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Arabidopsis WRKY46 coordinates with WRKY70 and WRKY53 in basal resistance against pathogen *Pseudomonas syringae*

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ABSTRACT

The WRKY transcription factors are involved in plant resistance against both biotrophic and necrotrophic pathogens. *Arabidopsis WRKY46* is specifically induced by salicylic acid (SA) and biotrophic pathogen *Pseudomonas syringae* infection. To determine its possible roles in plant defense and elucidate potential functional redundancy with structurally related WRKY70 and WRKY53, we examined loss-of-function T-DNA insertion single, double and triple mutants, as well as gain-of-function transgenic *WRKY46* over-expressing plants in response to *P. syringae*. *WRKY46* over-expressing plants were more resistant to *P. syringae*. In contrast, pathogen-infected *wrky46wrky70*, *wrky46wrky53* double mutants and *wrky46wrky70wrky53* triple mutants showed increased susceptibility to this pathogen, with increased bacterial growth and more severe disease symptoms. The contrasting responses of gain-of-function plants and loss-of-function mutants were correlated with increased or reduced expression of defense-related *PR1* gene. Expression studies of *WRKY46*, *WRKY70*, and *WRKY53* in various defense-signaling mutants suggested that they are partially involved in SA-signaling pathway. In addition, our findings demonstrated negative cross-regulation among these three genes. These results indicate that WRKY46, WRKY70, and WRKY53 positively regulate basal resistance to *P. syringae*; and that they play overlapping and synergetic roles in plant basal defense.

1. Introduction

To rapidly sense and defend against various microbial pathogens infection, plants have developed an effective innate immune system to protect themselves. There are two layers of innate immunity against pathogens' attack in plants. Following infection by a wide range of virulent pathogens, plants recognize microbes or pathogen-associated molecular patterns (PAMPs), such as chitin from fungi and flagellin from bacteria, *via* pattern-recognition receptors (PPRs) typically in plant plasma membranes [1]. For example, recent studies have revealed that the lysin motif (LysM)-containing chitin elicitor receptor kinase 1 (CERK1) directly binds chitin *in vitro* and functions as critical component for the immune responses to chitin in *Arabidopsis* [2,3]. The detection of PAMPs triggers plant basal resistance and subsequent defense responses, called PAMP-triggered immunity (PTI) [4]. Second, plant hosts recognize specific pathogen-secreted effectors by resistance (R) proteins. For instance, *Arabidopsis* resistance to *Ralstonia solanacearum* (RRS1-R) protein directly binds to the effector PopP2 from *R. solanacearum* [5]. Moreover, a recent study also characterizes an example of probably direct recognition between an R protein and an oomycete effector [6]. Such recognition activates a complex signal transduction cascade resulting in both locally induced defense responses (*e.g.*, the hypersensitive response or HR) and globally induced defense responses (*e.g.*, systemic acquired resistance or SAR) [7], collectively known as effector-triggered immunity (ETI) [4].

SAR is a long-lasting, systemic immunity to a broad spectrum of different pathogens. It is preceded by an increase in endogenous SA and accumulating SA induces a subset of pathogenesis-related (*PR*) genes [8,9]. Plants deficient in SA synthesis (*e.g.*, *sid2*, *eps1* and *mos1*) or its accumulation (*e.g.*, transgenic *nahG* plants) are compromised to mediate the responses [10–13]. Moreover, SAR was also blocked when SA methyl transferase (which converts SA to methyl salicylate) was silenced in primary infected leaves [14], which indicated

Abbreviations: SA, salicylic acid; JA, jasmonaic acid; MeJA, methyl jasmonate; ET, Ethylene; ACC, 1-aminocyclopropane-1-carboxylic acid; ABA, abscisic acid; BTH, benzothiadiazole S-methylester; PAMPs, pathogen-associated molecular patterns; PRRs, pattern-recognition receptors; LysM, lysin motif; CERK1, lysin motif (LysM)-containing chitin elicitor receptor kinase 1; PTI, PAMP-triggered immunity; R, resistance; RRS1-R, resistance to *Ralstonia solanacearum*; ETI, effector-triggered immunity; HR, hypersensitive response; SAR, systemic acquired resistance; NPR1, non-expressed of *PR* genes 1; PR1, pathogenesis-related gene 1; hpi, hours post-inoculation; dpi, days post-inoculation; PDF1.2, plant defensin 1.2; LOX2, lipoxygenase 2; VSP2, vegetative storage protein 2.

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that methyl salicylate also serves as a critical signal for SAR. The SA-induced SAR also depends on NPR1, a key node of SA-signaling. The *npr1* mutants were unable to activate the expression of *PR* genes [15]. Besides SA, JA and ethylene (ET) also play important roles in plant defense responses. The SA- and JA/ET-mediated defense pathways are often mutually antagonistic [16]. For example, blocking SA accumulation or signaling enhances JA-responsive gene expression (*e.g.*, *LOX2*, *VSP*, and *PDF1.2*) [17], while disruptions of JA signaling regulators such as COI1 show enhanced SA accumulation and *PR* expression [18].

The *Arabidopsis* WRKY transcription super-family consists of an estimated 74 members, and is sub-divided into three groups according to the number of WRKY domains and the features of their zinc finger-like motifs [19]. The WRKY domain has a high binding affinity to the W-box sequence [20]. Accumulating evidence has demonstrated that WRKY proteins are involved in regulating plant defense responses against both biotrophic and necrotrophic pathogens. For example, disruptions of the structurally related WRKY40 or WRKY60 show enhanced resistance against biotrophic pathogens *P. syringae* and *Golovinomyces orontii* [21,22]. Likewise, structurally related WRKY11 and WRKY17 also function as negative regulators of plant resistance against *P. syringae* [23]. A recent study suggests that WRKY51 may have an additive function as a positive regulator of basal defense against *P. syringae* [24]. In addition, WRKY25 and WRKY72 also were shown as regulators in response to biotrophs *Pseudomonas syringae* pv. *maculicola* strain ES4326 and *Hyaloperonospora arabidopsidis* [25,26]. Moreover, Hwang et al. recently showed that heterologous expression of *OsWRKY6* gene in *Arabidopsis* enhanced disease resistance to biotrophic pathogen *X. campestris pv. Campestris* [27]. Thus, members of WRKY proteins function as important regulators of plant disease resistance toward biotrophic pathogens.

Moreover, a majority of studies on *Arabidopsis WRKY* genes address their involvement in disease resistance toward necrotrophic pathogens. For example, disruption of WRKY33 enhances susceptibility to the necrotrophic fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola* [28]. Further investigation showed that WRKY33 physically interacts with ATG18a, SIB1 and SIB2, and regulates JA-mediated immunity against necrotrophic pathogens [29,30]. As well, WRKY3 and WRKY4 also function as positive regulators in plant resistance against *B. cinerea* [31]. Our previous report also demonstrated that mutations of *WRKY8* slightly decrease resistance to *B. cinerea* [32]. Thus, the WRKY transcription factors function as regulators involved in disease resistance against both biotrophs and necrotrophs.

