WRKY transcription factors involved in *PR-1* gene expression in Arabidopsis

Rana Muhammad Fraz Hussain

Rana Muhammad Fraz Hussain

WRKY transcription factors involved in *PR-1* gene expression in Arabidopsis

ISBN: 978-94-6203-162-3

Printed by: Wöhrmann Print Service

Cover art and designed by

RMF Hussain and HJM Linthorst

WRKY transcription factors involved in *PR-1* gene expression in Arabidopsis

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 17 oktober 2012
klokke 10:00 uur

door

Rana Muhammad Fraz Hussain

geboren te Rahim Yar Khan (Pakistan)

in 1986

Promotiecommissie

Promotor:	Prof.dr. J. Memelink
Co-promotor:	Dr. H.J.M. Linthorst
Overige leden:	Prof.dr. W. Dröge-Laser (Julius Maximilians Universität Würzburg, Germany)
	Prof.dr. H.P. Spaink
	Prof.dr. J.F. Bol
	Dr. M.C. van Verk (Utrecht University)

To My Parents, Sisters and Brother…!!!

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

R. Muhammad Fraz Hussain, Huub J.M. Linthorst

Institute of Biology, Leiden University, P.O. Box 9505, 2300 RA Leiden, the Netherlands

Plant defense gene regulation

Plants possess elaborate mechanisms to defend themselves against attack by pathogens and pests. During evolution different defense strategies have evolved against biotrophic and necrotrophic pathogens and insect attack. While defense against necrotrophic pathogens and insect attack involves a signaling pathway characterized by the plant hormone jasmonic acid (Howe, 2004), defense against biotrophic pathogens commonly involves a signal transduction pathway mediated by the plant compound salicylic acid (SA) (Dong, 1998). Both signaling pathways affect each other through extensive cross-talk occurring at different levels, while additional modulation of the defense response is brought about by the effects of a third signal transduction cascade triggered by ethylene (ET) (Koornneef and Pieterse, 2008; Leon-Reyes *et al.*, 2009; Reymond and Farmer, 1998; Spoel and Dong, 2008).

For the defense response launched after attack by biotrophic pathogens genetic data from Arabidopsis have led to a signal-transduction model in which SA plays a central role. Tissue colonization and pathogen proliferation are caused by pathogen effectors, also known as avirulence (Avr) proteins, which are targeted to the host tissues to promote pathogen virulence (Jones and Dangl, 2006). In incompatible plant–pathogen interactions these effectors are recognized by specific *R* gene-encoded receptors. Basal defense or innate immunity has significant overlap with *R* gene-mediated resistance responses, including production of SA and expression of SA-regulated defense genes (Tsuda *et al.*, 2008). In this case, pathogen-associated molecular patterns (PAMPs), such as conserved fragments of bacterial flagellin or elongation factor Tu, function as elicitors that are recognized by specific LRR receptor kinases (Kunze *et al.*, 2004; Mackey and Mcfall, 2006; Turner *et al.*, 2002; Zhao *et al.*, 2005), which subsequently transduce the signal through MAPK cascades,

ultimately leading to the establishment of immunity (Asai *et al.*, 2002; Chinchilla *et al.*, 2007).

In Arabidopsis, the biosynthesis of pathogen-induced SA depends on isochorismate synthase (ICS), the product of the *ICS1* gene that converts part of the ubiquitous chorismate into isochorismate. Isochorismate is an intermediate in the synthesis of phylloquinone (vitamin K1), which is an essential component of the plant's photosynthetic machinery (Verberne *et al.*, 2007; Wildermuth *et al.*, 2001). In non-infected cells SA is present only at very low concentrations, but upon pathogen attack its level increases rapidly. Apparently, after attack isochorismate is channeled away from phylloquinone synthesis toward synthesis of SA. Also bacteria synthesize SA from isochorismate in a single-step reaction involving the enzyme isochorismate pyruvate-lyase (IPL) (Gaille *et al.*, 2002). However, no such activity has yet been found in plants.

Genetic evidence has indicated that upstream of *ICS1*, several more genes are necessary to mount the defense response. Genes involved in the earliest steps of the signal-transduction pathway upstream of SA, that is, *PHYTOALEXIN DEFICIENT4* (*PAD4*) and *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) encode proteins with similarity to lipases. EDS1 is probably activated upon elicitor recognition by *R* gene-encoded cytoplasmic LRR receptors (Wirthmueller *et al.*, 2007). How exactly this activation is linked to induction of SA biosynthesis is not known. Possibly, hetero-dimerization of EDS1 and PAD4 and their nuclear localization may be important for subsequent steps in the signaling pathway (Feys *et al.*, 2001). Situated downstream of *EDS1* is *EDS5* (Rogers and Ausubel, 1997). Pathogen infection strongly induces the accumulation of the *EDS5* transcript in an *EDS1*- and *PAD4*-dependent manner. The increase in *EDS5* mRNA precedes SA accumulation, supporting a role for EDS5 in this process. *eds5* mutant plants are unable to accumulate high levels of SA (Nawrath and Métraux, 1999). Furthermore, *EDS5* gene expression is also

induced by treatment with exogenous SA, indicating a positive feedback loop for enhanced SA production during the defense response (Nawrath *et al.*, 2002). The increase in SA induces a state of enhanced defensive capacity, both locally, in the infected tissues as well as systemically in distal non-infected tissues. This last type of defense is known as systemic acquired resistance (SAR). SAR primes distal tissues for defense against secondary infections conferring broad-spectrum resistance to subsequent pathogen infection (Ross, 1961; Conrath *et al.*, 2006). Methyl SA (MeSA) was identified as a mobile signal that is critical for the development of SAR in tobacco. SA produced at the primary infection site is converted by a SA methyltransferase (SAMT) to MeSA and loaded into the vascular system for transport to distant plant tissues. Upon arrival in these systemic tissues, MeSA is converted back to active SA by the esterase SA-binding protein 2 (SABP2), which triggers defense gene expression in these tissues (Park *et al.*, 2007). However, a number of other compounds and proteins that may function as systemic signals for SAR have recently been put forward and as of yet, there is still no definite answer as to which (combination) of these molecules is the systemic signal. (Dempsey and Klessig, 2012).

One of the effects triggered by SA is the elicitation of an imbalance in the redox state of the cell, which results in reduction of specific disulfide bridges in the ankyrin-repeat protein NONEXPRESSOR OF PR GENES1 (NPR1). NPR1 plays a central role in defense responses and is required for the establishment of SAR and the expression of SA-dependent defense genes. NPR1 exists in the cytoplasm as a multimeric complex. Reduction results in release of NPR1 monomers and their subsequent translocation into the nucleus, where they interact with TGA transcription factors and activate defense gene expression (Kinkema *et al.*, 2000; Mou *et al.*, 2003). NPR1 contains an ankyrin-repeat domain, which facilitates protein–protein interactions (Cao *et al.*, 1997). Moreover, it harbors a BTB domain, which might be ubiquitinylated by an E3

ubiquitin ligase complex and targeted for degradation by the proteasome. Upon initiation of *PR* gene transcription by the TGA–NPR1 complex NPR1 is phosphorylated, possibly by a factor of the basal transcription machinery, and becomes inactive. Phosphorylation results in enhanced affinity for CUL3 to which it is bound via interaction with the SA-receptors NPR3 or NPR4 and consequently rapid degradation by the proteasome. This clears the promoter to reinitiate transcription, resulting in a pulse-wise activation of gene expression as long as nuclear NPR1 is available (Spoel *et al.*, 2009; Fu *et al.*, 2012). An alternative mechanism for NPR1's mode of action has been put forward by Wu *et al.* (2012), who found that NPR1 itself is the SA receptor. Binding of SA would result in a conformational change resulting in exposure of the activation domain and subsequent activation of gene expression. These results indicate that NPR1 acts as a co-activator that is recruited to the promoter by interaction with TGA transcription factors (Rochon *et al.*, 2006). However, it is possible that NPR1 is only necessary if a functional *SUPPRESSOR OF NPR1* (*SNI1*; Li *et al.*, 1999) allele is present. SNI1 is an armadillo repeat protein that may form a scaffold for interaction with proteins that modulate transcription (Mosher *et al.*, 2006), leading to transcriptional repression.

The defense response brought about by biotrophic pathogen attack ultimately leads to the local and systemic expression of genes encoding, amongst others, specific defense proteins with anti-microbial activities, collectively named pathogenesis-related, or PR proteins. PR proteins are conserved throughout the plant kingdom. The antimicrobial function of several classes of PR-proteins derives from their enzymatic activity as e.g. beta-1,3-glucanases (PR-2) or chitinases (PR-3), able to degrade fungal and oomycete cell-walls and thus preventing fungal growth. Although for the PR-1 proteins no specific anti-pathogen activity is known, the proteins and the induced expression of their genes are generally used as markers for SAR (Glazebrook,

2005; Grant and Lamb, 2006). As a model gene for SA-induced defense gene expression, the regulation of *PR-1* gene expression has been studied since more than two decades. These studies have indicated two types of DNA-binding proteins as important transcription factors involved in *PR-1* gene expression: TGA proteins and WRKY proteins.

TGA transcription factors

TGA proteins are members of the bZIP transcription factors, which are characterized by their basic leucine zipper (bZIP) domain (Jakoby *et al.*, 2002). This is a bipartite region enriched in basic amino acid residues that are in direct contact with the DNA and involved in DNA binding. In close proximity of this region is a leucine zipper region consisting of regularly spaced leucine residues. This region is important for the homo- and heterodimerization of the bZIP proteins (Schindler *et al.*, 1992).

The first TGA factor to be identified was the tobacco protein TGA1a, which binds to activation sequence-1 (*as-1*). This element, which is characterized by two TGACG motifs in a tandem arrangement, was first identified in the 35S promoter of cauliflower mosaic virus (CaMV) (Katagiri *et al.*, 1989). When acting independently of other enhancers, this element confers SA- and auxin-dependent expression in leaves (Qin *et al.*, 1994; Xiang *et al.*, 1996) and constitutive expression in roots (Benfey *et al.*, 1990). With the discovery of TGA factors interacting with NPR1, which has a central role in SA-regulated gene expression (see above), the importance of TGA factors in SA-regulated gene expression and their role in development of SAR were established (Després *et al.*, 2003; Zhang *et al.*, 1999). The Arabidopsis TGA family of transcription factors harbors 10 members of which six (TGAs 1 to 6), have been shown to be involved in defense responses against pathogen attack (Kesarwani *et al.*, 2007; Zhang *et al.*, 2003).

The Arabidopsis *PR-1* and the tobacco *PR-1a* promoters, which are studied as model systems to understand SA-induced transcriptional regulation, each contain an as-1-like element in a region of the promoter that is important for SA-inducible gene expression (Lebel *et al.*, 1998; Strompen *et al.*, 1998). In Arabidopsis, linker-scanning analysis revealed that one of the TGACG motifs is a positive regulatory element (LS7), whereas the other functions as a constitutive negative element (LS5) for induced expression (Lebel *et al.*, 1998). TGA2 and TGA3 were found to bind to the *PR-1* promoter in vivo (Johnson *et al.*, 2003; Rochon *et al.*, 2006), with TGA3 acting as a transcriptional activator of *PR-1* expression, whereas TGA2 represses expression in the non-induced state. Conflicting data concerning the mechanism of action of the TGA/NPR1 complex have been reported. Based on studies involving chromatin immunoprecipitation analysis (Johnson *et al.*, 2003), electrophoretic mobility shift assays (Després *et al.*, 2000) and transgenic plants expressing the C-terminal domain of TGA2 as a fusion with the DNA-binding domain of the yeast transcriptional activator protein Gal4 (Fan and Dong, 2002), it was first hypothesized that NPR1 serves to facilitate binding of TGA factors at the promoter. Later, it was found that at least TGA2 binds constitutively to the *PR-1* promoter and that yet unknown factors already recruit NPR1 to the promoter in the non-induced state. NPR1 interacts with TGA factors only under inducing conditions to form an enhanceosome, a protein complex that binds DNA in the enhancer region of the gene (Rochon *et al.*, 2006).

Although it is generally accepted that TGA factors are crucial for the regulation of many SA-dependent processes, the importance of the different members of the TGA family is controversial. First, it was reported that TGA2, TGA5, and TGA6 are redundant and essential activators of *PR-1* expression (Zhang *et al.*, 2003). Later, other studies documented that *PR-1* expression is only delayed in the *tga2 tga5 tga6* triple mutant (Blanco *et al.*, 2009), and that

additional mutation of TGA3 is necessary to get a more stringent knockout phenotype (Kesarwani *et al.*, 2007). TGA1 and TGA4 are essential for SA-dependent basal resistance (Kesarwani *et al.*, 2007). Disulfide bridges of Arabidopsis TGA1 are reduced after a SA-mediated redox change, which allows interaction with NPR1, while also S-nitrosylation of specific Cys-residues of TGA1 and NPR1 has been demonstrated to be important for TGA1-NPR1 interaction DNA-binding (Després *et al.*, 2003; Lindermayr *et al.*, 2010). However, more information is needed to unravel the in vivo function of TGA1 and TGA4 with respect to the regulation of SA-inducible genes (Pape *et al.*, 2010; Shearer *et al.*, 2012). Recently, it was found that tobacco NtWRKY12, a WRKY transcription factor required for high-level expression of *PR-1a*, specifically interacts in vitro and in vivo with tobacco TGA2.2 (Van Verk *et al.*, 2011a).

WRKY transcription factors

WRKY proteins are characterized by a stretch of the amino acids tryptophan (W), arginine (R), lysine (K), and tyrosine (Y), followed by a typical zinc-finger domain. They constitute a large class of DNA-binding proteins in plants (Zhang and Wang, 2005). In Arabidopsis, more than 70 *WRKY* genes have been identified. The first *WRKY*-cDNA clone was characterized from sweet potato (Ishiguro and Nakamura, 1994), and their description as a class of transcription factors followed soon afterwards (Eulgem *et al.*, 2000). Many WRKY proteins have specific binding affinity for the consensus W-box motif TTGAC (T/C). In parsley it was shown that clustering of W-boxes is important for a strong transcriptional response (Eulgem *et al.*, 1999; Rushton *et al.*, 1996). Based on their domain structure, WRKY proteins can be divided into three major groups. Proteins with two WRKY domains belong to group I. WRKY proteins containing one WRKY domain belongs to groups II or III, depending on the

type of zinc-finger motif (Eulgem *et al.*, 2000). The importance of WRKY factors for SA-mediated gene expression was first shown for the Arabidopsis SAR marker gene *PR-1*, in which a W-box motif conferred a strong negative effect on gene expression (Lebel *et al.*, 1998). W-box motifs are overrepresented in the promoters of Arabidopsis genes that are co-regulated with *PR-1*. Yet, TGA transcription factor-binding as-1 elements occur at statistically expected frequencies in these promoters (Rowland and Jones, 2001).

Besides the consensus W-box, WRKY factors have been identified to bind to other motifs. Recently, tobacco NtWRKY12 was identified as a WRKY protein with a variant WRKYGKK amino acid sequence in the WRKY domain instead of the WRKYGQK sequence of the majority of WRKY proteins (Van Verk *et al.*, 2008). NtWRKY12 is involved in transcriptional activation of the *PR-1a* promoter and binds to WK-boxes, TTTTCCAC, in this promoter, while it is unable to bind to the consensus W-box (Van Verk *et al.*, 2008). A WRKY protein from barley (SUSIBA) was found to bind to SURE, a sugar-responsive cis element in the promoter of the *ISOAMYLASE1* (*ISO1*) gene (Sun *et al.*, 2003). The authors did not further delineate the binding site of SUSIBA in SURE, although the presence of the sequence TTTTCCA in this element suggests that it could be a WK-like sequence.

WRKY proteins have been found as transcriptional activators at the end of the PAMP signaling cascade involved in the response of Arabidopsis to the flagellin fragment flg22. In this case, signal transduction via the MAPK cascade MEKK1–MKK4/MKK5 –MPK3/MPK6 leads to the activation of downstream WRKY22 and WRKY29. These WRKY factors are suggested to amplify their expression levels via multiple WRKY binding sites in their own promoters, thereby creating a positive feedback loop. The induced expression of these WRKY factors would then allow induction of resistance to both bacterial and fungal pathogens (Asai *et al.*, 2002). Activation of the WRKY factors could

possibly occur via targeted degradation of bound suppressors, as has been found for the activation of WRKY33. Another Arabidopsis MAPK cascade (MEKK1–MEK1/MKK2–MAPK4), induced by challenge inoculation with *Pseudomonas syringae* or treatment with flg22 leads to phosphorylation of MAP kinase substrate 1 (MKS1), through which WRKY33 and possibly WRKY25 are bound to MAPK4. Upon phosphory- lation of MKS1, WRKY33 is released in the nucleus to initiate positive regulation of JA-induced defense genes and negative regulation of SA-related defense genes. Also other WRKYs, like WRKY11 and WRKY17, act as negative regulators of basal resistance responses. Moreover, overexpression of the flagellin-inducible WRKY41 abolishes the inducibility of PDF1.2 by MeJA. In all these cases the mechanisms underlying these antagonistic effects are as yet unknown (Andreasson *et al.*, 2005; Brodersen *et al.*, 2006; Higashi *et al.*, 2008; Journot-Catalino *et al.*, 2006; Qiu *et al.*, 2008).

Activation of the MAPK pathway by flagellin leads to increased levels of SA, which is strongly dependent on the pathogen-inducible *ICS1*. Activation of *ICS1* gene expression is likely to occur via WRKY transcription factors. WRKY28 is rapidly induced to very high levels upon flg22 treatment (Navarro *et al.*, 2004). Van Verk *et al.* (2011b) have found that transient overexpression of WRKY28 in Arabidopsis protoplasts leads to induction of a *GLUCURONIDASE* (*GUS*) reporter gene under control of the 1 kb *ICS1* upstream promoter region, as well as elevated levels of endogenous *ICS1* mRNA. This points at a link between PAMP signaling and SA biosynthesis. From evaluation of microarray data it appears that WRKY28 is the only WRKY protein of which the expression is suppressed by both JA and ET. The 1 kb *ICS1* promoter lacks a consensus W-box, but WRKY28 was found to bind to two W-box-like sequences in the *ICS1* promoter (Van Verk *et al.*, 2011b). *AVRPPHB SUSCEPTIBLE 3* (*PBS3*), of which the pathogen-induced expression is highly correlated with *ICS1*, is acting downstream of SA. Accumulation of SA-glucoside and expression of *PR-1* are

drastically reduced in the *pbs3* mutant (Nobuta *et al.*, 2007). By a similar approach as described above, it was found that the 1 kb *PBS3* promoter directs reporter gene expression in Arabidopsis protoplasts upon transient expression of WRKY46 (Van Verk *et al.*, 2011b). WRKY46 is a transcription factor that is rapidly induced downstream of avirulence effectors. These results suggest an involvement of WKRY46 in the signaling cascade of avirulence effector recognition and the subsequent accumulation of SA (He *et al.*, 2006; Van Verk *et al.*, 2011b).

The important function of NPR1 in defense pathways is evident by the requirement of this cofactor for the development of SAR and *PR* gene expression. Eight WRKY genes (*AtWRKY18*, *-38*, *-53*, *-54*, *-58*, *-59*, *-66*, and *-70*) have been identified as direct targets of NPR1 (Spoel *et al.*, 2009; Wang *et al.*, 2006). Most of the encoded WRKYs play a role in the expression of *PR* genes and in SAR. Negative regulators are WRKY58, having a direct negative effect on SAR, and WRKYs 38 and 62, which through protein-protein interaction interfere with the function of histone deacetylase 19, which is required for *PR* gene expression (Kim *et al.*, 2008). WRKY62 also acts in the cross-talk between SA and JA signaling by repressing downstream JA targets such as *LOX2* and *VSP2* (Mao *et al.*, 2007). Both WRKY18 and WRKY53 are positive regulators of *PR*-gene expression and SAR. Functional WRKY18 is required for full induction of SAR and is linked to the activation of *PR-1* (Wang *et al.*, 2006). WRKY18, WRKY40 and WRKY60 play partly redundant roles in regulating disease resistance. These three WRKY proteins can interact physically and functionally in their responses to different microbial pathogens. While WRKY18 enhances resistance against *P. syringae*, co-expression of WRKY40 or WRKY60 renders plants more susceptible to this pathogen (Xu *et al.*, 2006). WRKY70 and its functional homolog WRKY54 have dual roles in SA-mediated gene expression and resistance. Upon high accumulation of SA, WRKY54/70 act as negative

regulators of SA biosynthesis, possibly by direct negative regulation of *ICS1*. Besides this negative role, they activate other SA-regulated genes (Kalde *et al.*, 2003; Wang *et al.*, 2006). WRKY70 also acts as a key regulator between the SA and JA defense pathways by inducing SA-dependent responses and repressing JA-dependent responses, such as expression of *VSP*, *LOX*, and *PDF1.2*. *WRKY70* expression is repressed by the JA-signaling regulator COI1 to overcome the negative effect of SA on JA signaling (Li *et al.*, 2004, 2006).

Tobacco NtWRKY12 activates *PR-1a* gene expression via the WK-box in its promoter. Mutation of this box has a far more severe effect on *PR-1a* gene expression than mutation of the nearby *as-1* element, implying that TGAs are not the predominant activators of *PR-1a* expression (Van Verk *et al.*, 2008). This is supported by the finding that in *npr1-1* mutant protoplasts NtWRKY12-induced *PR-1a* expression is still fully operative (Van Verk *et al.*, 2011a). NtWRKY12 gene expression is induced upon PAMP elicitation and tobacco mosaic virus infection. It is arguable that NtWRKY12 expression requires NPR1-dependent activation via TGAs, which would lend support for an indirect rather than a direct role of NPR1 in *PR-1a* expression.

As many WRKY transcription factors can bind similar cis elements, the question arises how the different WRKYs can specifically activate or suppress their respective target genes. Possibly, fine-tuning of specific gene regulation involves interactions between different transcription factors binding to proximal binding sites at the promoter. In previous studies of our group it was found that NtWRKY12 can specifically interact with tobacco TGA2.2 both in vitro and in vivo (Van Verk *et al.*, 2011a), suggesting a role of TGA2.2 in *PR-1a* expression as a recruiter of NtWRKY12 to the promoter or to stabilize its binding. Studies on the mechanisms underlying Arabidopsis *PR-1* gene expression have identified a number of elements in the promoter that are involved in the induction of gene expression. Several of these sequence

elements are similar to binding sites for WRKY transcription factors, but knowledge of which of Arabidopsis' 74 WRKYs bind to these putative binding sites is still lacking. This thesis deals with the identification of possible WRKY candidates.

Thesis Outline

Chapter 2 describes the results of a transactivation screening in Arabidopsis protoplasts of a large number of WRKYs, which resulted in the identification of AtWRKY50 as a potent activator of the *PR-1* promoter. The C-terminal half of AtWRKY50, containing the conserved DNA-binding domain appeared to bind at two positions in the promoter that were situated in close proximity to the binding sites of TGA transcription factors. The sequences of these binding sites differed considerably from the sequence of the W-box, the consensus-binding site of WRKY proteins.

In **Chapter 3**, AtWRKY50 was found to interact with TGA proteins 2 and 5 in yeast cells and also in Arabidopsis protoplasts where the interaction was found to occur in the nuclei. Furthermore, using electrophoretic mobility shift assays it was established that the two transcription factors were able to bind simultaneously to the promoter and that TGA2 and TGA5 predominantly bound to one of the two binding sites in the promoter that were previously proposed. Although transactivation experiments in Arabidopsis protoplasts derived from wild type, *npr1-1* and *tga256* mutant plants indicated that AtWRKY50 alone was able to induce expression of a *PR-1::β-glucuronidase* (GUS) reporter gene independent of TGAs or NPR1, co-expression of AtWRKY50 and TGA2 or TGA5 synergistically enhanced *PR-1* expression to high levels.

Chapter 4 describes results on AtWRKY28, which show that this WRKY factor 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, while the other binding site was a W-box previously identified to be 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.

Chapter 5 deals with a study of transgenic plants that overexpressed *AtWRKY50* and *AtWRKY28* or in which the *AtWRKY50* and *AtWRKY51* genes were knocked out. The plants did not have constitutive enhanced levels of *PR-1* mRNA, although *PR-1* mRNA accumulated to higher and lower levels, respectively, after treatment of the plants with SA. However, there was no clear-cut effect on resistance against infection with the biotrophic bacterial pathogen *Pseudomonas syringae* or with the necrotrophic fungal pathogen *Botrytis cinerea.*

Chapter 6 describes the effect of overexpression of several *WRKY* genes on the Arabidopsis metabolome. Transgenic plants were generated in which the coding sequence of the respective *WRKY* genes was fused to the Cauliflower mosaic virus *35S* promoter. Constitutive expression of several WRKYs had effects on the accumulation of metabolites as determined from multivariate analyses of ^{1}H NMR spectroscopy data. Especially *AtWRKY50* overexpressing plants accumulated higher levels of sinapic acid derivatives, suggesting that this transcription factor could be involved in stress-induced modifications of lignin.

