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Marcel Cristiaan van Verk

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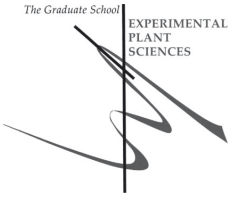

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Transcriptional Regulation of Plant Defense Responses

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ABSTRACT

Plants possess constitutive as well as inducible defense systems to oppose attack by pathogens and herbivores. Signal-transduction pathways mediated by the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are involved in regulating appropriate defense responses. Extensive cross-talk between these different signal-transduction pathways allows the plant to fine-tune its defenses against different types of pathogens and insect attackers. This review presents brief overviews of the separate JA, ET, and SA signal-transduction pathways, followed by a description of the main classes of transcription factors involved in defense gene activation. The last part is devoted to recent work highlighting the regulation of plant defense responses by transcriptional reprogramming at the chromosomal level.

I. PLANT IMMUNE SIGNALING PATHWAYS

As plants are sedentary organisms, they possess elaborate mechanisms to defend themselves against attack by pathogens and pests. Successful defense relies on early recognition of the attackers and activation of appropriate defense responses. Different defense strategies have evolved against biotrophic and necrotrophic pathogens and insect attack. Defense against biotrophic pathogens is typically mediated by a signal-transduction route in which the endogenous plant compound salicylic acid (SA) plays a prominent role (Dong, 1998). In contrast, attack by necrotrophic pathogens and herbivorous insects triggers a signal-transduction pathway that is characterized by the signal molecule jasmonic acid (JA) (Howe, 2004). 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) produced upon attack (Koornneef and Pieterse, 2008; Leon-Reyes *et al.*, 2009; Reymond and Farmer, 1998; Spoel and Dong, 2008).

Although this system of interacting signaling pathways may underscore the ability of the plant to specifically, efficiently, and effectively cope with the multitude of biotic threats from its environment, it is evident that the immense complexity of this signaling network stands in the way of an easy and clear-cut understanding of how exactly plant defense works. Nevertheless, the end result of the signal transduction is the induced production of defense proteins that directly or indirectly inhibit proliferation of the attacker. Upon infection or attack, various defense responses are induced, requiring these proteins to be newly synthesized. Figure 1 shows that in *Arabidopsis thaliana*, each of the signal-transduction pathways acts to activate a distinct set of defense genes. Marker genes are specifically expressed via a single pathway, as for example *VEGETATIVE STORAGE PROTEIN 1* (*VSP1*) by JA, *GLUTAREDOXIN 480* (*GRX480*) by SA, and *PATHOGENESIS-RELATED 3/CHITINASE B* (*PR-3/ChiB*) and

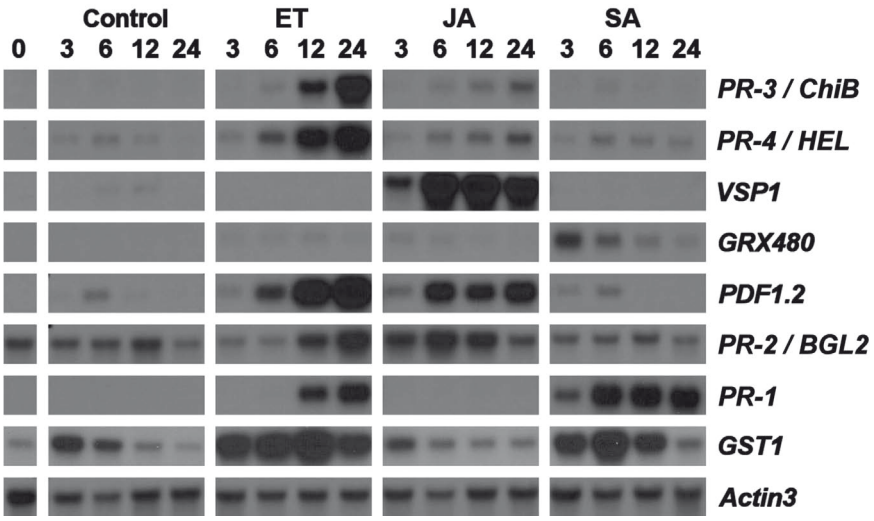


Fig. 1. Time course showing expression of ethylene-, jasmonic acid-, and salicylic acid-inducible defense-related genes in *Arabidopsis thaliana*. A total of five plants per time point per treatment were mock treated, or sprayed with 10 mM ethylene releasing ethephon (ET), 250 µm jasmonic acid (JA), or 5 mM salicylic acid (SA). After 3 h the plants were sprayed with tap water. Plants were harvested 3, 6, 12, or 24 h after treatment, after which RNA was extracted and loaded on denaturing agarose gels, subjected to electrophoresis and blotted. The blots were hybridized to cDNA probes corresponding to the various marker genes, as indicated. A cDNA probe for Actin 3 was used to check for equal loading. Abbreviations: *BGL, β-GLUCANASE; ChiB, CHITINASE B; GRX, GLUTAREDOXIN; GST, GLUTATHION-S-TRANSFERASE; HEL, HEVEIN-LIKE; PDF, PLANT DEFENSIN; PR, PATHOGENESIS- RELATED; VSP, VEGETATIVE STORAGE PROTEIN*.

