

Running head: WRKY53 regulates plant defense

Correspondence: Yonggen Lou

State Key Laboratory of Rice Biology

Institute of Insect Science

Zhejiang University

Hangzhou 310058

China

Tel. 0086 571 88982622

yglou@zju.edu.cn

The rice transcription factor WRKY53 suppresses herbivore-induced defenses by acting as a negative feedback modulator of map kinase activity

Lingfei Hu^{a, 1}, Meng Ye^{a, 1}, Ran Li^a, Tongfang Zhang^a, Guoxin Zhou^{a, b}, Qi Wang^a, Jing Lu^a, and Yonggen Lou^{a, 2}

Author affiliations

^a State Key Laboratory of Rice Biology, Institute of Insect Science, Zhejiang University, Hangzhou 310058, China

^b Key Laboratory for Quality Improvement of Agriculture Products of Zhejiang Province, Department of Plant Protection, Zhejiang Agriculture & Forestry University, Lin'an, 311300, China

¹ These authors contributed equally to this work.

² Corresponding author.

Summary: A transcription factor functions as a negative feedback modulator of MPK3/6 and thereby acts as an early suppressor of herbivore-induced defenses in rice.

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Present address: Lingfei Hu: Institute of Plant Sciences, University of Bern, 3013 Bern, Switzerland.

Corresponding author: Yonggen Lou (yglou@zju.edu.cn)

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Yonggen Lou (yglou@zju.edu.cn).

Abstract

The mechanisms by which herbivore-attacked plants activate their defenses are well studied. By contrast, little is known about the regulatory mechanisms that allow them to control their defensive investment and avoid a defensive overshoot. We characterized a rice (*Oryza sativa*) WRKY gene, *OsWRKY53*, whose expression is rapidly induced upon wounding and induced in a delayed fashion upon attack by the striped stem borer (SSB) *Chilo suppressalis*. The transcript levels of *OsWRKY53* are independent of endogenous jasmonic acid (JA), but positively regulated by the mitogen-activated protein kinases (MPKs), *OsMPK3/OsMPK6*. *OsWRKY53* physically interacts with *OsMPK3/OsMPK6* and suppresses their activity *in vitro*. By consequence, it modulates the expression of defensive, MPK-regulated WRKYs and thereby reduces JA, jasmonoyl-isoleucine (JA-Ile) and ethylene induction. This phytohormonal reconfiguration is associated with a reduction in trypsin protease inhibitor activity and improved SBB performance. *OsWRKY53* is also shown to be a negative regulator of plant growth. Taken together, these results show that *OsWRKY53* functions as a negative feedback modulator of MPK3/MPK6 and thereby acts as an early suppressor of induced defenses. *OsWRKY53* therefore enables rice plants to control the magnitude of their defensive investment during early signaling.

INTRODUCTION

To effectively combat herbivores, plants have evolved sophisticated mechanisms that provide several layers of constitutive and inducible defense responses. Constitutive defenses are physical and chemical defensive traits that plants express regardless of the presence of herbivores. By contrast, inducible defenses are mounted only after plants are attacked by an herbivore (Wu and Baldwin, 2010). Induced defensive responses are the result of highly coordinated sequential changes at the cellular level, changes that activate multiple signaling pathways. These pathways mainly include mitogen-activated protein kinase (MPK) cascades and signaling pathways mediated by phytohormones, such as jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile), salicylic acid (SA) and ethylene (ET) (van Loon et al., 2006; Bonaventure, 2012; Erb et al., 2012). Through cross-talk, both synergistic and antagonistic interactions, this signaling network plays a central role in herbivore-induced defense responses by activating transcription factors (TFs) and regulating the transcript levels of many genes (van Loon et al., 2006; Bonaventure, 2012; Erb et al., 2012).

MPK cascades in all eukaryotes including plants generally consist of three components: MPK kinase kinases (MEKK), MPK kinases (MEK) and MPKs; these components are sequentially activated by phosphorylation (Cristina et al., 2010) to transfer information from sensors to responses and are involved in diverse physiological functions, including cell division, development, hormone synthesis and signaling, and response to abiotic and biotic stresses (Nakagami et al., 2005; Cristina et al., 2010; Liu, 2012). A MPK cascade consisting of *MEKK1*, *MEK1/MEK2* and *MPK4* (Qiu et al., 2008; Cristina et al., 2010) for instance controls plant defenses by modulating defense-related signaling, WRKY TFs and other genes. Furthermore, MPK3/MPK6 in Arabidopsis mediate *FLS2-flg22* recognition and activate defense-related WRKYs (phosphorylation) as well as the biosynthesis of phytoalexins such as camalexin (Asai et al., 2002; Menke et al., 2004; Ren et al., 2008); they also modulate the ET signaling pathway and plant

resistance to pathogens (Kim et al., 2003; Kim and Zhang, 2004; Yoo et al., 2008; Han et al., 2010). In *Nicotiana attenuata*, wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK) (orthologs of AtMPK3 and AtMPK6) have been reported to regulate several WRKYs and to be involved in JA and SA signaling pathways, and herbivore-induced defense responses (Wu et al., 2007).

WRKYs, which specifically bind W-box sequences (TTGACC/T) in the promoter region of target genes, are one of the largest families of transcription factors in plants (Rushton et al., 2010). In *Arabidopsis* and rice, there are more than 70 and 100 WRKYs, respectively (Wu et al., 2005; Xie et al., 2005; Eulgem and Somssich, 2007). According to the number of WRKY domains and the features of their zinc-finger motifs, WRKY TFs are divided into three groups (Rushton et al., 2010). In addition to playing an important role in plant growth and development as well as in shaping plant responses to abiotic stresses, WRKYs, by acting as positive or negative regulators of the target genes, also figure in the regulation of plant defense responses to pathogens (Eulgem and Somssich, 2007; Pandey and Somssich, 2009; Rushton et al., 2010). WRKYs can function at different regulatory levels: besides being phosphorylated by protein kinases as stated above, they can also act up- and downstream of receptors and phytohormones as well as upstream of proteinase kinases (Ciolkowski et al., 2008; Bakshi and Oelmüller, 2014). In *Arabidopsis*, for example, small peptides encoded by *PROPEP* genes act as damage-associated molecular patterns that are perceived by 2 leucine-rich repeat receptor kinases, PEPR1 and PEPR2, to amplify defense responses. WRKY33 binds to the promoter of the *PROPEP* genes in a stimulus-dependent manner and regulates their expression (Logemann et al., 2013). *AtWRKY33* has also been found to regulate redox homeostasis, SA signaling, ethylene/JA-mediated crosscommunication and camalexin biosynthesis and to be essential for defense against the necrotrophic fungus *Botrytis cinerea* (Zheng et al., 2006; Birkenbihl et al., 2012). In rice, OsWRKY30, which may be phosphorylated by OsMPK3, positively regulates resistance to the rice sheath blight

fungus *Rhizoctonia solani* and the blast fungus *Magnaporthe grisea* (Peng et al., 2012; Shen et al., 2012). In *Nicotiana attenuata*, *NaWRKY3* and *NaWRKY6* control the biosynthesis of herbivore-induced JA and JA-Ile/-Leu, and, subsequently, herbivore-induced defenses (Skibbe et al., 2008). While the role of WRKYs as activators of plant defense against herbivores is established, the underlying molecular mechanisms remain unresolved. Furthermore, little is known about the potential of WRKYs to act as negative regulators of herbivory-induced defense responses.

Rice, *Oryza sativa*, one of the most important food crops worldwide, suffers heavily from insect pests (Cheng and He, 1996). The striped stem borer (SSB) *Chilo suppressalis* is one of the major lepidopteran pests of rice and causes severe yield losses in China (Chen et al., 2011). SSB larvae bore into and feed on rice stems, which results in “dead heart” and “white heads” symptoms at the vegetative and reproductive stage, respectively (Krishnaiah and Varma, 2011). SSB attack in rice induces the biosynthesis of a variety of phytohormones, including JA, SA and ethylene, which in turn regulate defense responses, such as the production of herbivore-induced volatiles and the accumulation of trypsin protease inhibitors (TrypPIs) (Lou et al., 2005; Zhou et al., 2009; Lu et al., 2011; Qi et al., 2011; Li et al., 2013; Wang et al., 2013). Given the importance of WRKYs in mediating signaling pathways and defense responses, we isolated the rice group I WRKY TF *OsWRKY53* and elucidated its roles in herbivore-induced defense responses. *OsWRKY53* localizes to the nucleus, has specific binding activity towards W-box elements and can be phosphorylated by the cascade OsMEK4-OsMPK3/OsMPK6 (Chujo et al., 2007, 2014; Yoo et al., 2014). *OsWRKY53* has also been found to positively modulate resistance to pathogens, such as *M. grisea* (Chujo et al., 2007) and is strongly induced by herbivore infestation (Zhou et al., 2011). However, whether and how *OsWRKY53* can regulate herbivore-induced defense in rice is unclear.

In this study, we reveal that *OsWRKY53* is rapidly induced by mechanical wounding, but only slowly induced by herbivore-attack. Through silencing and overexpressing

OsWRKY53, we show that it negatively regulates MPK3/MPK6 activity as well as the levels of herbivore-induced JA, JA-Ile and ethylene, which subsequently mediates the activity of trypsin protease inhibitors and resistance to SSB. Our study reveals that *OsWRKY53* is an important herbivore-responsive component that functions as a negative feedback modulator of MPK3/MPK6 which allows rice plants to control the magnitude of defensive investment against a chewing herbivore during early signaling.

RESULTS

cDNA Cloning and Expression Analysis of *OsWRKY53*

We screened rice plants for herbivore-induced transcripts using rice microarrays and found that one WRKY transcription factor, *OsWRKY53*, was up-regulated after SSB infestation (Zhou et al., 2011). Through reverse transcription PCR, we obtained the full-length cDNA of *OsWRKY53*, which includes an open reading frame (ORF) of 1464 bp (Supplemental Fig. S1). Phylogenetic analysis of the characterized group I-type WRKYs from different species revealed that *OsWRKY53* is homologous to *ZmWRKY33* in *Zea mays* (Li et al., 2013), *TaWRKY53-a* and *TaWRKY53-b* in *Triticum aestivum* (Van Eck et al., 2010), *NaWRKY6* in *Nicotiana attenuata* (Skibbe et al., 2008) and *AtWRKY33* in *Arabidopsis* (Zheng et al., 2006) (Supplemental Fig. S1), which share 69%, 67% and 64%, 51% and 51% amino acid sequence identity with *OsWRKY53*.

Quantitative real-time PCR (qRT-PCR) analysis revealed that the *OsWRKY53* gene is expressed at low levels in non-manipulated wild-type (WT) plants, whereas mechanical wounding rapidly and strongly enhanced the mRNA levels of *OsWRKY53* (Fig. 1). SSB larval feeding resulted in a slight increase in transcript levels in the stem after 1 and 2 h, and a significant increase in *OsWRKY53* transcript levels after 4 h (Fig. 1).

Overexpression and RNA Interference of *OsWRKY53*

To investigate the function of *OsWRKY53* in herbivore resistance, we obtained four T₂ homozygous lines consisting of two *OsWRKY53*-silenced lines (*ir-wrky* lines: *ir-14* and

ir-29) and two *OsWRKY53*-overexpression lines (oe-WRKY lines: oe-5 and oe-6), all of which contain a single T-DNA insertion (Supplemental Fig. S2). Transcription analysis showed that wound-induced transcript levels of *OsWRKY53* in the *ir-wrky* lines were approximately 30% of those in WT plants at 1 h after wounding (Supplemental Fig. S3). In contrast, transcript levels were significantly increased in the oe-5 (13.8- and 9.5-11.6-fold) and oe-6 (14.5- and 9.8-14.9-fold) lines without or with SSB infestation compared with transcript levels in equally-treated WT plants (Supplemental Fig. S3). In rice, genes whose nucleotide sequences have the highest similarity to *OsWRKY53* are *OsWRKY70* (69.96%, accession No. Os05g39720), *OsWRKY35* (66.58%, Os04g39570) and *OsWRKY24* (60.00%, Os01g61080)(Data not shown). Transcription analysis revealed that the RNAi construct did not co-silence the transcript accumulation of these genes (Fig. 4; Supplemental Fig. S4), suggesting that the specificity of the RNAi sequence is high. When grown in the greenhouse or the paddy, the overexpression lines consistently showed a semi-dwarf phenotype, and the root and stem lengths of oe-WRKY lines were almost half those of the WT plants (Supplemental Fig. S5 and Supplemental Fig. S6). In addition, the oe-WRKY lines were darker green than the WT plants owing to increased chlorophyll content (Supplemental Fig. S5 and Supplemental Fig. S6). Conversely, in *ir-wrky* lines, root length was slightly longer than in the oe-WRKY lines, whereas stem length and chlorophyll content were identical to those of WT plants (Supplemental Fig. S5 and Supplemental Fig. S6). Overexpressing plants showed a much higher leaf angle (Supplemental Fig. S7), delayed flowering time and produced fewer filled pollen grains (Data not shown).

***OsWRKY53* Negatively Regulates MPK Activity**

OsWRKY53 has been reported to be phosphorylated by the cascade *OsMEK4*-*OsMPK3*/*OsMPK6* and phosphorylation enhances its transactivation activity (Chujo et al., 2014; Yoo et al., 2014). We here confirm that *OsWRKY53* can physically interact with *OsMPK3* or *OsMPK6* *in vitro* and *in vivo* (Fig. 2; Supplemental Fig. S7).

We investigated the interactions between OsWRKY53 and OsMPK3 or OsMPK6 in a yeast two-hybrid (Y2H) assay system. Since the yeast transformed with full-length *OsWRKY53* fused to the GAL4 DNA-binding domain showed autoactivation, we constructed the N-terminal region of OsWRKY53 (WRKY₅₇₋₁₁₃), which contains the D domain and clustered Pro-directed Ser residues (SP cluster) and is sufficient for interaction with MPKs as bait based on the NbWRKY8 protein in *N. benthamiana* (Ishihama et al., 2011). Positive interactions, revealed by LacZ reporter activity (blue color) in the colonies, were observed only between one of the two MPKs and OsWRKY53, in addition to the positive control, which suggests that both OsMPK3 and OsMPK6 are capable of interacting with OsWRKY53 (Fig. 2A). To determine whether OsWRKY53 interacts with two MPKs in plant cells, bimolecular fluorescence complementation (BiFC) was performed in agro-infiltrated *N. benthamiana* leaves. Pair-wise expression of nYFP-WRKY53/cYFP-MPK3, cYFP-WRKY53/nYFP-MPK3 and nYFP-WRKY53/cYFP-MPK6, cYFP-WRKY53/nYFP-MPK6 resulted in a YFP fluorescence signal in the nucleus of agro-infiltrated cells at 72 h postinfiltration, whereas no fluorescence was detectable with combinations of nYFP-WRKY53/cYFP-GUS, cYFP-WRKY53/nYFP-GUS, nYFP-MPK3/cYFP-GUS, cYFP-MPK3/nYFP-GUS, nYFP-MPK6/cYFP-GUS, and cYFP-MPK6/nYFP-GUS (Fig. 2; Supplemental Fig. S7). From Supplemental Fig. S8, it is clear that OsMPK3/OsMPK6-WRKY53 interactions occur in the nucleus. These results show that OsWRKY53 and OsMPK3/OsMPK6 are co-localized in nucleus and interact directly at the protein level in plant cells.

To examine if this interaction also influences transcript levels of *OsWRKY53*, we investigated the expression of *OsWRKY53* in MPK mutants. We used the antisense expression lines *OsMPK3* (as-*mpk3*) and *OsMPK6* (as-*mpk6*), which had expression levels of 30 and 40% of *OsMPK3* and *OsMPK6* transcripts compared to WT plants (Lu et al., 2011; Wang et al., 2013). Transcript levels of *OsWRKY53* were significantly reduced in as-*mpk3* and as-*mpk6* plants compared with WT plants measured 30 and 60 min after

infestation with SSB larvae (Fig. 3).

