Physical and Functional Interactions between Pathogen-Induced *Arabidopsis* WRKY18, WRKY40, and WRKY60 Transcription Factors [W]

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Limited information is available about the roles of specific WRKY transcription factors in plant defense. We report physical and functional interactions between structurally related and pathogen-induced WRKY18, WRKY40, and WRKY60 transcription factors in *Arabidopsis thaliana*. The three WRKY proteins formed both homocomplexes and heterocomplexes and DNA binding activities were significantly shifted depending on which WRKY proteins were present in these complexes. Single *WRKY* mutants exhibited no or small alterations in response to the hemibiotrophic bacterial pathogen *Pseudomonas syringae* and the necrotrophic fungal pathogen *Botrytis cinerea*. However, *wrky18 wrky40* and *wrky18 wrky60* double mutants and the *wrky18 wrky40 wrky60* triple mutant were substantially more resistant to *P. syringae* but more susceptible to *B. cinerea* than wild-type plants. Thus, the three WRKY proteins have partially redundant roles in plant responses to the two distinct types of pathogens, with WRKY18 playing a more important role than the other two. The contrasting responses of these WRKY mutants to the two pathogens correlated with opposite effects on pathogen-induced expression of salicylic acid–regulated *PATHOGENESIS-RELATED1* and jasmonic acid–regulated *PDF1.2*. While constitutive expression of *WRKY18* enhanced resistance to *P. syringae*, its coexpression with *WRKY40* or *WRKY60* made plants more susceptible to both *P. syringae* and *B. cinerea*. These results indicate that the three WRKY proteins interact both physically and functionally in a complex pattern of overlapping, antagonistic, and distinct roles in plant responses to different types of microbial pathogens.

INTRODUCTION

Plants are constantly exposed to a variety of microbial pathogens and through evolution have developed a battery of active defense mechanisms to protect themselves. In resistance (*R*) gene–mediated resistance, direct or indirect recognition of race-specific elicitors (avirulence proteins) by the plant *R* gene product can often lead to rapid activation of hypersensitive response (HR) that confers specific and effective resistance against the races of the pathogen (Dangl and Jones, 2001). Pathogen-induced HR is often associated with activation of salicylic acid (SA)–regulated defense mechanisms in the surrounding or even distal parts of the plants, leading to development of systemic acquired resistance (SAR). HR and associated SA-dependent signaling pathways generally provide defense effective against

biotrophic pathogens that feed on living host tissues. Ethylene (ET)- and jasmonate (JA)–mediated signaling pathways, on the other hand, often mediate plant defense against necrotrophic pathogens that promote host cell death at early stages of infection (Glazebrook, 2004). It has been well documented that there is an extensive antagonism between SA-mediated and JA/ET-mediated defense signaling pathways (Kunkel and Brooks, 2002). As a result, blocking SA accumulation in pathogen-infected plants can promote JA signaling (Spoel et al., 2003), and mutations of important regulators of JA signaling, such as MPK4 and COI1, lead to enhanced SA accumulation and signaling in pathogen-infected plants (Petersen et al., 2000; Kloek et al., 2001).

Plant defense responses are associated with the transcriptional activation of a large number of plant host genes after pathogen infection (Rushton and Somssich, 1998). Induction of certain pathogenesis-related (PR) proteins is dependent upon SA-mediated defense signaling pathways and is associated with resistance to biotrophic and hemibiotrophic pathogens. The expression of *Arabidopsis thaliana PDF1.2*, which encodes a protein with antimicrobial activity, is regulated by JA/ET defense signaling pathways and often correlated with resistance to necrotrophic pathogens (Penninckx et al., 1998; Thomma et al., 1998). Other pathogen-induced genes encode enzymes involved in the biosynthesis of antimicrobial compounds (phytoalexins). Pathogen-induced plant genes also include those encoding regulatory proteins involved in signal transduction of plant

defense responses. Given that transcriptional regulation of plant host genes is central to plant defense responses, elucidating the complex regulatory mechanisms controlling the differential expression of plant genes is key to understanding the molecular basis of plant disease resistance.

Transcriptional regulation of gene expression is largely mediated by the specific recognition of *cis*-acting promoter elements by *trans*-acting sequence-specific DNA binding transcription factors. Among the several classes of transcription factors associated with plant defense responses are the DNA binding proteins containing WRKY domains (Ulker and Somssich, 2004). The WRKY domains contain a conserved WRKYGQK sequence followed by a Cys_2His_2 or $Cys_2HisCys$ zinc-finger binding motif (Ulker and Somssich, 2004). A number of studies have shown that WRKY proteins have regulatory functions in plant defense responses to pathogen infection. First, pathogen infection or treatment with pathogen elicitors or SA has been shown to induce rapid expression of WRKY genes from a number of plants (Rushton et al., 1996; Chen and Chen, 2000; Hara et al., 2000; Asai et al., 2002; Dong et al., 2003; Kalde et al., 2003; Turck et al., 2004). Secondly, a number of defense-related genes, including the well-studied *PR* genes, contain W-box elements in their promoter regions (Rushton et al., 1996; Chen and Chen, 2000; Hara et al., 2000; Asai et al., 2002; Dong et al., 2003; Kalde et al., 2003; Turck et al., 2004). A number of studies have shown that these W-box sequences are specifically recognized by WRKY proteins and are necessary for the inducible expression of these genes (Rushton et al., 1996; Chen and Chen, 2000; Hara et al., 2000; Asai et al., 2002; Dong et al., 2003; Kalde et al., 2003; Turck et al., 2004). A microarray study of the gene expression changes in *Arabidopsis* grown under 14 different SAR-inducing or -repressing conditions has shown that a group of 26 genes, including *PR1*, is coordinately induced during the establishment of SAR (Maleck et al., 2000). These genes have an average of 4.3 copies per promoter of binding site for WRKY proteins (W-boxes; TTGAC), compared with fewer than two W-boxes per promoter in a randomly selected set of genes (Maleck et al., 2000). More recent studies have provided direct evidence for the involvement of specific WRKY proteins in plant defense responses. For example, two *Arabidopsis* WRKY genes (*WRKY22* and *WRKY29*) have been shown to be induced by a mitogen-activated protein kinase MAPK pathway involved in plant responses to both bacterial and fungal pathogens, and expression of *WRKY29* in transiently transformed leaves led to reduced disease symptoms (Asai et al., 2002). Likewise, constitutive expression of *Arabidopsis WRKY18* and *WRKY70* led to constitutive or enhanced expression of defense-related genes, including SA-induced *PR1*, and increased resistance to virulent pathogens (Chen and Chen, 2002; Li et al., 2004). In tobacco (*Nicotiana tabacum*), virus-induced silencing of three *WRKY* genes compromises *N* gene–mediated resistance to *Tobacco mosaic virus* (Liu et al., 2004). In addition, the resistance gene *RRS1* that confers resistance to the bacterial pathogen *Ralstonia solanacearum* encodes a novel WRKY protein, WRKY52, that combines typical TIR–nucleotide binding site–leucine-rich repeat R protein motifs with a WRKY domain (Lahaye, 2002; Deslandes et al., 2003). These studies indicate that WRKY proteins can function as positive regulators of plant defense responses and disease resistance.

We have previously shown that constitutive expression of *Arabidopsis WRKY18* in transgenic plants leads to constitutive *PR* gene expression and enhanced disease resistance to a virulent strain of *Pseudomonas syringae* in mature transgenic plants (Chen and Chen, 2002). To study how WRKY18 regulates plant defense responses, we have performed yeast two-hybrid screens to identify proteins that interact with the pathogen-induced WRKY transcription factor. These studies demonstrated that *Arabidopsis* WRKY18 interacts with itself and with structurally related WRKY40 and WRKY60. Further studies revealed that the physical interactions of these WRKY proteins significantly change their sequence-specific DNA binding activities. To understand the functional significance of the interaction, we have isolated and characterized both loss-of-function mutants and gain-of-function constitutive overexpression transgenic lines for the three WRKY genes. Functional analysis of single, double, and triple combinations of these mutants for response to microbial pathogens indicated that these three pathogen-induced WRKY transcription factors also interact functionally and play complex overlapping, antagonistic, and distinct roles in plant disease resistance.

RESULTS

Structures, Expression, and Subcellular Localization

There are >70 genes encoding WRKY proteins in *Arabidopsis* (Eulgem et al., 2000; Dong et al., 2003). Based on the number and structures of the conserved WRKY zinc-finger motifs, these WRKY proteins can be classified into three groups (Eulgem et al., 2000). The first group contains two Cys_2His_2 zinc-finger motifs, the second group contains one Cys_2His_2 zinc-finger motif, and the third group contains one $Cys_2HisCys$ zinc-finger motif. Phylogenetic analyses of amino acid sequence of whole WRKY proteins or conserved WRKY zinc-finger motifs placed WRKY18, WRKY40, and WRKY60 into one of several distinct subgroups within the second group of the WRKY protein family (Eulgem et al., 2000; Dong et al., 2003). Multiple alignments of the amino acid sequences of the three proteins indicate that they share an overall 60 to 70% sequence identity (Figure 1). In addition to the conserved WRKY zinc-finger motifs, WRKY18, WRKY40, and WRKY60 share a potential Leu zipper motif at the N terminus and a conserved region at the C terminus. Structural divergence among these WRKY proteins is also obvious in a number of regions. Most notably, WRKY18 contains a His-rich region between the Leu zipper motif and the WRKY zinc-finger motif, but no such His-rich motif is found in WRKY40 or WRKY60 (Figure 1). Significant structural divergence is also found at the N terminus as well as in a region between the WRKY domain and the conserved C terminus where gaps of various numbers of amino acid residues are found (Figure 1).

A Leu zipper motif is a region of ∼30 to 35 amino acid residues where the sequence can be arranged in modules of seven residues (Landschulz et al., 1988). In each module, the fourth residue is usually Leu, and the first residue is frequently hydrophobic. According to this definition, the potential Leu zipper

Figure 1. Sequence Comparison of WRKY18, WRKY40, and WRKY60.

Amino acids identical in three proteins are blue, and residues similar in three proteins are green. The putative Leu zipper motifs with six modules at the N terminus of WRKY18, WRKY40, and WRKY60 are indicated. The fourth residues (Leu, Val, or Met) in the six modules of the Leu zipper motifs are magenta, and the first amino acid residues of these modules are cyan. The WRKY DNA binding motifs are also indicated with the highly conserved WRKYGQK sequences, and the residues forming the C_2H_2 zinc-fingers are red.

motifs at the N terminus of WRKY18, WRKY40, and WRKY60 can be divided into six modules (Figure 1). The fourth residues in four of the six modules are Leu, while the other two modules have Val or Met at this position. In addition, the hydrophobic Leu and Ile residues are also found at the first amino acid position of some of these modules. As shown below, the Leu zipper motifs are involved in the physical interaction of these WRKY proteins.

