CHAPTER 4

INVOLVEMENT OF AtWRKY28 IN EXPRESSION OF *PR-1*

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ABSTRACT

Several elements in the *PR-1* promoter have been identified to mediate positive and negative regulation of transcription. The previous two chapters of this thesis describe the identification of AtWRKY50 as a protein that specifically binds to the promoter and activates gene expression. This chapter describes results on AtWRKY28 showing that it also binds to the *PR-1* promoter. One of its binding sites was found to be the W-box overlapping with the binding site of AtWRKY50 in LS4, while the other binding site was a W-box previously identified to be also important for SA-induced *PR-1* expression. Transactivation assays in protoplasts proved that both W-boxes were important for full AtWRKY28-mediated expression of the *PR-1::GUS* reporter gene.

INTRODUCTION

The defense response of plants upon attack by pathogens involves the activation of specific signaling pathways tailored to the type of invader. Biotrophic pathogens trigger the salicylic acid (SA) signaling pathway, which ultimately leads to the induced expression of defense proteins and a state of enhanced resistance known as systemic acquired resistance (SAR). The group of the so-called pathogenesis-related (PR) proteins consists of members with antifungal activities, like β-1,3-glucanases (PR-2) and chitinases (PR-3), that are able to degrade fungal and oomycete cell-walls, thus preventing fungal growth. Although for the PR-1 proteins no specific anti-pathogen activity is known, the protein itself and the induced expression of its gene are generally used as markers for SAR (Glazebrook, 2005; Grant and Lamb, 2006). The SA signaling pathway involves the induced production of SA, which subsequently binds to NPR receptors (either NPR1 or NPR3 and NPR4), leading to the activation of

NPR1-mediated expression of defense genes (Wu *et al.*, 2012; Fu *et al.*, 2012). In Arabidopsis, SA is synthesized from isochorismate that is produced from chorismate by the enzyme isochorismate synthase (ICS), the product of the *ICS1* gene (Wildermuth *et al.*, 2001). Recent work of our group has identified AtWRKY28 as a transcriptional activator of *ICS1* gene expression (Van Verk *et al.*, 2011). Since the *AtWRKY28* gene is induced early after infection and both *AtWRKY28* and *ICS1* expression are co-regulated, it is expected that expression of AtWRKY28 is one of the early steps in the SA signaling pathway and thus ultimately leads to expression of the late defense genes, including *PR-1* (Van Verk *et al.*, 2011).

The *PR-1* promoter contains binding sites for TGA transcription factors that have been shown to regulate SA-mediated expression through interaction with the co-activator NPR1 (Dong, 2004). In addition, the promoter contains a number of W-boxes, consensus-binding sites for WRKY proteins, and mutational analyses have indicated that they are also important for regulation of *PR-1* expression (Lebel *et al.*, 1998; Pape *et al.*, 2010). In Chapter 2 we have identified AtWRKY50 as a transcriptional regulator of *PR-1*. However, AtWRKY50 binds to the DNA at sites different from the W-box, which suggests that other, W-box-binding WRKYs may also play roles in *PR-1* expression. As was shown in Chapter 2, protoplast transactivation assays with several other AtWRKYs, including AtWRKY42 and AtWRKY28, resulted in elevated expression of *PR-1* (Chapter 2, Table 1). Here we have investigated if these WRKYs are able to bind to the *PR-1* promoter and activate gene expression.

RESULTS AND DISCUSSION

AtWRKY28 binds to the *PR-1* promoter

In the large-scale protoplast transactivation screening presented in Chapter 2, Table 1, AtWRKY42, a subgroup IIb WRKY, was the second best activator of *PR-1*, enhancing luciferase activity 5.7-fold, while AtWRKY28 (subgroup IIc) was fourth in the line of activating WRKYs (see Chapter 2, Table 1). AtWRKY28 was previously identified in a screening for Arabidopsis WRKYs that were co-regulated with proteins involved in SA signaling. Another WRKY transcription factor that resulted from this co-expression analysis was AtWRKY46 (subgroup III) (Van Verk *et al.*, 2011). In the protoplast transactivation screening AtWRKY28 activated the *PR-1::Luc* reporter gene approximately 2.5-fold over the level obtained without co-expressed transcription factor, while AtWRKY46 enhanced expression 1.5-fold (Chapter 2, Table 1).