Arabidopsis group III WRKY transcription factors comprises 13 members, and can be further subdivided into two subgroups, group IIIa (*e.g.*, WRKY38 and WRKY62) and group IIIb (*e.g.*, WRKY46, WRKY70, and WRKY53) [33]. Previous studies have shown that group III members are responsive to SA and pathogen infection [33,34], and may be involved in plant defense responses. For example, disruption of WRKY38 or WRKY62 enhances plant basal defense against *P. syringae* [35]. Mutations in *WRKY70* increase susceptibility to biotrophs *Erysiphe cichoracearum* and *Hyaloperonospora parasitica*, while resistance to necrotroph *A. brassicicola* [36,37]. Moreover, Murray et al. showed that *wrky53* mutant plants decrease basal resistance against *P. syringae* [38]. To further clarify the functions of *Arabidopsis* group III WRKY factors in plant defense, we chose *WRKY46* for further investigation. Here, we show that *WRKY46* is specifically induced by SA and *P. syringae* infection. To determine its biological functions directly, we have isolated its loss-of-function T-DNA insertion mutants and cultured gain-of-function transgenic *WRKY46* over-expressing plants. Since *WRKY46* shares similar expression pattern with evolutionarily related *WRKY70* and *WRKY53* in response to SA and pathogen induction [33,34], we also generated double mutants (*wrky46wrky70* and *wrky46wrky53*) and triple mutants (*wrky46wrky70wrky53*) through genetic crossing, to elucidate possible functional cooperation among them. Functional analysis of the single, double and triple mutants and *35S:WRKY46* transgenic plants in response to *P. syringae* infection indicated that pathogen-induced *WRKY46* functions partially redundantly with *WRKY70* and *WRKY53* in plant disease resistance.

2. Materials and methods

2.1. Materials

The plant hormones SA, MeJA, ACC and ABA were purchased from Sigma Co. Ltd. (St. Louis); Taq DNA polymerases were purchased from TaKaRa Biotechnology (Dalian) Co. Ltd. (Tokyo); and other common chemicals were obtained from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). *Arabidopsis* plants were grown in an artificial growth chamber at 22 °C in a photoperiod of 10 h of light and 14 h darkness. The *Arabidopsis* mutants and wild-type plants used in this study are from the Columbia genetic background. The mutant lines *wrky46*, *wrky70*, *wrky53*, *npr1*, *sid2*, *coi1* and *ein2* were obtained from Prof. Zhixiang Chen (Purdue University, USA).

2.2. Pathogen infection and induction treatments

Pathogen inoculations were performed by infiltration of leaves of 8–10 plants for each treatment with the *P. syringae* pv. tomato DC3000 (*Pst*DC3000) strain containing the pVSP61 kanamycin-resistant empty plasmid vector (OD600 = 0.0001 in 10 mM $MgCl_2$). Inoculated leaves were harvested at pointed times after infection. Diluted leaf extracts were plated on King's B medium supplemented with rifampicin (100 μg/ml) and kanamycin (25 μg/ml) and incubated at 25 °C for 2 days before counting CFU. *B. cinerea* infection was performed as described in [32]. Induction treatments of defense-related hormones (SA, MeJA, ACC and ABA) were also performed as described in [32].

2.3. RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from *Arabidopsis* plants using the TRIZOL reagent (Invitrogen). The first-strand cDNA was obtained from

Table 1
List of qRT-PCR primer sequences.

Gene name	Gene number	Primer forward (5′ → 3′)	Primer reverse (5′ → 3′)
WRKY46	AT2G46400	TTCATGGATCCAAAAATCCTAGA (+350 → +372)	TGTGGTTTCCGAGATACTTCACT (+462 → +484)
WRKY70	AT3G56400	ACTTGAGGACGCATTTTCTTGGAGG (+354 → +378)	TGCTTTGTTGCCTTGCACCCTT (+452 → +473)
WRKY53	AT4G23810	GACGGGGATGCTACGGTTT (+600 → +619)	TTTTGGGTAATGGCTGGTTTG (+714 → +733)
PLANT DEFENSIN 1.2	AT5G44420	TCACCCTTATCTTCGCTGCTCT (+23 → +44)	ATGATCCATGTTTGGCTCCTTC (+172 → +193)
LIPOXYGENASE 2	AT3g45140	ATGAGCCTGTTATCAATGCTGC (+2495 → +2516)	AACACCAGCTCCAGCTCTATTCTT (+2590 → +2613)
VEGETATIVE STORAGE PROTEIN 2	AT5g24770	ACCCATCATACTCAGTGACCGT (+525 → +546)	GAGATGCTTCCAGTAGGTCACG (+594 → +615)
ACTIN2	AT3G18780	TGTGCCAATCTACGAGGGTTT (+492 → +512)	TTTCCCGCTCTGCTGTTGT (+610 → +628)

Primer locations are relative to the translation start (ATG) in coding sequences (CDS).