PR-4/HEVEIN-LIKE (*HEL*) by ET. Others respond to two signals, like *PLANT DEFENSIN 1.2* (*PDF1.2*) to ET and JA, or *PR-1* and *GLUTATHION-S-TRANSFERASE 1* (*GST1*) to ET and SA. From the increase in mRNA levels upon signal molecule application, it is evident that gene expression must require specific transcription factors that are activated or produced at the end of the signaling pathways.

Without trying to cover all the details that have accumulated in the past decades concerning transcription factors involved in biotic stress responses, this chapter aims at providing a timely overview of the most important classes of transcription factors engaged in the defenses that are mediated through the three signal-transduction pathways described above. Obviously, the model species *Arabidopsis* plays a central role in this review, although, where appropriate, results from other plant species are also described.

II. DEFENSE SIGNALING REGULATORY COMPOUNDS

A. JASMONATE SIGNAL TRANSDUCTION

Induced defense against necrotrophic pathogens and herbivorous insects involves a signal-transduction pathway in which the plant hormone JA plays a central role. Jasmonates are oxylipins that have an important function in the positive regulation of secondary metabolites like alkaloids, glucosinolates, phenylpropanoids, and terpenoids. Three types of stress activate the JA-signaling pathway. In tobacco, it was shown that perception of a primary wound results in accumulation of JA and its methyl ester, MeJA. An important factor in positive regulation of JA biosynthesis upon wounding is the wound-inducible protein kinase (WIPK), a member of the class of mitogen-activated protein kinases (MAPK). When *WIPK* is impaired, accumulation of JA or MeJA upon wounding no longer occurs (Seo *et al*., 1995). Overexpression of *WIPK* leads to accumulation of JA and proteinase inhibitor 2 (PIN2) (Seo *et al*., 1999). In *Arabidopsis*, a similar MAPK, MPK4, which is also rapidly activated upon wounding and is involved in JA signal transduction, was found (Ichimura *et al*., 2000).

Mutant screens for phenotypes showing impaired characteristics of JA signaling or impaired resistance against a variety of biotic stresses have revealed a number of genes involved in JA biosynthesis and signal transduction. In the *fatty acid desaturase* triple mutant *fad3-2 fad7-2 fad8*, no production of α-linolenic acid occurs. α-Linolenic acid is the precursor for jasmonates that are synthesized via the octadecanoid pathway (McConn and Browse, 1996). This pathway consists of a number of steps of which most enzymes have been identified. The α-linolenic acid is oxygenated by lipoxygenases (LOXs) to 13-hydroperoxy-linolenic acid (13-HPOT), which is then released from chloroplast membranes by lipases. In the plastids, 13-HPOT is converted by allene oxide synthase (AOS) and allene oxide cyclase (AOC) to 12-oxo-phytodienoic acid (OPDA). Next, OPDA is transported to the peroxisomes, where it is reduced by OPDA-reductase 3 (OPR3) and further converted by three cycles of β-oxidation by acyl-CoA oxidase (ACX), multifunctional protein (MFP), and 3-ketoacyl-CoA thiolase (KAT) to JA (Wasternack, 2007). JA can be metabolized in the cytoplasm to several derivatives. The best characterized are the volatile MeJA, synthesized by JA carboxyl methyltransferase (JMT) (Seo *et al*., 2001), and JA-amino acid conjugates, synthesized by the AMP-transferase activity of JA conjugate synthase Jasmonate resistant 1 (JAR1) (Staswick and Tiryaki, 2004). Active signaling molecules are the JA precursor OPDA, JA itself and its derivatives MeJA, and JA-isoleucine (JA-Ile). JA regulates its own synthesis positively by stimulating the expression of most of the JA biosynthetic genes. Overexpression of ORA47, an APETALA2/Ethylene-response factor (AP2/ERF) type transcription factor, results in an increased amount of the JA precursor OPDA. This accumulation of OPDA is caused by the enhanced expression of various JA biosynthetic genes (*LOX2, AOS, AOC2*) by ORA47, which is induced by JA, and therefore, having a positive feedback regulatory role for JA biosynthesis (Pré, 2006). Treatment of plants with JA overcomes mutations in any of the biosynthetic genes. The *constitutive expression* of VSP1 mutant (*cev1*) acts at an early step in the JA and ET signal-transduction

pathways, overproducing JA and ET and displaying constitutive expression of JA-inducible genes (Ellis *et al.*, 2002). *CEV1* encodes a cellulose synthase, indicating the involvement of the cell wall in defense responses.