We have previously shown that expression of *WRKY18*, *WRKY40*, and *WRKY60* are induced by infection of an avirulent strain of the bacterial pathogen *P. syringae* and treatment of SA (Dong et al., 2003). In this study, we have analyzed expression of the three WRKY genes in response to a virulent strain of the bacterial pathogen and the necrotrophic fungal pathogen *Botrytis cinerea*. As shown in Figure 2A, infection of the bacterial pathogen induced strong expression of *WRKY18*, whereas infiltration of an $MgCl_2$ buffer used for suspension of the bacterial pathogen caused only a low level of induction (Figure 2A). Expression of *WRKY40* and *WRKY60* was also stronger in pathogen-infected plants than in buffer-infiltrated plants (Figure 2A). Infection of *B. cinerea* also led to induced expression of the three WRKY genes (Figure 2B). Spraying a maltose buffer used for suspension of *B. cinerea* spores, on the other hand, induced little expression of the three WRKY genes (Figure 2B). Thus, expression of the three WRKY genes was induced by both the

virulent and avirulent strains of *P. syringae* and by the necrotrophic fungal pathogen *B. cinerea*.

WRKY18, WRKY40, and WRKY60 proteins, as putative transcription factors, are likely to be localized in the nucleus, and the presence of putative nuclear localization signals predicted by the PSORT II program is consistent with this possibility. To test this, we constructed green fluorescent protein (GFP) fusions of the three WRKY proteins and demonstrated that these transiently expressed GFP/WRKY fusion proteins were localized exclusively to the nuclei of onion (*Allium cepa*) epidermal cells (Figure 3). By contrast, the unfused GFP protein was found in both the nucleus and cytoplasm (Figure 3). Nuclear localization of these WRKY proteins supports their roles as transcriptional regulators.

Mutual and Self-Interactions

We previously showed that mature transgenic *Arabidopsis* plants constitutively expressing *WRKY18* exhibited enhanced resistance to a virulent strain of *P. syringae* (Chen and Chen, 2002). To understand how the WRKY protein enhances plant defense responses, we tried to identify its interacting proteins using yeast two-hybrid screens. In these screens, we used a fusion protein of WRKY18 with the Gal4 DNA binding domain as bait. The yeast strain transformed with the bait construct failed to grow in the
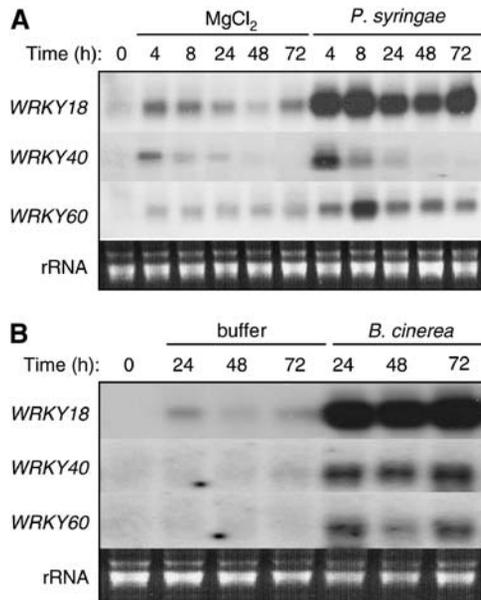
Figure 2. Pathogen-Induced Expression of *WRKY18*, *WRKY40*, and *WRKY60*.

Arabidopsis plants (4 to 5 weeks old) were infiltrated with the virulent strain of *P. syringae* pv *tomato* DC3000 (*Pst*DC3000) (OD$_{600}$ = 0.001 in 10 mM MgCl$_2$) or sprayed with the fungal pathogen *B. cinerea* (2 × 10^5 spores/mL in a maltose buffer). Total RNA was isolated from buffer-treated or pathogen-infected leaves that were harvested at indicated time points after treatment/inoculation. Two separate blots were prepared from the same preparation of total RNA, with one probed with the *WRKY18* probe and the other probed with the *WRKY40* probe. One of the two blots was subsequently stripped and reprobed with the *WRKY60* gene probe. The ethidium bromide stain of rRNA from one of the two blots is shown for each lane to allow assessment of equal loading. The experiments were repeated twice with similar results.

selective medium (data not shown), indicating that WRKY18 did not confer transcriptional activation activity to the fusion protein in yeast. We screened >2 × 10^7 independent transformants of a cDNA library generated from SA-treated *Arabidopsis* plants at a complexity of 2 × 10^6. The screens yielded two groups of cDNA fragments. The first group, which was isolated twice, encodes WRKY18, suggesting that this DNA binding protein can self-interact. The second group, which was isolated six times, encodes WRKY40.

WRKY18 and WRKY40 are structurally similar with >60% identical amino acid residues (Figure 1). These two proteins also form a subgroup with WRKY60 in phylogenetic studies based on WRKY DNA binding domains or full-length protein sequences (Eulgem et al., 2000; Dong et al., 2003). Since WRKY18 interacts with itself and with WRKY40, we investigated whether the mutual and self-interactions could happen to the other two members of the subgroup. Indeed, both WRKY40 and WRKY60 self-interacted based on yeast two-hybrid assays (Figures 4A and 4B). In addition, these three WRKY proteins interacted with each other: WRKY18 interacted with WRKY40 and WRKY60, and WRKY40 interacted with both WRKY18 and WRKY60 (Figures 4A and 4B). Thus, the three WRKY proteins not only self-interact

to form three different homocomplexes but also interact with each other to form heterocomplexes.

We next tested the role of two structural motifs of these three WRKY proteins in the protein–protein interactions. As shown in Figure 1, WRKY18 contains two conspicuous structural motifs in addition to the WRKY zinc-finger motif: a His-rich region (HRR) and a Leu zipper (LZ). To test the role of the HRR, we generated a construct encoding an HRR-less WRKY18 (W18ΔHRR) and used it as bait in yeast two-hybrid assays. As shown in Figure 4A, yeast cells transformed with both the bait and the full-length WRKY18, WRKY40, or WRKY60 prey construct were able to grow on the selective medium. Thus, the HRR-less WRKY18 was fully capable of interacting with itself and with WRKY40 and WRKY60, indicating that the HRR was not required for the protein–protein interaction. To analyze the role of LZ in protein–protein interaction, we generated LZ-less WRKY18 (W18ΔLZ) and used it as bait in the yeast two-hybrid assays. Yeast cells transformed with the combination of the LZ-less WRKY18 bait and the WRKY18, WRKY40, or WRKY60 prey construct were unable to grow on the selective medium (Figure 4A) and generated little Gal4-inducible
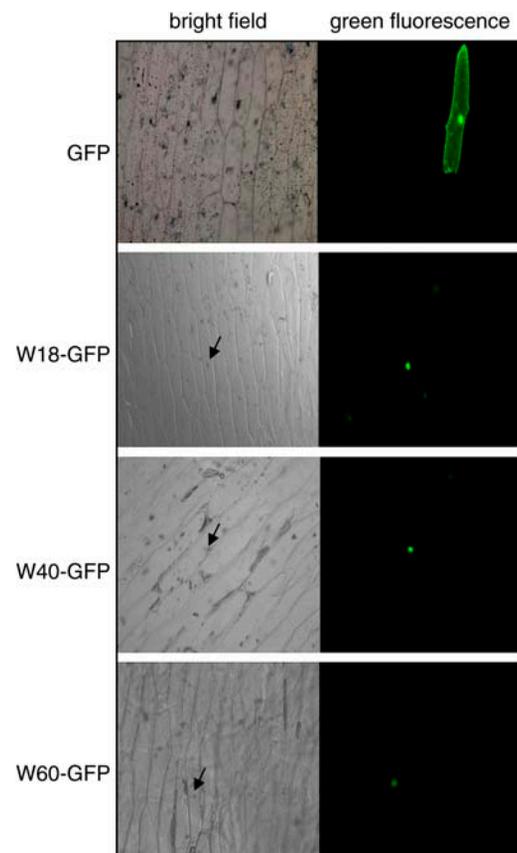

Figure 3. Nuclear Localization of WRKY18, WRKY40, and WRKY60.

WRKY18, WRKY40, and WRKY60 were fused to GFP to yield W18-GFP, WRKY40-GFP, and WRKY60-GFP, respectively. The fusion proteins are localized to the nucleus of onion epidermal cells. GFP alone is localized to both the nucleus and the cytoplasm because of its small size. Bright-field images of the onion epidermal cells are also shown. The arrows point to the nucleus of the cells where GFP fusion proteins are localized.
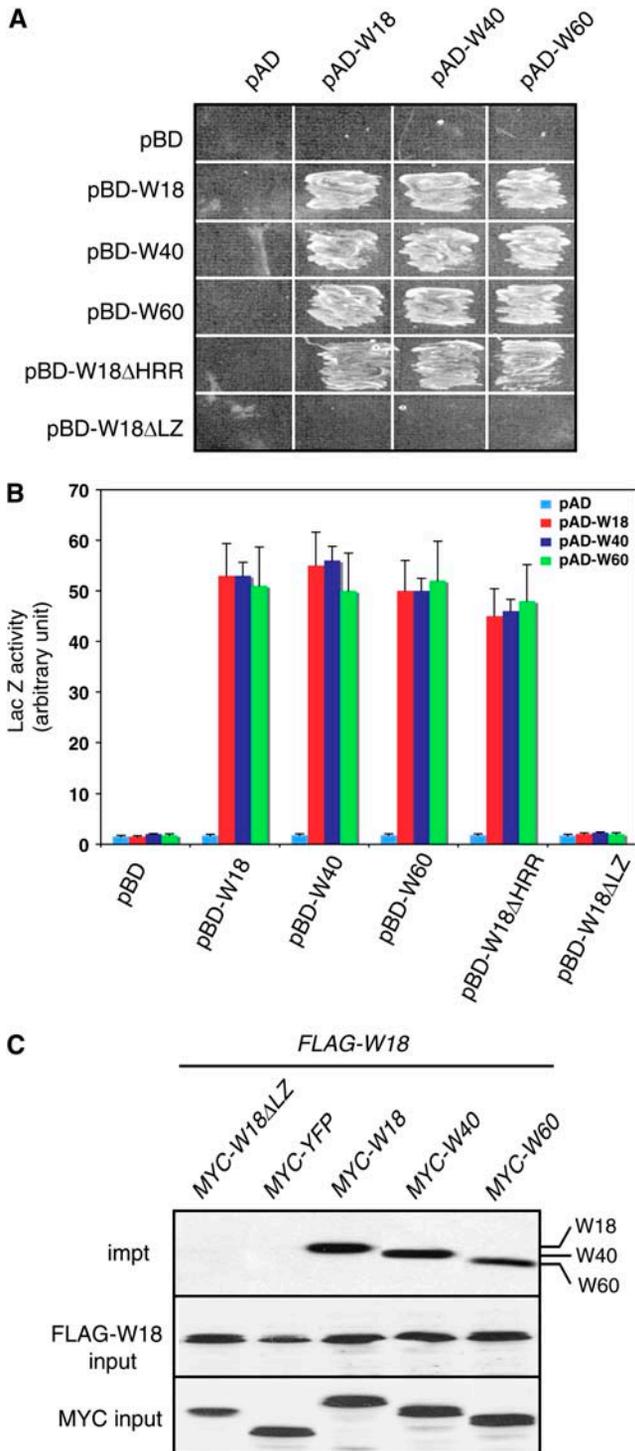
A

B

C

Figure 4. Mutual and Self-Interaction of WRKY18, WRKY40, and WRKY60.