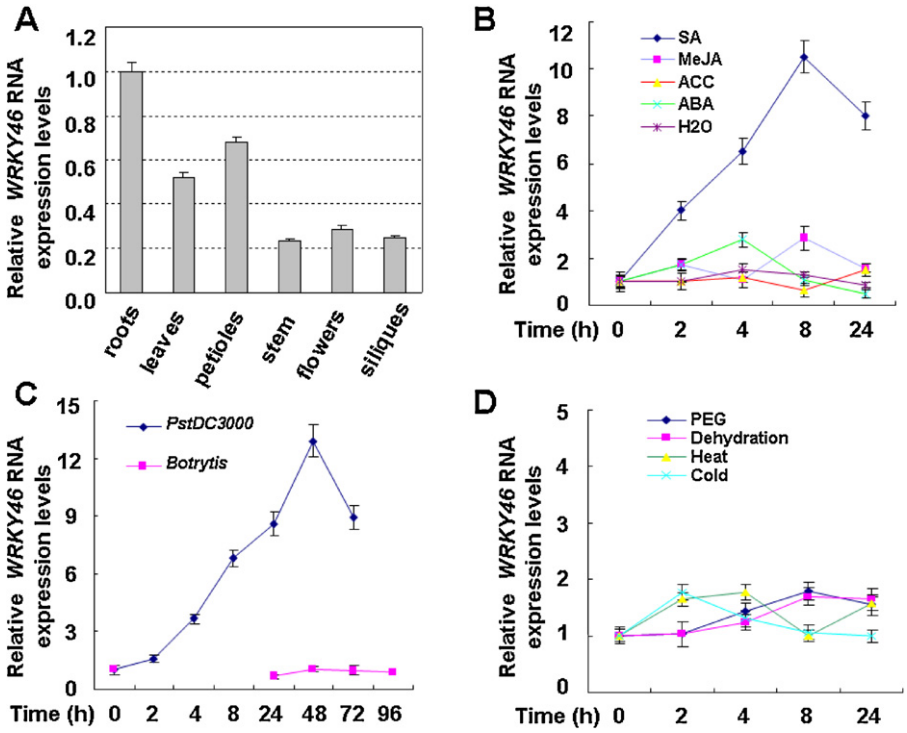

Fig. 1. Analysis of *WRKY46* expression in *Arabidopsis* plants using qRT-PCR analysis. The *ACTIN2* gene was used as an internal control of gene expression. (A) Basal expression of *WRKY46* in various organs. RNA samples were isolated from roots, leaves, petioles, stem, flowers and siliques of wild type grown at 22 °C. (B) Expression of *WRKY46* in response to defense-related hormones. RNA samples were prepared from 4-week-old wild type at given times after spraying with H_2O, SA (2 mM), MeJA (0.1 mM), ET (0.1 mM) and ABA (0.1 mM). (C) Expression of *WRKY46* after pathogens infection. For *P. syringae* treatment, 5-week-old wild type plants were infiltrated with a suspension of *P. syringae* (optical density at 600 nm = 0.0001 in 10 mM $MgCl_2$), and inoculated leaves were collected at indicated times. For *Botrytis* spp. treatment, 5-week-old wild-type plants were inoculated by spraying with a spore suspension (5×10^4 spores/ml). Plants were maintained under high humidity and whole seedlings were collected for isolation of RNA at given times. (D) Expression of *WRKY46* during various abiotic stresses. RNA samples were prepared from 4-week-old wild type at given times after treatment with dehydration, 25% PEG, cold (4 °C) and heat (42 °C). Error bars indicate standard deviations from three independent RNA extracts.

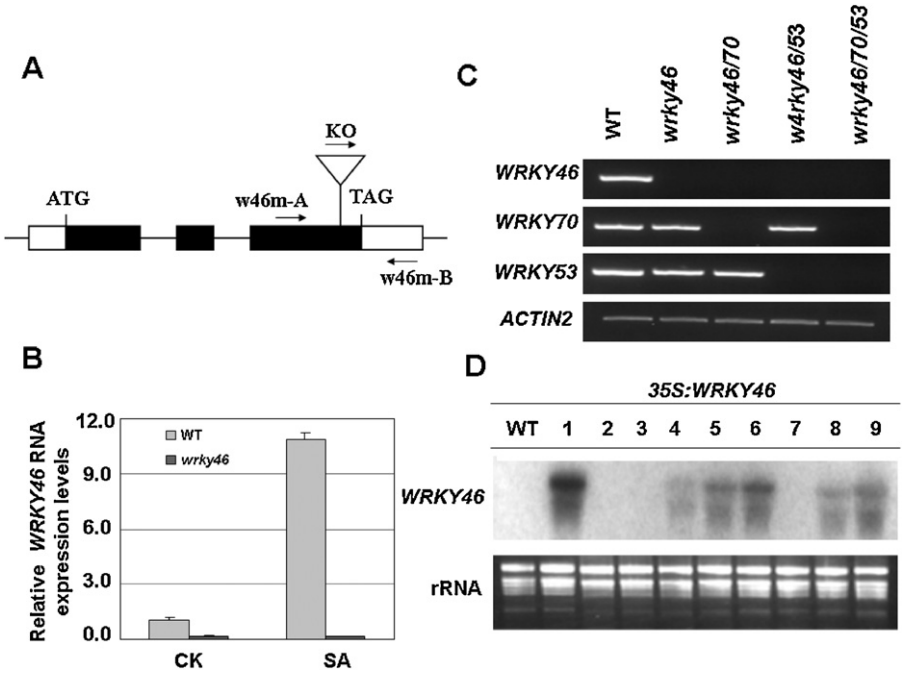

Fig. 2. T-DNA insertion mutants and over-expression lines for *WRKY46*. (A) Diagram of *WRKY46* gene and its T-DNA insertion mutant (SALK_134310). (B) qRT-PCR comparison of *WRKY46* RNA expression levels in wild type and *wrky46* mutants before and after SA induction. *WRKY46* RNA expression levels significantly increased in wild type after SA induction, while showed no distinct change in *wrky46* mutants. Error bars indicate standard deviations of three independent biological samples. (C) Northern blot analysis of *WRKY46* expression in transgenic plants over-expressing *WRKY46*. RNA samples were prepared from leaves of nine transgenic *35S:WRKY46* lines or wild type (WT). RNA was probed with *WRKY46* full length cDNA, and ethidium bromide-stained rRNA was used as a loading control. All the experiments described above were repeated three times with similar results. (D) Identification of homozygous *wrky46wrky70*, *wrky46wrky53* double mutants and *wrky46wrky70wrky53* triple mutants. RT-PCR was performed with total RNA isolated from wild type and double or triple mutants after SA treatment.

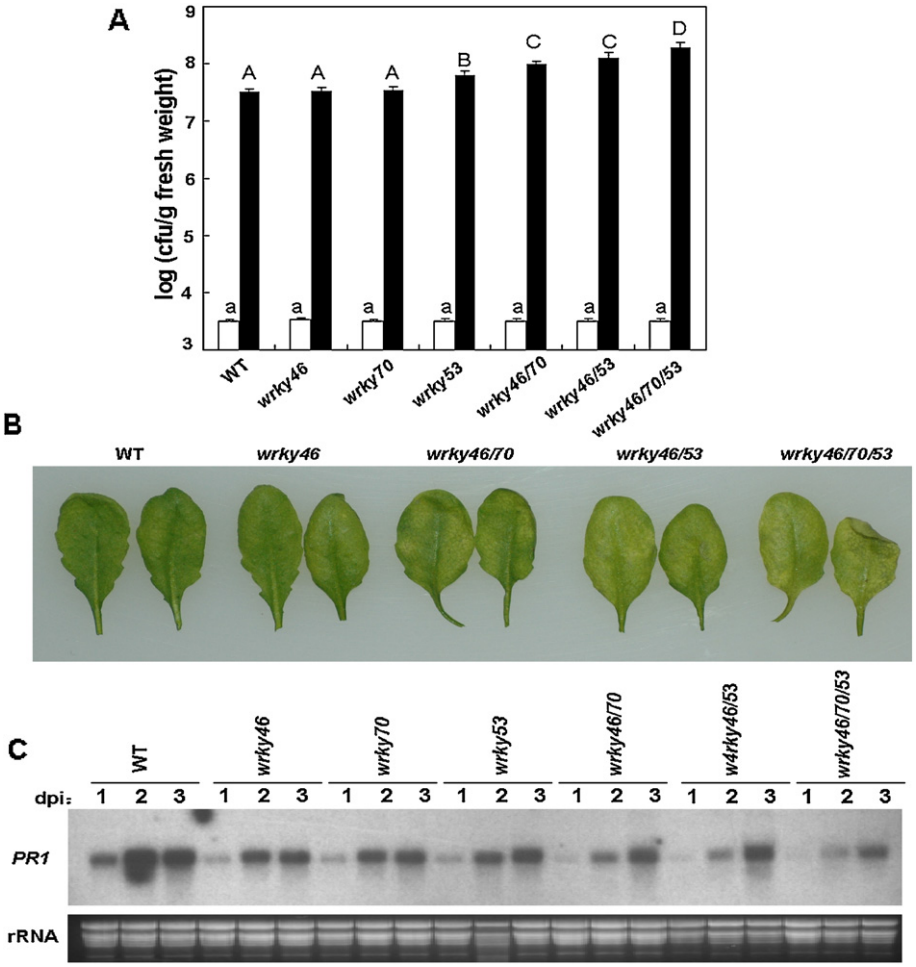

Fig. 3. Altered responses of loss-of-function mutants to *P. syringae*. (A) Altered bacterial growth. Single, double and triple mutants and WT were infiltrated with a suspension of *P. syringae* (optical density at 600 nm = 0.0001 in 10 mM $MgCl_2$). Growth of the bacterial pathogen was assessed at 0 and 60 hpi. Means and standard errors are shown (n = 8–10 plants per treatment). (B) Altered disease symptom development. Pathogen inoculation of single, double and triple mutants and WT was performed as in (A). Pictures of representative inoculated leaves taken at 60 hpi. (C) *PR1* expression. Pathogen inoculation of single, double and triple mutants and WT was performed as in (A). Total RNAs were isolated from inoculated leaves at 1, 2, and 3 dpi and probed with a *PR1* fragment. Ethidium bromide-stained ribosomal RNA was used as a loading control. These experiments described above were repeated three times with similar results.

