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Wounding-Induced WRKY8 Is Involved in Basal Defense in *Arabidopsis*

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The WRKY family of plant transcription factors controls several types of plant stress responses. *Arabidopsis* WRKY8, localized to the nucleus, is mainly induced by abscissic acid, H_2O_2, wounding, *Pseudomonas syringae* and *Botrytis cinerea* infection, and aphid and maggot feeding. To determine its biological functions, we isolated loss-of-function T-DNA insertion mutants and generated gain-of-function overexpressing *WRKY8* transgenic plants in *Arabidopsis*. Plants expressing the mutated *WRKY8* gene showed increased resistance to *P. syringae* but slightly decreased resistance to *B. cinerea*. In contrast, transgenic plants overexpressing *WRKY8* were more susceptible to *P. syringae* infection but more resistant to *B. cinerea* infection. The contrasting responses to the two pathogens were correlated with opposite effects on pathogen-induced expression of two genes; salicylic acid-regulated *PATHOGENESIS-RELATED1* (*PR1*) and jasmonic acid-regulated *PDF1.2*. Therefore, our results suggest that *WRKY8* is a negative regulator of basal resistance to *P. syringae* and positive regulator to *B. cinerea*.

During the long-term and constant interactions with various microbial pathogens or herbivores, resistant plants have successfully evolved sophisticated defense mechanisms to protect themselves. First, resistant plants recognize microbes or pathogen-associated molecular patterns (PAMP) via pattern recognition receptors, and then activate basal defense responses to halt pathogen infection. This is known as PAMP-triggered immunity (Jones and Dangl 2006). Second, plant hosts recognize specific effector molecules that are introduced into the plant by the pathogen, and then produce disease resistance proteins that activate highly efficient effector-triggered immunity (Jones and Dangl 2006).

Salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) are the main signaling molecules involved in defense responses. The SA signaling pathway is mainly linked to resistance to biotrophic pathogens. The JA and ET signaling pathways mediate resistance mainly to necrotrophic pathogens. Thus, there are complicated defense networks that are induced in response to different types of invading pathogens. Interestingly, the SA and ET/JA signaling pathways often interact in an antagonistic manner (Kunkel and Brooks 2002). For instance, disruptions of JA signaling regulators such as COI1 result in enhanced SA accumulation and signaling in pathogen-infected plants (Kloek et al. 2001), while blocking SA accumulation can promote JA signaling (Spoel et al. 2003). Besides SA, ET, and JA, abscissic acid (ABA) also plays negative or positive roles in plant responses to pathogen attack. For example, enhanced ABA levels are associated with increased susceptibility, whereas reduced ABA levels can increase resistance to many pathogens (Asselbergh et al. 2008; Mauch-Mani and Mauch 2005). Importantly, ABA also has a positive effect on virus infection by inhibiting the transcriptional level of a basic β-1,3-glucanase (*PR2*) (Rezzonico et al. 1998).

In *Arabidopsis*, the plant WRKY transcription superfamily consists of an estimated 74 members that fall into three major structural groups (Eulgem 2005). There is a large body of evidence demonstrating that specific WRKY proteins can function as positive or negative regulators in the basal defense responses of plants. For example, mutations in *Arabidopsis WRKY70* enhanced susceptibility to both biotrophic and necrotrophic pathogens, including the bacterial pathogen *Erwinia carotovora* and the fungal pathogens *Erysiphe cichoracearum* and *Botrytis cinerea* (Li et al. 2004, 2006; AbuQamar et al. 2006). In addition, *wrky70* mutants are compromised in both basal defense and full RPP4-mediated disease resistance to the oomycete *Hyaloperonospora parasitica* (Knoth et al. 2007). Disruption of *WRKY33* enhanced susceptibility to the necrotrophic fungal pathogens *B. cinerea* and *Alternaria brassicicola* (Zheng et al. 2006). Virus-induced silencing of three *WRKY* genes in tobacco compromised *N*-gene-mediated resistance to *Tobacco mosaic virus* (Liu et al. 2004). Overexpression of OsWRKY23 or OsWRKY45 in *Arabidopsis* conferred enhanced disease resistance against the bacterial pathogen *Pseudomonas syringae* (Jing et al. 2009; Qiu and Yu 2009). Thus, certain pathogen-induced WRKY proteins can positively regulate plant disease resistance.

In contrast, several recent studies have shown that a number of WRKY proteins can function as negative regulators of basal defense responses in plants. For example, mutations of *Arabidopsis WRKY7*, *WRKY11*, and *WRKY17* enhance basal resistance to virulent *P. syringae* strains (Park et al. 2005; Journot-Catalino et al. 2006; Kim et al. 2006). Likewise, the structurally related WRKY18, WRKY40, and WRKY60 also have partially redundant functions as negative regulators of plant resistance against the biotrophic bacterial pathogen *P. syringae* and the fungal pathogen *E. cichoracearum* (Xu et al. 2006;

Ligang Chen and Liping Zhang contributed equally to this work.

Nucleotide sequence data for the genes described in this article are available from The *Arabidopsis* Genome Initiative database under the following accession numbers: *WRKY8* (AT5G46350), *PR1* (At2g14610), and *PDF1.2* (At5G44420).

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*The *e*-Xtra logo stands for "electronic extra" and indicates that three supplementary figures are available online and that Figure 5 appears in color online.

Shen et al. 2007). Recently, WRKY48 and two structurally related proteins, WRKY38 and WRKY62, were shown to have additive functions as negative regulators of basal defense against the bacterial pathogen *P. syringae* (Kim et al. 2008; Xing et al. 2008). As well, barley HvWRKY1 and HvWRKY2 function as PAMP-inducible suppressors of basal defense (Shen et al. 2007). Thus, we can deduce that WRKY transcription factors act as both positive and negative regulators, and that they participate in the tight regulation and fine-tuning of the complex signaling and transcriptional networks of plant defense.