(A) Protein interactions in the yeast two-hybrid system. The activation domain (AD) fusions were cotransformed with various Gal4 DNA binding domain (BD) vectors into yeast cells and grown in the absence of Leu, Trp, and His.

(B) Quantitative assays of protein interactions in the yeast two-hybrid systems. The AD fusions were transformed with the various Gal4 DB

LacZ activity (Figure 4B). In addition, we generated LZ-less WRKY40 and WRKY60 and found that they were unable to interact with themselves or with the other two WRKY proteins (see Supplemental Figure 1 online). These results suggest that the LZ motifs in these WRKY proteins mediate protein–protein interactions with themselves and with each other. Further analysis with site-directed mutations would be required to confirm the role of the LZ motifs in interactions of the WRKY proteins.

We also performed coimmunoprecipitation to determine in vivo interactions of WRKY18 with itself and with WRKY40 and WRKY60. Through genetic crossing or double transformation, we generated transgenic plants harboring a combination of a FLAG-tagged *WRKY18* gene driven by the cauliflower mosaic virus (*CaMV*) *35S* promoter and a MYC-tagged WRKY gene driven by a steroid-inducible *Gal4* promoter (Aoyama and Chua, 1997). As shown in Figure 4C, the protein complexes immuno-precipitated by the anti-FLAG antibody from protein extracts of transgenic plants coexpressing the FLAG-tagged WRKY18 protein, and a MYC-tagged WRKY protein generated positive interactions to the anti-MYC antisera. By contrast, the immuno-precipitations from protein extracts of transgenic plants ex-pressing the FLAG-tagged WRKY18 protein and a MYC-tagged WRKY18(ΔLZ) or yellow fluorescent protein (YFP) produced no cross-reactivity to the antisera (Figure 4C). These results support in vivo interactions of WRKY18 protein with itself and with WRKY40 and WRKY60.

DNA Binding Activities

A number of WRKY proteins have been shown to bind the TTGACC/T sequence (W-box) (Eulgem et al., 2000; Yu et al., 2001; Dong et al., 2003; Miao et al., 2004). WRKY18 has also been shown to bind W-box sequences in vitro (Chen and Chen, 2002). To test the DNA binding activities of WRKY40 and WRKY60, we generated recombinant proteins in *Escherichia coli* and tested each for in vitro binding activity to a DNA molecule with a W-box sequence (Figure 5A). When the same amount of WRKY18 and WRKY40 was used in these binding assays, the intensities of the retarded bands were substantially higher with WRKY40 than with WRKY18 (Figure 5B). This result suggested that WRKY40 had a higher binding activity than WRKY18. Interestingly, when WRKY60 was tested for binding activity with the same DNA molecule as probe, we observed no retarded band in the gel retardation assays (Figure 5B). In fact, even when the level of the recombinant WRKY60 was increased five times

fusion vectors into yeast cells. Proteins were isolated from the yeast cells and assayed for the β-galactosidase activity using o-nitrophenyl-β-D-galactopyranose as substrate. Data represent means + SE (n = 5).

(C) Leaf protein extracts were prepared from transgenic plants coex-pressing FLAG-tagged WRKY18 and one of the following MYC-tagged proteins: WRKY18ΔLZ, YFP, WRKY18, WRKY40, or WRKY60. After incubation with a FLAG-specific antibody and precipitation with protein A-agarose, the immunoprecipitated complexes were analyzed with protein gel blotting using anti-MYC-peroxidase (top panel). Protein inputs for FLAG-WRKY18 in the immunoprecipitated complexes (middle panel) and MYC-tagged proteins in the extracts (bottom panel) were detected with anti-FLAG-peroxidase and anti-MYC-peroxidase, respectively.

higher than that of WRKY18 and WRKY40, we still observed no visible retarded band (data not shown). Thus, the recombinant WRKY60 showed little in vitro DNA binding to the W-box–containing DNA molecule.

Since the three WRKY proteins are likely to form both homo-complexes and heterocomplexes, we reasoned that they might also recognize composite DNA sequences containing more than one W-box. To test this possibility, we synthesized a series of oligo DNA molecules containing two W-box sequences separated by various numbers of nucleotides (Figure 6A). As shown in Figure 6B, combining WRKY18 and WRKY40 in the binding reactions generally produced much higher intensities of the retarded bands than the single WRKY18 or WRKY40 protein. Thus, interactions between WRKY18 and WRKY40 appeared to enhance their DNA binding activity.

WRKY60 alone had little DNA binding activities for W-box sequences. Mixing WRKY60 with WRKY18 did not alter DNA binding activities for a number of DNA sequences containing two W-boxes arranged in various spacings (Figure 6C). However, substantial enhancement of DNA binding activity of WRKY18 was observed by WRKY60 for a DNA molecule with two direct repeats of W-boxes spaced by three nucleotides (Figure 6C). This result
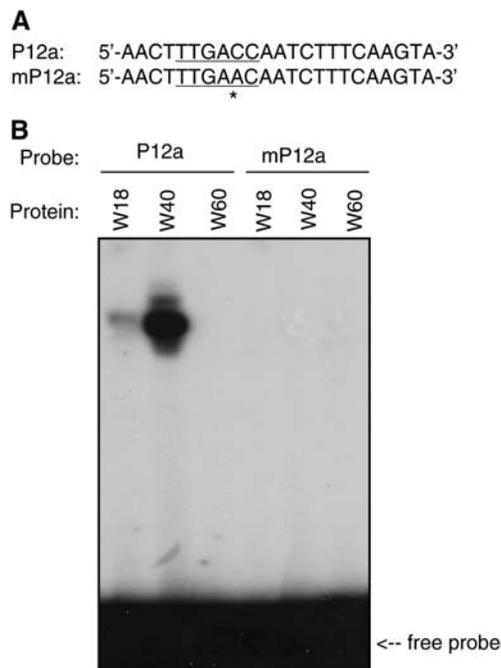
A

P12a: 5'-AACT TTGACC AATCTTTCAAGTA-3'
mP12a: 5'-AACT TTGAAC AATCTTTCAAGTA-3'
                        *

B

Figure 5. DNA Binding Activity of WRKY18, WRKY40, and WRKY60 Proteins.

(A) Nucleotide sequences of probes used for DNA binding assays. P12a contains a single TTGACC W-box sequence, which is mutated into TTGAAC in mP12a.

(B) Recombinant WRKY18, WRKY40, and WRKY60 were purified from *E. coli* cells and used for DNA binding assays with P12a and mP12a as probes. The binding reactions (20 μL) contained 2 ng labeled oligo DNA, 5 μg polydeoxyinosinic-deoxycytidylic acid, and 1 μg recombinant protein. The binding assays were repeated twice with independently prepared recombinant proteins with similar results.
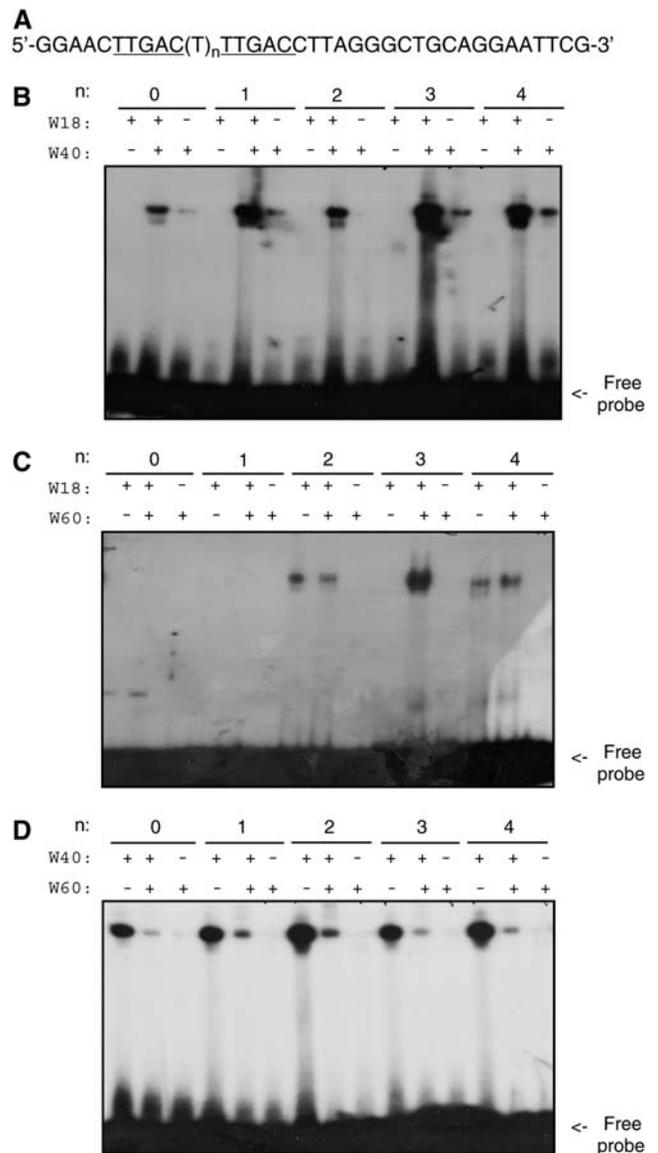
A

5'-GGAAC TTGAC (T)$_n$ TTGAC CTTAGGGCTGCAGGAATTCG-3'

Figure 6. Effects of Protein–Protein Interactions on DNA Binding Activity.

(A) Nucleotide sequences of the probes used for DNA binding assays. The probes contain two TTGAC W-box core sequences separated by a spacer of varying sizes.

(B) DNA binding activity of WRKY18, WRKY40, or a mixture of the two proteins.

(C) DNA binding activity of WRKY18, WRKY60, or a mixture of the two proteins.

(D) DNA binding activity of WRKY40, WRKY60, or a mixture of the two proteins.

The binding reactions and conditions were the same as those in Figure 5 except that the recombinant protein in each binding reaction was reduced to 0.2 μg. The reduced amount of protein was used to facilitate detection of possible effects of protein–protein interactions on their DNA binding activity. The binding assays were repeated twice with independently prepared recombinant proteins with similar results.

suggests that interaction of WRKY18 and WRKY60 selectively enhances their DNA binding activity to DNA molecules containing W-boxes arranged in a specific manner. By contrast, when WRKY40 was mixed with WRKY60, less DNA binding of WRKY40 was observed (Figure 6D). Thus, WRKY60 differentially affected the DNA binding activity of WRKY18 and WRKY40.