REFERENCES

Andreasson E, Jenkins T, Brodersen P, Thorgrimsen S, Petersen NHT, Zhu S, Qiu JL, Micheelsen P, Rocher A, Petersen M, Newman MA, Nielsen HB (2005) The MAP kinase substrate MKS1 is a regulator of plant defense responses. EMBO J **24**:2579–2589

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signaling cascade in Arabidopsis innate immunity. Nature **415**:977–983

Benfey PN, Ren L, Chua NH (1990) Tissue-specific expression from CaMV 35S enhancer subdomains in early stages of plant development. EMBO J **9**:1677–1684

Blanco F, Salinas P, Cecchini NM, Jordana X, Van Hummelen P, Alvarez ME, Holuigue L (2009) Early genomic responses to salicylic acid in Arabidopsis. Plant Mol Biol **70**:79–102

Brodersen P, Petersen M, Bjørn Nielsen H, Zhu S, Newman MA, Shokat KM, Rietz S, Parker J, Mundy J (2006) Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. Plant J **47**:532–546

Cao H, Glazebrook J, Clarke JD, Volko S, Dong X (1997) The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88**:57–63

Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nürnberger T, Jones JDG, Felix G, Boller T (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature **448**:497–500

Conrath U, Beckers GJM, Flors V, García-Agustín P, Jakab G, Mauch F, Newman M-A, Pieterse CMJ, Poinssot B, Pozo MJ, Pugin A, Schaffrath U (2006) Priming: getting ready for battle. Mol Plant-Microbe Interact **19**:1062–1071

Dempsey DA, Klessig DF (2012) SOS – too many signals for systemic acquired resistance? Trends Plant Sci, in press

Després C, De Long C, Glaze S, Liu E, Fobert PR (2000) The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. Plant Cell **12**:279–290

Després C, Chubak C, Rochon A, Clark R, Bethune T, Desveaux D, Fobert PR (2003) The Arabidopsis NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. Plant Cell **15**:2181–2191

Dong X (1998) SA, JA, ethylene, and disease resistance in plants. Curr Opin Plant Biol **1**: 316–323

Eulgem T, Rushton PJ, Schmelzer E, Hahlbrock K, Somssich IE (1999) Early nuclear events in plant defense signaling: rapid gene activation by WRKY transcription factors. EMBO J **18**:4689–4699

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. Trends Plant Sci **5**:199–206

Fan W, Dong X (2002) In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. Plant Cell **14**:1377–1389

Feys BJ, Moisan LJ, Newman MA, Parker JE (2001) Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. EMBO J **20**:5400–5411

Fu ZQ, Yan S, Saleh A, Wang W, Ruble J, Oka N, Mohan R, Spoel SH, Tada Y, Zheng N, Dong X (2012) NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature **486**:228-232

Gaille C, Kast P, Haas D (2002) Salicylate biosynthesis in Pseudomonas aeruginosa. Purification and characterization of PchB, a novel bifunctional enzyme displaying isochorismate pyruvate-lyase and chorismate mutase activities. J Biol Chem **277**:21768–21775

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Ann Rev Phytopathol **43**:205-227

Grant M, Lamb C (2006) Systemic immunity. Curr Opin Plant Biol **9**:414–420

He P, Shan L, Lin NC, Martin GB, Kemmerling B, Nürnberger T, Sheen J (2006) Specific bacterial suppressors of PAMP signaling upstream of MAPKKK in Arabidopsis innate immunity. Cell **125**:563–575

Higashi K, Ishiga Y, Inagaki Y, Toyoda K, Shiraishi T, Ichinose Y (2008) Modulation of defense signal transduction by flagellin-induced WRKY41 transcription factor in Arabidopsis thaliana. Mol Genet Genomics **279**:303–312

Howe GA (2004) Jasmonates as signals in the wound response. J Plant Growth Regul **23**:223–237

Ishiguro S, Nakamura K (1994) Characterization of a cDNA encoding a novel DNA-binding protein, SPF1, that recognizes SP8 sequences in the 50 upstream regions of genes coding for sporamin and ß-amylase from sweet potato. Mol Gen Genetic **244**:563–571

Jakoby M, Weisshaar B, Dröge-Laser W, Vicente-Carbajosa J, Tiedemann J, Kroj T, Parcy F (2002) bZIP transcription factors in Arabidopsis. Trends Plant Sci **7**:106–111

Johnson C, Boden E, Arias J (2003) Salicylic acid and NPR1 induce the recruitment of trans-activating TGA factors to a defense gene promoter in Arabidopsis. Plant Cell **15**:1846–1858

Jones JDG, Dangl JL (2006) The plant immune system. Nature **444**:323–329

Journot-Catalino N, Somssich IE, Roby D, Kroj T (2006) The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in Arabidopsis thaliana. Plant Cell **18**:3289–3302

Kalde M, Barth M, Somssich IE, Lippok B (2003) Members of the Arabidopsis WRKY group III transcription factors are part of different plant defense signaling pathways. Mol Plant-Microbe Interact **16**:295–305

Katagiri F, Lam E, Chua N-H (1989) Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. Nature **340**:727–730

Kesarwani M, Yoo J, Dong X (2007) Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in Arabidopsis. Plant Physiol **144**:336–346

Kim K-C, Lai Z, Fan B, Chen Z (2008) Arabidopsis WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. Plant Cell **20**:2357–2371

Kinkema M, Fan W, Dong X (2000) Nuclear localization of NPR1 is required for

activation of PR gene expression. Plant Cell **12**:2339–2350

Koornneef A, Pieterse CMJ (2008) Cross talk in defense signaling. Plant Physiol **146**:839–844

Kunze G, Zipfel C, Robatzek S, Niehaus K, Boller T, Felix G (2004) The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. Plant Cell **16**:3496–3507

Lebel E, Heifetz P, Thorne L, Uknes S, Ryals J, Ward E (1998) Functional analysis of regulatory sequences controlling PR-1 gene expression in Arabidopsis. Plant J **16**:223–233

Leon-Reyes A, Spoel SH, De Lange ES, Abe H, Kobayashi M, Tsuda S, Millenaar FF, Welschen RAM, Ritsema T, Pieterse CMJ (2009) Ethylene modulates the role of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 in cross talk between salicylate and jasmonate signaling. Plant Physiol **149**:1797–1809

Li J, Brader G, Palva ET (2004) The WRKY70 transcription factor: A node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell **16**:319–331

Li J, Brader G, Kariola T, Palva ET (2006) WRKY70 modulates the selection of signaling pathways in plant defense. Plant J **46**:477–491

Li X, Zhang Y, Clarke JD, Li Y, Dong X (1999) Identification and cloning of a negative regulator of systemic acquired resistance, SNI1, through a screen for suppressors of *npr1–1*. Cell **98**:329–339

Lindermayr C, Sell S, Müller B, Leister D, Durner J. (2010) Redox regulation of the NPR1-TGA1 system of Arabidopsis thaliana by nitric oxide. Plant Cell **22**:2894-907

Mackey D, McFall AJ (2006) MAMPs and MIMPs: proposed classifications for inducers of innate immunity. Mol Microbiol **61**:1365–1371

Mao P, Duan M, Wei C, Li Y (2007) WRKY62 transcription factor acts downstream of cytosolicNPR1 and negatively regulates jasmonate-responsive gene expression. Plant Cell Physiol **48**:833–842

Mosher RA, Durrant WE, Wang D, Song J, Dong X (2006) A comprehensive structure–function analysis of Arabidopsis SNI1 defines essential regions and transcriptional repressor activity. Plant Cell **18**:1750–1765

Mou Z, Fan W, Dong X (2003) Inducers of plant systemic acquired resistance regulates NPR1 function through redox changes. Cell **113**:935–944

Navarro L, Zipfel C, Rowland O, Keller I, Robatzek S, Boller T, Jones JDG (2004) The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. Plant Physiol **135**:1113–1128

Nawrath C, Métraux JP (1999) Salicylic acid induction-deficient mutants of Arabidopsis expresses *PR-2* and *PR-5* and accumulates high levels of camalexin after pathogen inoculation. Plant Cell **11**:1393–1404

Nawrath C, Heck S, Parinthawong N, Métraux J-P (2002) EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. Plant Cell **14**:275–286

Nobuta K, Okrent RA, Stoutemyer M, Rodibaugh N, Kempema L, Wildermuth MC, Innes RW (2007) The GH3 acyl adenylase family member PBS3 regulates salicylic acid-dependent defense responses in Arabidopsis. Plant Physiol

144:1144–1156

Pape S, Thurow C, Gatz C (2010) The Arabidopsis PR-1 promoter contains multiple integration sites for the coactivator NPR1 and the repressor SNI1. Plant Physiol **154**:1805-1818

Park SW, Kaimoyo E, Kumar D, Mosher S, Klessig DF (2007) Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. Science **318**:113–116

Qin X-F, Holuigue L, Horvath DM, Chua N-H (1994) Immediate early transcription activation by salicylic acid via the cauliflower mosaic virus as-1 element. Plant Cell **6**:863–874

Qiu J-L, Zhou L, Yun BW, Nielsen HB, Fiil BK, Petersen K, MacKinlay J, Loake GJ, Mundy J, Morris PC (2008) Arabidopsis mitogen-activated protein kinase kinases MKK1 and MKK2 have overlapping functions in defense signaling mediated by MEKK1, MPK4, and MKS1. Plant Physiol **148**:212–222

Reymond P, Farmer EE (1998) Jasmonate and salicylate as global signals for defense gene expression. Curr Opin Plant Biol **1**:404–411

Rochon A, Boyle P, Wignes T, Fobert PR, Després C (2006) The coactivator function of Arabidopsis NPR1 requires the core of its BTB/ POZ domain and the oxidation of C-terminal cysteines. Plant Cell **18**:3670–3685

Rogers EE, Ausubel FM (1997) Arabidopsis enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in *PR-1* gene expression. Plant Cell **9**:305–316

Ross AF (1961) Systemic acquired resistance induced by localized virus infections in plants. Virology **14**:340–358

Rowland O, Jones JDG (2001) Unraveling regulatory networks in plant defense using microarrays. Genome Biol **2**:1001.1–1001.3

Rushton PJ, Torres JT, Parniske M, Wernert P, Hahlbrock K, Somssich IE (1996) Interaction of elicitor-induced DNA binding proteins with elicitor response elements in the promoters of parsley *PR-1* genes. EMBO J **15**:5690–5700

Schindler U, Menkens AE, Beckmann H, Ecker JR, Cashmore AR (1992) Heterodimerization between light-regulated and ubiquitously expressed Arabidopsis GBF bZIP proteins. EMBO J **11**:1261–1273

Shearer HL, Cheng YT, Wang L, Liu J, Boyle P, Després C, Zhang Y, Li X, Fobert PR (2012) Arabidopsis clade I TGA transcription factors regulate plant defenses in an NPR1-independent fashion. Mol Plant Microbe Interact, in press

Spoel SH, Dong X (2008) Making sense of hormone crosstalk during plant immune responses. Cell Host Microbe **3**:348–351

Spoel SH, Mou Z, Tada Y, Spivey NW, Genschik P, Dong X (2009) Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. Cell **137**:860–872

Strompen G, Grüner R, Pfitzner UM (1998) An as-1-like motif controls the level of expression of the gene for the pathogenesis-related protein 1a from tobacco. Plant Mol Biol **37**:871–883

Sun C, Palmqvist S, Olsson H, Borén M, Ahlandsberg S, Jansson C (2003) A novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the iso1 promoter. Plant Cell **15**:2076–2092

Tsuda K, Sato M, Glazebrook J, Cohen JD, Katagiri F (2008) Interplay between MAMP-

triggered and SA-mediated defense responses. Plant J **53**:763–775
Turner JG, Ellis C, Devoto A (2002) The jasmonate signal pathway. Plant Cell **14**:S153–S164
Van Verk MC, Pappaioannou D, Neeleman L, Bol JF, Linthorst HJM (2008) A novel WRKY transcription factor is required for induction of PR-1A gene expression by salicylic acid and bacterial elicitors. Plant Physiol **146**:1983–1995
Van Verk MC, Neeleman L, Bol JF, Linthorst HJM (2011a) Tobacco Transcription Factor NtWRKY12 Interacts with TGA2.2 in vitro and in vivo. Front Plant Sci **2**:32
Van Verk MC, Bol JF, Linthorst HJM (2011b) WRKY transcription factors involved in activation of SA biosynthesis genes. BMC Plant Biol **11**:89
Verberne MC, Sansuk K, Bol JF, Linthorst HJM, Verpoorte R (2007) Vitamin K1 accumulation in tobacco plants overexpressing bacterial genes involved in the biosynthesis of salicylic acid. J Biotech **128**:72–79
Wang D, Amornsiripanitch N, Dong X (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathogens **2**:e123
Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414**:562–565
Wirthmueller L, Zhang Y, Jones JDG, Parker JE (2007) Nuclear accumulation of the Arabidopsis immune receptor RPS4 is necessary for triggering EDS1-dependent defense. Curr Biol **17**:2023–2029
Wu Y, Zhang D, Chu JY, Boyle P, Wang Y, Brindle ID, Luca VD, Després C (2012) The Arabidopsis NPR1 Protein Is a Receptor for the Plant Defense Hormone Salicylic Acid. Cell Rep **1**:639-647
Xiang C, Miao Z-H, Lam E (1996) Coordinated activation of as-1-type elements and a tobacco glutathion S-transferase gene by auxins, salicylic acid, methyl-jasmonate and hydrogen peroxide. Plant Mol Biol **32**:415–426
Xu X, Chen C, Fan B, Chen Z (2006) Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, andWRKY60 transcription factors. Plant Cell **18**:1310–1326
Zhang Y, Fan W, Kinkema M, Li X, Dong X (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. Proc Natl Acad Sci USA **96**:6523–6528
Zhang Y, Tessaro MJ, Lassner M, Li X (2003) Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. Plant Cell **15**:2647–2653
Zhang Y, Wang L (2005) The WRKY transcription factor superfamily: its origin in eukaryotes and expansion in plants. BMC Evol Biol **5**:1
Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotech Adv **23**:283–333

CHAPTER 2

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

R. Muhammad Fraz Hussain, Huub J.M. Linthorst

Institute of Biology, Leiden University, P.O. Box 9505, 2300 RA Leiden, the Netherlands

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.

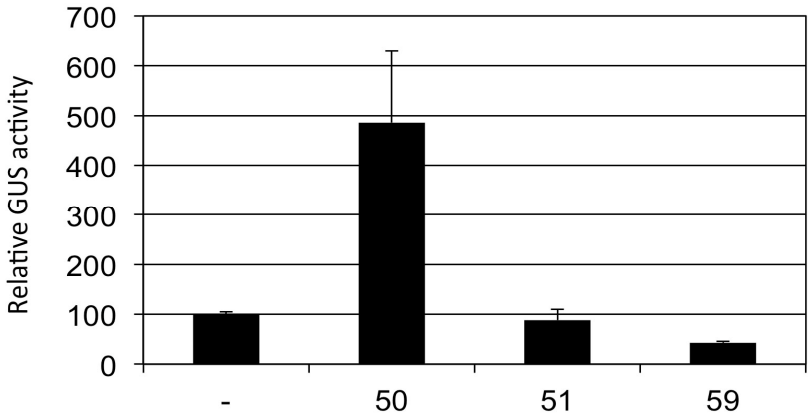

Figure 1. AtWRKY50 activates *PR-1* promoter. Arabidopsis protoplasts were co-transfected with *PR1::GUS* construct together with empty pRT101 expression plasmid (minus sign) or with plasmids containing *35S::AtWRKY50* (50), *35S::AtWRKY51* (51) or *35S::AtWRKY59* (59). After incubation GUS activity 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.

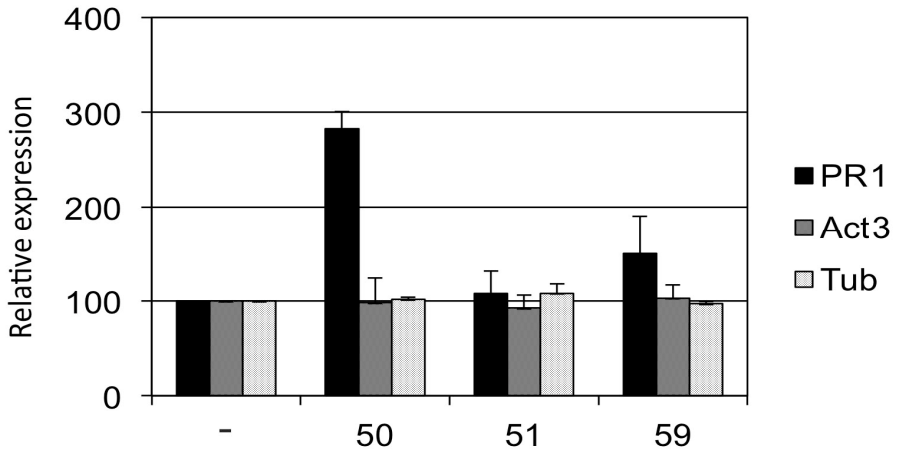

Figure 2. Effect of AtWRKY50, AtWRKY51 and AtWRKY59 on the expression of endogenous Arabidopsis genes. Expression of *PR-1*, *Act3* and *Tub* genes in Arabidopsis protoplasts was measured by qRT-PCR. Expression of each gene was measured in protoplasts transfected with the empty pRT101 vector (minus sign) 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

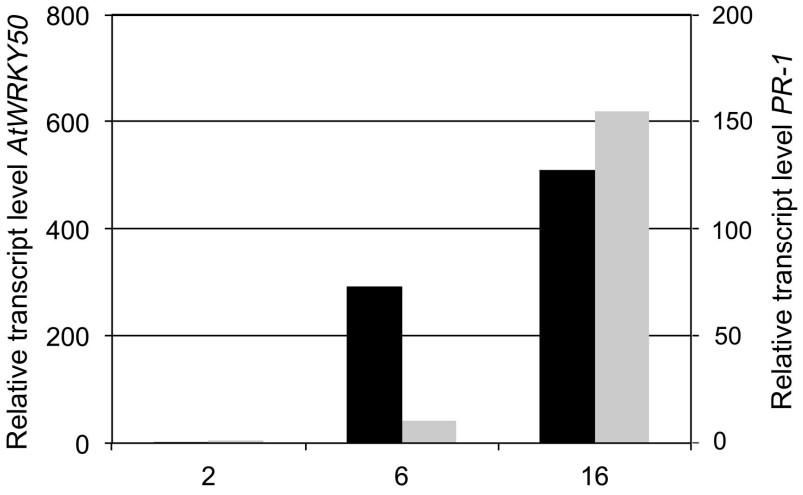

Figure 3. Salicylic acid-induced gene expression of *AtWRKY50* and *PR-1*. Expression of *AtWRKY50* (black bars) and *PR-1* (grey bars) was analyzed in Arabidopsis plants, incubated for the indicated times (hours) in medium containing 1mM salicylic acid. The expression was quantified by qRT-PCR.

(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.

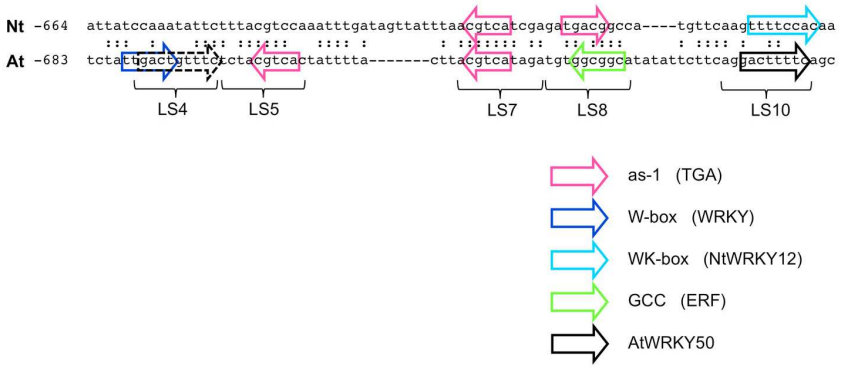

Figure 4. Comparison of sequences in the promoters of tobacco *PR-1a* (Nt) and Arabidopsis *PR-1* (At). Only the sequence of the top strands is given. The sequences of the promoter regions are shown with gaps to allow maximal alignment. The position of the leftmost nucleotide relative to the transcription 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

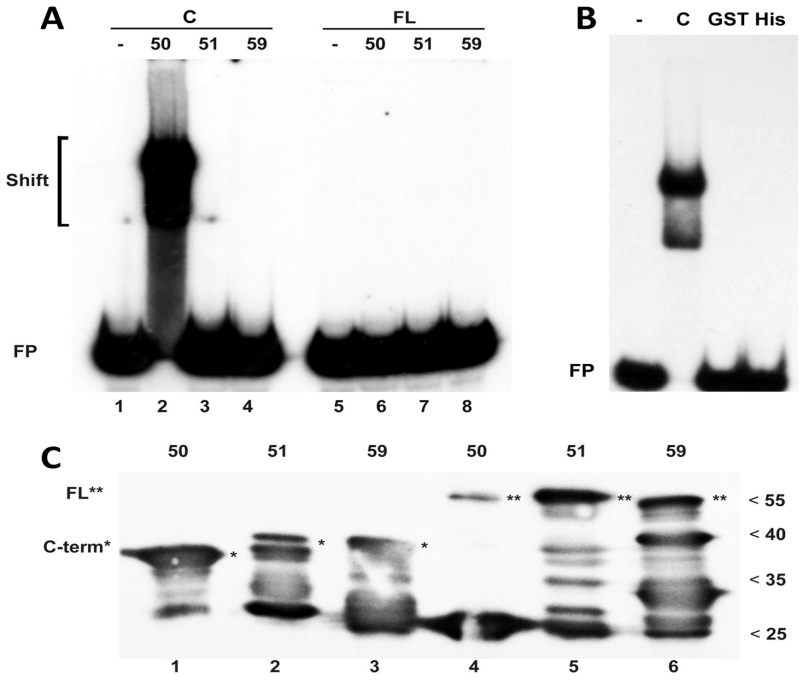

Figure 5. AtWRKY50 binds to the *PR-1* promoter. (A) EMSAs were performed with an 80-bp fragment of the *PR1* promoter and GST-tagged C-terminal halves (Lanes C) or full-length (Lanes FL) versions of AtWRKY50, -51 and -59, as indicated above the lanes. (B) EMSAs were performed with the same probe together 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).

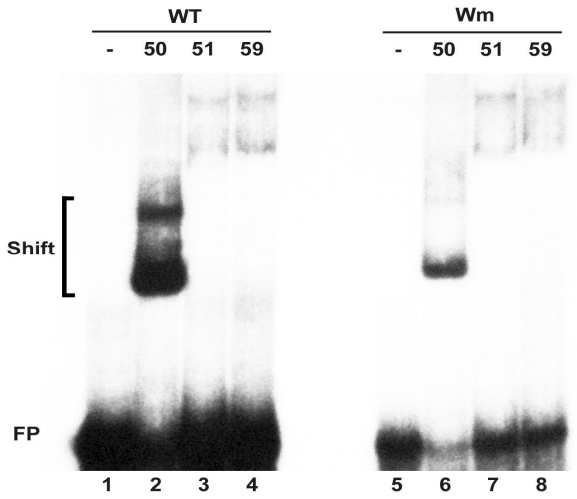

Figure 6. AtWRKY50 binds to the *PR-1* promoter at two positions. EMSAs were performed with wild-type 80-bp *PR-1* promoter fragment (WT) or with an 80-bp fragment with a mutation in the W-box (Wm) as probes together with the GST-tagged C-terminal halves of AtWRKY50, -51 and -59, as indicated above the 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.

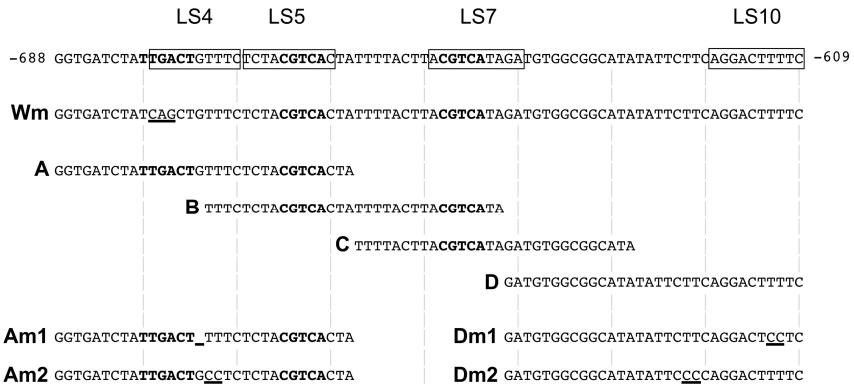

Figure 7. Sequences of *PR-1* promoter fragments used for EMSAs. Only the sequence of the upper strand is given. The top line displays the sequence of the 80-bp fragment corresponding to bp -688 to -609 upstream of the transcription start site. Regions LS4, LS5, LS7 and LS10, as used in the linker scanning 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).

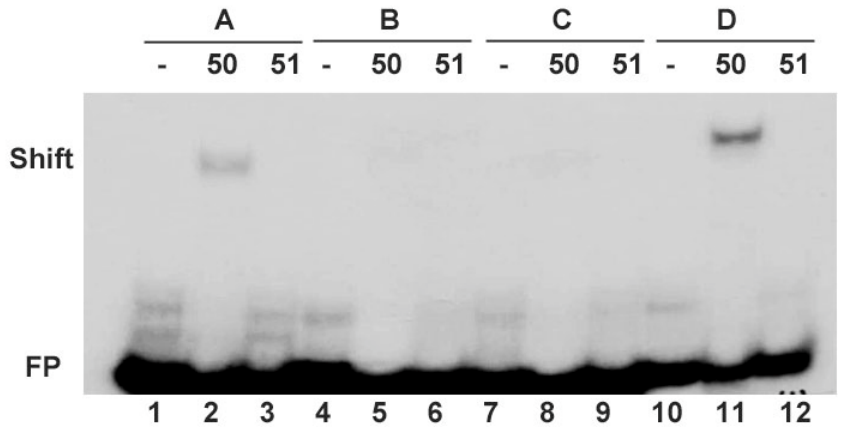

Figure 8. AtWRKY50 binds to the *PR-1* promoter at two positions. EMSAs were performed with overlapping *PR-1* promoter fragments A, B, C and D as probes and GST-tagged AtWRKY50-C or the C-terminal half of AtWRKY51, as indicated above the lanes. The positions of band shifts and the unbound probe (FP)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

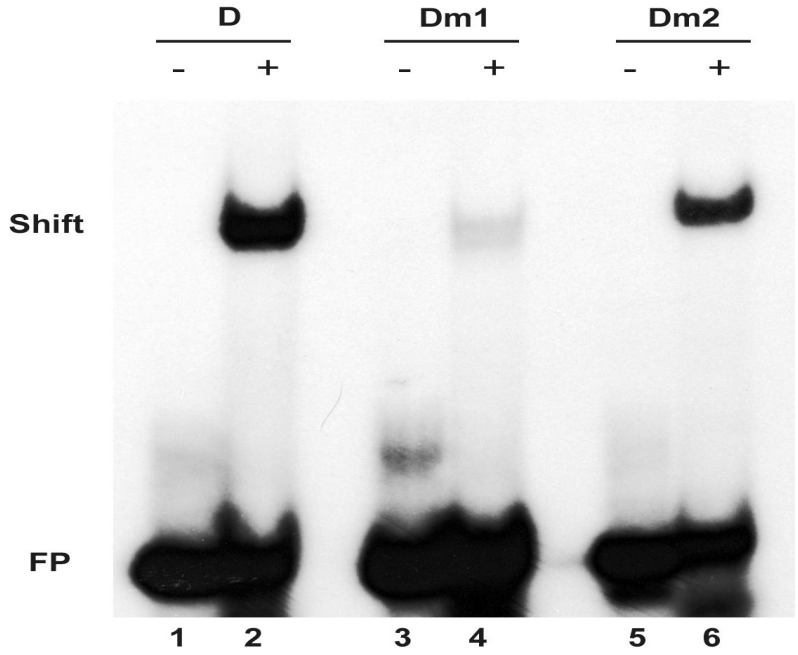

Figure 9. AtWRKY50 binds to the LS10 element in the *PR-1* promoter. EMSAs were performed with wild type (D) and mutant versions (Dm1, Dm2) of *PR1* promoter fragment D as probes and GST-tagged AtWRKY50-C, as indicated above the lanes. The positions of band shifts and the unbound probe (FP) are indicated.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

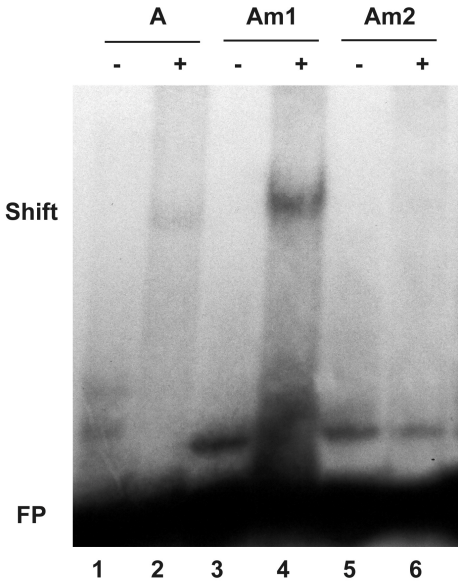

Figure 10. AtWRKY50 binds to the LS4 element in the *PR-1* promoter. EMSAs were performed with wild type (A) and mutant versions (Am1, Am2) of *PR-1* promoter fragment A as probes and GST-tagged AtWRKY50-C, as indicated above the lanes. The positions of band shifts and the unbound probe (FP) are indicated.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

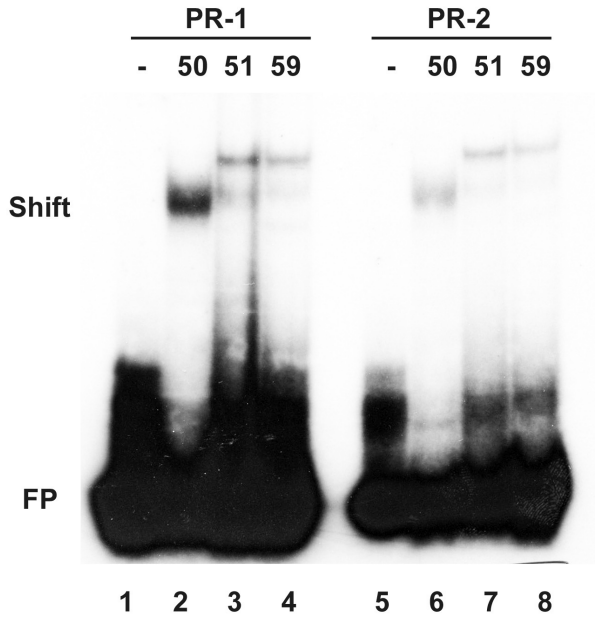

Figure 11. AtWRKY50 binds to an element in the *PR-2* promoter. EMSAs were performed with a 35-bp fragment from the *PR-1* promoter containing LS10 and a 35-bp fragment from the *PR-2* promoter containing the sequence GACTTTTC (-175 bp upstream of the transcription start site) as probes and GST-tagged 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.