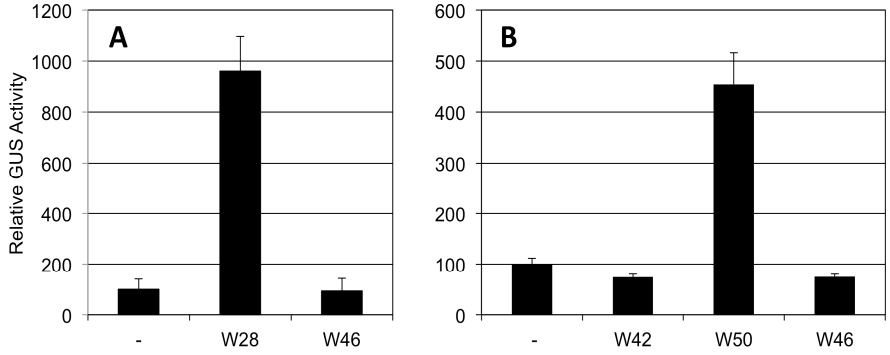

Figure 1. Protoplast transactivation assays. (A, B) Arabidopsis protoplasts were co-transfected with *PR-1::GUS* construct alone (minus sign) or together with expression plasmids *35S::AtWRKY28* (W28), *35S::AtWRKY46* (W46), *35S::AtWRKY42* (W42) or *35S::AtWRKY50* (W50). After incubation GUS activity was measured spectrophotometrically. The bars represent the percentage of GUS activity from triplicate experiments relative to that of the protoplasts co-transfected with *PR-1::GUS* construct and empty vector control. Error bars represent the SEM.

Although the difference between these activation levels seems trivial, Fig. 1A shows that in our protoplast transactivation assays using a *PR-1::GUS* reporter gene the difference in activation by the two WRKYs is highly significant. AtWRKY28 induced GUS expression almost 10-fold (Fig. 1A), while AtWRKY46

had no effect on GUS expression (Fig. 1A, B). Transactivation assays with a *35S::AtWRKY42* effector construct showed that AtWRKY42 did not activate the *PR-1* promoter in our protoplast system (Fig. 1B). As the GST-tagged version of the *AtWRKY42* open reading frame was properly expressed in *E. coli*, we have no reason to assume that the differences between the results of the protoplast transactivation assays presented here and in Table 1 of Chapter 2 were caused by cloning errors.

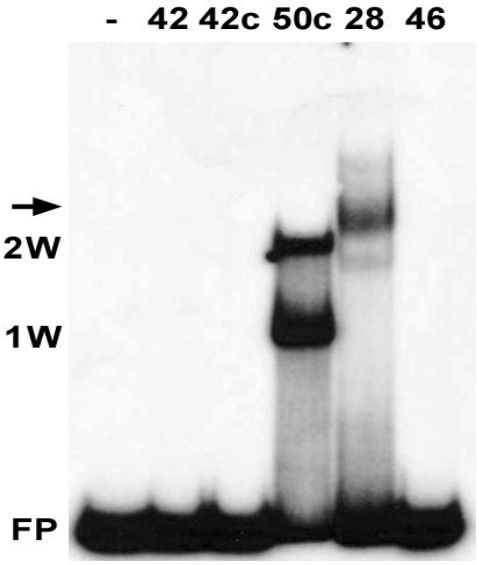

Figure 2. EMSAs of WRKYs with the 80-bp *PR-1* promoter fragment. EMSAs were performed with a frag-ment of the *PR-1* promoter ranging from -688 to -609 bp up-stream of the transcription start site incubated without (minus sign) or with GST-fusion proteins of full-length AtWRKY42 (42), C-terminal half of AtWRKY42 (42c), C-terminal half of AtWRKY50 (50c), AtWRKY28 (28) and AtWRKY46 (46). The positions of the unbound probe (FP: free probe), the band shifts correspond-ding to one (1W) and two (2W) AtWRKY50-C proteins bound to the probe, and the band shift produced with AtWRKY28 (arrow) are indicated at the left.

