



Genome-Wide Expression Profiling of *OsWRKY* Superfamily Genes during Infection with *Xanthomonas oryzae* pv. *oryzae* Using Real-Time PCR

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WRKY transcription factors (TFs) are involved in regulating a range of biological processes such as growth, development, and the responses to biotic and abiotic stresses. Genome-wide expression profiling of *OsWRKY* TF superfamily genes in rice after infection with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was performed to elucidate the function of *OsWRKY* TFs in the interaction between rice and *Xoo*. Of the 111 *OsWRKY* TF genes tested, the transcription of 94 genes changed after *Xoo* infection. The *OsWRKY* TF genes were classified into eight types according to their expression profiles. Eighty-two genes in Groups I, II, III, IV, VII were up-regulated after exposure to a compatible or an incompatible race of *Xoo*. Examination of salicylic acid (SA)-deficient rice lines revealed that SA was involved in Xa1-mediated resistance to *Xoo* infection. *OsWRKY* TF genes involved in Xa1-mediated resistance were classified according to their SA-dependent or -independent expression. In SA-deficient rice, the expression of 12 of 57 *OsWRKY* TF genes involved in Xa1-mediated resistance was compromised. Of these six *OsWRKY* TF genes were induced by SA. *OsWRKY88*, an example of a gene possibly involved in SA-dependent Xa1-mediated resistance, activated defense related genes and increased resistance to *Xoo*. Thus, expression profiling of *OsWRKY* TF genes may help predict the functions of *OsWRKY* TF genes involved in Xa1-mediated resistance.

Keywords: *OsWRKY* transcription factors superfamily, expression profiling, *Xanthomonas oryzae* pv. *oryzae*

INTRODUCTION

WRKY transcription factors (TFs) are characterized by the presence of one or two 60-amino-acid WRKY motifs, a very highly conserved WRKYGQK sequence, and zinc finger-like motifs [Cys(2)-His(2) or Cys(2)-HisCys] that bind to the W-box [TTGAC(C/T)] cis-element in target gene promoters (Sun et al., 2003; Cai et al., 2008; Ciolkowski et al., 2008; Pandey and Somssich, 2009). WRKY TFs are classified into three groups (Groups I–III) according to the similarity of their WRKY motifs (Zhang and Wang, 2005). WRKY TF families in *Arabidopsis* and rice contain 72 and 125 members, respectively (Eulgem et al., 2000; Rice WRKY Working Group, 2012).

Numerous WRKY TFs are involved in responding to biotic stresses in rice and *Arabidopsis* (Dong et al., 2003; Ryu et al., 2006; Berri et al., 2009). Several studies described expression

profiling of OsWRKY TF genes upon infection with pathogens such as *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in rice (Ryu et al., 2006; Bagnaresi et al., 2012; Wei et al., 2013). The first such study examined the expression of 45 OsWRKY TF genes in response to *M. oryzae* challenge and found that 15 OsWRKYs were up-regulated upon infection (Ryu et al., 2006). Later research with a custom OsWRKYARRAY found that 18 of 104 OsWRKY TF genes were up-regulated upon *M. oryzae* infection (Berri et al., 2009). A recent transcriptome study in rice noted that OsWRKY TF genes were significantly enriched in the group of genes up-regulated during the early defense response to *M. oryzae* infection and that OsWRKY47 played a critical role in rice blast resistance (Wei et al., 2013).

To date, relatively few studies have examined OsWRKY TF superfamily gene expression upon infection with *Xoo*, but several studies have examined individual OsWRKY TF genes in *Xoo*-mediated resistance. One study reported that 9 of 45 OsWRKY genes, namely, OsWRKY7, 10, 11, 30, 32, 67, 70, 83 [renamed 94 by the Committee on Gene Symbolization, Nomenclature, and Linkage (CGSNL)], and 85 (renamed 96 by CGSNL) were up-regulated upon infection with *Xoo* (Ryu et al., 2006). The expression profiles of some OsWRKY genes were summarized by Jimmy and Babu (Jimmy and Babu, 2015). Other studies observed up-regulation of OsWRKY6, 12, 13, 30, 45-1, 71, and 76 in response to *Xoo* infection (Liu et al., 2005, 2007; Chujo et al., 2008; Qiu and Yu, 2009; Tao et al., 2009; Hwang et al., 2011; Lee et al., 2013; Choi et al., 2015). However, no comprehensive genome-wide expression data are available for all 125 known OsWRKY superfamily genes.

SA has been shown to play a key role in plant immunity (Delaney et al., 1994; Jimmy and Babu, 2015). Many WRKY genes from a number of plants have also been shown to be induced by SA (Dong et al., 2003; Ryu et al., 2006). Transgenic plants expressing the bacterial *NahG* gene encoding a salicylate hydroxylase that converts SA to catechol are often used to evaluate the role of SA in plant immunity (Delaney et al., 1994; Yang et al., 2004). Pathogen mediated WRKY gene expression has also been examined in *NahG Arabidopsis* plants (Dong et al., 2003). To date there is no report on SA effect using *NahG* transgenic rice plants in pathogen mediated OsWRKY gene expression yet.

Nine resistance (R) genes for *Xoo*, namely, *Xa1*, *Xa3/26*, *xa5*, *Xa10*, *xa13*, *Xa21*, *Xa23*, *xa25*, and *Xa27*, were cloned previously (Song et al., 1995; Yoshimura et al., 1998; Iyer and McCouch, 2004; Sun et al., 2004; Gu et al., 2005; Chu et al., 2006; Liu et al., 2011; Tian et al., 2014; Wang C. et al., 2015). Most proteins encoded by plant disease R genes share common structural features such as nucleotide-binding site leucine-rich repeat (NBS-LRR) regions. Of the nine *Xoo* R genes cloned to date, only *Xa1* encodes a typical NBS-LRR-type protein. The mechanisms underpinning *Xa1*-mediated resistance remain largely unknown. In this study, genome-wide expression profiling of OsWRKY superfamily genes was used to investigate *Xa1*-mediated defense mechanisms and the function of OsWRKY88, an example of a gene possibly involved in SA-dependent *Xa1*-mediated resistance, was analyzed.

MATERIALS AND METHODS

Plant Material and Pathogen Treatment

Rice seedlings (*Oryza sativa* L. Japonica cv. Ilmi) carrying the *Xa1* R gene were grown in a greenhouse at 28°C for 3 weeks. Seedlings were inoculated with compatible (KACC10859) or incompatible (KXO42) *Xoo* strains grown on PSA medium (10 g/L peptone, 10 g/L sucrose, 1 g/L sodium glutamate, and 15 g/L agar) for 2 days until OD₆₀₀ = 0.5 (Kim et al., 2013). Leaves were harvested at 0, 6, 12, and 24 hpi. Seedlings were treated with 1 mM SA, and leaves were harvested at 0, 12, 24 h after treatment (hat). All samples were frozen in liquid nitrogen immediately after harvesting and stored at -80°C until used.