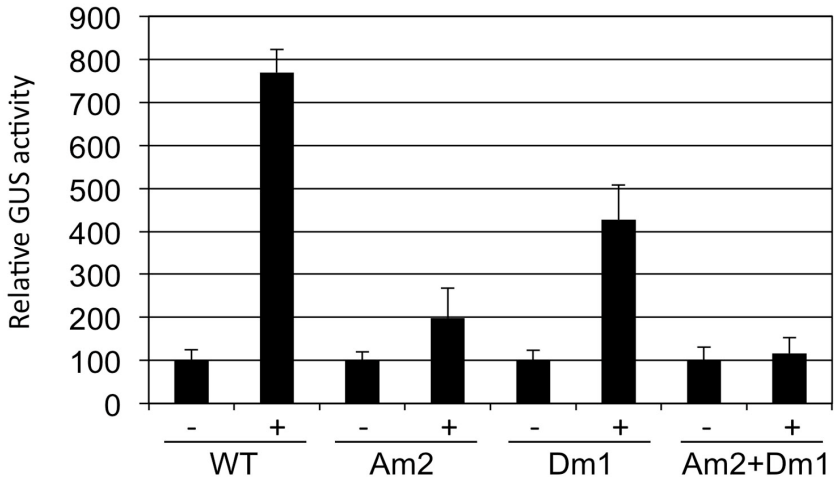

Figure 12. *PR-1* activation by AtWRKY50 requires intact binding sites. Arabidopsis protoplasts were co-transfected with wild type (WT) and mutant *PR1::GUS* construct alone (minus sign) or together with expression plasmids *35S::AtWRKY50*. Mutant promoters contained mutations as indicated in Fig. 7. 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.

ACKNOWLEDGEMENTS

We are grateful to Jasmin Göttler and Prof. Wolfgang Dröge-Laser for the protoplast transactivation assay screening of Arabidopsis WRKYs. We thank Zeshan Hassan and Imran Haider for assistance with the qRT-PCR. R. M. F. H. was supported by a grant from the Higher Education Commission of Pakistan.

REFERENCES

Axelos M, Curie C, Mazzolini L, Bardet C, Lescure B (1992) A protocol for transient gene expression in Arabidopsis thaliana protoplasts isolated from cell suspension cultures. Plant Physiol Biochem **30**:123–128

Brand LH, Kirchler T, Hummel S, Chaban C, Wanke D (2010) DPI-ELISA: a fast and versatile method to specify the binding of plant transcription factors to DNA in vitro. Plant Methods **6**:25.

Cao H, Glazebrook J, Clarke JD, Volko S, Dong X (1997) The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88**:57–63

Delaney TP, Friedrich L, Ryals JA (1995) Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. Proc Natl Acad Sci USA **92**:6602-6606

Després C, De Long C, Glaze S, Liu E, Fobert PR (2000) The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. Plant Cell **12**:279–290

Dong J, Chen C, Chen Z (2003) Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response. Plant Mol Biol **51**:21–37

Eulgem T, Somssich IE (2007) Networks of WRKY transcription factors in defense signaling. Curr Opin Plant Biol **10**:366–371

Fu ZQ, Yan S, Saleh A, Wang W, Ruble J, Oka N, Mohan R, Spoel SH, Tada Y, Zheng N, Dong X (2012) NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature **486**:228–232

Gao QM, Venugopal S, Navarre D, Kachroo A (2010) Low oleic acid-derived repression of jasmonic acid-inducible defense responses requires the WRKY50 and WRKY51 proteins. Plant Physiol **155**:464-476.

Green PJ, Kay SA, Lam E, Chua NH (1989) In vitro DNA footprinting. *In* SB Gelvin, RA Schilperoort, eds, Plant Molecular Biology Manual B11. Kluwer Academic Publishers, Dordrecht, the Netherlands, pp **1**–22.

Grüner R, Strompen G, Pfitzner AJP, Pfitzner UM (2003) Salicylic acid and the hypersensitive response initiate distinct signal transduction pathways in tobacco that converge on the *as-1*-like element of the *PR-1a* promoter. Euro J of Biochem **270**:4876–4886

Guan KL, Dixon JE (1991) Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal Biochem **192**: 262–267

Johnson C, Boden E, Arias J (2003). Salicylic acid and NPR1 induce the recruitment of *trans*-activating TGA factors to a defense gene promoter in Arabidopsis. Plant Cell **15**:1846–1858

Kesarwani M, Yoo J, Dong X (2007) Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in Arabidopsis. Plant Physiol **144**:336–346

Kinkema M, Fan W, Dong X (2000) Nuclear localization of NPR1 is required for activation of PR gene expression. Plant Cell **12**:2339–2350

Lebel E, Heifetz P, Thorne L, Uknes S, Ryals J, Ward E (1998) Functional analysis of regulatory sequences controlling PR-1 gene expression in Arabidopsis. Plant J **16**:223–233

Linthorst HJM (1991) Pathogenesis-related proteins of plants. Crit Rev Plant Sci **10**:123-150

Mou Z, Fan W, Dong X (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell **113**:935–944

Niggeweg R, Thurow C, Kegler C, Gatz C (2000a) Tobacco transcription factor TGA2.2 is the main component of as-1-binding factor ASF-1 and is involved in salicylic acid- and auxin-inducible expression of as-1-containing target promoters. J Biol Chem **275**:19897–19905.

Niggeweg R, Thurow C, Weigel R, Pfitzner U, Gatz C (2000b) Tobacco TGA factors differ with respect to interaction with NPR1, activation potential and DNA-binding properties. Plant Mol Biol **42**:775–788

Pandey SP, Somssich IE (2009) The Role of WRKY Transcription Factors in Plant Immunity. Plant Physiol **150**:1648-1655

Qin X-F, Holuigue L, Horvath DM, Chua N-H (1994) Immediate early transcription activation by salicylic acid via the cauliflower mosaic virus as-1 element. Plant Cell **6**:863–874

Rushton PJ, Somssich IE, Ringler P, Shen QJ (2010) WRKY transcription factors. Trends Plant Sci **15**:247-58

Schirawski J, Planchais S, Haenni AL (2000) An improved protocol for the preparation of protoplasts from an established Arabidopsis thaliana cell suspension culture and infection with RNA of a turnip yellow mosaic tymovirus: a simple and reliable method. J Virol **86**:85–94

Shen QH, Saijo Y, Mauch S, Biskup C, Bieri S, Keller B, Seki H, Ulker B, Somssich IE, Schulze-Lefert P (2007) Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. Science **315**: 1098–1103

Spoel SH, Mou Z, Tada Y, Spivey NW, Genschik P, Dong X (2009) Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. Cell **137**:860–872

Strompen G, Grüner R, Pfitzner UM (1998) An *as-1*-like motif controls the level of expression of the gene for the pathogenesis-related protein 1a from tobacco. Plant Mol Biol **37**:871–883

Töpfer R, Matzeit V, Gronenborn B, Schell J, Steinbiss HH (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res **15**, 5890

Van der Fits L, Memelink J (1997) Comparison of the activities of CaMV 35S and FMV 34S promoter derivatives in Catharanthus roseus cells transiently and stably transformed by particle bombardment. Plant Mol Bio **33**:943–946

Van Verk MC, Pappaioannou D, Neeleman L, Bol JF, Linthorst HJM (2008) A novel WRKY transcription factor is required for induction of PR-1A gene expression by salicylic acid and bacterial elicitors. Plant Physiol **146**:1983–1995

Van Verk MC, Neeleman L, Bol JF, Linthorst HJM (2011a) Tobacco Transcription Factor NtWRKY12 Interacts with TGA2.2 in vitro and in vivo. Front Plant Sci **2**:32

Wang D, Amornsiripanitch N, Dong X (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathogens **2**:e123

Wehner N, Hartmann L, Ehlert A, Böttner S, Oñate-Sánchez L, Dröge-Laser W (2011) High-throughput protoplast transactivation (PTA) system for the analysis of Arabidopsis transcription factor function. Plant J **68**:560-569

Xu X, Chen C, Fan B, Chen Z (2006) Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, and WRKY60 transcription factors. Plant Cell **18**:1310–1326

Yamasaki K, Kigawa T, Inoue M, Tateno M, Yamasaki T, Yabuki T, Aoki A, Seki E, Matsuda T, Tomo Y, Hayami N, Terada T, Shirouzu M, Tanaka A, Seki M, Shinozaki K, Yokoyama S (2005) Solution structure of an Arabidopsis WRKY DNA binding domain. Plant Cell **17**:944–956

Yanagisawa S (2004) Dof domain proteins: plant-specific transcription factors associated with diverse phenomena unique to plants. Plant and Cell Physiol **45**:386–391

Zhang Y, Fan W, Kinkema M, Li X, Dong X (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR-1* gene. Proc Natl Acad Sci USA **96**:6523–6528

Zhang Y, Tessaro MJ, Lassner M, Li X (2003) Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. Plant Cell **15**:2647–2653

Zheng Z, Qamar SA, Chen Z, Mengiste T (2006) Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J **48**: 592–605

Zhou J-M, Trifa Y, Silva H, Pontier D, Lam E, Shah J, Klessig DF (2000) NPR1differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the *PR-1* gene required for induction by salicylic acid. Mol Plant Microbe Interact **13**:191–202

CHAPTER 3

INTERACTION OF ATWRKY50 AND TGA TRANSCRIPTION FACTORS SYNERGISTICALLY ACTIVATES *PR-1* GENE EXPRESSION

R. Muhammad Fraz Hussain, Huub J.M. Linthorst

Institute of Biology, Leiden University, P.O. Box 9505, 2300 RA Leiden, the Netherlands

ABSTRACT

The promoter of the salicylic acid-inducible *PR-1* gene of Arabidopsis contains two binding sites for transcription factor AtWRKY50 that are located in close proximity to the binding sites for TGA transcription factors. Yeast-2-hybrid assays and bimolecular fluorescence complementation (BiFC) experiments revealed that AtWRKY50 could interact with TGA2 and TGA5. Using electrophoretic mobility shift assays (EMSA) it was established that AtWRKY50 and TGA2 or TGA5 were able to bind simultaneously to *PR-1* promoter fragments and that TGA2 and TGA5 predominantly bound to one of the two CGTCA motifs in the *as*-1-like element in the promoter. Transactivation experiments in Arabidopsis protoplasts derived from wild type, *npr1-1* and *tga256* mutant plants indicated that AtWRKY50 alone was able to induce expression of a *PR-1::β-glucuronidase* (GUS) reporter gene, independent of TGAs or NPR1. However, co-expression of TGA2 or TGA5 and AtWRKY50 synergistically enhanced expression to high levels.

INTRODUCTION

Plants possess sophisticated defense systems to counteract attack by microbial pathogens. This defense consists partly on pathogen-triggered local and systemic accumulation of specific defense proteins with anti-microbial activities, named pathogenesis-related, or PR proteins. PR proteins are conserved throughout the plant kingdom and the induction of their genes is mediated by the endogenous signaling compound salicylic acid (SA). The fact that their expression is also kingdom-wide conserved, suggests similar mechanisms of transcriptional regulation of the *PR* genes. The *PR-1* gene is generally used as a marker gene for the induced defense response. Although

the promoters of the tobacco and Arabidopsis *PR-1* genes do not share apparent sequence similarity, both contain a region approximately 600 bp upstream of the transcription start site responsible for induction of gene expression by SA. In both promoters this region contains two copies of a TGACG motif that are present as inverted repeats in tobacco and as direct repeats in Arabidopsis. In the *35S* promoter two direct TGACG repeats were characterized as *activating sequence-1* (*as-1*), required for SA-enhanced expression, and binding to TGA proteins of the bZIP family of transcription factors (Katagiri *et al.*, 1989; Qin *et al.*, 1994). Early analyses of the tobacco and Arabidopsis *PR-1* genes indicated that also the *as-1*-like elements in their promoters act as binding sites of TGA proteins and these sites are important for SA-induced expression (Strompen *et al.*, 1998; Lebel *et al.*, 1998; Niggeweg *et al.*, 2000; Grüner *et al.*, 2003; Durrant and Dong, 2004). In the years thereafter, cumulating results from several groups led to the adoption of a model in which the central defense regulator NPR1 functions as a transcriptional (co-)activator through interaction with TGA proteins bound at the promoters of SA-responsive genes (Després *et al.*, 2000; Zhang *et al.*, 1999; Zhou *et al.*, 2000; Fan and Dong, 2002; Johnson *et al.*, 2003; Rochon *et al.*, 2006; Spoel *et al.*, 2009; Fu *et al.*, 2012).

The seminal work of Lebel *et al.* (1998) on the characterization of the Arabidopsis *PR-1* promoter using a series of linker-scanning mutations introduced the "LS" naming for a sequential series of 10 bp mutations in the -700 to -600 bp region upstream of the transcription start site. Their results indicated that the TGACG motifs in LS5 and LS7 mediated negative and positive regulation of *PR-1* gene expression, respectively. Recent work of Pape *et al.* (2010) confirmed and extended the results of Lebel *et al.* (1998), although their additional data could be interpreted to suggest that the LS5 and LS7 elements are not the single prime determinants for INA-inducible expression of *PR-1*. In their studies, mutation of LS5 resulted in a relatively small (less than 2-

fold) enhancement of the level of inducible expression in comparison to the WT promoter, while mutation of LS7 modestly reduced expression (approximately 3-fold). Moreover, when both LS5 and LS7 were mutated, *PR-1* promoter-driven expression was 2-fold higher than that of the WT promoter, while in all these cases expression remained inducible by the SA-analog INA. On the other hand, mutation of LS10 had a more severe impact on inducible expression, reducing the expression level to approximately one fifth that of WT. Nevertheless, LS10 alone was not able to support high-level expression, as simultaneous mutation of LS4, LS5 and LS7 reduced inducible expression 5-fold. Furthermore, W-boxes downstream of the LS elements were also found to be important for expression of *PR-1* (Pape *et al.*, 2010). These results suggest that TGA proteins are not the only transcription factors important for *PR-1* expression, but that instead, regulation of expression is mediated by additional transcription factors binding to the intricate mosaic of elements in the *PR-1* promoter, and especially underline the importance of transcription factors binding to sites in LS4 and LS10.

In the previous chapter we identified AtWRKY50. AtWRKY50 is a member of the WRKY family of transcription factors of which Arabidopsis contains 74 genes. AtWRKY50 closely resembles NtWRKY12, which we characterized previously as an important transcriptional regulator of the tobacco *PR-1a* gene (Van Verk *et al.*, 2008). The C-terminal half of AtWRKY50 bound with high specificity to DNA sequences in the LS4 and LS10 elements, while AtWRKY50 enhanced expression of *PR-1::reporter* genes in protoplast transactivation assays. Here we found that AtWRKY50 interacts with TGA2 and TGA5 and we investigated the effects of combinations of AtWRKY50 and these TGAs in DNA binding assays and protoplast transactivation assays.

RESULTS

AtWRKY50 interacts with TGA2 and TGA5

Previously, we found that the close proximity of the binding sites for NtWRKY12 and TGAs in the promoter of the tobacco *PR-1a* gene may be functionally relevant for bringing both proteins together in order to direct full transcriptional activation. Further support for this came from studies that showed that NtWRKY12 interacted with TGA2.2 when expressed in yeast and in Arabidopsis protoplasts (van Verk *et al.*, 2011a). Similar to the *PR-1a* promoter of tobacco, the AtWRKY50 binding sites in LS4 and LS10 and the TGA-binding *as-1* element (in LS5 and LS7) of the *PR-1* promoter are in close proximity (Chapter 2, Fig. 2).

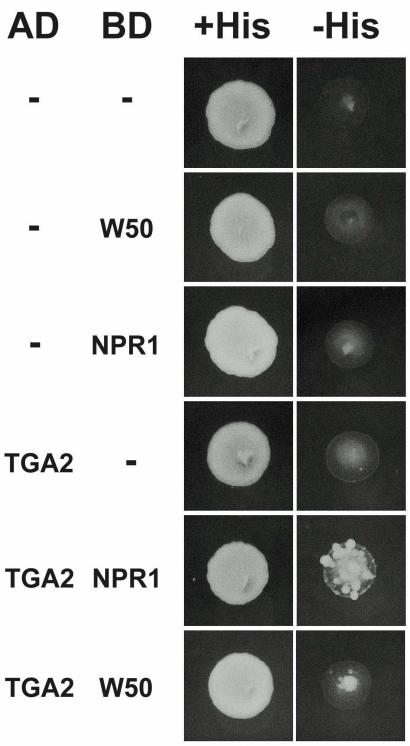

Figure 1. AtWRKY50 interacts with TGA2 in yeast. Yeast was transformed with expression plasmids pAS2.1 and pACT2, containing the coding regions of the binding domain (BD) and activation domain (AD) of GAL4, respectively. The BD domain was either fused to the coding regions of AtWRKY50 (W50), NPR1, or was not fused (minus sign); the AD domain was not fused (minus sign) or was fused to the coding region of TGA2. Growth of transformed yeast was evaluated on medium containing histidine (+His) or minus histidine (-His).

To investigate if Arabidopsis TGAs and AtWRKY50 can interact, we performed yeast-two-hybrid assays. Full-length coding sequences of AtWRKY50 fused to the binding domain (BD) of GAL4 were co-expressed in yeast containing a *Gal4::His* reporter gene with coding sequences for full-length TGA2, TGA3 and TGA5 fused to the GAL4 transcriptional activation domain (AD), after which

growth of yeast was scored on media lacking histidine. Fig. 1 shows the results of the two-hybrid assays with TGA2 and AtWRKY50. The control with TGA2-AD did not grow on medium lacking histidine. Moreover, also AtWRKY50-BD alone was not able to self-activate transcription of the *His* reporter gene. This last finding is in contrast to the finding that full-length tobacco NtWRKY12 fused to the Gal4-BD did activate *His* gene expression in the yeast-one-hybrid system. The positive control with the established interactors TGA2-AD and NPR1-BD resulted in growth of yeast colonies on medium lacking histidine (Després *et al.*, 2000). Co-expression of TGA2-AD and AtWRKY50-BD likewise resulted in growth of yeast colonies on medium without histidine, albeit that the colonies grew less fast than those of the positive control. This indicates that AtWRKY50 and TGA2 interacted in the yeast system. A possible interaction of AtWRKY50 with TGA5 could not be studied using the yeast-two-hybrid system as the fusion product of TGA5-AD allowed yeast to grow on medium without histidine, indicating that TGA5 activated the *Gal4::His* reporter gene presumably by the ability to bind to the *Gal4* promoter. Two-hybrid screenings with combinations of similar fusion products of TGA3 and AtWRKY50 and with TGA2 or TGA3 with AtWRKY51 did not indicate interactions between these proteins (data not shown).

Further support for interaction of AtWRKY50 with TGAs was obtained with bimolecular fluorescence complementation (BiFC) experiments. To this end, the AtWRKY50 and TGA2 or TGA5 coding sequences were fused at the N- or the C-terminus to the N- (YN) or C- (YC) terminal halves of the yellow fluorescent protein (YFP), respectively. Protein-protein interaction was analyzed 16 hours after co-transfection of Arabidopsis protoplasts with expression plasmids harboring these constructs, by determining the fluorescence of reconstituted YFP using confocal laser scanning microscopy. Typical results are shown in Fig. 2. For all combinations with YN- and YC-fused

versions of AtWRKY50 and TGA2 or TGA5, fluorescence was most strongly visible in nuclei, indicating a predominant nuclear presence of the AtWRKY50 and TGA fusion proteins. Controls with combinations of unfused YN and YC or with combinations of AtWRKY50 and TGA2 or TGA5 in which only one of the proteins was fused to a YFP half did not result in fluorescence (data not shown). The results indicate that AtWRKY50 interacts with both TGA2 and TGA5.

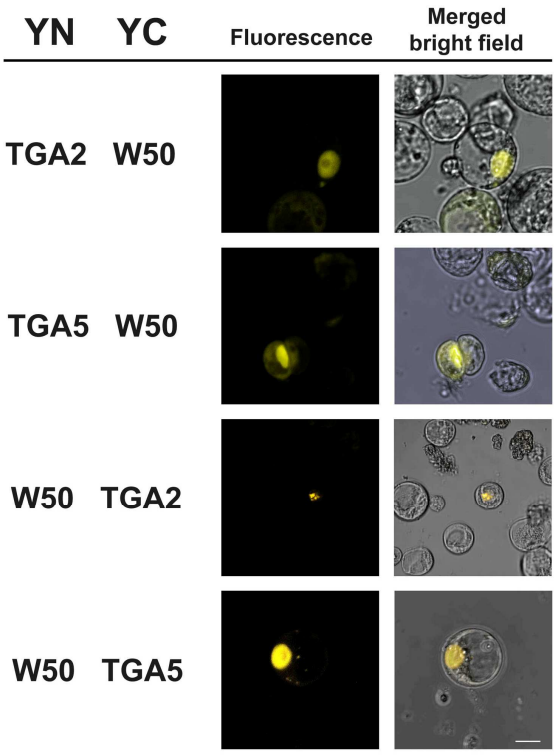

Figure 2. AtWRKY 50 interacts with TGA2 and TGA5 in Arabidopsis protoplasts. YFP fluorescence and merged bright field images of Arabidopsis cell suspension protoplasts co-transformed with expression plasmids containing constructs encoding TGA2, TGA5 and AtWRKY50 (W50) fused to the N-terminus (YN) or the C-terminus (YC) of yellow fluorescent protein. Scale bar = 10µm

AtWRKY50-C and TGA2 or TGA5 bind to the *PR-1* promoter simultaneously

Next, we investigated how combinations of AtWRKY50 and TGA2 or TGA5 influenced the binding to DNA. Therefore, EMSAs were done with purified, *E.*

coli-expressed GST-tagged AtWRKY50-C and His-tagged TGA2 and TGA5. The results are presented in Fig. 3. Lanes 2 and 8 show the double shifts resulting from the binding of one and two AtWRKY50-C peptides to the 80-bp *PR-1* promoter fragment that was used as a probe (compare with Chapter 2, Fig. 2, Lane 2 and Fig. 3, Lane 2). Fig. 3, Lanes 3 and 9 show the band shifts resulting from incubation of the probe with TGA2 and TGA5, respectively. A number of shifts are visible of which the intensity increases with decreasing mobility. The presence of multiple shifts with TGA proteins (notably with TGA2 and TGA5) has also been observed by others, who ascribed it to possible different degrees of occupancy of the binding sites present in the probe (Miao and Lam, 1994, 1995; Pontier *et al.*, 2001). However, we cannot exclude that some aggregate formation occurred during incubation, due to non-specific interactions of these TGAs. Indicative for this may also be the label remaining on top of the gel. Nevertheless, we speculate that the weak bands in Lanes 3 and 9 (indicated by single black asterisks) represent complexes in which only one of the CGTCA binding sites in either LS5 or LS7 was occupied by TGA, while the more slowly migrating bands in Lanes 3 and 9 (indicated by asterisks in white), represent higher order TGA shifts, possibly including shifts in which TGAs are bound at both the CGTCA sites in LS5 and LS7. Incubation of the 80-bp probe with combinations of AtWRKY50-C and TGA2 or TGA5 resulted in new bands indicated by the double asterisks in black and white (Fig. 3, Lanes 4 and 10). We hypothesize that these new bands represent complexes of the probe with both AtWRKY50-C and the respective TGA proteins, possibly with the proteins causing a supershift resulting from their interaction.

To investigate which of the two binding sites for AtWRKY50 allows formation of a complex containing both AtWRKY50 and TGA, promoter fragments consisting of the regions encompassing subfragments A, B and C (Fig. 4, ABC) and subfragments B, C and D (Fig. 4, BCD) were tested in EMSAs

with AtWRKY50-C and TGA2. The ABC and BCD promoter fragments each contain only one of the AtWRKY50 binding sites, which make the EMSA results easier to interpret. The results are shown in Fig. 5.

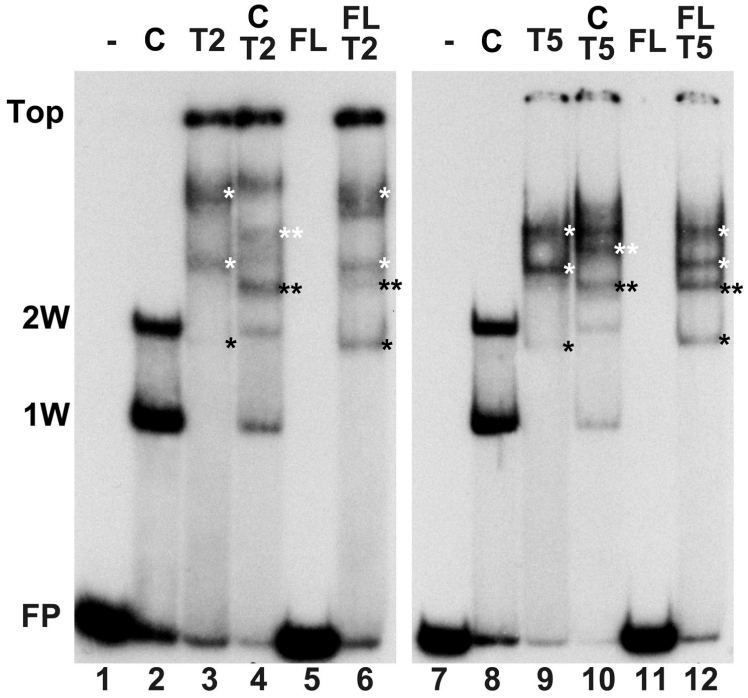

Figure 3. AtWRKY50 and TGA2 and TGA5 bind to the *PR-1* promoter. EMSAs were performed with an 80-bp fragment of the *PR-1* promoter without protein (minus signs) or with GST-tagged C-terminal half (C) or full-length (FL) versions of AtWRKY50, and His-tagged TGA2 (T2) or TGA5 (T5), and combinations of these proteins, as indicated above the lanes. The positions of the unbound probe (FP), the top of the gel (Top), and of band shifts caused by one (1W) or two (2W) AtWRKY50-C proteins are indicated at the left. Shifts caused by binding of single (single black asterisks) or multiple (single white asterisks) TGA proteins and shifts caused by a combination of TGA and AtWRKY50 (double black asterisks) are indicated.

