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Full Length Research Paper

Arabidopsis WRKY28 transcription factor is required for resistance to necrotrophic pathogen, *Botrytis cinerea*

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***Arabidopsis thaliana WRKY*28, which is strongly induced by H_2O_2 stress, is one of the group II *WRKY* proteins and responds to *Botrytis cinerea*. In this study, we successfully generated up and down-regulating *WRKY*28 transgenic *A. thaliana* lines. Our investigation into the action of *WRKY*28 demonstrated that the down-regulation of *WRKY*28 resulted in susceptible of plants. The enhanced susceptible in down-regulating plants was associated with the suppression of jasmonate (JA) and ethylene (ET) regulated $PDF_{1.2}$ gene by the necrotrophic fungal pathogen *B. cinerea*. In contrast, disease resistance and the expressions of JA and ET regulated $PDF_{1.2}$ genes are slightly enhanced in *WRKY*28 over-expressing plants. Thus, pathogen-induced *WRKY*28 transcription factor plays a key role in JA and ET mediated defense responses to *B. cinerea*. These results provide a genetic evidence of *WRKY28* in plant defense.**

Key words: Transcription factor, *Botrytis cinerea*, necrotroph fungus.

INTRODUCTION

In nature, plants are quaque threatened by a larger number of harmful pathogens, including viruses, bacteria and fungi. Therefore, plants use surface-localized pattern recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs) (Bittel and Robatzek, 2007). PAMP-triggered immunity (PTI) is considered an ancient form of plant immunity that acts as a first line of inducible defense against harmful pathogens (Chisholm et al., 2006; Jones and Dangl, 2006). However, pathogenic microorganism has evolved the means to suppress PTI by secreting effector proteins into the plant cell cytosol that presumably alter resistance signaling. To defend viruses, bacteria and fungi, plants have evolved some useful proteins to elicit strong immune responses called effector-triggered immunity (ETI) (Chisholm et al., 2006; Jones and Dangl, 2006). Many of plant hormones, including salicylic acid (SA), jasmonic acid and ethylene have been implicated in plants signaling pathways. Often mediate plant defense against biotrophic pathogens. On the other hand, JA and ET often mediate plant defense against necrotrophic pathogens (Glazebrook, 2005). $PDF_{1.2}$ and LOX_2 gene are molecular markers of the JA- and ET-mediated plants defense signaling pathways, the mainly function of $PDF_{1.2}$ gene encodes a protein with antimicrobial activity and correlates with plant defense response (Penninckx et al., 1996, 1998; Thomma et al., 1998), LOX_2 gene which encode enzymes of JA biosynthesis or derivatization correlates with wound-induced and have similar function in resistance to necrotrophic pathogens (Bell et al., 1995; Seo et al., 2001).

There is a large number of evidence demonstrating that some *WRKY* proteins can function as regulators of defense responses in plants, the family has 74 members and is a large family of regulatory proteins in *Arabidopsis thaliana* (Eulgem and Somssich, 2007). They show high binding affinity to a deoxyribonucleic acid (DNA) sequence of the W box (Chisholm et al., 2006), and are divided into three groups based on the number of *WRKY*

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Table 1. Primers used for Q-RT-PCR.

Gene name	Locus	Forward	Reverse
A. thaliana β-Tubulin4	At5g44340	GAGGGAGCCATTGACAACATCTT	GCGAACAGTTCACAGCTATGTTCA
B. cinerea β-Tubulin	Z69263	CCTCCAAGAGATACACCCAAA	CCGAGTCCAGAGAATCAGTCA
A. thaliana β-actin8	At1g49240	AGCTGCAGGGATCCACGAGA	TGCCTGGACCTGCTTCATCA
WRKY28	At5g46350	CATATCCATAATCCAAAGCCG	GAGATCGTTCCAAGATCCAAC
$PDF_{1.2}$	At5g44420	TCATGGCTAAGTTTGCTTCC	AATACACACGATTTAGCACC
LOX_2	At3g45140	GTCCAAACCTCAGAAGACGAT	CACCCATGACTCACATGTAA

domains and the Zn finger like motifs (Eulgem et al., 2000). *WRKY* super family seems to be involved in many plant physiological processes, such as the development of trichome and the biosynthesis of secondary metabolites.

