CHAPTER 2

ATWRKY50 SPECIFICALLY BINDS TO THE *PR-1* PROMOTER AND ACTIVATES GENE EXPRESSION

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ABSTRACT

Arabidopsis *PR-1* is a salicylic acid (SA)-inducible defense gene. Its promoter contains a number of consensus binding sites for WRKY transcription factors. In this study two promoter elements were identified that specifically bind the DNA-binding domain of AtWRKY50. AtWRKY50 belongs to a sub group of WRKY proteins containing a WRKYGKK domain that varies from the WRKYGQK domain present in the majority of WRKY proteins. *AtWRKY50* gene expression was induced by SA and preceded expression of *PR-1*. The binding sequences of AtWRKY50 (GACT[G]TTTC) deviated significantly from the consensus sequence (W box TTGAC[C/T]). Co-transfection of Arabidopsis protoplasts with *35S::AtWRKY50* and *PR-1::GUS* promoter fusions showed that expression of *AtWRKY12* resulted in a strong increase in GUS expression, which required functional binding sites in the *PR-1* promoter.

INTRODUCTION

Upon pathogen attack plants mobilize inducible defense systems. A classic example is the systemic acquired resistance (SAR) effective against a broad range of pathogens. The signal transduction route leading to SAR involves the induced synthesis of the endogenous signal molecule salicylic acid (SA). SAR is accompanied by the de novo synthesis of pathogenesis-related (PR) proteins of which many directly affect pathogen growth and disease proliferation. Although their exact function is still not fully characterized, the plant kingdom-wide conserved PR-1 proteins are generally considered as marker proteins for SAR. In most plant species expression of the genes encoding these proteins is under transcriptional control (Linthorst, 1991; van Verk *et al.*, 2009).

Early work by the group of Chua in tobacco (*Nicotiana tabacum*) has

indicated that gene expression controlled by the *35S* promoter from Cauliflower mosaic virus is enhanced by SA and that this effect depends on the presence of *activation sequence-1* (*as-1*), a DNA element in the 90 bp core promoter consisting of two TGACG tandem repeats (Qin *et al.,* 1994). The *as-1* element specifically binds to tobacco ASF-1, a DNA-binding complex containing basic leucine zipper (bZIP) type TGA proteins (Katagiri *et al.,* 1989; Qin *et al.,* 1994, Niggeweg *et al.,* 2000a).

Also promoters of several *PR* genes, such as *Arabidopsis thaliana PR-1* and tobacco *PR-1a* contain *as-1*-(like) elements in promoter regions important for SA-induced expression. In tobacco the *as-1*-like element in the *PR-1a* promoter consists of a set of inverted TGACG motifs which were found to bind TGA transcription factors, while mutation of the element in a *PR-1a-promoter::GUS* reporter gene affected SA-induced GUS expression (Strompen *et al.,* 1998; Niggeweg *et al.,* 2000b; Grüner *et al.,* 2003). Likewise, a linker scanning analysis of the region of the Arabidopsis *PR-1* promoter responsible for induced expression by the SA analog 2,6-dichloroisonicotinic acid (INA) revealed the presence of an *as-1* element with two TGACG direct repeats in inverted orientation, of which one is a positive regulatory element (-645 to -636 upstream of the transcription start site; for convenience this region will further be referred to with LS7, the name of the linker that was used to mutate this element), while the other (LS5, -665 to -656) mediates negative regulation of *PR-1* expression (Lebel *et al.,* 1998). Through knock-out analyses it was shown that the Arabidopsis bZIP transcription factors TGA2, TGA5 and TGA6 act as redundant but essential activators of *PR-1* expression and SAR (Zhang *et al.,* 2003; Kesarwani *et al.,* 2007).

The ankyrin repeat protein NPR1 plays a central role in defense responses and is required for induction of *PR* gene expression and the establishment of SAR (Cao *et al.,* 1997; Delaney *et al.,* 1995; Wang *et al.,* 2006).

Pathogen-induced accumulation of SA effects a change of the redox state of the cell, resulting in release of reduced NPR1 monomers from multimeric complexes residing in the cytoplasm, which subsequently translocate to the nucleus where they interact with TGA transcription factors to activate gene expression (Mou *et al.,* 2003; Kinkema *et al.,* 2000; Després *et al.,* 2000; Zhang *et al.,* 1999; Zhou *et al.,* 2000). Recently, it was shown that coactivation by NPR1 occurs in a pulse-wise manner and is regulated by degradation of NPR1 via the proteasome (Spoel *et al.,* 2009; Fu *et al.*, 2012).

In addition to TGAs, WRKY transcription factors are important for transcriptional programs induced in response to environmental signals (Eulgem and Somssich, 2007; Pandey and Somssich, 2009). WRKY transcription factors are classified as a family of plant-specific DNA-binding proteins characterized by the occurrence of the peptide sequence Trp-Arg-Lys-Tyr (WRKY) followed by a Zn-finger domain (Rushton *et al.*, 2010). An ever-increasing number of research publications indicate the involvement of WRKY transcription factors in SAR. Unlike the TGA transcription factors that are present at steady state levels (Johnson *et al.,* 2003), many of the WRKY genes are transcriptionally activated upon biotic and abiotic stress. Various WRKY proteins positively regulate resistance against necrotrophic pathogens, like AtWRKY33 (Zheng *et al.*, 2006), others positively regulate defense against biotrophs, like AtWRKY53 and AtWRKY70 (Wang *et al.*, 2006). In addition, there are numerous reports describing that particular WRKY proteins have dual effects on plant defense, either enhancing defense against biotrophic pathogens and diminishing defense against necrotrophs, or *vice versa*. Examples are the closely related AtWRKYs -18, -40 and -60 (Xu *et al.*, 2006; Shen *et al.*, 2007; Wang *et al.*, 2006). Of the 74 WRKY genes in Arabidopsis, 49 were differentially expressed upon *Pseudomonas syringae* infection or treatment with SA (Dong *et al.,* 2003). Many WRKY proteins bind to the W-box, a DNA motif with the core sequence

TTGAC(T/C) and the overrepresentation of this motif in several WRKY genes suggests their expression is regulated by WRKY transcription factors (Eulgem and Somssich, 2007). Furthermore, for several WRKY genes, SA-induced expression is dependent on NPR1 and TGAs, suggesting a similar activation strategy as was originally suggested for *PR-1* (Dong *et al.,* 2003; Wang *et al.,* 2006). Despite the fact that extensive genetic information has been obtained on the physiological processes in which specific WRKYs are involved, surprisingly little is known about which specific genes they regulate.