Root growth is inhibited by JA. Therefore, it is a useful selection feature for mutant screens to identify genes involved in JA signaling. In an *ethylene-insensitive* (*ein3*) mutant background, where ET is not able to repress JA-regulated responses to stress, five *JA-insensitive* (*jai1-5*) mutants were identified (Lorenzo *et al.*, 2004). JAI1, also known as JIN1, encodes the basic helix-loop-helix (bHLH) transcription factor AtMYC2, which is rapidly induced by JA. AtMYC2 controls two main branches of JA signaling. The first branch consists of genes that are activated by AtMYC2 in the systemic responses to wounding or chewing herbivores. The second branch results in repression of genes involved in defense responses against pathogens and in this way acts as an integrator of different environmental stress responses (Lorenzo *et al.*, 2004). The JAI2 locus corresponds to JAR1, as described above.

In a screen for *Arabidopsis* mutants insensitive to coronatine and MeJA-mediated growth inhibition, the mutant *coronatine insensitive 1* (*coi1-1*) was isolated (Feys *et al.*, 1994). *COI1* encodes a JA-receptor protein that is required for responses mediated through JA-Ile. Coronatine is an effector produced by several pathovars of *Pseudomonas syringae* (Mitchell and Young, 1978). An example of a fungus that directly triggers *COI1* without inducing the JA biosynthetic pathway is *Fusarium oxysporum*. It has been proposed that *F. oxysporum* is capable of producing an oxylipin-like chemical just as *Ps. syringae* (Thatcher *et al.*, 2009). By mimicking JA-Ile it induces JA signal transduction resulting in suppression of SA-mediated defense against the biotrophic *Ps. syringae*. The *COI1* gene was mapped to a small region of the genome and located by complementation. *COI1* corresponds to the *JAI5* locus. The amino acid sequence of the COI1 protein contains an F-box motif and has similarity with Transport inhibitor response 1 (TIR1), an F-box protein that is part of an SCF complex and functions as an auxin receptor (Ruegger *et al.*, 1998; Xie *et al.*, 1998). F-box proteins associate with Cullin (Cul1), Rbx1, and Skp1 to form an Skp, Cullin, F-box (SCF) complex, also known as E3 ubiquitin ligase. The F-box protein in this complex functions as a receptor to target interacting proteins to be ubiquitinated and degraded by the 26S proteasome. *In vivo* interaction of the COI1 F-box protein with Cul1, Skp1, and Rbx1 was shown by co-immunoprecipitation, linking COI1 to the SCF complex. Genetic and molecular analyses show the involvement of *AUXIN RESISTANT 1* (*AXR1*), *CONSTITUTIVE PHOTOMORPHOGENIC 9* (*COP9*) and *SUPPRESSOR OF THE G2 ALLELE OF SKP1 VARIANT B* (*SGT1b*) (corresponding to the *JAI4* locus) as regulators of the SCF complex. Mutations in these regulators result in a reduced response in JA signaling (Feng *et al.*, 2003; Lorenzo and Solano, 2005; Tiryaki and Staswick, 2002). The *coi1-1* mutant fails to express JA-regulated genes and is defective in resistance against necrotrophic pathogens and insects (Turner *et al.*, 2002). This indicates the importance of ubiquitination in the JA-signaling pathway. The function of COI1 is specific to the JA pathway, whereas other parts of the SCF^{COI1} complex (SGT1b/JAI4 and AXR1) are shared by other pathways.

A breakthrough in understanding how COI1 mediates JA signaling via the SCF^{COI1} complex came with the discovery of JA ZIM-domain (JAZ) repressor proteins. One member of this group, JAZ3

(corresponding to the *JAI3* locus), interacts directly with MYC2 and acts as a negative regulator of MYC2-dependent gene expression. JA-Ile produced after biotic stress or coronatine are proposed to bind the leucine-rich repeat (LRR) domain of COI1, thereby generating a high affinity-binding site for JAZ3. Polyubiquitinylation of JAZ3 by the SCF^{COI} complex results in its degradation through the 26S proteasome. The release of the JAZ3 repressor frees MYC2 to activate transcription of its target genes. As one of MYC2 target genes is *JAZ3*, this process constitutes a direct negative regulatory loop to dampen MYC2 activity in cells with low levels of JA (Chini *et al.*, 2007; Thines *et al.*, 2007).

B. ETHYLENE SIGNAL TRANSDUCTION

The simplest hormone in plants is the gaseous ET. ET is involved in various developmental processes, such as plant growth and fruit ripening. Besides these processes, ET is also involved in environmental stress signaling upon wounding or pathogen attack.