Quantitative RT-PCR

The 125 OsWRKY TF superfamily genes were reported by CGSNL (Rice WRKY Working Group., 2012; Table S1). Among them we could not obtain the data for the 14 OsWRKY TFs in this study due to various reasons. Primers for qRT-PCR were designed using the rice genome annotation database (<http://rice.plantbiology.msu.edu>; Tables S1, S2). Nucleotide sequences for OsWRKY44 and OsWRKY59 were not found in the database. Some OsWRKY TFs are duplicated genes: OsWRKY46 (91), 61 (103), 4 (122). NCBI primer-blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) was used to optimize the design of specific primers for each OsWRKY TF gene. For nine OsWRKY TFs (OsWRKY24, 33, 35, 57, 61, 92, 99, 116, 124) we could not optimize the condition to get PCR products. Specific qRT-PCR primers for OsWRKY TF genes are listed in Table S1.

Total RNA was extracted from harvested leaves using Trizol reagent (Invitrogen, USA). M-MLV reverse transcriptase (Promega, USA) was used to synthesize cDNA from total RNA (2 µg). qRT-PCR was conducted as described by Kim et al. (2013). OsActin was used as a calibration control to determine OsWRKY gene expression levels (Schmittgen and Livak, 2008). The 2^{-ΔΔct} method was used for analysis of relative gene expression. Three biological replicates were performed for each qRT-PCR analysis. Above 2-fold difference at each time point in each expression profile was considered as either increase or decrease of OsWRKY expression.

Generation of Transgenic Rice Plants

Gateway cloning using pB2GW7 (Karimi et al., 2002) was used to generate constructs 35S::NahG from *Arabidopsis NahG* plants and 35S::OsWRKY88. *Agrobacterium tumefaciens* LBA4404 was transformed with the 35S::NahG construct and the 35S::OsWRKY88 construct. *A. tumefaciens* carrying both constructs was used to transform rice embryogenic calli (Kim et al., 2009). Transgenic rice T₀ plants were selectively screened using 0.1% BastaTM spray. Integration of the construct was confirmed by PCR of genomic DNA with bar-specific primer sets (Table S2). Homozygous *NahG* transgenic rice plants were confirmed by 0.1% BastaTM spray through several generations and used for disease assays and qRT-PCRs. In the case of OsWRKY88 ox lines we used T2 lines selected by 0.1% BastaTM spray.

Plant Disease Assays

Xoo KXO42 and KACC10859 bacteria were grown overnight at 28°C in PSA medium and resuspended at 10⁸ cells/mL in 10 mM MgCl₂. The scissors-dip method (Kauffman et al., 1973) was used to challenge WT and ten SA-deficient rice plants with *Xoo* (KXO42) and WT and ten OsWRKY88 ox lines (T₂) with *Xoo* (KACC10859). Disease was assessed as the average lesion length in leaves of individual plants 14 days post-inoculation (dpi). Bacterial growth was assessed at 0, 7, and 14 dpi (Shimono et al., 2007).

Promoter Transient Expression Assays

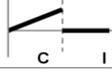
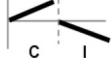
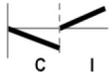
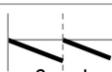
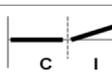
A 2 kb region of the *CHITINASE 2* (*CHIT2*; Os04g41620) promoter was amplified by PCR using promoter-specific primers (Table S2) and introduced into an entry vector, pENTR/d-TOPO (Invitrogen, Carlsbad, CA, USA). The *pCHIT2::GFP-GUS* construct was made an LR reaction between the entry clones containing the promoter and the destination vector pBGWFS7 (Karimi et al., 2002). Transient expression assays in *Nicotiana benthamiana* were performed using the protocol reported previously (Li, 2011). *N. benthamiana* was infiltrated with an *Agrobacterium* carrying *pCHIT2::GFP-GUS* and *pPR10a::GFP-GUS* (Hwang et al., 2008) constructs alone, or with a mixture of *Agrobacterium* carrying *35S::OsWRKY88* and either *pCHIT2::GFP-GUS* or *pPR10a::GFP-GUS*. Infiltrated leaves were collected 2 days post-infiltration, and promoter activities in each sample were visualized using β-glucuronidase (GUS) activity assay and staining.

RESULTS

Expression Profiles of OsWRKY TF Superfamily Genes during *Xoo* Infection Were Analyzed

The roles of *OsWRKY* TF superfamily genes in the defense pathway mediated by Xa1, a bacterial blight resistance protein (Yoshimura et al., 1998), were investigated using expression profiling. *Oryza sativa* L spp. *japonica* cv. Ilni, which carried the *Xa1* gene, was profiled after challenge with an incompatible (KXO42) or a compatible (KACC10859) race of *Xoo*. *OsWRKY* TF superfamily genes used in this study were named according to recently re-defined nomenclature (Rice WRKY Working Group., 2012; Table S1). Expression profiles of *OsWRKY* TF superfamily genes were shown in eight different types and the results were summarized in **Table 1** (Types I–VIII). The expression profile of a representative *OsWRKY* TF in each type was shown in **Figure 1** and the rest of them were shown in Figure S1. Most of the *OsWRKY* TFs (57 genes) were up-regulated in response to both the compatible and incompatible *Xoo* races (Type I). Of these, *OsWRKY58* transcription was particularly elevated upon challenge with *Xoo* (**Figure 1A**). *OsWRKY* TFs (*OsWRKY107*, *118*) showed Type II pattern that were up-regulated only in response to the compatible *Xoo* race (**Table 1**). Of these, transcription of *OsWRKY118* was noticeably elevated 6 hpi (**Figure 1B**). Fourteen *OsWRKY* TFs

TABLE 1 | Expression profiles of *OsWRKY* TF genes in response to *Xoo* infection were summarized.

Types	Expression profile	OsWRKY TFs
I		<i>OsWRKY1</i> , 2, 3, 6, 7, 8, 9, 10, 11, 12, 13, 16, 18, 22, 23, 25, 26, 29, 30, 36, 38, 40, 42, 43, 45, 47, 49, 50, 51, 52, 54, 55, 58, 63, 64, 65, 66, 69, 70, 71, 76, 80, 81, 84, 87, 88, 90, 94, 96, 105, 106, 108, 112, 113, 114, 115, 123
II		<i>OsWRKY107</i> , <i>118</i>
III		<i>OsWRKY4</i> , 5, 14, 19, 28, 48, 78, 79, 82, 85, 86, 98, 101, 121
IV		<i>OsWRKY56</i> , 74, 77, 110, 119, 120
V		<i>OsWRKY72</i> , <i>117</i>
VI		<i>OsWRKY15</i> , 17, 20, 21, 27, 31, 34, 37, 39, 41, 54, 56, 62, 67, 68, 73, 75, 83, 93, 95, 104, 109
VII		<i>OsWRKY32</i> , 46, 60
VIII		<i>OsWRKY53</i> , 72, 111, 102, 125

No data were generated for the following *OsWRKY* TF genes.