Previous study has shown that numerous *WRKY* genes were induced by *P. syringae* infection at early stages (Dong et al. 2003). However, it is unclear whether *WRKY* genes' expression was affected by *P. syringae* infection at later stages. Based on the obviously stronger expression of *WRKY8* in the wild type 2 days after *P. syringae* infection, we choose *WRKY8* for further analysis. Here, we show that *Arabidopsis* WRKY8, which is localized in the nucleus, is induced by mechanical wounding, aphid and maggot feeding, ABA treatment, and pathogen infection. To directly determine the biological functions of *WRKY8*, we have isolated loss-of-function T-DNA insertion mutants and generated gain-of-function transgenic plants overexpressing *WRKY8*. Functional analysis of *wrky8* mutants and transgenic *35S:WRKY8* plants for response to microbial pathogens indicated that the pathogen-induced *WRKY8* transcription factor plays important roles in plant disease resistance.

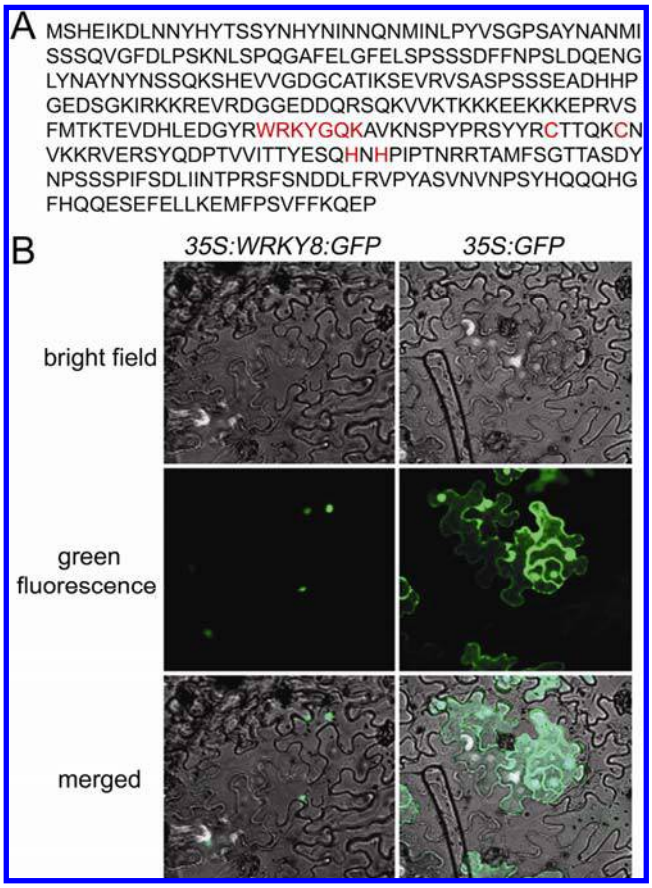

Fig. 1. Sequence and subcellular localization of *WRKY8.* **A,** Amino acid sequence of WRKY8. Highly conserved WRKYGQK sequences and residues forming C_2H_2 zinc-fingers are shown in red. **B,** Subcellular localization of WRKY8. WRKY8 was fused to green fluorescent protein (GFP) to yield *WRKY8:GFP*. The chimeric protein was localized to the nucleus of *Nicotiana benthamiana* leaf cells (left) whereas GFP alone was detected in both the nucleus and the cytoplasm due to its small size (right).

RESULTS

Protein structure, subcellular localization, and expression pattern.

AtWRKY8 (AT5G46350) encodes a protein of 326 amino acids with a molecular weight of 37293.8 Da and an isoelectric point of 8.0888 (Fig. 1A). Sequence analyses revealed that the *WRKY8* protein contains one DNA-binding domain (WRKY domain) located at amino acid positions 182 to 241, and is classified as a group II WRKY protein (Eulgem et al. 2000). As a putative transcription factor, WRKY8 is likely to be localized in the nucleus. To determine the subcellular localization of the WRKY8 protein, we fused the full-length *WRKY8* cDNA to the green fluorescent protein (GFP) gene, then infiltrated the construct into *Nicotiana benthamiana* leaves via *Agrobacterium* spp. infiltration. We analyzed transient expression of the construct using reverse-transcription polymerase chain reaction (RT-PCR) (*data not shown*). The transiently expressed *pWRKY8:GFP* fusion protein was localized exclusively to the nucleus (Fig. 1B). In contrast, free GFP was found in both the nucleus and cytoplasm (Fig. 1B). These results indicate that *WRKY8* protein is localized to the nucleus, supporting its predicted role as a transcriptional regulator.

Next, we examined the expression pattern of *WRKY8* in *Arabidopsis*. First, we used RNA gel blot analyses to investi-

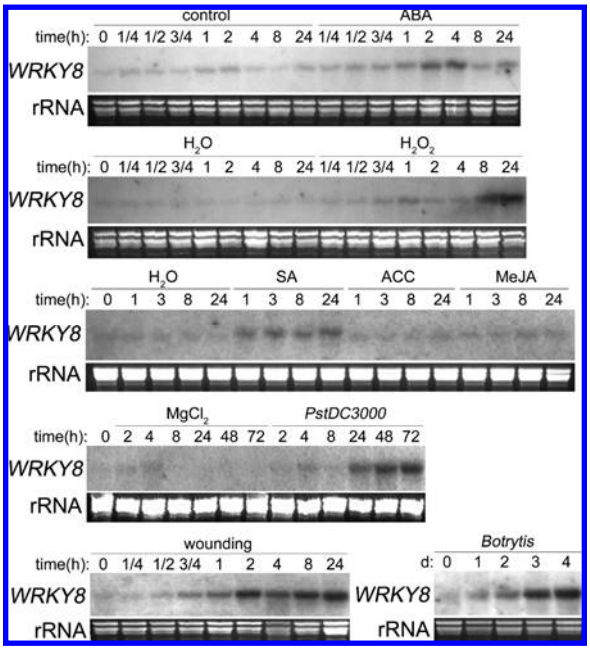

Fig. 2. Expression of *WRKY8*. RNA samples were prepared from 4-week-old *Arabidopsis* plants (Col-0) at given times (hours or days) after spraying with H_2O, abscisic acid (ABA) (0.1 mM), jasmonic acid (JA) (0.1 mM), ethylene (ET) (0.1 mM), salicylic acid (SA) (1 mM), or H_2O_2 (5 mM). Isolated RNAs were probed with a *WRKY8* cDNA fragment. Ethidium bromide–stained ribosomal RNA was used as a loading control. For wounding treatments, leaves of 4-week-old plants were squeezed with forceps two or three times per leaf (approximately 50% of the leaf area) and RNA was isolated from wounded leaves at indicated times. Northern blot analyses were carried out to reveal expression of *AtWRKY8*. For *Pseudomonas syringae* pv. *tomato* DC3000 treatment, 5-week-old wild-type (Col-0) plants were infiltrated with a suspension of *P. syringae* pv. *tomato* DC3000 (optical density at 600 nm = 0.0001 in 10 mM $MgCl_2$). Inoculated leaves were collected at indicated times, then RNAs were isolated and Northern blot analyses were carried out. 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 indicated times. Expression of *WRKY8* was determined by Northern blot analysis.