The left panels show the EMSAs with the wild type probes ABC and BCD. The single shifts (1W) in Lanes 2 correspond to AtWRKY50-C binding to the sites in LS4 of probe ABC and LS10 of probe BCD, respectively. Lanes 3 show that TGA2 predominantly bound to only one binding site in ABC and BCD (1T),

while the presence of weak, high shifts in Lanes 3 suggests that binding of multiple TGA2 proteins occurred at low frequency. This is in contrast to the EMSAs with the longer 80-bp probe, which indicated that the TGAs preferentially bound as multimeric complexes (Fig. 3, Lanes 3 and 9). Yet, the ABC and BCD probes were only 18 bp shorter than the 80-bp probe, while all contained the same two CGTCA boxes. This suggests that the size of the probe contributes to the efficiency and number of TGA2 proteins it can bind. Evidently, the EMSAs with TGA2 alone resulted in a single prominent shift, suggesting that only one of the two CGTCA boxes in the fragments efficiently

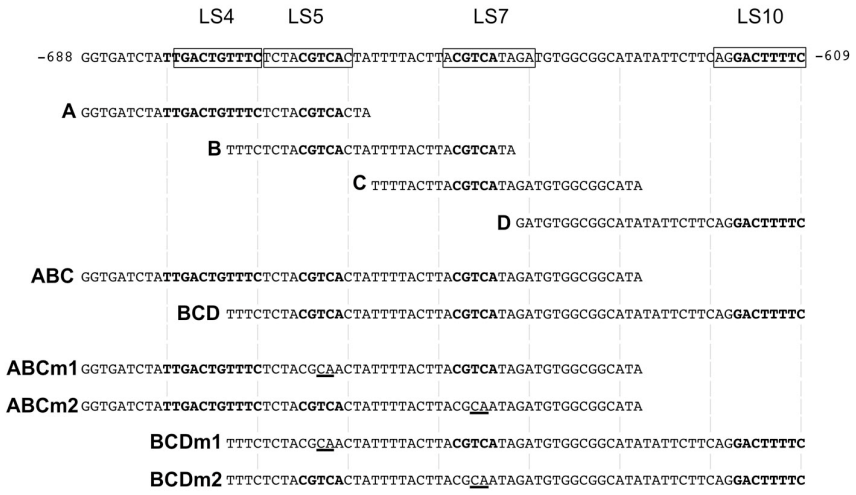

Figure 4. Sequences of *PR-1* promoter fragments used for electromobility shift assays. Only the sequence of the upper strand is given. The top line displays the sequence of the 80-bp fragment corresponding to bp -688 to -609 upstream of the transcription start site. Regions LS4, LS5, LS7 and LS10 as used in the linker scanning analysis of Lebel *et al.* (1998) are blocked. Overlapping subfragments A, B, C and D, and subfragments ABC and BCD are aligned with the sequence of the 80-bp fragment. The overlapping W-box (TTGACT) and AtWRKY50 binding sequence (GACTGTTTC) in LS4, the CGTCA boxes of the *as-1* element in LS5 and LS7, and the AtWRKY50 binding sequence (GACTTTTC) in LS10 are indicated in bold. Subfragments ABCm1, ABCm2, BCDm1 and BCDm2 represent variants of fragments ABC and BCD with mutations (underlined) in the CGTCA boxes in LS5 and LS7, respectively.

bound TGA2. To find out which of the CGTCA boxes is bound by TGA2,

fragments ABCm1, BCDm1, ABCm2 and BCDm2 (Fig 4) with mutations in LS5 (m1) and LS7 (m2), respectively, were used as probes in EMSAs (Fig. 5, middle and right panels). Evidently, mutation of the CGTCA box in LS7 interfered with TGA2 binding to the fragments (right panels), while mutation of the CGTCA motif in LS5 had no effect on binding of TGA2 (middle panels). These results were confirmed by the EMSAs of Fig. 6 that show that fragment A, which contains the CGTCA motif in LS5, did not produce a shift upon incubation with TGA2, whereas predominantly single TGA shifts were present with probe B, containing both CGTCA motifs of LS5 and LS7, and with probe C that contains only the TGA binding site in LS7. As expected, probes A and D bind AtWRKY50-C. These results indicate that the CGTCA box in LS7 is the main binding site of TGA2. Furthermore, the shifts indicated by the double asterisks in Fig.5 Lanes 4 in the panels with probes BCD and BCDm1 show that these fragments are able to bind AtWRKY50-C and TGA2 simultaneously and with higher efficiency than probe ABCm1

AtWRKY50 stimulates binding of TGA2 and TGA5 to the *PR-1* promoter

. In Chapter 2 it was shown that full-length AtWRKY50 was unable to bind to the *PR-1* promoter. As AtWRKY50-C binds highly efficiently and specifically, we speculated that a conformational change is required to release the N-terminal half of AtWRKY50 of blocking the DNA-binding domain. To see if such a change could be brought about by the interaction of TGA2 and TGA5, EMSAs were performed with mixtures of full-length AtWRKY50 and TGA2 or TGA5. The results are shown in Fig. 3. Surprisingly, EMSAs with the 80-bp probe and a combination of full-length AtWRKY50 and TGA2 or TGA5 (Fig. 3, Lanes 6 and 12, respectively) resulted in an extra band shift (indicated by the double asterisks) that co-migrated with the band shifts in Lanes 4 and 10, respectively. It is unlikely that the band shifts in Lanes 6 and 12 are the result of

the binding of a combination of the TGA and the WRKY protein, as the larger size of full-length AtWRKY50 would likely result in a lower mobility of such a protein-DNA complex than the ones of TGA and AtWRKY50-C present in Lanes 4 and 10. The fusion product of full-length AtWRKY50 and GST (44 kD) used in these EMSAs has approximately the same size as the TGA-His fusion protein (41 kD), implicating that a band shift produced by the binding of a

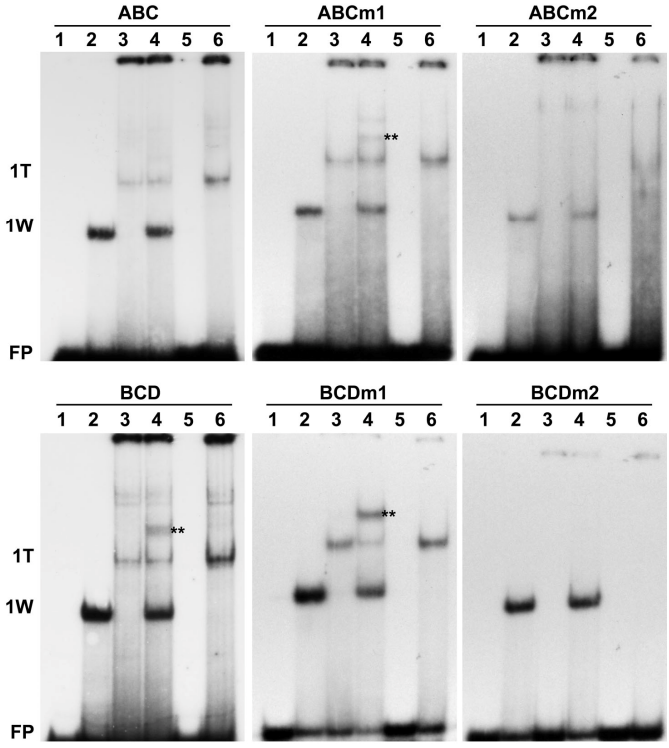

Figure 5. AtWRKY50 and TGA2 bind to the *PR-1* promoter. EMSAs were performed with probes corresponding to *PR1* promoter fragments ABC and BCD and their mutated versions ABCm1, ABCm2, BCDm1, BCDm2, as indicated above the panels. EMSA incubation mixtures contained no protein (Lanes 1), AtWRKY50-C (Lanes 2), TGA2 (Lanes 3), AtWRKY50-C and TGA2 (Lanes 4), full-length AtWRKY50 (Lanes 5), and full-length AtWRKY50 and TGA2 (Lanes 6). The positions of the unbound probe (FP) and of band shifts caused by AtWRKY50-C (1W) or TGA2 (1T) are indicated at the left. Shifts caused by binding of a combination of TGA and AtWRKY50 (double black asterisks) are indicated.

single AtWRKY50 protein to the probe would migrate to approximately the same position as one caused by a single TGA2 or TGA5 protein. This suggests that the extra band shifts in Lanes 6 and 12 could be the result of binding of full-length AtWRKY50 and that this was caused by the presence of TGA2 or TGA5 in the EMSA incubation mixtures. Speculative as the above may be, the EMSA results also show that the presence of full-length AtWRKY50 influenced the binding of TGA. Although binding of full-length AtWRKY50 could not be directly demonstrated, its addition to TGA2 or TGA5 resulted in an enhancement of the bands corresponding to the single TGA shifts (Fig. 3, compare the bands indicated by the single black asterisks in Lanes 6 and 12 with those in Lanes 3 and 9 and in Fig. 5, compare Lanes 6 and 3 in the leftmost panels). This effect was not observed with combinations of AtWRKY50-C and the TGAs (Fig. 3, Lanes 4 and 10; Fig. 5, left panels, Lanes 4). Apparently, full-length AtWRKY50 promotes binding of the TGA protein to the 80-bp probe and to the ABC and BCD probes. This effect of AtWRKY50 on TGA binding does not require AtWRKY50's binding site on the DNA. When combinations of full-length AtWRKY50 and TGA2 were incubated with promoter fragments lacking AtWRKY50's binding site, the stimulating effect on TGA binding was still present. This can be seen in Fig. 6, where fragments B and C, containing two (LS5 and LS7) and one (LS7) CGTCA motifs, respectively, but lacking the AtWRKY50 binding site in LS4 or LS10, show an increased intensity of the TGA2 shifts in Lanes 6 of panels B and C.

AtWRKY50 induced *PR-1* expression does not depend on NPR1, TGA2, TGA5 or TGA6

SA-induced expression of *PR-1* genes in plants is dependent on NPR1 and it is generally assumed that NPR1 activates expression through its interaction with TGAs binding to the promoter.

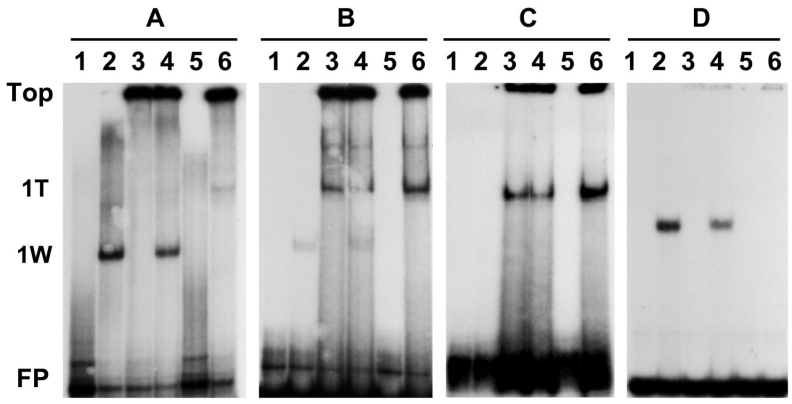

Figure 6. AtWRKY50 and TGA2 bind to the *PR-1* promoter. EMSAs were performed with *PR-1* promoter fragments A, B, C, and D as probes, as indicated above the panels. EMSA incubation mixtures contained no protein (Lanes 1), AtWRKY50-C (Lanes 2), TGA2 (Lanes 3), AtWRKY50-C and TGA2 (Lanes 4), full-length AtWRKY50 (Lanes 5), and full-length AtWRKY50 and TGA2 (Lanes 6). The positions of the unbound probe (FP), the top of the gel (Top) and of band shifts caused by AtWRKY50-C (1W) or TGA2 (1T) are indicated at the left.

To investigate if the activation of the *PR-1* promoter by AtWRKY50 requires NPR1 or TGAs, transactivation assays were performed with protoplasts derived from *npr1-1* mutant and *tga256* triple mutant Arabidopsis plants. The results are shown in Fig. 7. Evidently, AtWRKY50 was able to activate the *PR-1::GUS* reporter gene in these mutant backgrounds to similar relative levels as in wild type plants. Apparently, activation by AtWRKY50 does not require the TGAs or their co-activator NPR1. It also suggests that none of these proteins is the factor(s) presumed to enable full-length AtWRKY50 to bind to the promoter.

Activation of *PR-1* expression by AtWRKY50 is enhanced by TGA2 and TGA5

Although AtWRKY50 alone is able to activate the *PR-1* promoter, TGA factors may function in further modulation of gene expression and this may possibly occur via their interaction with AtWRKY50. To determine if there is an effect of

the AtWRKY50-TGA interaction on *PR-1* expression, we performed a series of co-expression experiments. Arabidopsis protoplasts were co-transfected with the *PR-1::GUS* reporter construct and plasmids containing *35S*-driven *AtWRKY50*, *TGA2*, *TGA3* and *TGA5* genes. After overnight incubation GUS expression was determined. The results are shown in Fig. 8. While AtWRKY50 enhanced GUS expression approximately 7-fold (compare the first two bars in Fig. 8), the TGA proteins enhanced expression only 2-fold at the most. However, combinations of AtWRKY50 and TGA2 or TGA5 resulted in an up to 14-fold boosted expression of the reporter gene, while co-expression of TGA3 did not further enhance AtWRKY50-dependent GUS expression. The results indicate that TGA2 and TGA5 act synergistically with AtWRKY50 to maximize activation of the *PR-1* promoter.

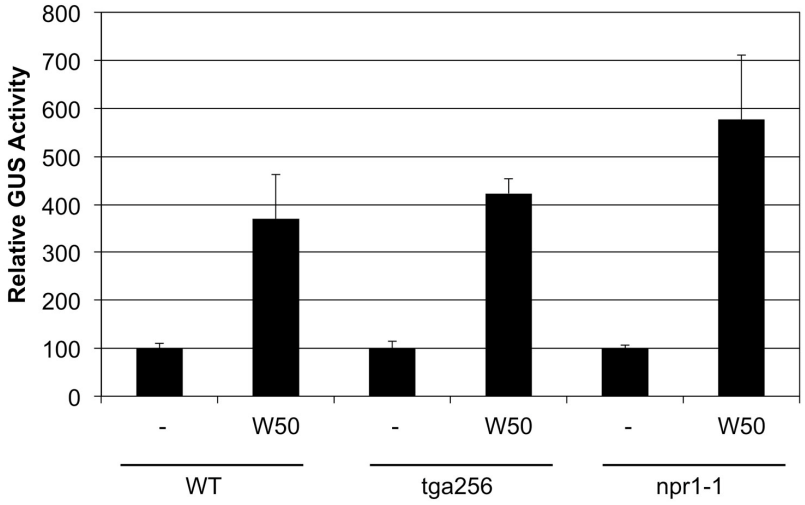

Figure 7. AtWRKY50 induced expression is independent of NPR1 and TGAs. Protoplasts from wild type (WT), *tga2-1 tga5-1 tga6-1* triple mutant (tga256) and *npr1-1* mutant (npr1) Arabidopsis plants, were co-transfected with *PR1::GUS* reporter construct alone (minus sign) or together with expression plasmids containing *35S::AtWRKY50* (W50), as indicated. 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 *PR-1::GUS* construct and empty vector control. Error bars represent the SEM.

DISCUSSION

In the previous chapter we showed that AtWRKY50 is an activator of *PR-1* gene expression. The C-terminal half of the protein, characterized by the conserved amino acid sequence WRKYGKK and a proximal zinc-finger region, bound at two positions in the 80-bp region of the *PR-1* promoter essential for SA-inducible expression. This region also contains an *as-1* element, consisting of two direct CGTCA motifs, that acts as a binding site for TGA transcription factors.

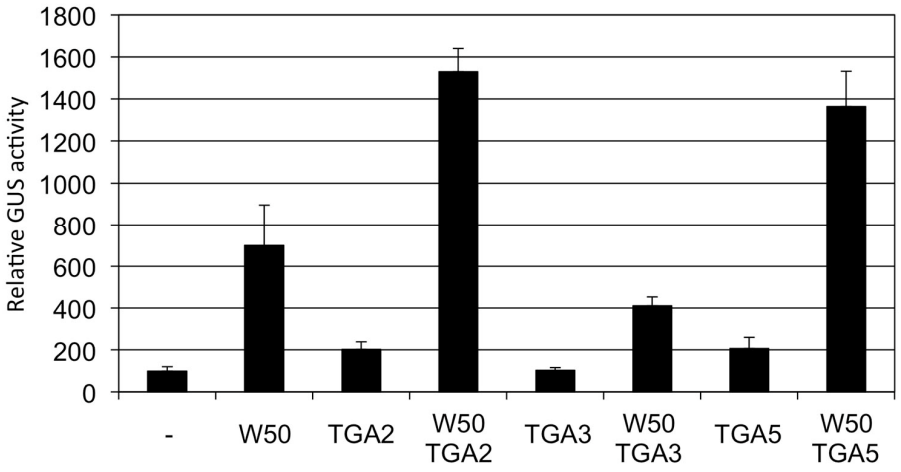

Figure 8. Synergistic effect of AtWRKY50 and TGA2 or TGA5 on *PR-1* expression. Arabidopsis protoplasts were co-transfected with *PR-1::GUS* reporter construct alone (minus sign) or together with expression plasmids containing *35S* promoter-controlled genes encoding AtWRKY50, TGA2, TGA3, TGA5, or combinations, as indicated. After incubation GUS activity was measured spectrophotometrically. Expression levels (%) are given relative to the expression the level without expression plasmid. 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.

Here we have shown that AtWRKY50 interacts with TGAs 2 and 5 in the nucleus. Using in vitro DNA binding assays we showed that especially the rightmost CGTCA motif of the *as-1* element was able to bind TGA2 with high

efficiency and that simultaneous binding of AtWRKY50-C and TGA2 to the promoter occurred. Furthermore, although protoplast transactivation assays demonstrated that AtWRKY50 alone was able to activate the *PR-1* promoter, and that this did not require transcription factors TGA2, TGA5 or TGA6 or their co-activator NPR1, expression of the GUS reporter gene was greatly enhanced when also TGA2 or TGA5 were present. We have not investigated if this synergistic effect of TGA2 and TGA5 on AtWRKY50-induced expression required NPR1.

Our finding that TGA2 did not efficiently bind to the CGTCA motif in LS5 is at variance with the results of Després *et al.* (2000) who found that TGA2 bound to both LS5 and LS7. However, these authors used DNA probes containing either the LS5 or the LS7 element, which precludes a comparison of the relative strengths with which the two elements are bound. LS5 has been identified as a DNA element conferring a negative effect on *PR-1* gene expression.

Table 1. Primers used for cloning and EMSAs

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. ABC	F	ATCGGATCCGGTGATCTATTGACTGTTTCTCTAC

	R	TATATGCCGCCACATCTATGACGTAAGTAAAA
Frag. BCD	F	TTTCTCTACGTCACTATTTTACTTACGTCATA
	R	GCCTAGATCTGAAAAGTCCTGAAGAATATATGCC
Frag. ABC LS5m	F	ATCGGATCCGGTGATCTATTGACTGTTTCTCTAC
	R	CATCTATGACGTAAGTAAAATAGTTGCGTAGAG
Frag. ABC LS7m	F	ATCGGATCCGGTGATCTATTGACTGTTTCTCTAC
	R	CATCTATTGCGTAAGTAAAATAGTGACGTAGAG
Frag. BCD LS5m	F	CTCTACGCAACTATTTTACTTACGTCATAGATG
	R	GCCTAGATCTGAAAAGTCCTGAAGAATATATGCC
Frag. BCD LS7m	F	CTCTACGTCACTATTTTACTTACGCAATAGATG
	R	GCCTAGATCTGAAAAGTCCTGAAGAATATATGCC
Frag. ABC LS5+7m	F	ATCGGATCCGGTGATCTATTGACTGTTTCTCTAC
	R	CATCTATTGCGTAAGTAAAATAGTTGCGTAGAG
Frag. BCD LS5+7m	F	CTCTACGCAACTATTTTACTTACGCAATAGATG
	R	GCCTAGATCTGAAAAGTCCTGAAGAATATATGCC

The proximity of the TGA and AtWRKY50 binding sites in the *PR-1* promoter, together with the ability of the proteins to interact, suggest that such an interaction could also take place when the transcription factors are bound to the promoter and that this could be relevant for *PR-1* expression. Indeed, although AtWRKY50 expressed in protoplasts activates a co-transfected *PR-1::GUS* gene, co-expression of TGA2 or TGA5 further enhanced GUS expression considerably. This synergistic effect was specific for TGA2 and TGA5, which both interacted with AtWRKY50, while TGA3, which did not interact with AtWRKY50, was not able to enhance gene expression.

TGA2 on its own is not a transcriptional activator but requires binding of NPR1. In the absence of NPR1, TGA2 has been suggested to act as a repressor of *PR-1* gene expression (Zhang *et al.*, 2003; Rochon *et al.*, 2006). TGA2, 5 and 6 belong to the same subclass of TGAs (clade II). There is accumulating evidence that in addition to NPR1, TGAs are able to interact with other proteins.

Recently, glutaredoxin was shown to interact with Arabidopsis TGA2 and tobacco TGA2.2 (Ndamukong *et al.,* 2007), while Arabidopsis TGA2 and TGA5 were found to interact with SCL14, a protein mediating regulation of genes involved detoxification processes (Fode *et al.*, 2008).

Based on their findings, the authors suggested that clade II TGAs could act as sequence-specific anchor proteins to recruit other transcription regulatory proteins, like SCL14 and DELLA proteins, to the promoters of their target genes. In this perspective, we speculate that TGA2 could likewise assist in recruiting AtWRKY50 to the *PR-1* promoter. Also WRKYs have been found to interact with other proteins. E.g., Arabidopsis WRKY7 has been found to interact with calmodulin (CaM) through a CaM binding domain in the N-terminal half of the protein that is conserved in other members of the WRKY IId group (Park *et al.*, 2005). Other examples are WRKY70 interacting with the EAR domain repressor ZAT7 (Ciftci-Yilmaz *et al.*, 2007), WRKY53 interacting with mitogen activated protein kinase kinase kinase1 (MEKK1; Miao *et al.*, 2007), WRKY33 interacting with mitogen activated protein kinase 4 (MAPK4; Andreasson *et al.*, 2005), and WRKYs 38 and 62 that have been found to interact with histone deacetylase19 (HDA19; Kim *et al.*, 2008). In our EMSAs, the new band shifts produced upon incubation of combinations of AtWRKY50-C and TGA2 or TGA5 with the 80-bp promoter fragment (Fig. 3, double asterisks) or with probes ABC and BCD (Fig. 5, double asterisks) likely represent supershifts produced by the simultaneous binding of both proteins to the probe, possibly as a complex of interacting transcription factors.

MATERIALS AND METHODS

Expression of TGA Fusion Proteins

The full-length coding sequence of Arabidopsis *TGA2* and *TGA5* were cloned in frame in front of the His-tag open reading frame of expression vector pASK-IBA45Plus (IBA Biotechnology, Göttingen, Germany). The PCR was amplified by sets of primer corresponding to the sequence of TGA2: 5′-TAG CGA ATT CGA TGG CTG ATA CCA GTC CGA G-3′ and 5′- TGA CCT CGA GGG CTC TCT GGG TCG AGC AAG C-3′ and TGA5: 5′-TAG CGA ATT CGA TGG GAG ATA CTA GTCCAA G-3′ and 5′-TGA CCT CGA GGG CTC TCT TGG TCT GGC AAG C-3′, digested with EcoRI and XhoI and cloned in pASK-IBA. These plasmids were transformed into E. coli BL21-(DE3) pLysS (Novagen). For induction of protein expression, cultures were grown to mid-log phase at 37°C, after which 2mg/ml anhydrotetracyclin was added to a final concentration of 0.4 mM and incubation continued for 3 h at 29°C. The cells were harvested by centrifugation, resuspended in 20ml binding buffer (5mM imidazole, 0.5M NaCl, 20mM Tris-HCl pH 8). The samples were sonicated until viscosity was low. The fusion proteins were purified using Ni-NTA agrose beads (Qiagen), which were eluted at 4°C with elution buffer (1M imidazole, 0.5M NaCl, 20mM Tris-HCl, pH8), 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.

Bimolecular fluorescence complementation Assays:

Primer sets used for BiFC cloning were: 5′-GAT CGT CGA CAA TGA ATG ATG CAG ACA CAA ACT TG-3′ and 5′-CAG TAG ATC TGT TAG TTC ATG CTT GAG TGA TTG TG-3′, for *WRKY50* cloning with SalI and BglII in pRTL2-YNEE and –YCHA; 5′-GAT CGT CGA CAA TGA ATG ATG CAG ACA CAA ACT TG-3′ and 5′-CGT AAG CGG CCG CGT GTT CAT GCT TGA GTG ATT GT-3′ for *WRKY50* cloning with SalI and NotI in pRTL2-EEYN and –HAYC; 5′ GAT CGT CGA CAA TGG CTG ATA CCA GTC CGA GAA CT 3′ and 5′ CAG TAG ATC TGT CAC TCT CTG GGT CGA GCA AGC CA 3′ for TGA2 cloning with Sal1 and BglII in pRTL2-YNEE and –YCHA; 5′ GAT CGT CGA CAA TGG CTG ATA CCA GTC CGA GAA CT 3′ and 5′ CGT AAG CGG CCG CGT CTC TCT GGG TCG AGC AAG CC 3′ for TGA2 cloning with SalI and NotI in pRTL2-EEYN and –HAYC; 5′ GAT CGT CGA CAA TGG GAG ATA CTA GTC CAA GAA CA 3′ and 5′ GAT CGT CGA CAA TGG GAG ATA CTA GTC CAA GAA CA 3′ for TGA5 cloning with Sal1 and BglII in pRTL2-YNEE and –YCHA; 5′ GAT CGT CGA CAA TGG GAG ATA CTA GTC CAA GAA CA 3′ and 5′

CGT AAG CGG CCG CGT CTC TCT TGG TCT GGC AAG CC 3′ for TGA5 cloning with SalI and NotI in pRTL2-EEYN and –HAYC. PCR-amplified inserts were digested with the restriction enzymes mention above and cloned in the respective pRTL2 derivates (Bracha-Drori *et al.*, 2004) digested with the corresponding enzymes. Protoplasts were isolated and transformed with PEG as described above. Images of transfected protoplasts were acquired with a Leica DM IRBE confocal laser-scanning microscope equipped with an Argon laser line of 488nm (excitation) and a band pass emission filter of 500-550nm.

Yeast two hybrid assays

Full-length *AtWRKY50* (At5g26170) and *AtNPR1* (At1g64280) cloned in pAS2.1 with primer sets WRKY50: 5′-ATA GGA ATT CGT ATG AAT GAT GCA GAC ACA AAC TTG-3′ and 5′-GCC GGA TCC CGA GTC TTA GTT CAT GCT TGA GTG ATT GTG-3′ digested with EcoRI and BamHI, AtNPR1: 5′-TAG CGA ATT CTA ATG GAC ACC ACC ATT GAT GG-3′ and 5′-TGA CGG ATC CTC ACC GAC GAC GAT GAG AG-3′, digested with EcoRI and BamHI were co-transformed with empty pACT2 to yeast strain PJ69-4A (James *et al.*, 1996). The *TGA2* (At5g06950) ORF was PCR-amplified from Arabidopsis cDNA library using the primer sets 5′- TAG CGA ATT CGA TGG CTG ATA CCA GTC CGA G-3′ and 5′-TGA CGG ATC CGG TCA CTC TCT GGG TCG AGC AAG C 3′, digested with EcoRI and BamHI and cloned into pACT2. The *TGA5* (At5g06960) ORF was PCR-amplified from Arabidopsis CDNA library using the primer sets 5′- TAG CGA ATT CGA TGG GAG ATA CTA GTCCAA G-3′ and 5′-TGA CGG ATC CGG TCA CTC TCT TGG TCT GGC AAG C 3′, digested with EcoRI and BamHI and cloned into pACT2. For auto activation assays, transformants were plated on minimal synthetic defined (SD)-glucose medium supplemented with Met/Ura/His and lacking Leu and Trp (-LT). Ability to activate transcription in yeast was evaluated by monitoring growth after 7 days on selective SD medium

lacking Leu, Trp and His (-LTH). Interaction assays were performed by co-transformation of bait and prey plasmids into yeast strain PJ69-4A and plated on SD-LT medium. As control, empty pAS2.1 and pACT2 were used. Transformants were allowed to grow for 4-5 days. Subsequently, cells were incubated for 16 hours in liquid SD-LT and 10µL of 10-fold dilutions were spotted on SD-LTH medium. Yeast cells were allowed to grow for 7 days at 30^0C.