In the same linker scanning study that identified the *as-1*-like regulatory element in the Arabidopsis *PR-1* promoter, a nearby consensus W-box motif (LS4, -675 to -666) with a strong negative effect was identified, suggesting that WRKY factors are important for SA-mediated *PR-1* gene expression (Lebel *et al.,* 1998). The tobacco *PR-1a* promoter does not harbor a consensus W-box, however, NtWRKY12, a WRKY protein with a variant DNA binding domain, was found to bind to a WK-box (TTTTCCAC) in the *PR-1a* promoter that was located 13 bp from the *as-1*-like element (van Verk *et al*., 2008). Mutation of the WK-box sharply reduced SA-mediated *PR-1a::GUS* expression (van Verk *et al*., 2008). Furthermore, pull-down assays and Fluorescence Resonance Energy Transfer analysis showed that NtWRKY12 specifically interacted with tobacco TGA2.2 (van Verk *et al*., 2011). These results indicate that NtWRKY12 and TGA2.2 interact in the regulation of the tobacco *PR-1a* promoter activity.

In addition to the *as-1* element and the W-box, the Arabidopsis *PR-1* promoter contains another nearby element that influences *PR-1* expression. Mutation of sequence of element LS10 (-615 to -606) resulted in loss of INA-inducible expression, indicating the sequence as a positive regulatory element. Based on the presence of the sequence TTTC, LS10 has been suggested as a potential binding site for DOF transcription factors, although there are no

experimental data to support this (Yanagisawa, 2004). In the present study we identified AtWRKY50 as an activator of *PR-1* gene expression and investigated its binding sites in the promoter.

RESULTS

AtWRKY50 is the most effective WRKY activator of *PR-1*

Previously, we identified NtWRKY12 as a transcriptional activator of tobacco *PR-1a* gene expression (van Verk *et al.*, 2008). NtWRKY12 bound to the WK-box (TTTTCCAC) in the tobacco *PR-1a* gene, which differed from the W-box consensus-binding site of WRKY proteins (TTGACT/C). To investigate if WRKY transcription factors are also involved in activation of Arabidopsis *PR-1* gene expression a protoplast transactivation assay (PTA) was set up with 40 of the Arabidopsis WRKY proteins (Wehner *et al.*, 2011). Therefore, a fragment containing approximately 1000 bp upstream of the transcription start site of the *PR-1* gene was cloned in front of the coding sequence for firefly luciferase (LUC) in vector pBT10. After parallel co-transfections of Arabidopsis protoplasts with this reporter plasmid and an expression vector containing one of the *35S*-driven Arabidopsis WRKY genes, luciferase expression was measured. The results of the screening are shown in Table 1. AtWRKY50 and AtWRKY42 were the two most effective activators of the *PR-1::LUC* reporter gene. Both proteins are characterized by the presence of a single WRKY domain and an adjacent Cys-Cys/His-His zinc finger domain. AtWRKY50 belongs to a small subgroup of WRKY proteins in which the domain that interacts with the DNA is characterized by the sequence WRKYGKK as opposed to WRKYGQK present in most other WRKY proteins (Yamasaki *et al.*, 2005). Also NtWRKY12 belongs to this GKK subgroup (van Verk *et al.*, 2008). In addition to AtWRKY50, only two other Arabidopsis WRKY proteins, AtWRKY51 and AtWRKY59,

possess the WRKYGKK sequence and of these three, AtWRKY50 has the highest homology to tobacco NtWRKY12 (68% sequence similarity). This prompted us to further investigate the involvement of these WRKYGKK proteins in Arabidopsis *PR-1* gene expression.

AtWRKY51 and AtWRKY59 do not transactivate *PR-1* expression in protoplasts

The results of the PTA presented in Table 1 indicated that AtWRKY50 is an efficient activator of *PR-1::LUC* reporter gene expression in Arabidopsis protoplasts. Since constructs corresponding to AtWRKY51 and AtWRKY59

Table 1. Protoplast transactivation assays

Name	Gene	Fold Induction
AtWRKY50	At5g26170	6.03
AtWRKY42	At4g04450	5.74
AtWRKY26	At5g07100	2.51
AtWRKY28	At4g18170	2.38
AtWRKY10	At1g55600	2.33
AtWRKY35	At2g34830	2.23
AtWRKY25	At2g30250	2.07
AtWRKY47	At4g01720	2.02
AtWRKY06	At1g62300	1.98
AtWRKY17	At2g24570	1.78
AtWRKY38	At5g22570	1.74
AtWRKY22	At4g01250	1.52
AtWRKY44	At2g37260	1.49
AtWRKY12	At2g44745	1.49
AtWRKY46	At2g46400	1.49
AtWRKY75	At5g13080	1.48
AtWRKY43	At2g46130	1.44
AtWRKY72	At5g15130	1.42
AtWRKY21	At2g30590	1.42
AtWRKY29	At4g23550	1.38
AtWRKY45	At3g01970	1.37

Name	Gene	Fold Induction
AtWRKY33	At2g38470	1.36
AtWRKY55	At2g40740	1.36
AtWRKY41	At4g11070	1.34
AtWRKY09	At1g68150	1.34
AtWRKY69	At3g58710	1.28
AtWRKY70	At3g56400	1.26
AtWRKY01	At2g04880	1.23
AtWRKY15	At2g23320	1.19
AtWRKY61	At1g18860	1.13
AtWRKY20	At4g26640	1.13
AtWRKY56	At1g64000	1.12
AtWRKY23	At2g47260	1.11
AtWRKY13	At4g39410	1.10
AtWRKY67	At1g66550	1.08
AtWRKY65	At1g29280	1.05
AtWRKY40	At1g80840	1.03
AtWRKY07	At4g24240	0.97
AtWRKY62	At5g01900	0.97
AtWRKY53	At4g23810	0.90
AtWRKY60	At2g25000	0.84

were not available in the panel of WRKYs tested in the PTA, transactivation assays in Arabidopsis protoplasts were done with separate 35S expression plasmids for these WRKYs co-expressed with *PR-1::GUS* reporter constructs. The results are shown in Fig. 1.

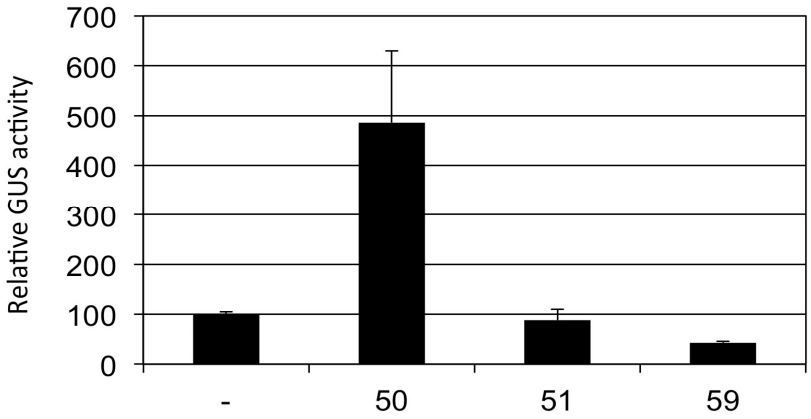

Figure 1. AtWRKY50 activates *PR-1* promoter. Arabidopsis protoplasts were co-transfected with *PR1::GUS* construct together with empty pRT101 expression plasmid (minus sign) or with plasmids containing *35S::AtWRKY50* (50), *35S::AtWRKY51* (51) or *35S::AtWRKY59* (59). After incubation GUS activity was measured spectrophotometrically. Expression levels (%) are given relative to expression level without WRKY effector.