gate expression of the *WRKY8* gene in response to various treatments. Expression of *WRKY8* was rapidly induced by ABA treatment (100 μM) and peaked at 4 h (Fig. 2), which is consistent with the existence of ABRE elements upstream the *WRKY8* promoter (Supplementary Fig. 1). Expression of *WRKY8* was also induced by H_2O_2. Expression of *WRKY8* was slightly induced by SA but not by methyl jasmonate (MeJA) or 1-aminocyclopropane-1-carboxylate (ACC) treatments (Figs. 2 and 3Ai). We also examined expression of the *WRKY8* gene during biotic and abiotic stress treatments. Expression of *WRKY8* was strongly induced in *P. syringae*-infected plants at 1, 2, and 3 days postinoculation (dpi), compared with $MgCl_2$-infiltrated plants (Fig. 2). Infection of *B. cinerea* also induced strongly expression of *WRKY8* (Fig. 2). Accumulation of *WRKY8* messenger RNA (mRNA) was detected as early as 2 dpi and levels of expression remained high during the infection of *B. cinerea*. Mechanical wounding also induced expression of *WRKY8.* Together, these results indicate that the *WRKY8* gene is involved in responses to multiple stresses.

To determine the expression patterns of *WRKY8* more precisely, we generated two homozygous T_3 lines of *pWRKY8*:*GUS* transgenic plants. Once we confirmed that *WRKY8* was upregulated by ABA, H_2O_2, *B. cinerea*, *P. syringae,* and wounding by Northern blotting analysis (Fig. 2), we next tested whether the *WRKY8* promoter was responsible for these upregulations. β-glucuronidase (GUS) activities were observed in leaves after ABA, H_2O_2, wounding or rubbing treatments, and *B. cinerea* infection (Fig. 3A). Interestingly, *AtWRKY8* was also expressed in response to aphid or maggot feeding because *GUS* expression was observed at the sites of feeding (Fig. 3Ae and f). In addition to the abovementioned pattern of GUS activity, the infection of *P. syringae* induced more strongly *GUS* expression in infected plants than in $MgCl_2$-infiltrated plants, especially at 2 and 3 dpi (Fig. 3B).

Together, the Northern blot and GUS staining results show that *WRKY8* is mainly induced by SA, ABA, H_2O_2, wounding, *P. syringae* and *B. cinerea* infection, and aphid and maggot feeding.

To determine whether wounding- and pathogen-induced *WRKY8* expression is influenced by the SA, ET, or JA signaling pathways, wounding- and pathogen-induced *WRKY8* expression was monitored in various signaling mutants. Induced *WRKY8* expression was modestly reduced in the *npr1-3* and *sid2-3* mutants, which are defective in SA signaling and biosynthesis, respectively (Cao et al. 1997; Wildermuth et al. 2001) (Fig. 4). By contrast, in the JA-insensitive *coi1-1* and ET-insensitive *ein2-1* mutants (Xie et al. 1998; Alonso et al. 1999), there were slightly higher expression levels of *WRKY8* transcripts than those in wild-type plants (Fig. 4). These results suggest that wounding- and pathogen-induced *WRKY8* expression is positively regulated by the SA signaling pathway but negatively regulated by the JA and ET signaling pathways.

Isolation of three *AtWRKY8* T-DNA insertion mutants.

To study the functions of WRKY8 directly, we first characterized three T-DNA insertion alleles of WRKY8 from Salk T-DNA populations (Alonso et al. 2003). The T-DNA insertions in the mutants, designated *wrky8-1* (SALK_107668), *wrky8-2* (SALK_050194), and *wrky8-3* (SALK_117175), were first confirmed by PCR using primers specific to the *WRKY8* gene and the T-DNA insertions in the three alleles (*data not shown*). The three *wrky8* mutant alleles carry T-DNA insertions in the first intron (720 bp from the translation start), the second intron (2,123 bp from the translation start), and the third exon (2,347 bp from the translation start), respectively (Fig. 5A). Northern blotting and RT-PCR analysis confirmed the absence of the normal, full-length WRKY8 transcripts in wounding-treated *wrky8-1* homozygous lines, and a very faint band in the *wrky8-2*