1. Nucleotide sequences for *OsWRKY44* and *OsWRKY59* were not found.

2. Duplicated genes: *OsWRKY91* (46), 103 (61), 122 (4).

3. No PCR products were obtained: *OsWRKY24*, 33, 35, 57, 61, 92, 99, 116, 124.

(*OsWRKY4*, 5, 14, 19, 28, 48, 78, 79, 82, 85, 86, 98, 101, 121) showed Type III pattern that up-regulated in response to the compatible *Xoo* race and down-regulated in response to the incompatible *Xoo* race. Of these transcription of *OsWRKY4* was noticeably elevated 6 hpi (**Figure 1C**). Six *OsWRKY* TFs (*OsWRKY74*, 77, 110, 119, 120) showed Type IV pattern that were down-regulated in response to the compatible *Xoo* race and up-regulated in response to the incompatible *Xoo* race. Of these *OsWRKY110* was decreased at 3 hpi upon the infection of the compatible *Xoo* race and increased at 12 hpi upon the infection of the incompatible *Xoo* race (**Figure 1D**). Two *OsWRKY* TFs showed Type V pattern that were down regulated only upon infection of the compatible *Xoo* race. Of these *OsWRKY117* was decreased at 6 hpi (**Figure 1E**). Twenty two *OsWRKY* TFs showed Type VI pattern that was down regulated both upon infection of the compatible and incompatible *Xoo* race. Of these, *OsWRKY27* was dramatically down-regulated 6 hpi with both the compatible and incompatible interactions (**Figure 1F**). Type VII contained three *OsWRKY* TFs (*OsWRKY32*, 46, 60) that were up-regulated only in response

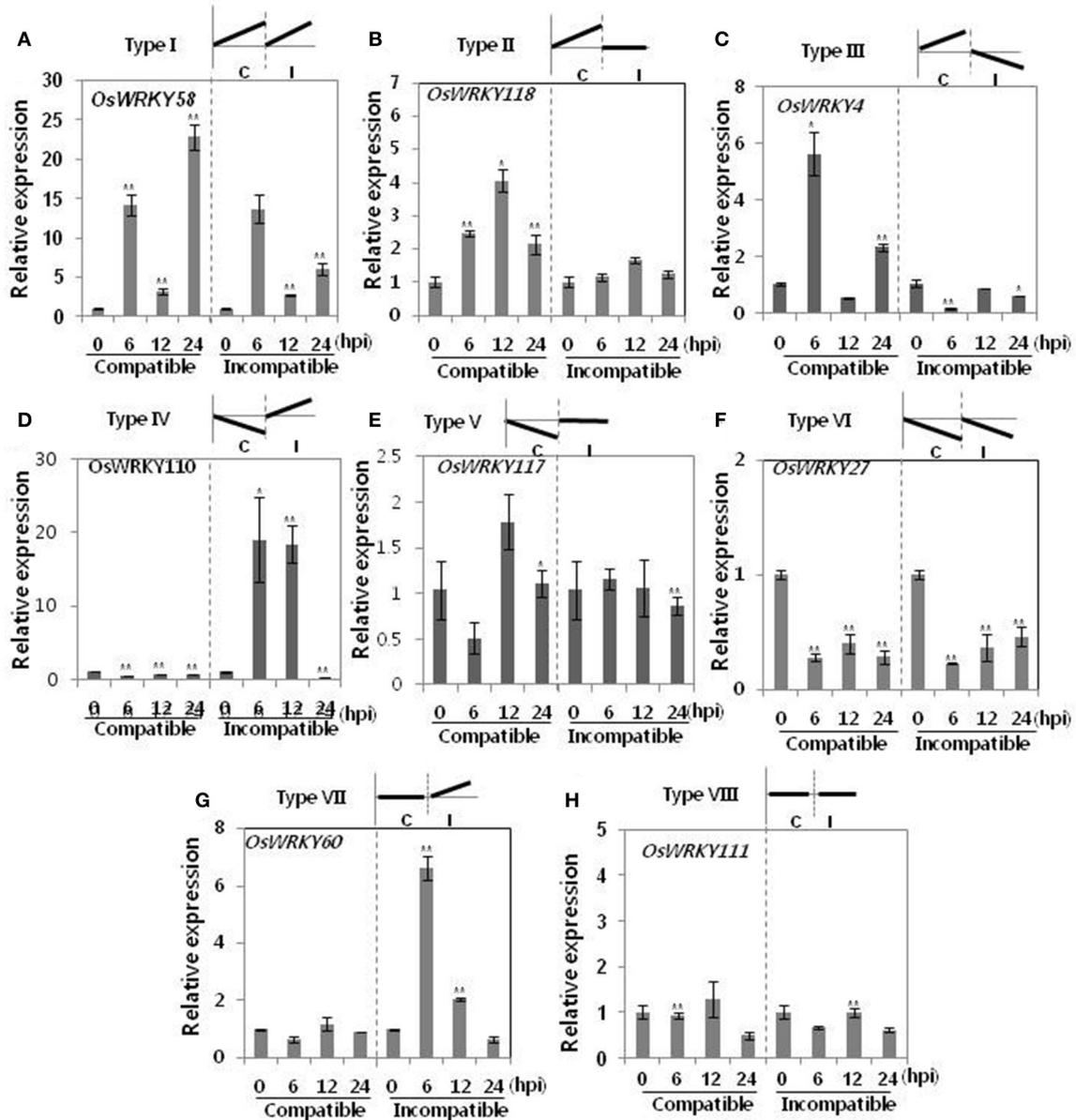


FIGURE 1 | Expression profiling of *OsWRKY* TF genes upon infection with compatible and incompatible races of *Xoo*. **(A)** Type I: Genes up-regulated after challenge with both compatible and incompatible *Xoo* races. **(B)** Type II: Genes up-regulated only after challenge with a compatible *Xoo* race. **(C)** Type III: Genes up-regulated after challenge with a compatible *Xoo* race and down-regulated by infection of incompatible *Xoo* race. **(D)** Type IV: Genes down-regulated after challenge with a compatible *Xoo* race and up-regulated by infection of incompatible *Xoo* race. **(E)** Type V: Genes down-regulated only after challenge with an incompatible *Xoo* race. **(F)** Type VI: Genes up-regulated after challenge with both compatible and incompatible *Xoo* races. **(G)** Type VII: Genes up-regulated only after challenge with an incompatible *Xoo* race. **(H)** Type VIII: Genes exhibiting no expression response upon challenge with either a compatible or an incompatible *Xoo* race. Asterisks indicate significant differences (** $P < 0.01$; * $P < 0.05$).

to only the incompatible *Xoo* race. Of these, the *OsWRKY60* transcript reached maximum levels 6 h post-inoculation (hpi; **Figure 1G**). Finally, Type VIII contained five *OsWRKY* TFs (*OsWRKY53*, 72, 102, 111, 125) whose transcription profiles were unaffected by *Xoo* infection (**Figure 1H**). For the reasons described in the Materials and Methods section of qRT-PCR and **Table 1**, no expression profile data were obtained for 14 *OsWRKY*

genes (*OsWRKY24*, 33, 35, 44, 57, 59, 61, 91, 92, 99, 103, 116, 122, 124).