In *A. thaliana* and other plants, accumulating evidence indicates that *WRKY* proteins are involved in biotic stresses responses (Dong et al., 2003; Eulgem and Somssich, 2007; Kim et al., 2006; Li et al., 2006; Ulker and Somssich, 2004; Wang et al., 2006). For instance, in *A. thaliana*, knock down of *AtWRKY23* expression was shown to enhance resistance to the parasitic cyst nematode (Grunewald et al., 2008). *WRKY48* negatively regulates basal resistance toward virulent *Pseudomonas syringae* in *A. thaliana* (Xing et al., 2008), because the expression of *A. thaliana* transcription factor *WRKY*28 was induced by *P. syringae* (Dong et al., 2003), suggesting that transcription factor *WRKY*28 may be correlated with plants defense response and its function can be identified by transgenic technology. In the present study, we found that antisense suppression of *WRKY28* led in reduced resistance to disease and $PDF_{1.2}$ gene expression. Over expression of *WRKY28*, on the other hand, increases resistance to the necrotrophic fungal pathogen *Botrytis cinerea*. The increase resistance of *WRKY28* over expressing plants to *B. cinerea* is associated with enhanced expression of the JA AND ET-regulated $PDF_{1.2}$ gene.

MATERIALS AND METHODS

Materials and stress-induction treatments

A. thaliana (ecotype RLD) were kept in a growth cabinet at 22°C under a 10 h light/14 h dark cycle in soil. H_2O_2 was diluted with water and a 5 mM solution was sprayed onto *A. thaliana*. Leaves were harvested at time points 0, 1, 2, 4, 8, 16 and 24 h during treatment, samples were flash frozen in liquid nitrogen and kept at -80ºC until use. H_2O_2 and DNA polymerase were purchased from Sigma, while other common chemicals were purchased from Shanghai Sangon Biotechnology (Shanghai, China).

Construction of transgenic *WRKY28* over expressing and antisense suppression plants

To generate the *WRKY28* over expressing construct, an approximately 960-bp DNA fragment of *WRKY28* was amplified with primers (5′-GAGGGAGCCATTGACAACATCTT-3′ and 5′ GCGAACAGTTCACAGCTATGTTCA-3′) and digested with *Bam*HI/*Sma*I then subcloned into the *Bam*HI/*Ecl*136II restriction sites of pBI121 in the sense orientation behind the 35S promoter. Amplifying an approximately 960-bp DNA fragment from the *WRKY28* over-expressing construct by using two gene specific primers (5′-CCCCCGGGGGTCATCTCTTCTTCTTTGT-3′ and 5′-CGGGATCCCGAACTGTCTCATTCTTTCA-3′) was subcloned into the *Bam*HI/*Ecl*136II sites of pBI121, resulting in the construct of antisense suppression of *WRKY28*. The reliability of the constructs was confirmed by restriction and sequence analysis. *A. thaliana* transformation was performed by the floral-dip procedure (Clough and Bent, 1998). The transformed lines were selected for resistance to kanamycin (50 µg/ml). And confirmed by PCR using primers (*WRKY28* over expressing plants: 5′-ACCTAACAGAACTCGCCGTAAA-3′ and 5′-GGAAGAGGGAGAAAGACCAAAA-3′: antisense suppression of *WRKY28*: 5′-ATGGACCCCCACCCACGAG-3′ and 5′ -GATTGCCGCCAAGACACCG3′).