While we identified AtWRKY50, the best activator in the protoplast transactivation assay, as a direct activator of *PR-1* expression (Chapters 2 and 3), we were interested to know if WRKYs -28, -42 and -46 were able to bind to the *PR-1* promoter and activate transcription directly. Therefore, EMSAs were done with purified WRKY-GST fusion proteins produced in *Escherichia coli*. The proteins were incubated with the 80-bp *PR-1* promoter fragment comprising the region of -688 to -609 bp upstream of the transcription start site shown to be

important for SA-induced expression, which was also used in the previous chapters. Fig. 2 shows the results of the EMSAs with AtWRKY42, AtWRKY28 and AtWRKY46. The two band shifts produced in the EMSA of the positive control shown in Lane 50c correspond to one and two AtWRKY50-C peptides, respectively, binding to the probe (see Chapter 2). The band shift produced by AtWRKY28 indicates that also this WRKY protein is able to bind to the 80-bp fragment. AtWRKY46 and AtWRKY42 did not produce band shifts and neither did a shorter version of AtWRKY42, consisting of only the C-terminal DNA-binding domain, indicating that AtWRKY42 and AtWRKY46 did not bind to the 80-bp promoter fragment. To further identify the binding site of AtWRKY28 in the 80-bp fragment, EMSAs were done with the series of overlapping subfragments A, B, C and D used previously (See Chapter 2, Fig. 5). The relative location and the sequences of the various promoter fragments used as probes are shown in Fig. 3.

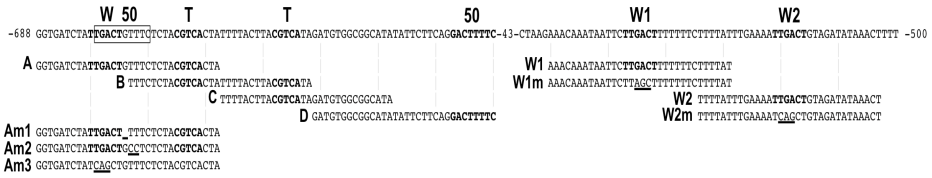

Figure 3. Sequences of *PR-1* promoter fragments used for EMSAs. Only the sequence of the upper strand is given. The top line displays the sequence of the 80-bp fragment corresponding to bp -688 to -609 and -565 to -500, upstream of the transcription start site. The gap of 43 bp between the two sequence stretches is indicated. Promoter element LS4 in the 80-bp fragment is boxed. W, W1 and W2 indicate the W-boxes in the sequence, with the consensus sequence indicated in bold. For comparison, also the positions of the TGA binding sites (T) and the AtWRKY50 binding sites (50) are indicated. Overlapping subfragments A, B, C and D, and mutant versions Am1, Am2, and Am3 are aligned with the sequence of the 80-bp fragment. Subfragments W1 and W2 and the mutant versions W1m and W2m are aligned with the sequence of the region from -565 to -500. Nucleotide changes in the mutant fragments are underlined.

The results of the EMSAs are shown in Fig. 4. As expected, AtWRKY46 did not yield band shifts with any of the fragments, while incubation of the probes with

AtWRKY50-C resulted in single band shifts with both fragments A and D, each containing an AtWRKY50 binding site (Chapter 2). Evidently, AtWRKY28 only produced a band shift with fragment A. Because fragment A contains a consensus WRKY binding site (W-box) in the LS4 element, we tested the binding of AtWRKY28 to a series of fragment A mutants. Mutant probe Am1 has a deletion of a G-residue immediately 3' to the W-box, while Am2 has two T to C changes in the binding site of AtWRKY50 situated to the right of the W-box (Fig. 2). Neither of these mutations affects the W-box consensus sequence TTGACT. In mutant fragment Am3 however, the W-box is mutated (Fig. 3). The results of EMSAs of AtWRKY28 with the wild type and mutant fragment A probes are shown in Fig. 5. Both probes Am1 and Am2 yielded band shifts upon incubation with AtWRKY28, indicating that mutations outside of the W-box do not interfere with AtWRKY28's ability to bind to the probe.