Salicylic Acid Plays a Role in Xa1-Mediated Resistance

Transcription of 66 *OsWRKY* TF genes (Groups I, IV, and VII) was up-regulated during the incompatible interaction of

rice cultivar Ilmi carrying Xa1 with *Xoo*, suggesting that many OsWRKY TFs could be involved in the Xa1-mediated defense pathway. Salicylic acid (SA) has a role in plant defense response, and SA-deficient transgenic rice plants were therefore used to investigate the involvement of SA in Xa1-mediated defense. Bacterial salicylate hydroxylase, which is encoded by the *NahG* gene, degrades SA to catechol. SA-deficient transgenic rice plants were generated by introduction of the *NahG* gene into rice via *Agrobacterium*-mediated transformation. Nineteen independent *NahG*-expressing transgenic lines were produced. Lines #15 and #19 were selected for further analysis based on their *NahG* transcript levels (data not shown). Transgenic lines challenged with incompatible *Xoo* race KXO42 exhibited longer lesions (~13 cm) than wild-type (WT) Ilmi plants (~0.2 cm; **Figures 2A,B**), indicating that Xa1-mediated resistance was compromised in the transgenic plants. Bacterial populations in the two SA-deficient lines were approximately higher than in the WT at 3, 7, and 11 dpi, respectively (**Figure 2B**). Activation of the Xa1-mediated defense pathway in the SA-deficient lines was examined using qRT-PCR transcriptional analysis of the genes encoding *NHI/OsNPR1* and *chitinase 2 (CHIT2)*. Compared with the WT, induction of both genes was partially compromised in line #15 and severely compromised in line #19 (**Figure 2C**). These observations correlated with lesion length and bacterial growth data. On the other hand, some studies claim that catechol results in susceptibility to *Magnaporthe grisea* but not to *Striga hermonthica* (Yang et al., 2004; Mutuku et al., 2015). We therefore examined the effect of catechol on *Xoo* infection (**Figure S1**), which revealed it had no effect on compatible or incompatible *Xoo*-rice interactions. Thus, impaired defense in SA-deficient lines is not due to catechol production. Overall, these results suggest that SA plays a role in Xa1-mediated resistance.

SA-Dependent Transcriptional Induction of OsWRKY TFs Up-regulated in the Xa1-Mediated Defense Pathway

66 OsWRKY TF genes (Groups I, IV, and VII) were up-regulated after challenge with the incompatible *Xoo* race (**Table 1**). Type I expression pattern is further divided into Type Ia and Ib. OsWRKY TF genes in Type Ia exhibited more pronounced up-regulation (either a faster response or a more abundant response) with the incompatible interaction than with the compatible interaction, whereas OsWRKY TF genes in Type Ib exhibited a more pronounced effect with the compatible interaction than with the incompatible interaction. OsWRKY TF genes in Type Ia (**Table 2**) exhibited expression profiles typical of defense response genes, and this group was therefore used to further investigate SA-dependent Xa1-mediated resistance. Expression of OsWRKY TF genes in Type Ia was investigated in SA-deficient rice plants using qRT-PCR (**Figure 3**). Gene expression during the incompatible interaction was not compromised in the SA-deficient rice lines for the majority of Type Ia OsWRKY TF genes (34 genes). However, induction of *OsWRKY9*, *11*, *22*, *23*, *47*, *58*, *60*, *64*, *88*, *106*, *113*, and *114* expression during the incompatible interaction was reduced in the two SA-deficient rice lines. This suggests that *OsWRKY9*, *11*, *22*, *23*, *47*, *58*, *60*, *64*, *88*, *106*, *113*, and *114* were induced in a SA-dependent manner as part of the

Xa1-mediated defense pathway but that other OsWRKY TF genes in Type Ia were induced as part of the Xa1 mediated response in a SA-independent manner.

To investigate whether these OsWRKY TFs are induced by SA we performed qRT-PCR (**Figure 4**). Among 12 OsWRKY TFs *OsWRKY11*, *23*, *47*, *58*, *60*, *88* were induced by SA. However, *OsWRKY9*, *22*, *64*, *106*, *113*, *114* were not induced by SA. Taken together the six OsWRKY TFs appears to play roles in Xa1 mediated resistance.

Ectopic Expression of OsWRKY88, a Type I OsWRKY TF That Enhanced Resistance to Xoo

We showed that six OsWRKY TFs were induced as part of SA-dependent Xa1-mediated resistance. Among them the transgenic plants overexpressing *OsWRKY88* were generated and analyzed. We found that *OsWRKY88* transcript levels were higher in #22 lines than that in #14 lines (**Figure 5A**). *OsWRKY88* ox lines were challenged with the compatible *Xoo* strain KACC10859 by the leaf clip method. After challenge, *OsWRKY88* ox lines #14 and #22 exhibited shorter lesion lengths (~4 cm) than those (~12 cm) of WT plants (**Figures 5B,C**), suggesting that *OsWRKY88* plays a role in *Xoo* resistance. We also performed qRT-PCR of defense related genes such as *CHIT2* and *OsPR10a*. Unlike in WT plants, *CHIT2* and *OsPR10a* were constitutively expressed in *OsWRKY88* ox lines #14 and #22 (**Figures 5D,E**), indicating that *OsWRKY88* positively regulates the defense response to *Xoo*.

OsWRKY88 Directly Regulates the OsPR10a Promoter But Not the CHIT2 Promoter

Defense related genes *OsPR10a* and *CHIT2* were expressed at a high level in *OsWRKY88* ox lines. To examine whether *OsWRKY88* directly regulates *OsPR10a* and *CHIT2*, we examined its effect on transcription from the *OsPR10a* and *CHIT2* gene promoters using the promoter transient assay (**Figure 6**). *Agrobacterium*-mediated transient assays for *pOsPR10a::GFP-GUS* (Hwang et al., 2008) were performed in *N. benthamiana* leaves (**Figures 6A,C**). The trans-activation activity of *OsWRKY88* at the *OsPR10a* promoter was assessed using GUS staining and GUS enzyme activity. GUS activity was stronger in leaves co-infiltrated with *pOsPR10a::GFP-GUS* and *35S::OsWRKY88* than in leaves infiltrated with either *pOsPR10a::GFP-GUS* or *35S::OsWRKY88*. We also performed a *CHIT2* promoter transient assay (**Figures 6B,D**), which revealed no significant difference in GUS staining and GUS enzyme activity between the sample co-infiltrated with a mixture of *pCHIT2::GFP-GUS* and *35S::OsWRKY88* and infiltrated with either *pCHIT2::GFP-GUS* or *35S::OsWRKY88*. These results suggest that *OsWRKY88* trans-activates the *OsPR10a* promoter but not the *CHIT2* promoter.

