPR1bF                    | LOC_Os01g28450| TACGCCAGCGCACAGGAGG                   |
| qPR1bR                    |               | GCCGAACCCCAAGAAGA                     |
| qPR5F                     | LOC_Os12g43430| TACAAACTGCACCAGAAGGCTC                |
| qPR5R                     |               | ACTTGCGTGCTGGGTGTCGCG                 |
| qPR10F                    | LOC_Os12g36830| CCAATGAACGCTTAAACCCGATG               |
| qPR10R                    |               | AGCTTGCACCTTGTG                      |
| Primers for yeast two-hybrid |               |                                          |
| SAPK10-EcoRI-F            | LOC_Os03g41460| CGGAATTCATGGAGACCCGCGCGCTGACGGTG     |
|
| Primers for pull-down |  |
|-----------------------|------------------|
| WRKY72-BamHI-F        | GAGGAGATCCCATGGGAACAATTCGCCGACTCTCTTTTG |
| WRKY72-Smal-R         | AAACCCGGGAACTACTGGAAACATGTGGGAGCAGCA |
| SAPK10-EcoRI-F        | CGGAATTCCATGGACCGGGCGGCGCTGACGGGTGG |
| SAPK10-XhoI-R         | CGGCTCGAGTCACATAGCGTATACTATCCCCA |

| Primers for co-IP |  |
|-------------------|------------------|
| WRKY72-XbaI-F     | GCTCTAGAGATTGGGAACCTTCCCGGATACCTCTTTTG |
| WRKY72-Smal-R     | TCCCCCGGGCTGGAACATGTGGGAGCAGCA |
| SAPK10-KpnI-F     | GGGGTACCATGGACCGGGCGGCGCTGACGGTCG |
| SAPK10-XbaI-R     | GCTCTAGACATAGCGTATACTATCTCCCA |

| Primers for WRKY72 mutation (overlap-PCR) |  |
|------------------------------------------|------------------|
| WRKY72-S71A-mutation-F                  | GGAGGAGCTCGCCAATTCCACAAAGCACAG |
| WRKY72-S71A-mutation-R                  | GCTCTAGAGATTGGGAACCTTCCCGGATACCTCTCAT |
| WRKY72-S73A-mutation-F                  | GCTCTAGAGATTGGGAACCTTCCCGGATACCTCTCAT |
| WRKY72-S73A-mutation-R                  | GCTCTAGAGATTGGGAACCTTCCCGGATACCTCTCAT |
| WRKY72-T86A-mutation-F                  | CGGTGGTGCAGCCAGGAGCCCACAC |
| WRKY72-T86A-mutation-R                  | GCTCTAGAGATTGGGAACCTTCCCGGATACCTCTCAT |
| WRKY72-T129A-mutation-F                 | CGGCTCCGAGACCGAGCCGAGGAGGAGGAGGAG |
| WRKY72-T129A-mutation-R                 | CGGCTCCGAGACCGAGCCGAGGAGGAGGAGGAG |
| WRKY72-T129D-mutation-F                 | CGGCTCCGAGACCGAGGAGGAGGAGGAGGAGGAG |
| WRKY72-T129D-mutation-R                 | CGGCTCCGAGACCGAGGAGGAGGAGGAGGAGGAG |

| Primers for EMSA |  |
|-----------------|------------------|
| AOS1probeF1     | AAGGTTGGTGAGCCCCCAATCACCACTAACTTACCTGTT |
| AOS1probeR1     | GGCCAGCGGGAGGCTCTCGGCCAGCAACACACCAAC |
| AOS1probeF2     | AAGGTTGGTGAGCCCCCAATCACCACTAACTTACCTGTT |
| AOS1probeR2     | GGCCAGCGGGAGGCTCTCGGCCAGCAACACACCAAC |
| AOS1probeF3     | AAGGTTGGTGAGCCCCCAATCACCACTAACTTACCTGTT |
| AOS1probeR3     | GGCCAGCGGGAGGCTCTCGGCCAGCAACACACCAAC |
| AOS1probeF4     | AAGGTTGGTGAGCCCCCAATCACCACTAACTTACCTGTT |
| Primers for dual luciferase transcriptional activity assay |  |
|----------------------------------------------------------|--------------------------------------------------|
| AOS1-XhoI-F                                              | AATCTCGAGGGAGTACTAGCAGCAGCAG                     |
| AOS1-SalI-R                                              | TAAGTCGACTTCATGTCCATCTCGTGCCC                    |
| WRKY72-XbaI-F                                            | GCTCTAGAATGGAGAACTTCCCGATATCTCTTTG              |
| WRKY72-KpnI-R                                            | GGGGTACCTACTGGAACATGTGGGAAAGCACAGA              |