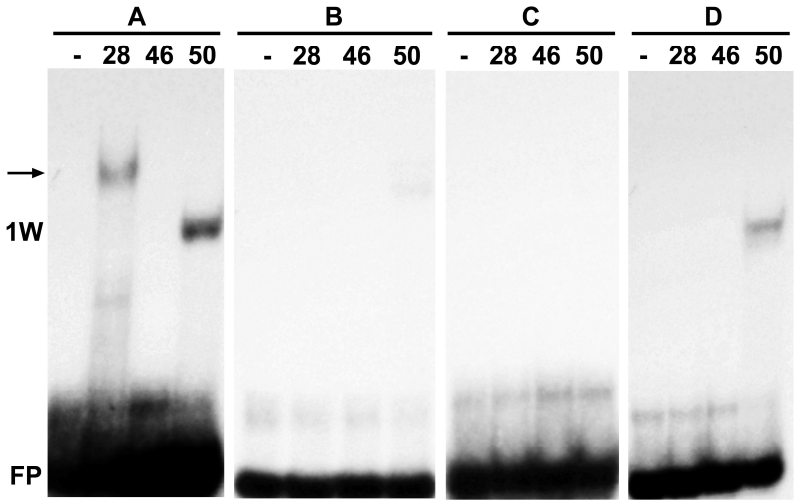

Figure 4. Binding of AtWRKY28 to subfragments of the 80-bp *PR-1* promoter fragment. EMSAs were performed with a series of overlapping fragments (A to D) of the 80-bp *PR-1* promoter incubated without (minus sign) or with N-terminal GST-fusion proteins of full-length AtWRKY28 and AtWRKY46 and the C-terminal binding region of AtWRKY50 as indicated above the lanes. The positions of the unbound probe (FP: free probe), the band shifts corresponding to AtWRKY50-C (1W) and the band shift produced with AtWRKY28 (arrow) are indicated at the left.

However, the absence of a band shift with probe Am3 indicates that mutation of the W-box itself disrupts the binding. This indicates that the W-box in LS4 is the binding site of AtWRKY28 in the 80-bp fragment. Surprisingly, deletion of the G-residue immediately downstream of the W-box resulted in a much more intense band shift (Fig. 5, compare the band shifts of fragments WT and Am1), suggesting that nucleotides outside of the consensus-binding site have a strong effect on the binding affinity for AtWRKY28. In addition to the W-box in LS4 (indicated as with W in Fig. 3), the *PR-1* promoter contains two other consensus W-boxes. These are located at positions -546 (W1) and -520 (W2) upstream of the transcription start site. Deletion of W-box W1 was shown to result in greatly reduced activity of the *PR-1* promoter, which was not further reduced when the deletion was extended to also remove W-box W2 (Pape *et al.*, 2010).

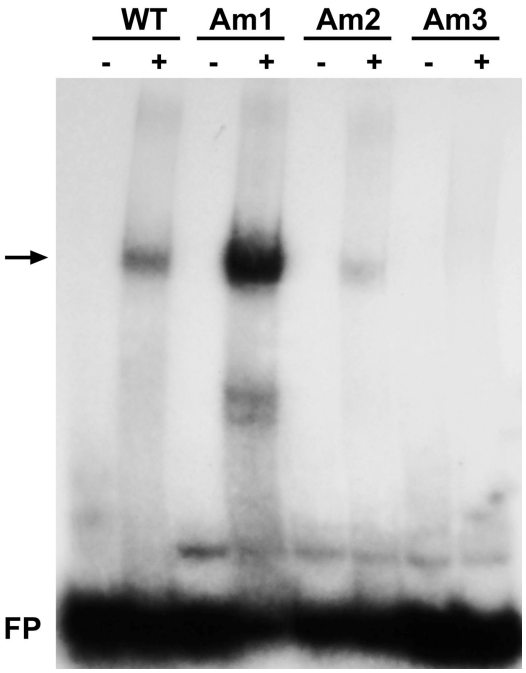

Figure 5. Mutational analysis of AtWRKY28's binding site in subfragment A. EMSAs were performed with wild type (WT) and mutant (Am1, Am2, Am3) versions of fragment A, without (minus sign) or with (plus signs) the N-terminal GST-fusion protein of AtWRKY28. The positions of the unbound probe (FP: free probe) and the band shifts produced with AtWRKY28 (arrow) are indicated at the left.