While AtWRKY50 enhanced GUS expression approximately 5-fold, AtWRKY51 and AtWRKY59 did not increase expression over the background level. Fig. 2 shows that in protoplasts transformed with *35S::WRKY* constructs, expression of AtWRKY50 also results in activation of endogenous *PR-1* gene expression. In agreement with the co-expression experiment of Fig. 1, expression of AtWRKY51 and AtWRKY59 did not result in enhanced *PR-1* mRNA accumulation.

***AtWRKY50* gene expression is induced upon treatment with SA**

In tobacco, *NtWRKY12* gene expression was induced to high levels upon inoculation with tobacco mosaic virus and after spraying the plants with SA,

while the time course of the expression coincided with that of *PR-1a*. To determine if *AtWRKY50* expression was SA-inducible in Arabidopsis, accumulation of *AtWRKY50* and *PR-1* mRNA was determined through quantitative reverse transcriptase PCR. The result is shown in Fig. 3. It is evident that *AtWRKY50* expression is induced by SA treatment, and leads to high accumulation of mRNA at 6h and 16h of treatment, shortly preceding the accumulation of the *PR-1* transcript.

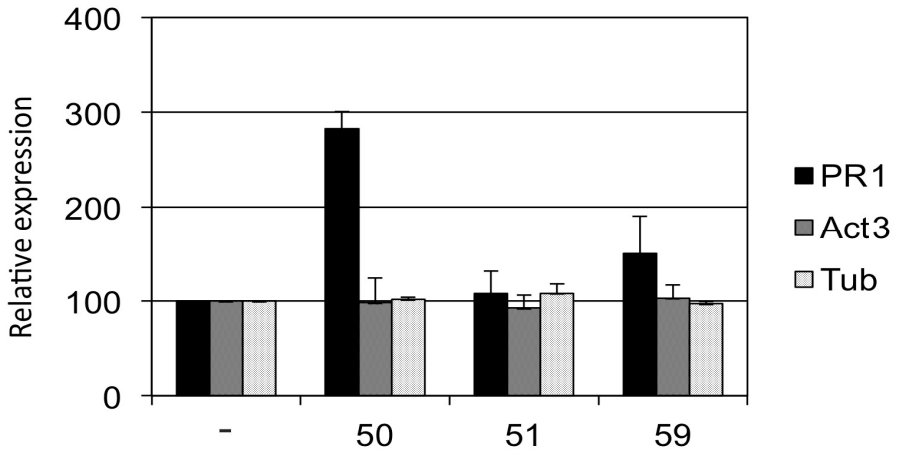

Figure 2. Effect of AtWRKY50, AtWRKY51 and AtWRKY59 on the expression of endogenous Arabidopsis genes. Expression of *PR-1*, *Act3* and *Tub* genes in Arabidopsis protoplasts was measured by qRT-PCR. Expression of each gene was measured in protoplasts transfected with the empty pRT101 vector (minus sign) or with the pRT101 vector containing *35S::AtWRKY50* (50), *35S::AtWRKY51* (51) or *35S::AtWRKY59* (59) expression constructs. Bars represent the average level of mRNA accumulation observed in three experiments. mRNA levels in protoplasts transfected with the empty pRT101 vector were taken as 100%. Error bars represent the SEM.

AtWRKY50's C-terminal half binds to the *PR-1* promoter

Previous work on the Arabidopsis *PR-1* promoter had shown that the region between approximately -700 and -600 bp upstream of the transcription start site was important for inducible gene expression upon treatment with the SA analog INA (Lebel *et al.*, 1998). In addition to two inverted TGACG motifs

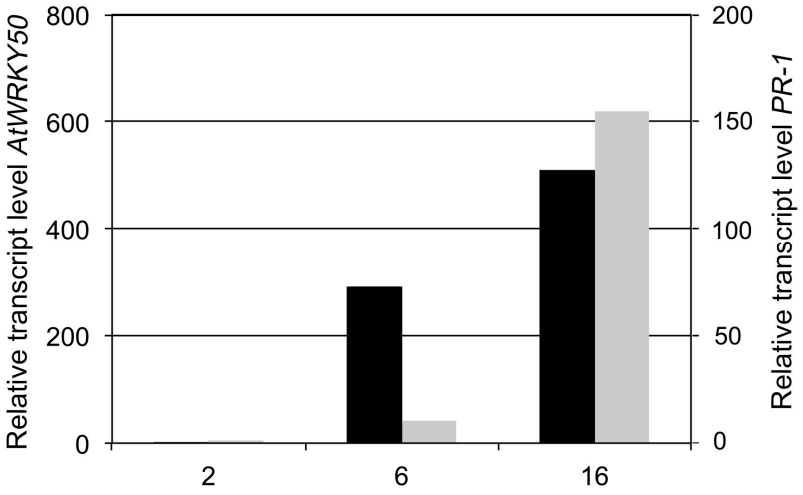

Figure 3. Salicylic acid-induced gene expression of *AtWRKY50* and *PR-1*. Expression of *AtWRKY50* (black bars) and *PR-1* (grey bars) was analyzed in Arabidopsis plants, incubated for the indicated times (hours) in medium containing 1mM salicylic acid. The expression was quantified by qRT-PCR.

(CGTCA in LS5 and LS7) comprising the *as-1*-like element, this region contains a consensus WRKY binding W-box (in LS4) and an additional sequence stretch (LS10). A mutational analysis revealed that all these elements are involved in INA-inducible expression. For reference, Fig. 4 shows a schematic representtation of the Arabidopsis *PR-1* promoter and a comparison to the tobacco *PR-1a* promoter. To analyze if AtWRKY50, AtWRKY51 and AtWRKY59 are able to specifically interact with this region of the promoter, we set up electromobility shift assays (EMSA) with an 80-bp fragment of the *PR-1* promoter, corresponding to the region of -685 to -606, which covers all of the above elements. EMSAs were performed with affinity purified glutathion-S-transferase (GST)-coupled fusion products of the respective WRKY proteins expressed in *E. coli.* Both full-length WRKYs and WRKY domain-containing C-terminal halves were produced (Fig. 5C). The results of the EMSAs are shown in Fig. 5A. The right panel of Fig. 5A (Lanes 5-8) shows that none of the three

GST-tagged full-length WRKY proteins produced a shift with the 80-bp *PR-1* probe.