The biosynthesis of ET proceeds via a short pathway. First, methionine is activated by ATP through the action of methionine adenosyltransferase, resulting in S-adenosylmethionine (SAM). In the next step SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). Finally, production of ET from ACC is catalyzed by ACC oxidase (ACO). The biosynthesis of ET is regulated through a range of positive and negative factors. Formation of ACC is the rate-limiting step in the pathway. *Arabidopsis* contains nine *ACS* genes encoding three types of enzymes, which are under strict regulatory control. Enzymers encoded by *ACS2* and *ACS6* can be phosphorylated by MAPK 6 (MPK6). This phosphorylation stabilizes the protein, which results in increased ET production. Presumably, phosphorylation of ACS2 and ACS6 protects these proteins from recognition and breakdown by the 26S proteasome pathway (Liu and Zhang, 2004). ACS4, ACS5, and ACS9 are members of the second type of ACSs, while ACS7 comprises the third type. The type II ACSs are under control of *ETHYLENE OVERPRODUCER 1* (*ETO1*) and *ETO1-LIKE1* and *2* (*EOL1/EOL2*). ETO1 is a member of the Broad complex/Tramtrack/Bric-a-brac (BTB) proteins, and together with Cul3a/b and Rbx1 forms an E3 ubiquitin protein ligase. Binding of type II ACSs by ETO1 (and EOL1/EOL2) targets them for ubiquitination and degradation by the 26S proteasome pathway, thereby negatively regulating ET production. It is likely that type I ACSs can also be recognized by a BTB protein and, vice versa, that the type II enzymes can be phosphorylated to prevent them from being targeted for degradation (Christians *et al.*, 2009; Guzmán and Ecker, 1990; Wang *et al.*, 2004).

After production, ET is perceived by a group of (hybrid) histidine kinases that are membrane-bound ET receptors: Ethylene response 1 (ETR1)/Ethylene insensitive 1 (EIN1), ETR2, EIN4, Ethylene-response sensor 1 (ERS1) and ERS2 (Bleecker *et al.*, 1988; Chang *et al.*, 1993; Hua *et al.*, 1995, 1998; Roman *et al.*, 1995; Sakai *et al.*, 1998). Pull-down experiments and yeast two-hybrid interaction assays show that Constitutive triple response 1 (CTR1), a Ser/Thr kinase, is in the same signaling complex as ETR1 and can interact with ERS1 and ETR2 (Cancel and Larsen, 2002; Clark *et al.*, 1998; Gao *et al.*, 2003; Kieber *et al.*, 1993). CTR1 has a negative regulatory function on ET

signaling by actively suppressing the signaling pathway in the absence of ET. Upon binding of ET to the receptors, CTR1 is no longer capable of repressing EIN2. EIN2 is a membrane-bound protein that directly or indirectly prevents the key ET response transcription factors EIN3 and EIN3-like 1 (EIL1) to bind to EIN3 binding F-box protein 1 and 2 (EBF1, EBF2) that are part of a SCF E3 ligase complex ($SCF^{EBF1/2}$), with the result that EIN3 and EIL1 are no longer degraded through the 26S proteasome pathway (Binder *et al.*, 2007; Guo and Ecker, 2003; Potuschak *et al.*, 2003). EIN3 and EIL1 regulate the downstream targets of the ET-signaling pathway among which is the Ethylene-response factor 1 (ERF1) (Solano *et al.*, 1998). Besides directly targeting downstream targets, EIN3 and EIL1 also induce transcription of EBF1 and EBF2 (Konishi and Yanagisawa, 2008). This results in a negative feedback loop that targets EIN3 and EIL1 for degradation when ET levels decrease. This feedback loop is under control of EIN5, a 5'→ 3' exoribonuclease (XRN4) that acts downstream of CTR1. In the presence of ET, EIN5 promotes *EBF1* and *EBF2* mRNA decay, which allows the accumulation of EIN3 (Olmedo *et al.*, 2006).

In addition to being regulated by the proteasome pathway, EIN3 can, just like the ACSs, be stabilized by phosphorylation. This occurs via a MAPK cascade consisting of CTR1 (a MAPK kinase kinase), MKK9, and MPK3/MPK6. It remains unclear how CTR1, a dominant negative regulator, can positively activate the phosphorylation of EIN3 via MKK9—MPK3/MPK6 (Yoo *et al.*, 2008).

C. SA SIGNAL TRANSDUCTION

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, previously named avirulence (Avr) proteins, which are targeted (in)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. Examples are, for instance, the *Ps. syringae* effector AvrRps4, which is recognized by the *Arabidopsis* Toll/Interleukin1 receptor-nucleotide binding site-leucine-rich repeat (TIR-NBS-LRR) receptor RPS4 (Aarts *et al.*, 1998).

Innate immunity or basal defense has been found to have 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, or microbe-induced molecular patterns (MIMPs), that are released from the host by pathogen activity, function as elicitors that are recognized by LRR receptor kinases, that is, the flagellin receptor Flagellin sensing 2 (FLS2) and the EF-Tu receptor EFR (Kunze *et al.*, 2004; Mackey and Mcfall, 2006; Turner *et al.*, 2002; Zhao *et al.*, 2005). Subsequent signal transduction from the flagellin-activated FLS2 receptor involves MAPK cascades with intricate positive and negative regulation on the establishment of immunity (Asai *et al.*, 2002; Chinchilla *et al.*, 2007).