We investigated the ability of AtWRKY28 to bind to W-boxes W1 and W2. The

results of EMSAs with 32-bp probes corresponding to the *PR-1* sequence surrounding W-boxes W1 and W2 are shown in Fig. 6. AtWRKY28 produced band shifts with both probes, although the band shift with the W1 probe was more intense than the one with the W2 probe. Furthermore, mutation of three central base pairs of W-box W1 abolished the binding to AtWRKY28. A similar mutation of W-box W2 did not result in a diminished binding of AtWRKY28, suggesting that the band shift produced with this promoter region is independent of the W-box. The TTGACT consensus sequence of W-box W1 is followed by a series of six T-residues. This is reminiscent of the W-box in Am1, in which deletion of the G-residue led to a stretch of three T-residues 3′ of the consensus sequence, which resulted in a strongly enhanced binding of AtWRKY28 (Fig. 5). W-box W2 conforms to the consensus sequence, but has no 3′ stretch of T-residues. Apparently, the extra T-residue(s) 3′ of the consensus sequence enhance the binding affinity or the binding specificity for AtWRKY28. Our results are in good agreement with the results of Van Verk *et al.* (2011), who characterized the AtWRKY28 binding sites in the *ICS1* promoter and found that nucleotides upstream and downstream of the W-box core sequence were important for binding to AtWRKY28. With a C-residue immediately 5′ of the W-box and the absence of G-residues in the three nucleotides 3′ to the W-box, the W1 binding site in the *PR-1* promoter matches AtWRKY28's binding sequence deduced by Van Verk *et al.* (2011). Although we have not performed EMSAs of fragments W1 or W2 with a more extensive set of WRKYs, neither of the probes produced band shifts with full-length AtWRKY42, nor with its C-terminal DNA binding domain AtWRKY42-C (data not shown), indicating that probably, in addition to the 6-bp W-box, residues outside of the W-box also determine the binding to specific WRKY proteins. To determine the contribution of the W-boxes in LS4 (W) and at position -546 (W1) to AtWRKY28's activation of *PR-1* expression, mutations of these W-boxes as in

Am3 (Wm) and W1m (Fig. 3) were introduced in the 1000 bp *PR-1* promoter. Fig. 7 shows that mutation of the W-box in LS4 (Wm) reduced the level of AtWRKY28-activated GUS expression approximately 50% compared to expression directed by the WT promoter. Mutation of W-box W1 resulted in an even larger reduction of GUS expression (75%), while combination of the two mutations did not further reduce expression (Fig. 7).

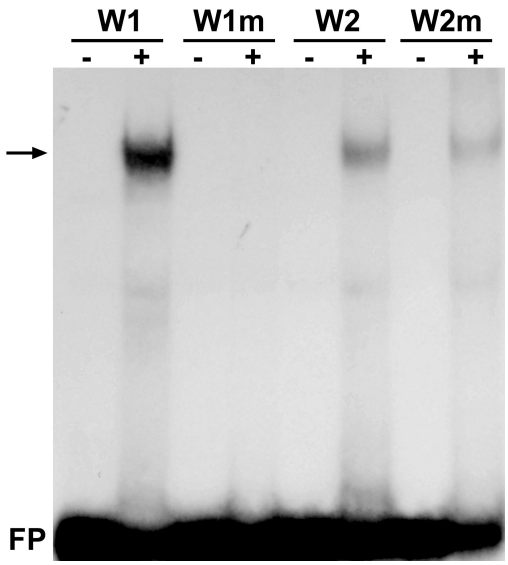

Figure 6. Binding of AtWRKY28 to W-boxes W1 and W2 in the *PR-1* promoter. EMSAs were performed with 35-bp promoter fragments containing wild type (W1, W2) and mutant (W1m, W2m) versions of the W-boxes at positions -546 and -540 bp upstream of the transcription start site, in the absence (minus signs) and presence (plus signs) of the GST-AtWRKY28 fusion protein. The arrow indicates the position of the band shifts. FP: free probe.

Apparently, AtWRKY28 activates low levels of expression through other binding sites in the promoter. These may include the non-specific binding site in fragment W2, although we have not further investigated this. Nevertheless, the results show that the W-boxes in LS4 and W1 contribute to AtWRKY28-activated expression of *PR-1*.As stated above, we have previously identified

AtWRKY28 as an activator of *ICS1* gene expression (Van Verk *et al.*, 2011). *ICS1* is expressed early after pathogen attack and so is the *AtWRKY28* gene, which is already highly expressed 2 hours after inoculation with avirulent *Pseudomonas syringae*, long before SA begins to accumulate (Wildermuth *et al.*, 2001; Dong *et al.*, 2003; Navarro *et al.*, 2004; Van Verk *et al.*, 2011). However, Fig. 8 shows that *AtWRKY28* gene expression is also induced upon exogenous application of SA. At 6h after SA application *AtWRKY28* transcript accumulation is maximal and after that gradually declines. The time course of SA-induced *PR-1* expression follows that of *AtWRKY28*, which supports a role for AtWRKY28 as a direct activator of *PR-1* expression.