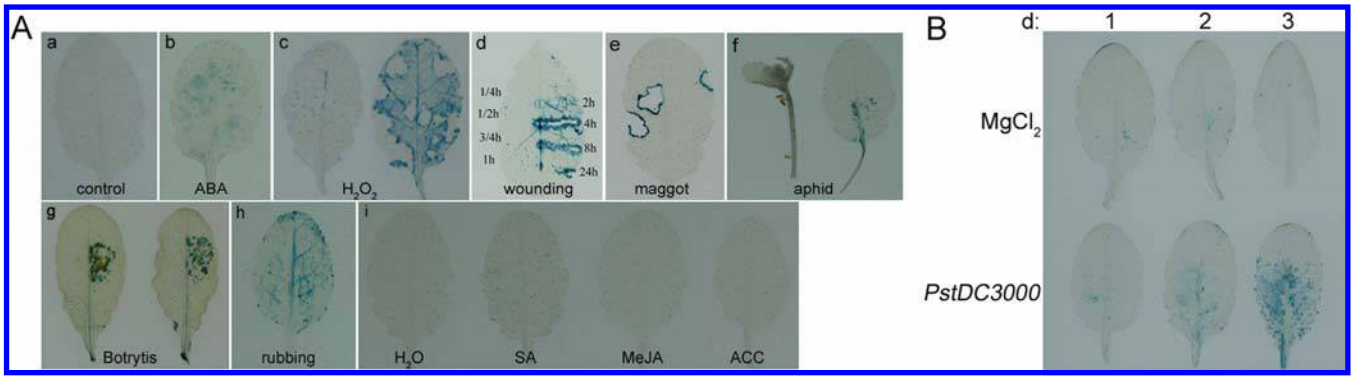

Fig. 3. β-Glucuronidase (GUS) staining of *WRKY8*. **A,** GUS staining of *AtWRKY8* after treatment with abscisic acid (ABA), H_2O_2, wounding, maggot or aphid feeding, *Botrytis* infection; Aa, H_2O; Ab, ABA (4 h); Ac, H_2O_2 (left, 4 h; right 24 h); Ad, wounding (time course of 24, 8, 4, 2, 1, three-quarter, half, and one-quarter h, respectively); Ae, maggot feeding; Af, aphid feeding; Ag, *Botrytis* infection(3 days); Ah, rubbing (24 h); Ai, salicylic acid (SA), methyl jasmonate (MeJA), and 1-aminocyclopropane-1-carboxylate (ACC) (3 h). **B,** GUS staining of *AtWRKY8* at indicated times after inoculation with 10 mM $MgCl_2$ or *Pseudomonas syringae* pv. *tomato* DC3000 (optical density at 600 nm = 0.0001 in 10 mM $MgCl_2$). These experiments were performed two times with similar results.

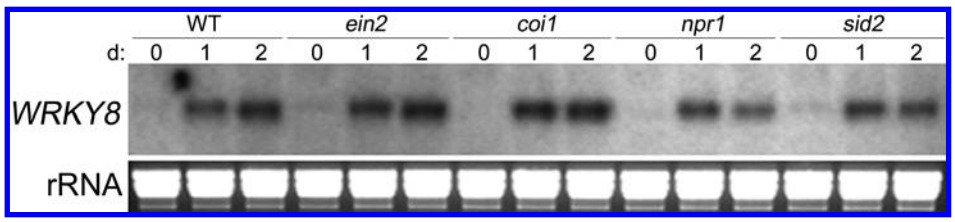

Fig. 4. Expression of *WRKY8* in defense signaling mutants infected with *Pseudomonas syringae* pv. *tomato* DC3000. Five-week-old *Arabidopsis* wild-type (WT) (Col-0) and mutant plants were infiltrated with *P. syringae* pv. *tomato* DC3000 (optical density at 600 nm = 0.0001 in 10 mM $MgCl_2$). The infiltrated leaves were collected at indicated times after inoculation for RNA isolation. Northern blot analyses were carried out to reveal expression of *AtWRKY8*.

and *wrky8-3* mutant plants (Fig. 5B; Supplementary Fig. 2).The *wrky8* mutants grew at the same rate and flowered at the same time as wild-type plants, and showed normal plant morphology.

To characterize the role of *WRKY8* more precisely, we generated transgenic *Arabidopsis* plants constitutively expressing *WRKY8* under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. Northern blotting showed that several transgenic plants constitutively expressed high levels of *WRKY8* transcripts, even in the absence of pathogen infection or wounding treatments. Similar to transgenic *Arabidopsis* plants that constitutively express *WRKY18* or *WRKY48* (Chen and Chen 2002; Xing et al. 2008), plants constitutively expressing *WRKY8* were smaller in size and had slightly serrated leaves compared with the wild type (Fig. 5D). Finally, we chose two transgenic lines (Fig. 5C, nos. 4 and 7) for further study. These lines constitutively expressed *WRKY8* at elevated levels and contained a single T-DNA locus in their genomes based on the ratio of antibiotic resistance phenotypes.

Disrupted or altered *WRKY8* expression affected responses to *P. syringae.*

To determine the possible roles of the *WRKY8* gene in plant defense, we first examined the response of wild-type and *wrky8* mutant plants to a virulent strain of *P. syringae*. Plants were inoculated with the bacteria and the growth of the pathogen was monitored. The homozygous *wrky8* mutants had an approximately two- to threefold reduction in the growth of the bacterial pathogen at 3 dpi, compared with the wild-type (Fig. 6A). Moreover, the inoculated leaves of the *wrky8* mutant showed less chlorosis than wild-type plants at 3 dpi (Fig. 6B). Thus, disruption of *WRKY8* enhanced resistance to the bacterial pathogen.

To further characterize the role of *WRKY8* in defense against *P. syringae*, we compared bacterial growth in *35S:WRKY8* transgenic plants with that in wild-type plants. Conversely to *wrky8* mutants, there was a marked increase (seven- to 10-fold) in bacterial growth in *35S:WRKY8* transgenic plants compared with wild-type plants (Fig. 6A). The transgenic plants also developed more severe disease symptoms than the wild-type plants after infection with *P. syringae* (Fig. 6B). Thus, constitutive overexpression of *WRKY8* led to increased growth of the bacterial pathogen and enhanced development of disease symptoms in the transgenic plants.