In *Arabidopsis*, the biosynthesis of pathogen-induced SA depends on iso-chorismate 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 K_1), which is an essential component of the plant's photosynthetic machinery (Verberne *et al.*, 2007; Wildermuth *et al.*, 2001). In noninfected 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 DEFICIENT 4* (*PAD4*) and *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*) encode proteins with similarity to lipases. *EDS1* is probably activated upon elicitor recognition by R gene-encoded cytoplasmic TIR-NB-LRR receptors (Wirthmueller *et al.*, 2007). How exactly this activation is linked to induction of SA biosynthesis is not known, however, heterodimerization of EDS1 and PAD4 and their nuclear localization may be important for subsequent steps in the signaling pathway (Feys *et al.*, 2001). Recently, it was found that *EDS1* expression is repressed by the Ca^{2+}/calmodulin-binding transcription factor Serine/threonine protein kinase 1 (AtSR1) binding to a conserved CGCG element in the *EDS1* promoter, indicating that SA levels are regulated by Ca^{2+} (Du *et al.*, 2009).

Situated downstream of *EDS1*, but upstream of SA synthesis 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. Furthermore, *EDS5* gene expression is also induced by treatment with exogenous SA, suggesting a positive feedback loop for enhanced SA production during the defense response (Nawrath *et al.*, 2002). Increased levels of SA induce a state of enhanced defensive capacity, called systemic acquired resistance (SAR), that confers broad-spectrum resistance to subsequent pathogen infection (Ross, 1961). *eds5* mutant plants cannot mount the SAR response and are unable to accumulate high levels of SA (Nawrath and Métraux, 1999).

The EDS5 protein has homology to bacterial multidrug and toxin extrusion (MATE) antiporters that export toxic compounds across the plasma membrane in a process energized by H^+ or Na^+ electrochemical gradients. EDS5 contains a number of transmembrane domains suggesting that the protein is membrane-localized and might likewise function as a transmembrane transporter of small compounds (Nawrath *et al.*, 2002). Chloroplast localization of EDS5 was recently confirmed by transient transformation experiments with *GREEN FLUORESCENT PROTEIN* (*GFP*)-tagged *EDS5* (Ishihara *et al.*, 2008). Based on its homology to MATE transporters and the initial placement of the *EDS5* gene before *ICS1* in the SA signaling pathway, Nawrath *et al.* (2002) suggested that EDS5 functions as a transporter of precursors of SA synthesis. Alternatively, EDS5 could be the chloroplast-cytosol translocator of SA. This would equally rightfully explain the inability of the *eds5* mutant to mount the SAR response due to lack of sufficient SA in the cytosol.

AVR_{PPHB} SUSCEPTIBLE 3 (*PBS3*), of which the pathogen-induced expression is highly correlated

with *ICS1*, is acting downstream of SA. In the *pbs3* mutant accumulation of SA-glucoside and expression of *PR-1* are drastically reduced. PBS3 is a member of the auxin-responsive GH3 family of acyl-adenylate/thioester forming enzymes of which some have been shown to catalyze hormone–amino acid conjugation, like JAR1 in the JA pathway. This has led to speculation that SA–amino acid conjugates are involved in SA signal transduction (Jagadeeswaran *et al.*, 2007; Nobuta *et al.*, 2007).

Upon a local primary infection with a necrotizing pathogen, SAR primes distal tissues for defense against secondary infections (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 methyl transferase (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). Recently, it was found that in *Arabidopsis* MeSA is not the systemic signal for SAR. Instead, azelaic acid was identified as a mobile signal for priming defense, for which also the gene *AZELAIC ACID INDUCED 1* (*AZI1*) is required (Attaran *et al.*, 2009; Jung *et al.*, 2009).

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* genes 1 (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 like *PR-1*. 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. Recently, it was found that 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 and consequently rapid degradation by the proteasome. This will clear 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). Based on these results, NPR1 seems to act as a co-activator that is recruited to the promoter by interaction with TGA transcription factors (Rochon *et al.*, 2006). However, it still has to be considered 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 Whirly protein AtWhy1 is a transcription factor that is also involved in SA-dependent basal resistance. Interestingly, AtWhy1 acts independently of NPR1 (Després *et al.*, 2000).

In the *npr1-1* mutant not only the SA signaling pathway is disrupted, but also JA/ET signaling is affected, indicating a role of NPR1 in both SA and JA/ET signal transduction (Pieterse *et al.*, 1998).

III. TRANSCRIPTION FACTORS REGULATING PLANT DEFENSE GENE EXPRESSION

As indicated in the above sections, the transcription factors involved in the various defense pathways mostly belong to five main groups. Many transcription factors involved in JA and ET signal transduction are members of the AP2/ERF group, while for example, SA signal transduction involves mostly WRKY and bZIP members. Venn diagrams have been created using publicly available microarray datasets of SA-, ET-, and MeJA-treated *Arabidopsis* plants (Fig. 2). Although the numbers of genes of which the expression was found to change are rather small, it is evident that considerable overlap occurs in the induction characteristics of these transcription factors. This overlap allows integration of different signals and, thereby, the fine-tuning of plant defense responses to attackers activating different signal transduction pathways. The diagrams show that there is no strict correlation between signaling pathway and transcription factor type and that transcription of the genes encoding these transcription factors can be either up- or downregulated by the treatments. In the next sections these main types of transcription factors are being discussed.