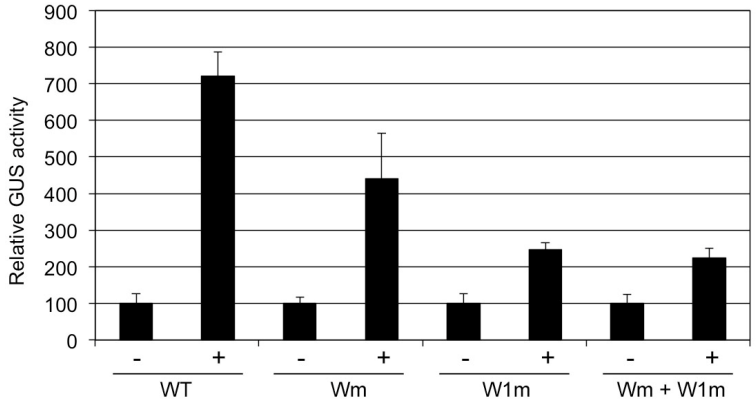

Figure 7. Protoplast transactivation assays. Arabidopsis protoplasts were transfected with *PR-1::GUS* constructs in which the promoter had no mutations (WT) or had mutations in the W-box in LS4 (Wm) or the W-box at position -546 (W1m), or a combination of the two mutations (Wm + W1m). Minus signs indicate samples that were co-transfected with an empty effector construct, plus signs indicate samples that were co-transfected with *35S::AtWRKY28* effector plasmid. After incubation GUS activity was measured spectrophotometrically. The bars represent the percentage of GUS activity from triplicate experiments relative to that of the protoplasts co-transfected with the corresponding *PR-1::GUS* construct and empty vector control. Error bars represent the SEM.

In conclusion, we have shown that the *PR-1* promoter contains a number of W-boxes that are able to specifically bind to AtWRKY28 with different affinities.

Mutation of the consensus W-boxes in LS4 and W1 abolished binding of AtWRKY28 and resulted in reduced, AtWRKY28-mediated *PR-1* expression. As *AtWRKY28* gene expression is induced by SA and precedes *PR-1* expression, it likely plays a role as a direct transcriptional activator of *PR-1* expression.

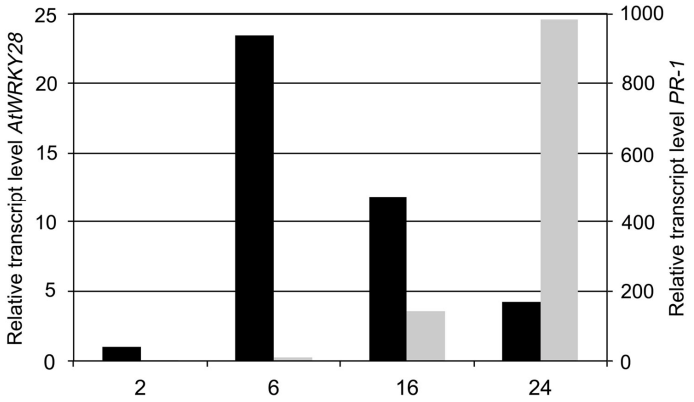

Figure 8. Time course of salicylic acid induced *AtWRKY28* and *PR1* expression. Accumulation of *AtWRKY28* (black bars) and *PR-1* (grey bars) mRNA at the indicated times (hours) after incubation of plants in 1mM salicylic acid is relative to the level of the transcripts measured at 2 h post treatment.

Table 1.