Quantitative RT-PCR analysis

Material for RNA analysis was ground in liquid nitrogen, and total RNA was isolated using the Nucleospin RNA plant kit (Promega) according to the manufacturer's recommendations. Reverse transcription (RT) was performed with SuperScriptTMIII Reverse Transcriptase (*Invitrogen*).

Q-RT-PCR using a 29 SYBR Green I master mix in 7500 fast real-time PCR system (Applied Biosystems) according to the manufacturer's instruction with the following conditions: 1 cycle of 5 min at 95°C, and 45 cycles of 5 s at 95°C, 10 s at 57°C, and 20 s at 72°C. The primer sets used in the experiments are listed in Table 1. Efficiency of the amplification was verified by the analysis of standard curves. The specificity of the amplifications was verified by melting curve analysis and by analysis of the PCR products on agarose gels to ensure that only a single band was present in the experiments.

Production of recombinant protein

To generate the *WRKY*28 recombinant protein, its full-length cDNA was cloned into pET28a (Novagen) and transformed into *Escherichia coli* strain BL21 (DE3). Induction of expression of recombinant His tagged *WRKY*28 protein were performed according to the protocol provided by Novagen.

Fungal culture and disease assays

The *B. cinerea* used in these experiments was cultured on 2×V8 agar (36% V8 juice, 0.2% $CaCO_3$, and 2% agar) and incubated at

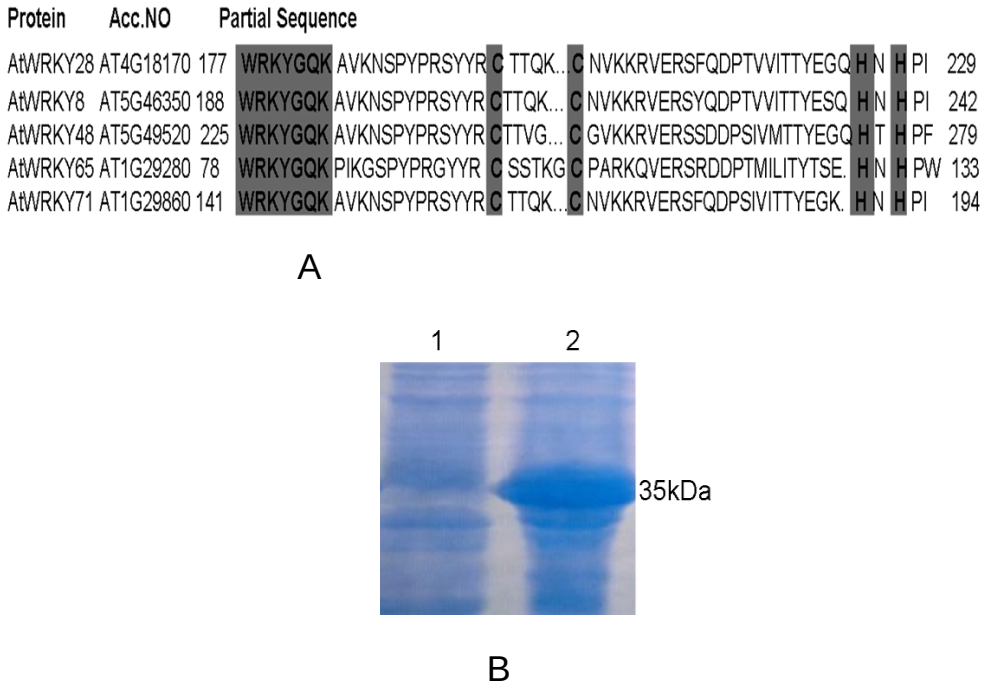


Figure 1. The sequence alignment and protokaryon expression of *WRKY*28. A. Sequence alignment of *WRKY*28. B. SDS-PAGE analysis of the recombinant *WRKY*28. 1: control; 2: culture of the induced by 1 mM isopropylthio-*β*-galactoside (IPTG).