Knockout Mutants

The *wrky18-1* and *wrky60-1* insertion mutants obtained from the Salk T-DNA populations (Alonso et al., 2003) have a T-DNA insertion within the first exon of their respective genes (Figure 7A). The *wrky40-1* mutant, obtained from Cold Spring Harbor Laboratory gene and enhancer trap lines (Sundaresan et al., 1995), contains a Ds transposon inserted within the second exon of *WRKY40* (Figure 7A). In wild-type plants, *WRKY18*, *WRKY40*, and *WRKY60* transcripts of expected sizes accumulated to significant levels after infiltration with a virulent strain of *P. syringae* (Figure 7B). In the insertion mutants, no transcripts of expected sizes were detected after the pathogen infection (Figure 7B). No obvious abnormal phenotypes in plant growth or development were displayed by any of these mutants. Double and triple mutants were generated through genetic crossing and progeny screening by PCR genotyping. From the segregating populations derived from the genetic crossings, we observed that homozygous single, double, and triple mutants appeared at rates similar to the expected ratios, further supporting that mutations of these genes had no significant effect on plant growth and reproduction. RNA gel blot analysis indicated that no transcripts of expected sizes for these genes were detected in
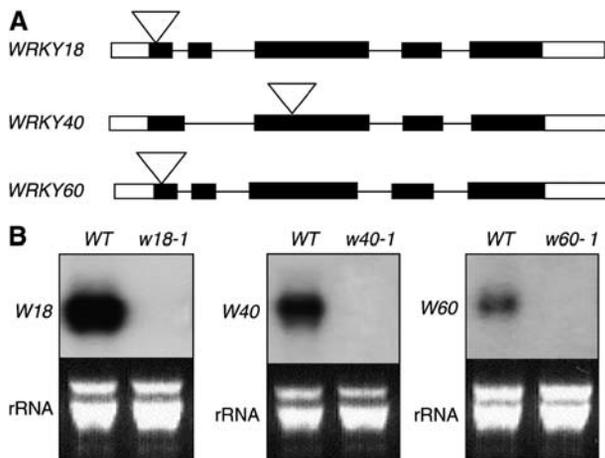

Figure 7. Loss-of-Function Mutants for the WRKY Genes.

(A) Diagram of *WRKY18*, *WRKY40*, and *WRKY60* and their T-DNA insertion or transposon tagging mutants.
(B) RNA gel blot analysis of *wrky18-1*, *wrky40-1*, and *wrky60-1* mutants. Wild-type and mutant plants were infiltrated with *Pst*DC3000 (OD$_{600}$ = 0.01). Two hours after the inoculation, the inoculated leaves were harvested and total RNA was isolated. After separation on the gel and blotting to a nylon membrane, the blot was probed with gene-specific DNA fragments. The RNA gel blotting was repeated at least three times with independently isolated RNA with similar results.

their respective homozygous double mutants and the homozygous triple mutant (data not shown).

To examine the roles of these three WRKY genes in plant defense, we first analyzed the mutant plants for response to a virulent strain of *Pst*DC3000. As shown in Figure 8A, the homozygous *wrky18-1* mutant had an approximately twofold to three-fold reduction in the growth of the bacterial pathogen, while the homozygous *wrky40-1* and *wrky60-1* mutants displayed no such reduction. The homozygous *wrky40 wrky60* double mutant also had little difference in the bacterial growth from those in wild-type plants and their parental single mutants (Figure 8A). On the other hand, reduction in bacterial growth was observed in the homozygous *wrky18 wrky60* (approximately fivefold) and *wrky18 wrky40* (approximately sixfold) double mutants (Figure 8A). An even greater reduction (approximately ninefold) of bacterial growth was observed in the homozygous *wrky18 wrky40 wrky60* triple mutant relative to the wild-type plants (Figure 8A). These results suggest that WRKY18, WRKY40, and WRKY60 function redundantly with a negative role in resistance to the hemibio-trophic bacterial pathogen. However, since only the double and triple mutants containing the *wrky18* mutant allele displayed altered responses (Figure 8A), this functional redundancy is only partial, with *WRKY18* playing a more important role than the other two WRKY genes.

Some of the mutants developed more chlorotic symptoms than wild-type plants after infection of the bacterial pathogen (Figure 8B). Surprisingly, the extent of chlorosis on the inoculated leaves was negatively correlated with bacterial growth. Thus, the wild type, *wrky40*, *wrky60*, and *wrky40/60* mutants supported the highest bacterial growth and had the least chlorosis (Figure 8B). The *wrky18 wrky40* and *wrky18 wrky60* double mutants had modest levels of reduction in the bacterial growth and were accompanied with modest levels of chlorosis (Figure 8B). The *wrky18 wrky40 wrky60* triple mutant had the largest reduction in bacterial growth and developed the most extensive chlorosis (Figure 8B).

To determine how the reduction in the bacterial growth was associated with enhanced chlorosis in the mutants, we compared them with the *npr1-3* mutant (Cao et al., 1997) and transgenic *nahG* plants (Delaney et al., 1994). As shown in Figure 8A, the *npr1-3* and transgenic *nahG* plants had increased growth (~7- to 12-fold) of the bacterial pathogen relative to wild-type plants. When compared with the *wrky18 wrky40 wrky60* triple mutant, the *npr1-3* and transgenic *nahG* plants had ~70- to 100-fold increase in the bacterial growth. The *npr1-3* and transgenic *nahG* plants also developed severe disease symptoms when compared with the wild-type plants (Figure 8B). However, there was a marked difference between the symptoms on the *npr1* and transgenic *nahG* plants and those on the WRKY mutants with reduced bacterial growth (Figure 8B). In the transgenic *nahG* plants, which had the highest bacterial growth (Figure 8A), the inoculated leaves first developed water-soaking symptoms followed by necrotic and wilting leaves with little chlorosis after the infection (Figure 8B). The *npr1* mutant plants also developed extensively necrotic and slightly chlorotic symptoms, and the wild-type plants had mixed chlorotic and necrotic symptoms (Figure 8B). Inoculated leaves of the WRKY double and triple mutants with reduced bacterial growth, particularly the *wrky18*
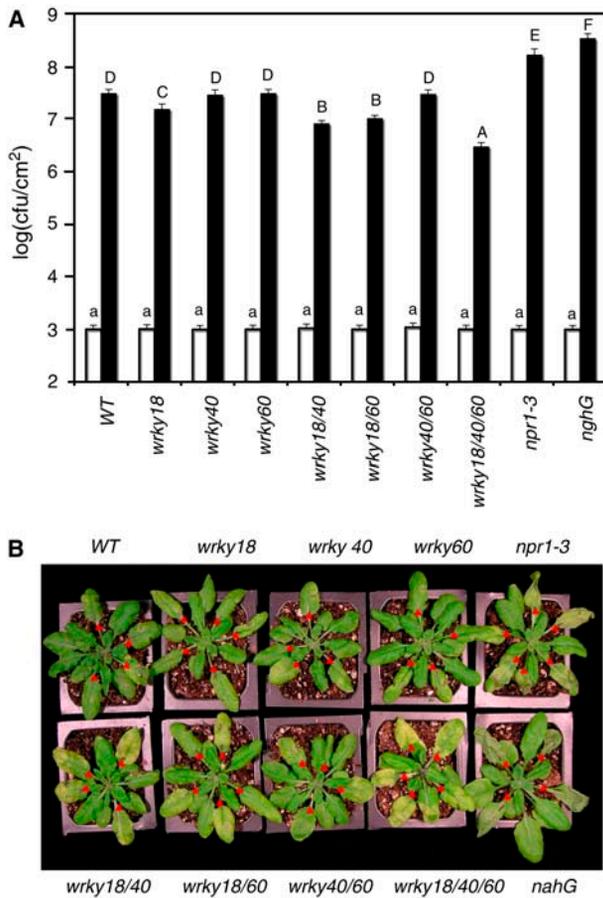
Figure 8. Altered Response of WRKY Mutants to *P. syringae*.

(A) Wild-type, single, double, and triple mutants were inoculated through infiltration with a suspension of *Pst*DC3000 (OD_{600} = 0.001 in 10 mM $MgCl_2$). Samples were taken at 0 d (open bars) or 3 d (closed bars) after inoculation (DAI) to determine the growth of the bacterial pathogen. The means and standard errors were calculated from 10 plants for each treatment. According to Duncan's multiple range test (P = 0.05), means of colony-forming units (cfu) at 0 DAI do not differ significantly if they are indicated with the same lowercase letter; means of cfu at 3 DAI do not differ significantly if they are indicated with the same capital letter.
(B) Wild-type and *wrky* mutants were inoculated through infiltration with a suspension of *Pst*DC3000 (OD_{600} = 0.001 in 10 mM $MgCl_2$). Pictures of representative inoculated leaves were taken 3 DAI. Infiltrated leaves are indicated with red triangles.
These experiments were repeated twice with similar results.

wrky40 wrky60 triple mutant, had extensive yellowing with little necrotic collapsing or wilting (Figure 8B).

We also analyzed the responses of the WRKY mutants to the necrotrophic fungal pathogen *B. cinerea*. Based on both visual inspection of the infected plants and quantification of biomass of the infecting fungal pathogen, the *wrky60* and *wrky40/60* mutants had no significant alteration in response to the pathogen, while the *wrky18* mutant displayed a slight increase in susceptibility to the fungal pathogen (Figure 9). The *wrky18 wrky40* and *wrky18 wrky60* double mutants and the *wrky18 wrky40 wrky60* triple mutant were also more susceptible to the fungal pathogen

(Figure 9). Thus, the extent of susceptibility to the necrotrophic fungal pathogen was generally correlated with the extent of resistance to the hemibiotrophic bacterial pathogen *P. syringae* among these WRKY mutants.