PR-1 80BP	F	ATCGGATCCGGTGATCTATTGACTGTTTCTCTAC
	R	GCCTAGATCTGAAAAGTCCTGAAGAATATATGCC
PR-1 Frag. A	F	GGTGATCTATTGACTGTTTCTCTACGTCACTA
	R	TAGTGACGTAGAGAAACAGTCAATAGATCACC
PR-1 Frag. B	F	TTTCTCTACGTCACTATTTTACTTACGTCATA
	R	TATGACGTAAGTAAAATAGTGACGTAGAGAAA
PR-1 Frag. C	F	TTTTACTTACGTCATAGATGTGGCGGCATATA
	R	TATATGCCGCCACATCTATGACGTAAGTAAAA
PR-1 Frag. D	F	GATGTGGCGGCATATATTCTTCAGGACTTTTC
	R	GAAAAGTCCTGAAGAATATATGCCGCCACATC
Frag.Am1	F	GGGGGTGATCTATTGACTTTTCTCTACGTCACTAT
	R	GGGATAGTGACGTAGAGAAAAGTCAATAGATCACC
Frag. Am2	F	GGGGGTGATCTATTGACTGCCTCTCTACGTCACTA
	R	GGGTAGTGACGTAGAGAGGCAGTCAATAGATCACC
Frag. Am3	F	GGGGGTGATCTATCAGCTGTTTCTCTACGTCACTA
	R	GGGTAGTGACGTAGAGAAACAGCTGATAGATCACC
Frag. W1	F	GGGAAACAAATAATTCTTGACTTTTTTTCTTTTAT

	R	GGGATAAAAGAAAAAAAGTCAAGAATTATTTGTTT
Frag. Wm1	F	GGGAAACAAATAATTCTCAGCTTTTTTTCTTTTAT
	R	GGGATAAAAGAAAAAAAGCTGAGAATTATTTGTTT

MATERIALS AND METHODS

Expression of AtWRKY28, 42 and 46 Fusion Proteins

The full-length coding sequence of *AtWRKY28* (At4g18170), *AtWRKY42* (At4g04450) and *AtWRKY46* (At2g46400) were cloned in frame behind the GST open reading frame of expression vector pGEX-KG (Guan and Dixon, 1991). The ORF was PCR-amplified from Arabidopsis cDNA library using primer sequence of AtWRKY28 5′-GTC ATC TAG ACA TGT CTA ATG AAA CCA GAG ATC TCT AC-3′ and 5′-GTC ACT CGA GTC AAG GCT CTT GCT TAA AGA AAA TTG-3′ digested with XbaI and XhoI to clone into pGEX-KG. The ORF of AtWRKY42 was PCR-amplified by the sets of primer 5′-ATA GGG ATC CGT ATG TTT CGT TTT CCG GTA AGT CTT GGA-3′ and 5′-GCC AAG CTT CGA GTC TTA TTG CCT ATT GTG AAC GTT GCT-3′, digested with BamHI and HindIII to clone into pGEX-KG. To clone the c-terminal half of the open reading frame we use these sets of primer for AtWRKY42C: 5′-ATA GGG ATC CGT GTC ATT GAG CAA GCG GCC G-3′ and 5′-GCC AAG CTT CGA GTC TTA TTG CCT ATT GTG AAC GTT GCT-3′, digested with BamHI and HindII to clone into pGEX-KG. The ORF of AtWRKY46 was PCR-amplified by the sets of primer 5′-GTC ATC TAG ACA TGA TGA TGG AAG AGA AAC TTG TG-3′ and 5′-GTC AAA GCT TCT ACG ACC ACA ACC AAT CCT GTC-3′, digested with XbaI and HindIII to clone into pGEX-KG. These plasmids were transformed into *E. coli BL21-DE3*. For induction of protein expression, cultures were grown to mid-log phase at 37°C, after which isopropyl-β-thiogalacto-pyranoside was added to a final concentration of 0.1 mM and incubation continued for 3 h at 22°C. The cells were harvested by centrifugation,

resuspended in 1/20th volume sonication buffer (1x phosphate-buffered saline containing 2% [v/v] Tween 20, 0.1% [v/v] Triton X-100, 5 mM dithiothreitol [DTT], and 1 mg mL-1 lysozyme) and lysed by sonication (Vibracell). The fusion proteins were purified using glutathione-Sepharose 4B columns (Amersham), which were eluted overnight at 4°C with 10mM reduced glutathione, after which 1/50th volume Complete (Roche) protease inhibitors were added. Expressed fusion proteins were analyzed using 12% SDS-PAGE.

Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed essentially as described by Green et al. (1989). DNA probes for the EMSA assays were obtained by slowly cooling down mixtures of equimolar amounts of complementary oligonucleotides from 95°C to room temperature. Annealed oligonucleotides were subsequently labeled using T4-nucleotide kinase and [γ-32P] ATP or kelnow fragment and [α-32P] dCTP, after which unincorporated label was removed by Autoseq G-50 column chromatography (Amersham-Pharmacia Biotech). Different sets of oligo's and their mutated version can be found in table (1). EMSA reaction mixtures contained 0.5 µg purified protein, 3 µL 5x gel shift binding buffer [20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.25 mg mL-1 poly (dI-dC) x poly(dIdC) (Promega)] in a total volume of 14 µL. After 10-min incubation at room temperature, 1 µL containing 60,000 cpm of labeled probe was added and novel WRKY factor in defense signaling incubation was continued for 60 min at ice. The total mixture was loaded onto a 5% polyacrylamide gel in Tris-borate buffer and electrophoresed at 4°C. After electrophoresis, the gel was dried; auto radiographed, and analyzed using a Bio-Rad Phosphoimager/developer system.

qRT-PCR

Total RNA was isolated from pulverized frozen transgenic Arabidopsis plants

by phenol extraction and LiCl precipitation. Oligo (dT) -primed cDNA for PCR was obtained using M-MLV reverse transcriptase. Subsequently, qPCR was performed during 40 cycles with primers corresponding to PR-1: 5′-CTC GGA GCT ACG CAG AAC AAC T-3′ and 5′-TTC TCG CTA ACC CAC ATG TTC A-3′; WRKY28: 5′-CAA GAG CCT TGA TCG ATC ATT G-3′ and 5′-GCA AGC CCA ACT GTC TCA TTC-3′; and the control gene At1g13320: 5′-TAA CGT GGC CAA AAT GAT GC-3′ and 5′-GTT CTC CAC AAC CGC TTG GT-3′. To quantify we used 2x Syber green super mix from Bio-Rad (cat# 170-8882).

Plasmid construction and Transactivation Experiments

The *AtWRKY28* (At4g18170), and *AtWRKY46* (At2g46400) open reading frames was PCR-amplified from Arabidopsis cDNA library (6h SA treated Arabidopsis) using the primer sets to cloned into pRT101. The primer sequences was WRKY28: 5′-GTC ACT CGA GAT GTC TAA TGA AAC CAG AGA TCT CTA C-3′and 5′-CAG TGG ATC CTC AAG GCT CTT GCT TAA AGA AAA TTG-3′; WRKY46: 5′-GTC ACT CGA GAT GAT GAT GGA AGA GAA ACT TGT TG-3′ and 5′-CAG TTC TAG ACT ACG ACC ACA ACC AAT CCT GTC-3′. .In order to get PR-1 promoter fuse with GUS we used genomic DNA from Arabidopsis to PCR with primer set 5′-GTC AAA GCT TCT GAT TCG GAG GGA GTA TAT GTT ATT G-3′ and 5′-CGA TGG ATC CTTTTC TAA GTT GAT AAT GGT TAT TGT TGT G-3′, digested with BamHI and HindIII to put into pT7:GUS vector. Protoplasts were prepared from Arabidopsis ecotype Columbia-0 cell suspension according to Axelos *et al*. (1992) with lab suited modifications. A 5-days old cell suspension culture was diluted 5 fold in 50 mL medium (cell culture media-3.2 g/L Gamborg B5 basal medium with minimal organics [Sigma-Aldrich], 3% Suc, 1 µM naphthylacetic acid [NAA], pH 5.8) and incubated overnight at 25^0C at 250rpm. Cells were harvested and cell walls digested with 20mL of enzyme mix (0.4% macerozyme R-10, 1.5% cellulose R-10, 12% sorbitol, pH 5.8) for 3h at 28^0C with minimal shaking. The protoplasts

were filtered with a 65-µm steel sieve and washed two times in 50mL of protomedium (Gamborg B5 basal medium, 0.1 M glucose, 0.25 M mannitol, 1 µM NAA, pH 5.8). The volume of the protoplast suspension was adjusted to 4 x 10^6 cells/mL. Protoplasts were cotransfected with 2 µg of plasmid carrying one of the PR-1 promoter:GUS constructs and 6 µg of effector plasmid pRT101 (Töpfer *et al*., 1987) carrying 35S::AtWRKY28 and 46. As a control, co-transformation of PR-1 promoter::GUS fusions with the empty expression vector pRT101 was carried out. Protoplasts were transformed using PEG as described previously (Schirawski *et al*., 2000). The protoplasts were harvested 16 h after transformation and frozen in liquid nitrogen. For protoplast experiments, GUS activity was determined as described (van der Fits and Memelink, 1997), with minor modifications. GUS activities from triplicate experiments were normalized against total protein level.

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