20 to 25°C. Plant inoculation and disease assays were done as described by Mengiste et al. (2003). However, inoculated plants were maintained in high humidity by keeping them under a transparent cover.

Accession numbers

The genes from this article are under accession numbers: At5g46350 (*AtWRKY28*), At5g44340 (*Arabidopsis β-Tubulin4*), At2g14610 (*PR*$_1$), At3g45140 (*LOX*$_2$), Z69263 (*Botrytis cinerea β-Tubulin*), At5g44420 (*PDF*$_{1.2}$), At1g49240 (*Arabidopsis β-actin8*).

RESULTS AND DISCUSSION

Protein structure and expression pattern

Analyses of protein sequence reveals that *A. thaliana* *WRKY*28 has 319 amino acids containing one *WRKY*GQK domain (177 to 183 amino acid) and one Cys_2His_2 zinc-finger motifs (Figure 1A), which is classified as a group IId *WRKY* protein (Dong et al., 2003) and share similar structure with *WRKY*8, *WRKY*48, *WRKY*65, and *WRKY*71 (Figure 1A). To determine the molecular weight of the *WRKY*28 protein, its full-length cDNA was cloned into pET28a in the *Bam*HI/*Xho*I restriction sites and transformed into *E. coli* strain BL21 (DE3). As shown in Figure 1B, *WRKY*28 protein is approximately 35 kDa.

Next, we examined the expression pattern of *WRKY28* in *A. thaliana*. By quantitative real-time PCR (Q-RT-PCR) analyses, we investigated expression of the *WRKY28* gene in response to abiotic treatments. *WRKY28* is weakly expressed in leaves treated with H_2O, as shown in Figure 2A, *WRKY28* transcript levels increased rapidly in wild-type plants after spraying with H_2O_2 (5 mM) and peaked at 1 h.

To elucidate the role of the *WRKY*28 proteins in the regulation of *A. thaliana* defense responses of pathogen, the expression of the *WRKY28* was analyzed after challenge with sabouraud maltose buffer (SMB) with *B. cinerea* spore suspension at a density of 5×10^4 spores/ml by quantitative real-time PCR. After inoculation with *B. cinerea*, the expression of *WRKY28* is obviously induced and peaks at 3 days post-inoculation (dpi). On the other hand, *WRKY28* is weakly expressed in leaves treated with SMB (Figure 2B). Together, the quantitative real-time PCR analyses show that *WRKY28* is strongly induced by H_2O_2 and *B. cinerea* infection.

Overexpression or antisense suppression of *WRKY28* modulates plant disease resistance

To evaluate the putative function of *WRKY28* during plant basal disease resistance, we generated *A. thaliana* anti-sense suppression of *WRKY28*; homozygous progenies T_3 were used for further disease resistance analysis. We detected 3 independent lines with the transcript levels that were slightly and significantly lower than those of the wild-type plants by quantitative real-time PCR. At the same time, we generated transgenic *A. thaliana* that