We also measured transcription levels of *OsMPK3*, *OsMPK6*, and *OsMEK4* in *ir-wrky* and *oe-WRKY* lines. Surprisingly, compared with WT plants, silencing *OsWRKY53* increased the mRNA accumulation of *OsMPK3* and *OsMPK6*, whereas overexpressing *OsWRKY53* decreased their levels; moreover, the effect from *oe-WRKY* lines was bigger than that from *ir-wrky* lines and the effect was stronger on *OsMPK3* than on *OsMPK6* (Fig. 3). To determine if this influence affects the activity of MPK3/MPK6, we used immunoblot analysis with an anti-phosphoERK1/2 (anti-pT-E-pY) antibody to measure the activity of MPKs in WT and transgenic lines after SSB infestation. The result showed that SSB infestation quickly induced the activation of MPK3/MPK6 in WT plants. As the transcription results predicted, the activity of MPKs was lower in the *oe-5* line and slightly higher in the *ir-14* line than in WT plants (Fig. 3; Supplemental Fig. S9). These data show that *OsWRKY53* functions as a repressor of MPK cascades.

***OsWRKY53* Regulates Other Defense-Related WRKYs**

Auto-regulation and cross-regulation are common features of WRKY action (Ishihama and Yoshioka, 2012). Thus, we examined the transcript levels of *OsWRKY70*, *OsWRKY24*, *OsWRKY30*, *OsWRKY45*, *OsWRKY13*, *WRKY35* and *OsWRKY33*, all of which have been reported to be involved in defense responses in rice (Qiu et al., 2007; Shimono et al., 2007; Koo et al., 2009; Li, 2012; Shen et al., 2012), in *ir-wrky*, *oe-WRKY* and WT plants after SSB infestation. The results showed that silencing *OsWRKY53* did not strongly change the elicited expression levels of the other WRKYs, whereas overexpression of *OsWRKY53* altered WRKY mRNA levels, except the expression of *OsWRKY35* (Fig. 4; Supplemental Fig. S4). Moreover, of the four WRKYs that were strongly influenced, *OsWRKY33* was induced, but *OsWRKY70*, *OsWRKY24* and *OsWRKY30* were suppressed by overexpression of *OsWRKY53* (Fig. 4).

***OsWRKY53* is a Regulator of SSB-elicited JA, JA-Ile, SA and Ethylene**

Plant hormones play major roles in plant defense (Ahuja et al., 2012; Erb et al., 2012;

Nomura et al., 2012). The importance of JA, SA and ethylene in rice defense against herbivores has also been reported previously (Zhou et al., 2009; Lu et al., 2011; Li et al., 2013; Wang et al., 2013). To evaluate whether the altered expression of *OsWRKY53* affected the production of JA, JA-Ile, SA and ethylene, levels of these phytohormones were quantified in *ir-wrky*, *oe-WRKY* and WT plants after SSB infestation. Basal JA and JA-Ile levels were similar between the *ir-wrky* lines and WT plants, whereas JA and JA-Ile levels in the *ir-wrky* lines were significantly increased (by about 95-110% and 52-82% at 1.5 h after SSB infestation), compared with those of WT plants in response to SSB attack. In agreement with this finding, overexpression lines showed significantly decreased constitutive (in one line *oe-6*) and SSB-induced JA and JA-Ile levels (reduced by 42-61% and 43-56%, respectively) (Fig. 5). Consistent with the JA and JA-Ile levels, the transcript levels of JA biosynthesis-related genes, a 13-lipoxygenase gene *OsHI-LOX* (Zhou et al., 2009) and two putative allene oxide synthase (AOS) genes, *OsAOS1* and *OsAOS2* (Supplemental Fig. S10), were decreased in *oe-WRKY* lines and slightly enhanced in *ir-wrky* lines (Fig. 5).

The WT plants and transgenic lines (*ir-wrky* and *oe-WRKY* lines) showed similar constitutive SA levels, whereas the SA levels were increased in *oe-WRKY* lines and decreased in *ir-wrky* lines after SSB infestation, although SSB infestation did not induce the biosynthesis of SA in WT plants (Supplemental Fig. S11). The transcript levels of an isochorismate synthase (ICS) gene *OsICS1* that is involved in herbivore-induced SA biosynthesis in rice (Wang, 2012) were also positively regulated by *OsWRKY53* (Supplemental Fig. S11). A significantly lower accumulation of ethylene in the *oe-WRKY* lines and higher production in the *ir-wrky* lines compared with WT plants were observed at 24 and 48 h after infestation with SSB larvae (Fig. 6). The different levels of ethylene accumulation in transgenic plants compared with in WT plants correlate with distinct transcript levels of the *OsACS2* gene, which encodes the ethylene biosynthetic enzyme, 1-aminocyclopropane-1-carboxylic acid synthase (ACS) (Lu et al.,

2014) (Fig. 6).

To explore the notion that OsWRKY53 may be an upstream component that regulates the biosynthesis of these signals, we investigated the expression of *OsWRKY53* in transgenic plants with impaired JA or ethylene biosynthesis. We used our previous transgenic lines with antisense expression of *OsHI-LOX* (*as-lox*) (Zhou et al., 2009), *OsAOS1* (*as-aos1*) and *OsAOS2* (*as-aos2*) (Supplemental Fig. S10), all of which lines produced remarkably lower JA levels compared with those found in WT plants when infested by SSB larvae, as well as with antisense expression of *OsACS* (*as-acs*), which produced significantly less SSB-elicited ethylene than was found in WT plants (Lu et al., 2014). The levels of constitutive and induced *OsWRKY53* transcripts in *as-lox*, *as-aos1*, and *as-aos2* plants were identical to those in WT plants, whereas levels of constitutive and induced *OsWRKY53* transcripts in *as-acs* plants were significantly lower than in WT plants (Fig. 7). These results indicate that OsWRKY53 is induced upstream of the JA pathway but may form a negative feedback loop with the ethylene pathway.

OsWRKY53 Lowers Levels of TrypPIs and Resistance to SSB

Trypsin protease inhibitors (TrypPIs) are important direct defense proteins that help plants resist herbivores that chew on rice, and their activity was regulated by JA- and ET-mediated signaling pathways (Zhou et al., 2009; Lu et al., 2014). Therefore, we investigated TrypPI activity and SSB performance on transgenic lines and on WT plants. SSB-induced TrypPI activity was enhanced in the *ir-wrky* lines and suppressed in the *oe-WRKY* lines unlike in WT plants (Fig. 8). Consistent with the TrypPI activity, larvae of SSB gained less mass on the *ir-wrky* lines than on WT plants. By day 12, the mass of larvae that fed on the *ir-wrky* lines was about 65% of larvae that fed on WT plants (Fig. 8). In contrast, the mass of SSB larvae that fed on the *oe-WRKY* lines *oe-5* and *oe-6* was 1.93- and 1.67-fold higher than the mass of SSB larvae that fed on WT plants (Fig. 8). Moreover, the *oe-WRKY* lines were more severely damaged by SSB larvae than were the WT plants, whereas the *ir-wrky* lines were less damaged (Fig. 8).

To determine whether impaired resistance to herbivores and compromised defense responses in *oe-WRKY* plants could be due to lower JA and JA-Ile levels, we treated the overexpression lines with 100 μ g methyl jasmonate (MeJA). This direct JA complementation restored TrypPI activity in *oe-WRKY* plants to the levels observed in WT plants (Fig. 8). Larvae of SSB that fed on MeJA-treated *oe-WRKY* plants showed the same low growth rate as larvae that fed on WT plants (Fig. 8). These results show that the attenuated TrypPI accumulation and resistance to SSB of the *oe-WRKY* lines is probably largely caused by defective jasmonate signaling, which is negatively mediated by *OsWRKY53*.

DISCUSSION

In this study, we elucidate the mechanism by which *OsWRKY53* acts as a negative regulator of rice defenses and growth. Several lines of evidence point to a key role of *OsWRKY53* in controlling induced rice defense responses against SSB. First, the expression levels of *OsWRKY53* are induced when plants are wounded or infested with a chewing herbivore (Fig. 1). Second, *OsWRKY53* interacts directly with the MPK proteins *OsMPK3* and *OsMPK6* (Fig. 2) in a feedback-loop (Fig. 3). Third, altering expression of *OsWRKY53* affects the elicited accumulation of JA, JA-Ile, SA and ethylene and the expression of their biosynthesis genes (Fig. 5, Fig. 6 and Supplemental Fig. S11). Fourth, mutants with impaired JA pathway do not influence the levels of *OsWRKY53* transcripts, but the ethylene biosynthesis mutant decreases the expression of *OsWRKY53* (Fig. 7). Finally, *OsWRKY53* regulates the production of defense compounds, such as TrypPIs, and resistance in rice to SSB (Fig. 8).

***OsWRKY53* Functions as a Negative Feedback Modulator of MPK3/MPK6-Mediated Plant Defense Responses**

WRKYs can act as positive or negative regulators of the target genes and function at different regulatory levels (Ciolkowski et al., 2008; Rushton et al., 2010; Bakshi and

Oelmuller, 2014), and MPKs can mediate the activity of WRKYs via transcriptional and translational regulation (Ishihama et al., 2011; Li et al., 2012). Both OsMPK3 and OsMPK6 have been reported to phosphorylate OsWRKY53 (Yoo et al., 2014). Here, we found that OsWRKY53 negatively influenced the activity of OsMPK3 and OsMPK6 in turn. OsWRKY53 overexpression in particular strongly suppressed MPK activity (Fig. 3F). The relatively weak influence of OsWRKY53 silencing on MPK activities, which is also reflected in weaker phytohormone and gene expression patterns, may be caused by functional redundancy with other homologous WRKY genes or non-complete silencing of OsWRKY53. Our results suggest that OsWRKY53 and OsMPK3/6 form an interactive loop: OsMPK3 and OsMPK6 elicit the activity of OsWRKY53, whereas the activated OsWRKY53 suppresses the activity of MPK3 and MPK6, acting as a negative feedback regulator. It has been reported that WIPK and SIPK in *N. attenuata*, the homologs of MPK3 and MPK6 in rice, can regulate each other at the transcriptional level (Wu et al. 2007). Thus it is possible that OsWRKY53 directly suppresses the activity of one of the two MPKs and then influences the activity of the other indirectly by the interaction between the two MPKs. The mechanism on how OsWRKY53 inhibits MPK3/MPK6 activities might be related to OsWRKY53 regulation of MPK3/6 phosphorylation: By interacting physically with MPK3/6, OsWRKY53 may prevent access of MAPK phosphatases to the MPKs. Further experiments will be required to test these hypotheses.

Given the fact that MPK3 and MPK6 play an important role in plant defense responses by regulating defense-related signaling pathways, such as JA, SA, and ethylene (Schweighofer et al., 2007; Li et al., 2012; Tsuda et al., 2013; Wang et al., 2013) and that herbivore infestation induced the expression of *OsWRKY53* at later time points (Fig. 1), we propose that *OsWRKY53* may function mainly as a regulator for herbivore-induced defense responses and may allow plants to control the strength of their defense response and investment during early signaling. SSB infestation elicits a MPK3 dependent JA burst (Wang et al., 2013) that reaches a maximum at 3 h after infestation and **subsides** to

control levels at 8 h (Zhou et al., 2011). The early expression pattern of *OsWRKY53* upon SSB attack fits its role as a negative regulator that contributes to bringing JA signaling down after the initial burst (Fig. 1B). In rice, other negative modulators of herbivore-induced defenses, such as *Osr9-LOX1* (Zhou et al., 2014) and *OsNPRI* (Li et al., 2013) have been described. In other plants, SA signaling as well as jasmonate catabolism have been shown to be involved in attenuating herbivore defenses (Pieterse and Van Loon, 2004; Campos et al., 2014). This suggests that plants possess a set of mechanisms to control the magnitude of herbivore-induced defenses in space and time. Because of its involvement upstream of phytohormone signaling, *OsWRKY53* is among the earliest modulators described so far in this context. Interestingly, we also found that the expression level of *OsWRKY53* was continuously up-regulated by SSB infestation up to 48 h (Fig. 1B). Since low JA levels impair resistance of rice to SSB (Zhou et al., 2009), this phenomenon opens questions that need to be elucidated in the future. Especially the role of *OsWRKY53* at later stages of SSB infestation should be addressed.

***OsWRKY53* and Its Regulation on Other WRKYs and Phytohormones**

Increasing evidence shows that both MPKs and WRKYs can modulate the biosynthesis of JA, JA-Ile, SA, and ethylene by directly regulating the activity of related enzymes (Li et al., 2006; Wu et al., 2007; Skibbe et al., 2008; Birkenbihl et al., 2012; Li et al., 2012). In *Arabidopsis thaliana*, for example, AtMPK6 can directly phosphorylate AtACS2 and AtACS6, which subsequently elevates ACS activities and the production of ethylene (Liu and Zhang, 2004); WRKY33 modulates the expression of ACS2 and ACS6 by binding to the W-boxes in the promoters of the two genes (Liu and Zhang, 2004; Li et al., 2012). We found that *OsWRKY53* negatively modulated the production of elicited JA, JA-Ile and ethylene as well as the transcript levels of JA and ethylene biosynthesis-related genes, such as *OsHI-LOX* and *OsACS2* (Fig. 5 and Fig. 6), whereas it positively influenced the accumulation of SA after SSB infestation, including the transcript level of a SA biosynthesis-related gene *ICS1* (Supplemental Fig. S11); Since SSB infestation did not

elicit the production of SA in WT plants, the latter suggests that OSWRKY53 plays a role in SA homeostasis. Moreover, OsWRKY53 also affected transcript levels of other WRKYs (Fig. 4). In rice, OsMPK3/OsMPK6 and these OsWRKYs are known to be involved in regulating signaling pathways and defense responses, and it seems that OsWRKY53 negatively mediates the components activating JA and ET pathways, but positively regulates the components activating the SA pathway. OsWRKY13 and OsWRKY33 (Qiu et al., 2007; Koo et al., 2009), for instance - both of which suppress the JA-dependent but activate the SA-dependent pathway by regulating the transcript levels of JA biosynthesis- or SA biosynthesis-related genes, such as *AOS2*, *LOX*, and *ICS1* - were positively modulated by OsWRKY53. OsMPK3 (Wang et al., 2013), OsWRKY30 (Peng et al., 2012), and OsWRKY70 and OsWRKY24 (Li, 2012), all of which have been reported to positively regulate the JA and ET pathways, were negatively regulated by OsWRKY53. Given the fact that MPKs can modulate the activity of WRKYs as stated above and that WRKYs can regulate each other (Xu et al., 2006; Chen et al., 2009; Besseau et al., 2012; Chi et al., 2013), the influence of OsWRKY53 on these WRKYs and on phytohormone biosynthesis might occur via its direct and indirect (by mediating MPKs and other WRKYs) regulation. Here we observed some synchronized changes between OsMPK3/OsMPK6 and some WRKYs, such as OsWRKY70 and OsWRKY30, both of which have been reported to be positively regulated by these MPKs (Li, 2012; Shen et al., 2012). Therefore, the indirect regulation of OsWRKY53, i.e. its functioning as a negative feedback regulator of OsMPK3/6 as stated above, may also play an important role in regulating the biosynthesis of phytohormones. Further research should investigate the direct target genes of OsWRKY53 and elucidate which OsWRKYs and/or OsMPKs can directly mediate the activity of phytohormone biosynthesis-related enzymes.