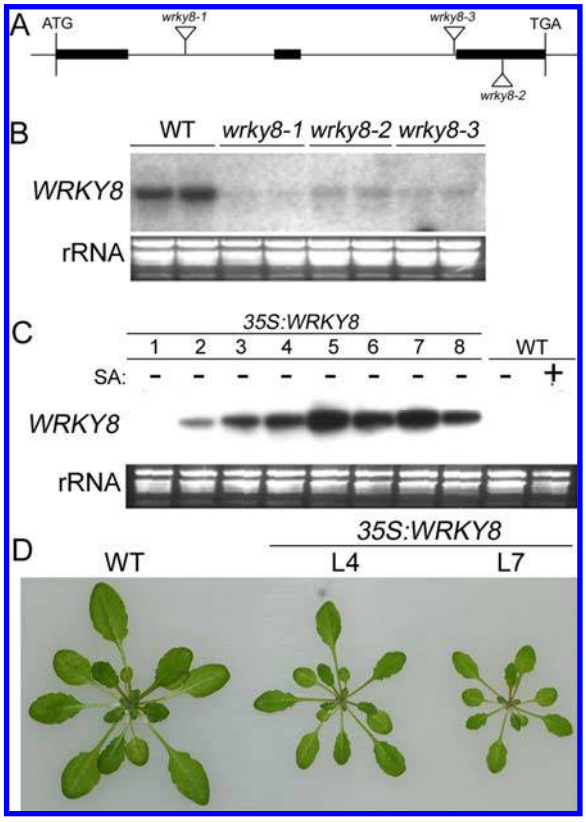

Fig. 5. T-DNA insertion mutants and overexpression lines for *WRKY8*. **A,** Diagram of *WRKY8* gene and T-DNA insertions in *AtWRKY8* mutants. **B,** Northern blot analysis of *wrky8* mutants. RNA samples were prepared from 24-day-old wild-type (WT) *Arabidopsis* and *wrky8* mutant leaves 4 h after wounding treatment. RNAs were probed with a *WRKY8* cDNA fragment. Ethidium bromide–stained ribosomal RNA (rRNA) was used as a loading control. **C,** Northern blot analysis of *WRKY8* expression in transgenic plants constitutively overexpressing *WRKY8*. RNA samples were prepared from leaves of eight transgenic *35S:WRKY8* lines or leaves of WT plants (Col-0) (treated with 2 mM salicylic acid or untreated). RNAs were probed with a *WRKY8* cDNA fragment, and ethidium bromide–stained rRNA was used as a loading control. **D,** Phenotype of representative 32-day-old WT (Col-0) and *35S:W8* lines 4 (L4) and 7 (L7). Both L4 and L7 contained a single T-DNA insertion in the genome and stably expressed *WRKY8*. Homozygous F3 progeny plants were used in all experiments.

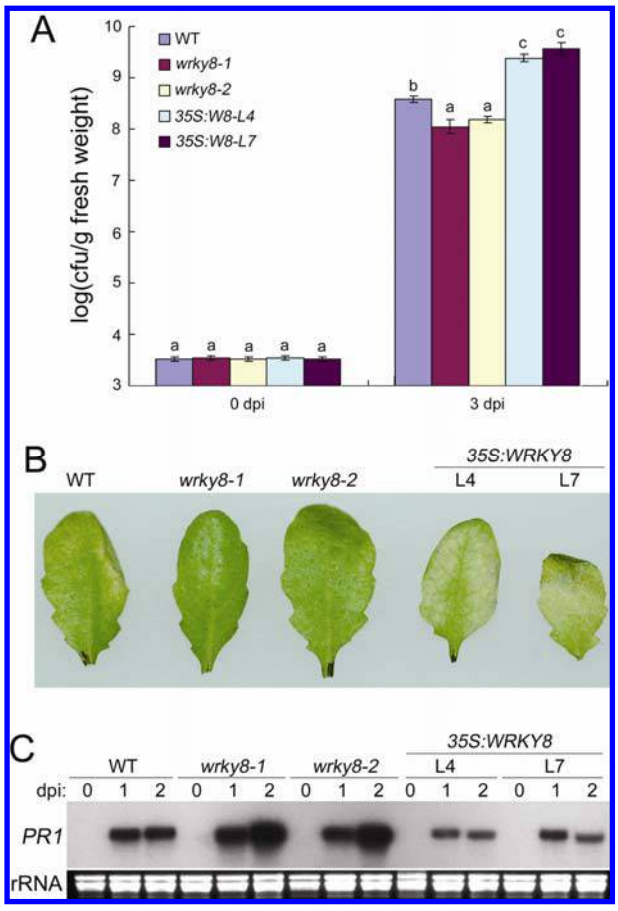

Fig. 6. Altered responses of T-DNA insertion mutants and overexpression plants to *Pseudomonas syringae* pv. *tomato* DC3000. **A,** Altered bacterial growth. Wild-type (WT) (Col-0), *wrky8* mutants, and *WRKY8* overexpression lines were infiltrated with a suspension of *P. syringae* pv. *tomato* DC3000 (optical density at 600 nm = 0.0001 in 10 mM $MgCl_2$). Growth of the bacterial pathogen was assessed at 0 and 3 days postinoculation (dpi). Means and standard errors are shown (n = 6 to 8 plants per treatment). **B,** Disease symptom development. Pathogen inoculation of WT (Col-0), *wrky8* mutants, and *WRKY8* overexpression lines was performed as in A. Pictures of representative inoculated leaves taken at 3 dpi. **C,** *PR1* expression. Pathogen inoculation of WT (Col-0), *wrky8* mutants, and *WRKY8* overexpression lines 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. The experiments were repeated twice with similar results.

Disrupted or altered *WRKY8* expression affected the response to *B. cinerea*.

To determine whether *wrky8* mutant plants are also resistant to a necrotrophic fungal pathogen, we challenged wild-type and *wrky8* mutant plants with *B. cinerea*. Five-week-old plants were inoculated with a *B. cinerea* spore suspension at a density of 5×10^4 spores/ml. At 6 dpi, leaves showing necrotic symptoms were evaluated for disease severity. *B. cinerea* infection caused necrotic symptoms but necrosis remained at localized sites in the wild-type (Col-0) plants (Fig. 7A). Only 20% of the leaves of wild-type plants had disease symptoms at 6 dpi (Fig. 7A and B). However, necrotic symptoms rapidly increased in severity during infection in *wrky8* mutant plants, and approximately 50% of the leaves were severely decayed at 6 dpi (Fig. 7A and B). In addition, higher levels of *β-tubulin* mRNA of *B. cinerea* had accumulated at 4 dpi (Fig. 7C). Thus, disruption of *WRKY8* slightly enhanced susceptibility to the fungal pathogen.