Plasmid construction and protoplast assays

The *AtWRKY50* (At5g26170) open reading frame was PCR–amplified from an Arabidopsis cDNA library (6h SA-treated) using the primer set 5′- ATA GCT CGA GGT ATG AAT GAT GCA GAC ACA AAC TTG 3′ and 5′- GCC TCT AGA CGA GTC TTA GTT CAT GCT TGA GTG ATT GTG 3′, digested with XhoI and XbaI and cloned into pRT101. The *TGA2*(At5g06950) ORF was PCR-amplified from Arabidopsis cDNA library using the primer sets 5′- TAG CGA ATT CGA TGG CTG ATA CCA GTC CGA G-3′ and 5′-TGA CGG ATC CGG TCA CTC TCT GGG TCG AGC AAG C 3′, digested with EcoRI and BamHI and cloned into pRT101. The *TGA3* (At1g22070) open reading frame was PCR-amplified from Arabidopsis cDNA library using the primer sets 5′- `TAG CGA ATT CGA TGG AGA TGA TGA GCT CTT C 3′and 5′- TGA CGG ATC CGG TCA AGT GTG TTC TCG TGG ACG TG 3′, digested with EcoRI and BamHI and cloned into pRT101. The *TGA5* (At5g06960) ORF was PCR-amplified from Arabidopsis cDNA library using the primer sets 5′- TAG CGA ATT CGA TGG GAG ATA CTA GTCCAA G-3′and 5′-TGA CGG ATC CGG TCA CTC TCT TGG TCT GGC AAG C 3′, digested with EcoRI and BamHI and cloned into pRT101. Protoplasts were prepared from Arabidopsis ecotype Columbia-0 cell suspension according to Axelos *et al.* (1992) with minor 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, and 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 the *PR-1 promoter::GUS* constructs and 6 µg of effector plasmid pRT101 (Töpfer *et al.*, 1987) carrying *35S::AtWRKY50* or *35S::TGA* alone and in combinations. As a control, co-transformation of *PR-1 promoter::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 16h 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.

ACKNOWLEDGEMENTS

R. M. F. H. was supported by a grant from the Higher Education Commission of Pakistan.

REFERENCES

Andreasson E, Jenkins T, Brodersen P, Thorgrimsen S, Petersen NHT, Zhu S, Qiu JL, Michelsen P, Rocher A, Petersen M, Newman MA, Nielsen HB (2005) The MAP kinase substrate MKS1 is a regulator of plant defense responses. EMBO J **24**:2579–2589

Axelos M, Curie C, Mazzolini L, Bardet C, Lescure B (1992) A protocol for transient gene expression in Arabidopsis thaliana protoplasts isolated from cell suspension cultures. Plant Physiol Biochem **30**:123–128

Bracha-Drori K, Shichrur K, Katz A, Oliva M, Angelovici R, Yalovsky S, Ohad N (2004) Detection of protein-protein interactions in plants using bimolecular fluorescence complementation. Plant J **40**:419-427

Ciftci-Yilmaz S, Morsy MR, Song L, Coutu A, Krizek BA, Lewis MW, Warren D, Cushman J, Connolly EL, Mittler R (2007). The EAR-motif of the Cys2/His2-type zinc finger protein Zat7 plays a key role in the defense response of Arabidopsis to salinity stress. J Biol Chem **282**:9260-9268

Després C, De Long C, Glaze S, Liu E, Fobert PR (2000) The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. Plant Cell **12**:279–290

Durrant WD, Dong X (2004). Systemic acquired resistance. Annu Rev Phytopathol **42**:185–209

Fan W, Dong X (2002) In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. Plant Cell **14**:1377–1389

Fode B, Siemsen T, Thurow C, Weigel R, Gatz C (2008). The Arabidopsis GRAS Protein SCL14 Interacts with Class II TGA Transcription Factors and Is Essential for the Activation of Stress-Inducible Promoters. Plant Cell **20**:3122-3135

Fu ZQ, Yan S, Saleh A, Wang W, Ruble J, Oka N, Mohan R, Spoel SH, Tada Y, Zheng N, Dong X (2012) NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature **486**:228–232

Green PJ, Kay SA, Lam E, Chua NH (1989) In vitro DNA footprinting. *In* SB Gelvin, RA Schilperoort, eds, Plant Molecular Biology Manual B11. Kluwer Academic Publishers, Dordrecht, the Netherlands, pp 1–22.

Grüner R, Strompen G, Pfitzner AJP, Pfitzner UM (2003) Salicylic acid and the hypersensitive response initiate distinct signal transduction pathways in tobacco that converge on the *as-1*-like element of the *PR-1a* promoter. Eur J Biochem **270**:4876–4886

James P, Halladay J, Craig EA (1996) Genomic libraries and a host strain designed for highly efficient two hybrid selection in yeast. Genetics **144**:1425-1436

Johnson C, Boden E, Arias J (2003) Salicylic acid and NPR1 induce the recruitment of *trans*-activating TGA factors to a defense gene promoter in Arabidopsis. Plant Cell **15**:1846–1858

Katagiri F, Lam E, Chua N-H (1989) Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. Nature **340**:727–730

Kim K-C, Lai Z, Fan B, Chen Z (2008) Arabidopsis WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. Plant Cell **20**:2357–2371

Lebel E, Heifetz P, Thorne L, Uknes S, Ryals J, Ward E (1998) Functional analysis of regulatory sequences controlling PR-1 gene expression in Arabidopsis. Plant J **16**:223–233

Miao ZH, Liu X, Lam E (1994) TGA3 is a distinct member of the TGA family of bZIP transcription factors in Arabidopsis thaliana. Plant Mol Biol **25**:1–11

Miao Y, Laun TM, Smykowski A, Zentgraf U (2007) Arabidopsis MEKK1 can take a short cut: it can directly interact with senescence-related WRKY53 transcription factor on the protein level and can bind to its promoter. Plant Mol Biol **65**:63-76

Ndamukong I, Al Abdallat A, Thurow C, Fode B, Zander M, Weigel R, Gatz C (2007) SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription. Plant J **50**:128–139

Niggeweg R, Thurow C, Kegler C, Gatz C (2000a) Tobacco transcription factor TGA2.2 is the main component of as-1-binding factor ASF-1 and is involved in salicylic acid- and auxin-inducible expression of as-1-containing target promoters. J Biol Chem **275**:19897–19905.

Niggeweg R, Thurow C, Weigel R, Pfitzner U, Gatz C (2000b) Tobacco TGA factors differ with respect to interaction with NPR1, activation potential and DNA-binding properties. Plant Mol Biol **42**:775–788

Pape S, Thurow C, Gatz C (2010) The Arabidopsis PR-1 promoter contains multiple integration sites for the coactivator NPR1 and the repressor SNI1. Plant Physiol **154**:1805-1818

Park CY, Lee JH, Yoo JH, Moon BC, Choi MS, Kang YH, Lee SM, Kim HS, Kang KY, Chung WS, Lim CO, Cho MJ (2005) WRKY group IId transcription factors interact with calmodulin. FEBS Lett **579**:1545-50

Pontier D, Miao ZH, Lam E (2001) Trans-dominant suppression of plant TGA factors reveals their negative and positive roles in plant defense responses. Plant J **27**:529–538

Rochon A, Boyle P, Wignes T, Fobert PR, Després C (2006) The coactivator function of Arabidopsis NPR1 requires the core of its BTB/ POZ domain and the oxidation of C-terminal cysteines. Plant Cell **18**:3670–3685

Schirawski J, Planchais S, Haenni AL (2000) An improved protocol for the preparation of protoplasts from an established Arabidopsis thaliana cell suspension culture and infection with RNA of a turnip yellow mosaic tymovirus: a simple and reliable method. J Virol **86**:85–94

Spoel SH, Mou Z, Tada Y, Spivey NW, Genschik P, Dong X (2009) Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. Cell **137**:860–872

Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J. and Steinbiss, H. H. (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res **15**:5890.

Van der Fits L, Memelink J (1997) Comparison of the activities of CaMV 35S and FMV 34S promoter derivatives in Catharanthus roseus cells transiently and stably transformed by particle bombardment. Plant Mol Biol **33**:943–946

Van Verk MC, Pappaioannou D, Neeleman L, Bol JF, Linthorst HJM (2008) A novel WRKY transcription factor is required for induction of PR-1A gene expression by salicylic acid and bacterial elicitors. Plant Physiol **146**:1983–1995

Van Verk MC, Neeleman L, Bol JF, Linthorst HJM (2011a) Tobacco Transcription Factor NtWRKY12 Interacts with TGA2.2 in vitro and in vivo. Front Plant Sci **2**:32

Zhang Y, Fan W, Kinkema M, Li X, Dong X (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. Proc Natl Acad Sci USA **96**:6523–6528

Zhang Y, Tessaro MJ, Lassner M, Li X (2003) Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. Plant Cell **15**:2647–2653

Zhou J-M, Trifa Y, Silva H, Pontier D, Lam E, Shah J, Klessig DF (2000). NPR1differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the *PR-1* gene required for induction by salicylic acid. Mol Plant-Microbe Interact **13**:191–202

CHAPTER 4

INVOLVEMENT OF AtWRKY28 IN EXPRESSION OF *PR-1*

R. Muhammad Fraz Hussain, Huub J.M. Linthorst

Institute of Biology, Leiden University, P.O. Box 9505, 2300 RA Leiden, the Netherlands

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).

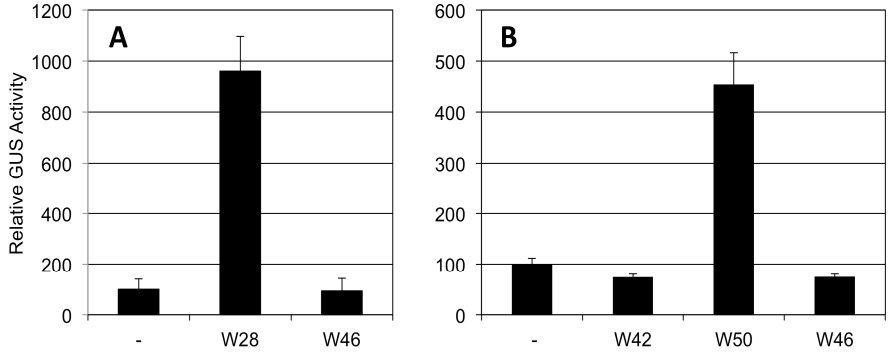

Figure 1. Protoplast transactivation assays. (A, B) Arabidopsis protoplasts were co-transfected with *PR-1::GUS* construct alone (minus sign) or together with expression plasmids *35S::AtWRKY28* (W28), *35S::AtWRKY46* (W46), *35S::AtWRKY42* (W42) or *35S::AtWRKY50* (W50). After incubation GUS activity 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.

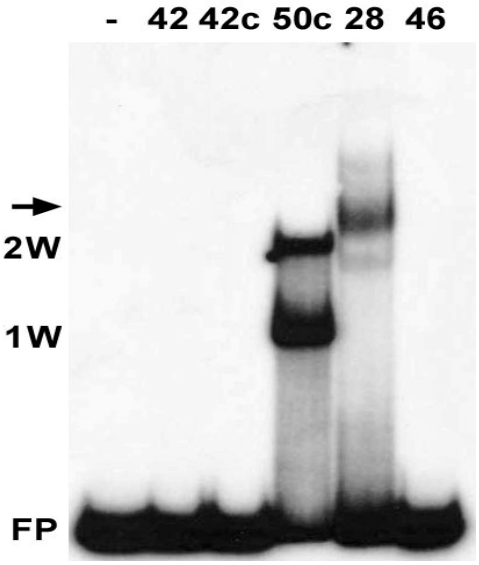

Figure 2. EMSAs of WRKYs with the 80-bp *PR-1* promoter fragment. EMSAs were performed with a frag-ment of the *PR-1* promoter ranging from -688 to -609 bp up-stream of the transcription start site incubated without (minus sign) or with GST-fusion proteins of full-length AtWRKY42 (42), C-terminal half 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.

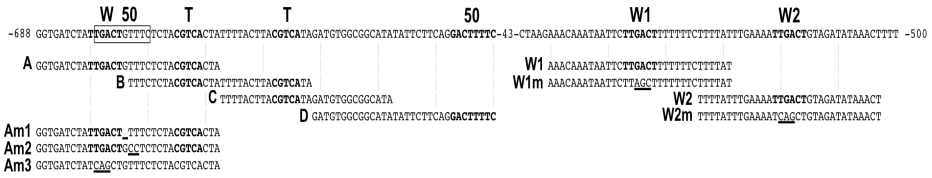

Figure 3. Sequences of *PR-1* promoter fragments used for EMSAs. Only the sequence of the upper strand is given. The top line displays the sequence of the 80-bp fragment corresponding to bp -688 to -609 and -565 to -500, upstream of the transcription start site. The gap of 43 bp between the two sequence 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.

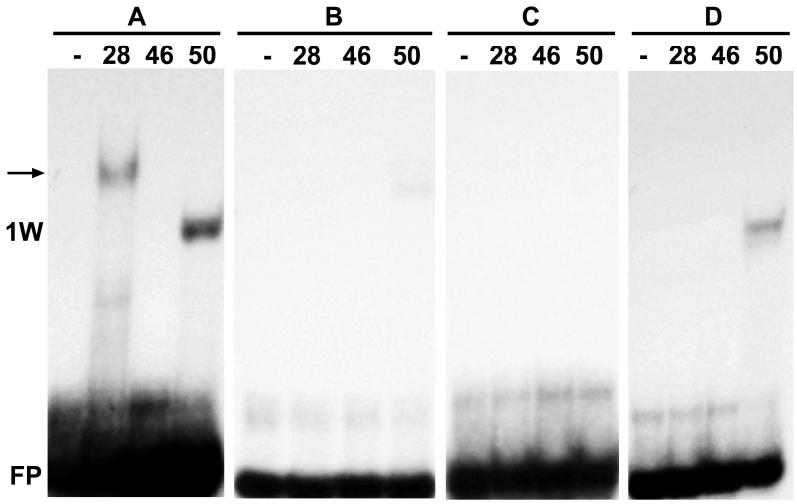

Figure 4. Binding of AtWRKY28 to subfragments of the 80-bp *PR-1* promoter fragment. EMSAs were performed with a series of overlapping fragments (A to D) of the 80-bp *PR-1* promoter incubated without (minus sign) or with N-terminal GST-fusion proteins of full-length AtWRKY28 and AtWRKY46 and the C-terminal 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).

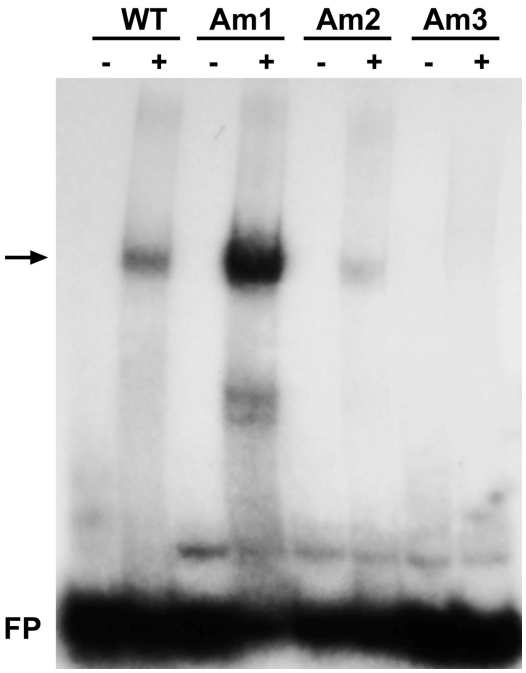

Figure 5. Mutational analysis of AtWRKY28's binding site in subfragment A. EMSAs were performed with wild type (WT) and mutant (Am1, Am2, Am3) versions of fragment A, without (minus sign) or with (plus signs) the N-terminal GST-fusion protein of AtWRKY28. The positions of the unbound probe (FP: free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

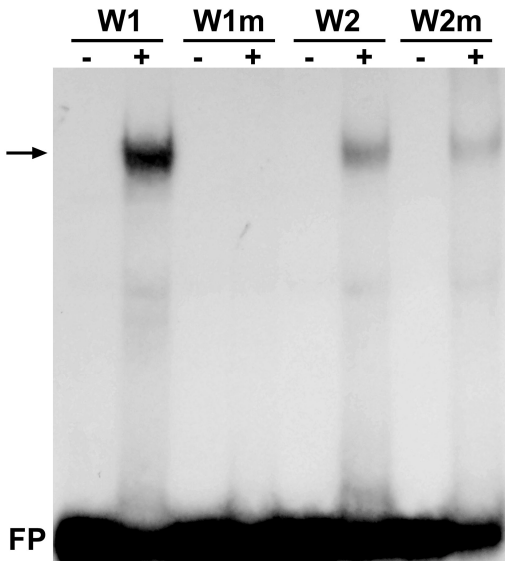

Figure 6. Binding of AtWRKY28 to W-boxes W1 and W2 in the *PR-1* promoter. EMSAs were performed with 35-bp promoter fragments containing wild type (W1, W2) and mutant (W1m, W2m) versions of the W-boxes at positions -546 and -540 bp upstream of the transcription start site, in the absence (minus signs) 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.

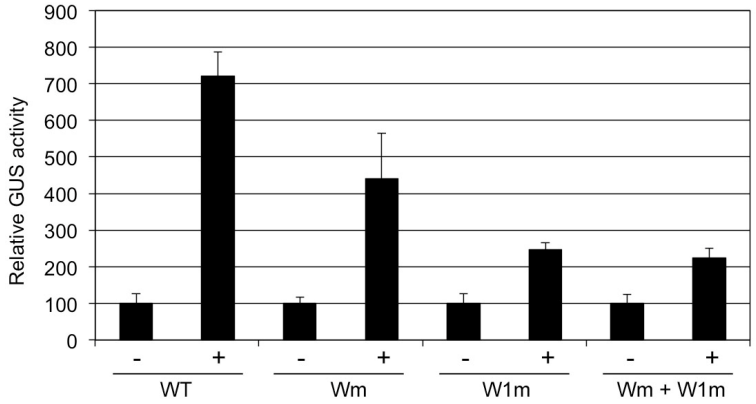

Figure 7. Protoplast transactivation assays. Arabidopsis protoplasts were transfected with *PR-1::GUS* constructs in which the promoter had no mutations (WT) or had mutations in the W-box in LS4 (Wm) or the W-box at position -546 (W1m), or a combination of the two mutations (Wm + W1m). Minus signs indicate 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.

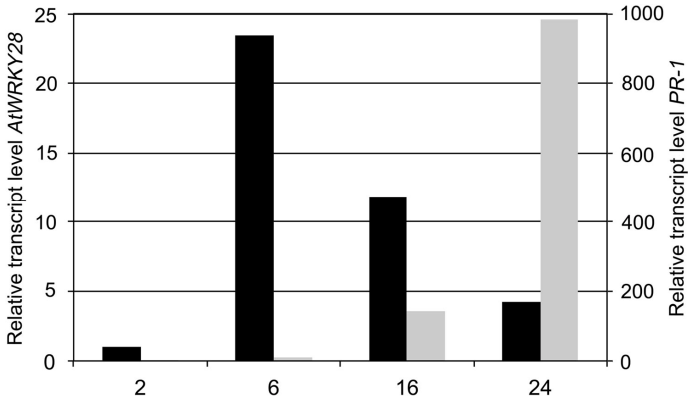

Figure 8. Time course of salicylic acid induced *AtWRKY28* and *PR1* expression. Accumulation of *AtWRKY28* (black bars) and *PR-1* (grey bars) mRNA at the indicated times (hours) after incubation of plants in 1mM salicylic acid is relative to the level of the transcripts measured at 2 h post treatment.

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.

ACKNOWLEDGEMENTS

We thank Zeshan Hassan and Imran Haider for assistance with the qRT-PCR experiments. R. M. F. H. was supported by a grant from the HEC of Pakistan.

REFERENCES

Dong J, Chen C, Chen Z (2003) Expression profile of the Arabidopsis WRKY gene superfamily during plant defense response. Plant Mol Biol **51**:21–37

Dong X (2004) NPR1, all things considered. Curr Opin Plant Biol **7**:547–552

Fu ZQ, Yan S, Saleh A, Wang W, Ruble J, Oka N, Mohan R, Spoel SH, Tada Y, Zheng N, Dong X (2012) NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature **486**:228-232

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annual Rev Phytopathol **43**:205-227

Grant M, Lamb C (2006) Systemic immunity. Curr Opin Plant Biol **9**: 414–420

Green PJ, Kay SA, Lam E, Chua NH (1989) In vitro DNA footprinting. *In* SB Gelvin, RA Schilperoort, eds, Plant Molecular Biology Manual B11. Kluwer Academic Publishers, Dordrecht, the Netherlands, pp **1**–22.

Guan KL, Dixon JE (1991) Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal Biochem **192**: 262–267

Lebel E, Heifetz P, Thorne L, Uknes S, Ryals J, Ward E (1998) Functional analysis of regulatory sequences controlling PR-1 gene expression in Arabidopsis. Plant J **16**:223–233

Navarro L, Zipfel C, Rowland O, Keller I, Robatzek S, Boller T, Jones JDG (2004) The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. Plant Physiol **135**:1113–1128

Pape S, Thurow C, Gatz C (2010) The Arabidopsis PR-1 promoter contains multiple integration sites for the coactivator NPR1 and the repressor SNI1. Plant Physiol **154**:1805-1818

Schirawski J, Planchais S, Haenni AL (2000) An improved protocol for the preparation of protoplasts from an established Arabidopsis thaliana cell suspension culture and infection with RNA of a turnip yellow mosaic tymovirus: a simple and reliable method. J Virol **86**:85–94

Töpfer R, Matzeit V, Gronenborn B, Schell J, Steinbiss HH (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res **15:**5890

Van der Fits L, Memelink J (1997) Comparison of the activities of CaMV 35S and FMV 34S promoter derivatives in Catharanthus roseus cells transiently and stably transformed by particle bombardment. Plant Mol Biol **33**:943–946

Van Verk MC, Bol JF, Linthorst HJM (2011) WRKY transcription factors involved in activation of SA biosynthesis genes. BMC Plant Biol **11**:89

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414**:562–565

Wu Y, Zhang D, Chu JY, Boyle P, Wang Y, Brindle ID, Luca VD, Després C (2012) The Arabidopsis NPR1 Protein Is a Receptor for the Plant Defense Hormone Salicylic Acid. Cell Rep **1**:639-647

CHAPTER 5

EFFECTS OF KNOCKOUT AND OVEREXPRESSION OF ATWRKY50 AND ATWRKY28 IN TRANSGENIC PLANTS

R. Muhammad Fraz Hussain[1], Marcel C. Van Verk[2] and Huub J.M. Linthorst[1]

[1] Institute of Biology, Leiden University, P.O. Box 9505, 2300 RA Leiden, The Netherlands

[2] Department of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands

ABSTRACT

Transcription factors AtWRKY50 and AtWRKY28 specifically bind to the promoter of the *PR-1* gene and activate *PR-1::GUS* reporter genes in protoplast transactivation assays. Here we have studied the effects of overexpression or T-DNA knockouts of the WRKY genes in transgenic Arabidopsis plants. Overexpression of the genes did not result in enhanced expression of *PR-1* in non-induced plants, but salicylic acid (SA) treatment resulted in higher levels of *PR-1* mRNA accumulation in plants overexpressing *AtWRKY50* than wild type plants. For the plants overexpressing *AtWRKY28,* SA treatment had the opposite effect. No conclusive results were obtained for the effect of overexpression or knockout of the *WRKY* genes on resistance against *Botrytis cinerea* and *Pseudomonas syringae*.

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 characterized, the plant-wide conserved PR-1 proteins are generally considered as marker proteins for SAR. In most plant species expression of the *PR-1* genes is under transcriptional control (Van Loon and Van Strien, 1999).

The 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. A linker scanning analysis of the region of the Arabidopsis *PR-1* promoter responsible for induced expression by the SA analog INA revealed the presence of an as-1 element with two TGACG direct repeats of which one is a positive regulatory element (LS7), while the other (LS5) mediates negative regulation of *PR-1* expression (Lebel *et al*., 1998). Through knockout analyses it was shown that the Arabidopsis bZIP transcription factors TGA2, TGA3, TGA5 and TGA6 act as redundant but essential activators of *PR-1* expression and SAR (Zhang *et al*., 2003; Kesarwani *et al*., 2007). 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). 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. 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. However, for several *WRKY* genes, SA-induced expression is dependent on NPR1 and TGAs, suggesting a similar activation strategy as was originally proposed for *PR-1* (Dong *et al*., 2003; Wang *et al*., 2006).

In the same linker scanning study that identified the two as-1-like regulatory elements in the Arabidopsis *PR-1* promoter, a consensus W-box motif with a strong negative effect was identified, suggesting WRKY factors to be important for SA-mediated *PR-1* gene expression (Lebel *et al*., 1998). In the previous chapters AtWRKY50 and AtWRKY28 were identified as WRKY transcription factors that specifically bound and activated the *PR-1* promoter.

Here we generated transgenic plants overexpressing AtWRKY50 and AtWRKY28, and *AtWRKY50* T-DNA knockout plants to study the effects of the WRKYs on *PR-1* gene expression and on infection by necrotrophic and biotrophic pathogens.

RESULTS

The *AtWRKY50* and *AtWRKY28* coding regions were amplified by RT-PCR on total RNA isolated from SA-treated Arabidopsis and cloned behind the *35S* promoter. After flower-dip transformation of Arabidopsis Col-0, 20 primary, hygromycin-resistant seedlings were selected for further analysis. The seedlings were transferred to soil and grown through flowering and seed set. T2 generation plants were grown for 18 of the AtWRKY50 lines and 15 AtWRKY28 lines. None of the lines produced plants that were phenotypically different from wild type Arabidopsis. The Northern blots of Fig. 1 show the expression levels of the transgenes in the plants of the T2 generation. The absence of bands in wild type Arabidopsis indicates that the expression levels of the *AtWRKY50* (Fig. 1, Panel A) and *AtWRKY28* (Fig. 1, Panel B) genes are below the level of detection, whereas a band corresponding to *AtWRKY50* mRNA is visible in all AtWRKY50 overexpression lines and similarly is *AtWRKY28* mRNA present in most of the AtWRKY28 overexpression lines. This demonstrates that the transgenes are expressed in most lines, although the expression levels vary considerably. For further analyses *AtWRKY50* overexpression lines W50#2, #8, #12 and #13, and *AtWRKY28* overexpression lines W28#2, #4 and #12 were selected for further analyses.

In addition to plants overexpressing *AtWRKY50*, homozygous plants were generated in which the gene was knocked-out through a T-DNA insertion; plants of knockout line w50#2 were used in this study. Because of the high

similarity between AtWRKY50 and AtWRKY51, we also crossed homozygous T-DNA insertion lines of both genes to obtain double homozygous plants; plants of lines w50w51#2 and #12 were used here. For all these lines the presence of the T-DNA insert and the absence of alleles containing intact genes was confirmed by PCR (data not shown).

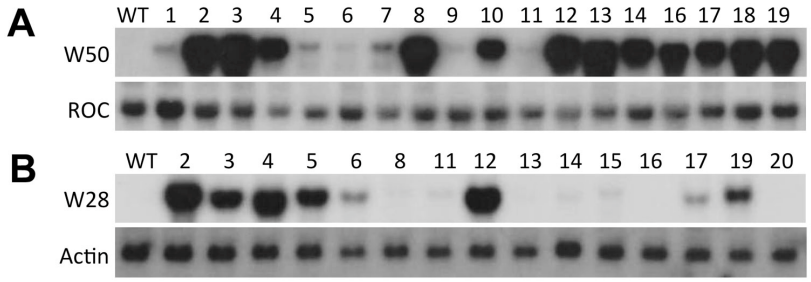

Figure 1. Transgene expression levels in transgenic plants. Northern blots containing total RNA extracted from hygromycin resistant seedlings generated from flower-dip transformed Arabidopsis were hybridized to a cDNA probe corresponding to *AtWRKY50* (A) and *AtWRKY28* (B). To check equal loading, identical blots were hybridized with probes corresponding to constitutive house-keeping genes *At4G38740* encoding rotamase cyclophilin (ROC) and *At3G18780* encoding actin 2 (Actin), respectively. Numbers above the lanes indicate the transgenic line. Samples from non-transformed Arabidopsis were electrophoresed in lanes WT.