1.5 μg of DNase-treated RNA in a 20 μl reaction volume using M-MuLV reverse transcriptase (Fermentas, EU) with oligo(dT)18 primer. qRT-PCR were performed with 2× SYBR Green I master mix on a Roche LightCycler 480 real-time PCR machine, according to the manufacturer's instructions. All PCRs were performed under the following conditions: 3 min at 95 °C followed by 50 cycles of 30 s at 95 °C, 30 s at 60 °C, and 20 s at 72 °C in LightCycler capillaries (Roche Diagnostics). The specificity of amplicons was verified by melting curve analysis (60–95 °C) after 50 cycles. At least three biological replicates for each sample were used for qRT-PCR analysis and at least two technical replicates were analyzed for each biological replicate. *ACTIN2* (At3g18780) was used as the reference gene internal control for comparison of the target gene transcripts amplified per cDNA sample. Gene-specific primers used to detect transcripts are listed in Table 1. All primers for qRT-PCR were designed and synthesized by TaKaRa.

2.4. Probe making and Northern blot analysis

Transcripts for *WRKY46* were detected using full-length *WRKY46* coding sequence (885 bp) as a probe. DNA probe for *PR1* (352 bp) was obtained from PCR amplification using the following gene-specific primers: 5′-TCTTCCCTCGAAAGCTCAAG-3′ and 5′-ACACCTCACTTTGGCACATC-3′. Probes were labeled by [α-^{32}P] dATP using the TaKaRa Random Primers DNA Labeling System. For northern blot analysis, total RNA (20 μg) was separated on agarose-formaldehyde gels and then transferred onto nylon membranes, which were hybridized and washed following standard procedures [39]. Briefly, the membranes were hybridized with (α-^{32}P)-dATP-labeled DNA probes. Hybridization was performed in PerfectHyb Plus hybridization buffer (Sigma) for 16 h at 68 °C. The membranes were washed once for 10 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate (SDS), twice for 20 min with 0.5× SSC and 0.1% SDS, once for 20 min with 0.1× SSC and 0.1% SDS at 68 °C, and then exposed to X-ray films at −80 °C.

2.5. Identification of T-DNA insertion mutants

wrky46 (salk_134310), *wrky70* (salk_025198) and *wrky53* (salk_034157) were kindly provided by Prof. Zhixiang Chen (Purdue University, USA). At first, we confirmed the T-DNA insertions by PCR using a combination of a gene-specific primer and a T-DNA border primer (5′-AAACGTCCGCAATGTGTTAT-3′). Homozygosity of the mutants was identified by PCR using a pair of primers corresponding to sequences flanking the T-DNA insertion

sites (w46m-A: 5′-ATCTCGGAAACCACACTTGTAA-3′, w46m-B: 5′-TGCATCACTTTTACAAAGCGT-3′; w70m-A: 5′-TTGGCTACTTATGATTCTTGC-3′, w70m-B: 5′-AAATGGGTGGATAAGTTCAA-3′; w53m-A: 5′-ATCAGGGAACGAGAAAACGT-3′, w53m-B: 5′-AGAGAGGCTTAGAAGGACCTCA-3′). The double and triple mutants were generated through genetic crosses of single mutants and identified through PCR genotyping. All of the insertion mutants were further confirmed by RT-PCR.

2.6. Construction of WRKY46 over-expressing transgenic lines

The *WRKY46* full cDNA linked to a vector pUNI [40] was obtained from the *Arabidopsis* Resource Center (ABRC). To generate the *35S:WRKY46* construct, the cDNA fragment containing the full coding sequence was sub-cloned into the same restriction sites of pOCA30 [41] in the sense orientation behind the CaMV 35S promoter. The recombinant plasmids were introduced into *Agrobacterium tumefaciens* GV3101 and used to transform *Arabidopsis* by the floral dip method [42]. Transformed lines were selected for resistance to kanamycin (50 μg/ml). Northern blot analyses were performed to select the transgenic plants.

2.7. Accession numbers

Arabidopsis genome initiative numbers for the genes discussed in this article are as follows: *WRKY46*, AT2G46400, *WRKY70*, AT3G56400, *WRKY53*, AT4G23810, *PR1*, AT2g14610, *PDF1.2*, AT5G44420, *LOX2*, AT3g45140, *VSP2*, AT5g24770 and *ACTIN2*, AT3G18780.

3. Results

3.1. Expression profiles of WRKY46

Arabidopsis WRKY46 (AT2G46400) encodes a protein with 295 amino acids, containing one WRKY domain and one C_2-HC zinc-finger motif, and is classified as a group III WRKY protein [43]. To investigate functions of *WRKY46*, we examined its expression in *Arabidopsis*. First, the basic expression of *WRKY46* in different organs was analyzed by qRT-PCR. As shown in Fig. 1A, the roots exhibited higher *WRKY46* expression than other organs. Induced expressions of *WRKY46* in response to various defense-related hormones were also measured. Expression of *WRKY46* was rapidly and strongly induced by SA treatment (2 μM) and peaked at 8 h (Fig. 1B). However, *WRKY46* is not responsive to other defense-related hormones, such as methyl jasmonate (MeJA), 1-aminocyclopropane-1-carboxylate (ACC) and abscisic acid (ABA) (Fig. 1B). To determine the expression profiles of *WRKY46* more precisely, we also examined its expression during various biotic and abiotic stresses. Interestingly, *WRKY46* mRNA accumulated high levels in *P. syringae*-infected plants (Fig. 1C). *WRKY46* transcripts did not change after infection by the necrotrophic pathogen *B. cinerea* (Fig. 1C) or treatments under abiotic stress (*e.g.* dehydration, PEG, cold or heat) (Fig. 1D). Together, these results indicate that the *WRKY46* gene specifically responds to SA and *P. syringae*-infection and may be involved in disease resistance against biotrophic pathogens.