In addition, we also found that altering OsWRKY53 expression influenced the growth phenotype of plants, especially *oe*-WRKY lines (Supplemental Fig. S5 and

Supplemental Fig S6). In *Arabidopsis*, *WRKY53* regulates leaf senescence and leaf development (Zentgraf et al., 2010; Xie et al., 2014). Moreover, in rice, the homologs of *OsWRKY53*, *OsWRKY70* (Li, 2012) and *OsWRKY24* (Zhang et al., 2009) negatively mediate the biosynthesis of gibberellins and/or abscisic acid and their signaling. Thus, the effect of *OsWRKY53* on plant growth may be related to its influence on these phytohormones. Interestingly, the characteristics of the effect of *OsWRKY53* on plant growth we observed here contradict to what Chujo et al. (Chujo et al., 2007) found. This difference might be related to different levels of *OsWRKY53* transcripts in mutants and the different genetic backgrounds. It has been reported that different transcription levels of a target gene caused different growth phenotypes (Kang et al., 2006). The mechanism of *OsWRKY53* underlying rice morphological alterations is worthy of elucidation in the future.

CONCLUSIONS

In summary, our results demonstrate that *OsWRKY53* is a regulator of herbivore-induced defense responses in rice (Fig. 9). When infested by an herbivore, rice plants perceive the signals from the herbivore and immediately activate MPKs, such as *OsMPK3* and *OsMPK6*; these subsequently increase the activity of some *OsWRKYs* except for *OsWRKY53*, such as *OsWRKY70*. The activated MPKs and *WRKYs* then regulate the biosynthesis of defense-related signal molecules, including JA, JA-Ile and ethylene. Moreover, the activated *OsMPK3* and *OsMPK6* also gradually activate *OsWRKY53* and then enhance its transcript level, which in turn inhibits *OsMPK3* and *OsMPK6* directly and indirectly by the interaction of the two MPKs, and thereby controls the magnitude of the plant's defense response. This system likely enables plants to fine-tune the activity of their defensive investment in space and time in a highly coordinated fashion.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The rice genotypes used in this study were Xiushui 11 WT and transgenic lines of *ir-wrky*, *oe-WRKY*, *as-acs* (Lu et al., 2014), *as-aos1*, *as-aos2* (Supplemental Fig. S10), *as-mpk3*, *as-mpk6* (Wang et al., 2013), and *as-lox* (Zhou et al., 2009). Pre-germinated seeds of the different lines were cultured in plastic bottles (diameter 8 cm, height 10 cm) in a greenhouse ($28 \pm 2^{\circ}\text{C}$, 14 h light, 10 h dark). 10-day-old seedlings were transferred to 20-L hydroponic boxes with a rice nutrient solution (Yoshida et al., 1976). After 40 d, seedlings were transferred to individual 500 mL hydroponic plastic pots. Plants were used for experiments 4–5 d after transplanting.

Insects

An SSB colony was originally obtained from rice fields in Hangzhou, China, and maintained on rice seedlings of TN1, a rice variety that is susceptible to infestation by SSB. All of the plants were kept in a controlled climate chamber at $26 \pm 2^{\circ}\text{C}$, with a 12 h photoperiod and 80% relative humidity.

Isolation and Characterization of *OsWRKY53* cDNA

The full-length cDNA of *OsWRKY53* was PCR-amplified. The primers WRKY-F (5'-CGTTCTCGTCTCCGATCACT-3') and WRKY-R (5'-ATACGGCGAGGCGAAAATAC-3') were designed based on the sequence of rice *OsWRKY53*. The PCR products were cloned into the pMD19-T vector (TaKaRa, <http://www.takara-bio.com/>) and sequenced.

Phylogenetic Analysis

For the phylogenetic analysis, the program MEGA 6.0 (Tamura et al., 2013) was used. The protein sequences aligned using the ClustalW method in MEGA 6.0 (pairwise alignment: gap opening penalty 10, gap extension penalty 0.1; multiple alignment: gap opening penalty 10, gap extension penalty 0.2, protein weight matrix using Gonnet). The residue-specific and hydrophilic penalties were ON, the end gap separation and the use negative separation matrix were OFF. Gap separation distance was 4, and the delay divergence cutoff (%) was at 30. This alignment (available as Supplemental Data Set 1)

was then used to generate an unrooted tree with statistical tests (parameters for phylogeny reconstruction were neighbor-joining method (Saitou and Nei, 1987), bootstrap (Felsenstein, 1985), $n = 1000$, amino acid, Poisson model, rate among sites: uniform rates gaps/missing, data treatment: complete deletion, traditional tree without modification for graphics) using MEGA 6.0.

Quantitative Real-Time PCR

For qRT-PCR analysis, five independent biological samples were used. Total RNA was isolated using the SV Total RNA Isolation System (Promega, <http://www.promega.com/>) following the manufacturer's instructions. One microgram of each total RNA sample was reverse-transcribed using the PrimeScript[®] RT-PCR Kit (TaKaRa, Dalian, China). The qRT-PCR assay was performed on CFX96[™] Real-Time system (Bio-RAD, California, USA) using the SsoFast[™] probes supermix (Bio-RAD, <http://www.bio-rad.com/>). A linear standard curve, threshold cycle number versus log (designated transcript level), was constructed using a series dilutions of a specific cDNA standard and the relative levels of the transcript of the target gene in all unknown samples were determined according to the standard curve. A rice actin gene OsACT (TIGR ID Os03g50885) was used as an internal standard to normalize cDNA concentrations. The primers and probes used for qRT-PCR for all tested genes are listed in Supplemental Table S1.

Generation and Characterization of Transgenic Plants

The full-length cDNA sequence and a 333 bp fragment of *OsWRKY53* were inserted into the pCAMBIA-1301 transformation vector to yield an overexpression and an RNAi construct, respectively (Supplemental Fig. S12). Both vectors were inserted into the rice variety Xiushui 11 using *Agrobacterium tumefaciens*-mediated transformation. The transformation of rice, the screening of the homozygous T₂ plants and the identification of the number of insertions followed the same method as described in Zhou et al. (2009). Two T₂ homozygous lines (ir-14 and ir-29) of *ir-wrky* and two lines (oe-5 and oe-6) of *oe-WRKY*, each harboring a single insertion (Supplemental Fig. S2), were used in

subsequent experiments.

Plant Treatments

For mechanical wounding, the lower portion of plant stems (about 2 cm long) were individually pierced 200 holes with a needle. Control plants (Con) were not pierced. For SSB treatment, plants were individually infested by a third-instar SSB larva that had been starved for 2 h. Control plants (Con) were not infested. For MeJA treatment, plant stems were individually treated with 100 µg MeJA in 20 µL lanolin paste. Controls (lanolin) were similarly treated with 20 µL pure lanolin.

Yeast Two-Hybrid Assay

The *OsWRKY53*₅₇₋₁₁₃ cDNA fragment was cloned into the pGBKT7 vector in-frame with the GAL4 binding domain. Full-length *OsMPK3* and *OsMPK6* were cloned into the pGADT7 vector, in the in-frame next to the activation domain (Clontech). The combinations of bait and prey plasmids (see in Fig. 2) were co-transformed into yeast Y2H Gold (Clontech). The interactions were tested on selective medium (SD-LWAH) lacking Leu, Trp, Ade and His, and containing X-α-gal and 0.25 µg ml⁻¹ aureobasidin A, according to Matchmaker TM Gold Yeast Two-Hybrid System User Manual (Clontech, <http://www.clontech.com>). Serial 1:10 dilutions were prepared in water, and 4 µL of each dilution was used to yield one spot. Plates were incubated at 30°C for about 72h before the scoring and capturing of photographs took place. SV40 T-antigen with p53 or Lamin C (Clontech) was used as the positive and negative control, respectively.

Bimolecular Fluorescence Complementation (BiFC) Assay

For BiFC studies, full-length *OsWRKY53*, *OsMPK3*, *OsMPK6*, and *GUS* were cloned into the pCV-nYFP or pCV-cYFP vector (Lu et al., 2011) to produce fused N- or C-terminal half of YFP, i.e. pCV-nYFP-WRKY53, pCV-nYFP-MPK3, pCV-nYFP-MPK6, pCV-nYFP-GUS, pCV-cYFP-WRKY53, pCV-cYFP-MPK3, pCV-cYFP-MPK6 and pCV-cYFP-GUS, respectively. Constructed plasmids were separately transformed into *Agrobacterium* EHA105. The plasmid-containing *Agrobacterium* were co-infiltrated into

tobacco (*N. benthamiana*) leaves at OD₆₀₀ 0.5:0.5 (see combinations in Fig. 2 and Supplemental Fig. S7). Small living pieces of tobacco leaves were cut from the infected area at 72 h after infiltration and mounted in water for microscopic observation. YFP fluorescence was observed and photographed by using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) with an argon laser.

Detection of MPK Activity

To detect MPK activities, one-month-old plants of different genotypes were randomly assigned to SSB treatment. Plant stems were harvested at 0, 15 and 30 min after treatment. Five replicates at each time point were pooled together and total proteins were extracted using the method described by (Wu et al., 2007). Forty µg total proteins were separated by SDS-PAGE and transferred onto Bio TraceTM pure nitrocellulose blotting membrane (PALL, <http://www.pall.com/main/home.page>). Immunoblotting was performed using rabbit anti-pTEpY (Cell Signaling Technologies, <http://www.cellsignal.com>), or plant-actin rabbit polyclonal antibody (EarthOx, <http://www.earthox.net/>). Chemiluminescence-based detection (Thermo Scientific, <http://www.thermoscientificbio.com>) was performed using horseradish peroxidase-conjugated goat anti-rabbit antibody (Sigma).

JA, JA-Ile, SA and Ethylene Analysis

Plants of the different genotypes were randomly assigned to SSB and control treatments. For JA, JA-Ile or SA analysis, plant stems were harvested at 0, 1.5, 3, 8 h after the start of SSB infestation. Samples were ground in liquid nitrogen, and JA and JA-Ile were extracted with ethyl acetate spiked with labeled internal standards (¹³C₂-JA and ¹³C₆-JA-Ile, each with 100 ng) and analyzed with high performance liquid chromatography/mass spectrometry/mass spectrometry system following the method as described in Lu et al. (2015). SA levels were analyzed by gas chromatography-mass spectrometry using labeled internal standards as described previously (Lou and Baldwin, 2003). For ethylene analysis, infested and control plants were covered with sealed glass

cylinders (diameter 4 cm, height 50 cm). Ethylene production was determined using the method described by (Lu et al., 2006). Each treatment at each time interval was replicated five times.

Analysis of TrypPI Activity

The stems of WT plants and transgenic lines (*ir-wrky* and *oe-WRKY*) (0.12-0.15 g sample⁻¹) were harvested with or without SSB treatment for 3 days. The TrypPI concentrations were measured using a radial diffusion assay as described by (Van Dam et al., 2001). Each treatment at each time interval was replicated five times.

Herbivore Resistance Experiments

The performance of SSB larvae on different genotypes (*ir-14*, *ir-29*, *oe-5*, and *oe-6*) and WT plants was determined by infesting with freshly hatched larvae. For testing the effect of MeJA on SSB larval performance, the second-instar SSB larvae which had been weighed and starved for 2 h were placed individually on each transgenic (*oe-5*, and *oe-6*) plant that had been treated with MeJA (20 μ L of lanolin containing 100 μ g MeJA). Sixty replicate plants from each line and treatment were used. Larval mass (to an accuracy of 0.1 mg) was measured 12 d after the start of the experiment. For the effect of MeJA, the increased percentage of larval mass on each line or treatment was calculated.

To determine differences in the tolerance of plants to herbivore attack, the different genotypes were individually infested with individual SSB third-instar larvae. The damage levels of plants were checked and photographs were taken.

Data Analysis

Differences in herbivore performance, expression levels of genes, and levels of herbivore-induced JA, SA, and ethylene, in different treatments, lines, or treatment times were determined by analysis of variance (or Student's *t*-test for comparing two treatments). All tests were carried out with Statistica (SAS Institute, Inc., <http://www.sas.com/>).

Accession Numbers

Sequence data from this article can be found in the Rice Annotation Project under the following accession numbers OsWRKY53 (Os05g27730), OsWRKY70 (Os05g39720), OsWRKY45 (Os05g25770), OsWRKY35 (Os04g39570), OsWRKY33 (Os03g33012), OsWRKY30 (Os08g38990), OsWRKY24 (Os01g61080), OsWRKY13 (Os01g54600), OsMEK4 (Os2g54600), OsMPK3 (Os03g17700), OsMPK6 (Os06g06090), OsHI-LOX (Os08g39840), OsAOS1 (Os03g55800), OsAOS2 (Os03g12500), OsICS1 (Os09g19734), OsACS2 (Os04g48850), OsACTIN (Os03g50885).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Sequences of nucleotides and deduced amino acids of *OsWRKY53* and phylogenetic analysis of group I-type WRKYs from different plant species.

Supplemental Figure S2. DNA gel-blot analysis of *ir-wrky*, *oe-WRKY* lines and WT plants.

Supplemental Figure S3. *OsWRKY53* expression levels of *ir-wrky*, *oe-WRKY* lines and WT plants.

Supplemental Figure S4. *OsWRKY35* expression levels of *ir-wrky*, *oe-WRKY* lines and WT plants.

Supplemental Figure S5. Growth phenotypes of *ir-wrky* and *oe-WRKY* lines and WT plants.

Supplemental Figure S6. *OsWRKY53* influences the phenotype of rice plants.

Supplemental Figure S7. *OsWRKY53* interacts with MPK3/6 in vivo.

Supplemental Figure S8. High resolution photos of interactions between *OsWRKY53* and MPK3/6 in the nucleus.

Supplemental Figure S9. Activity of *OsMPK3* and *OsMPK6* in *ir-wrky*, *oe-WRKY* lines and WT plants.

Supplemental Figure S10. *OsAOS1* and *OsAOS2* mediate herbivore-induced jasmonic acid (JA) biosynthesis in rice.

Supplemental Figure S11. OsWRKY53 mediates salicylic acid (SA) accumulation in rice after infestation with the striped stem borer (SSB).

Supplemental Figure S12. Transformation vectors were used in this study.

Supplemental Table S1. Primers and probes used for qRT-PCR of target genes.

Supplemental Table S2. Accession numbers of the amino acid sequences included in the phylogenetic tree.

Supplemental Data Set 1. Text file of alignments used for the phylogenetic analysis in Supplemental Figure S1B.

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AUTHOR CONTRIBUTIONS

LH, MY and YL designed the research. LH, MY, RL, GZ performed experiments. LH, MY, QW, JL and YL analyzed the data. YL, LH and MY prepared and wrote the paper.

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Figure Legends

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Mean transcript levels (+SE, $n = 5$) of *OsWRKY53* in rice stems that were mechanically wounded (A), infested by rice striped stem borer (SSB) (B). Con, control plants. Transcript levels were analyzed by quantitative RT-PCR. Asterisks indicate significant differences in transcript levels between treatments and controls (* $P < 0.05$; ** $P < 0.01$, Student's t-tests).

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B and C, BiFC visualization of WRKY53-MPK3 and WRKY53-MPK6 interactions. *N. benthamiana* leaves were cotransformed with the N-terminal part of YFP-fused WRKY53 or GUS (nYFP-WRKY53, nYFP-GUS) and the C-terminal part of YFP-fused MPKs or GUS (cYFP-MPK3, cYFP-MPK6, cYFP-GUS) by agro-infiltration.

Figure 3. *OsWRKY53* was downstream of MPK cascades, but negatively regulated *OsMPK3* and *OsMPK6*.

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C to E, Mean transcript levels (+SE, $n = 5$) of *OsMPK3* (C), *OsMPK6* (D) and *OsMEK4* (E) in *ir-wrky* and *oe-WRKY* lines, and WT plants that were individually infested by a third-instar SSB larva.