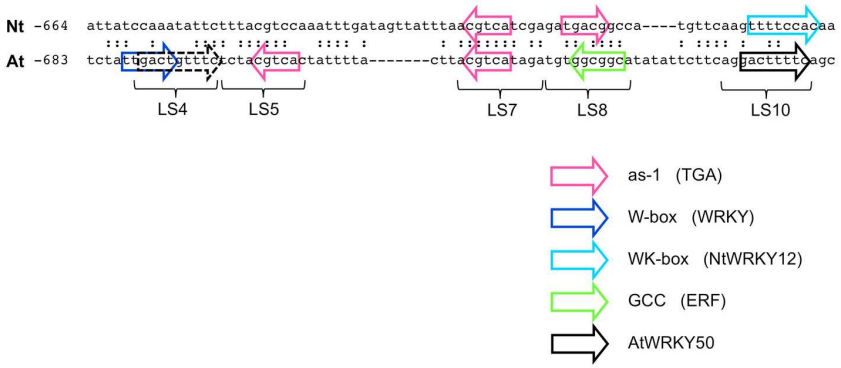

Figure 4. Comparison of sequences in the promoters of tobacco *PR-1a* (Nt) and Arabidopsis *PR-1* (At). Only the sequence of the top strands is given. The sequences of the promoter regions are shown with gaps to allow maximal alignment. The position of the leftmost nucleotide relative to the transcription start site is indicated. Corresponding nucleotides are indicated by colons. Colored block arrows mark consensus binding sites for various transcription factors, as indicated. The direction of the arrow indicates whether the consensus sequence is in the top (right-pointing arrow) or bottom strand. The dashed and solid black arrows mark the binding sites for AtWRKY50. The positions of sequence elements used in the linker scanning analysis of the *PR-1* promoter by Lebel *et al.* (1998) are indicated (LS).

However, a GST-tagged version of the 88-amino acid long C-terminal half of AtWRKY50 (AtWRKY50-C), containing the DNA-binding domain comprising the WRKYGKK sequence and the zinc finger region (Fig. 5A, lane 2), efficiently bound to the probe. This is similar to NtWRKY12 of which the C-terminal binding domain also bound the tobacco *PR-1a* promoter much more efficiently than full-length NtWRKY12 (van Verk *et al.*, 2008). A reason for this lack of binding of full-length AtWRKY50 could be that the relatively large GST-tag fused at the N-terminus of the full-length protein masks the WRKY's DNA-binding domain for interaction with the DNA, while when fused to the C-terminal half, it leaves the binding domain exposed. However, an EMSA with full-length AtWRKY50 fused to the much smaller His-tag neither produced a

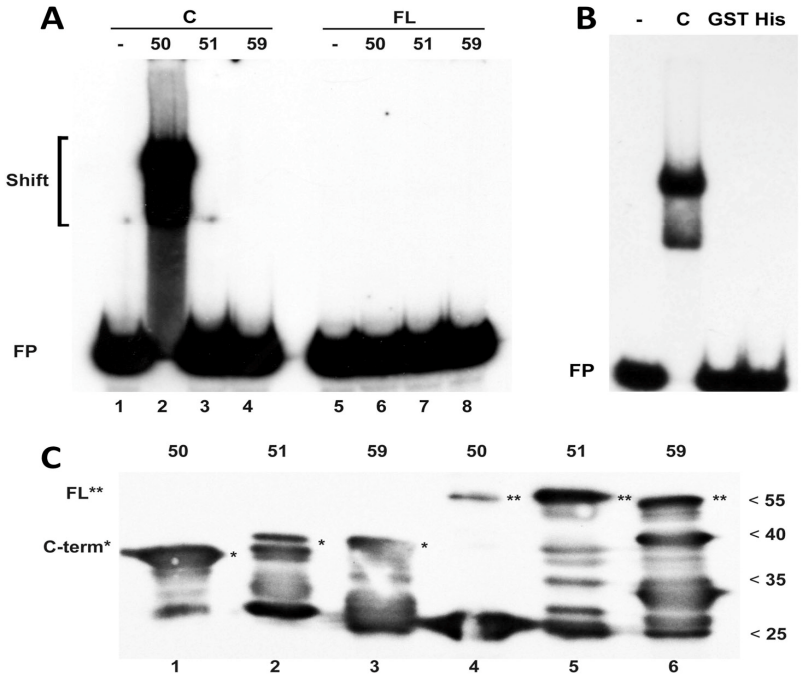

Figure 5. AtWRKY50 binds to the *PR-1* promoter. (A) EMSAs were performed with an 80-bp fragment of the *PR1* promoter and GST-tagged C-terminal halves (Lanes C) or full-length (Lanes FL) versions of AtWRKY50, -51 and -59, as indicated above the lanes. (B) EMSAs were performed with the same probe together with the GST-tagged C-terminal half (Lane C) and GST-tagged (Lane GST) and His-tagged (Lane His) full-length versions of AtWRKY50. In (A) and (B), lanes labeled with the minus sign were loaded with the probe only. The positions of shifts and the unbound probe (FP) are indicated. (C) Western blot with GST-tagged C-terminal and full-length AtWRKY50, -51 and -59, as indicated above the lanes. Bands corresponding to the respective longest peptides are indicated with single (C-termini) or double (full-length) asterisks and with C-term* and FL** to the left of the panel. The size (x10^{-3}) of molecular weight markers is indicated to the right of the panel.

shift with the 80-bp promoter fragment (Fig. 5B). The corresponding C-terminal halves of AtWRKY51 and AtWRKY59 (Fig. 5A, Lanes 3 and 4, respectively) did not produce shifts, indicating that amino acids outside of the conserved WRKYGKK domain are also important determinants for binding to the 80-bp *PR-1* promoter fragment.

Characterization of AtWRKY50's binding site

To investigate if the WRKY protein-binding consensus W-box in LS4 is the binding site for WRKY50, a mutant version of the 80-bp fragment was constructed in which the TTGACT sequence of the W-box was changed to TCAGCT (Fig. 7, probe Wm).

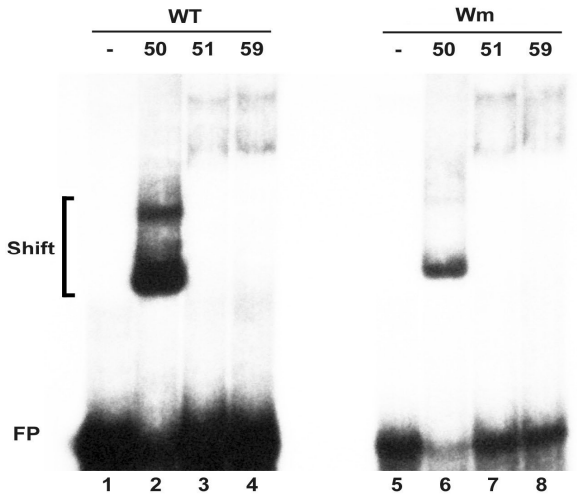

Figure 6. AtWRKY50 binds to the *PR-1* promoter at two positions. EMSAs were performed with wild-type 80-bp *PR-1* promoter fragment (WT) or with an 80-bp fragment with a mutation in the W-box (Wm) as probes together with the GST-tagged C-terminal halves of AtWRKY50, -51 and -59, as indicated above the lanes. The positions of band shifts and the unbound probe (FP) are indicated. Lanes labeled with the minus sign were loaded with probe only.