In the previous chapters it was shown that AtWRKY50 and AtWRKY28 were each able to specifically interact with the *PR-1* promoter and activate gene expression in protoplasts. Fig. 2 shows the results of *PR-1* gene expression analyses in plants of lines W50#2 and W28#2. As was shown before, expression of *AtWRKY50* and *AtWRKY28* was below the detection level in non-induced wild type plants, but expression was induced by treatment with SA and accumulation of the corresponding mRNAs reached high levels at 6h (*AtWRKY28*) and 24h (*AtWRKY50*) after application of SA, preceding and concomitantly with *PR-1* gene expression, respectively (Chapters 2 and 4). The Northern blot in Fig. 2A shows that constitutive expression of AtWRKY50 in

the transgenic W50#2 plants, did not result in a measurable increase in *PR-1* expression, but that accumulation of *PR-1* mRNA upon SA treatment reached higher levels in the transgenic plants. Similarly, constitutive expression of AtWRKY28 did not lead to *PR-1* expression in non-treated W28#2 plants. However, in these plants SA treatment led to reduced accumulation of *PR-1* mRNA at 16h post treatment. For a more quantitative result, *PR-1* mRNA accumulation upon SA treatment was measured by qRT-PCR. The results are shown in Fig. 2B and 2C. For each of the samples the accumulation of *PR-1* transcript was calculated based on the cycle threshold (Ct) values at the indicated time points relative to that of the transcript of housekeeping gene *At1G13320* (Czechowski *et al.*, 2005).

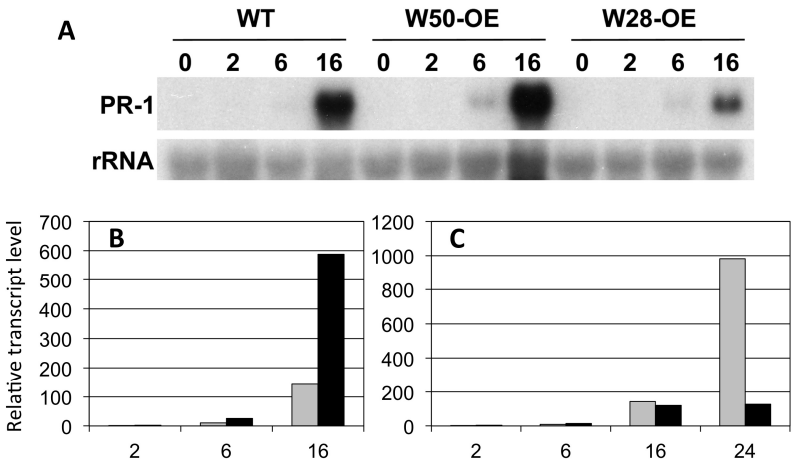

Figure 2. *PR-1* expression in WRKY overexpressing plants. (A) Time course (hours) of salicylic acid-induced *PR-1* mRNA accumulation in wild type Arabidopsis and in transgenic plants overexpressing *AtWRKY50* (W50-OE) or *AtWRKY28* (W28-OE). The band corresponding to ribosomal 25S RNA is shown as a loading control. (B, C) *PR-1* mRNA accumulation in wild type (grey bars) and transgenic (black bars) plants overexpressing *AtWRKY50* (B) or *AtWRKY28* (C) after incubation for the indicated times in 1mM salicylic acid. Transcript levels are given as 2^-ΔΔCt values relative to that of the *At1G13320* reference gene.

The results show that *PR-1* mRNA accumulation was higher in the W50#2 plants at all-time points and increased to 4-fold the level in wild type plants at 16h post treatment. Interestingly, in the W28#2 plants *PR-1* expression was higher than in wild type plants at early time points (2 and 6h post treatment), but at later time points *PR-1* mRNA accumulation leveled off. *PR-1* expression is induced upon infection by biotrophic pathogens and correlated with SAR. While the role of the PR-1 protein in enhanced defense is not clear, other PR proteins that are co-regulated with PR-1 possess antifungal activities that have been suggested to contribute to SAR (Ferreira *et al.*, 2007).

To investigate the possible role of AtWRKY50 in defense against pathogen attack, we investigated the effect of infection with the necrotrophic fungal pathogen *Botrytis cinerea* and the biotrophic bacterial pathogen *Pseudomonas syringae* of plants that constitutively express *AtWRKY50* or *AtWRKY28*, or that contain knockout *AtWRKY50* genes. The results of the *B. cinerea* assay are shown in Fig. 3. In Panel B the disease severity was scored three days after inoculation of the plants on the basis of the disease symptom index shown in Panel A. While W50#2 and W28#2 plants did not show disease scores that were statistically different from wild type Arabidopsis, the number of leaves of W50#8 and W28#4 plants showing symptoms was significantly less than that of wild type plants. However, the fact that for both types of overexpressors one line appeared less sensitive to infection with *Botrytis*, while the other did not, does not permit drawing conclusions on the role of the WRKYs on defense against *Botrytis*. Results of the infection assays with the biotrophic bacterial pathogen *P. syringae* pv. tomato DC3000 are shown in Fig. 4. In this experiment the scoring index was limited to either chlorotic symptoms or absence of symptoms (Fig. 4A). The disease scoring between the lines ranged between 30% and 70%, with the double knock out w50w51 lines and the overexpressing W28 lines showing somewhat less symptoms than wild type

plants and lines overexpressing *AtWRKY50* (Fig. 4B). The level of infection was also scored by determining the bacterial multiplication in leaf extracts of the infected plants at 3 days after inoculation. The results are shown in Fig. 5. Over all, no big differences were apparent between the different lines; although knock-out line w50w51#7 and overexpressor line W28#12 had slightly lower colony counts than the wild type, differing significantly at a P of ≤0.05 by one-way ANOVA.

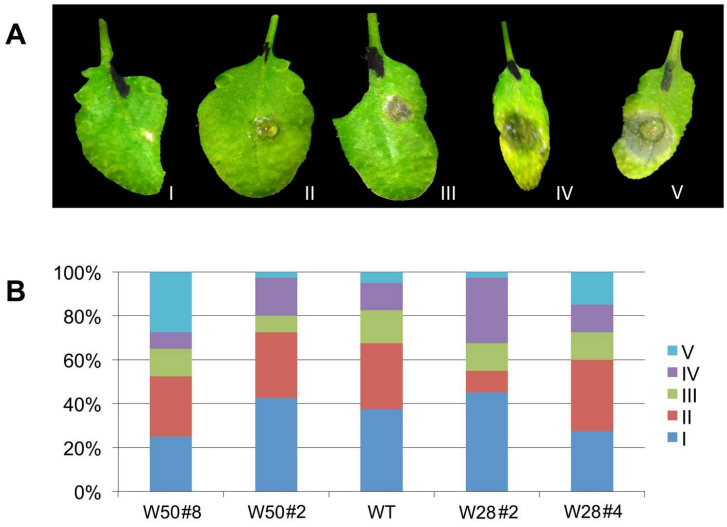

Figure 3. *Botrytis cinerea* infection assays. Disease ratings were assigned to the inoculated leaves of each plant, as described by Ton *et al.* (2002). (A) Intensity of disease symptom and lesion size was classified: 1, no visible disease symptom; 2, non-spreading lesion; 3, spreading lesion; 4, spreading lesion surrounded by a chlorotic halo; and 5, spreading lesion with extensive tissue maceration. (B) Symptoms of infection were scored 3 days after inoculation in wild type (WT) and transgenic plants of two lines each overexpressing *AtWRKY50* or *AtWRKY28*. Ratings are graphically displayed as 100% stacked columns. The differences between the genotypes were analyzed by Pearson Chi-square test.

DISCUSSION

As markers for SAR, the PR-1 proteins have since long been considered to be

involved in induced plant defense against attack by biotrophic pathogens, like many other PR proteins that are induced during the defense response. However, although other members of the group of PR proteins have been characterized as chitinases, β-1,3-glucanases or membrane leakiness provoking proteins with antifungal activities, a function for PR-1 proteins has yet to be determined (Linthorst *et al.*, 1989; Cutt *et al.*, 1989; Alexander *et al.*, 1993; Van Loon and Van Strien, 1999).

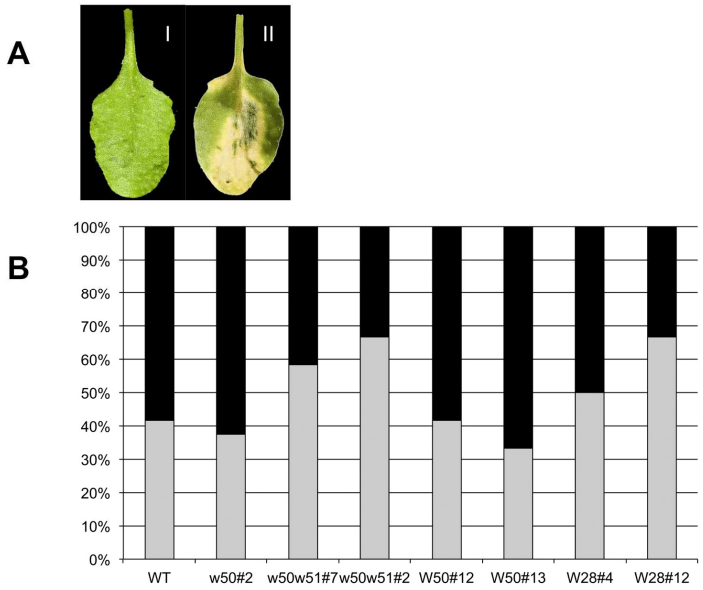

Figure 4. *Pseudomonas syringae* infection assay. Disease ratings were assessed at day 3 after infiltration. (A) Leaves were either scored as symptomless (I) or as chlorotic symptoms (II). (B) Disease ratings were assigned to each of three infiltrated leaves of 8 wild type plants (WT) and 8 plants each of lines over-expressing AtWRKY50 (W50#12 and W50#13), AtWRKY28 (W28#4 and W28#12), or knockout lines of AtWRKY50 (w50#2), or the combination of AtWRKY50 and AtWRKY51 (w50w51#2 and w50w51#7). Ratings are graphically displayed as 100% stacked columns (grey bars: no symptoms, black bars: symptoms). The differences between the genotypes were analyzed by Pearson Chi-square test

In the previous chapters we identified AtWRKY50 and AtWRKY28 as DNA-binding proteins that specifically bound to the *PR-1* promoter and activated the

expression of reporter genes under the control of this promoter in Arabidopsis protoplasts. The genes encoding these WRKY proteins were induced by treatment with SA, the signal molecule that mediates the defense response. Together these findings prompted further functional analyses of AtWRKY50 and AtWRKY28, of which we have described initial results in this chapter.

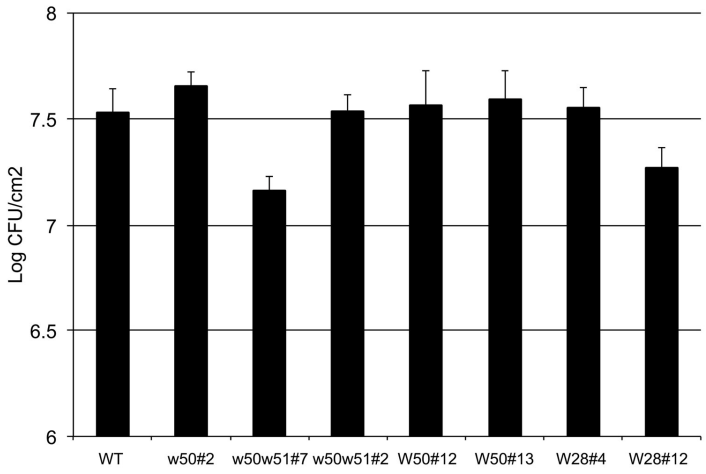

Figure 5. *Pseudomonas syringae* infection assay. Colony-forming units (CFU) of infected leave extracts from wild type plants and from transgenic plants of lines overexpressing WRKY genes or knockout lines as used in Fig. 4 were scored three days after infiltration with bacterial inoculum. Significance was assessed using One-way ANOVA.

Transgenic plants transformed with *AtWRKY50* and *AtWRKY28* genes under the control of the strong, constitutive *35S* promoter expressed high levels of the corresponding mRNAs. Under laboratory conditions, these plants appeared not phenotypically different from wild type plants. Assuming that the respective *WRKY* mRNAs were translated, this suggests that the transcription factors did not interfere with normal plant functions. In any case, the expression of the *WRKY* genes did not result in enhanced levels of *PR-1* gene expression under non-inducing conditions. However, although we haven't yet confirmed this with more transgenic lines, overexpression of *AtWRKY50* in line W50#2

resulted in a higher expression of *PR-1* at 2h, 6h and 16h of treatment with SA. This indicates that on its own, AtWRKY50 cannot trigger *PR-1* expression, but once expression is initiated, the high levels of AtWRKY50 in the transgenic plant augment *PR-1* expression. In Chapter 2 we identified two sites in the *PR-1* promoter that specifically bound the DNA-binding domain of AtWRKY50. One of these sites was located in promoter element LS10, which has a strong positive effect on *PR-1* expression (Lebel *et al.*, 1998; Pape *et al.*, 2010), suggesting that the enhanced expression of *PR-1* in the overexpression plants is mediated through AtWRKY50's binding to this element.

In Chapter 4 we found that AtWRKY28 strongly enhanced *PR-1::GUS* expression in protoplast transactivation assays. Here we observed that in transgenic W28#2 plants overexpressing AtWRKY28, SA-induced *PR-1* mRNA accumulation was reduced in comparison to the expression in wild type plants, suggesting a role for AtWRKY28 as a transcriptional repressor of *PR-1*. An explanation for these apparently contradictory results could possibly be related to the different conditions of the two in vivo assay systems. However, other WRKYs have also been found to have opposite effects on gene expression. Examples are AtWRKY6 and AtWRKY53, which dependent on the promoter context, activate or repress gene expression (Robatzek and Somssich, 2002; Miao *et al.*, 2008). The W-box in LS4 is one of the binding sites of AtWRKY28 in the *PR-1* promoter (Chapter 4). Since the LS4 element was previously found to have a repressing effect on *PR-1* expression (Lebel *et al.*, 1998; Pape *et al.*, 2010), this suggests that the effect of AtWRKY28 could be mediated through this element. Future analyses with more overexpression and knockout lines are required to confirm these results.

Although several studies have failed to discover a clear anti-pathogen activity for PR-1 proteins of tobacco, as far as we know, a possible anti-pathogen effect of Arabidopsis PR-1 has not yet been extensively investigated.

Furthermore, AtWRKY50 and/or AtWRKY28 could possibly also be involved in regulation of other genes functioning in defense. Indeed, the sequence GACTTTTC is present in the promoter of the Arabidopsis *BGL2* gene encoding PR-2 and we determined that the region of the BGL2 promoter that contains this sequence binds AtWRKY50 in EMSA (data not shown). Nevertheless, the infection assays failed to convincingly show enhanced resistance to the necrotrophic fungal pathogen *B. cinerea* or the biotrophic bacterial pathogen *P. syringae* (Figs. 3, 4, 5). Whether the WRKYs play a role in defense against other pathogens or stresses awaits further studies.

MATERIALS AND METHODS

Construction of T-DNA mutant Plants

T-DNA knockout lines for *wrky50* (GK-650F10.01) and *wrky51* (SALK_022198) were obtained from NASC. Pollen from homozygous *wrky50* plants were used to pollinate emasculated homozygous *wrky51* flowers. F1 seedlings were grown without selection and genotyped with GABI-LB for *wrky50* and LBb1.3 for *wrky51*. The primer for genotyping was GABI-LB: 5′-GGG CTA CAC TGA ATT GGT AGC TC-3′ and for LBb1.3: 5′-ATT TTG CCG ATT TCG GAA C-3′. The gene primers used to check for homozygosity were for wrky50: 5′-GGA GGG ATG AAT AAT CCA TGG-3′ and for wrky51: 5′-TTG CTT TCA AAC CAT GCT TTG-3′. Both sets of primer were used to identify double homozygous (*wrky50wrky51*) individuals.

Construction of T-DNA plasmids and transformation of Arabidopsis

For the construction of transgenic lines constitutively overexpressing AtWRKY50 (At5g26170) The PCR was amplified by using these primer sets; 5′-ATA GCT CGA GGT ATG AAT GAT GCA GAC ACA AAC TTG-3′ and 5′-

GCC TCT AGA CGA GTC TTA GTT CAT GCT TGA GTG ATT GTG-3′ and Arabidopsis cDNA library (6h SA treated) was used a template, digested with XhoI and XbaI to clone in pRT101. The AtWRKY28 (At4g18170) open reading frame was PCR–amplified from Arabidopsis cDNA library (6h SA treated Arabidopsis) using the primer sets to cloned into pRT101. The primer sequences were 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′. The Cauliflower Mosaic Virus (*CaMV*) 35S cassette containing the *WRKY's* ORF in sense orientation was digested from pRT101 and cloned in pCAMBIA1300 (Acc. No. Af234296). The binary vector pCAMBIA1300-*WRKYs* was introduced into *Agrobacterium tumefaciens* strain containing the Vir plasmid. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on solid MA medium containing 100 mg/L timentin and 20 mg/L hygromycin. Transgenic plants from T2 generations were selected on MA medium containing only 20 mg/L hygromycin.

Growth of plant materials

Arabidopsis thaliana ecotype Columbia (Col-0) seeds were surface sterilized by incubation for 1 min in 70% ethanol, 15 min in 50% bleach, and five rinses with sterile water. Alternatively, seeds were surface-sterilized in a closed container with chlorine gas for 3-4 hours. Surface-sterilized seeds were grown on plates containing MA (Masson and Paszkowski, 1992) medium supplemented with 0.6% agar. Following stratification for 3 days at 4^0C, seeds were incubated at 21^0C in a growth chamber (16h light / 8h dark, 2500 lux) for 10-12 days. Immediately after harvesting, the material was frozen in liquid nitrogen and kept at -80°C until use.

RNA extraction and Northern blot analyses

Total RNA was extracted from the 10-12 days old seedlings that were frozen in liquid nitrogen by phenol/chloroform extraction followed by overnight precipitation with 8M lithium chloride, washed with 70% ethanol, and re-suspended in water. For RNA blot analysis 10µg RNA samples were subjected to electrophoresis on 1.5% agarose, 1% formaldehyde gels and blotted to Gene Screen nylon membranes (Perkin-Elmer Life Sciences). All probes were ^{32}P-labelled by random priming. Pre-hybridization of blots, hybridization and subsequent washings were performed as described (Memelink *et al.*, 1994), with minor modifications. Blots were exposed on X-ray films (Fuji, Tokyo). DNA fragments used as probes were PCR amplified using the sets of primers used to clone the gene for overexpressing.

***B. cinerea* infection assay**

B. cinerea was grown on potato dextrose agar plates for 2 weeks at 22°C. Spores were harvested as described by Broekaert *et al.* (1990). Plant seedlings germinated on plates were transferred to individual pots containing sterile soil and randomly distributed in trays. Seedlings were cultivated for another 3 weeks in a growth chamber with an 8 h day (1400 lux at 24°C) and 16 h night (20°C) cycle at 65% humidity. For inoculation with fungal pathogens, 5 µL droplets of spore suspension were deposited on two matured leaves of each plant. Inocula consisted of 7.5 X 10^5 spores/mL. *B. cinerea* spores were incubated in half strength potato dextrose broth for 2 hours prior to inoculation. After inoculation, plants were maintained under high relative humidity with the same temperature and photoperiod conditions. In each experiment, 20 plants per genotype were inoculated. Control plants were not inoculated but kept under the same growing conditions. Disease ratings were assessed at day 2 and day 3 after inoculation with *B. cinerea*. Disease ratings were assigned to the

inoculated leaves of each plant, as described by Ton *et al.* (2002). Intensity of disease symptom and lesion size was classified: 1, no visible disease symptom; 2, non-spreading lesion; 3, spreading lesion; 4, spreading lesion surrounded by a chlorotic halo; and 5, spreading lesion with extensive tissue maceration. Disease resistance test were performed at the same time for all genotypes. The differences between the genotypes were analyzed by Pearson Chi-square test.

***P. syringae* infection assay**

Inoculations with the bacterial leaf pathogen *P. syringae* pv. tomato DC3000 were performed as described previously (Van Wees *et al.*, 1999). Briefly, *P. syringae* pv. tomato DC3000 with the plasmid pV288 carrying avirulence gene avrRpt2 (Kunkel *et al.*, 1993) was cultured overnight at 28°C in liquid King's medium B (King *et al.*, 1954), supplemented with kanamycin at 25 mg L^{-1} to select for the plasmid. Subsequently, bacterial cells were collected by centrifugation and resuspended in 10mM $MgSO_4$ to a final density of 10^7 CFU ml^{-1}. Wild-type Col-0 plants were inoculated by pressure infiltrating a suspension of *P. syringae* at 10^7 CFU ml^{-1} into fully expanded leaves of 5-week-old plants. After infiltration, plants were maintained under high relative humidity at the same temperature and photoperiod conditions. In each experiment, 3 leaves of 8 plants per genotype were infiltrated. Control plants were infiltrated with 10 mM $MgSO_4$ and kept under the same growing conditions. Disease ratings were assessed at day 2 and day 3 after infiltration. Disease ratings were assigned to the infiltrated leaves of each plant. Disease resistance tests were performed at the same time for all genotypes. The differences between the genotypes were analyzed by Pearson Chi-square test. The CFU scores from the leaf extracts at three days after infiltrations were assessed with One-way ANOVA.

ACKNOWLEDGEMNTS

We are grateful to Prof. Corné Pieterse for providing the opportunity to perform the infection assays in his laboratory. R. M. F. H. was supported by a grant from the Higher Education Commission of Pakistan.

REFERENCES

Alexander D, Goodman RM, Gut-Rella M, Glascock C, Weymann K, Friedrich L, Maddox D, Ahl-Goy P, Luntz T, Ward E, Ryals J (1993) Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein la. Proc Natl Acad Sci USA **90**:7327-7331

Broekaert WF, Terras FRG, Cammue BPA, Van Der Leyden J (1990) An automated quantitative assay for fungal growth. FEMS Microbiol Lett **69**:55–60

Clough SJ, Bent AF (1998) floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J **16**:735-743

Cutt JR, Harpster MH, Dixon DC, Carr JP, Dunsmuir P, Klessig DF (1989) Disease response to tobacco mosaic virus in transgenic tobacco plants that constitutively express the pathogenesis-related PR1b gene. Virology **173**:89-97

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R (2005) Genome-Wide Identification and Testing of Superior Reference Genes for Transcript Normalization in Arabidopsis. Plant Physiol **139**:5-17

De Vos M, Van Oosten VR, Van Poecke RMP, Van Pelt JA, Pozo MJ, Mueller MJ, Buchala AJ, Métraux J-P, Van Loon LC, Dicke M, Pieterse CMJ (2005) Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. Mol Plant-Microbe Interact **18**:923-937

Dong J, Chen C, Chen Z (2003) Expression profile of the Arabidopsis WRKY gene superfamily during plant defense response. Plant Mol Biol **51**:21–37

Eulgem T, Somssich IE (2007) Networks of WRKY transcription factors in defense signaling. Curr Opin Plant Biol **10**:366–371

Ferreira RB, Monteiro S, Freitas R, Santos CN, Chen Z, Batista LM, Duarte J, Borges A, Teixeira AR (2007) The role of plant defence proteins in fungal pathogenesis. Mol Plant Pathol **8**:677-700

Johnson C, Boden E, Arias J (2003) Salicylic acid and NPR1 induce the recruitment of *trans*-activating TGA factors to a defense gene promoter in Arabidopsis. Plant Cell **15**:1846–1858

Kesarwani M, Yoo J, Dong X (2007) Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in Arabidopsis. Plant Physiol **144**:336–346

King EO, Ward MK, Raney DE (1954) Two simple media for the demonstration of phycocyanin and fluorescin. J. Lab. Clin. Med.**44**:301-307

Kunkel BN, Bent AF, Dahlbeck D, Innes RW, Staskawicz BJ (1993) RPS2, an Arabidopsis disease resistance locus specifying recognition of Pseudomonas syringae strains expressing the avirulence gene AvrRpt2. Plant Cell **5**:865-875

Lebel E, Heifetz P, Thorne L, Uknes S, Ryals J, Ward E (1998) Functional analysis of regulatory sequences controlling PR-1 gene expression in Arabidopsis. Plant J **16**:223–233

Linthorst HJM, Meuwissen RLJ, Kauffmann S, Bol JF (1989) Constitutive Expression of Pathogenesis-Related Proteins PR-1, GRP, and PR-S in Tobacco Has No Effect on Virus Infection. Plant Cell **1**:285-291

Masson J, Paszkowski J (1992) the culture response of Arabidopsis thaliana protoplasts is determined by the growth conditions of donor plants. Plant J **2**:829-833

Memelink J, Swords KMM, Staehelin LA, Hoge JHC (1994) Southern, Northern and Western blot analysis. In Plant Molecular Biology Manual, S.B. Gelvin, R.A. Schilperoot, and D.P.S verma, Eds (Dordrecht: Kluwer academic Publishers), pp. **F1**-F23

Miao Y, Smykowski A, Zentgraf U (2008) A novel upstream regulator of WRKY53 transcription during leaf senescence in Arabidopsis thaliana. Plant Biol **10** (Suppl 1):110-20

Pandey SP, Somssich IE (2009) The Role of WRKY Transcription Factors in Plant Immunity. Plant Physiol **150**:1648-1655

Pape S, Thurow C, Gatz C (2010) The Arabidopsis PR-1 promoter contains multiple integration sites for the coactivator NPR1 and the repressor SNI1. Plant Physiol **154**:1805-1818

Robatzek S, Somssich IE (2002) Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes Dev **16**:1139–1149

Ton J, Van Pelt JA, Van Loon LC, Pieterse CMJ (2002) Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in Arabidopsis. Mol Plant-Microbe Interact **15**:27-34

Van Wees SCM, Luijendijk M, Smoorenburg I, Van Loon LC, Pieterse CMJ (1999) Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis is not associated with a direct effect on expression of known defense-related genes, but stimulates the expression of the jasmonate-inducible gene Atvsp upon challenge. Plant Mol Biol **41**: 537-549

Van Loon LC, Van Strien EA (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol **55**:85-97

Wang D, Amornsiripanitch N, Dong X (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathogens **2**:e123

Zhang Y, Tessaro MJ, Lassner M, Li X (2003) Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. Plant Cell **15**:2647–2653

CHAPTER 6

OVEREXPRESSION OF ATWRKY50 IS CORRELATED WITH ENHANCED PRODUCTION OF SINAPIC DERIVATIVES IN ARABIDOPSIS

R. Muhammad Fraz Hussain, Hye Kyong Kim, Huub J.M. Linthorst

Institute of Biology, Leiden University, P.O. Box 9505, 2300 RA Leiden, the Netherlands

ABSTRACT

Several WRKY proteins, members of a plant-specific class of transcription factors, were overexpressed in Arabidopsis. To investigate their influences on the metabolites of Arabidopsis, an NMR spectroscopy-based metabolomic approach was applied. Multivariate data analysis, such as principal component analysis, hierarchical cluster analysis and partial least square-discriminant analysis of ^{1}H NMR data have been conducted. The results showed that the metabolome of transgenic Arabidopsis overexpressing AtWRKY50 was quite different from wild type Arabidopsis and transgenic Arabidopsis overexpressing other WRKY genes. Amongst other metabolites, especially sinapic acid and sinapoyl glucose were the most prominent differentiating metabolites and increased to levels 2 to 3 fold higher in the AtWRKY50 overexpressors. Our results indicate a possible involvement of AtWRKY50 on secondary metabolite production in Arabidopsis, in particular hydroxycinnamates such as sinapic acid and sinapoyl glucose. However, whether regulation takes place at the level of the genes encoding enzymes of the biosynthesis pathway or at higher levels of signal transduction is not clear and requires further study.