F, SSB-elicited MPK activation in *ir-wrky*, *oe-WRKY* lines and WT plants. *Ir-wrky* and *oe-WRKY* lines, and WT plants were treated with or without SSB larva, and stems from

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Asterisks indicate significant differences in *ir-wrky*, *oe-WRKY*, *as-mpk3* and *as-mpk6* lines compared with WT plants (* $P < 0.05$, **, $P < 0.01$, Student's t-tests).

Figure 4. OsWRKY53 mediates the expression levels of defense-related *OsWRKY* genes. Mean transcript levels (+SE, $n = 5$) of *OsWRKY70* (A), *OsWRKY24* (B), *OsWRKY30* (C), *OsWRKY45* (D), *OsWRKY13* (E), *OsWRKY30* (F) in *ir-wrky* (insert), *oe-WRKY* lines and wild-type (WT) plants that were individually infested by a third-instar rice striped stem borer (SSB) larva. Asterisks indicate significant differences in *ir-wrky* and *oe-WRKY* lines compared with WT plants (* $P < 0.05$, **, $P < 0.01$, Student's t-tests).

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C to E, Mean transcript levels (+SE, $n = 5$) of JA biosynthesis-related genes *OsHI-LOX* (C), *OsAOS1* (D) and *OsAOS2* (E) in *ir-wrky* and *oe-WRKY* lines, and WT plants that were individually infested by a third-instar SSB larva. Asterisks indicate significant differences in *ir-wrky* and *oe-WRKY* lines compared with WT plants (* $P < 0.05$, **, $P < 0.01$, Student's t-tests).

F, A SSB larva.

Figure 6. OsWRKY53 mediates rice striped stem borer (SSB)-induced ethylene accumulation.

A, Mean levels (+SE, $n = 5$) of ethylene in *ir-wrky* (insert) and *oe-WRKY* lines, and wild-type (WT) plants that were individually infested by a third-instar SSB larva.

B, Mean transcript levels (+SE, $n = 5$) of *OsACS2* in *ir-wrky* (insert) and *oe-WRKY* lines, and WT plants that were individually infested by a third-instar SSB larva. Asterisks indicate significant differences in *ir-wrky* and *oe-WRKY* lines compared with WT plants (* $P < 0.05$, **, $P < 0.01$, Student's t-tests).

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A and B, Mean trypsin protease inhibitor (TrypPI) activities (+SE, $n = 5$) in *ir-wrky*, *oe-WRKY* lines, and WT plants that were individually infested by a third-instar SSB larva for 3 days.

C and D, Mean larval mass (+SE, $n = 60$) of SSB that fed on *ir-wrky* and *oe-WRKY* lines or wild-type (WT) plants for 12 days.

E, Mean activities (+SE, $n = 5$) of TrypPIs in *oe-WRKY* lines, and WT plants that were individually treated with 100 μg of MeJA in 20 μL of lanolin paste (MeJA) or with 20 μL of pure lanolin (insert), followed by a SSB larva feeding for 3 days.

F, Mean increased larval mass (+SE, $n = 60$) of SSB larvae 12 days after they fed on *oe-WRKY* lines, and WT plants that were individually treated with 100 μg of MeJA in 20 μL of lanolin paste (MeJA) or with 20 μL of pure lanolin (insert).

G, Damaged phenotypes of *ir-wrky*, *oe-WRKY* lines and WT plants that were individually infested by a third-instar SSB larva for 14 or 7 days ($n = 20$).

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Plants recognize signals from wounding and herbivore infestation and quickly transduce these to MPK cascades, which leads to the activation of OsMPK3/OsMPK6. Active OsMPK3/OsMPK6 activates some WRKYs and thus both OsMPK3/OsMPK6 and WRKYs regulate the biosynthesis of defense-related signals, such as JA, JA-Ile and ethylene. The activated OsMPK3/OsMPK6 gradually elicits OsWRKY53 by phosphorylating it. Moreover, the ethylene pathway may also positively mediate the activity of OsWRKY53. OsWRKY53 can inhibit the activity of OsMPK3/OsMPK6 directly and indirectly by the interaction of the two MPKs, and may mediate other WRKYs with an unknown way, which keeps the defense response at an appropriate level. Arrows represent regulation negatively or positively; barred lines represent negative regulation; arrows with “+” represent positive regulation, respectively. Lines in green represent the OsWRKY53-ET regulation loop.

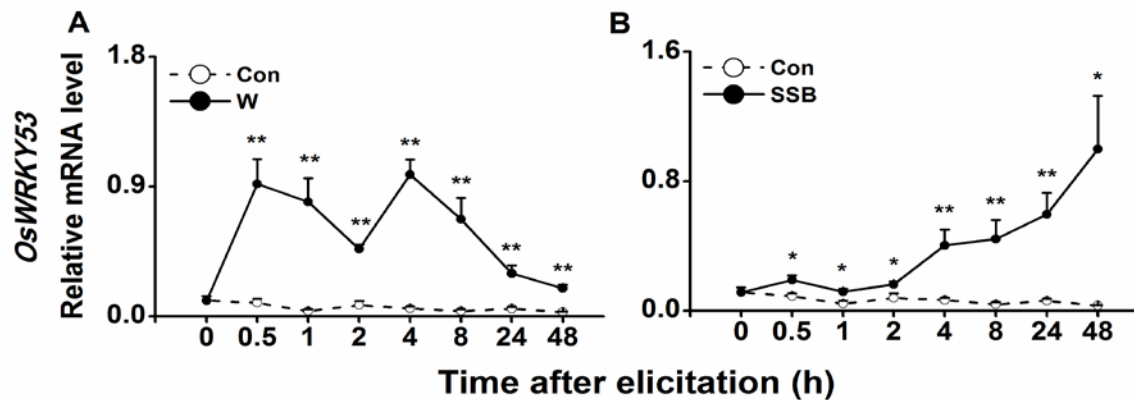


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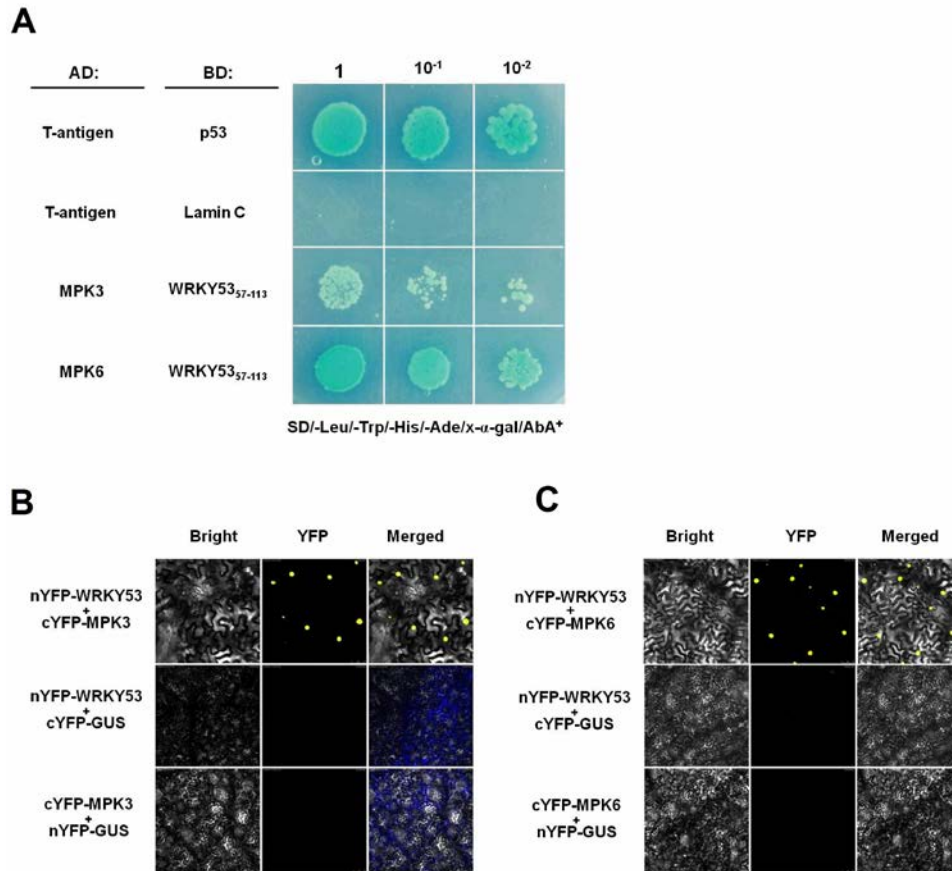


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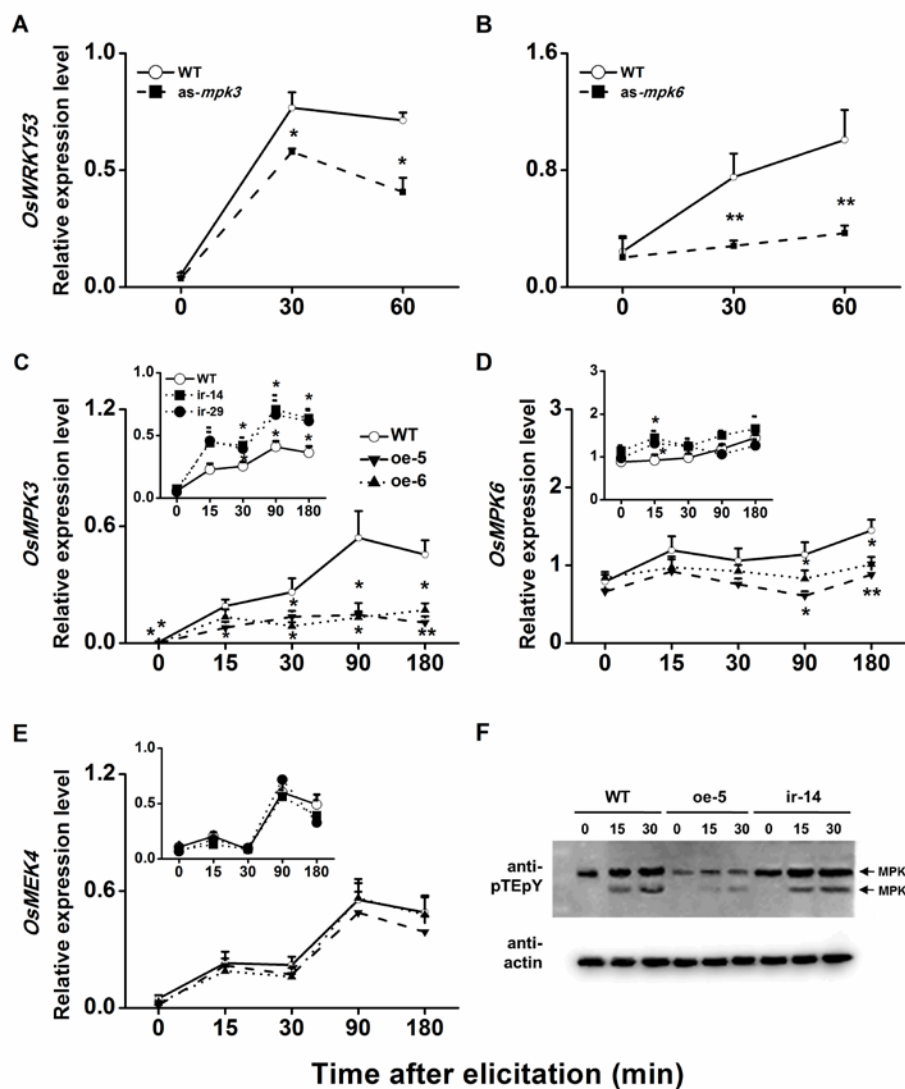


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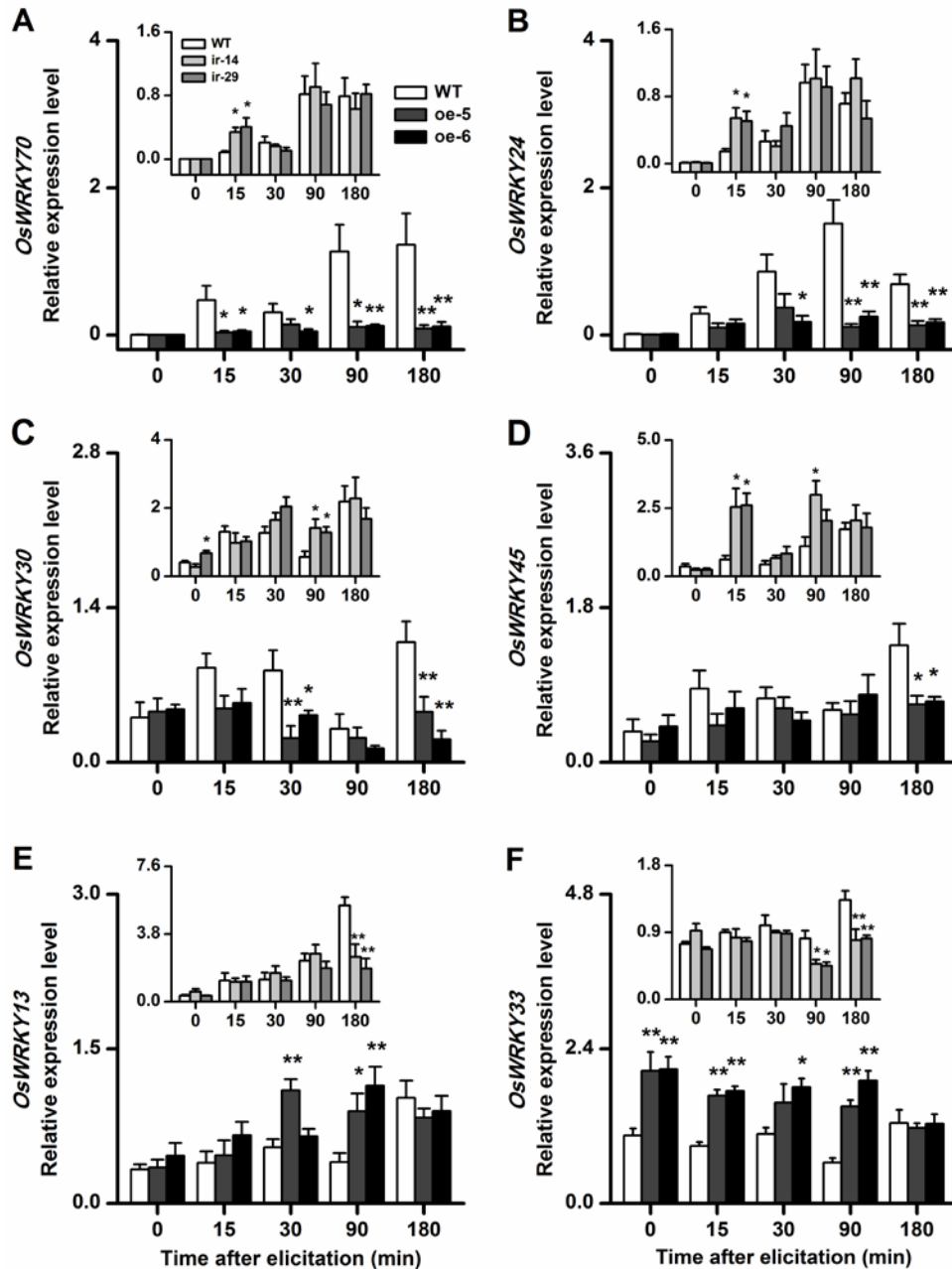