EMSAs with this mutant 80-bp probe are shown in Fig. 6. While incubation of the wild type and mutant 80-bp probes with the C-terminal halves of AtWRKY51 and AtWRKY59 did not result in shifts (Fig. 6, Lanes 3, 4, 7, 8), AtWRKY50-C produced shifts with both probes (Fig. 6, Lanes 2, 6). Interestingly, a double shift is produced with the wild type probe, while with the mutant probe the higher shift is lost. This suggests that AtWRKY50-C has two binding sites in the 80-bp *PR-1* promoter fragment of which one overlaps with the W-box in LS4. The shift with the mutant probe indicates that

AtWRKY50-C also binds to a second site in the 80-bp promoter fragment, which is different from the W-box consensus.

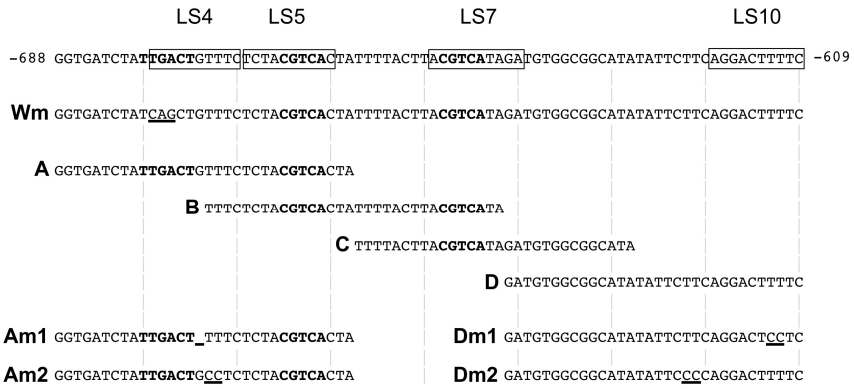

Figure 7. Sequences of *PR-1* promoter fragments used for EMSAs. Only the sequence of the upper strand is given. The top line displays the sequence of the 80-bp fragment corresponding to bp -688 to -609 upstream of the transcription start site. Regions LS4, LS5, LS7 and LS10, as used in the linker scanning analysis of Lebel *et al.* (1998), are blocked. Wm indicates an 80-bp fragment with a mutation (TTGACT to TCAGCT) in the W-box in LS4. Overlapping subfragments A, B, C and D, and their mutant versions Am1, Am2, Dm1 and Dm2 are aligned with the sequence of the 80-bp fragment. The W-box (TTGACT) and the CGTCA boxes of the *as-1* element are indicated in bold. Mutations in Wm, Am1, Am2, Dm1 and Dm2 are underlined.

To further delimit the AtWRKY50 binding sites in the 80-bp fragment, a series of overlapping subfragments (A to D) was generated as shown in Fig. 7. The results of EMSAs performed with these subfragments after incubation with the C-terminal halves of AtWRKY50 and AtWRKY51 are shown in Fig. 8. As expected, incubation with the AtWRKY51-C peptide did not result in shifts with any of the four subfragments (Fig. 8, Lanes 3, 6, 9, 12). However, AtWRKY50-C produced shifts with subfragments A and D (Fig. 8, Lanes 2 and 11, respectively). The shift with subfragment A supports the result from the EMSA shown in Fig. 5, suggesting that the sequence overlapping with the W-box in LS4 facilitates AtWRKY50-C binding. The shift with fragment D indicates that an additional AtWRKY50 binding site is present, which is

different from the W-box. The finding that of all tested Arabidopsis proteins, AtWRKY50 was the most efficient activator of *PR-1* (Table 1) and that its DNA-binding domain binds to fragment D that contains the LS10 element previously found to be required for inducible expression of *PR-1*, suggested that a sequence in the LS10 element could be the binding site for AtWRKY50. To test this, double-stranded oligonucleotides corresponding to fragment D, containing mutations in the LS10 element (Dm1, Fig. 6) and upstream of the LS10 element (Dm2, Fig. 6) were used as probes in EMSAs with AtWRKY50-C. The results are shown in Fig. 9. Whereas the mutation of two nucleotides immediately upstream of the LS10 element (Dm2) did not change the ability of the probe to bind (compare Fig. 9, Lanes 2 and 6), mutation of two central T nucleotides in LS10 (Dm1) almost completely abolished binding of AtWRKY50-C (Fig. 9, Lane 4). This indicates that LS10 indeed contains a binding site for AtWRKY50, which is distinct from the consensus WRKY binding site (W-box).

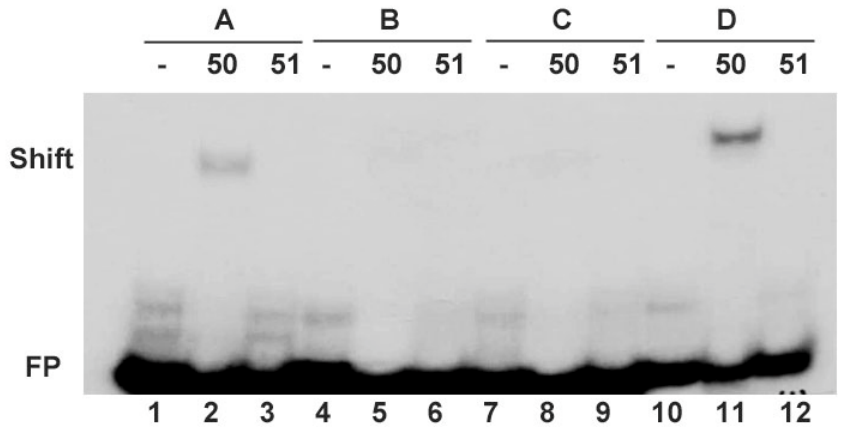

Figure 8. AtWRKY50 binds to the *PR-1* promoter at two positions. EMSAs were performed with overlapping *PR-1* promoter fragments A, B, C and D as probes and GST-tagged AtWRKY50-C or the C-terminal half of AtWRKY51, as indicated above the lanes. The positions of band shifts and the unbound probe (FP) are indicated. Lanes labeled with the minus sign were loaded with probe only.

Almost an exact copy of the sequence GACTTTTC of LS10 is present in LS4, partly overlapping with the W-box and with only a G inserted between the first and second T. Fig. 10 shows the results of an EMSA in which this G was removed from subfragment A (Am1, Fig. 7). It is evident that this results in a much-increased binding of AtWRKY50-C (Fig. 10, Lane 4). Moreover, we speculate that the binding of AtWRKY50-C to fragment A (Fig. 8, Lane 2) is actually caused by the presence of this LS10-like GACTGTTTC sequence, rather than by the W-box, as mutation of GACTGTTTC to GACTGCCTC (Am2, Fig. 7), which leaves the W-box intact, completely abolished binding to AtWRKY50-C (Fig. 10, Lane 6).