A. AP2/ERF TRANSCRIPTION FACTORS

With over 140 predicted members in *Arabidopsis* the AP2/ERF family of plant transcriptional regulators is one of the largest. AP2/ERF transcription factors are characterized by a 58- to 60-amino acid DNA-binding domain first identified in APETALA2 (AP2) and the Ethylene-response factors (ERF) (Jofuku *et al*., 1994; Ohme-Takagi and Shinshi, 1995). It has been demonstrated that members of this family have important functions in a broad range of biological processes, from growth and development to the response to environmental stimuli (Nakano *et al*., 2006, and references therein). Within the AP2/ERF family, members can be divided into AP2-like transcription factors containing two AP2 domains, and ERF-like factors with a single AP2 domain. In the last subfamily, the proteins Related to ABI/VP1 1 (RAV1) and RAV2 are classified as a separate group because in addition to the AP2 domain they contain a second DNA-binding domain, B3. Both domains bind autonomously to the DNA motifs CAACA and CACCTG, respectively, and together they achieve a high DNA-binding affinity of the protein (Kagaya *et al*., 1999). The other ERF-like members are separated into a class that is responsive to drought and/or low temperature. They bind the *C-repeat* (*CRT*) or *dehydration-responsive element* (*DRE*) in the promoters with the core sequence CCGAC. The other class of AP2/ERF proteins with a single AP2-domain is responsive to ET and bind *ethylene-response elements* (*ERE*), also known as the GCC-box (GCCGCC) (Allen *et al*., 1998). The GCC-box is found in many promoters of biotic stress genes that are inducible by ET. The GCC-box also occurs in the promoters of SA-inducible PR genes, indicative of possible cross-talk between ET and SA.

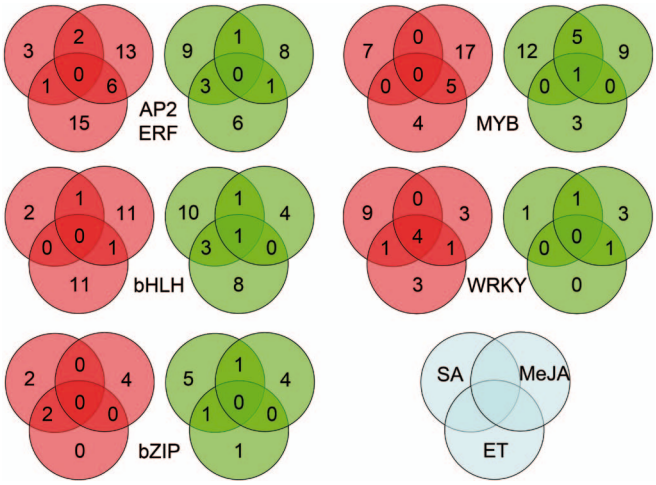


Fig. 2. Venn diagrams of transcription factor-encoding genes in *Arabidopsis thaliana* responsive to treatment of the plants with salicylic acid (SA), methyl jasmonate (MeJA), or ethylene (ET). Separate Venn diagrams for AP2/ERF-, bHLH-, bZIP-, MYB-, and WRKY-encoding genes were generated. Transcription factor gene IDs were obtained from the Database of *Arabidopsis* transcription factors (DATF) (Guo *et al.*, 2005) and loaded in Genevestigator V3, where they were used in a bicluster analysis, sorted on stimulus, and analyzed using the bimax algorithm (Hruz *et al.*, 2008). Expression data from 3 h SA, 3 h ET, or 2 h MeJA treatment were selected and the numbers of upregulated or downregulated genes determined. For each set of genes the left diagrams (red) correspond to number of genes that are upregulated by the various treatments, the diagrams at the right (green) show the number of genes that were downregulated by the treatments.

AP2/ERF proteins are also involved in JA-inducible gene expression. *STRICTOSIDINE SYNTHASE* (*STR*) gene expression in *Catharanthus roseus* depends on the Octadecanoid-responsive *C. roseus* AP2/ERF transcription factors ORCA2 and ORCA3. These transcription factors bind to a GCC-like box that is a *JA- and elicitor-responsive element* (*JERE*) in the promoter of the *STR* gene (Menke *et al.*, 1999). STR is an important enzyme in the terpenoid indole alkaloid (TIA) biosynthetic pathway. TIAs offer the plant protection against pathogens and UV radiation (Meijer *et al.*, 1993; Van der Fits and Memelink, 2000). In *Arabidopsis* the subgroup of AP2/ERF transcription factors that are rapidly induced by JA is known as Octadecanoid-responsive *Arabidopsis* AP2/ERF (ORA).