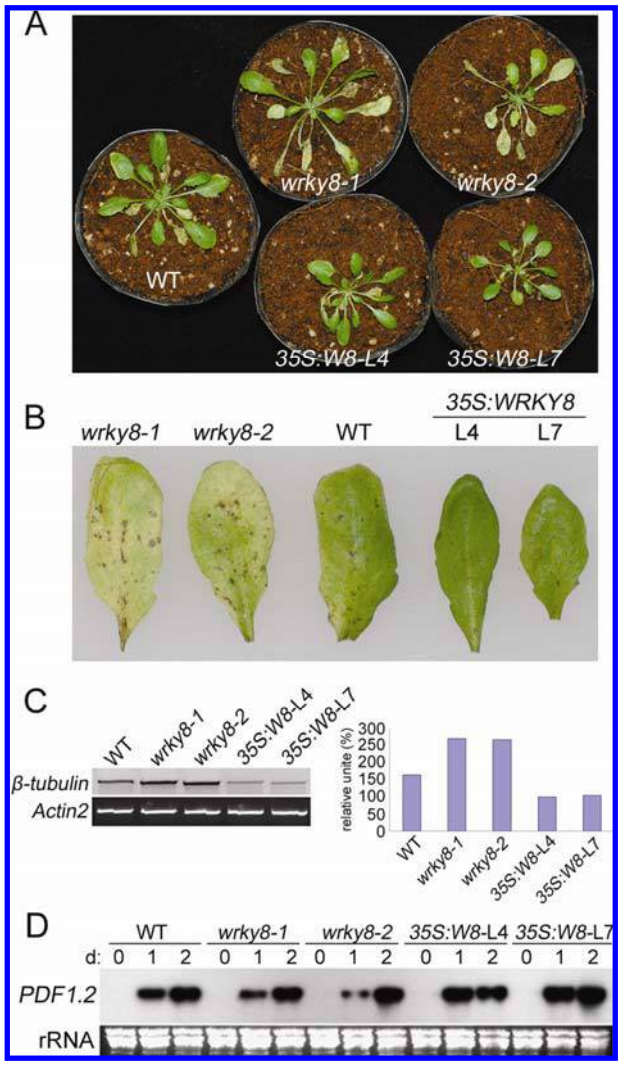

Fig. 7. Responses of T-DNA insertion mutants and *WRKY8* overexpression lines to *Botrytis cinerea* infection. **A** and **B,** Disease symptom development. Leaves of wild-type (WT) (Col-0), *wrky8* mutants, and *WRKY8* overexpression lines were inoculated by spraying with a spore suspension (5×10^4 spores/ml). Plants were maintained at high humidity and disease symptoms were photographed at 6 days postinoculation (dpi). **C,** Accumulation of β-tubulin. Total RNA was isolated from inoculated plants at 4 dpi and reverse-transcription polymerase chain reaction was performed with *Botrytis* spp. β-tubulin gene-specific primers. Quantification of *Botrytis* spp. β-tubulin messenger RNA enabled measurement of fungal biomass on infected plants. The accumulation of various transcripts was quantified by densitometry using Bio-Profile Bio1D software. **D,** *PDF1.2* expression. Pathogen inoculation of WT (Col-0), mutants, and overexpression lines was performed as in A. Total RNAs were isolated from inoculated leaves harvested at 0, 1, and 2 dpi and probed with a *PDF1.2* fragment. Ethidium bromide–stained ribosomal RNA was used as a loading control. The experiments were repeated twice with similar results.

To further characterize the role of *WRKY8* in defense against the fungal pathogen, we compared pathogen growth in *35S:WRKY8* transgenic plants with that in wild-type plants. Conversely to *wrky8* mutants, only 10% of the leaves of *35S:WRKY8* transgenic plants had disease symptoms at 6 dpi, and lower levels of *β-tubulin* mRNA of *B. cinerea* accumulated by 4 dpi (Fig. 7A, B, and C). Thus, constitutive overexpression of *WRKY8* enhanced tolerance toward the fungal pathogen *B. cinerea* and decreased development of disease symptoms in the transgenic plants.

Expression of defense-related genes.

Plants with mutations or constitutive overexpression of *WRKY8* showed contrasting phenotypes with respect to *P. syringae* and *B. cinerea* resistance. This may reflect an antagonistic relationship between SA- and JA/ET-regulated defense pathways. To test this possibility, we analyzed pathogen-induced expression of SA-regulated *PR1* and JA-regulated *PDF1.2* in both loss-of-function and overexpressing transgenic plants (Glazebrook 2005). After inoculation with *P. syringae*, *wrky8* mutants showed enhanced expression of the *PR1* gene (Fig. 6C). Transgenic plants constitutively overexpressing *WRKY8* showed decreased expression of *PR1* at 1 and 2 dpi compared with that in wild-type plants. In contrast, after *Botrytis* spp. infection, there were lower levels of *PDF1.2* transcripts in *wrky8* mutants and higher levels of *PDF1.2* transcripts in *WRKY8*-overexpressing lines when compared with the wild-type plants, especially at 1 dpi (Fig. 7D).

DISCUSSION

Arabidopsis WRKY8 is a stress- and pathogen-induced *WRKY* gene that encodes a nuclear localized WRKY transcriptional factor. To determine its roles in plant defense against microbial pathogens, we analyzed both knockout lines and constitutive overexpression lines with respect to pathogen infection. Two pathogens were tested: the bacterial pathogen *P. syringae* and the fungal pathogen *B. cinerea*.

P. syringae is a biotrophic bacterial pathogen in the early stages of infection but can become necrotrophic at later stages of infection. Thus, it should probably be considered to be a hemibiotroph. Numerous studies have demonstrated that SA-mediated signaling mechanisms play vital roles in limiting *P. syringae* growth. For example, *Arabidopsis* mutants defective in SA biosynthesis or signaling, such as *eds1*, *pad4*, *eds5*, *sid2*, and *npr1*, allow increased growth of *P. syringae* (Glazebrook et al. 1996; Rogers and Ausubel 1997; Aarts et al. 1998; Zhou et al. 1998; Nawrath and Metraux 1999). Mutants with constitutively high SA levels, such as *accelerated cell death 6* (*acd6*) and *aberrant growth and death 2* (*agd2*) (Rate et al. 1999; Rate and Greenberg 2001), displayed increased resistance to *P. syringae*. Plants with defective *wrky8* exhibited a two- to threefold reduction in bacterial growth compared with wild-type plants (Fig. 6A). In contrast, constitutive overexpression of *WRKY8* led to enhanced susceptibility to the bacterial pathogen *P. syringae*, as manifested by enhanced growth of the bacterial pathogen and increased and more rapid development of disease symptoms (Fig. 6A and B). Similar to *WRKY18* and *WRKY48* (Chen and Chen 2002; Xing et al. 2008), overexpression of *WRKY8* resulted in transgenic plants that were smaller in size and showed a slightly higher degree of serration in the leaves (Fig. 5D).

However, *WRKY8* and *WRKY48* have effects opposite to that of *WRKY18* with respect to defense against *P. syringae*. Thus, we can deduce that the more susceptible phenotype of the transgenic *35S:WRKY8* plants are unlikely to result from their altered growth and morphology. In addition, we observed that the enhanced resistance or susceptibility of *wrky8* mutant plants and transgenic plants to *P. syringae* was associated with increased or reduced expression of *PR1*, a defense-related gene often associated with SA-mediated defense responses. Thus, *WRKY8* might play a negative role in SA-mediated signaling pathways. Overexpression of the gene could have a negative impact on pathogen-induced, SA-mediated defense mechanisms and, thus, compromise plant resistance to *P. syringae*.