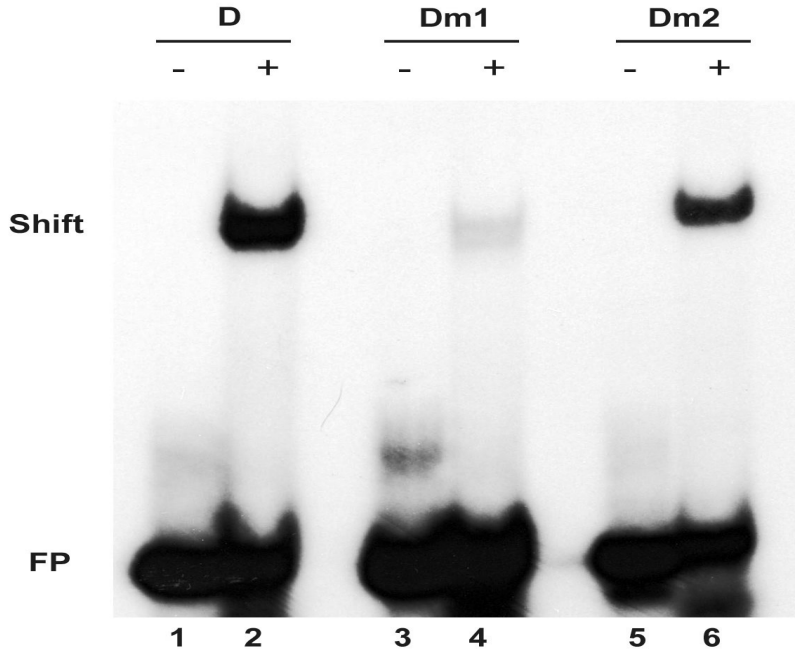

Figure 9. AtWRKY50 binds to the LS10 element in the *PR-1* promoter. EMSAs were performed with wild type (D) and mutant versions (Dm1, Dm2) of *PR1* promoter fragment D as probes and GST-tagged AtWRKY50-C, as indicated above the lanes. The positions of band shifts and the unbound probe (FP) are indicated. Lanes labeled with the minus sign were loaded with probe only; lanes labeled with the plus sign were loaded with the probe and AtWRKY50-C.

The reduced binding of AtWRKY50-C observed upon mutation of the W-box (Fig. 6, Lane 6) could thus be attributable to the fact that the W-box mutation changes the two left nucleotides of the LS10-like element. Taken together, the results of these experiments support the notion that GACT(G)TTTC is a binding site of AtWRKY50.

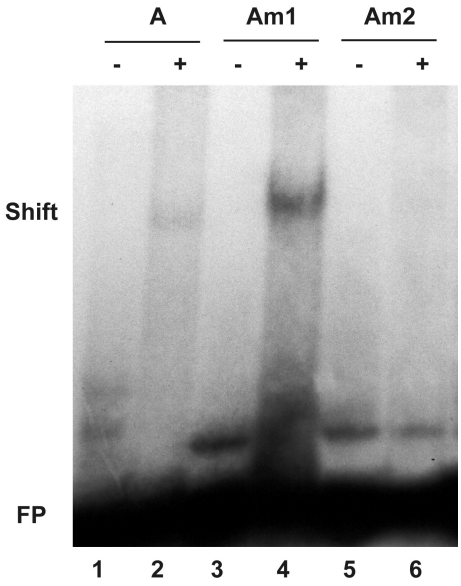

Figure 10. AtWRKY50 binds to the LS4 element in the *PR-1* promoter. EMSAs were performed with wild type (A) and mutant versions (Am1, Am2) of *PR-1* promoter fragment A as probes and GST-tagged AtWRKY50-C, as indicated above the lanes. The positions of band shifts and the unbound probe (FP) are indicated. Lanes labeled with the minus sign were loaded with probe only; lanes labeled with the plus sign were loaded with the probe and AtWRKY50-C.

Like *PR-1*, the *BGL2* gene encoding the β-1,3-glucanase PR-2 is SA-inducible. Although the two promoters have no obvious sequence similarity, the *BGL2* promoter does contain a GACTTTTC sequence element at -175 bp upstream of the transcription start site. Fig. 11 shows that 35 bp long probes corresponding to the relevant regions of the *PR-1* and *BGL2* promoters produce similar shifts after incubation with AtWRKY50-C (Fig. 11, Lanes 2 and 6), indicating that AtWRKY50 is able to bind to the SA-inducible *BGL2* gene.

Activation of *PR-1* gene expression by AtWRKY50 requires intact binding sites

Above, we identified the GACTGTTTC and GACTTTTC sequences in LS4 and

LS10 as binding sites of AtWRKY50. To test whether these sites are necessary for activation of gene expression by AtWRKY50, mutations Am2 and Dm1 (Fig. 6) were incorporated into the 1000bp promoter of *PR-1::GUS* reporter gene constructs.

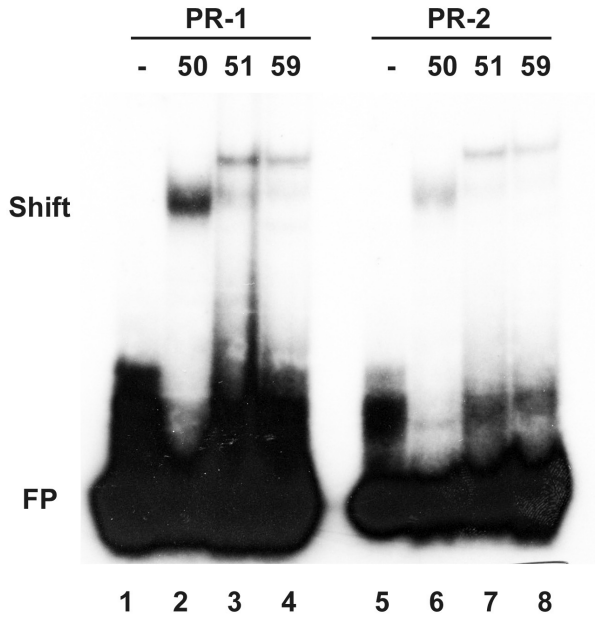

Figure 11. AtWRKY50 binds to an element in the *PR-2* promoter. EMSAs were performed with a 35-bp fragment from the *PR-1* promoter containing LS10 and a 35-bp fragment from the *PR-2* promoter containing the sequence GACTTTTC (-175 bp upstream of the transcription start site) as probes and GST-tagged C-terminal domains of AtWRKY50, -51 and -59, as indicated above the lanes. The positions of band shifts and the unbound probe (FP) are indicated. Lanes labeled with the minus sign were loaded with probe only.