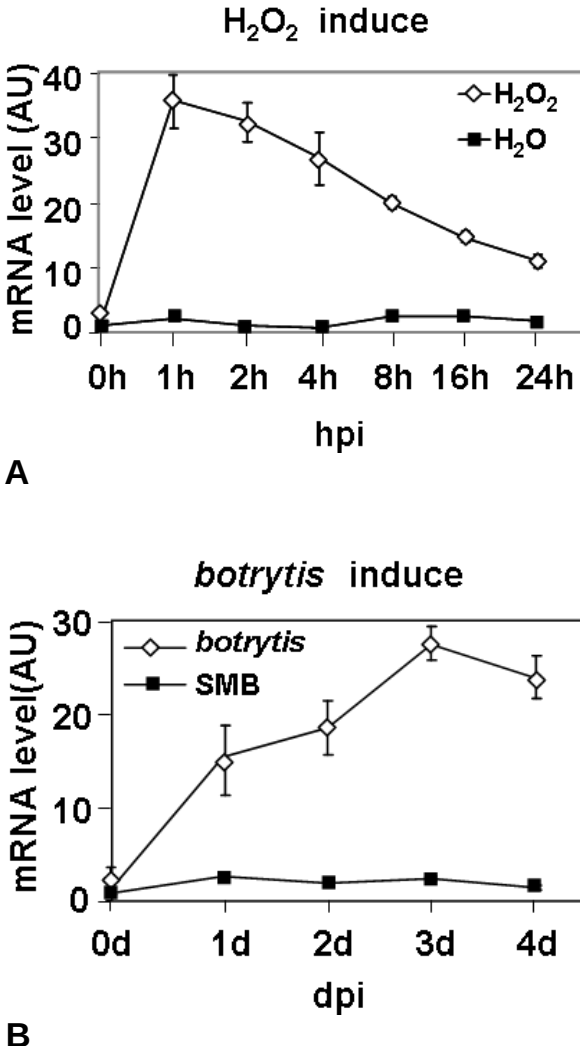
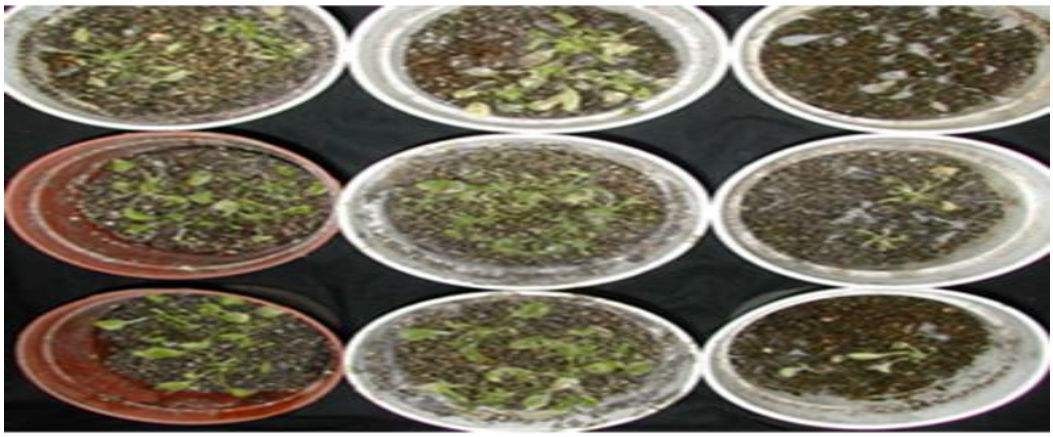


Figure 2. The stess-responsive expression pattern of *WRKY28*. A. Induced by spraying H_2O_2 (5mM). B. Induced by spraying *Botrytis cinerea* sopres. Transcript levels of the *WRKY28* were determined by Q-RT-PCR. The internal standard was the expression level of *Arabidopsis thaliana β-Tubulin4*. The expriments were repeated three times with similar results. dpi, days post-inoculation; hpi, hours post-inoculation; AU, arbitrary units.