To confirm that the altered disease resistance of the mutants was due to disruption of the WRKY genes, we performed genetic complementation of the *wrky18 wrky40 wrky60* triple mutant because of its strongest phenotypes in disease assays. Full-length cDNA clones for the three WRKY genes were placed behind their native promoters and transformed into the mutant. T1 transformants were identified through Basta selection and analyzed for expression of the WRKY transgenes and responses to *P. syringae* and *B. cinerea*. Transformation of the mutant with *WRKY18* resulted in enhanced growth of *P. syringae* but reduced susceptibility to *B. cinerea* to the levels close to those in wild-type plants, while transformation of the same mutant with *WRKY40* and *WRKY60* led to partial restoration of the altered phenotypes in the pathogen assays (see Supplemental Figure 2 online). These results indicate that the altered disease resistance of the *wrky18 wrky40 wrky60* mutant was attributed to mutations of the WRKY genes.
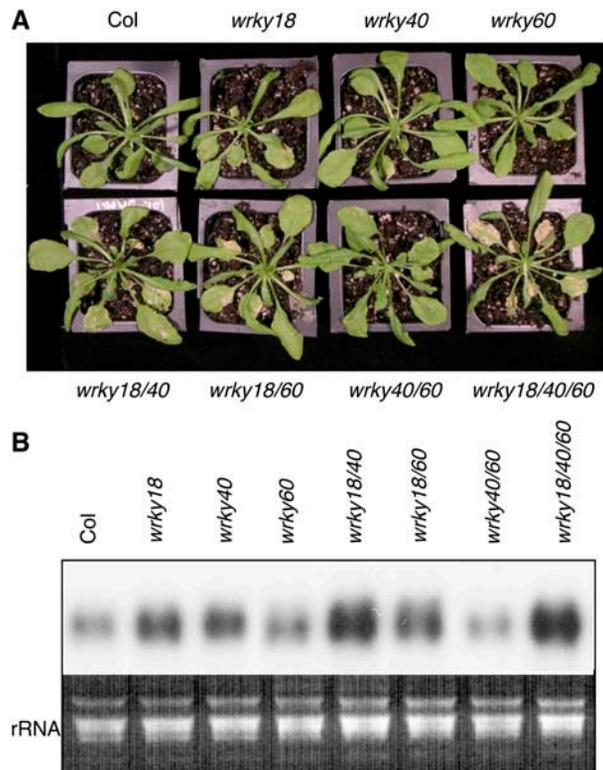

Figure 9. Altered Response of WRKY Mutants to *B. cinerea*.

(A) Wild-type, single, double, and triple mutants were inoculated by spraying spore suspension at a density of 2×10^4 spores/mL and kept at high humidity. The pictures of the plants were taken 4 DAI.
(B) Total RNA was isolated from inoculated plants 3 DAI and probed with a *Botrytis* β-tubulin gene probe to determine the accumulation of the *Botrytis* β-tubulin mRNA for measurement of the biomass of the fungal pathogen on infected plants.
The experiments were repeated twice with similar results.

Constitutive Expression in Transgenic Plants

Transgenic plants constitutively expressing *WRKY18* are significantly smaller in size and have more serrated leaves than wild-type plants (Chen and Chen, 2002). In this study, we generated transgenic *Arabidopsis* plants that constitutively expressed *WRKY40* and *WRKY60* under control of the *CaMV 35S* promoter (Figure 10A). As shown in Figure 10B, the plants that constitutively express *WRKY40* also exhibited more serrated leaves than wild-type plants, while transgenic *35S:WRKY60* plants exhibited no such altered phenotype. To determine the functional interactions between these WRKY genes, we generated transgenic plants constitutively expressing more than one WRKY gene through genetic crossing. The F1 progeny of homozygous transgenic *35S:WRKY18* plants crossed with homozygous transgenic *35S:WRKY60* plants expressed both *WRKY18* and *WRKY60* constitutively (Figure 10C). These plants were substantially smaller than parental plants and displayed less leaf serration than the transgenic *35S:WRKY18* parent plants (Figure 10B). Crossing transgenic *35S:WRKY18* plants with transgenic *35S:WRKY40* plants resulted in F1 plants that were either greatly stunted (Figure 10B) or unable to survive beyond a few weeks after germination. By contrast, the F1 progeny plants from the cross between transgenic *35S:WRKY40* plants and transgenic *35S:WRKY60* plants had sizes similar to those of the wild-type plants and exhibited no significantly altered leaf morphology (Figure 10B). We also attempted but failed to generate transgenic plants expressing all these three *WRKY* genes. Progeny expressing all three WRKY genes died within several weeks after germination, indicating that constitutive overexpression of all three WRKY genes is very detrimental.

To determine the impact of constitutive expression of the *WRKY* genes on plant disease resistance, we analyzed these transgenic plants for responses to *P. syringae*. As previously reported (Chen and Chen, 2002), mature transgenic *35S:WRKY18* plants exhibited enhanced resistance to the bacterial pathogen as manifested by decreased bacterial growth when compared with the wild-type plants (Figure 11A; Chen and Chen, 2002). Transgenic plants constitutively expressing *WRKY40* or *WRKY60* showed no significant difference in the bacterial growth (Figure 11A). Unlike the transgenic *WRKY18* plants that were more resistant to *P. syringae*, transgenic plants coexpressing *WRKY18* and *WRKY60* had bacterial growth significantly higher than that observed in wild-type plants (Figure 11A). In addition, transgenic plants coexpressing *WRKY18* and *WRKY40* supported 10 times more bacterial growth than wild-type plants (Figure 11A). Thus, constitutive overexpression of *WRKY40* or *WRKY60* antagonizes the positive effects of *WRKY18* overexpression on plant resistance to the bacterial pathogen.

We also analyzed the responses of the transgenic overexpression plants to *B. cinerea*. Based on symptom development (Figure 11B) and quantification of biomass of the fungal pathogen (Figure 11C), constitutive expression or coexpression of *WRKY40* or *WRKY60* had no significant effect on plant response to the pathogen. Constitutive expression of *WRKY18*, on the other hand, resulted in a substantial increase in the susceptibility to the fungal pathogen (Figures 11B and 11C). Coexpression of *WRKY40* or *WRKY60* with *WRKY18* resulted in further enhanced
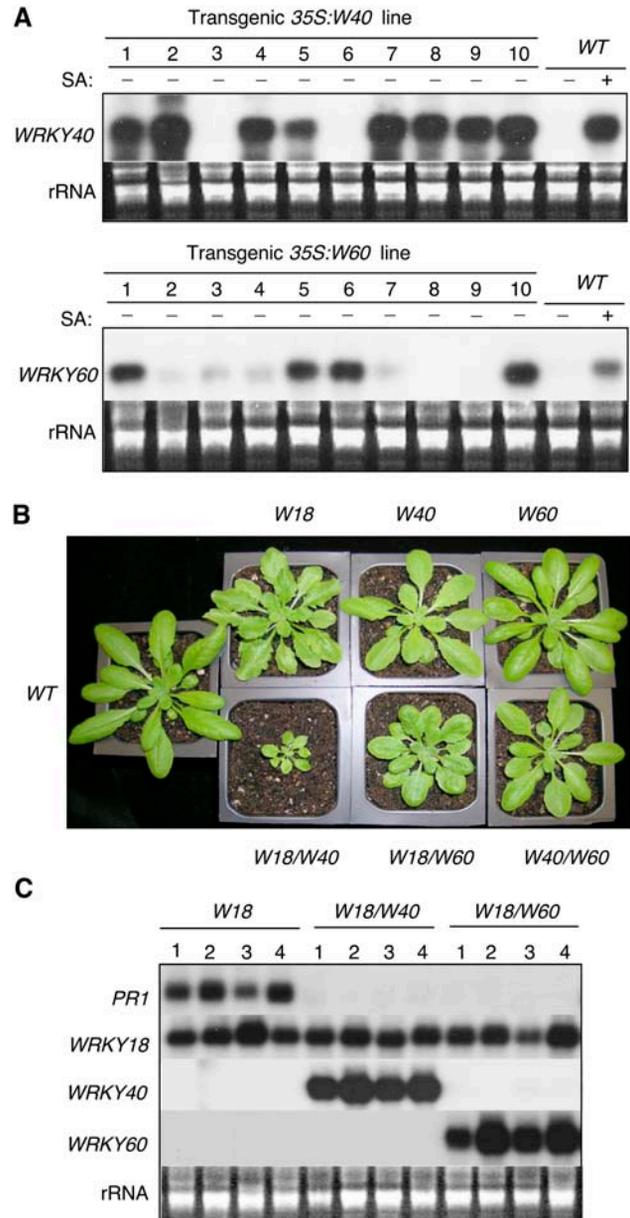

Figure 10. Construction of Overexpression Lines.

(A) RNA gel analysis of *WRKY40* and *WRKY60* expression in transgenic plants. RNA samples were prepared from leaves of 32-d-old wild-type and transgenic plants and probed with WRKY gene-specific DNA fragments. Transgenic *WRKY40* line 7 and transgenic *WRKY60* line 1 contained a single T-DNA insertion in their genomes and exhibited stable expression of their respective transgenes. Their F3 homozygous progeny plants were used in all the experiments in this study.

(B) Morphology of 5-week-old wild-type and transgenic plants overexpressing one or two *WRKY* genes.

(C) Total RNA was isolated from 5-week-old transgenic plants expressing *WRKY18* alone or coexpressing *WRKY18* and *WRKY40* or *WRKY18* and *WRKY60*. Two separate blots were prepared from the same preparation of total RNA, with one probed with the *PR1* probe and the other probed with the *WRKY18* probe. The two blots were subsequently stripped and reprobed with the *WRKY40* and *WRKY60* gene probes, respectively.
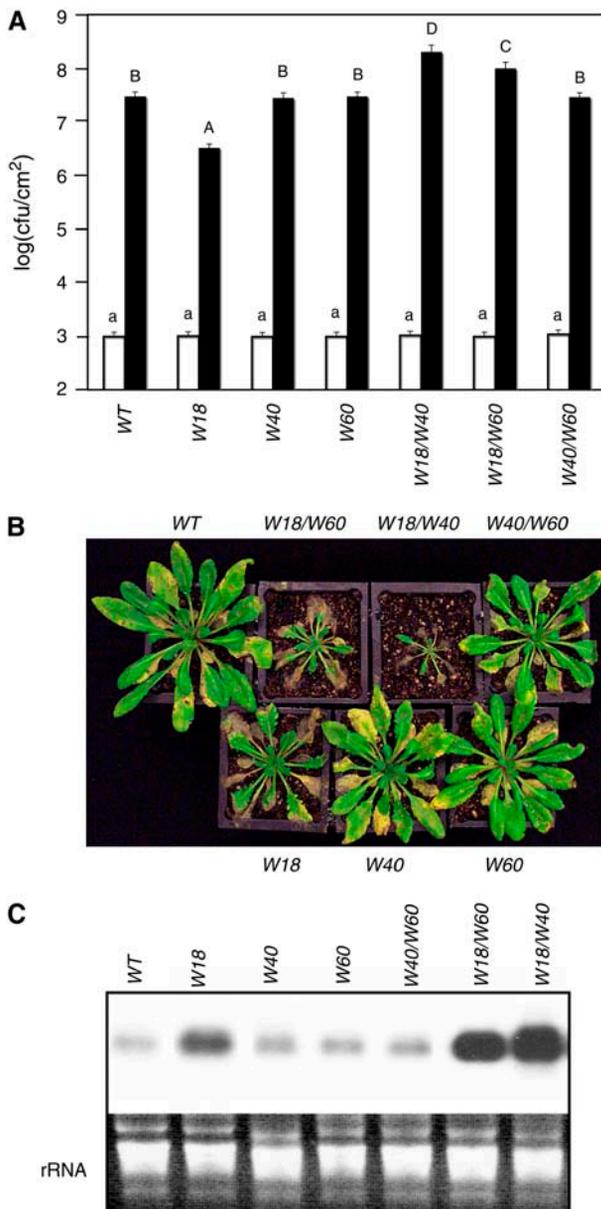
Figure 11. Altered Responses of Overexpression Lines to *P. syringae* and *B. cinerea*.