3.2. Identification of mutants and construction of over-expressed transgenic Arabidopsis plants

To characterize the role of WRKY46 in plant defense, we first identified one loss-of-function T-DNA insertion mutant for *WRKY46*. *wrky46* mutant (salk_134310) harbored a T-DNA insertion at the third exon (752 bp from the translation start, Fig. 2A). The T-DNA insertion was confirmed by PCR using primers specific

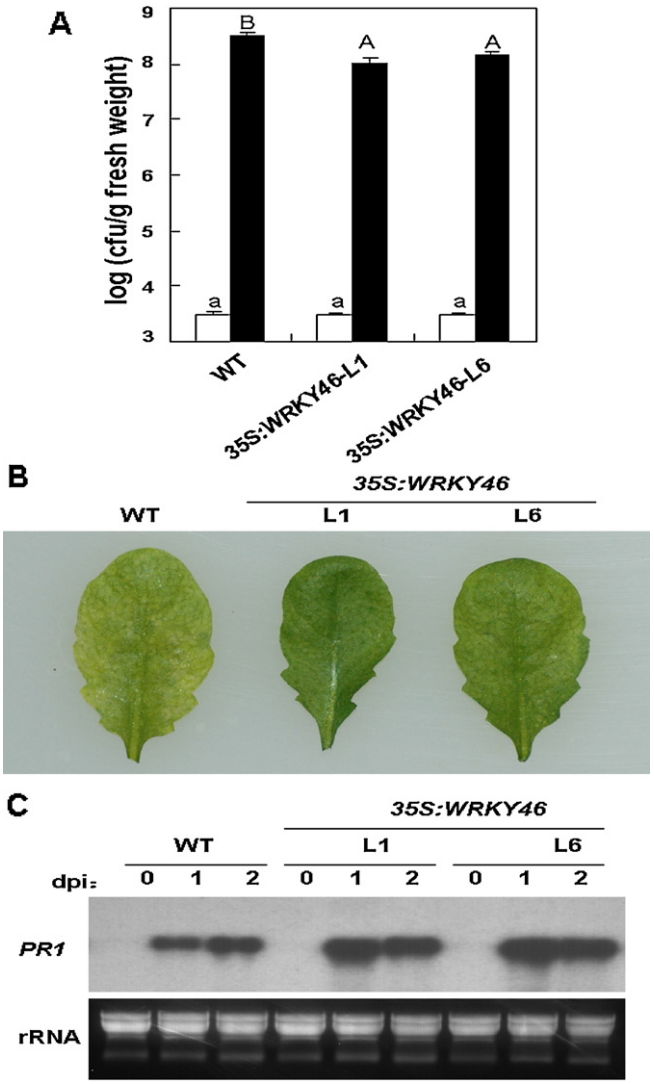

Fig. 4. Altered responses of *WRKY46* over-expression lines to *P. syringae*. (A) Altered bacterial growth. *WRKY46* over-expression lines and WT were infiltrated with a suspension of *P. syringae* (optical density at 600 nm = 0.0001 in 10 mM $MgCl_2$). Growth of the bacterial pathogen was assessed at 0 and 3 dpi. Means and standard errors are shown (n = 8–10 plants per treatment). (B) Altered disease symptom development. Pathogen inoculation of *WRKY46* over-expression lines and WT was performed as in (A). Pictures of representative inoculated leaves taken at 3 dpi. (C) Defense-related *PR1* expression. Pathogen inoculation of *WRKY46* over-expression lines and WT was performed as in (A). Total RNAs were isolated from inoculated leaves at 0, 1, and 2 dpi and probed with a *PR1* fragment. Ethidium bromide-stained ribosomal RNA was used as a loading control. These experiments described above were repeated three times with similar results.

to the *WRKY46* gene and the T-DNA insertion (data not shown). Additional qRT-PCR and RT-PCR were performed to compare the wild-type plants and *wrky46* mutants for SA-induced accumulation of *WRKY46* transcripts. *WRKY46* transcripts of the expected induction level were observed in wild-type plants; however, SA did not induce *WRKY46* expression in *wrky46* mutants (Fig. 2B and D). To further investigate the function of WRKY46, we cultured and analyzed transgenic *Arabidopsis* plants constitutively expressing *WRKY46* under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. Northern blot analysis showed that several transgenic plants constitutively expressed elevated levels of *WRKY46* transcripts, even without SA or pathogen treatments (Fig. 2C). Two transgenic lines (lines 1 and 6) were selected for further study. Line 1 showed a markedly higher expression of *WRKY46* compared with the wild type, and line 6 also showed elevated *WRKY46* expression.

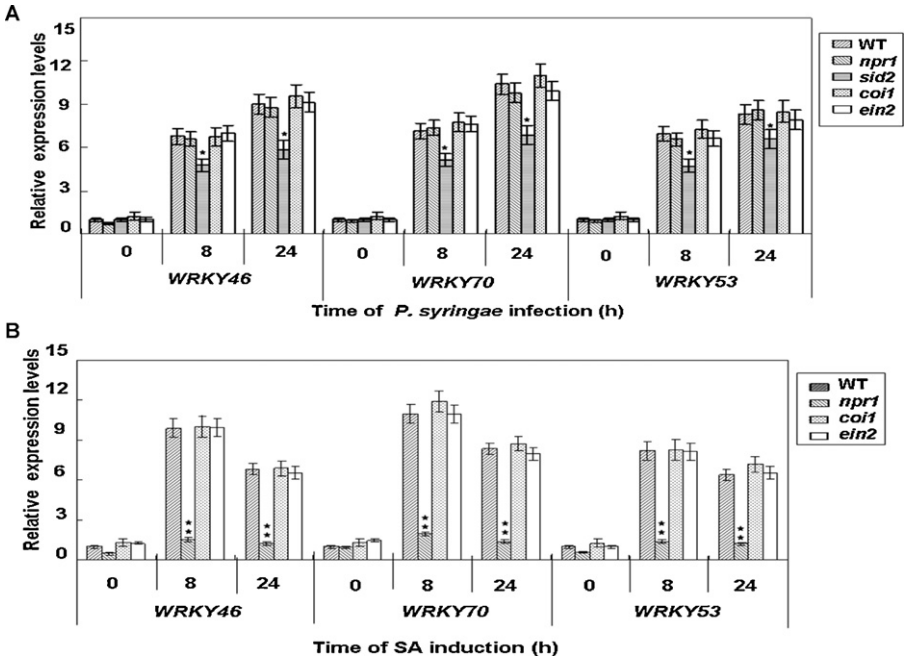

Fig. 5. Partial involvement of WRKY46, WRKY70, and WRKY53 in the SA-signaling pathway. (A) Pathogen-induced *WRKY46*, *WRKY70*, and *WRKY53* expression in WT, *npr1*, *sid2*, *coi1* and *ein2*. 5-Week-old plants were infiltrated with a suspension of *P. syringae* (optical density at 600 nm = 0.0001 in 10 mM $MgCl_2$), and inoculated leaves were collected at indicated times. (B) Exogenous SA-induced *WRKY46*, *WRKY70*, and *WRKY53* expression in WT, *npr1*, *coi1* and *ein2*. RNA samples were prepared from 4-week-old plants at given times after spraying with SA (2 mM). Error bars indicate standard deviations from three independent RNA extracts. **Differences for the mutants compared with corresponding wild type are highly significant ($P < 0.01$). *Differences for the mutants compared with corresponding wild type are significant ($P < 0.05$).

T3 homozygous plants from both lines showed the same morphology as wild-type plants (data not shown).