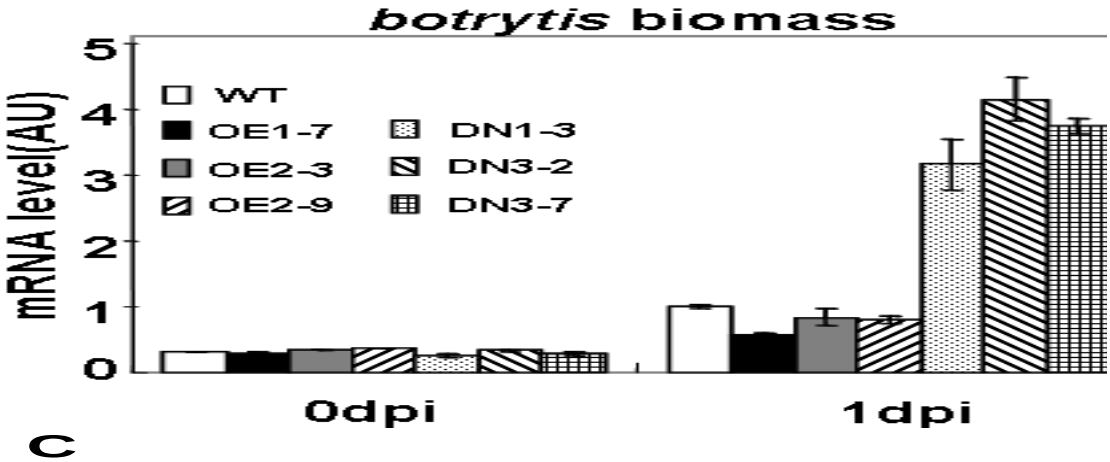
express their cDNAs under the control of the CaMV35S promoter. By quantitative real-time PCR, we selected three of ten independent lines that showed the highest accumulation of transcripts and subjected the selected lines to further experiments.

To investigate possible changes of the *WRKY28* anti-sense suppression lines and over expressing transgenic *A. thaliana* in defense to pathogens, we challenged plants with *B. cinerea* spore suspension at a density of 5 × 10^4 spores/ml. The *WRKY28* over expressing *A. thaliana* developed slightly less disease symptoms than those in the wild-type *A. thaliana* at 5 dpi. On the contrast, antisense suppression of *WRKY28* is susceptible for the *B. cinerea* (Figure 3A). When leaves of transgenic *A. thaliana* of *WRKY*28 were drop-inoculated with *B. cinerea* spores, a single 3 µl drop of suspension of 5 × 10^4 spores/ml in sabouraud maltose broth buffer was placed on each leaf. The disease lesions expanded faster in the antisense suppression of *WRKY28* compared to wild-type plants at 4 dpi. On the contrast, the disease lesions of the *WRKY28* over expressing plants expanded slowly (Figure 3B). The transcript levels of the *B. cinerea β-tubulin* gene correlated with *B. cinerea* biomass (Benito et al., 1998). We investigated the accumulation of the *B. cinerea β-tublin gene* in inoculated *A. thaliana*. The results shown that *WRKY28* over expressing *A. thaliana* had lower levels of *β-tubulin* mRNA of *B. cinerea* accumulated than



A

B



C

Figure 3. Altered responses of *WRKY28* antisense suppression lines and over expressing plants to *B. cinerea*. A. Disease responses of spray-inoculated plants at 5 dpi. B. Disease responses of drop-inoculated plants at 4 dpi. C. Accumulation of the *B. cinerea β-Tubulin* mRNA in spray-inoculated plants. The accumulation of the *B. cinerea β-Tubulin* mRNA for measurement of the biomass of the *B. cinerea* on infected plants and total RNA was isolated from inoculated plants 1 dpi. The expression values of genes were normalized using the expression level of *A. thaliana β-actin8* as an internal standard. Mean expression values were calculated from three independent experiments. dpi, days post-inoculation ; AU, arbitrary units; WT, wild-type, OE, over expressing 1 to 7, 2 to 3 and 2 to 9, DN, antisense suppression 1 to 3, 3 to 2 and 3 to 7.