(A) Wild-type and transgenic overexpression plants were inoculated with *Pst*DC3000 ($OD_{600} = 0.001$). Samples were taken at 0 (open bars) and 3 (closed bars) DAI to determine the growth of the bacterial pathogen. The means and standard errors were calculated from 6 to 10 plants. According to Duncan's multiple range test (P = 0.05), means of cfu at 0 DAI do not differ significantly if they are indicated with the same lowercase letter; means of cfu at 3 DAI do not differ significantly if they are indicated with the same capital letter.

(B) Wild-type and transgenic overexpression lines constitutively expressing one or two WRKY genes were inoculated by spraying spore suspension at a density of 10^5 spores/mL and kept at high humidity. The pictures of the plants were taken 4 DAI.

(C) Total RNA was isolated from inoculated plants 3 DAI and probed with a *Botrytis* β-tubulin gene probe to determine the accumulation of the

susceptibility to the fungal pathogen (Figures 11B and 11C). Thus, while constitutive overexpression and knockout mutations of some of these WRKY genes had opposite effects on response to *P. syringae*, both overexpression and disruption of some of the WRKY genes resulted in enhanced susceptibility to *B. cinerea*.

Defense-Related Gene Expression

The contrasting phenotypes of the loss-of-function WRKY mutants in resistance to *P. syringae* and *B. cinerea* 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 these mutants. The *wrky18 wrky40 wrky60* triple mutant had the highest level of *PR1* transcripts that correlated with the lowest growth of the bacterial pathogen in the mutant, while the *wrky18 wrky40* and *wrky18 wrky60* double mutants had modest reduction in the bacterial growth and also had enhanced levels of *PR1* transcripts relative to that in wild-type plants (Figures 8 and 12A). Induction of *PDF1.2* by *P. syringae* infection was low in the wild type and WRKY mutants (Figure 12A). *B. cinerea* also induced *PR1* expression, but the expression levels of this SA-regulated gene were negatively correlated with the extents of resistance to the fungal pathogen among the wild type and WRKY mutants (Figures 9 and 12B). Expression of JA-regulated *PDF1.2*, on the other hand, was positively correlated with the resistance to *B. cinerea* (Figure 12B). The *wrky18 wrky40 wrrky60* triple mutant, being most susceptible to the fungal pathogen, had the lowest level of *PDF1.2* transcripts after infection by the fungal pathogen. The *wrky18 wrky40* and *wrky18 wrky60* double mutants also exhibited enhanced susceptibility to *B. cinerea* and had reduced expression of *PDF1.2* as well when compared with wild-type plants.

Enhanced resistance to *P. syringae* in transgenic plants constitutively expressing *WRKY18* is associated with constitutive expression of *PR1* genes (Figure 10C). Coexpression of *WRKY40* or *WRKY60* rendered transgenic *WRKY18* plants more susceptible to the bacterial pathogen and, at the same time, abolished the constitutive *PR1* expression in the transgenic plants (Figure 10C). Pathogen infection did not further enhance *PR1* expression in the transgenic *WRKY18* plants (Figure 12C), as previously reported (Chen and Chen, 2002). The levels of *PR1* transcripts in transgenic plants expressing *WRKY40* or *WRKY60* were similar to those observed in the wild-type plants after infection by *P. syringae* (Figure 12C), consistent with their unaltered resistance to the bacterial pathogen (Figure 11A). On the other hand, pathogen-induced *PR1* expression was substantially reduced in the transgenic plants coexpressing *WRKY18* and *WRKY60* or *WRKY18* and *WRKY40* (Figure 12C), consistent with their enhanced susceptibility to the bacterial pathogen (Figure 11A).

Botrytis β-*tubulin* mRNA for measurement of the biomass of the fungal pathogen on infected plants.
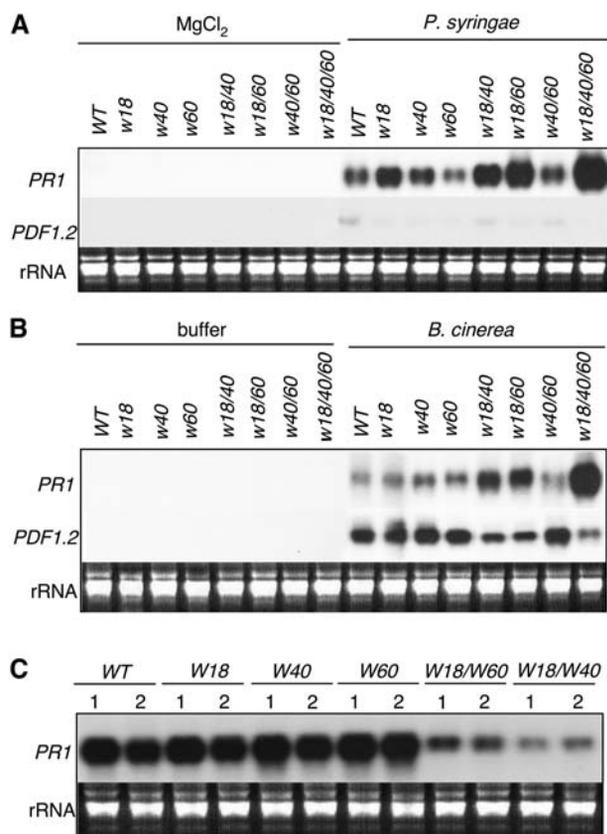
The experiments were repeated twice with similar results.

Figure 12. Defense-Related Gene Expression.

(A) Plants of wild-type, single, double, and triple mutants were infiltrated with 10 mM MgCl₂ or *Pst*DC3000 (OD$_{600}$ = 0.001 in 10 mM MgCl₂). Total RNA was isolated from infiltrated leaves harvested at 24 h after infiltration and probed with a *PR1* probe. The blot was stripped and reprobed with a *PDF1.2* probe.

(B) Plants of wild-type, single, double, and triple mutants were sprayed with a maltose buffer or with *B. cinerea* spore suspension at a density of 10^5 spores/mL and kept at high humidity. Total RNA was isolated from leaves harvested 24 h after spraying and probed with a *PR1* probe. The blot was stripped and reprobed with a *PDF1.2* probe.

(C) Three leaves from wild-type and transgenic plants constitutively expressing *WRKY18*, *WRKY40*, and *WRKY60* alone or coexpressing *WRKY18* and *WRKY60* or *WRKY18* and *WRKY40* were inoculated with *Pst*DC3000 (OD$_{600}$ = 0.001). Total RNA was isolated from the inoculated leaves harvested at 48 h after inoculation, separated on an agarose (1.2%)–formaldehyde gel, and probed with a *PR1* gene fragment.

DISCUSSION

Roles in Defense Responses

Arabidopsis WRKY18, *WRKY40*, and *WRKY60* are pathogen-induced and encode three structurally related WRKY proteins (Dong et al., 2003). To determine their roles in plant defense against microbial pathogens, we have analyzed both their knockout mutants and constitutive overexpression lines to the bacterial pathogen *P. syringae* and the fungal pathogen *B. cinerea*. *P. syringae* is biotrophic in the early stages of infection

but can become necrotrophic at late stages of infection and should probably be considered a hemibiotroph. *Arabidopsis* mutants defective in SA biosynthesis or signaling, including *eds1* (Aarts et al., 1998), *pad4* (Zhou et al., 1998), *eds5* (Rogers and Ausubel, 1997), *sid2* (Nawrath and Metraux, 1999), and *npr1* (Glazebrook et al., 1996), allow increased growth of *P. syringae*, indicating that SA-mediated signaling mechanisms play a vital role in limiting *P. syringae* growth. Among the three single knockout mutants analyzed, only *wrky18* exhibited a small reduction in bacterial growth, while *wrky40* and *wrky60* displayed no significant phenotype after infection of the bacterial pathogen (Figure 8A). Two double knockout mutants, *wrky18 wrky40* and *wrky18 wrky60*, on the other hand, supported modestly reduced bacterial growth, while the *wrky18 wrky40 wrky60* triple knockout mutant had the most reduced bacterial growth when compared with wild-type plants (Figure 8A). In transgenic overexpression plants, although constitutive expression of *WRKY18* led to constitutive expression of the SA-related *PR1* gene and enhanced resistance to *P. syringae*, its coexpression with *WRKY40* or *WRKY60* resulted in increased growth of the bacterial pathogen (Figure 11A). Based on these results, *WRKY18*, *WRKY40*, and *WRKY60* function redundantly and cooperatively as negative regulators in *Arabidopsis* defense that limits the growth of the bacterial pathogen.

B. cinerea is a necrotrophic fungal pathogen that promotes host cell death at very early stages in infection. *Arabidopsis* resistance to *B. cinerea* depends on JA and ET signaling pathways since mutations that block JA signaling, including *coi1* and *jar1*, or ET signaling, including *ein2*, result in enhanced susceptibility (Glazebrook, 2004). Our analyses revealed that loss-of-function mutants for the three WRKY genes exhibited altered phenotypes in response to the necrotrophic fungal pathogen, and these altered phenotypes were opposite to those observed with the bacterial pathogen *P. syringae*. Among the three single knockout mutants, only the *wrky18* mutant exhibited slightly reduced growth of *P. syringae* (Figure 8) and was also slightly more susceptible to *B. cinerea* than the wild-type plants (Figure 9). Double knockout mutants *wrky18 wrky40* and *wrky18 wrky60* but not *wrky40 wrky60* exhibited modestly reduced growth of *P. syringae* (Figure 8) and were modestly more susceptible to the fungal pathogen than wild-type plants (Figure 9). The *wrky18 wrky40 wrky60* triple knockout mutant had the most reduced bacterial growth (Figure 8) and was most susceptible to *B. cinerea* among all these mutants tested (Figure 9).