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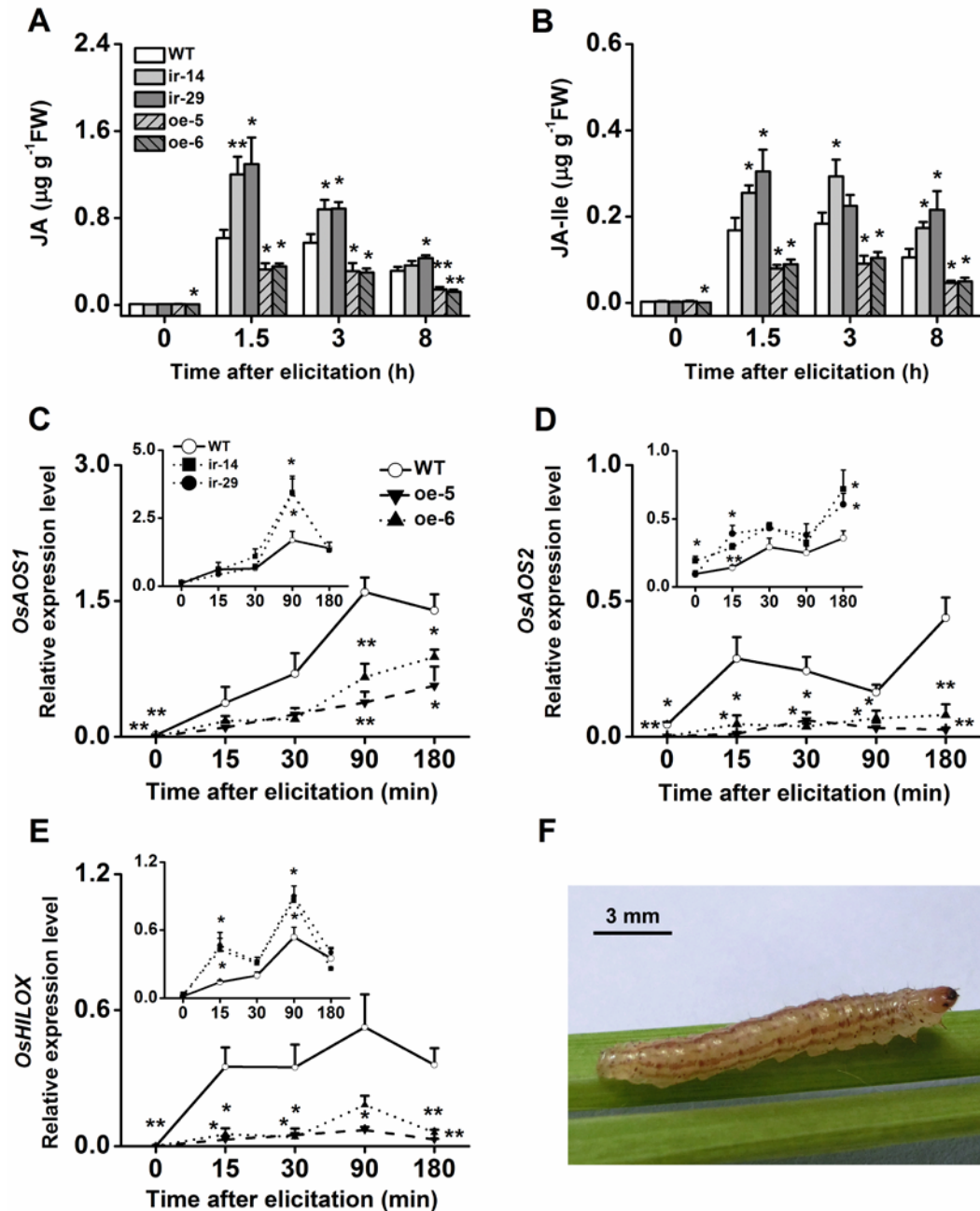


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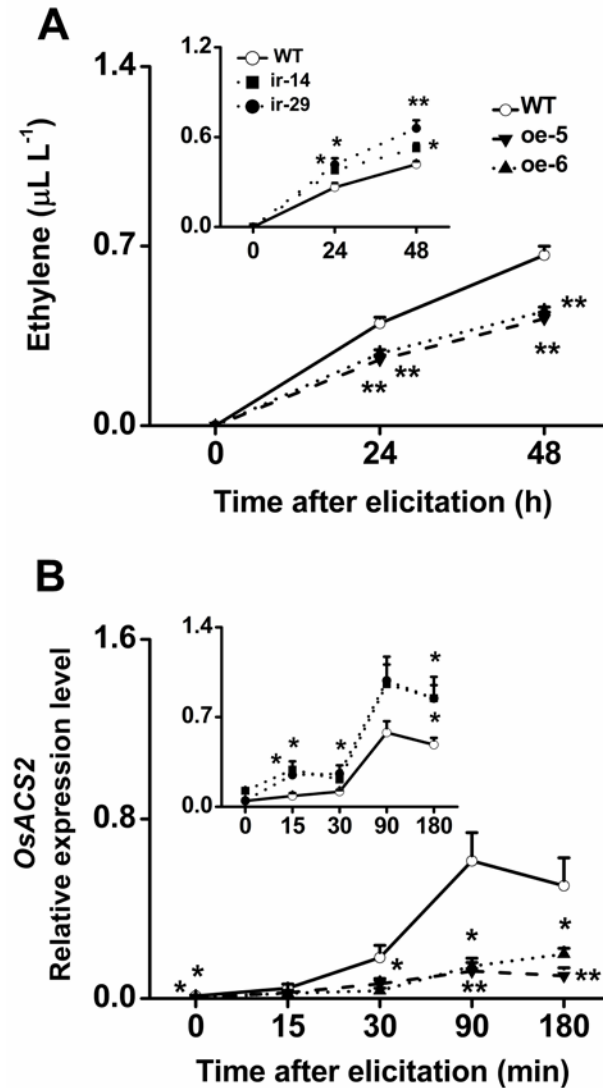


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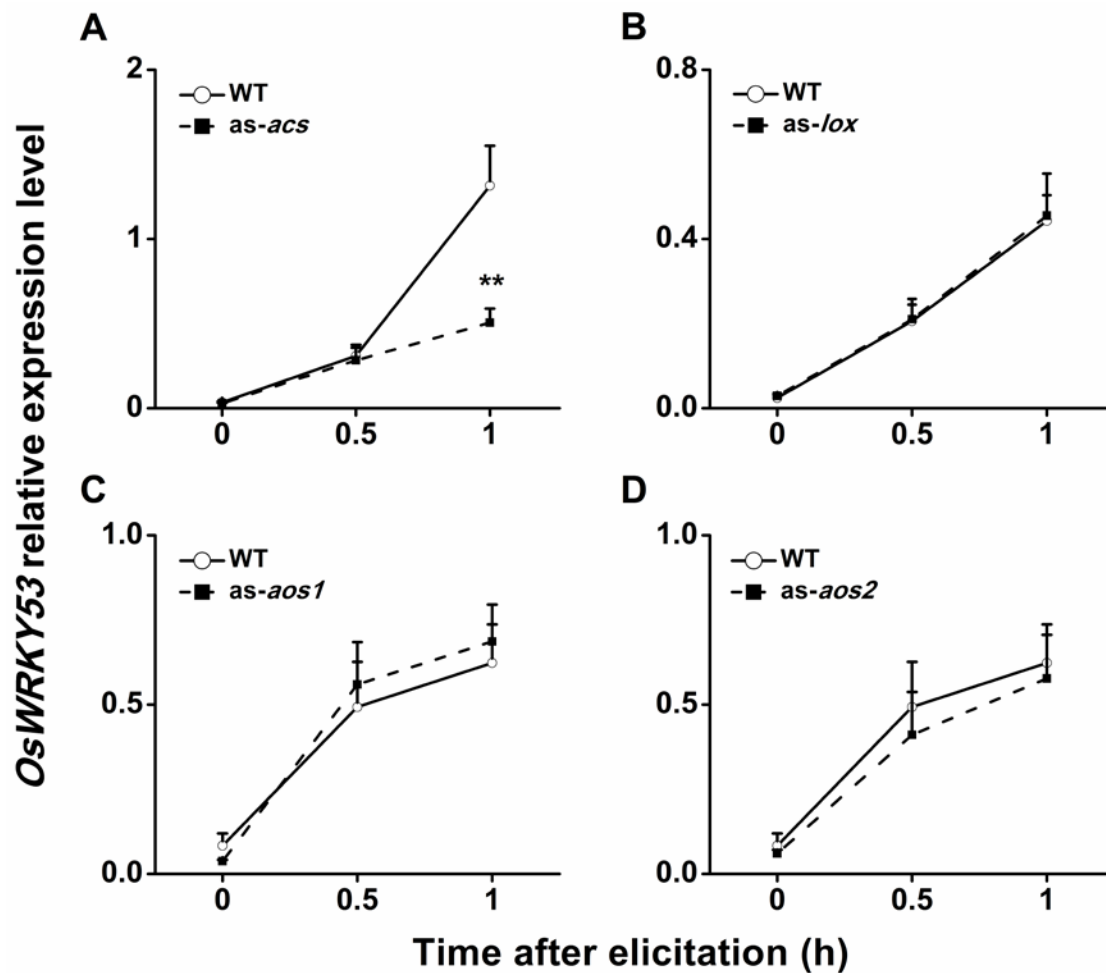


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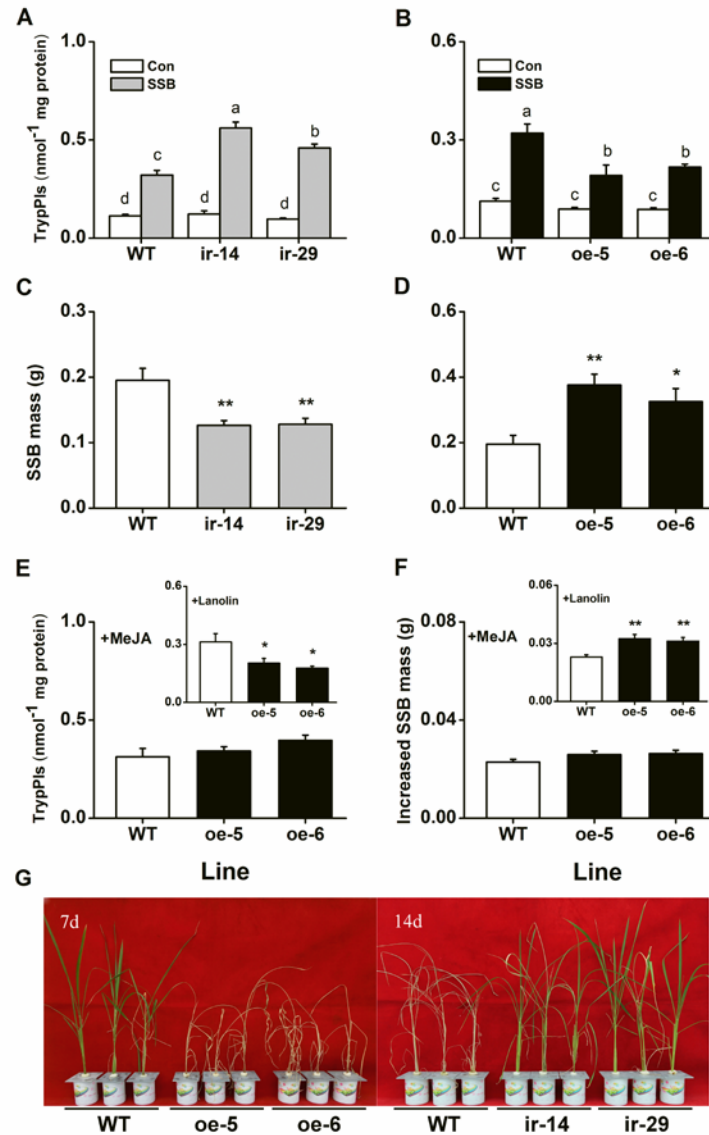


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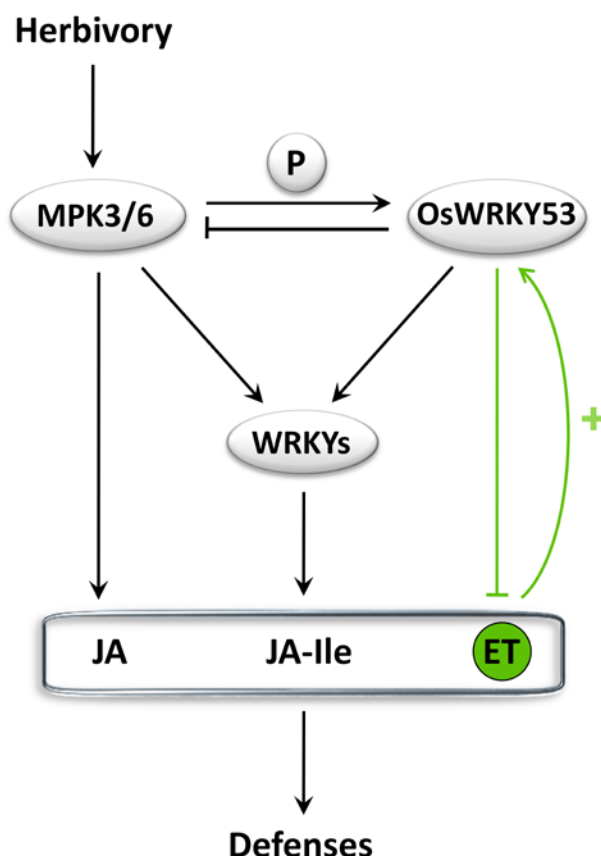


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Supplemental Figure Legends

Supplemental Figure S1. Sequences of nucleotides and deduced amino acids of *OsWRKY53* and phylogenetic analysis of group I-type WRKYs from different plant species.

A, Nucleotide sequence and the deduced amino acid sequence of the cloned *OsWRKY53*. The positions of the D domain (the black frame), SP clusters (red character) and conserved WRKY domains (underlined) were shown. B, Phylogenetic analysis of Group I-type WRKYs from different species. The unrooted tree was constructed with a neighbor-joining method on the basis of the alignment of protein sequences and confirmation of the tree topology by bootstrap analysis (1,000 replicates) were performed with MEGA software (default settings except the replicates of the bootstrap value). Species acronyms are included before the protein name: At, *Arabidopsis thaliana*; Ca, *Capsicum annuum*; Na, *Nicotiana attenuata*; Ta, *Triticum aestivum*; Nb, *Nicotiana benthamiana*; Os, *Oryza sativa*; Zm, *Zea mays*; Hb, *Hevea brasiliensis*; Gh, *Gossypium hirsutum*; SUSIBA2, sugar signaling in barley. The bootstrap values for the branches are shown. Accession numbers are shown in Supplemental Table S2. The scale bar represents 0.1 amino acid substitutions per site in the primary structure.

Supplemental Figure S2. DNA gel-blot analysis of *ir-wrky*, *oe-WRKY* lines and WT plants. Genomic DNA was digested with *EcoR* I or *Xba* I. The blot was hybridized with a probe specific for reporter gene *gus*. All transgenic lines have a single insertion of the transgene.

Supplemental Figure S3. *OsWRKY53* expression levels of *ir-wrky*, *oe-WRKY* lines and WT plants.

Mean transcript levels (+SE, $n = 5$) of *OsWRKY53* in *ir-wrky* lines, *oe-WRKY* lines and WT plants that were individually wounded for 1h (W, A) or infested by a third-instar SSB larva (B). Transcript levels were analyzed by qRT-PCR. Asterisks indicate significant differences in *ir-wrky* and *oe-WRKY* lines compared to WT plants (*, $P < 0.05$; **, $P < 0.01$, Student's t-tests).

Supplemental Figure S4. *OsWRKY35* expression levels of *ir-wrky*, *oe-WRKY* lines and WT plants.