The results of protoplast co-expression experiments with these mutant promoter constructs are shown in Fig. 12. Mutation of the binding site in LS10 resulted in a reduction of GUS expression to approximately 50%, while mutation of the binding site in LS4 reduced the expression to less than 20%. When both mutations were incorporated in the *PR-1* promoter, AtWRKY50 no longer activated GUS expression. These results indicate that both AtWRKY50

binding sites are required for maximal activation of the *PR-1* promoter by AtWRKY50.

DISCUSSION

The present results parallel our previous findings that in tobacco, NtWRKY12 is involved in expression of the *PR-1a* gene (van Verk *et al.*, 2008). Of all 74 Arabidopsis WRKY proteins, AtWRKY50 has the highest similarity to NtWRKY12, including the aberrant G-K-K sequence instead of G-Q-K immediately following the conserved W-R-K-Y sequence present in the majority of WRKY proteins. In the WRKY protein-DNA complex the amino acids of the WRKY domain have been shown to be in direct contact with the DNA (Yamasaki *et al.*, 2005). This could explain why the WK-box, NtWRKY12's binding site in the DNA, is different from the consensus W-box. Also AtWRKY59, one of the two other Arabidopsis WRKYs with a W-R-K-Y-G-K-K sequence was reported to lack binding specificity for the W-box (Dong *et al.*, 2003). Here we found that also AtWRKY50 binds at DNA sequences that are different from the W-box. We identified *PR-1* promoter fragments A and D (Fig. 7) to specifically bind the DNA-binding domain of AtWRKY50.Although we haven't performed an extensive mutational analysis to determine the minimal binding sequence, changing the two central T-residues in the TTTTC stretch in LS10 or in the GTTTC stretch in LS4 to C's severely reduced the binding of AtWRKY50-C, indicating that these base pairs are important for AtWRKY50's binding. It is worthy to note that NtWRKY12 and AtWRKY50, although their binding sites are different (TTTTCCAC and GACT[G]TTTC, respectively), both contain a TTTC stretch. Our results seem to be in conflict with the recent finding that a C-terminal region of AtWRKY50 bound to a W-box-containing probe (Brand *et al.*, 2010). However, in this study a mutated version of the W-box probe was also bound with significant efficiency, while the probe also

contained the sequence ACTTTT, which is identical to part of the binding sequence we characterized in LS10. Furthermore, the authors used a 77-amino acid long C-terminal peptide, while our AtWRKY50-C consists of the C-terminal 88 amino acids. We previously found that the corresponding region of NtWRKY12 is important for binding to the promoter of tobacco *PR-1a* (Van Verk *et al.*, 2011). This makes it conceivable that the extra amino acids in AtWRKY-C contribute to the binding specificity.

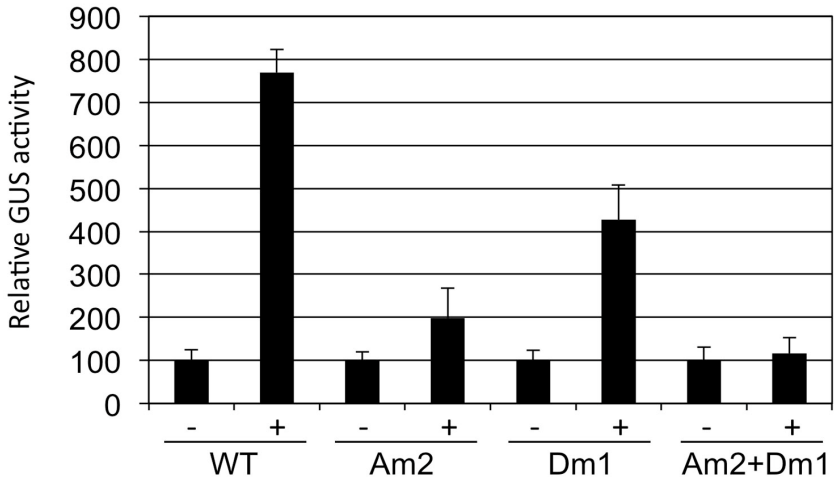

Figure 12. *PR-1* activation by AtWRKY50 requires intact binding sites. Arabidopsis protoplasts were co-transfected with wild type (WT) and mutant *PR1::GUS* construct alone (minus sign) or together with expression plasmids *35S::AtWRKY50*. Mutant promoters contained mutations as indicated in Fig. 7. After incubation GUS activity was measured spectrophotometrically. Expression levels (%) are given relative to expression level without WRKY effector.

Table 2. Primers used for cloning and EMSAs

AtWRKY50-BD::GST	F	CTAGAATTCCTGCCGACAACCAAAACAAG
	R	GCCAAGCTTCGAGTCTTAGTTCATGCTTGAGTGATTGTG
AtWRKY50-FL::GST	F	ATAGGAATTCGTATGAATGATGCAGACACAAACTTG
	R	GCCAAGCTTCGAGTCTTAGTTCATGCTTGAGTGATTGTG

AtWRKY51-BD::GST	F	CTAGAATTCGAGGAAGTAAAGAGAGTGATCAG
	R	GATGAAAGCTTTGGATTAAGATCGAAGAAGAGAGTGTTGG
AtWRKY51-FL::GST	F	AAACGAATTCAAATGAATATCTCTCAAAACCCTAGC
	R	GATGAAAGCTTTGGATTAAGATCGAAGAAGAGAGTGTTGG
AtWRKY59-BD::GST	F	CTAGAATTCGGAAGAGACACAAAGAAGATCCG
	R	CTACAAGCTTTCAATATGGAGCAGAATGAGAGAAAC
AtWRKY59-FL::GST	F	GAGAGAATTCAAATGAACTATCCTTCAAACCCTAACC
	R	CTACAAGCTTTCAATATGGAGCAGAATGAGAGAAAC
AtWRKY50 pRT101	F	ATAGCTCGAGGTATGAATGATGCAGACACAAACTTG
	R	GCCTCTAGACGAGTCTTAGTTCATGCTTGAGTGATTGTG
AtWRKY51 pRT101	F	AAACGAATTCAAATGAATATCTCTCAAAACCCTAGCC
	R	GATGAGGTACCTGGATTAAGATCGAAGAAGGTGTTG
AtWRKY59 pRT101	F	GAGACTCGAGAAATGAACTATCCTTCAAACCCTAACC
	R	CTACTCTAGATCATTATGGAGCAGAATGAGAGAGAAAC
PR-1::GUS	F	GTCAAAGCTTCTGATTCGGAGGGGTATATGTTATTG
	R	CGATGGATCCTTTTCTAAGTTGATAATGGTTATTGTTGTG
PR-1 80BP::GUS	F	ATCGGATCCGGTGATCTATTGACTGTTTCTCTAC
	R	GCCTAGATCTGAAAAGTCCTGAAGAATATATGCC
PR-1 fragA	F	GGTGATCTATTGACTGTTTCTCTACGTCACTA
	R	TAGTGACGTAGAGAAACAGTCAATAGATCACC
PR-1 fragB	F	TTTCTCTACGTCACTATTTTACTTACGTCATA
	R	TATGACGTAAGTAAAATAGTGACGTAGAGAAA
PR-1 fragC	F	TTTTACTTACGTCATAGATGTGGCGGCATATA
	R	TATATGCCGCCACATCTATGACGTAAGTAAAA
PR-1 fragD	F	GATGTGGCGGCATATATTCTTCAGGACTTTTC
	R	GAAAAGTCCTGAAGAATATATGCCGCCACATC
Frag Am1	F	GGGGGTGATCTATTGACTTTTCTCTACGTCACTAT
	R	GGGATAGTGACGTAGAGAAAAGTCAATAGATCACC
Frag Am2	F	GGGGGTGATCTATTGACTGCCTCTCTACGTCACTA
	R	GGGTAGTGACGTAGAGAGGCAGTCAATAGATCACC