Based on analyses of pathogen-induced expression of SA-induced *PR1* and JA-regulated *PDF1.2* in these mutants (Figures 12A and 12B), the opposite effects of mutations of these WRKY genes on plant responses to the two pathogens probably reflected the antagonism between SA- and JA-mediated defense signaling pathways (Kunkel and Brooks, 2002; Li et al., 2004; Takahashi et al., 2004). After infection by *P. syringae*, enhanced induction of SA-regulated *PR1* expression was observed in some of these WRKY mutants, including *wrky18 wrky40*, *wrky18 wrky60*, and *wrky18 wrky40 wrky60* (Figure 12A), and was correlated with increased resistance to the bacterial pathogen (Figure 8). After infection by *B. cinerea*, on the other hand, reduced expression of JA-regulated *PDF1.2* was observed in the *wrky18 wrky40*, *wrky18 wrky60*, and *wrky18*

wrky40 wrky60 mutants (Figure 12B) and was correlated with enhanced susceptibility of these mutants to the necrotrophic fungal pathogen (Figure 9). Furthermore, the induction level of JA-regulated *PDF1.2* by the fungal pathogen was inversely correlated with that of SA-regulated *PR1* among the wild type and WRKY mutants (Figure 12B). These results suggest that these three WRKY proteins may function redundantly as negative regulators in SA-dependent pathways but play a positive role in JA-mediated pathways. Recently, *Arabidopsis WRKY70* has also been shown to be involved in the crosstalk between SA and JA signaling. However, studies with both overexpression and antisense lines indicated that WRKY70 plays a positive role in SA signaling and functions as a negative regulator of JA-inducible genes (Li et al., 2004). In a recent study using yeast two-hybrid screening, *Arabidopsis* mitogen-activated protein kinase 4 (MPK4), a repressor of SA-dependent resistance (Petersen et al., 2000), was found to interact with a MPK4 substrate MKS1 that in turn interacts with *Arabidopsis* WRKY25 and WRKY33 (Andreasson et al., 2005). In addition, WRKY25 and WRKY33 were shown to be in vitro substrates of MPK4, and a *wrky33* knockout mutant was found to express enhanced levels of the *PR1* gene under a short-day growth condition (Andreasson et al., 2005). These results suggest that WRKY25 and WRKY33 may function as downstream components of the MPK4-mediated signaling pathway and also act as repressors of SA-dependent disease resistance. Thus, different WRKY proteins play distinct roles in various signaling pathways of plant defense responses.

A recent study using the β-glucuronidase reporter gene driven by the *Arabidopsis WRKY18* gene promoter has shown that this gene is expressed in vascular bundles throughout the plant but not in very young leaves, suggesting that WRKY18 may play a role in vascular development (Ohashi-Ito et al., 2005). However, no obvious phenotype in vascular development was observed in the knockout mutants or overexpression plants for *WRKY18*. Since *WRKY18* is induced by pathogens and defense-inducing molecules such as SA, its constitutive expression in vascular bundles in the absence of pathogen may be caused by certain defense-mimic molecules or conditions such as lignification and reactive oxygen species that are associated with vascular tissues. The preferential expression of WRKY18 in the vascular bundles might contribute to plant defense as these tissues may be important for systemic defense signaling.

Analysis of the response of the overexpression and knockout lines for the three WRKY genes to *P. syringae* and *B. cinerea* have also revealed intriguing phenotypes that might reflect the complex nature of plant defense mechanisms. First, both mutations of these WRKY genes and constitutive expression of *WRKY18* and its coexpression with *WRKY40* or *WRKY60* resulted in enhanced susceptibility to *B. cinerea* (Figures 9 and 11). Thus, the roles of these WRKY genes in plant responses to the necrotrophic fungal pathogen may be more complex. It appeared that resistance to the fungal pathogen was associated with certain expression levels of the three WRKY genes. It has been proposed that the cell death induced by *B. cinerea* is a form of HR and is important for the virulence of the fungal pathogen (Govrin and Levine, 2000). HR is a form of programmed cell death that can be activated or positively influenced by a variety of signaling mechanisms, including those that normally antagonize each other. In plants, for example, SA and ET can activate signaling pathways antagonistic to each other, but they both are also known to induce or enhance pathogen-induced cell death (Shirasu et al., 1997; Devadas et al., 2002; Samuel et al., 2005). Therefore, it is possible that mutations of the WRKY genes may lead to activation or suppression of signaling mechanisms different from those in the transgenic overexpression lines, but these different signaling events may then converge to enhance cell death upon infection by the necrotrophic pathogen and promote its virulence.

Second, we observed reduced necrosis but enhanced chlorosis in the WRKY mutants with reduced bacterial growth after infection by *P. syringae* (Figure 8B). This was in contrast with the extensive necrosis but limited chlorosis in the *npr1* mutant and transgenic *nahG* plants with increased growth of the bacterial pathogen (Figure 8B). *P. syringae* is a biotroph during early stages of infection but after a period of multiplication in the plant becomes necrotrophic (Collmer and Bauer, 1994). Population density–dependent sensing is important for the expression of virulence in plant pathogens (Pierson et al., 1998) and may also be important for the transition from biotrophic to necrotrophic phases of *P. syringae*. In the *npr1* mutants, suppressed SA-mediated defense mechanisms support enhanced growth of the bacterial pathogen and may promote initiation of quorum sensing and switch to the necrotrophic phase. Likewise, expression of *nahG* in transgenic plants results in pleiotropic changes in defense pathways, including those mediated by elevated SA accumulation (Heck et al., 2003; van Wees and Glazebrook, 2003), and may also promote switch of the bacterial pathogen to necrotrophic phase by supporting enhanced bacterial growth. In the WRKY double and triple mutants with reduced bacterial growth, necrosis was limited probably because the bacterial density remained low and the initiation of quorum sensing and switch to the necrotrophic phase might be inhibited.

Physical and Functional Interactions

Despite similar structures and expression patterns, WRKY18, WRKY40, and WRKY60 were found to be distinct in a number of molecular and functional characteristics. For example, the three WRKY proteins differed in in vitro binding activity for DNA molecules containing the TTGACC/T W-box sequences: WRKY18 and WRKY40 had binding activity, while WRKY60 had little binding activity for the same DNA probes containing the W-box sequences (Figures 5 and 6). The sequence-specific DNA binding activity of WRKY18 and WRKY40 was correlated with altered growth phenotypes (e.g., reduced growth and enhanced serration of leaves) in the transgenic overexpression plants constitutively expressing *WRKY18* or *WRKY40* (Figure 10B). In addition, mature transgenic *35S:WRKY18* plants constitutively expressed the *PR1* gene and became more resistant to *P. syringae* but more susceptible to *B. cinerea* (Figures 10 and 11). No such alteration in defense responses was observed in transgenic plants overexpressing *WRKY40* or *WRKY60* (Figures 10 and 11). Likewise, the *wrky18* single mutant has a weak, but detectable, phenotype in response to the bacterial and fungal pathogens, while no such phenotype was observed in the *wrky40* or *wrky60* mutant (Figures 8 and 9). These observations suggest

that while the DNA binding activity of the WRKY proteins may be important for altered growth and defense phenotypes, there are additional properties that may differ among these WRKY proteins and that can influence their roles in plant growth and defense.

Using yeast two-hybrid assays and immunoprecipitation, we have shown that these three WRKY proteins interact with themselves and with each other to form both homocomplexes and heterocomplexes (Figure 4). Pathogen-induced expression of all three WRKY genes was detected in leaves (Figure 2), and it is likely that they can interact with each other in the leaf tissues, although additional experiments would be required to confirm that they are expressed in the same cells. Physical interactions have been widely reported between structurally related DNA binding transcription factors with comparable affinities to the same DNA sequences in vitro, and such interactions are often required for achieving the necessary selectivity and specificity of DNA binding in vivo. For example, the animal steroid hormone receptors bind exclusively as homodimers to response elements where the half-sites are organized in palindromic orientation (Freedman and Luisi, 1993). Other nuclear receptors, such as those for vitamin D, thyroid hormone, or trans-retinoic acid, form heterodimers with the *cis*-retinoic acid receptor and recognize response elements with half-sites arranged as direct repeats with different heterodimers recognizing response elements with different spacer lengths (Freedman and Luisi, 1993; Towers et al., 1993). Our analysis of in vitro DNA binding with mixed WRKY proteins indicated that such protein–protein interactions did not result in the high selectivity or specificity of DNA binding observed in other DNA binding proteins. For example, WRKY18 and WRKY40, alone or in combination, could bind DNA molecules containing two W-box sequences separated by various numbers of nucleotides (Figure 6B). On the other hand, distinct effects of protein–protein interactions on the DNA binding activity and specificity of these WRKY proteins were observed. Interactions between WRKY18 and WRKY40 had a positive effect on their DNA binding activity (Figure 6B). WRKY60, a protein with little in vitro DNA binding activity for W-box sequences, differentially influenced the DNA binding activity of the two other WRKY proteins. When mixed with WRKY18, WRKY60 enhanced the binding to a DNA molecule containing two specifically arranged W-box repeats (Figure 6). When mixed with WRKY40, WRKY60 reduced the DNA binding activity of WRKY40 (Figure 6). The altered DNA binding activities due to formation of heterocomplexes of two WRKY proteins could result from cooperative binding of the proteins or from a conformational change of one WRKY protein by the other interacting WRKY protein. These protein–protein interactions might even affect other properties important for their regulatory functions in transcription.

The physical interactions among these three WRKY proteins could explain some of the functional interactions observed in our analysis of their roles in plant defense responses. For example, there was a striking difference in the phenotype between transgenic plants expressing a single WRKY gene and those coexpressing two WRKY genes. In mature transgenic plants expressing *WRKY18*, there was constitutive expression of *PR1* and enhanced resistance to *P. syringae* (Figures 10C and 11A). When the plants were crossed with plants expressing *WRKY40* or *WRKY60*, the progeny plants were more stunted and the constitutive expression of *PR1* and enhanced resistance to the bacterial pathogen was abolished or even reversed (Figures 10C and 11A). This observation indicated that at least in these transgenic plants, *WRKY40* and *WRKY60* antagonized *WRKY18* in the activation of defense responses to the bacterial pathogen. However, since the transgenic plants expressing *WRKY40* or *WRKY60* alone did not have phenotypes opposite to those observed in the transgenic *WRKY18* plants (Figures 10C and 11A), it appeared that the phenotypes of transgenic *WRKY18/40* and *WRKY18/60* plants were the result of not only simple antagonistic actions but also functional cooperation of these WRKY proteins. Functional interactions were also obvious from the loss-of-function mutants in that the mutation of *WRKY40* or *WRKY60* was less consequential when it was single or in combination (Figures 8 and 9). However, when either mutation was combined with the *wrky18* mutation, an enhanced phenotype was observed (Figures 8 and 9). The functional interactions among the three WRKY proteins in influencing disease resistance are consistent with the changes in DNA binding activity. WRKY18 has a DNA binding activity (Figure 5) and can itself influence plant defense and disease resistance based on the altered phenotypes of its transgenic overexpression plants and the T-DNA insertion mutant (Figures 8, 9, and 11). Interaction of WRKY18 with WRKY40 and/or WRKY60 results in enhanced DNA binding activity and specificity (Figure 6) that may contribute to their increased roles in plant defense and disease resistance as exhibited from the stronger phenotypes of T-DNA double and triple mutants than those of the single mutants (Figures 8 and 9). The changes in DNA binding activity as a result of physical interactions may also explain the apparent antagonism of WRKY18 by WRKY40 and WRKY60 in transgenic overexpression plants. The enhanced *PR1* gene expression and resistance to *P. syringae* in the transgenic WRKY18 expression plants (Figure 11) is apparently not consistent with the role of WRKY18 in plant defense deduced from the similar phenotypes of its T-DNA insertion mutant (Figure 8). It is, therefore, possible that the observed phenotypes in the transgenic *WRKY18* overexpression plants might result from pleiotropic effects of *WRKY18* overexpression that could lead to nonspecific activation or repression of genes that are not normally regulated by WRKY18. Coexpression of WRKY40 or WRKY60 may enhance binding specificity of WRKY18 and reduce its nonspecific effects on gene expression. On the other hand, WRKY40 interaction with WRKY60 resulted in decreased DNA binding activity (Figure 6), and no positive functional interactions were observed between the two WRKY genes since their coexpression or double knockout did not affect disease resistance phenotypes (Figures 8, 9, and 11). When the cognate target genes of these WRKY proteins are identified in future studies, they will serve as more sensitive and reliable markers for determining the phenotypes of the WRKY single, double, and triple mutants. These molecular phenotypes can then be used for more rigorous analysis of the functional significance of the physical interactions between the WRKY proteins. These studies should provide important insights into the mode of action of the large number of plant WRKY proteins in plant defense and other biological processes.