Multiple ORA transcription factors have a key role in disease resistance and signaling. As described above, ORA47 plays a role in the positive feedback regulation of JA biosynthetic genes by JA. ORA37/AtERF4 acts as a negative regulator of various defense genes, including *PDF1.2*, *ChiB* and *β-GLUCOSIDASE 2* (*BGL2*) upon treatment with JA and/or ET (McGrath *et al.*, 2005; Pré, 2006; Yang *et al.*, 2005). Contrary to ORA37, ORA59 positively regulates expression of these defense genes, integrating both JA and ET signals. Overexpression of ORA59 results in increased resistance against the necrotrophic fungus *Botrytis cinerea*. Induction of defense genes *PDF1.2* and *HEL* in ORA59-overexpressing *Arabidopsis* is also observed in the *coi1-1* background, placing ORA59

downstream of COI1. Silencing of ORA59 using RNA interference (RNAi) results in reduced resistance against *B. cinerea*. Besides ORA59, also ERF1 has been reported to integrate JA and ET signals and to synergistically induce *PDF1.2* downstream of COI1 (Lorenzo *et al.*, 2003). Although ORA59 and ERF1 appear to have similar functions, RNAi-silenced lines of *ORA59* that still activate ERF1 upon application of JA or ET, nevertheless are impaired in *PDF1.2* expression, indicating an essential role of ORA59 in this signaling branch. Another difference between ERF1 and ORA59 is that after induction, ERF1 represses JA-related marker genes such as *VSP*, while ORA59 does not (Pré *et al.*, 2008).

Apart from factors that activate gene expression by binding to the GCC-box (AtERF1, 2, and 5), the AtERF group of transcription factors also contains GCC-box-binding repressors (AtERF3, 4, and 7–12; cf. ORA37/AtERF4 described above) (Fujimoto *et al.*, 2000; Yang *et al.*, 2005). AtERF14 induces *PDF1.2* relatively late in comparison to ORA59 and ERF1; its expression is induced only by ET. This indicates that Arabidopsis AP2/ERF transcription factors can be divided into a group that integrates JA and ET pathways to activate defense gene expression, a group that selectively represses JA-responsive genes, and a group that induces gene expression through ET only (Onãte-Sánchez *et al.*, 2007).

Interactions of AP2/ERF proteins with other transcription factors may also play an important role in defense gene regulation. In a screen for interactors of the bZIP transcription factor TGA4, AP2/ERF transcription factor ERF72 was identified. ERF72 binds a GCC-box in the promoter of *PRB-1b*, encoding a basic PR-1 type protein from tobacco (Büttner and Singh, 1997; Sessa *et al.*, 1995). Another screen for interactors of the tomato Ser/Thr kinase Pto, the product of the *R* gene recognizing the *Ps. Syringae* effector AvrPto, using the yeast two-hybrid system resulted in several AP2/ERF proteins, which were named Pto-interacting (Pti) proteins Pti4, Pti5, and Pti6 (Tang *et al.*, 1996; Zhou *et al.*, 1997). ET treatment resulted in rapid induction of Pti4. Pti4 can be phosphorylated by the Pto kinase, enhancing its ability to activate defense gene expression (Chakravarthy *et al.*, 2003; Gu *et al.*, 2000). Overexpression of Pti4 in transgenic *Arabidopsis* resulted in increased gene expression of GCC-box containing *PR* genes (Wu *et al.*, 2002). That phosphorylation can be important for AP2/ERF transcription factor activity was also shown for the rice AP2/ERF transcription factor Ethylene-responsive element binding protein (OsEREBP1), which after phosphorylation showed an enhanced binding to GCC-boxes (Cheong *et al.*, 2003).

B. MYB TRANSCRIPTION FACTORS

The first MYB factor identified was v-MYB from *Avian myeloblastosis virus*. It probably originated by capture from a vertebrate gene, which was subsequently converted into an oncogene. Vertebrates contain three *Myb* genes (*c-Myb*, *A-Myb*, and *B-Myb*) that all have MYB DNA-binding domains. The MYB DNA-binding domain contains up to three repeats that each form a helix-turn-helix structure characterized by a series of regularly spaced tryptophan residues. In c-MYB there are three different versions of these repeats, referred to as R1, R2, and R3. Other MYB proteins are characterized based

upon their similarity with these repeats. MYB factors that have one repeat are referred to as MYBR1, MYB proteins with two repeats named R2R3-MYB, and proteins with three repeats named MYBR3. Plants have very large MYB families; for example, *Arabidopsis* contains 125 *MYB* genes. Most plant MYB factors belong to the R2R3 group, which is divided in two types that can bind different DNA sequences. Type I binds the DNA sequence (T/C)AAC(T/G)G, while type II binds to G(G/T)T(A/T)G(G/T)T (Eulgem, 2005; Stracke *et al.*, 2001). In *Arabidopsis*, only a few R2R3-MYB proteins are involved in defense-related pathways.

