CHAPTER 1

GENERAL INTRODUCTION

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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.

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