To clarify possible functional cooperation between WRKY46 and structurally related WRKY70 and WRKY53 in plant defense, we generated double and triple mutants through genetic crossing with *wrky70* (salk_025198) and *wrky53* (salk_034157). The *wrky46wrky70*, *wrky46wrky53* double mutants and *wrky46wrky70wrky53* triple mutants progenies' homozygosity were confirmed by RT-PCR (Fig. 2D). Besides the changes in gene expression, no other obvious differences in morphology were observed between the wild type and any mutants under normal growth conditions (data not shown).

3.3. Enhanced resistance to P. syringae in WRKY46 over-expression lines

To determine the functions of WRKY46 in plant defense, we first examined the responses of wild type and *wrky46* mutants to the biotrophic pathogen *P. syringae*. Plants were inoculated with the pathogen and bacteria growth was monitored. The homozygous *wrky46* single mutants showed no differences in bacterial growth compared to wild-type plants (Fig. 3A). Moreover, the inoculated leaves of *wrky46* mutants showed similar chlorosis as wild-type plants (Fig. 3B). This finding was similar to the previous report that *wrky70* T-DNA insertion mutants (salk_025198) also did not show altered resistance to *P. syringae* [36].

To further examine the roles of WRKY46 against *P. syringae*, wild-type plants and *WRKY46* over-expression lines (lines 1 and 6) were also challenged with this pathogen. Bacterial growth decreased three- to four-fold in *35S:WRKY46* transgenic lines compared to wild-type plants (Fig. 4A). Meanwhile, the inoculated leaves of *35S:WRKY46* transgenic plants showed less chlorosis than wild-type plants at 3 days post-inoculation (dpi) (Fig. 4B). Since *35S:WRKY46* transgenic plants acquired more resistance against *P. syringae*, the pathogen-induced expression of *PR1* was analyzed by northern blotting. Total RNA was isolated from the inoculated leaves at 0, 1 and 2 dpi and probed with a *PR1* probe. Compared to wild-type plants, *PR1* transcripts were increased in *WRKY46* over-expression lines at 1 and 2 dpi (Fig. 4C). Thus, constitutive over-expression of *WRKY46* enhances tolerance against *P. syringae*.

3.4. Enhanced susceptibility to P. syringae in wrky46wrky70, wrky46wrky53 double mutants and wrky46wrky70wrky53 triple mutants

To illustrate potential functional redundancy between WRKY46 and structurally related WRKY70 and WRKY53 in disease resistance, we also examined the responses of wild-type plants and *wrky46wrky70*, *wrky46wrky53* double mutants and *wrky46wrky70wrky53* triple mutants to *P. syringae*. In contrast to *WRKY46* over-expression lines, bacterial growth increased by three- or four-fold in *wrky46wrky70* and *wrky46wrky53* double mutants, compared with wild-type plants at 60 h post-inoculation (hpi) (Fig. 3A). An even greater increase in bacterial growth was observed in the *wrky46wrky70wrky53* triple mutants (six-fold) (Fig. 3A). The *wrky46wrky70*, *wrky46wrky53* double mutants and *wrky46wrky70wrky53* triple mutants also developed more severe disease symptoms than wild type (Fig. 3B). Thus, disruption of *WRKY46*, *WRKY70*, and *WRKY53* increases susceptibility to *P. syringae* and their function against this pathogen may be redundant to some degree.

To further analyze the defense responses in various loss-of-function mutants, we compared their defense gene expression with that of wild-type plants following infection of the biotrophic pathogen. After inoculation with *P. syringae*, there were lower levels of *PR1* transcripts at 1 and 2 dpi in the *wrky46wrky70* and *wrky46wrky53* double mutants than in the wild-type plants. In addition, the *wrky46wrky70wrky53* triple mutants had the lowest *PR1* transcripts at 1, 2, and 3 dpi (Fig. 3C).

3.5. WRKY46, WRKY70, and WRKY53 partially involved in SA-signal transduction pathway

Since WRKY46, WRKY70, and WRKY53 participate in plant defense responses, we analyzed which defense-signaling pathways they are involved in. Their expression was monitored in a set of mutants that are defective in various defense response pathways, including SA biosynthesis (*sid2*), SA signaling (*npr1*), JA signaling (*coi1*) and ET response (*ein2*) [10,44–46]. As shown in Fig. 5A, *P. syringae*-induced expression of *WRKY46*, *WRKY70*, and *WRKY53* was not affected in *npr1*, *coi1* and *ein2* compared with the wild-type plants. However, reduced expression of *WRKY46*, *WRKY70*, and *WRKY53* occurred in SA-synthesis defective mutant *sid2* plants (Fig. 5A). Thus, induction of *WRKY46*, *WRKY70*, and *WRKY53* by the biotrophic pathogen is positively regulated by endogenous SA. Since endogenous SA is required for pathogen responsive expression of *WRKY46*, *WRKY70*, and *WRKY53*, we also analyzed their expression in those signaling mutants after exogenous SA treatment. Unlike in pathogen-inoculated plants, the levels of *WRKY46*, *WRKY70*, and *WRKY53* transcripts were significantly reduced in *npr1* after SA treatment, compared to wild-type plants (Fig. 5B). However, the accumulation of *WRKY46*, *WRKY70*, and *WRKY53* mRNA was similar between *coi1*, *ein2* mutants and wild type (Fig. 5B). Thus, SA-induced expression of *WRKY46*, *WRKY70*, and *WRKY53* is mainly dependent on NPR1.

3.6. Cross-regulation among WRKY46, WRKY70, and WRKY53

Previous studies have shown that promoters of multiple WRKY transcription factors are enriched in W-boxes, and they can interact with their own promoters or other WRKY genes' [34,47]. To test this possibility for mutual regulation among *WRKY46*, *WRKY70*, and *WRKY53*, we analyzed *WRKY46* expression in *wrky70* and *wrky53* single mutants, *WRKY70* expression in *wrky46*, *wrky53* single mutants and *wrky46wrky53* double mutants, and *WRKY53* expression in *wrky46*, *wrky70* single mutants and *wrky46wrky70* double mutants before and after *P. syringae* infection.

As shown in Fig. 6, non-infected *wrky70* mutants exhibited 2.6-fold increase of *WRKY46* expression compared to wild type, while pathogen-induced expression of *WRKY46* in *wrky70* mutants also showed 3.0-fold increase. Likewise, *wrky46* and *wrky46wrky53* mutants slightly increased their basal expression (1.7- and 2.8-fold) of *WRKY70* compared to wild type; and *WRKY70* expression in pathogen-infected *wrky46* and *wrky46wrky53* mutants was also 2.0- and 2.4-fold higher than in wild-type plants (Fig. 6). Without *P. syringae* infection, *WRKY53* expression in *wrky70* and *wrky46wrky70* mutants was 2.8- and 3.1-fold increased, compared to wild type (Fig. 6). After treatment, *WRKY53* expression in *wrky70* and *wrky46wrky70* mutants was 2.2-and 2.4-fold higher than in wild-type plants (Fig. 6). Taken together, basal and pathogen-induced expression of *WRKY46* or *WRKY53* was partially repressed by WRKY70, while *WRKY70* was repressed by WRKY46 to some degree.