A and B, Mean transcript levels (+SE, $n = 5$) of *OsWRKY35* in *ir-wrky* and *oe-WRKY* lines and WT plants that were individually infested by a third-instar SSB larva. Asterisks indicate significant differences in *ir-wrky* and *oe-WRKY* lines compared to WT plants (*, $P < 0.05$; **, $P < 0.01$, Student's t-tests).

Supplemental Figure S5. Growth phenotypes of *ir-wrky* and *oe-WRKY* lines and WT plants.

A, 12-days-old seedlings. B, 30-days-old seedlings. C and D, Plants at heading stage.

Supplemental Figure S6. OsWRKY53 influences the phenotype of rice plants.

A to D, Mean lengths (+SE, $n = 20$) of stems ([A] and [B]) and roots ([C] and [D]) of WT plants and plants of *ir-wrky* and *oe-WRKY* lines at 30 or 60 d. (E) and (F) Mean chlorophyll contents (+SE, $n = 10$), measured by soil-plant analysis development (SPAD) meter, of WT plants and plants of *ir-wrky* and *oe-WRKY* lines at 30 or 60d. Asterisks indicate significant differences in *ir-wrky* and *oe-WRKY* lines compared to WT plants (*, $P < 0.05$; **, $P < 0.01$, Student's t-tests).

Supplemental Figure S7. OsWRKY53 interacts with MPK3/6 in vivo.

A and B, BiFC visualization of WRKY53-MPK3 and WRKY53-MPK6 interactions. *N. benthamiana* leaves were cotransformed with the C-terminal part of YFP-fused WRKY53 or GUS (cYFP-WRKY53, cYFP-GUS) and the N-terminal part of YFP-fused MPKs or GUS (nYFP-MPK3, nYFP-MPK6, nYFP-GUS) by agro-infiltration.

Supplemental Figure S8. High resolution photos of interactions between OsWRKY53 and MPK3/6 in nucleus.

High resolution photos of BiFC visualization of WRKY53-MPK3 and WRKY53-MPK6 interactions. *N. benthamiana* leaves were cotransformed with the N-terminal part of YFP-fused WRKY53 (nYFP-WRKY53) and the C-terminal part of YFP-fused MPKs (cYFP-MPK3, cYFP-MPK6) by agro-infiltration. The red circle indicates the position of the nucleus of tobacco cell.

Supplemental Figure S9. The activity of OsMPK3 and OsMPK6 in *ir-wrky*, *oe-WRKY* lines and WT plants.

A, SSB-elicited MPK activation in *ir-wrky*, *oe-WRKY* lines and WT plants. *Ir-wrky*, *oe-WRKY* lines, and WT plants were treated with or without SSB larva, and stems from five replicate plants were harvested at the indicated times. Immunoblotting was performed using either α -pTEpY antibody (upper panel) to detect phosphorylated MPKs, or actin antibody (lower panel) as a loading control.

B and C, The relative activity of OsMPK3 and OsMPK6 based on the signal intensity in A. The activity for the WT sample at 0 min was set to 1. The relative activity of all other samples at each time point was expressed relative to the WT sample at 0 min.

D, The relative amount of the loading OsActin based on the signal intensity in A. The quantity for the WT sample at 0 min was set to 1. The relative amount of all other samples at each time point were expressed relative to the WT sample at 0 min.

Supplemental Figure S10. *OsAOS1* and *OsAOS2* mediate herbivore-induced jasmonic acid (JA) biosynthesis in rice.

A and B, Mean transcriptional levels (+SE, $n = 5$) of *OsAOS1* (A) and *OsAOS2* (B) in anti-sense (as)-*aos1* (A) and as-*aos2* (B) lines, and WT plants that were individually infested by a third-instar striped stem borer (SSB) larva for 1h. Transcript levels were analyzed by qRT-PCR.

C and D, Mean levels (+SE, $n = 5$) of JA in as-*aos1* (C) and as-*aos2* (D) lines and WT plants that were individually infested by a third-instar SSB larva. Asterisks indicate significant differences in as-*aos1* and as-*aos2* lines compared to WT plants (*, $P < 0.05$; **, $P < 0.01$, Student's t-tests).

Supplemental Figure S11. OsWRKY53 mediates salicylic acid (SA) accumulation in rice after infestation with the striped stem borer (SSB)

A to B, Mean levels (+SE, $n = 5$) of SA in ir-*wrky* (A) and oe-WRKY (B) lines, and wild-type (WT) plants that were individually infested by a third-instar SSB larva.

C, Mean transcript levels (+SE, $n = 5$) of *OsICS1* in ir-*wrky* and oe-WRKY lines, and WT plants that were individually infested by a third-instar SSB larva. Asterisks indicate significant differences in ir-*wrky* and oe-WRKY lines compared with WT plants (* $P < 0.05$, **, $P < 0.01$, Student's t-tests).

Supplemental Figure S12. Transformation vectors were used in this study.

Supplemental Table S1. Primers and probes used for qRT-PCR of target genes.

Supplemental Table S2. Accession numbers of the amino acid sequences included in the phylogenetic tree.

Sequence data in the phylogenetic tree can be found in the GenBank/EMBL data libraries under accession numbers: AtWRKY1 (NP_178565.1); AtWRKY2 (NP_200438.1); AtWRKY3 (NP_178433.1); AtWRKY4 (NP_172849.1); AtWRKY25 (NP_180584.1); AtWRKY26 (NP_196327.1); AtWRKY33 (NP_181381.2); AtWRKY34 (NP_194374.1); CaWRKY2 (ABD65255.1); CaWRKY58 (AAW67002.1) NaWRKY3 (AAS13439.1); NaWRKY6 (AAS13440.1); NbWRKY8 (BAI63296.1); OsWRKY4 (DAA05069.1); OsWRKY24 (AAT84156.1); OsWRKY30 (ADK13079.1); OsWRKY35 (DAA05100.1); OsWRKY53

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93 (NP_001147551.1); HbWRKY1 (AEE81757.1); GhWRKY44 (AJA30422.1); GhWRKY3
94 (ADI52618.1); SUSIBA2 (AAQ63880.1).

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Supplemental Figures

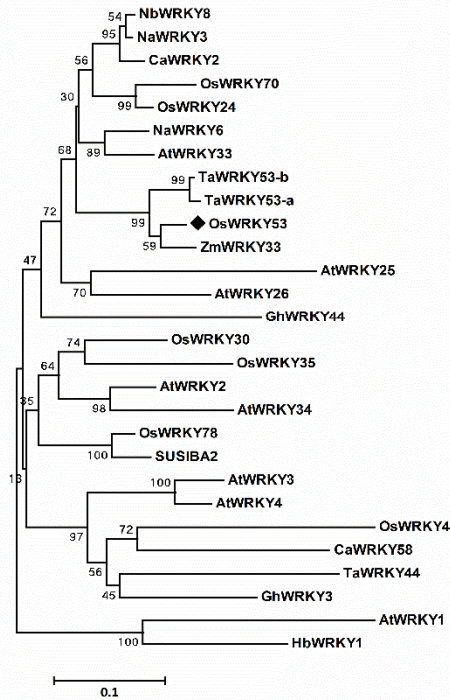
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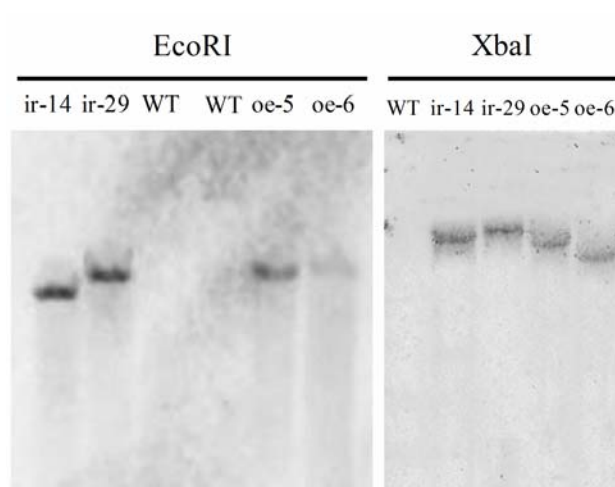
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Supplemental Figure S1. Sequences of nucleotides and deduced amino acids of *OsWRKY53* and phylogenetic analysis of group I-type WRKYs from different plant species.

A, Nucleotide sequence and the deduced amino acid sequence of the cloned *OsWRKY53*. The positions of the D domain (the black frame), SP clusters (red character) and conserved WRKY domains (underlined) were shown. B, Phylogenetic analysis of Group I-type WRKYs from different species. The unrooted tree was constructed with a neighbor-joining method on the basis of the alignment of protein sequences and confirmation of the tree topology by bootstrap analysis (1,000 replicates) were performed with MEGA software (default settings except the replicates of the bootstrap value). Species acronyms are included before the protein name: At, *Arabidopsis thaliana*; Ca, *Capsicum annuum*; Na, *Nicotiana attenuata*; Ta, *Triticum aestivum*; Nb, *Nicotiana benthamiana*; Os, *Oryza sativa*; Zm, *Zea mays*; Hb, *Hevea brasiliensis*; Gh, *Gossypium hirsutum*; SUSIBA2, sugar signaling in barley. The bootstrap values for the branches are shown. Accession numbers are shown in Supplemental Table S2. The scale bar represents 0.1 amino acid substitutions per site in the primary structure.

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130 **Supplemental Figure S2.** DNA gel-blot analysis of *ir-wrky*, *oe-WRKY* lines and WT plants.
131 Genomic DNA was digested with *EcoR* I or *Xba* I. The blot was hybridized with a probe specific
132 for reporter gene *gus*. All transgenic lines have a single insertion of the transgene.

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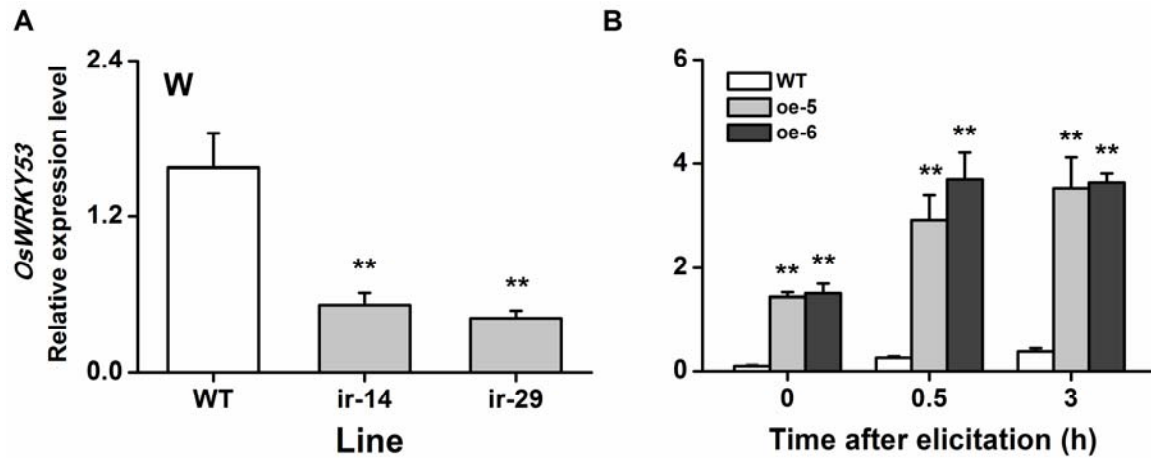
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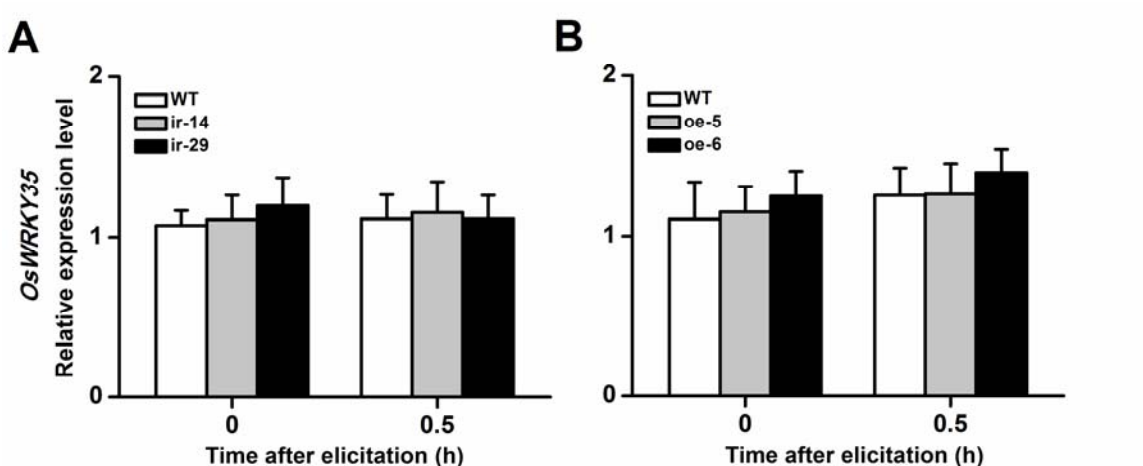
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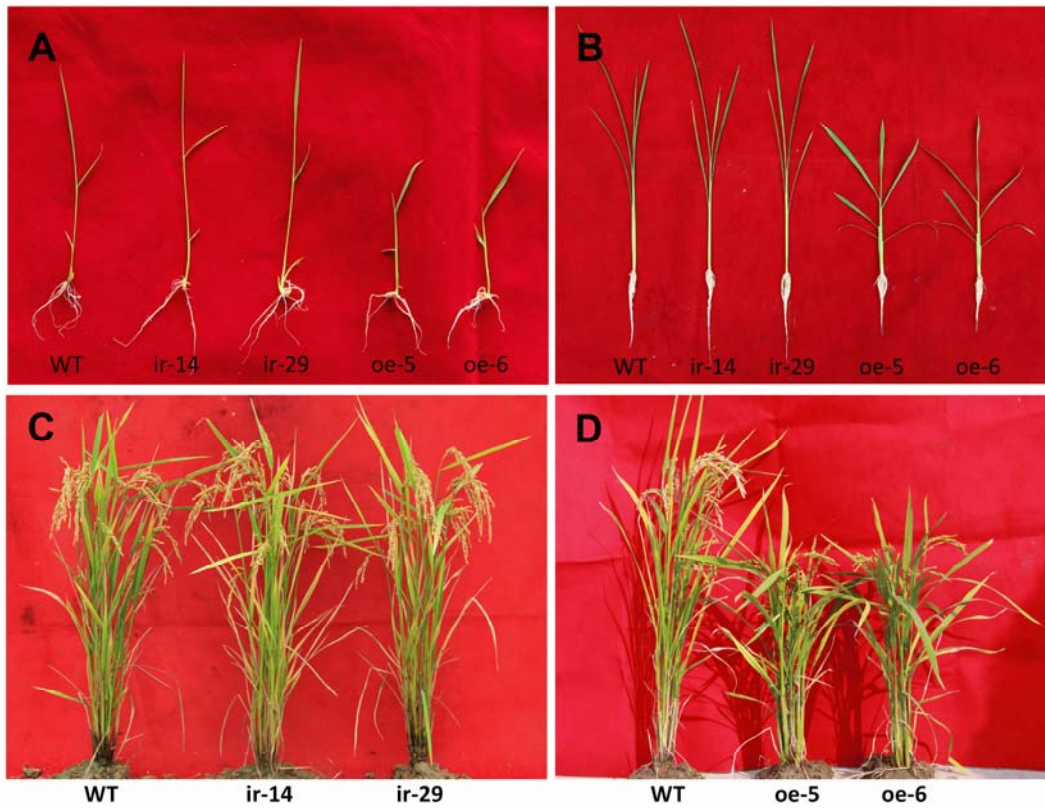
Supplemental Figure S3. *OsWRKY53* expression levels of *ir-wrky*, *oe-WRKY* lines and WT plants.