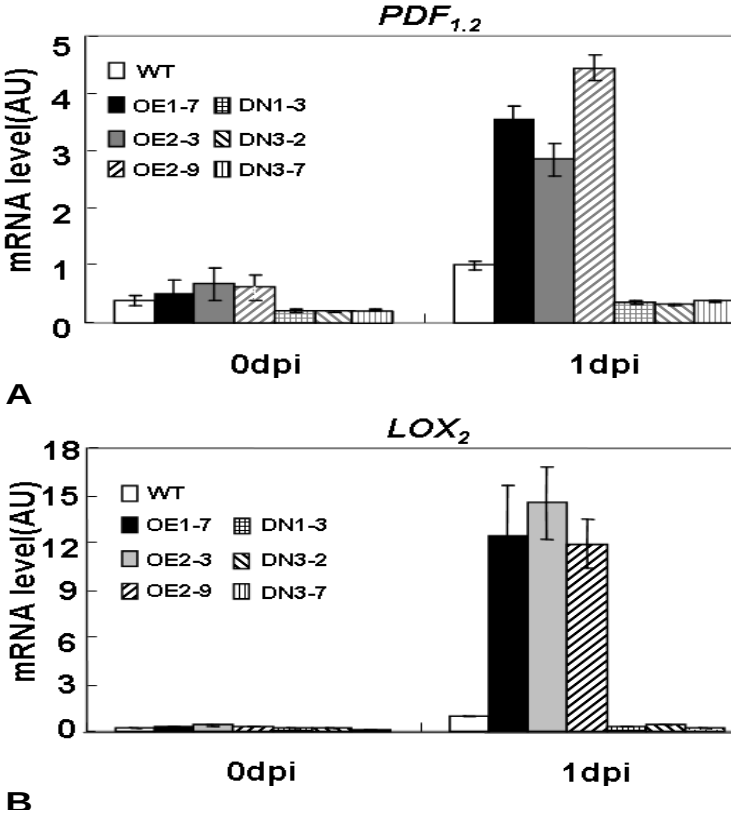


Figure 4. Defense-related gene expression. A. The transcript levels of $PDF_{1.2}$. B. The transcript levels of LOX_2. Plants of wild-type, *WRKY28* over expressing, and antisense suppression lines were sprayed with a sabouraud maltose buffer with *B. cinerea* spore suspension at a density of 5×10^4 spores/ml. Total RNA was isolated from leaves of 4-week-old *A. thaliana* harvested 24h after spraying. The transcript levels of LOX_2 and $PDF_{1.2}$ were determined by Q-RT-PCR. The expression values of the individual genes were normalized using the expression level of *A. thaliana β-Tubulin4* as an internal standard. The experiments were repeated at least three times with similar results. dpi, days post-inoculation; WT, wild-type, OE, over-expressing 1 to 7, 2 to 3 and 2 to 9, DN, antisense suppression 1 to 3, 3 to 2 and 3 to 7; AU, arbitrary units.

those in wild-type *A. thaliana* by 1 dpi. As shown in Figure 3C, antisense suppression of *WRKY28* had higher transcript levels for the *B. cinerea β-tubulin* gene. Thus, both growth of the *B. cinereas* and symptom development confirmed that the antisense suppression of *WRKY28* were more susceptible to *B. cinerea*, on the contrast, *WRKY28* over expressing transgenic plants were increase resistance to *B. cinerea*.

Defense-related gene expression

To investigate the molecular basis of the disease resistance for *WRKY28*, we study the expression level of the pathogen-responsive marker genes by Q-RT-PCR. After *B. cinerea* infection, $PDF_{1.2}$ and LOX_2 transcripts levels were reduced in the antisense suppression of *WRKY28* than the wild-type plants (Figure 4). On the other hand, the levels of $PDF_{1.2}$ and LOX_2 transcripts in the *WRKY28* over expressing plants were highly related to those in wild-type plants (Figure 4). These results suggest that *WRKY28* is a positive regulator of JA and ET-regulated LOX_2 and $PDF_{1.2}$ gene.