Frag Dm1	F	GGGGATGTGGCGGCATATATTCCCCAGGACTTTTC
	R	GGGGAAAAGTCCTGGGGAATATATGCCGCCACATC
Frag Dm2	F	GGGGATGTGGCGGCATATATTCTTCAGGACCCTTC
	R	GGGGAAGGGTCCTGAAGAATATATGCCGCCACATC
PR-2 LS10	F	GGGCATATTGTTAGACTTTTCAAAGCGTATATT
	R	GGGAATATACGCTTTGAAAAGTCTAACAATATG

Intriguingly, despite the strong and specific binding of the C-terminal half of the AtWRKY50 protein to the *PR-1* promoter, our EMSAs failed to reveal binding of full-length AtWRKY50. This was also the case with NtWRKY12 (Van Verk *et al.*, 2008). The fact that a C-terminal His-tagged full-length AtWRKY50 protein neither produced a shift of the 80-bp promoter fragment makes it unlikely that the inability of full-length AtWRKY50 to bind is caused by masking of the binding domain by the relatively large GST-tag at the N-terminus of the protein. Possibly, the N-terminal halves of the full-length WRKYs themselves prevent binding to the DNA under EMSA conditions. The fact that this is the case with both the tobacco and Arabidopsis homologs could indicate that this is a functionally relevant property, e.g. to prevent promiscuous binding of the WRKY protein to DNA regions with consensus binding sequences that are not in the correct structural context. It could be speculated that interaction with other factors is required to change the configuration of the full-length WRKYs to release the binding domains for binding to the DNA.

Our studies in Arabidopsis protoplasts showed that AtWRKY50 enhanced expression of co-transfected *PR-1::Luc* and *PR-1::GUS* reporter genes and also of the endogenous *PR-1* gene, suggesting that the protein acts as a transcriptional activator. This was also the case for its tobacco homolog NtWRKY12 (Van Verk *et al.*, 2008). However, while the full-length NtWRKY12-GAL4BD fusion protein activated the *His* reporter gene in yeast, AtWRKY50

showed no transcriptional activity in this system. We speculate that either, the BD part of the fusion protein interferes with the correct folding of AtWRKY50, or that yeast lacks specific factors necessary for its activating function.

Recently, it was found that AtWRKY50 and AtWRKY51 are involved in repression of jasmonic acid (JA)-dependent defense responses, including *PDF1.2* marker gene expression (Gao *et al.*, 2010). Although it was not investigated if this was the effect of a direct interaction of the WRKYs with the *PDF1.2* promoter, the authors contemplated that the WRKYs might act as transcriptional repressors, possibly by binding to specific binding sequences in the promoters of JA-responsive genes. In this context it is significant to note that the *PDF1.2* promoter lacks W-boxes, but contains the AtWRKY50 binding element GACTGTTTC.

In conclusion, we have shown that AtWRKY50 is an activator of Arabidopsis *PR-1* expression in Arabidopsis protoplasts. It's C-terminal DNA-binding domain specifically binds to two GACT(G)TTTC elements that are located at -675 and -616 bp upstream of the transcription start site in the *PR-1* promoter.

MATERIALS AND METHODS

Vector construction

The *PR-1*: LUC was constructed as a reporter. The 1000bp upstream region of *PR-1* (At2g14610) was PCR-amplified using Arabidopsis Col-0 genomic DNA as template and the primers with the following sequence: 5'-GTG GAA TTC CTG ATT CGG AGG GAG TAT ATG TTA TTG- 3' and 5'-CGA TCC ATG GTT TTC TAA GTT GAT AAT GGT TAT TG-3'. The DNA-fragments were inserted into the vector pBT10-LUC by using NcoI and EcoRI restriction enzymes. The screening was done according to Wehner *et al.* (2011).

Bacterial Expression of AtWRKY50 Fusion Proteins

The full-length and C-terminal coding sequence of AtWRKY50, AtWRKY51 and AtWRKY59 were amplified by PCR (for primer sequences, see Table 2) and cloned in-frame behind the GST open reading frame of expression vector pGEX-KG (Guan and Dixon, 1991). 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-thiogalactopyranoside 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-^{32}P]ATP or using Klenow fragment and [α-^{32}P]dCTP, after which unincorporated label was removed by Autoseq G-50 column chromatography (Amersham-Pharmacia Biotech). Different sets of oligonucleotides and their mutated versions are presented in Table 2. 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 incubation was continued for 60 min on 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.

qRT-PCR

Total RNA was isolated from pulverized frozen Arabidopsis tissue 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′-GTT CTT CCC TCG AAA GCT CAA GAT-3′ and 5′-CAC CTC ACT TTG GCA CAT CCG-3′, tubulin7: 5′-GGA AGA AGC TGA GTA CGA GCA-3′ and 5′-GCA ACT GGA AGT TGA GGT GTT-3′, and actin3: 5′-CCT CAT GCC ATC CTC CGT CT-3′ and 5′-CAG CGA TAC CTG AGA ACA TAG TGG-3′.

Plasmid construction and Transactivation Experiments:

The *AtWRKY50* (At5g26170), *AtWRKY51* (At5g64810) and *AtWRKY59* (At2g21900) open reading frames were amplified by PCR using corresponding primer sets (Table 2) from a cDNA library obtained from Arabidopsis plants 6h after treatment with SA, and cloned into pRT101. The *PR-1* promoter was obtained by PCR on genomic DNA and cloned in front of the GUS coding region in pT7:GUS. Protoplasts were prepared from Arabidopsis ecotype Columbia-0 cell suspension according to Axelos *et al.* (1992) with some 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% Sucrose, 1 µM naphthylacetic acid [NAA], and pH 5.8) and incubated overnight at 25^0C at 250 rpm. Cells were harvested and

cell walls digested with 20 mL 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 Glc, 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::AtWRKY50*, *35S::AtWRKY51* or *35S::AtWRKY 59*. As a control, co-transformation of *PR-1::GUS* fusions with the empty expression vector pRT101 was carried out. Protoplasts were transformed using polyethylene glycol 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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