INTRODUCTION

Plants are under continuous threat of attack by fungal, viral and bacterial pathogens. Upon pathogen attack, the plant may respond by activating defense measures through signaling hormones including salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Malamy et al., 1990; Vlot et al., 2009). SA is typically involved in mediating defense against biotrophic pathogens, while JA

and ET mediate resistance responses against necrotrophic pathogens (Glasebrook et al., 2005). The accumulation of SA in systemic leaves leads to the onset of systemic acquired resistance (SAR). SAR is a broad-spectrum plant defense mechanism that is engaged upon a diversity of plant/pathogen interactions (Ryals et al., 1996). SAR is tightly correlated with the expression of several classes of genes, including genes encoding proteins collectively called pathogenesis-related (PR) proteins (Uknes et al., 1992; Uknes et al., 1993b; Ward et al., 1991). *PR* genes are conserved across the entire plant kingdom, including tobacco and Arabidopsis (Uknes et al., 1992). The enzymatic activity of various PR proteins is well-characterized and functionally correlates with resistance against pathogens, although the function of other PR proteins is still not understood. Among these is PR-1, which is generally used as marker for SAR.
WRKY proteins belong to a plant-specific class of transcription factors. There are 74 *WRKY* genes identified in Arabidopsis (Eulgem et al., 2000). In Arabidopsis and other plants many WRKY proteins are involved in responses to stress, especially to biotic stress. They may act either as transcriptional activators or as repressors of genes that play roles in the stress response (Asai et al., 2002; Dong et al., 2003; Journot-Catalino et al., 2006; Kim et al., 2006; Li et al., 2006; Wang et al., 2006; Eulgem and Somssich, 2007). 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. The WRKY proteins are characterized by the presence of the WRKY domain, a DNA-binding domain consisting of a conserved WRKYGQK sequence followed by a zinc-finger (Eulgem et al., 2000).

We are interested in the transcriptional activation of defense during SAR and have used the tobacco and Arabidopsis *PR-1* genes as model genes in our studies. Previous work by others has indicated the importance of a region in the promoter of the *PR-1* gene for SA-induced expression (Lebel et al., 1998,

Pape et al., 2010). The region contains several potential binding sites for transcription factors that could be involved in the induced expression. Members of the TGA proteins, a group of proteins with conserved amino acid sequences that belong to the class of bZIP transcription factors, were found to be important for transcriptional regulation of the *PR-1* genes. However, evidence indicates that also other transcription factors are important for regulation of gene expression (Lebel et al., 1998; Pape et al., 2010). A number of studies have suggested the involvement of Arabidopsis WRKY transcription factors in induced *PR* gene expression, although no direct evidence has been presented for specific WRKY-*PR* promoter interactions (Chen and Chen, 2002; Robatzek and Somssich, 2002; Kim et al., 2006). Recently, our studies on the transcriptional activation of the tobacco *PR-1a* gene have indicated that tobacco WRKY transcription factor NtWRKY12 is important for gene expression (van Verk et al., 2008). Based on these results we started a study of the putative WRKY proteins that might be involved in expression of the Arabidopsis *PR-1* gene. Arabidopsis WRKY50 is the closest homologs of NtWRKY12. The *AtWRKY50* gene is induced by pathogen infection and SA, and the protein localizes to the nucleus, supporting a role as transcription factor. We have shown that AtWRKY50 binds to the *PR-1* promoter at two positions in close proximity to the TGA binding sites. Protoplast transactivation assays have indicated that AtWRKY50 is able to activate *PR-1* gene expression and that co-expression with TGA2 or TGA5 further enhances expression. Together with the finding that AtWRKY50 physically interacts with the TGAs, this supports the idea that the transcription factors co-operate in the regulation of *PR-1* expression.

As mentioned above, the function of PR-1 in defense is not known. Overexpression of the protein in transgenic plants did not elevate defense against virus infection and also defense against other types of pathogens has

not been substantiated (Linthorst et al., 1989; Alexander et al., 1993; Niderman et al., 1995; Hussain et al., this thesis). Although PR-1 proteins are produced at high levels in infected plants and partly accumulate in the apoplast, properties that are not in support of an enzymatic function, we cannot exclude that their expression has direct or indirect effects on the metabolome. Furthermore, in addition to its function in activation of *PR-1* gene expression, AtWRKY50 may act in the transcriptional regulation of other genes involved in defense, like genes encoding enzymes of biosynthesis pathways for metabolites with defense properties, cell-wall strengthening, etc. Here we have investigated the effects of overexpression of several WRKY proteins, including AtWRKY50, on the metabolome using NMR spectroscopy.

RESULTS AND DISCUSSION

Transgenic WRKY overexpressor plants

This study was aimed at investigating the effects of WRKY transcription factors on metabolite production in Arabidopsis. Therefore we made use of the *AtWRKY50* and *AtWRKY28* overexpression lines that were generated in the previous chapter and in addition generated transgenic Arabidopsis lines overexpressing *AtWRKY51* and *AtWRKY46*. To this end, the *AtWRKY51* and *AtWRKY46* coding regions were amplified by RT-PCR on total RNA isolated from SA-treated Arabidopsis and cloned behind the *35S* promoter. After flower-dip transformation of Arabidopsis Col-0, 20 primary, hygromycin-resistant seedlings were selected for further analysis. The seedlings were transferred to soil and grown through flowering and seed set. T2 generation plants were grown for 19 of the AtWRKY51 lines and 20 AtWRKY46 lines. None of the lines produced plants that were phenotypically different from wild

type Arabidopsis. The Northern blots of Fig. 1 show the expression levels of the transgenes in the plants of the T2 generation. The absence of bands in wild type Arabidopsis indicates that the expression levels of the *AtWRKY51* (Fig. 1, Panel A) and *AtWRKY46* (Fig. 1, Panel B) genes are below the level of detection, whereas a band of varying intensity corresponding to *AtWRKY51* mRNA is visible in most AtWRKY51 overexpression lines and similarly is *AtWRKY46* mRNA present in most of the AtWRKY46 overexpression lines. This demonstrates that the transgenes are expressed in most lines, although the expression levels vary considerably. For further analyses *AtWRKY51* overexpression lines W51 #1, #8 and #11 and AtWRKY46 overexpressor lines W46 #5, #8 and #9 were selected. In addition to these WRKY overexpressor lines also overexpressor lines of *AtWRKY50* and *AtWRKY28* described in the previous chapter were used for the metabolomic analyses. These were lines W50#2, #3 and #8, and W28#2, #4 and #12.

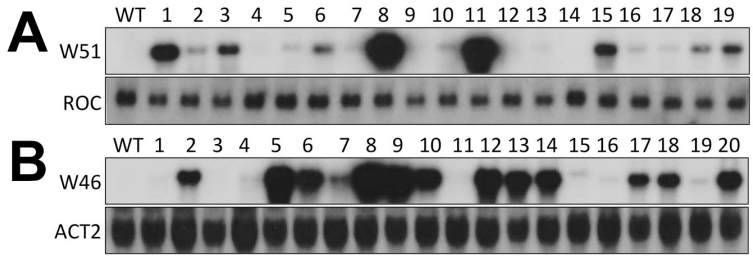

Figure 1. Transgene expression levels in transgenic plants. Northern blots containing total RNA extracted from hygromycin resistant seedlings generated from flower-dip transformed Arabidopsis were hybridized to cDNA probes corresponding to the respective transgenes *AtWRKY51* (A) and *AtWRKY46* (B). To check equal loading, identical blots were hybridized with probes corresponding to constitutive house-keeping genes *At4G38740* encoding rotamase cyclophilin (ROC) and *At3G18780* encoding actin 2 (Actin), respectively. Numbers above the lanes indicate the transgenic line. Samples from non-transformed Arabidopsis were electrophoresed in lanes WT.

Principal component analysis

Selected transgenic Arabidopsis lines overexpressing *WRKY* genes (*AtWRKY28*, *AtWRKY46*, *AtWRKY50*, *and AtWRKY51*) were examined for their metabolites using ^{1}H NMR spectroscopy. ^{1}H NMR spectroscopy can detect all metabolites containing hydrogen, and therefore it is suitable to obtain broad range metabolome snapshots of the given samples. In general ^{1}H NMR spectra produce large numbers of variables (usually more than 200 signals), which makes it necessary to perform multivariate data analysis. The most common unsupervised multivariate data analysis is principal component analysis (PCA). As the first step of multivariate data analysis, PCA of ^{1}H NMR spectra was performed to discriminate the transgenic Arabidopsis WRKY overexpression plants. The PCA score plot showed that PC1 and PC2 explained 34% and 31%, respectively (Fig. 2). Most prominent differences among the tested Arabidopsis plants were found in the two lines that overexpressed AtWRKY50, notably lines W50#3 and W50#8. Line W50#3 was separated by PC1 compared to wild type Arabidopsis, while line W50#8 separated both PC1 and PC2 (Fig. 2a).

The loading plot of PC1 (Fig. 2b) indicated that sinapic acid, sinapoylglucose, sucrose, glucose, alanine and threonine had high levels in both W50#3 and W50#8 in comparison to wild type. In contrast, levels of other phenolic compounds were decreased in the W50#3 and W50#8 plants. The loading plot of PC2 (Fig. 2b) indicated that glucose, glutamate and the signals of δ 2.70, δ 2.95, δ 3.10 were increased in W50#3, compared to W50#8, while hydroxycinnamates and amino acids, in particular, threonine, were higher in W50#8, compared to wild type and W50#3 plants.

Hierarchical cluster analysis

Although the PCA analysis provides some clues for the differences between the Arabidopsis overexpression lines, the available PCs are limited, because only

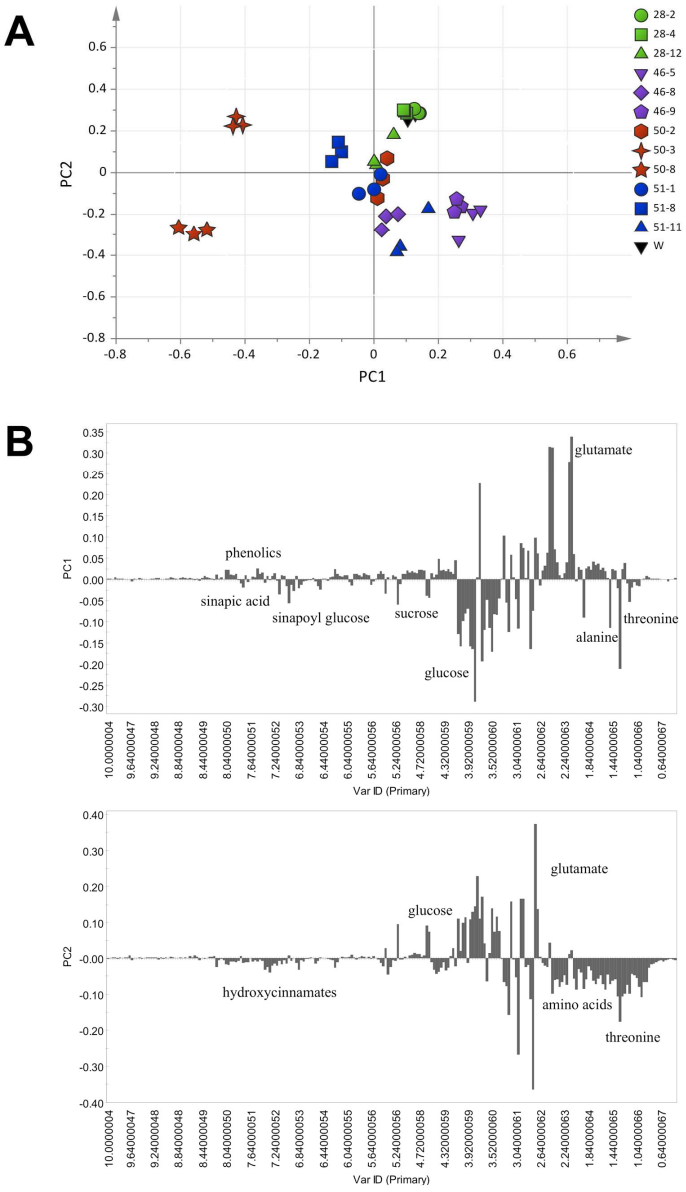

Figure 2. Principal component analysis of ^{1}H NMR spectra from Arabidopsis overexpressing WRKY28, WRKY46, WRKY50 and WRKY51 and wild type Arabidopsis. Score plot (a) and loading plots (b) of PC1 and PC2.

two or three PCs can be visualized. Besides, the score plot does not provide detailed information on the closeness between differently overexpressed plants and wild type plants. Applying hierarchical cluster analysis (HCA) allows obtaining further information on these aspects. For the HCA, 7 PCs reduced from the original ^{1}H NMR signals were used, which explained almost 95% of variables. The HCA showed that Arabidopsis overexpressing AtWRKY28 is very similar to wild type (group A), while plants overexpressing AtWRKY46 and AtWRKY51 were clustered in a different group (group B) (Fig. 3). It was obvious that Arabidopsis overexpressing AtWRKY50 was quite different (group C) from wild type Arabidopsis and other WRKY overexpressors, as was also shown in PCA.

Partial least square–discriminant analysis

To further analyze which metabolites contribute for the differentiation of each group, partial least square–discriminant analysis (PLS-DA) was applied. As a type of PLS, PLS-DA uses discrete class matrix, in contrast to PCA, which only uses the information of one matrix (Berrueta et al., 2007). In the PLS-DA, only two groups (group A and B) were used as Arabidopsis overexpressing AtWRKY50 most obviously differed from wild type by the PCA analysis. In the PLS-DA score plot, Arabidopsis overexpressing AtWRKY46 and AtWRKY51 were clearly differentiated by PLS component 1 (Fig. 4a). The model diagnostics for the first component showed an explained variation (R^2Y) of 0.85 and a goodness of fit (Q^2Y) of 0.76. Model validation using 200 permutations showed a negative slope of the regression line suggesting that there is no model overfit. To find out precisely which metabolites contributed to the discrimination between two groups, a PLS-DA loading plot was generated (Fig. 4b). In the loading plot, positive values of wc*[1] were associated with group A, while negative values were associated with group B. Investigation of the loading plots

suggested that glucose (δ 5.20, δ 4.60), sucrose (δ 5.40), glutamate (δ 2.12, δ 2.16, δ 2.48), and *cis*-sinapic acid (δ 5.96) were higher in group A, whereas flavonoids and other hydroxycinamates (*trans*-sinapic acid, sinapoyl glucose) and amino acids (alanine, phenylalanine) were higher in group B.

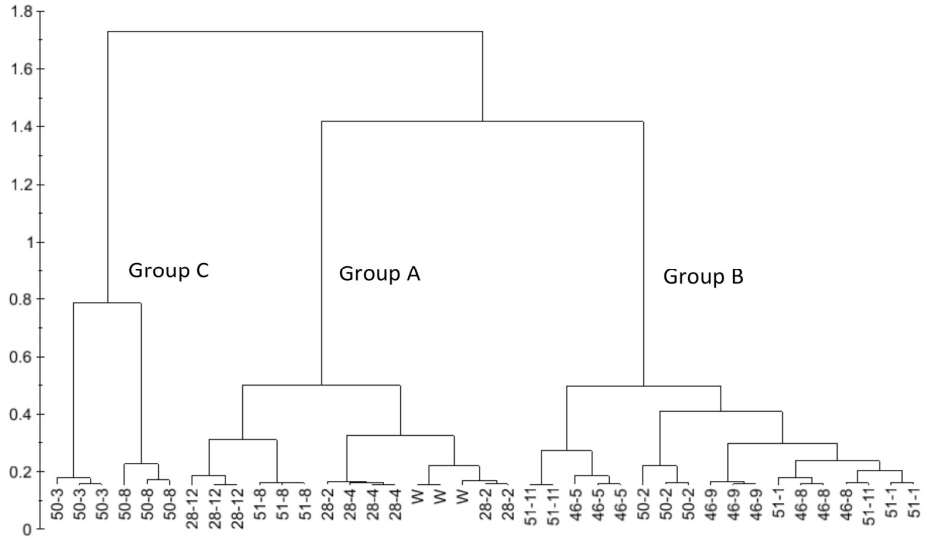

Figure 3. Dendrogram resulting from hierarchical cluster analysis showing three distinct groups A, B and C.

To summarize the results, Arabidopsis overexpressing AtWRKY50 (W50#3, W50#8) had increased amounts of sinapic acid, sinapoyl glucose, glucose, sucrose and amino acids (especially threonine in W50#8), compared to wild type. Group B plants overexpressing AtWRKY46 and AtWRKY51 (W51#1, W51#11) showed increased levels of sinapoyl glucose, sinapic acid, flavonoids and amino acids compared to wild type. The metabolome's of Arabidopsis overexpressing AtWRKY28 and AtWRKY51 (W51#8) were similar to wild type Arabidopsis. This study shows that the levels of sinapic acid and sinapoyl glucose were increased in Arabidopsis plants overexpressing AtWRKY50.

Quantitative analysis of sinapic acid and sinapoyl glucose of all samples clearly indicated the level of both compounds in Arabidopsis plants overexpressing AtWRKY50 were increased by 2-3 folds compared to wild type (Fig. 5).

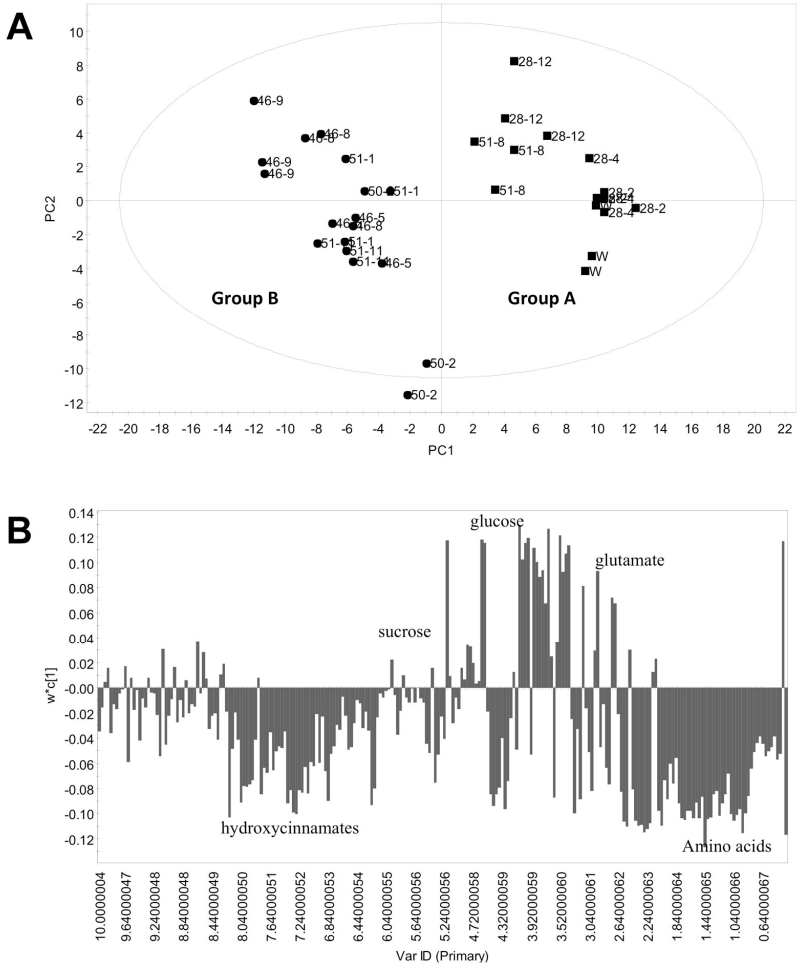

Figure 4. Partial least square-discriminant analysis of overexpressors of WRKY28, WRKY46, WRKY50 and WRKY51. The score plot (a) and loading plot (b) of PC1. Only two groups, group A (green) and group B (blue) have been analyzed for PLS-DA. Validation has been tested using permutation methods.

Sinapic acid is a common hydroxycinammate found in many plants, including Arabidopsis and Brassica, and is mostly present in a conjugated form. In the leaves, sinapoyl malate is the dominant form and sinapoyl glucose and sinapoyl choline mostly occur in the seeds of Arabidopsis (Chappel et al., 1992). Sinapoyl glucose is synthesized from sinapate by UDP-glucose:sinapic acid glycosyltransferase (Wolfram et al., 2010) and it is known to function in protection against UV radiation (Landry et al., 1995; Sheahan, 1996).

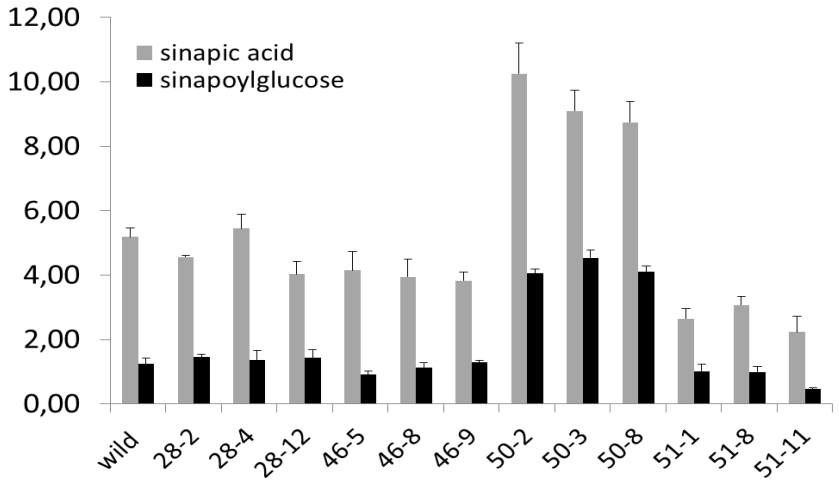

Figure 5. Quantitative analysis of sinapic acid and sinapoyl glucose in Arabidopsis plants. Peak intensities of the signals of sinapic acid (δ 7.00) and sinapoyl glucose (δ 7.02) were expressed relative to the peak area of the internal standard TSP. Mean value of each peak intensity was shown with standard deviation.

Moreover, derivatives of the related compounds sinapic acid, coumaric acid and ferulic acid are constituents of the cell wall strengthening phenolic polymer lignin, emphasizing the importance of these hydroxycinnamates for plant defense. Indeed, plants are able to adapt lignin structure to remedy particular types of stress by regulating expression of genes encoding enzymes involved in hydroxycinnamate biosynthesis. An example is the gene for ferulate-5-hydroxylase (F5H), the enzyme involved in the conversion of ferulate to

sinapate, which is regulated by biotic and abiotic types of stress (Kim et al., 2006; Hruz et al., 2008). Although WRKY transcription factors have been suggested to be especially involved in defense, a function of WRKYs in the biosynthesis of hydroxycinnamates has not been reported thus far. In this context it is interesting to note that the promoter of the Arabidopsis *F5H* gene contains the sequence GACTTTTC that we identified as an AtWRKY50 binding site in the *PR-1* promoter.

In conclusion, our results show that WRKY transcription factors effect secondary metabolite production, such as sinapic acid and sinapoyl glucose in Arabidopsis. However, whether regulation takes place at the level of the genes encoding enzymes of the biosynthesis pathway or at higher levels of signal transduction is not clear and requires further study.

MATERIALS AND METHODS

Plant vectors and transformation

For the construction of transgenic lines constitutively overexpressing WRKYs, the Cauliflower mosaic virus (*CaMV*) *35S* cassette containing the *WRKY's* ORF in sense orientation was obtained from pRT101 and cloned in pCAMBIA1300 (acc. no. Af234296). Binary vectors pCAMBIA1300-*WRKY* was introduced into *Agrobacterium tumefaciens*. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on solid MA medium containing 100 mg/L timentin and 20 mg/L hygromycin. Transgenic plants from T2 generations were selected on MA medium containing only 20 mg/L hygromycin.

Growth of plant materials

Arabidopsis thaliana ecotype Columbia (Col-0) seeds were surface sterilized by incubation for 1 min in 70% ethanol, 15 min in 50% bleach, and five rinses with sterile water. Alternatively, seeds were surface-sterilized in a closed container with chorine gas for 3-4 hours. Surface-sterilized seeds were sown on plates containing MA (Masson and Paszkowski, 1992) medium supplemented with 0.6% plant agar. Following stratification for 3 days at 4^0C, seeds were incubated at 21°C in a growth chamber (16 h light/8 h dark, 2500 lux) for 10-12 days. Immediately after harvesting, the material was frozen in liquid nitrogen and kept at -80^0C until use.

RNA extraction and Northern blot analyses

Total RNA was extracted from the 10-12 days old seedlings that were frozen in liquid nitrogen by phenol/chloroform extraction followed by overnight precipitation with 8M lithium chloride, washed with 70% ethanol, and re-suspended in water. For RNA blot analysis 10µg RNA samples were subjected to electrophoresis in 1.5% agrose/1% formaldehyde gels and blotted to Gene Screen nylon membranes (Perkin-Elmer Life Sciences). All probes were ^{32}P-labelled by random priming. Pre-hybridization of blots, hybridization of probes and subsequent washings were performed as described (Memelink et al., 1994) with minor modifications. Blots were exposed to X-ray film (Fuji, Tokyo). DNA fragments used as probes were PCR-amplified from sets of primers shown in Table 1.

Sample extraction for NMR analysis

Twenty mg of freeze-dried material were transferred to a microtube (2 ml) to which 1.5 ml of 50% methanol-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) containing 0.05% TSP (trimethyl silyl propionic acid sodium salt, w/v) was added. The mixture was vortexed at room temperature for 1 min, ultrasonicated for 20 min,

and centrifuged at 13,000 rpm at room temperature for 5 min. Eight hundred μL of the supernatant was transferred to a 5 mm NMR tube.

Table 1.

AtWRKY28 (At4g18170)	F	GTCACTCGAGATGTCTAATGAAACCAGAGATCTCTAC
	R	CAGTGGATCCTCAAGGCTCTTGCTTAAAGAAAATTG
AtWRKY46 (A2g46400)	F	GTCACTCGAGATGATGATGGAAGAGAAACTTGTG
	R	CAGTTCTAGACTACGACCACAACCAATCCTGTC
AtWRKY50 (At5g26170)	F	ATAGCTCGAGGTATGAATGATGCAGACACAAACTTG
	R	GCCTCTAGACGAGTCTTAGTTCATGCTTGAGTGATTGTG
AtWRKY51 (At5g64810)	F	AAACGAATTCAAATGAATATCTCTCAAAACCCTAGCC
	R	GATGAGGTACCTGGATTAAGATCGAAGAAGGTGTTG
Actin2 (At3g18780)	F	CTGTGCCAATCTACGAGGGTT
	R	GGAAACCTCAAAGACCAGCTC
ROC1 (At4g38740)	F	CGGGAAGGATCGTGATGGA
	R	CCAACCTTCTCGATGGCCT

NMR measurements

^{1}H NMR spectra were recorded at 25^{o}C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. MeOH-d_4 was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 sec acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30^o (11.3 μsec), and relaxation delay (RD) = 1.5 sec. A presaturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to TSP at 0.0 ppm, using XWIN NMR (version 3.5, Bruker). 2D J-resolved NMR spectroscopy, ^{1}H-^{1}H-correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600 MHz Bruker DMX-600 spectrometer (Bruker). 2D J-resolved NMR spectra were acquired using 8 scans per 128 increments for F1 and 8 k for F2 using spectral widths of

5000 Hz in F2 (chemical shift axis) and 66 Hz in F1 (spin-spin coupling constant axis). A 1.5 sec relaxation delay was employed, giving a total acquisition time of 56 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. J-resolved spectra tilted by 45^{o}, was symmetrized about F1, and then calibrated, using XWIN NMR (version 3.5, Bruker). The COSY spectra were acquired with 1.0 sec relaxation delay, 6361 Hz spectral width in both dimensions. Window function for COSY spectra was sine-bell (SSB = 0). The HSQC spectra were obtained with 1.0 sec relaxation delay, 6361 Hz spectral width in F2 and 27 164 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HSQC. The HMBC spectra were recorded with the same parameters as the HSQC spectrum except for 30183 Hz of spectral width in F2. The optimized coupling constants for HSQC and HMBC were 145 Hz and 8 Hz, respectively.