Mean transcript levels (+SE, $n = 5$) of *OsWRKY53* in *ir-wrky* lines, *oe-WRKY* lines and WT plants that were individually wounded for 1h (W, A) or [infested by a third-instar SSB larva \(B\)](#). Transcript levels were analyzed by qRT-PCR. Asterisks indicate significant differences in *ir-wrky* and *oe-WRKY* lines compared to WT plants (*, $P < 0.05$; **, $P < 0.01$, Student's t-tests).

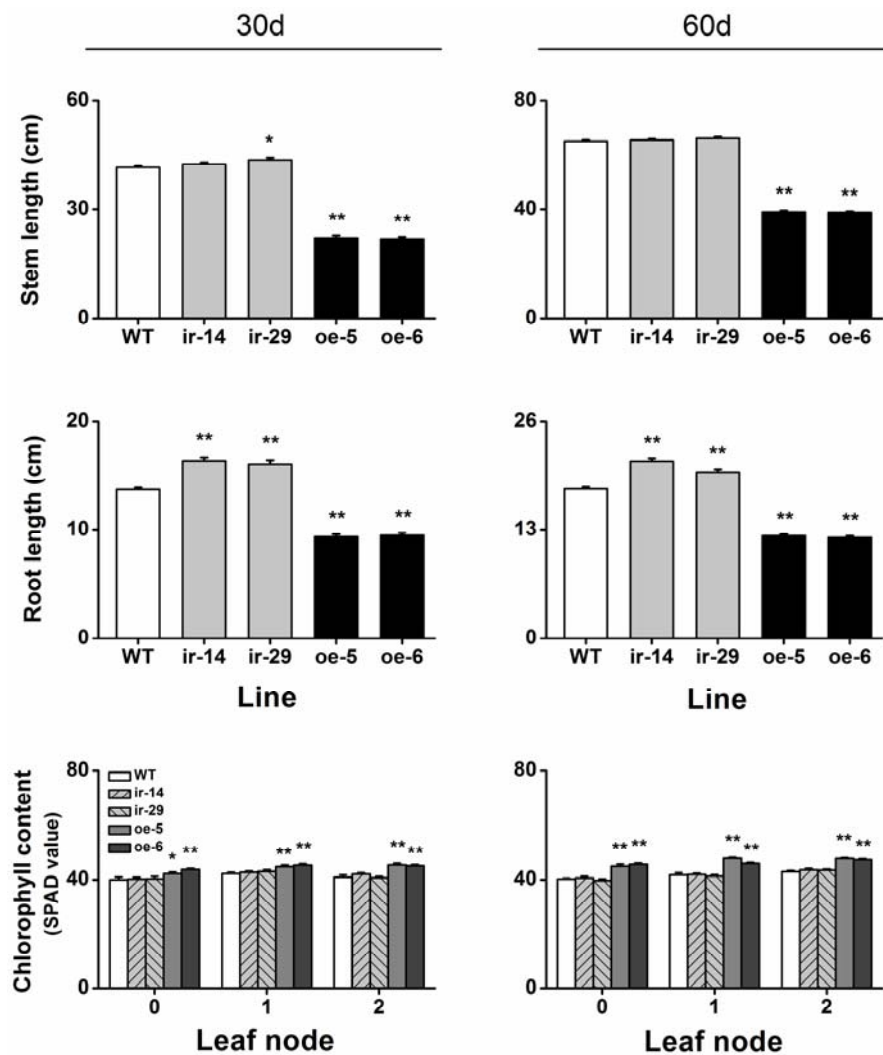


Supplemental Figure S4. *OsWRKY35* expression levels of *ir-wrky*, *oe-WRKY* lines and WT plants.

A and B, Mean transcript levels (+SE, $n = 5$) of *OsWRKY35* in *ir-wrky* and *oe-WRKY* lines and WT plants that were individually infested by a third-instar SSB larva. Asterisks indicate significant differences in *ir-wrky* and *oe-WRKY* lines compared to WT plants (*, $P < 0.05$; **, $P < 0.01$, Student's t-tests).



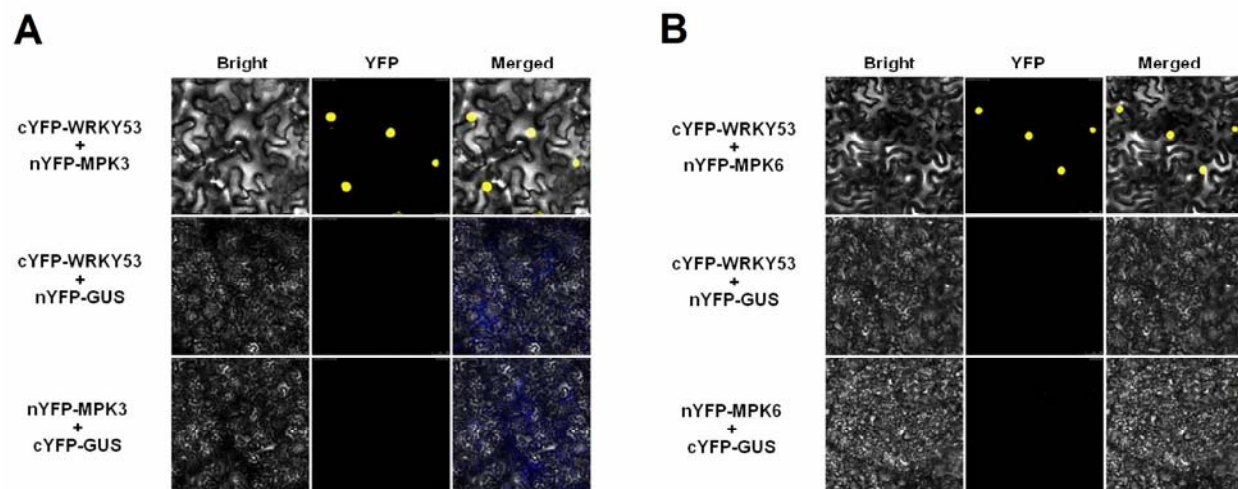
Supplemental Figure S5. Growth phenotypes of *ir-wrky* and *oe-WRKY* lines and WT plants. A, 12-days-old seedlings. B, 30-days-old seedlings. C and D, Plants at heading stage.



Supplemental Figure S6. OsWRKY53 influences the phenotype of rice plants.

A to D, Mean lengths (+SE, $n = 20$) of stems ([A] and [B]) and roots ([C] and [D]) of WT plants and plants of *ir-wrky* and *oe-WRKY* lines at 30 or 60 d. (E) and (F) Mean chlorophyll content (+SE, $n = 10$), measured by soil-plant analysis development (SPAD) meter, of WT plants and plants of *ir-wrky* and *oe-WRKY* lines at 30 or 60d. Asterisks indicate significant differences in *ir-wrky* and *oe-WRKY* lines compared to WT plants (*, $P < 0.05$; **, $P < 0.01$, Student's t-tests).

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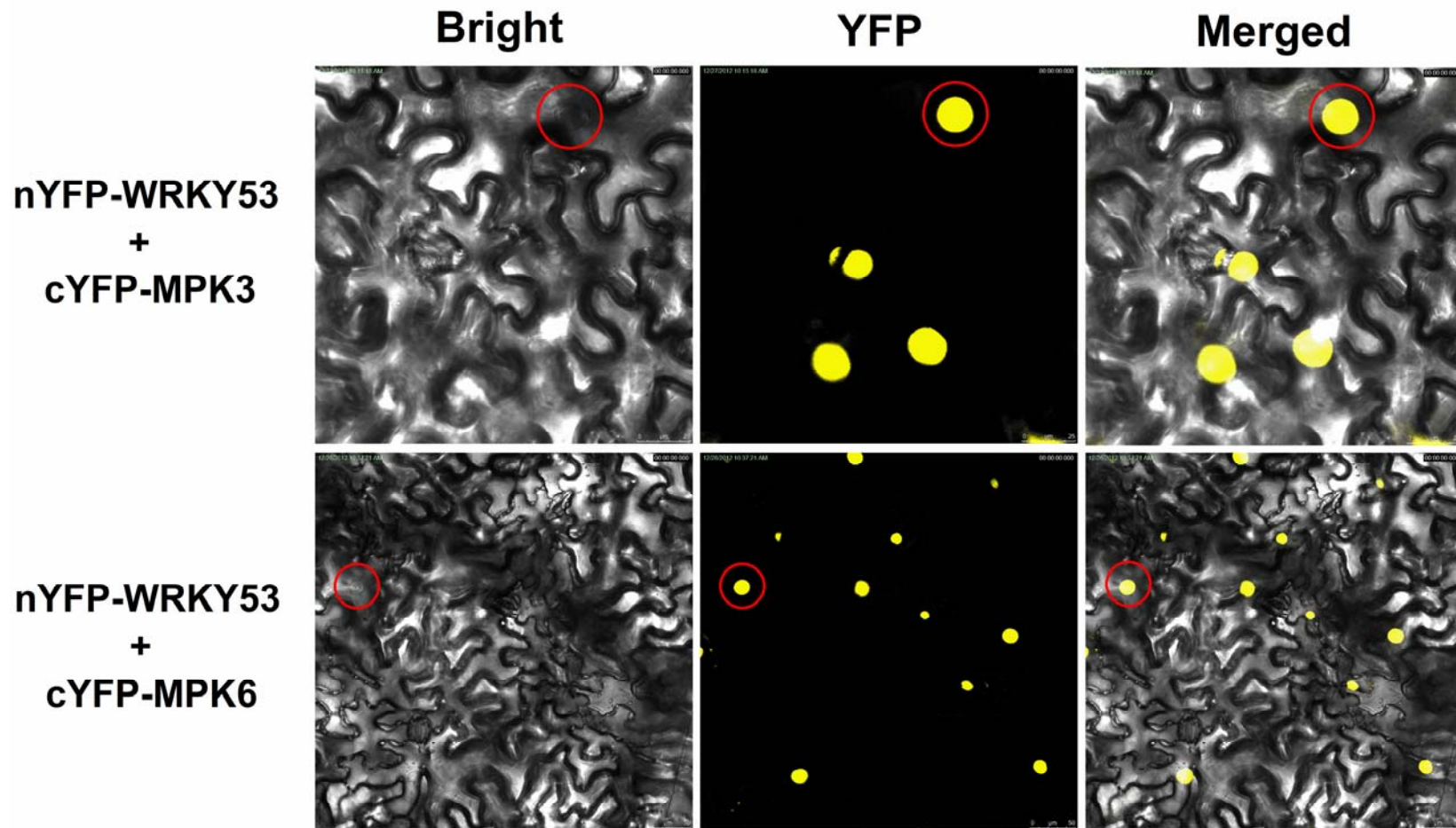
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207 **Supplemental Figure S7.** OsWRKY53 interacts with MPK3/6 in vivo.

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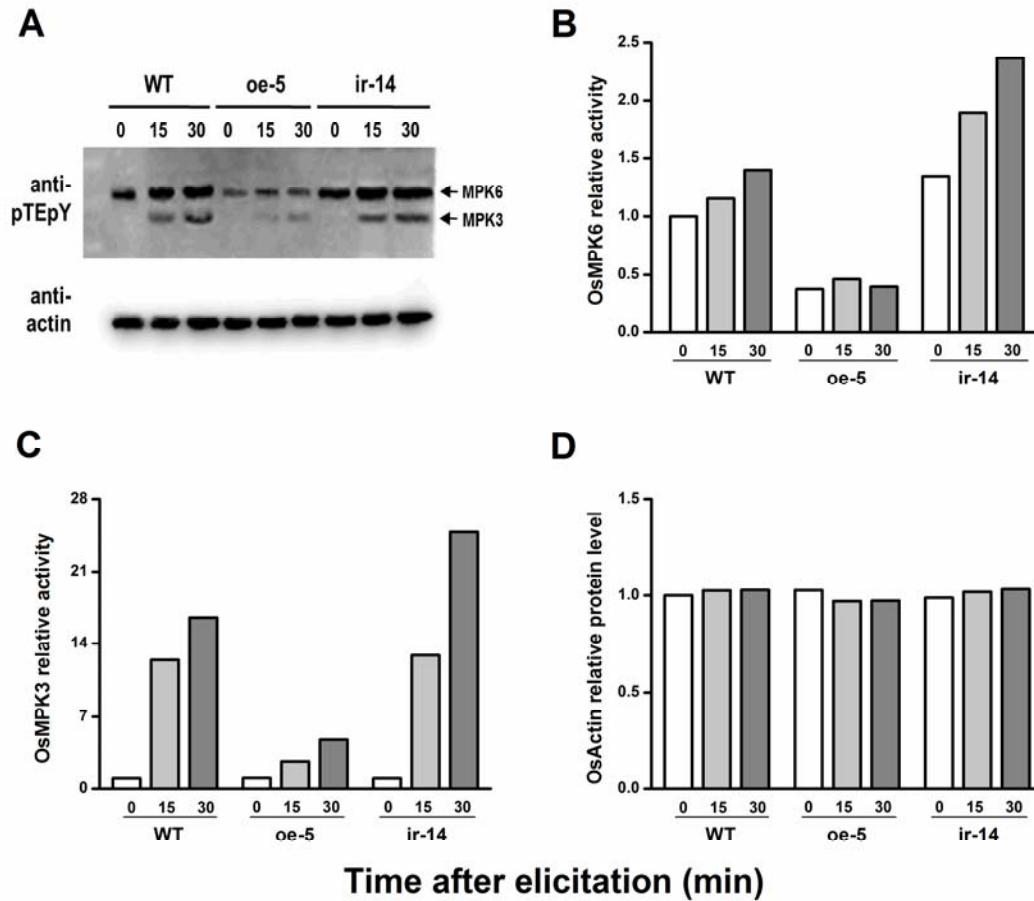
209 A and B, BiFC visualization of WRKY53-MPK3 and WRKY53-MPK6 interactions. *N.*
 210 *benthamiana* leaves were cotransformed with the C-terminal part of YFP-fused WRKY53 or
 211 GUS (cYFP-WRKY53, cYFP-GUS) and the N-terminal part of YFP-fused MPKs or GUS
 212 (nYFP-MPK3, nYFP-MPK6, nYFP-GUS) by agro-infiltration.

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Supplemental Figure S8. High resolution photos of interactions between OsWRKY53 and MPK3/6 in nucleus.

High resolution photos of BiFC visualization of WRKY53-MPK3 and WRKY53-MPK6 interactions. *N. benthamiana* leaves were cotransformed with the N-terminal part of YFP-fused WRKY53 (nYFP-WRKY53) and the C-terminal part of YFP-fused MPKs (cYFP-MPK3, cYFP-MPK6) by agro-infiltration. The red circle indicates the position of the nucleus of tobacco cell.