DISCUSSION

Plants have developed unique immune systems for defense against a range of stresses. WRKY TFs have roles in biological processes such as growth, development, and responses to abiotic

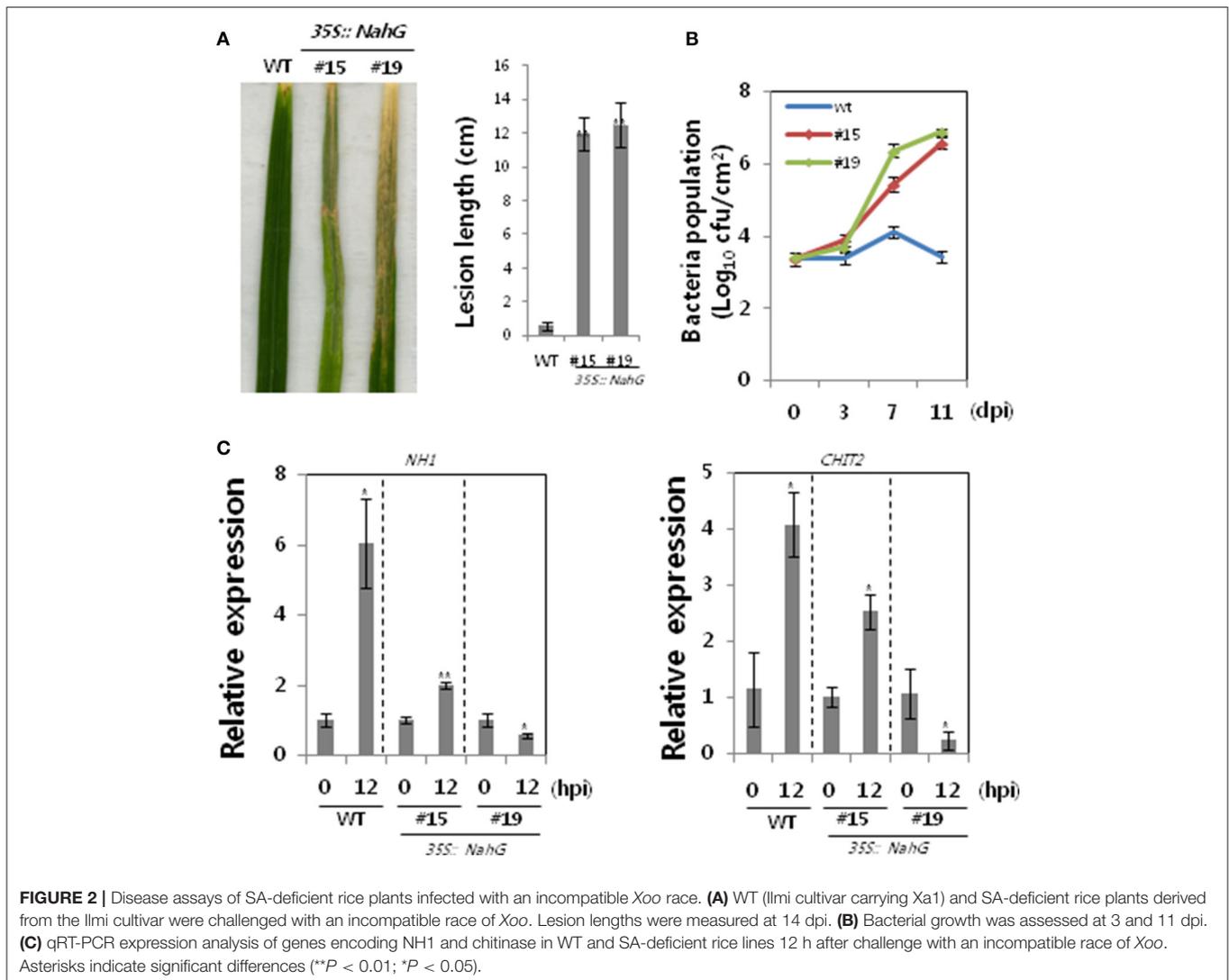


TABLE 2 | Expression of *OsWRKY* TF genes in SA-deficient rice after challenge with an incompatible race of *Xoo* assessed by qRT-PCR.

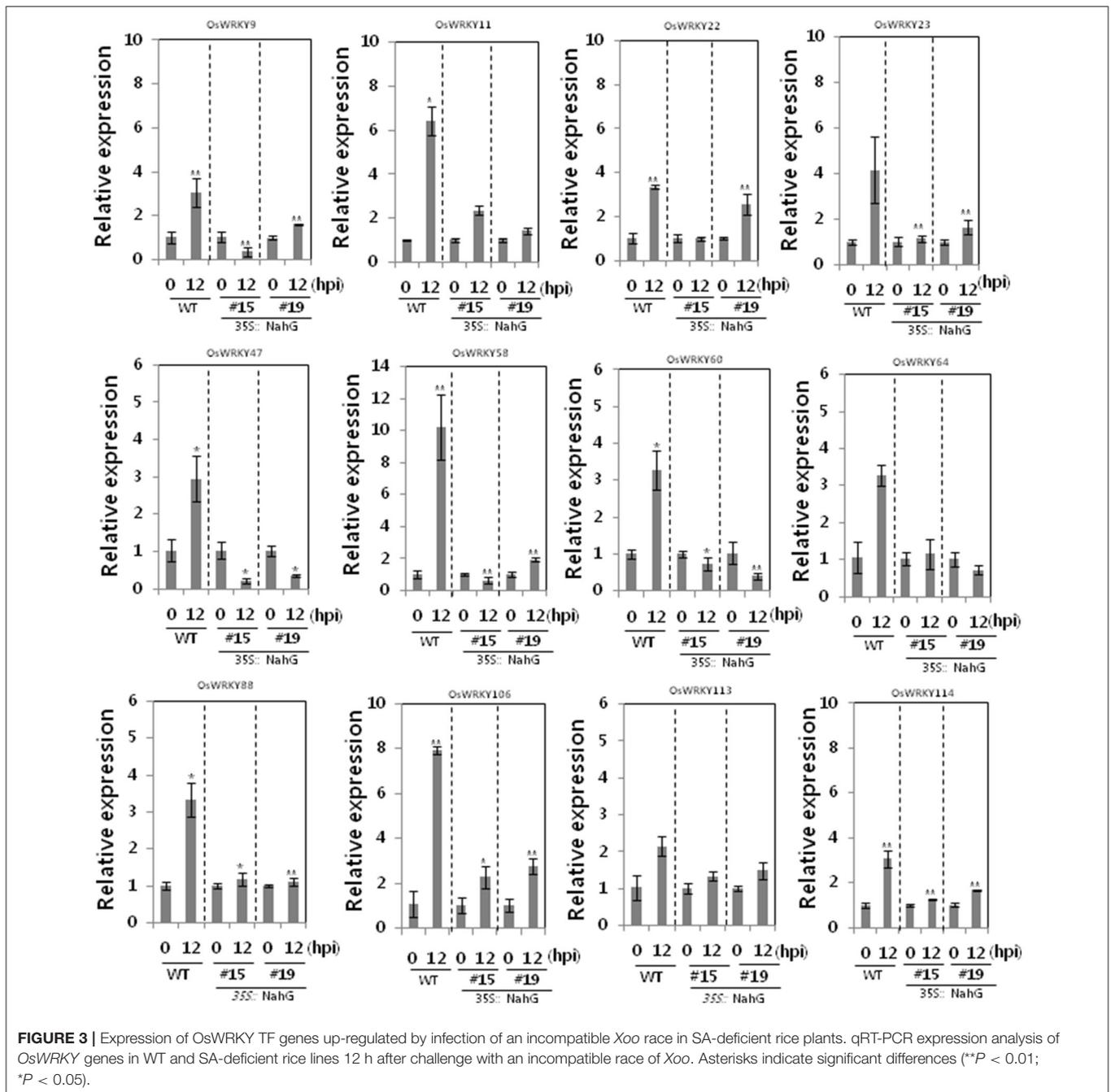
Type I	Expression pattern	Gene
OsWRKY2, 6, 7, 8, 9, 11, 12, 18, 22, 23, 25, 26, 29, 42, 45, 47, 50, 52, 55, 58, 60, 64, 65, 66, 76, 80, 84, 88, 105, 106, 108, 112, 113, 114	Compromised in SA-deficient rice compared with the WT	OsWRKY9, 11, 22, 23, 47, 58, 60, 64, 88, 106, 113, 114
	Unchanged in SA-deficient rice compared with the WT	OsWRKY2, 7, 12, 18, 25, 26, 42, 45, 50, 52, 55, 65, 66, 76, 80, 84, 105, 108, 112