NMR Data Analysis

The ^{1}HNMR spectra were automatically reduced to ASCII file. Spectral intensities were scaled to the total intensity and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.4 – δ 10.0. The region of δ 4.75 – δ 4.90 and δ 3.28 – δ 3.34 was excluded from the analysis because of the residual signal of HDO and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker) with scaling on total intensity. Principal component analysis (PCA) was performed with the SIMCA-P software (v. 12.0, Umetrics, Umea, Sweden) with scaling based on Pareto method. PLS-DA was performed same way except using UV scaling method.

ACKNOWLEDGEMNTS

We thank Muzamal Iqbal for assistance with the NMR measurements. R. M. F. H. was supported by a grant from the Higher Education Commission of Pakistan.

REFERENCES

Alexander D, Goodman RM, Gut-Rella M, Glascock C, Weymann K, Friedrick L, Maddox D, Ahl-Goy P, Luntz T, Ward E, Ryals JA (1993) Increased tolerance to two Oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc Natl Acad Sci USA **90**:7327–7331

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signaling cascade in Arabidopsis innate immunity. Nature **415**:977–983

Berrueta LA, Alonso-Salces RM, Heberger K (2007) Supervised pattern recognition in food analysis. J. Chromatography **A 1158**:196-214

Chappel CCS, Vogt T, Ellis BE, Somerville CR (1992) An Arabidopsis mutant defective in general phenylpropanoid pathway. Plant Cell **4**:1413-1424

Chen C, Chen Z (2002) Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced Arabidopsis transcription factor. Plant Physiol **129:**706–716

Dong J, Chen C, Chen Z (2003) Expression profile of the Arabidopsis WRKY gene superfamily during plant defense response. Plant Mol Bio **51**:21–37

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. Trends Plant Sci **5**:199–206

Eulgem T, Somssich IE (2007) Networks of WRKY transcription factors in defense signaling. Curr Opin Plant Bio **10**:366–371

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Ann Rev Phytopathol **43**:205-227

Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L, Widmayer P, Gruissem W and P Zimmermann (2008) Genevestigator V3: a reference expression database for the meta-analysis of transcriptomes. Adv Bioinformatics **2008**:420747

Journot-Catalino N, Somssich IE, Roby D, Kroj T (2006) The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in Arabidopsis thaliana. Plant Cell **18**:3289–3302

Kim KC, Fan B, Chen Z (2006) Pathogen-induced Arabidopsis WRKY7 is a transcriptional repressor and enhances plant susceptibility to *Pseudomonas syringae*. Plant Physiol **142**:1180–1192

Kim YJ, Kim DG, Lee SH, Lee I (2006) Wound-induced expression of the ferulate 5-hydroxylase gene in Camptotheca acuminata. Biochim Biophys Acta **1760**:182-90

Landry LG, Chapple CC, Last RL (1995) *Arabidopsis* mutants lacking phenolic sunscreens exhibit enhanced ultraviolet-B injury and oxidative damage. Plant Physiol **109**:1159–1166

Lebel E, Heifetz P, Thorne L, Uknes S, Ryals J, Ward E (1998) Functional analysis of regulatory sequences controlling PR-1 gene expression in Arabidopsis. Plant J **16**:223–233

Li J, Brader G, Kariola T, Palva ET (2006) WRKY70 modulates the selection of signaling pathways in plant defense. Plant J **46**:477–491

Linthorst HJM, Meuwissen RLJ, Kauffman S, Bol JF (1989) Constitutive expression of pathogenesis-related proteins PR-1, GRP, and PR-S in tobacco has no effect on virus infection. Plant Cell **1**:285–291

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic Acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science **250**:1002-1004

Niderman T, Genetet I, Bruyère T, Gees R, Stintzi A, Legrand M, Fritig B, Mosinger E. (1995) Pathogenesis-related PR-1 proteins are antifungal: Isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against Phytophthora infestans. Plant Physiol **108**:17-27

Pape S, Thurow C, Gatz C (2010) The Arabidopsis PR-1 promoter contains multiple integration sites for the coactivator NPR1 and the repressor SNI1. Plant Physiol **154**:1805-1818

Robatzek S, Somssich IE (2002) Targets of AtWRKY6 regulation during plant senescense and pathogen defense. Genes & Develop **16**:1139–1149

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner H-Y, Hunt MD (1996) Systemic Acquired Resistance. Plant Cell **8**:1809-1819

Sheahan JJ (1996) Sinapate esters provide greater UV-B attenuation than flavonoids in Arabidopsis thaliana (Brassicaceae). Am J Bot **83**:679-686

Uknes S, Dincher S, Friedrich L, Negrotto D, Williams S, Thompson-Taylor H, Potter S, Ward E, Ryals J (1993) Regulation of pathogenesis-related protein-1a gene expression in tobacco. Plant Cell **5**:159–169

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward E, Ryals J (1992) Acquired resistance in Arabidopsis. Plant Cell **4**:645–656

Van Verk MC, Pappaioannou D, Neeleman L, Bol JF, Linthorst HJM (2008) A novel WRKY transcription factor is required for induction of PR-1A gene expression by salicylic acid and bacterial elicitors. Plant Physiol **146**:1983–1995

Vlot AC, Dempsey DMA, Klessig DF (2009) Salicylic acid, a multifaceted hormone to combat disease. Ann Rev Phytopathol **47**:177-206

Wang D, Amornsiripanitch N, Dong X (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathogens **2**:e123

Ward ER, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Métraux J-P, Ryals JA (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3**:1085–1094 resistance response of tobacco to viral infection. Sci **250:**1002-1004

Wolfram K, Schmidt J, Wray V, Milkowski C, Schliemann W, Strack (2010) Profiling of phenylpropanoids in transgenic low-sinapine oilseed rape (*Brassica napus*). Phytochemistry **71**:1076–1084

SUMMARY

The survival rate of plants depends on their efficient mechanism to handle the adverse conditions present in the natural environment. In addition to abiotic types of stress, like drought, saline soil, temperature, or high intensity light, plants are under continuous threat of attack by a variety of pathogens. Upon pathogen attack, the plant may respond by activating defense measures through signaling hormones including salicylic acid (SA). SA is typically involved in mediating defense against biotrophic pathogens. The current knowledge of the SA-mediated signaling pathway and the transcriptional regulation of defense responses mediated through this signal molecule is reviewed in **Chapter 1.**

The accumulation of SA in systemic leaves leads to the onset of systemic acquired resistance (SAR). SAR is a broad-spectrum plant defense mechanism that is engaged upon a diversity of plant/pathogen interactions. The enzymatic activity of various PR proteins is well characterized and functionally correlates with resistance against pathogens, although the function of other PR proteins is still not understood. Among these is PR-1, which is generally used as marker for SAR. WRKY proteins belong to a plant-specific class of transcription factors. There are 74 WKRY genes identified in Arabidopsis. In Arabidopsis and other plants many WRKY proteins are involved in responses to stress, especially to biotic stress. WRKY proteins bind to DNA at so-called W-boxes, DNA elements with the sequence TTGAC(T/C), and they may act either as transcriptional activators or as repressors of genes that play roles in the stress response. We are interested in the transcriptional activation of defense during SAR and have used the tobacco and Arabidopsis *PR-1* genes as model genes in our studies.

Previous work by others has indicated the importance of a region in the promoter of the *PR-1* gene for SA-induced expression. This region contains

several potential binding sites for transcription factors that could be involved in the induced expression of *PR-1*.

In **Chapter 2** we have shown that WRKY50 binds to the *PR-1* promoter at two specific positions in close proximity to binding sites of TGA proteins, members of the bZIP class of DNA-binding transcription factors. The two WRKY50 binding sites were highly similar in sequence, but surprisingly, they did not resemble the consensus W-box. To validate the role of the WRKY50 binding sites in the promoter protoplast transactivation assays were performed, which showed that WRKY50 is able to activate *PR-1* gene expression. Combined mutations of these two binding site completely abolished expression of *PR-1*-controlled reporter gene expression in Arabidopsis protoplasts.

Chapter 3 deals with the effects of combinations of WRKY50 and TGA proteins on *PR-1* gene expression. We found that WRKY50 interacts with TGA2 and TGA5 in protein-protein interaction studies in yeast and in planta. Co-expression of AtWRKY50 with TGA2 or TGA5 synergistically enhanced *PR-1* gene expression in protoplast transactivation assays. These findings support the idea that WRKY50 and TGA2 and TGA5 co-operate in the regulation of *PR-1* expression. In addition to the two binding sites for WRKY50, the *PR-1* promoter contains a number of W-boxes that have been shown to be also important for expression. In **Chapter 4** we describe that AtWRKY28 is able to bind to these W-boxes and to activate *PR-1* gene expression in the protoplast transactivation assay.

Chapter 5 describes the effects of overexpression of the WRKY50 and WRKY28 proteins in transgenic plants. Also T-DNA insertion mutants of *WRKY50* were characterized. High constitutive expression of the *WRKY50* gene resulted in higher accumulation of *PR-1* mRNA when the plants were treated with SA, but without SA treatment *PR-1* expression was not enhanced. This indicates that on its own WRKY50 cannot trigger *PR-1* expression, but once

expression is initiated by SA, WRKY50 supports high level expression. WRKY28 had an opposite effect on *PR-1* expression. SA-induced *PR-1* expression in the WRKY28 overexpression plants was lower than in wild type plants. This supports a role for WRKY28 as a repressor of *PR-1* expression. The overexpression of WRKY50 and WRKY28 had no clear-cut effect on the plants' resistance to the biotrophic bacterial pathogen *Pseudomonas syringae* or the necrotrophic fungal pathogen *Botrytis cinerea*, and neither was this the case with WRKY50 T-DNA knockout plants.

The effects of various WRKYs on the metabolome were investigated in **Chapter 6**. In addition to the overexpression lines of WRKY50 and WRKY28 described in the previous chapter, transgenic plants Arabidopsis plants overexpressing WRKY51 and WRKY46 were generated. To investigate the WRKY's influences on metabolite composition, a ^{1}H NMR spectroscopy-based metabolomic approach was applied. Multivariate data analyses, such as principal component analysis, hierarchical cluster analysis and partial least square-discriminant analysis of the NMR data showed that the metabolome of Arabidopsis overexpressing AtWRKY50 differed from wild type Arabidopsis and transgenic Arabidopsis overexpressing the other WRKY genes. The AtWRKY50 overexpression plants contained two- to three-fold more sinapic acid and sinapoyl glucose. This indicates a possible involvement of AtWRKY50 on secondary metabolite production in Arabidopsis, in particular of hydroxycinnamates such as sinapic acid and sinapoyl glucose. As these compounds are components of lignin, this may point to a role of AtWRKY50 in stress-induced lignin modification.

SAMENVATTING

Planten staan doorlopend bloot aan bedreigingen uit de omgeving. Deze kunnen van abiotische aard zijn, zoals veroorzaakt door het klimaat (koude, hitte, droogte), de omgeving (grond met hoge gehaltes aan zout) of weefselschade door verwonding, of ze komen van insecten die plantenweefsels eten of microbiële ziekteverwekkers (schimmels, bacteriën, virussen). In de laatste gevallen spreken we van biotische stress. Deze is weer onder te verdelen in stress veroorzaakt door enerzijds, insectenvraat en necrotrofe pathogenen en anderzijds, biotrofe pathogenen, waarbij de eerste leven van de vrijkomende suikers en andere celbestanddelen van gedood plantenweefsel, terwijl de biotrofe pathogenen parasiteren op levende cellen.

Om deze bedreigingen het hoofd te bieden beschikken planten over een uitgebreid arsenaal aan verdedigingsmechanismen. Sommige van deze mechanismen zijn continu aanwezig, zoals de celwand en de waslaag op stengels en bladeren, die de plant beschermen tegen uitdroging, mechanische schade en opportunistische schimmels en bacteriën die anders een gemakkelijk maaltje zouden hebben aan de onbeschermde cel. Andere voorbeelden van continue verdediging zijn de al dan niet met afweerstoffen gevulde bladhaartjes en blaasjes, die het insecten moeilijk maken zich op de plant te verplaatsen, of antimicrobiële verbindingen (phytoalexines) die ophopen in de weefsels van sommige planten. Daartegenover staan verdedigingsmechanismen die pas worden geactiveerd op het moment dat de plant wordt bedreigd. Deze geïnduceerde afweer resulteert veelal in de productie van eiwitten die een direct of indirect effect hebben op het vermogen van de pathogenen om zich door de plant te verspreiden. Een voorbeeld van eiwitten die worden geproduceerd tijdens de geïnduceerde afweer tegen biotrofe pathogenen zijn de zogenaamde PR-eiwitten. PR-eiwitten worden niet alleen geproduceerd in de directe omgeving van het binnendringende pathogeen, maar ook ver van de infectiehaard, in weefsels waar geen pathogeen aanwezig is. De ophoping van

PR-eiwitten is gecorreleerd met de productie van het plantenhormoon salicylzuur (SA) en gaat gepaard met een verhoging van de resistentie tegen een breed scala aan pathogenen in alle weefsels van de plant. Deze zogenaamde systemische verworven resistentie wordt aangeduid met SAR (*systemic acquired resistance*). PR-eiwitten zijn binnen het hele plantenrijk geconserveerd. Er worden zo'n 15 verschillende subgroepen onderscheiden. Tot deze groepen behoren o.a. β-1,3-glucanases (PR-2) en verschillende typen chitinases (PR-3, PR-4, PR-8, PR-11), enzymen die de celwanden van bepaalde schimmels kunnen afbreken, en thaumatine-achtige eiwitten (PR-5) met een antischimmel activiteit. Ondanks het feit dat na infectie de PR-1 eiwitten in grote hoeveelheden worden aangemaakt, waardoor ze wereldwijd worden gebruikt als markers voor de geïnduceerde resistentie, is er nog niet veel bekend over hun functie. Het in dit proefschrift beschreven onderzoek was gericht op de rol van transcriptiefactoren, met name WRKY-eiwitten, in de expressie van het gen dat codeert voor het PR-1 eiwit in de modelplant Arabidopsis.

Hoofdstuk 1 geeft een overzicht van de huidige kennis op het gebied van de signaaltransductie en van de transcriptionele regulatie van de afweermechanismen die door het plantenhormoon salicylzuur (SA) worden beïnvloed. Hierin wordt het gedeeltelijk in kaart gebrachte signaaltransductieproces beschreven dat leidt van herkenning van het binnendringende pathogeen tot SAR en de expressie van de genen die coderen voor de PR-eiwitten. Naast SA, dat een centrale plaats inneemt in de signaaltransductie, speelt ook het eiwit NPR1 een belangrijke rol. Als gevolg van de verhoogde productie van SA worden NPR1 monomeren vrijgemaakt uit een multimeer NPR1 complex in het cytoplasma, waarna ze verhuizen naar de celkern om daar een interactie aan te gaan met zgn. TGA eiwitten, transcriptiefactoren die binden aan de promoters van o.a. *PR* genen. Deze interactie leidt vervolgens tot activering van de transcriptie van de genen. De

promoter van het *PR-1* gen van Arabidopsis bevat inderdaad bindingsplaatsen voor TGA eiwitten en eerder onderzoek heeft duidelijk gemaakt dat deze plaatsen belangrijk zijn voor SA-geïnduceerde genexpressie. Echter, hetzelfde onderzoek heeft ook aangetoond dat andere sequenties in de promoter eveneens een belangrijke rol spelen. Voor een deel zijn deze sequenties identiek aan de W-box, een DNA element dat is gekarakteriseerd als bindingsplaats voor WRKY eiwitten, transcriptiefactoren die alleen bij planten voorkomen en gerelateerd zijn aan stress responsen.

In **Hoofdstuk 2** is beschreven dat AtWRKY50 de sterkste activator was van de *PR-1* expressie in Arabidopsis na screening van 41 van de 74 Arabidopsis WRKY transcriptiefactoren met behulp van transactivatie experimenten in protoplasten. AtWRKY50 verschilt van de meeste andere WRKY eiwitten door de aanwezigheid van een lysine in plaats van een glutamine in het DNA-bindende domein van het eiwit. Voorafgaand onderzoek van de groep had aangetoond dat een bepaalde WRKY transcriptiefactor in de tabaksplant, NtWRKY12, een rol speelt bij de expressie van *PR-1*. Ook tabaks NtWRKY12 heeft een lysine in het DNA-bindingsdomein en van alle Arabidopsis WRKY's heeft AtWRKY50 de hoogste homologie met NtWRKY12. Om te zien of deze lysine bepalend is voor het vermogen *PR-1* expressie te activeren, is ook onderzocht of de twee andere WRKY factoren van Arabidopsis met een lysine in plaats van glutamine in het DNA-bindingsdomein *PR-1* kunnen activeren. Dat bleek niet het geval. De expressie van het *AtWRKY50* gen zelf bleek na behandeling van planten met SA te worden geactiveerd en deze activering ging iets vooraf aan die van *PR-1*, wat een functie van AtWRKY50 als activator van *PR-1* expressie ondersteunt. Vervolgens is met behulp van *electromobility shift assays* (EMSA) de bindingssequentie van AtWRKY50 in de *PR-1* promoter onderzocht. AtWRKY50 bleek te binden aan de DNA sequentie GACT(G)TTTC, die op twee plaatsen in de promoter voorkomt. Een van deze

plaatsen bleek overeen te komen met een sequentie die uit eerder onderzoek was gebleken noodzakelijk te zijn voor geïnduceerde expressie; de andere plaats overlapte gedeeltelijk met een W-box (TTGACT), waarvan eerder was gevonden dat deze een remmende werking op de expressie had. Het feit dat in de bindingsproeven alleen het C-terminale domein van AtWRKY50 in staat was aan het DNA te binden en niet het volledige AtWRKY50, suggereert dat in vivo, de configuratie van het eiwit zodanig wordt gemodificeerd dat de N-terminale helft van AtWRKY50 het C-terminale DNA-bindingsdomein niet kan afschermen.

De bindingsplaatsen van AtWRKY50 in de *PR-1* promoter liggen op korte afstand van twee bindingsplaatsen voor TGA transcriptiefactoren. Een dergelijke topografie bestaat ook in de tabaks *PR-1* promoter. Dit suggereert dat de WRKY en TGA eiwitten op de promoter wellicht een interactie aangaan, zoals ook is gevonden bij NtWRKY12 en tabaks TGA2.2. Dat dit inderdaad het geval is werd aangetoond in **Hoofdstuk 3**. In het *yeast two-hybrid* systeem bleek dat AtWRKY50 een eiwit-eiwit interactie aanging met TGA2 en TGA5 van Arabidopsis. Dit resultaat kon worden bevestigd met behulp van *bimolecular fluorescence complementation* (BiFC) experimenten in protoplasten van Arabidopsis, waaruit bovendien bleek dat deze interactie plaats vond in de celkern. DNA bindingsproeven met gezuiverd TGA2 en TGA5 toonden aan dat deze eiwitten voornamelijk bonden aan een van de twee veronderstelde TGA bindingsplaatsen in de promoter van *PR-1*, terwijl bij combinatie van TGA2 of TGA5 en het C-terminale domein van AtWRKY50 beide eiwitten tegelijk aan het promoter DNA bonden. Tevens kon uit de experimenten worden afgeleid dat de combinatie van intact AtWRKY50 met TGA2 of TGA5 de binding van beide eiwitten aan de promoter verhoogt. Tenslotte bleek uit co-expressie experimenten in protoplasten dat TGA2 en TGA5 zelf nauwelijks *PR-1* genexpressie activeerden, maar dat ze een sterk synergistisch effect hadden op

de activering door AtWRKY50. Deze resultaten ondersteunen een model waarin AtWRKY50 en TGA2 en TGA5 samenwerken in de regulatie van de *PR-1* expressie.

Zoals boven beschreven, geldt de W-box als consensus WRKY bindingsplaats, waar veel WRKY eiwitten aan kunnen binden. Uit het onderzoek beschreven in de voorgaande hoofdstukken bleek echter dat AtWRKY50 niet bond aan de W-box, maar aan een element dat er gedeeltelijk mee overlapt. In **Hoofdstuk 4** is onderzocht of andere WRKY eiwitten van Arabidopsis aan deze W-box in de *PR-1* promoter konden binden en mogelijk een effect hadden op de expressie. AtWRKY28 was in eerder onderzoek van de groep gekarakteriseerd als transcriptiefactor betrokken bij de expressie van een gen dat codeert voor een SA biosynthese enzym. In de WRKY screening beschreven in Hoofdstuk 2 was al gebleken dat AtWRKY28 ook *PR-1* expressie activeerde. Door middel van EMSA bindingsexperimenten werd aangetoond dat AtWRKY28 inderdaad bond aan de W-box naast de bindingsplaats van AtWRKY50. AtWRKY42, de op één na sterkste activator van *PR-1* in bovenvermelde screening, en AtWRKY46 bonden echter niet aan deze W-box. AtWRKY28 bond bovendien aan een tweede W-box in de *PR-1* promoter waarvan eerder was gevonden dat deze een effect had op de genexpressie. Transactivatie experimenten in protoplasten bevestigden dat beide W-boxen nodig zijn voor activering van de *PR-1* expressie door AtWRKY28. Een mogelijke rol van AtWRKY28 in de *PR-1* expressie werd verder ondersteund door de vaststelling dat het *AtWRKY28* gen wordt geïnduceerd door SA en dat dit voorafgaat aan de SA-geïnduceerde *PR-1* genexpressie.

In **Hoofdstuk 5** worden de effecten beschreven van overexpressie van AtWRKY50 en AtWRKY28 in Arabidopsis getransformeerd met constructen waarin de coderende sequenties van de WRKY's onder controle staan van de sterke constitutieve *35S* promoter van Bloemkoolmozaïekvirus. De hoge

constitutieve expressie van AtWRKY50 resulteerde in een hogere accumulatie van *PR-1* mRNA dan in wild type planten, maar alleen wanneer de planten waren behandeld met SA; zonder SA bleek de *PR-1* expressie niet verhoogd in de AtWRKY50 overexpressor planten. Dit toont aan dat AtWRKY50 in zijn eentje de *PR-1* expressie niet kan induceren, maar dat na een SA-afhankelijke inductie, AtWRKY50 een hoog niveau van *PR-1* expressie ondersteunt. AtWRKY28 had een tegengesteld effect. SA-behandeling van AtWRKY28 overexpressor planten resulteerde in een lagere expressie dan in wild type planten. Dit suggereert een rol van AtWRKY28 als repressor van *PR-1* expressie, mogelijk als gevolg van zijn binding aan de W-box die eerder was gevonden een negatief effect te hebben op de expressie van *PR-1*. Infectietesten met de transgene overexpressor planten en met T-DNA knock-out mutanten waarin het *AtWRKY50* gen was uitgeschakeld, brachten geen duidelijke effecten aan het licht van de respectievelijke WRKY's op resistentie tegen de biotrofe pathogene bacterie *Pseudomonas syringae* en de necrotrofe schimmel *Botrytis cinerea*.

Hoofdstuk 6 tenslotte, beschrijft onderzoek gedaan naar de effecten van een aantal WRKY's op het metaboloom. Daarvoor werden transgene WRKY overexpressor planten met behulp van ^{1}H NMR spectroscopie geanalyseerd. Uit multivariate data analyses van de NMR gegevens, zoals *principal component analysis*, *hierarchical cluster analysis* en *partial least square-discriminant analysis* bleek dat het metaboloom van AtWRKY50 overexpressor planten aanzienlijk verschilde van dat van wild type planten en de meeste andere WRKY overexpressor planten. Naast verschillen in de hoeveelheid van sommige suikers en aminozuren, waren met name sinapinezuur en sinapoyl glucose 2 tot 3 keer verhoogd in de AtWRKY50 overexpressor planten. Derivaten van sinapinezuur en andere hydroxy-kaneelzuren vormen componenten van lignine en het is aannemelijk dat een verandering in de relatieve hoeveelheden

van deze verbindingen gevolgen heeft voor de lignine structuur. Of AtWRKY50 een rol speelt in dergelijke stress-geïnduceerde lignine modificaties, bijvoorbeeld door de regulatie van de expressie van genen coderend voor enzymen betrokken bij lignine synthese, verdient nader onderzoek.

ACKNOWLEDGEMENTS

All praises to Allah Almighty who created the universe and knows whatever is in it, hidden or evident, and who bestowed upon me the intellectual ability and wisdom to search for its secrets.

Starting from the beginning, I want to thank the Higher Education Commission (HEC) of Pakistan, whose scholarship showed me the way to the Clusius lab in Leiden. There I got an acceptance from Prof. Dr. Johan Memelink, who opened the doors of his lab and to whom I am thankful for the opportunity to learn science in Leiden. The Netherlands Organization for International Cooperation in Higher Education (NUFFIC) is greatly acknowledged for managing my scholarship and health insurance during my stay in the Netherlands.

I feel much pleasure in expressing my heartiest gratitude to my ever-affectionate supervisor Dr. Huub Linthorst for his dynamic and friendly supervision and whose inspiring attitude made it very easy to undertake this project. It was only through his guidance that my efforts were fruitful.

I express my heartiest and sincerest gratitude to my lab colleagues Marcel, Ana, Hongtao, Dr. Guillaume, Dr. Fang, Meiliang, Karel, Dalia, Kaixuan and Yingjie for their valuable suggestions, sympathetic attitude, and work discussions during the course of my studies. Some experiments would not have been possible without the technical help of the very experienced professionals Ward and Gerda. I especially appreciate your help to turn long hours of work into a very pleasant experience.

To me, there is nothing better than having good friends, so great pride is with me while thanking my friends in and outside Leiden: Aamir Aslam, Aamir Bhai, Farooq, Imran, Kashif, Khurrum, Majid, Muzamal, Nadeem, Qammar, Shabaz, Soban Bhai, Sohail, Tariq Bhai, Yahya, and Waseem Bhai. I would like to thank all the Pakistani families in Leiden for their friendly company and for offering nice dishes away from home. Special thanks to

Hasnain Bhai for his care in and outside the lab and to his wife for taking care of all the good food. I want to say special thanks to my all-time blooming flowers, Muhammad Khurshid, Muhammad Tayyab Akhtar and Zeshan Hassan for their good wishes, inspiring and excellent cooperation and encouraging attitude. When back in Pakistan, I would like to thank Jam Tayyib, Mian Mehmood, Adnan Akhter, Qaiser Farid and Ikram Anwar for their true and long lasting friendship.

Words are lacking to express my humble obligation for my affectionate sisters for their sacred prayers and gracious behavior. I would like to say special thanks to my special friend in the USA for nice and valuable gossips, caring and cheering behave, and ever ready smiling face.

Last but certainly not least, deepest appreciations are extended to my beloved Parents, for my success is really the fruit of their devoted prayers and encouragement. I am greatly indebted and submit my earnest thanks to my brother, Jawad. May Almighty Allah bless them with good health and prosperous long lives and be a source of prayer for me.

Rana Muhammad Fraz Hussain

CURRICULUM VITAE

Rana Muhammad Fraz Hussain was born on December 08, 1986 in Rahim Yar Khan, Pakistan. He attended primary school and high school at Tamer-e-Millet high school in R.Y. Khan. In 2000 he started at Khawja Fareed College in R.Y. Khan. In 2003 he started his bachelor honours in Agriculture at the University of Agriculture Faisalabad (UAF). In January 2006 he started a six-month internship at the Soil Salinity Research Institute (SSRI) in Pakistan, where he worked on soil analysis, the effects of nutrients on wheat, and experimental design. He received his B.Sc. (HONS) Agri. degree from UAF in June 2006. After obtaining his Bachelor degree he joined the National Institute of Biotechnology and Genetic Engineering (NIBGE) for his Master studies in biotechnology, where he finished the course work of his Masters. In September 2007 he was awarded an overseas PhD scholarship from the Higher Education Commission of Pakistan. He started his first year with an internship as a Master student in plant cell physiology with a research project on transcription factors involved in defense gene expression in Arabidopsis under the supervision of Dr. Huub Linthorst at Leiden University, the Netherlands. From October 2008 until September 2012 he worked as a PhD student under the supervision of Dr. Huub Linthorst at the Institute of Biology (IBL) at Leiden University in the Netherlands.