3.7. WRKY46, WRKY70, and WRKY53 redundantly suppress the JA-induced expression of PDF1.2

SA and JA are two essential defense-related hormones, and SA- and JA-mediated defense responses can be mutually antagonistic. Previous studies have shown that several members of group III WRKY proteins negatively regulate the expression of JA-induced genes in *Arabidopsis* [48–50], prompting us to analyze whether WRKY46, WRKY70, and WRKY53 are also involved in repressing JA-responsive gene expression. Because *PDF1.2*, *LOX2*, and *VSP2* are well-characterized JA-regulated genes, we explored their

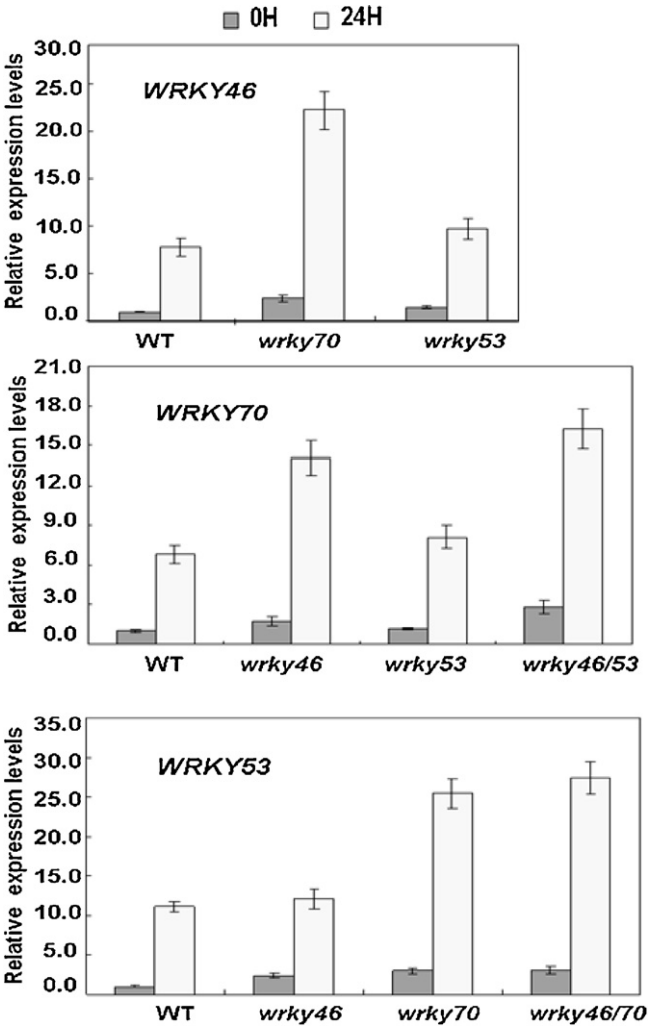

Fig. 6. Cross-regulation among WRKY46, WRKY70, and WRKY53. The transcripts of *WRKY46*, *WRKY70*, and *WRKY53* were determined by qRT-PCR using cDNA generated from leaves of 35-day-old wild type and mutants before and after *P. syringae* infection for 24 h. *WRKY46* expression was first checked in WT, *wrky70* and *wrky53* single mutants; and then *WRKY70* expression was detected in WT, *wrky46*, *wrky53* single mutants and *wrky46wrky53* double mutants. At last, *WRKY53* expression in WT, wrky46, *wrky70* single mutants and *wrky46wrky70* double mutants was tested. Error bars indicate standard deviations from three independent RNA extracts.

expression in various knock-out mutants and *35S:WRKY46* transgenic lines.

As shown in Fig. 7, *wrky46*, *wrky70* and *wrky53* single mutants, *wrky46wrky70*, *wrky46wrky53* double mutants and *wrky46wrky70wrky53* triple mutants showed almost the same *PDF1.2* basal expression as wild-type plants before 0.1 mM MeJA treatment. Furthermore, *wrky46*, *wrky70* and *wrky53* single mutants had similar MeJA-induced expression of *PDF1.2* as wild type. *PDF1.2* expression in *wrky46wrky70*, *wrky46wrky53* double mutants and *wrky46wrky70wrky53* triple mutants, however, was 2.2-, 2.7-, and 6.0-fold higher than that in wild-type plants after treatment with 0.1 mM MeJA, respectively (Fig. 7). By contrast, MeJA-induced expression of *PDF1.2* in two *35S:WRKY46* transgenic lines showed 2.4- and 1.6-fold reductions (Fig. 7). Our results also showed that the expression of *LOX2* and *VSP2* were not affected in all knock-out mutants, while decreased in *35S:WRKY46* transgenic lines after MeJA treatment compared to wild type (Fig. 7). Taken together, these results indicate that WRKY46, WRKY70, and WRKY53 function synergistically to suppress the expression of JA-responsive *PDF1.2*.