Type I *OsWRKY* TF genes were further classified into Type Ia and Ib. Type Ia contained *OsWRKY* TF genes that exhibited more pronounced up-regulation with the incompatible interaction than with the compatible interaction. Type Ib contained *OsWRKY* TF genes that exhibited more pronounced up-regulation with the compatible interaction than with the incompatible interaction.

stress, but are also involved in regulating plant immune responses (Jimmy and Babu, 2015). In this study, expression of *OsWRKY* TF genes was profiled during pathogenic challenge with *Xoo* to elucidate the roles of *OsWRKY* TFs in the defense response.

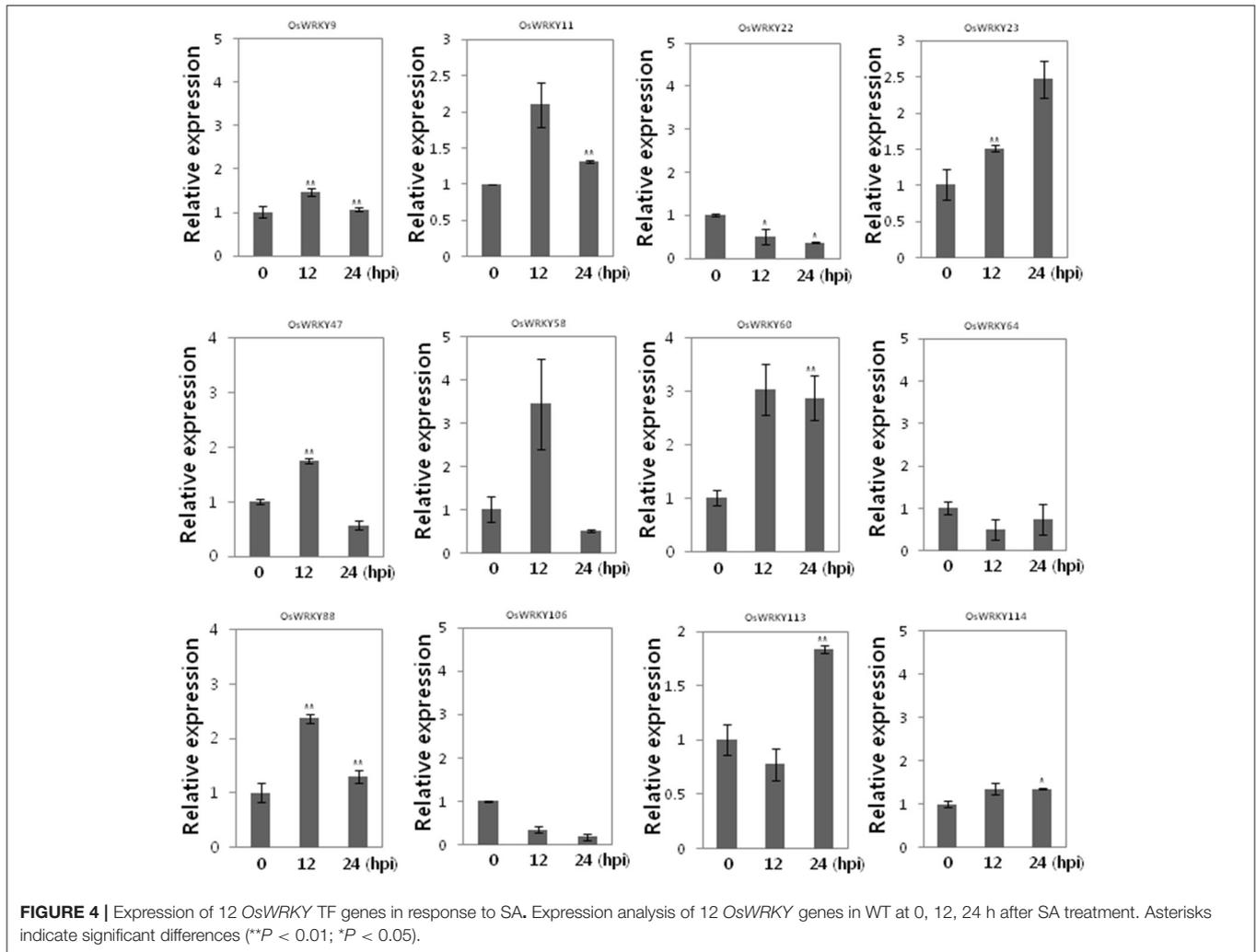
Genes encoding WRKY superfamily TFs in *Arabidopsis* and rice were previously expression profiled in response to biotic stresses (Dong et al., 2003; Ryu et al., 2006). In this study, gene

expression was modulated in 106 of the 111 *OsWRKY* TF genes examined in response to *Xoo* infection (Table 1). Approximately 96% of *OsWRKY* TFs were up- or down-regulated upon *Xoo* infection. The *OsWRKY* TF genes showed 8 different types of expression profiles (Table 1). Genes in type I, II, III, IV, and V (81 *OsWRKY* TF genes in total; ~73% of the 111 *OsWRKY* TF genes) were up-regulated after challenge with a compatible



or an incompatible race of *Xoo*. In *Arabidopsis* leaves, gene expression was modulated in 49 of 53 *AtWRKY* TF genes (~90%) as assessed by northern blotting (Dong et al., 2003). Of these, 43 *AtWRKY* TF genes (~81%) were up-regulated after challenge with an incompatible pathogen. Research in rice reported that 9 of 15 (60%) *OsWRKY* TF genes tested were induced by an incompatible race of *Xoo* (Ryu et al., 2006). However, the study examined only 15 of the 125 known *OsWRKY* TF genes. In this study, 66 of 111 *OsWRKY* TF genes (type I, IV, and VII; 60%) were up-regulated by an incompatible race of *Xoo*. This

discrepancy may be due to the sensitivity of methods such as RT-PCR and qRT-PCR when used for expression analysis. The previous study noted that *OsWRKY7*, *10*, *11*, *30*, *32*, *67*, *70*, *83* (renamed *94* by CGSNL), and *85* (renamed *96* by CGSNL) were induced by an incompatible race of *Xoo* (Ryu et al., 2006). Of these, *OsWRKY7*, *10*, *11*, *30*, *32*, *70*, *94*, and *96* were also induced by an incompatible race of *Xoo* in our results. However, in contrast with the previous study (Ryu et al., 2006), our results showed clear down-regulation of *OsWRKY67* after exposure to incompatible and compatible *Xoo* races. This