B. cinerea is a necrotrophic fungal pathogen that promotes host cell death at very early stages in infection. In *Arabidopsis*, resistance to *B. cinerea* depends on JA and ET signaling pathways because mutations that block JA signaling, including *coi1* and *jar1*, or ET signaling, including *ein2*, result in enhanced susceptibility (Glazebrook 2005). Our analyses revealed that loss-of-function mutants for the WRKY8 gene were slightly more susceptible to *B. cinerea* than the wild-type plants, as measured by enhanced disease symptoms and increased pathogen growth in inoculated plants (Fig. 7A and B). On the other hand, constitutive overexpression of *WRKY8* led to slightly decreased susceptibility to the fungal pathogen. Interestingly, these altered phenotypes were opposite to those observed with the bacterial pathogen *P. syringae*. Furthermore, expression of *PDF1.2,* a molecular marker of the JA- and ET-mediated defense response signaling pathways, differed between the two transgenic lines. Expression of *PDF1.2* was decreased in *wrky8* mutants after *Botrytis* spp. infection but increased in *WRKY8*-overexpressing transgenic plants.

Our results show that *wrky8* mutants and *WRKY8*-overexpressing transgenic plants showed opposite responses with respect to pathogen-induced expression of SA-induced *PR1* and JA-regulated *PDF1.2* (Glazebrook 2005). These opposite responses to the two pathogens probably reflect the antagonism between SA- and JA-mediated defense signaling pathways (Kunkel and Brooks 2002; Li et al. 2004; Takahashi et al. 2004; Pieterse et al. 2009). Together, our results suggest that the WRKY8 protein may function as a negative regulator in SA-dependent pathways but as a positive regulator in JA-mediated pathways. The expression of *SID2*, *NPR*1, *JAR1*, or *JAZ1* were not affected in *WRKY8*-overexpressing or mutant plants, which suggested that *WRKY8* may work at a point downstream of both SA and JA to repress or activate defense responses (Supplementary Fig. 3). Similarly to WRKY8, WRKY33 functions as a positive regulator in JA-mediated defense response signaling and also acts as a repressor of SA-dependent disease resistance (Andreasson et al. 2005; Zheng et al. 2006). In contrast, *WRKY70* plays a positive role in SA signaling and functions as a negative regulator of JA-inducible genes (Li et al. 2004). These examples support the existing trade-off between SA-dependent and JA-dependent defense against biotrophic and necrotrophic pathogens.

We carried out expression analyses using the GUS reporter gene fused to the *Arabidopsis WRKY8* gene promoter. Our results show that this gene is expressed mainly in vascular bundles in response to H_2O_2, wounding, or rubbing treatments. This suggests that reactive oxygen species (ROS) may be candidates as signaling molecules that mediate pathogen-induced expression of the *WRKY8* gene. Pathogen infection results in generation of ROS and causes oxidative stress in plants (Lamb and Dixon 1997). The induction of *WRKY8* through ROS-mediated signaling mechanisms is also consistent with its demonstrated role in defense against hemibiotrophic or necrotrophic pathogens. These pathogens promote ROS generation, which can contribute to induction of cell death. The preferential expression of *WRKY8* in the vascular bundles might contribute to plant defense because these tissues may be important for systemic defense signaling.

Several previous studies have suggested that different WRKY proteins played distinct roles in various signaling pathways of plant defense responses. Interestingly, most of the reported WRKY transcriptional factors, such as WRKY7, WRKY11, WRKY17, WRKY25, WRKY48, WRKY38, and WRKY62, function as negative regulators of plant basal defense against *P. syringae* (Park et al. 2005; Journot-Catalino et al. 2006; Kim et al. 2006, 2008; Zheng et al. 2007, Xing et al. 2008). At the same time, the W-box sequences upstream of the *PR1* gene promoter act as a negative *cis*-acting element in the expression of the defense-related gene (Lebel et al. 1998). This implied that defense genes, such as *PR1*, can be directly repressed by certain WRKY proteins. By contrast, some WRKY transcriptional factors, such as WRKY4 and WRKY33, play positive roles in the basal defense against *Botrytis* spp. (Zheng et al. 2006; Lai et al. 2008). The diverse roles of WRKY proteins may reflect the complex signaling and transcriptional networks of plant defense that require tight regulation and fine-tuning. The underlying mechanisms for the antagonistic interactions between SA-dependent and JA-dependent defense signaling have not been clearly understood. Identification of WRKY transcription factors that affect plant resistance in ways opposite to different types of microbial pathogens suggest that the regulation of the crosstalk between these defense-signaling pathways occurs at the transcription level and also contribute to further elucidate the mechanisms of the antagonistic actions between defense-response pathways.

MATERIALS AND METHODS

Materials.

We obtained [^{32}P]-dATP (>3,000 Ci/mmol) from the Beijing Furui Biotechnology Co., Ltd. ABA, SA, MeJA, ACC, and 5-bromo-4-chloro-3-indolyl b-D-glucuronic acid (X-gluc) were purchased from Sigma Co. Ltd. (St. Louis); *Taq* DNA polymerase from TaKaRa Biotechnology (Dalian) Co. Ltd. (Tokyo); and agarose and agar from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China) *Arabidopsis thaliana* plants were grown in an artificial growth chamber at 22°C under 180 μE m^2 s^{-1} light with a photoperiod of 10 h of light and 14 h of darkness. Columbia-0 (Col) was used as the wild type. We obtained *wrky8* mutants in the Col background from the *Arabidopsis* Biological Resource Center.

Induction treatments.

SA was dissolved in water as a 100-mM stock solution and adjusted to pH 6.5 with KOH. Plants were sprayed with a 2-mM SA solution diluted from the stock. MeJA was dissolved in 50% ethanol as a 10-mM stock solution. The MeJA stock solution was diluted to 100 μM with water and sprayed onto plants. ACC was dissolved in water, and a 2-mM solution was sprayed onto plants. ABA (14.1 mg) was first dissolved in 90 μl of ethanol and then water was added to obtain a 10-mM stock solution. The ABA stock solution was diluted to 100 μM with water and sprayed onto plants. H_2O_2 was diluted with water and a 5-mM solution was sprayed onto plants. Wound lesions were generated with forceps by squeezing rosette leaves two to three times, which wounded approximately 50% of the leaf area. In all cases, the aerial parts of 4-week-old plants grown in soil were used.