METHODS

Plant Materials

The [α-^{32}P]dATP (>3000 Ci/mmol) was obtained from New England Nuclear; other common chemicals were purchased from Sigma-Aldrich. *Arabidopsis thaliana* plants were grown in a growth chamber at 22°C with 150 μE m^{-2} s^{-1} light in a photoperiod of 12 h light and 12 h dark.

cDNA Library Screening

The cDNA library was prepared from *Arabidopsis* plants harvested for 4 h after spraying with 2 mM SA. The library (in ZAP Express λ-vector from Stratagene) of 10^6 phages was screened using ^{32}P-labeled PCR-amplified products with primers designed from the genomic sequences of the *WRKY* genes. The hybridization was performed in the buffer of 5× SSPE, 0.5% SDS, 5× Denhardt's solution, and 100 μg/mL salmon sperm DNA for 16 h at 55°C. The filters were washed three to four times for 30 min each with 0.5× SSPE and 0.5% SDS at 55°C. The phagemid for each isolated clone was obtained through in vivo excision according to the manufacturer's instructions. DNA sequencing was performed by the dideoxynucleotide chain-termination method at the Genomics Center of Purdue University.

RNA Gel Blot Analysis

For RNA gel blot analysis of plant gene expression, *Arabidopsis* plants were treated and two fully expanded leaves from each plant were harvested at each time point for total RNA isolation using TRIZOL reagent (BRL Life Technologies). The RNA was separated on agarose (1.2%)–formaldehyde gels and blotted onto nylon membrane. Hybridization was performed using random-primed ^{32}P-labeled DNA probes in PerfectHyb plus hybridization buffer (Sigma-Aldrich) overnight at 68°C. The membranes were washed at 68°C once in 2× SSC and 0.1% SDS for 5 min, twice in 0.5× SSC and 0.1% SDS, and twice in 0.1× SSC and 0.1% SDS.

Subcellular Localization

Onion (*Allium cepa*) epidermal cell layers were peeled and placed inside up on the Murashige and Skoog (MS) plates. Plasmid DNAs of appropriate fusion genes (0.5 μg) were introduced to the onion cells using a pneumatic particle gun (PDS 1000; DuPont). The condition of bombardment was vacuum of 28-inch Hg, helium pressure of 1100 or 1300 psi, and 6 cm of target distance using 1.1 μm of tungsten microcarriers. After bombardment, tissues were incubated on the MS plates for 24 h at 22°C. Samples were observed directly or transferred to glass slides.

Yeast Two-Hybrid Screens

WRKY18-interacting proteins were identified using a *Gal4*-based two-hybrid system as described by the manufacturer (Stratagene). The *Arabidopsis* HybridZAP-2.1 two-hybrid cDNA library was prepared from *Arabidopsis* plants harvested for 4 h after spraying with 2 mM SA. The DNA fragments corresponding to different WRKY proteins were amplified by PCR and confirmed by sequencing. Overlapping PCR as previously described (Chen et al., 2003) was used to generate WRKY deletion constructs according to the following WRKY protein amino acids: HRR of WRKY18, 126 to 134; LZ of WRKY18, 44 to 80; LZ of WRKY40, 30 to 64; LZ of WRKY60, 42 to 76. The fragments were inserted into the *pBD-Gal4Cam* plasmid to generate bait plasmids. The HybridZAP cDNA library and corresponding bait plasmids were used to transform yeast strain YRG-2. Yeast transformants were plated onto the selection medium lacking Trp, Leu, and His. β-Galactosidase activity was assayed using *o*-nitrophenyl-β-D-galactopyranose as substrate.

Plasmid DNA was recovered from positive yeast colonies, transformed into *Escherichia coli* strain DH5α, and isolated for DNA sequencing.

Immunoprecipitation

To generate the FLAG-tagged WRKY18, a *WRKY18* cDNA fragment was generated by PCR amplification and subsequently subcloned into a tagging plasmid behind the FLAG tag sequence. The tagged gene was subcloned into pTA7002 behind the steroid-inducible *Gal4* promoter (Aoyama and Chua, 1997) and transformed into *Arabidopsis* plants. To analyze expression of the tagged transgene, transformants were treated with 5 μM dexamethasone, a steroid, and leaves were harvested 24 h later for RNA gel blot and protein gel blot analyses. MYC-tagged WRKY18, WRKY40, WRKY60, WRKY18(ΔLZ), and YFP constructs were generated using a similar approach, subcloned into the plant transformation vector pOCA30, and transformed into *Arabidopsis* plants. Transformants expressing the tagged transgenes were identified by protein gel blotting using anti-MYC-peroxidase (Invitrogen). Plants expressing FLAG-tagged WRKY18 and a MYC-tagged protein were generated through genetic crossing or double transformation and identified through protein gel blotting using epitope-recognizing antibodies after treatment with 5 μM dexamethasone.

Protein extracts (100 μg) from control plants and transgenic plants expressing FLAG-tagged WRKY18 and a MYC-tagged protein were incubated with the anti-FLAG epitope antibody at 4°C for 5 h on a shaker. Protein A-agarose (50 μL packed volume) was added and incubated for another 2 h. Agarose-protein complexes were collected by brief centrifugation. After washing three times with a wash buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 0.5% Triton X-100, and 10 μg/mL phenylmethylsulfonyl fluoride), the protein complexes were boiled for 5 min in 1× SDS-PAGE sample buffer. After centrifugation, the supernatant was separated by SDS-PAGE, and MYC-tagged WRKY protein in the immunoprecipitated FLAG-tagged WRKY18 complexes were detected with protein gel blot analysis using anti-MYC-peroxidase (Invitrogen).

Recombinant Protein and DNA Binding

Preparation of recombinant WRKY proteins and DNA binding assays were performed as previously described (Yu et al., 2001).

Identification and Genotyping of Knockout Mutants

The *wrky18-1* (Salk_093916) and *wrky60-1* (Salk_120706) T-DNA insertion mutants were identified from the Salk *Arabidopsis* T-DNA insertion population (Alonso et al., 2003) and are in the Columbia (Col) ecotype. The *wrky40* mutant was a Ds transposon insertion mutant obtained from Cold Spring Harbor Laboratory gene trap lines (Sundaresan et al., 1995) and is in the Landsberg *erecta* (L*er*) background. Confirmation of the insertions was done by performing PCR using a combination of a border primer of T-DNA (5′-GCTTGCTGCAACTCTCTCAG-3′) or Ds transposon (5′-ACC-CGACCGGATCGTATCGGT-3′) and a gene-specific primer (W18, 5′-TCA-TAGAAATTGAAGGGATACAAAAA-3′; W40, 5′-CGGAGATGCTAACTTTG-ATGTG-3′; W60, 5′-GCTGAAGCCGAGACTTCTCTT-3′). Another PCR was performed to identify plants homozygous for the insertions using the above gene-specific primers and respective reverse primers (W18R, 5′-TGATTTTTCATTTTCGTTAAAGC-3′; W40R, 5′-GGAGCACAAGCAC-ATTTGAA-3′; W60R, 5′-ATTTTCCACCCAAATCGTCA-3′). To remove additional insertions or mutations from the mutant, backcrosses to wild-type plants were performed, and plants homozygous for the insertion were again identified. Since the *wrky40* mutant was in the L*er* ecotype, it was backcrossed to Col wild-type plants three times, and the progeny plants from each cross were genotyped with a collection of 22 simple sequence length polymorphism markers spaced evenly over the entire genome in order to identify plants that contained the most Col

genetic background. Double and triple mutants were generated from genetic crosses of single mutants and identified through PCR genotyping.

To generate WRKY genes for genetic complementation, their cDNA clones were placed behind their native promoter fragments (~1.2 kb) that were amplified by PCR. The genes were subcloned into a modified plant transformation vector pKMB (Mylne and Botella, 1998) and directly transformed into the *wrky18 wrky40 wrky60* triple mutants. T1 transformants were identified based on their resistance to spraying with 1.384% (v/v) Finale (Farnam Companies) in soil and used for analysis of disease resistance.

Construction of Transgenic Plants

The transgenic line constitutively expressing *WRKY18* (W18Δ-1) has been previously described (Chen and Chen, 2002) and was used in this study. The cDNA fragments that contain the full coding sequences and 3′ untranslated regions of *WRKY40* or *WRKY60* were excised from the cloning plasmid and subcloned into the *Agrobacterium tumefaciens* 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 seeds were collected from the infiltrated plants and selected in MS medium containing 50 μg/mL kanamycin. Kanamycin-resistant plants were transferred to soil 9 d later and grown in a growth chamber.

Pathogen Infection

Pathogen inoculations were performed by infiltration of leaves of 6 to 10 plants for each treatment with the *Pst*DC3000 strain that contains the pVSP61 kanamycin-resistant empty plasmid vector (OD_{600} = 0.001 in 10 mM $MgCl_2$). Inoculated leaves were harvested 3 DAI 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 d before counting the colony-forming units.

Botrytis cinerea was grown on 2xV8 agar as described previously (Mengiste et al., 2003). To infect plants, conidia were collected from 10-d-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 3 to 4 d after the inoculation. Biomass of the fungal pathogen was quantified by RNA gel blotting of total RNA isolated from inoculated plants. A *Botrytis* β-*tubulin* gene fragment amplified from the *Botrytis* genome DNA was used as a probe as described previously (Mengiste et al., 2003).

Accession Numbers

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: *WRKY18*, At4g31800; *WRKY40*, At1g80840; *WRKY60*, At2g25000; *PR1*, At2g14610; *PDF1.2*, At5g44420.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Quantitative Assays of the Effect of the Leu Zipper Motif on WRKY Protein Interactions.

Supplemental Figure 2. Genetic Complementation of the *wrky18 wrky 40 wrky60* Mutant.

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