*WRKY*28 as a positive regulator of JA and ET-mediated plant defense response

Plant defense responses are associated with the transcriptional activation of many of plant host genes when they are challenged with microbe pathogens (Rushton and Somssich, 1998). Transcriptional regulation of gene expression is largely mediated by transcription factors which can specify recognition of cis-acting promoter

elements. Among the several classes of transcription factors associated with plant defense responses are the *WRKY* super family (Ulker and Somssich, 2004). The recently accumulating evidence directly confirmed the involvement of *WRKY* super family in plant defense responses. For example, over expression of *OsWRKY71* produced an enhanced resistance to virulent bacteria in rice (Liu et al., 2007). In *A. thaliana*, the expression of *AtWRKY7* enhanced plant susceptibility to *P. syringae* (Kim et al., 2006). Over expression of *WRKY25* resulted in increased disease symptoms to *P. syringae* infections by negatively regulating salicylic acid-mediated disease resistance in *A. thaliana* (Zheng et al., 2007). In the present study, we analyzed the roles of the *A. thaliana WRKY28* gene in resistance of plant to diseases; our results suggest that *WRKY28* is important for resistance to necrotrophic fungal *B. cinerea*.

Necrotrophic fungal pathogen *B. cinerea* causes severe disease in a wide range of crops in the field, and result in significant economic losses. The genetic control and molecular basis involved in plant resistance to *B. cinerea* are poorly understood. Accumulating evidence implies that JA and ET and their related compounds play a key role in regulating plant defense responses to necrotrophic fungal pathogen infection (Turner et al., 2002). Through analysis of both transgenic over expression and antisense suppression of *WRKY28*, we have known that *WRKY28* gene is an important regulator of defense responses. Antisense suppression of *WRKY28* enhanced susceptibility to the necrotrophic pathogens *B. cinerea*. On the contrast, over expression of *WRKY28* gene enhanced resistant to *B. cinerea* than those in wild-type plants. From the results we have known that *A. thaliana* transcription factor *WRKY28* plays a key role in defense against necrotrophic pathogens *B. cinerea*. Furthermore, expression of $PDF_{1.2}$ and LOX_2 were decreased in antisense suppression of *WRKY28* after *B. cinerea* infection, on the contrast, expression of $PDF_{1.2}$ and LOX_2 were increased in *WRKY28* over expressing plants. Thus, the key role for *WRKY28* in plant defense against *B. cinerea* may be mediated by its action as a positive regulator of JA and ET-mediated defense response signaling.

Is *WRKY*28 a regulator of SA-mediated plant defense response?

Salicylic acid and jasmonic acid signaling pathways have a complicated relationship of interactions, including antagonism and synergism (Kunkel and Brooks, 2002). PR_1 is marker genes which regulated SA-mediated plants defense response (Ward et al., 1991; Yang et al., 1997). To further determine the molecular mechanisms of the plant defense responses in the future, we will compare their SA-mediated defense gene PR_1 expression with that of wild-type plants after *P. syringae*. If the defense gene PR_1 expression have changed by *B. cinerea* or *P. syringae*, then it is possible that the function of *WRKY28* gene is involved in both salicylic acid and jasmonic acid signaling pathways.

WRKY28 gene is rapidly induced by H_2O_2 in *A. thaliana*, the stress-induced expression implys that reactive oxygen species (ROS) may be candidates as signaling molecules that mediate expression of *WRKY28* gene. In the previous study, pathogen infection is known to generate ROS and cause oxidative stress in plants (Lamb and Dixon, 1997). In some *A. thaliana* mutants, susceptibility to *B. cinerea* correlated with sensitivity to oxidative stress (Mengiste et al., 2003; Tierens et al., 2002; Veronese et al., 2004). The induction of *WRKY28* through ROS-mediated signaling mechanisms is also consistent with its key role in defense against necrotrophic pathogens *B. cinerea*, which is known to promote ROS generation (Govrin and Levine, 2000).

In *A. thaliana*, some of *WRKY* transcription factors have been associated with plant defense responses. More recent studies have provided some direct evidence for their significance as positive and negative regulators of plant defense (Thomas and Imre, 2007). In the present study, our results demonstrate *WRKY28* genes in the regulation of *A. thaliana* disease resistance. Further identification of downstream target genes of *WRKY28* will provide valuable insights into how the *WRKY* transcription factor regulates plant gene expressions and disease resistance to *B. cinerea*.

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