Subcellular localization.

WRKY8 cDNA was cloned into a GFP vector and subcloned into the *Agrobacterium* spp. transformation vector pOCA30

(Chen and Chen 2002) in the sense orientation behind the CaMV 35S promoter. The construct was then transformed into *Agrobacterium tumefaciens* GV3101. For transient expression in *N. benthamiana*, leaves of wild-type *N. benthamiana* were infiltrated with the bacterial cell suspensions (optical density at 600 nm [OD_{600}] = 0.05, 10 mM MES, 10 mM $MgCl_2$, and 100 mM acetosyringone). Leaves were sectioned 24 to 48 h after infiltration and localization was observed under a confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

Northern blotting and RT-PCR analysis.

For Northern blotting analyses, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). Approximately 20 μg of RNA was separated on an agarose-formaldehyde gel and then blotted onto nylon membranes following standard procedures. 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. DNA probes for WRKY8 were isolated from its full-length cDNA clone. DNA probes for *PR1* and *PDF1.2* were obtained from PCR amplifications using the following gene-specific primers: *PR1*, 5′-TCTTCCCTCGAA AGCTCAAG-3′ and 5′-ACACCTCACTTTGGCACATC-3′; and *PDF1.2*, 5′-ACGGGAAGATGATGTCTGTTT-3′ and 5′-TTCAGTGGTCCTGTTGTAGACA-3′.

For RT-PCR analysis, total RNA was extracted using TRIzol reagent (Invitrogen) and was treated with RNase-free DNase (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed in a 20-μl reaction mixture using the Superscript II (Invitrogen). After the reaction, 1-μl aliquots were used as a template for PCR amplification with the following primers: 5′-ATGATCTCTTCCGTGTGCCA-3′ and 5′-ATCATCAAGGC TCTTGTTTGAAGA-3′ for *WRKY8*, 5′-TGTGCCAATCTAC GAGGGTTT-3′ and 5′-TTTCCCGCTCTGCTGTTGT-3′ for *ACTIN2*, 5′-AGCCTACGATCAAGCTGCTTT-3′ and 5′-TTC ACCAAGTCCCACTATTTTCA-3′ for *ERF1*, 5′-CCATTGA CAGAACTTCCTATTGCT-3′ and 5′-GGTTAGCATTCATA TTTCAGCTGC-3′ for J*AZ1*, 5′-AAGACTATTGGAGCCTT GGAGC-3′ and 5′-AGTGAGTCAAAACGCTGTGCT-3′ for *JAR1*, 5′-AATTTGCAGTCGGGATCAGAT-3′ and 5′-ACTC TTTCTTCAATTAATCGCCTGT-3′ for *SID2*, 5′-ACACTAA AGAAGGCCTTTAGTGAGGA-3′ and 5′-TAAGAGGCAAG AGTCTCACCGA-3′ for *NPR1*.

PCR amplification was then performed with *Taq* DNA polymerase (TaKaRa-Bio).

Isolation of *wrky8* T-DNA insertion mutants.

The *wrky8-1* (SALK_107668), *wrky8-2* (SALK_ 050194), and *wrky8-3* (SALK_117175) lines contain a T-DNA insertion in the first intron, second intron, and third exon, respectively, of the *WRKY8* gene. We confirmed the T-DNA insertions by PCR using a combination of a gene-specific primer and a T-DNA border primer. Homozygous *wrky8* mutant plants were identified by PCR using a pair of primers corresponding to sequences flanking the T-DNA insertion sites (pW8-A, 5′-GG AGAAGATGATCAACGCTCT-3′ and pW8-B, 5′-TCTTAAG GTGTAGTCTGCCAAAA-3′ for *wrky8-1*; and pW8-C, 5′-AA GTCGAAGGGACTCCATATTT-3′ and pW8-D, 5′-CCATTCA TCATGATATGGACTCT-3′ for *wrky8-2* and *wrky8-3*). *wrky8* T-DNA insertion mutants were further confirmed by Northern blot analysis and RT-PCR.

Construction of transgenic overexpression lines.

To generate the *35S:WRKY8* construct, the cDNA fragment containing the full coding sequence and 3′ untranslated region of *WRKY8* was excised from a cloning plasmid and subcloned into the same restriction sites of the *Agrobacterium* transformation vector pOCA30 (Chen and Chen 2002) in the sense orientation behind the CaMV 35S promoter. *Arabidopsis* transformation was performed by the floral dip procedure (Clough and Bent 1998). The seed were collected from the infiltrated plants and selected on half-strength Murashige Skoog medium containing kanamycin at 50 μg/ml. Kanamycin-resistant plants were transferred to soil 8 days after germination and were grown in a growth chamber.

GUS staining.

Histochemical detection of GUS activity was performed with X-gluc as the substrate. Plant tissues were first prefixed in ice-cold 90% (vol/vol) acetone for 20 min, then washed three times with GUS staining buffer (without X-gluc) before incubation in X-gluc solution (1 mM X-Gluc, 50 mM $NaPO_4$ [pH 7], 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, and 0.05% Triton X-100) under vacuum for 10 min at room temperature, then incubated overnight at 37°C. Chlorophyll was removed using several changes of 70% ethanol; then, tissues were photographed.

Pathogen infection.

For each treatment, leaves of six to eight plants were inoculated by infiltration with the *Pseudomonas syringae* pv. *tomato* DC3000 strain containing the pVSP61 kanamycin-resistant empty plasmid vector (OD_{600} = 0.0001 in 10 mM $MgCl_2$). Inoculated leaves were harvested 3 dpi and homogenized in 10 mM $MgCl_2$. 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 was grown on 2× V8 agar as described previously (Mengiste et al. 2003). To infect plants, conidia were collected from a 10-day-old culture and the spore density was adjusted in Sabouraud Maltose Broth and sprayed using a Preval sprayer. Inoculated plants were maintained at high humidity with a transparent cover in a growth chamber, and symptom development was observed from 5 to 7 dpi. Biomass of the fungal pathogen was quantified by RT-PCR of total RNA isolated from inoculated plants. For GUS staining, a single 2.5-μl drop of a suspension of 2×10^5 spores ml^{-1} in Sabouraud maltose broth (SMB) buffer was placed on each leaf.

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