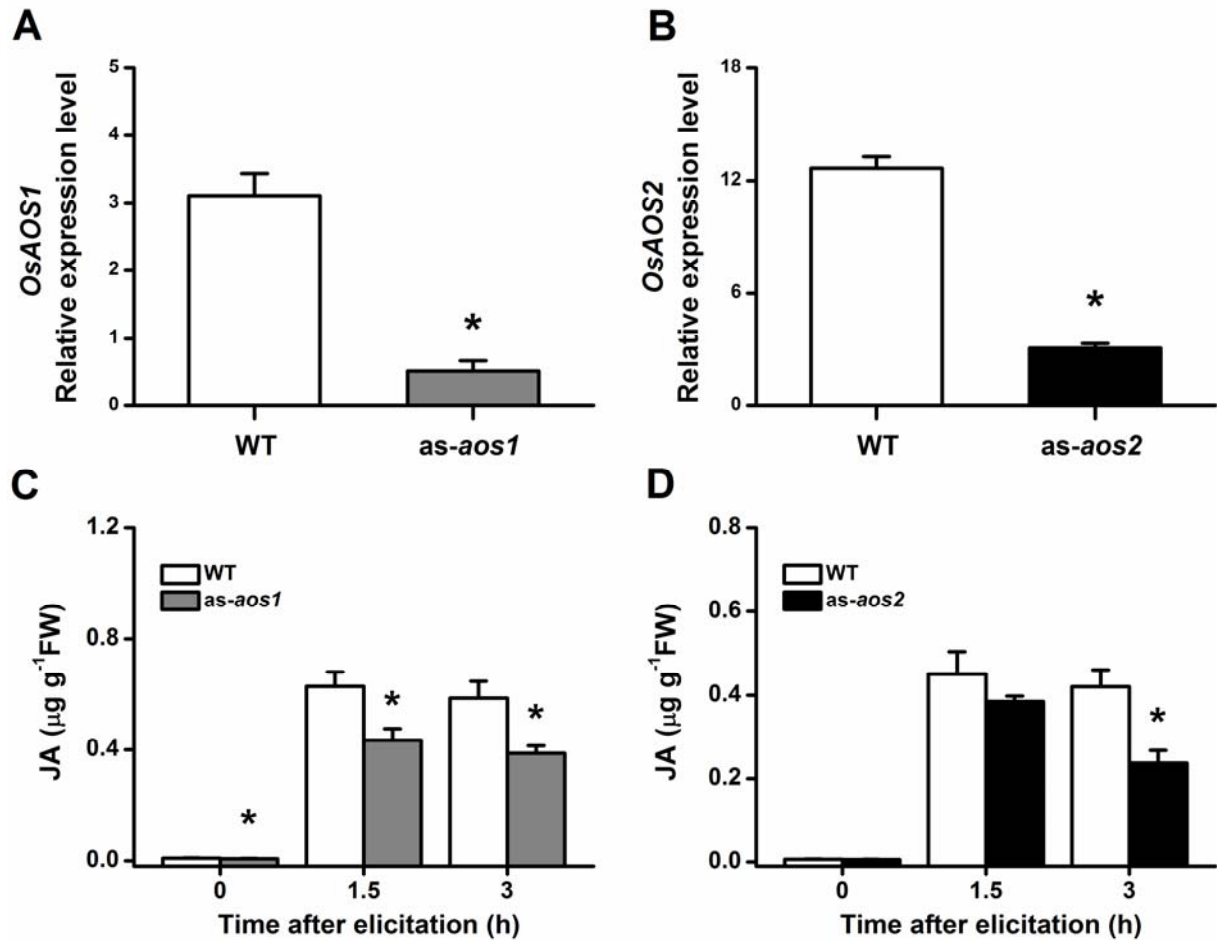


Supplemental Figure S9. The activity of OsMPK3 and OsMPK6 in *ir-wrky*, *oe-WRKY* lines and WT plants.

A, SSB-elicited MPK activation in *ir-wrky*, *oe-WRKY* lines and WT plants. *Ir-wrky*, *oe-WRKY* lines, and WT plants were treated with or without SSB larva, and stems from five replicate plants were harvested at the indicated times. Immunoblotting was performed using either α -pTEpY antibody (upper panel) to detect phosphorylated MPKs, or actin antibody (lower panel) as a loading control.

B and C, The relative activity of OsMPK3 and OsMPK6 based on the signal intensity in A. The activity for the WT sample at 0 min was set to 1. The relative activity of all other samples at each time point was expressed relative to the WT sample at 0 min.

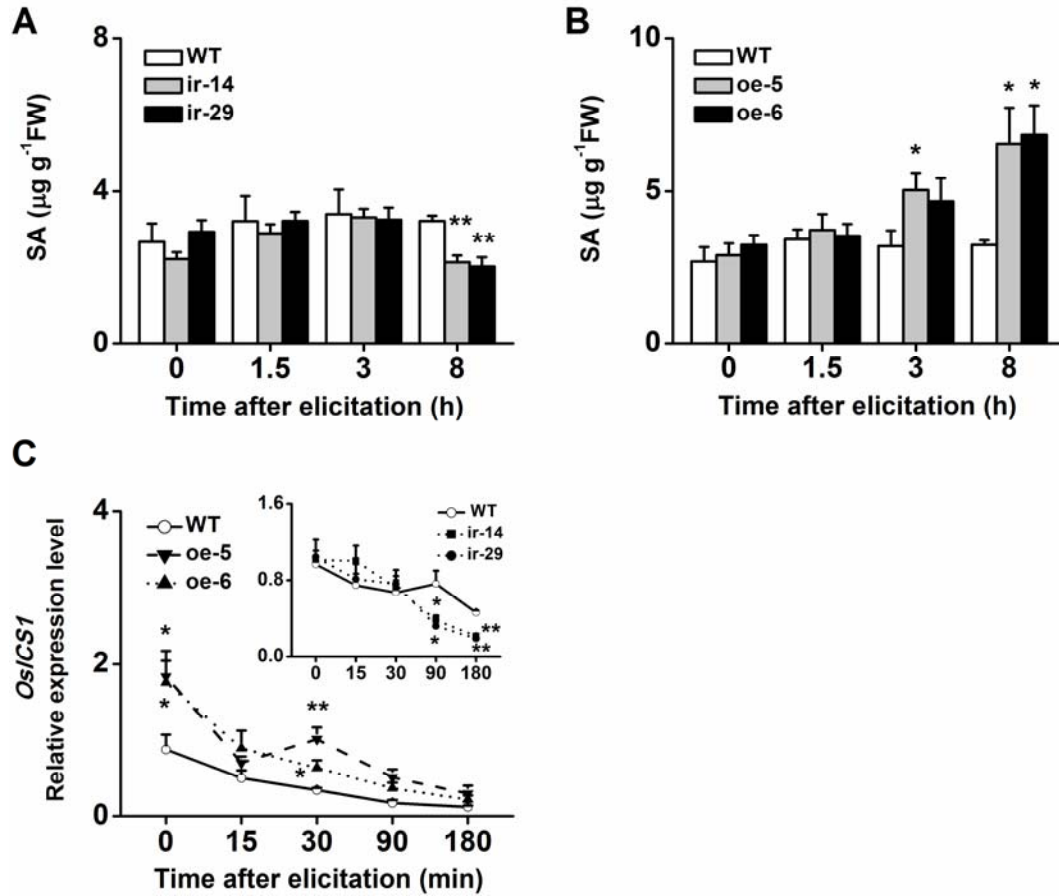
D, The relative amount of the loading OsActin based on the signal intensity in A. The quantity for the WT sample at 0 min was set to 1. The relative amount of all other samples at each time point were expressed relative to the WT sample at 0 min.



Supplemental Figure S10. *OsAOS1* and *OsAOS2* mediate herbivore-induced jasmonic acid (JA) biosynthesis in rice.

A and B, Mean transcriptional levels (+SE, $n = 5$) of *OsAOS1* (A) and *OsAOS2* (B) in anti-sense (*as-aos1* (A) and *as-aos2* (B) lines, and WT plants that were individually infested by a third-instar striped stem borer (SSB) larva for 1h. Transcript levels were analyzed by qRT-PCR.

C and D, Mean levels (+SE, $n = 5$) of JA in *as-aos1* (C) and *as-aos2* (D) lines and WT plants that were individually infested by a third-instar SSB larva. Asterisks indicate significant differences in *as-aos1* and *as-aos2* lines compared to WT plants (*, $P < 0.05$; **, $P < 0.01$, Student's t-tests).

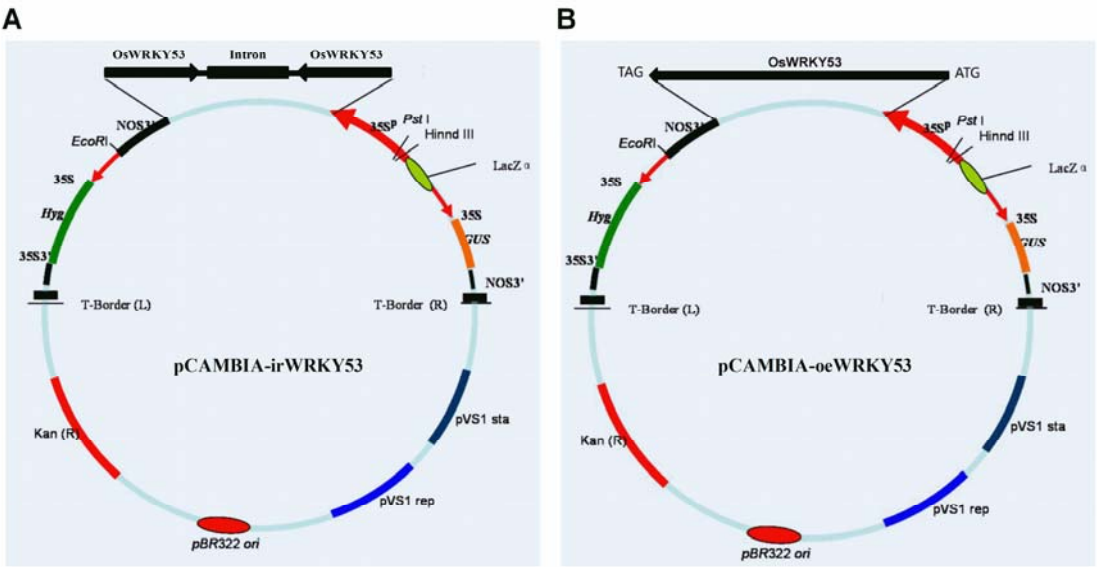


Supplemental Figure S11. OsWRKY53 mediates salicylic acid (SA) accumulation in rice after infestation with the striped stem borer (SSB)

A and B, Mean levels (+SE, $n = 5$) of SA in *ir-wrky* (A) and *oe-WRKY* (B) lines, and wild-type (WT) plants that were individually infested by a third-instar SSB larva.

C, Mean transcript levels (+SE, $n = 5$) of *OsICS1* in *ir-wrky* and *oe-WRKY* lines, and WT plants that were individually infested by a third-instar SSB larva. Asterisks indicate significant differences in *ir-wrky* and *oe-WRKY* lines compared with WT plants (* $P < 0.05$, **, $P < 0.01$, Student's t-tests).

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261 **Supplemental Figure S12.** Transformation vectors were used in this study.

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277 **Supplemental Table S1.** Primers and probes used for qRT-PCR of target genes.

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Gene name	TIGR ID	Forward primer (5'---3')	Reverse primer (5'---3')	Probe (5'---3')
<i>OsWRKY53</i>	Os05g27730	AACGGCTGCTCCATGAAGAA	TTGTGTGCGCCCTTGTAGAC	CTCGCCGACGGCCGCATC
<i>OsMEK4</i>	Os2g54600	ATGGTTGGAGCAAGCAAATCT	TCATTGATCATTCTCCCCTC	TTTCCTCCTCCTCTTCCTGAATCCTG
<i>OsMPK3</i>	Os03g17700	CGACTTCGAGCAGAAGGCTCTA	GTTTCATCTCGATCGCTTCGTT	ACGAGGACCAAATGAAGCAGCTGAT
<i>OsMPK6</i>	Os06g06090	CGCACGCTCAGGGAGATC	GGTATGATATCCCTTATGGCAACAA	CTCCGCCACATGGACCACGAGAA
<i>OsHI-LOX</i>	Os08g39840	CCGAGCTTGACGCGAAGA	GATCGTCGTCGTCCACATTGT	CGGGAAGGAGAAAGCAACTGTGCG
<i>OsAOS1</i>	Os03g55800	CGAGCTCTTCCTCCGATACG	GTCAGAAGGTGGCCTTCTTGAG	ACCTCCACGCTCGGCTCATCTGTC
<i>OsAOS2</i>	Os03g12500	TGCCCATGATCATCGAGGAT	TGTAGTCGGAGCTGATGAGGAA	CTCCTCCACACGCTGCCGCTG
<i>OsACS2</i>	Os04g48850	CACCCCGAGGCATCCAT	ATTGGCGATCCTCTTGAACTG	TGCACACCGGAGGGCGTCT
<i>OsACTIN</i>	Os03g50885	TGGACAGGTTATCACCATTGGT	CCGCAGCTTCCATTCCCTATG	CGTTTCCGCTGCCCTGAGGTCC
<i>OsWRKY70</i>	Os05g39720	CCGCTGCTGTTTTGATCATCT	GGAGCTAAGCTAACTCACTCCACA	ATCGGGCCGTCAATTTGATCAGCA
<i>OsWRKY45</i>	Os05g25770	CGACCGGAAGGCCAACT	GAAGTAGGCCTTTGGGTGCTT	ACGCAGCAATCGTCCGGGAATTC
<i>OsWRKY35</i>	Os04g39570	CCACTGGGAAGCTGCACAT	TGAGGATGCGAGATTCAGAGG	TCCAATCAGATTTGAATCCCCTCGG
<i>OsWRKY33</i>	Os03g33012	AGGCAAGCACAGCCATGAC	GAAGACGATACGTTGGCATTAGC	CCAGCAGCCAGGAACAGTAGCCATTC
<i>OsWRKY30</i>	Os08g38990	AACAGTGGCCACCCAAGCT	GTTTCAGGTCTCCGGTGAAGAAG	AGGCTCAGCCGCTGCACCACA
<i>OsWRKY24</i>	Os01g61080	AAGAGATGGAGGAAAGACGGTG	TGTCGATGTCGCTCATGGTT	AGGGGATCTCCATGGCTGGCAA
<i>OsWRKY13</i>	Os01g54600	GCGCAAGTACGGCCAGAA	CCTTGAGCTACTGCACCTGTA	CCCATCAAGGGCTCTCCCTACCCA
<i>OsICS1</i>	Os09g19734	ACCAATTATGTTTCCGATCAATCA	CGTCGCCTTCTTGGATTTATG	ACACCTCCTACATACGCAGCCGCTGA

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Supplemental Table S2. Accession numbers of the amino acid sequences included in the phylogenetic tree.

Sequence data in the phylogenetic tree can be found in the GenBank/EMBL data libraries under accession numbers: [AtWRKY1 \(NP_178565.1\)](#); [AtWRKY2 \(NP_200438.1\)](#); [AtWRKY3 \(NP_178433.1\)](#); [AtWRKY4 \(NP_172849.1\)](#); [AtWRKY25 \(NP_180584.1\)](#); [AtWRKY26 \(NP_196327.1\)](#); [AtWRKY33 \(NP_181381.2\)](#); [AtWRKY34 \(NP_194374.1\)](#); [CaWRKY2 \(ABD65255.1\)](#); [CaWRKY58 \(AAW67002.1\)](#); [NaWRKY3 \(AAS13439.1\)](#); [NaWRKY6 \(AAS13440.1\)](#); [NbWRKY8 \(BAI63296.1\)](#); [OsWRKY4 \(DAA05069.1\)](#); [OsWRKY24 \(AAT84156.1\)](#); [OsWRKY30 \(ADK13079.1\)](#); [OsWRKY35 \(DAA05100.1\)](#); [OsWRKY53 \(NP_001055252.1\)](#); [OsWRKY70 \(ABC02812.1\)](#); [OsWRKY78 \(DAA05640.1\)](#); [TaWRKY53-a \(ABN43178.1\)](#); [TaWRKY53-b \(ABN43185.1\)](#); [TaWRKY44 \(ALC04261.1\)](#); [ZmWRKY33 \(NP_001147551.1\)](#); [HbWRKY1 \(AEE81757.1\)](#); [GhWRKY44 \(AJA30422.1\)](#); [GhWRKY3 \(ADI52618.1\)](#); [SUSIBA2 \(AAQ63880.1\)](#).