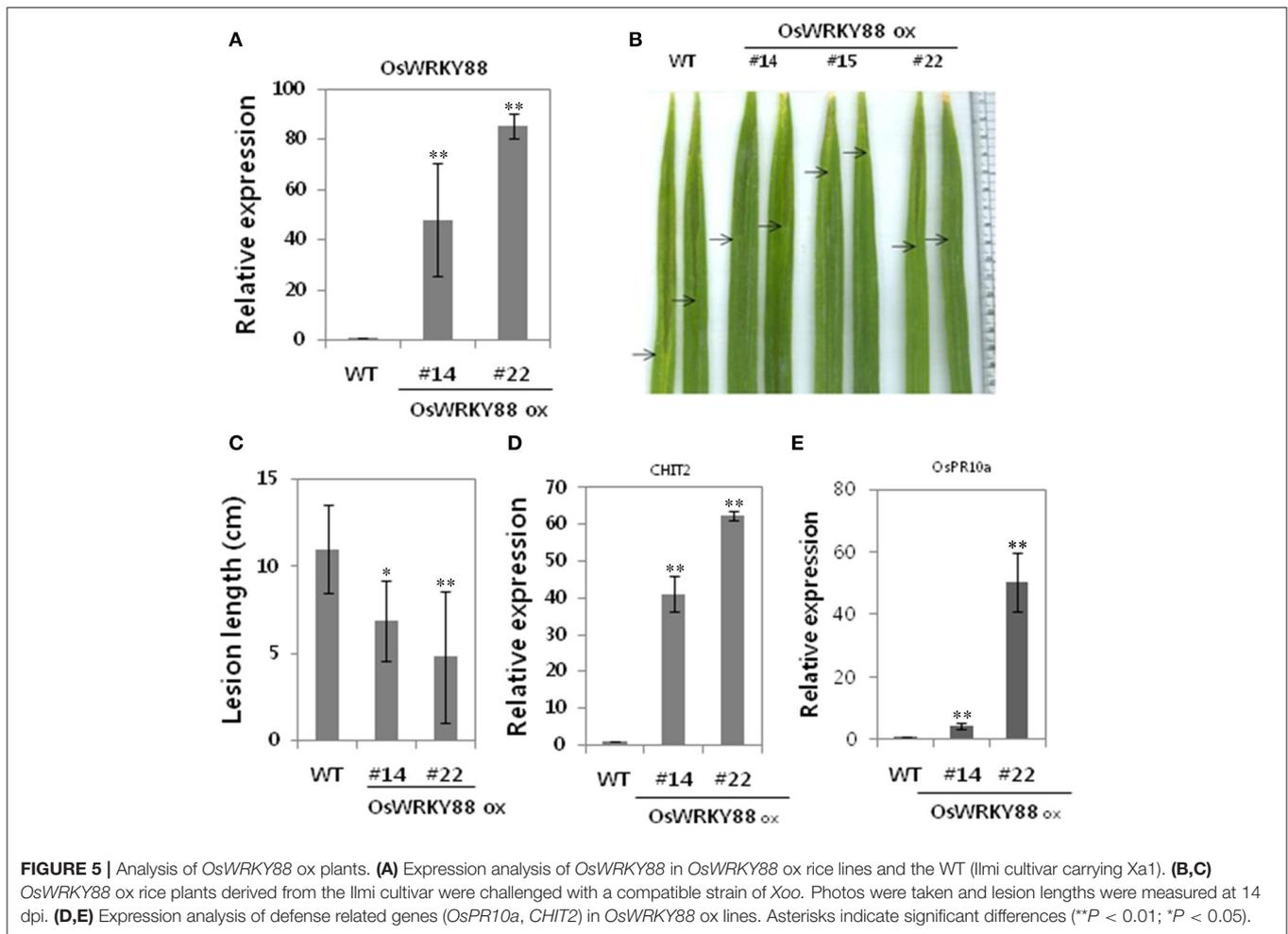


discrepancy is also predicted to be caused by the different methods used for expression analysis of *OsWRKY* TF genes in the two studies. Consistent with our data, previous studies showed that *OsWRKY03* (renamed *12* by CGSNL), 6, 13, 30, 45, and 76 were induced by *Xoo* and were involved in regulation of *Xoo*-mediated resistance (Liu et al., 2005, 2007; Chujo et al., 2008; Qiu and Yu, 2009; Tao et al., 2009; Hwang et al., 2011; Han et al., 2013; Lee et al., 2013; Choi et al., 2015).

Studies exploring *OsWRKY* TF gene expression after infection with a compatible race of *Xoo* are scarce. Expression profiles of defense-related genes induced by compatible and incompatible pathogens have been identified (Jimmy and Babu, 2015). However, to our knowledge, the expression profiles of genes induced only by compatible pathogens have not been published. In this study, *OsWRKY* TF gene expression profiles were generated in response to challenge with a compatible race of *Xoo*. Sixteen *OsWRKY* TF genes were induced by compatible infection. Of these, 14 *OsWRKY* TFs (*OsWRKY4*, 5, 14, 19, 28, 48, 78, 79, 82, 85, 86, 98, 101 and 121) were down-regulated by incompatible infection, suggesting that some *OsWRKY* TFs might function as negative regulators in the defense response,

resulting in increased disease susceptibility to *Xoo*. Previous research indicated that *OsWRKY4* enhanced resistance to a necrotrophic pathogen, *Rhizotonia solani*, and was involved in the jasmonic acid (JA)-mediated pathway (Wang H. et al., 2015). This suggests that *OsWRKY4* has a negative role in responding to challenge with biotrophic pathogens such as *Xoo*. Another TF, *OsWRKY28*, was found to act as a negative regulator in innate immunity, consistent with the gene expression profile seen in this study (Qiu et al., 2007). *Xoo* produces transcription activator-like (TAL) effectors that activate transcription of susceptibility (S) genes such as *OsSWEET14* and *OsTFX1* (Huang et al., 2016). Type II and III *OsWRKY* TFs might therefore be targets of TAL effectors.