| Primers for ChIP-qPCR |
|-----------------------|
| cAOS1F1 | ACGCACTGGGCGTAAAAG |
| cAOS1R1 | GAGGAGCCTAAAGCACCC |
| cAOS1F2 | TCTCTCCACTTTAAAAC |
| cAOS1R2 | CTAAGCTCAACTGAAGT |
| cAOS1F3 | TTGCTACCTCGTTCCGTCC |
| cAOS1R3 | AAGTACTCGTACCTGTC |
| cAOS1F4 | TCTCCACCGGCACTTCA |
| cAOS1R4 | AGAAGAGGAGGTCTTTGA |
| cAOS1F5 | TGCACGACAGCTGCG |
| cAOS1R5 | TCAGAAGGTCGGCCTTTTG |

| Primers for DNA bisulfite conversion |
|--------------------------------------|
| mAOS1F | TACTGGTAGGTGTGAGATGTTGTTA |
| mAOS1R | CTCACCTACTAACTAATACTC |

| Primers for dual luciferase transcriptional activity assay |  |
|----------------------------------------------------------|--------------------------------------------------|
| AOS1probeR4                                              | GGCCAGCGGAGCGGTCTTCGCCAAGCCCTTTACCCAGGGAAGGGGTCTTCCTAATATGAGT |
| AOCprobeF                                                | TTGGAAAGGTACGATGTCAAAAAATAAAATTTGACCATTATTTCTATTATATTATGAT |
| AOCprobeR                                                | ATACATATTATAATGAAAATAATGGTCATAATTTTTGACACATTCCAGTCCCAA |
| LOX1probeF                                               | TCGGTCCGATCGATCGAGTCCACGCGCATGAGGACTGCTTCATAATTAGGCCC |
| LOX1probeR                                               | AGACTGGAAGTTCCGGGGCTAATTAAGCTACGTGCATGACAAAGCTAAGCTCATGCGTCGATC |

| Primers for ChIP-qPCR |
|-----------------------|
| cAOS1F1 | ACGCACTGGGCGTAAAAG |
| cAOS1R1 | GAGGAGCCTAAAGCACCC |
| cAOS1F2 | TCTCTCCACTTTAAAAC |
| cAOS1R2 | CTAAGCTCAACTGAAGT |
| cAOS1F3 | TTGCTACCTCGTTCCGTCC |
| cAOS1R3 | AAGTACTCGTACCTGTC |
| cAOS1F4 | TCTCCACCGGCACTTCA |
| cAOS1R4 | AGAAGAGGAGGTCTTTGA |
| cAOS1F5 | TGCACGACAGCTGCG |
| cAOS1R5 | TCAGAAGGTCGGCCTTTTG |

| Primers for DNA bisulfite conversion |
|--------------------------------------|
| mAOS1F | TACTGGTAGGTGTGAGATGTTGTTA |
| mAOS1R | CTCACCTACTAACTAATACTC |

| Primers for dual luciferase transcriptional activity assay |  |
|----------------------------------------------------------|--------------------------------------------------|
| AOS1probeR4                                              | GGCCAGCGGAGCGGTCTTCGCCAAGCCCTTTACCCAGGGAAGGGGTCTTCCTAATATGAGT |
| AOCprobeF                                                | TTGGAAAGGTACGATGTCAAAAAATAAAATTTGACCATTATTTCTATTATATTATGAT |
| AOCprobeR                                                | ATACATATTATAATGAAAATAATGGTCATAATTTTTGACACATTCCAGTCCCAA |
| LOX1probeF                                               | TCGGTCCGATCGATCGAGTCCACGCGCATGAGGACTGCTTCATAATTAGGCCC |
| LOX1probeR                                               | AGACTGGAAGTTCCGGGGCTAATTAAGCTACGTGCATGACAAAGCTAAGCTCATGCGTCGATC |

| Primers for ChIP-qPCR |
|-----------------------|
| cAOS1F1 | ACGCACTGGGCGTAAAAG |
| cAOS1R1 | GAGGAGCCTAAAGCACCC |
| cAOS1F2 | TCTCTCCACTTTAAAAC |
| cAOS1R2 | CTAAGCTCAACTGAAGT |
| cAOS1F3 | TTGCTACCTCGTTCCGTCC |
| cAOS1R3 | AAGTACTCGTACCTGTC |
| cAOS1F4 | TCTCCACCGGCACTTCA |
| cAOS1R4 | AGAAGAGGAGGTCTTTGA |
| cAOS1F5 | TGCACGACAGCTGCG |
| cAOS1R5 | TCAGAAGGTCGGCCTTTTG |

| Primers for DNA bisulfite conversion |
|--------------------------------------|
| mAOS1F | TACTGGTAGGTGTGAGATGTTGTTA |
| mAOS1R | CTCACCTACTAACTAATACTC |
**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 *KpnI* and *BamHI* 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 *BsaI* 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°C. The filtered protoplasts were washed with 10 mL ice cold W5 solution (154 mM NaCl, 125 mM CaCl₂, 2 mM KH₂PO₄, 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 MgCl₂, 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 CaCl₂, 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 pGBKKT7 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™ 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™ 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, WRKY72S71A, WRKY72S73A, WRKY72T86A, and WRKY72T129A) 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 MgCl₂, 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 effect or, 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 from 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
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.

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