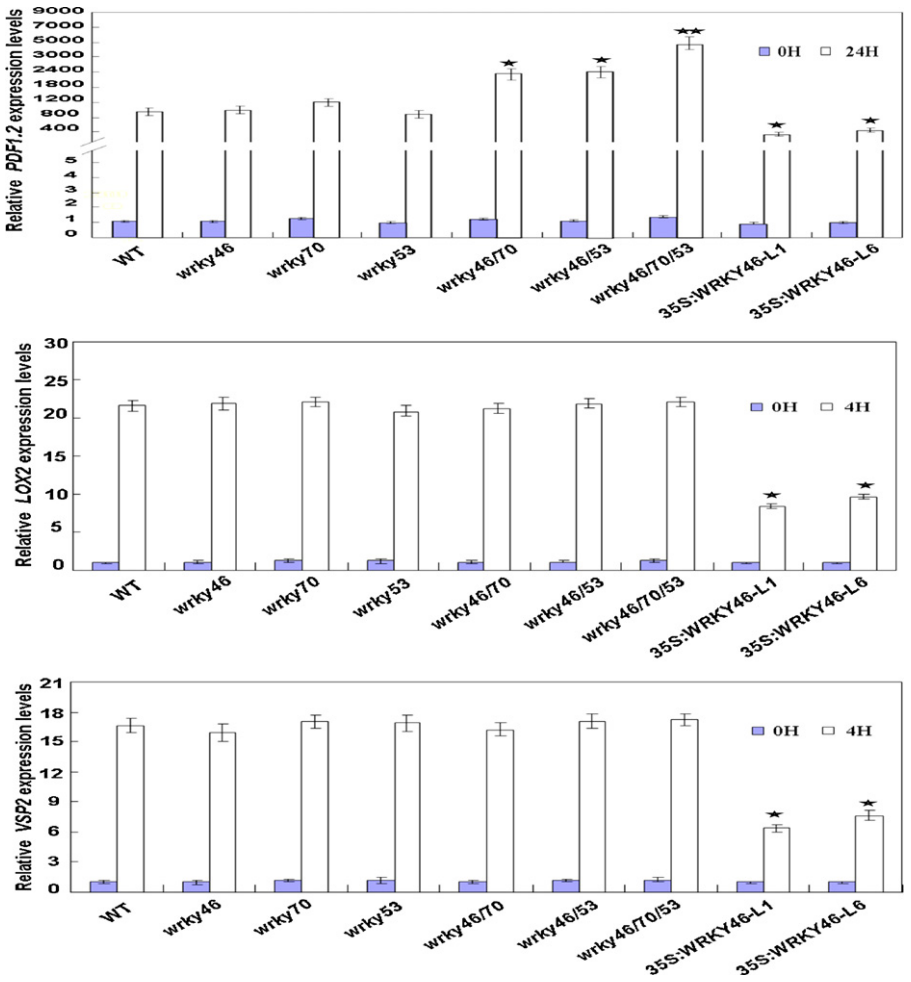

Fig. 7. qRT-PCR analysis of JA-responsive gene expression. The transcript levels of well-characterized JA-regulated genes *PDF1.2*, *LOX2*, and *VSP2* were determined by qRT-PCR. RNA samples were prepared from leaves of 30-day-old WT, various loss-of-function mutants and *WRKY46* over-expression lines before and after JA treatment (0.1 mM). Error bars indicate standard deviations from three independent RNA extracts. **Differences for the mutants compared with corresponding wild type are highly significant ($P < 0.01$). *Differences for the mutants compared with corresponding wild type are significant ($P < 0.05$).

4. Discussion

Arabidopsis WRKY46, encoding a group III WRKY protein, is a SA- and *P. syringae*-responsive gene (Fig. 1B and C). It shares a similar expression pattern with evolutionarily related *WRKY70* and *WRKY53* in response to SA and pathogen induction, which suggests they may function cooperatively in defense responses. Among the three single-knockout mutants analyzed, only *wrky53* suffered a small increase in bacterial growth, while *wrky46* and *wrky70* showed similar phenotype as wild type after *P. syringae* infection (Fig. 3A). This observation is partly consistent with previous findings [24,36,38]. Two double knockout mutants, *wrky46wrky70* and *wrky46wrky53*, supported slightly increased bacterial growth, while the *wrky46wrky70wrky53* triple mutants had the greatest bacterial growth compared to wild-type plants (Fig. 3A). An earlier study also showed that the *wrky70wrky53* double mutants exhibited an enhanced disease symptoms phenotype [51]. In contrast to their knockout mutants, *35S:WRKY46* and *35S:WRKY70* transgenic plants had more resistance to *P. syringae* (Fig. 4, [48]). The role of WRKY46, WRKY70, and WRKY53 in plant defense can also be deduced from expression of pathogen-induced *PR1* marker gene (Figs. 3C and 4C, [48]). These data indicate WRKY46, WRKY70, and WRKY53 may function cooperatively as positive regulators in basal defense against *P. syringae*.

Mutations in most other reported *WRKY* genes, such as *WRKY40*, *WRKY60*, *WRKY11*, *WRKY17*, *WRKY8*, *WRKY38*, *WRKY62*, and *WRKY48* enhanced resistance against *P. syringae* [21,23,32,35,52]. This means that they are negative regulators of basal defense against *P. syringae*. The multiple roles of WRKY proteins may suggest that complex signaling and transcriptional networks of plant defense require tight regulation and fine-tuning. We speculate that WRKY proteins are important for maintaining proper balance of different signaling networks in response to *P. syringae*, resulting in appropriate defense responses against pathogens parasitism while minimizing detrimental effects on plant growth and development. Moreover, increasing evidence has demonstrated that WRKY factors are also involved in regulating various abiotic stresses [53]. However, the mechanisms of WRKY proteins involvement in different regulatory pathways still remain unclear. Further experiments are needed to find putative proteins interacting with WRKY transcription factors and identify their downstream target genes.

Arabidopsis resistance to biotrophic pathogens mainly depends on SA-signaling pathways, as mutations that block SA biosynthesis (*sid2*) or signaling (*npr1*) result in enhanced susceptibility [10,44]. We observed that, after pathogen infection, the *sid2* mutants showed reduced expression of *WRKY46*, *WRKY70*, and *WRKY53* compared to wild type (Fig. 5A). This observation suggested that pathogen-induced expression of *WRKY46*, *WRKY70*, and *WRKY53* is partially dependent on endogenous SA. However, the accumulation

of *WRKY46*, *WRKY70*, and *WRKY53* transcripts was similar in *npr1* mutants and wild-type plants after pathogen infection (Fig. 5A), indicating that they may function in NPR1-independent pathways in response to *P. syringae* infection. Unlike in pathogen-infected plants, exogenous SA-induced expression of *WRKY46*, *WRKY70*, and *WRKY53* was significantly reduced in *npr1* compared to wild type (Fig. 5B). This means that the NPR1-dependent pathway is responsible for SA-induced expression of these three WRKY genes. This result is consistent with a recent report that *WRKY70* and *WRKY53* expression was markedly reduced in the *npr1* mutants after benzothiadiazole S-methylester (BTH; a functional analog of SA) induction, compared to wild type [51]. Thus, there appears to be NPR1-independent and NPR1-dependent pathways for regulated expression of *WRKY46*, *WRKY70*, and *WRKY53*.

Dong et al. showed that the promoters of multiple *WRKY* genes are enriched in W-boxes [34], which indicates that cross-regulation may be a general characteristic of the WRKY super-family. Our results suggest that cross-regulation of WRKY46, WRKY70, and WRKY53 maybe comprise a negative feedback loop during pathogen infection (Fig. 6). Thus, the pathogen induction and mutual suppression appear to consist of positive and negative control elements possibly allowing for an efficient and balanced amplification and diversification of plant defense signals. Other examples of such interaction have been reported in the literature. Chen et al. showed that WRKY40 and WRKY60 appear to play cooperatively a negative role in the ABA-induction of WRKY18 [54]. The group II f member WRKY11 also down-regulates structurally and functionally related *WRKY17* expression during pathogen infection [23]. Nevertheless, there are also positive feedback loops among WRKY proteins. We recently showed that expression of *WRKY25*, *WRKY26*, or *WRKY33* during heat stress partially depended on expression of the other two related genes [55]. Furthermore, the expression of several group III *WRKY* genes showed a sustained expression pattern in *wrky54* mutant after induction [33]. However, the mechanisms of these cross-regulations remain unclear and further work is needed to investigate the interaction of WRKY factors and direct targets promoter region.

Our results showed that JA-induced *PDF1.2* was up-regulated in *wrky46wrky70*, *wrky46wrky53* double mutants and *wrky46wrky70wrky53* triple mutants, while no significant differences were observed between three single mutants and wild-type plants (Fig. 7). This suggests that WRKY46, WRKY70, and WRKY53 function synergistically to suppress the expression of JA-responsive *PDF1.2*. Interestingly, the expression of two early JA-induced genes (*LOX2* and *VSP2*) was not affected in *wrky46wrky70*, *wrky46wrky53* double mutants and *wrky46wrky70wrky53* triple mutants, though they were also down-regulated in *35S:WRKY46* transgenic plants. It is possible that other homologies compensate for the absence of WRKY46, WRKY70, and WRKY53. Mao et al. provided evidence that WRKY62, another group III WRKY protein, also negatively regulates the expression of *LOX2* and *VSP2* [49]. In a time-course experiment, *wrky62* mutants showed enhanced *LOX2* and *VSP2* accumulation compared with wild-type plants.

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