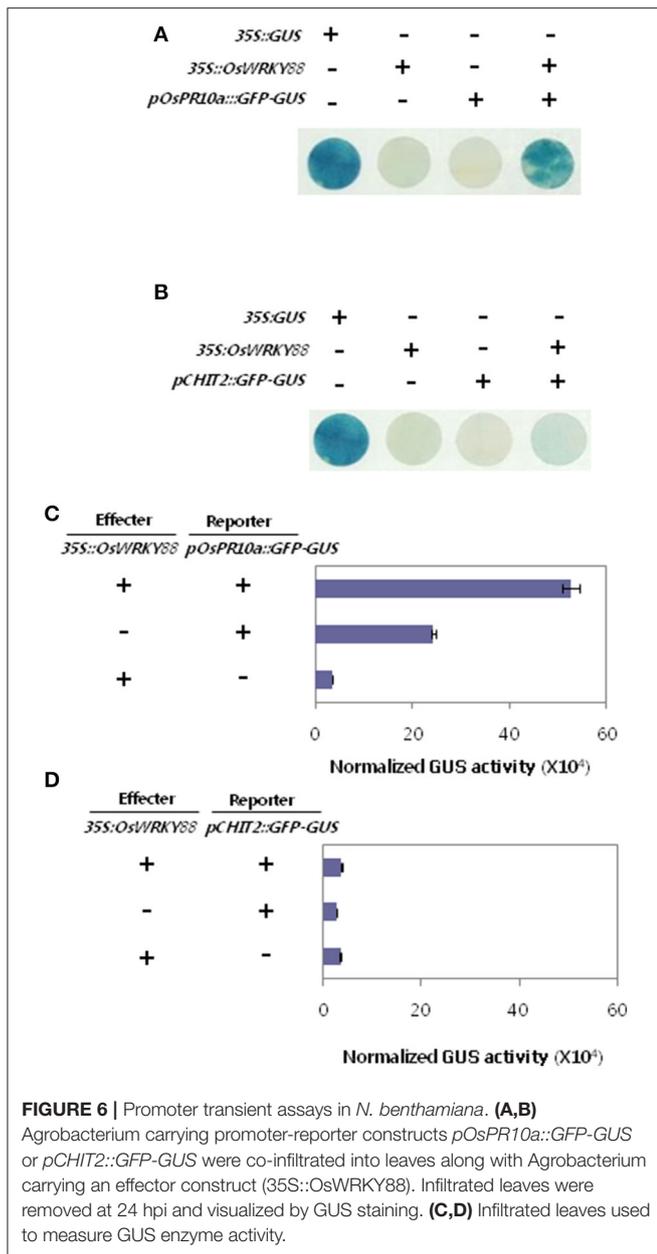
Previous studies showed that SA was involved in *Xoo*-mediated resistance (Chujo et al., 2013; Choi et al., 2015). However, the incompatible interactions of *Xoo* with Mudanjiang 8 and Minghui 63 rice varieties were due to the *Xa3/26* resistance factor, which is a typical pattern recognition receptor, rather than a NBS-LRR-type resistance factor. This resistance mechanism resembled pattern-triggered immunity, and it therefore remained unclear whether SA was involved in canonical R-gene-mediated



resistance against *Xoo* in rice. In this study, we demonstrated that SA-deficient rice exhibited disease susceptibility when exposed to an incompatible race of *Xoo*, while the WT plant (Ilmi, carrying Xa1) exhibited hypersensitive-response-type resistance. This result suggested that SA was involved in Xa1-mediated resistance. *OsWRKY* TF genes induced during the Xa1-mediated resistance response were classified based on their expression profiles in response to SA. Of the 34 *OsWRKY* TF genes in Types I, III, and V, induction of 12 *OsWRKY* TF genes (*OsWRKY9*, 11, 22, 23, 47, 58, 60, 64, 88, 106, 113, and 114) by *Xoo* was compromised in SA-deficient rice lines generated by over-expression of *NahG*. Among them 6 *OsWRKY* TF genes (*OsWRKY11*, 23, 47, 58, 60, 88) were induced by SA. We therefore propose that Xa1-mediated resistance involves both SA-dependent and SA-independent pathways. Induction of *OsWRKY* TF genes such as *OsWRKY10*, 30, 45, 62, 82 (renamed 89 by CGSNL), and 83 (renamed 94 by CGSNL) by SA was reported previously (Ryu et al., 2006). An additional study reported that *OsWRKY19*, 45, 62, and 76 were induced by the SA analog BTH (Shimono et al., 2007). Studies of individual *OsWRKY* TF genes indicated that *OsWRKY03* (renamed 12 by CGSNL), *OsWRKY6*, *OsWRKY33* (renamed 81 by CGSNL),

OsWRKY51, and *OsWRKY77* were induced by SA (Liu et al., 2005; Koo et al., 2009; Hwang et al., 2011, 2016; Lan et al., 2013). However, 12 *OsWRKY* TF genes profiled in this study (*OsWRKY9*, 11, 22, 23, 47, 58, 60, 64, 88, 106, 113, and 114) were not described previously, possibly because only *OsWRKY* TF genes involved in Xa1 mediated resistance were examined in this study. Overall, these results suggest that *OsWRKY* TFs regulate both SA-dependent and SA-independent Xa1-mediated resistance.

In this study, SA-deficient rice plants were generated by transforming rice plants with *NahG*, which degrades SA to catechol. However, several studies have questioned using *NahG* rice to study the effect of SA on plant resistance to infection (van Wees and Glazebrook, 2003; Yang et al., 2004; Mutuku et al., 2015). van Wees and Glazebrook (2003) arrived at false conclusions concerning the role of SA in plants defense because catechol affected non-host resistance in the *Arabidopsis* plants expressing *NahG*. Another study claimed that catechol had no effect on resistance to *S. hermonthica* (Mutuku et al., 2015). In this study, we demonstrated that catechol had no effect on Xa1-mediated resistance, confirming that the effects observed in SA-deficient rice were not due to catechol production. The effect



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of catechol on plant resistance would appear to depend on the pathosystem studied.

The function of *OsWRKY88*, a gene whose expression was altered during SA-dependent Xa1-mediated resistance, was analyzed. *OsWRKY88* ox lines showed increased resistance to *Xoo* and activation of the defense response. We demonstrated that *OsWRKY88* directly activated the *OsPR10a* promoter but not the *CHIT2* promoter. The higher level of the *CHIT2* transcript might be due to the presence of another transcription factor induced by *OsWRKY88*. Some studies claim that transcription regulatory cascades of TFs are required for disease resistance (Cheng et al., 2015). *OsWRKY22* and *OsWRKY23* (previously known as *OsWRKY31*) have been previously reported to be positive regulators of resistance to *M. oryzae*, although the effect of *OsWRKY22* and *OsWRKY23* on *Xoo* infection was not investigated (Zhang et al., 2008; Abbruscato et al., 2012). Expression profiling of *OsWRKY* TF genes was performed after the infection of rice with *Xoo*, which revealed changes in expression of most of the genes. *OsWRKY* TF gene expression in Xa1-mediated resistance appeared to involve both SA-dependent and SA-independent pathways. Therefore, we propose that *OsWRKY* TF genes are involved in Xa1-mediated resistance.

AUTHOR CONTRIBUTIONS

NC and DH wrote the manuscript. DH designed the experiments. NC, SL, and CC performed qRT-PCR. EL analyzed the transgenic plants. SP, IA, and SB identified database *OsWRKY* TF gene sequences and designed primers. CH critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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