Many MYB transcription factors are involved in the regulation of the biosynthesis of both primary and secondary metabolites. As an example of secondary metabolites, the group of the glucosinolates contains important defense compounds against herbivores and microorganisms. There are two main branches in glucosinolate biosynthesis. One leads to the formation of aliphatic glucosinolates derived from methionine, while the other branch leads to indole glucosinolates, which are derived from tryptophan. MYB 28, also known as Production of methionine-derived glucosinolate 1 (PMG1), plays a key role in MeJA-induced biosynthesis of the aliphatic glucosinolates. In addition, MYB29/PMG2 has a modest role in regulation of this pathway (Hirai *et al.*, 2007). MYB34/*Arabidopsis* P450 reductase (ATR1) has a similar function for the tryptophan-derived glucosinolates as MYB29/PMG2 has for the methionine-derived glucosinolates (Celenza *et al.*, 2005). Pathways for other secondary metabolites are also regulated by MYB transcription factors. For instance, the flavonoid biosynthetic pathway is positively regulated by AtMYB75 (Borevitz *et al.*, 2000).

During the hypersensitive response (HR) to the bacterial pathogen *Xanthomonas campestris* pv. *campestris*, *AtMYB30* is activated early and independently of *NPR1*. Overexpression of *AtMYB30* results in a stronger HR response against avirulent bacterial pathogens and increases the resistance against a variety of bacterial pathogens, while silencing of *AtMYB30* in *Arabidopsis* using antisense lines strongly decreases the HR response against avirulent bacterial pathogens. This indicates a role for AtMYB30 in promoting HR-related cell death and resistance against bacterial pathogens (Daniel *et al.*, 1999; Raffaele *et al.*, 2006; Vailleau *et al.*, 2002). Besides resistance against HR-inducing pathogens, MYB transcription factors also play an important role in resistance against necrotrophic pathogens like *B. cinerea* and *Alternaria brassicicola*, for example, *BOTRYTIS SUSCEPTIBLE 1* (*BOS1*)/*AtMYB108*. This pathogen-induced MYB factor functions to reduce spread of the pathogen through the plant tissue. The expression of *AtMYB108* is severely impaired in the *coi1-1* mutant, indicating an important role for the JA-signaling pathway in this defense response (Mengiste *et al.*, 2003). *AtMYB72* has been shown to be an essential component of rhizobacteria-mediated induced systemic resistance (ISR). Induction of this gene in roots by nonpathogenic *Pseudomonas fluorescens* WCS417r bacteria is necessary for priming of systemic JA/ET-dependent defense responses against various pathogens (Van der Ent *et al.*, 2008).

Defense responses regulated by MYB transcription factors seem to cover all signaling pathways and act against many types of pathogens. MYB transcription factors also play roles in the defense response against insects. Caterpillars of the small cabbage white, *Pieris rapae*, induce local expression of *AtMYB102*. Overexpression of *AtMYB102* results in upregulation of a large number of genes that are involved in cell wall modifications. On T-DNA insertion lines lacking AtMYB102 the development

of *Pi. Rapae* proceeds faster than on wild-type plants. Possibly, plants lacking AtMYB102 can no longer support the induction of cell wall modifications that interfere with *Pi. rapae* feeding (De Vos *et al.*, 2006).

C. MYC TRANSCRIPTION FACTORS

The MYC family of transcription factors is part of a large transcription factor family that consists of more than 120 basic helix-loop-helix (bHLH) proteins, which has been intensively studied in mammals. The most characteristic attribute of the family is the presence of a bipartite bHLH domain consisting of about 60 amino acids. This domain contains a region with a large number of basic residues at the N-terminal side, which is involved in DNA binding. The HLH part located at the C-terminal part of the domain consists of two hydrophobic regions that play a role in homo-and/or heterodimerization. Binding to DNA occurs at the core DNA hexamer sequence CANNTG, named the E-Box or G-Box after the most frequently observed variant (CACGTG). Other less frequently occurring variants of these motifs are known as H-Box, N-Box, and Z-Box (Murre *et al.*, 1994; Toledo-Ortiz *et al.*, 2003). Of the current 162 annotated bHLH transcription factor genes in *Arabidopsis* not many have been studied to an extent that a function is known. The bHLH proteins that have been characterized function mainly in anthocyanin biosynthesis, phytochrome signaling, seed globulin expression, fruit dehiscence, and carpel and epidermal development (Buck and Atchley, 2003).

Only a limited number of bHLH transcription factors characterized so far have been found to be involved in defense against pathogens. One important member is conserved in many plant species and in *Arabidopsis* is named AtMYC2/JIN1, which plays a central role in both JA- and abscisic acid (ABA)-regulated signaling. AtMYC2 is induced by wounding and herbivory. The response to these types of stresses is mediated through the JA pathway and results in the induced expression of a subset of JA-responsive genes, including *VSP1*, *LOX*, and *THIONIN 2.1* (*THI2.1*). Upon infection with necrotrophic pathogens, genes like *PDF1.2*, *ChiB*, and *HEL* are regulated by both the JA- and ET-signaling pathways. AtMYC2 negatively regulates the induced expression of these genes. This negative regulation is suggested not to be a direct effect of AtMYC2 on the downstream targets, and might be caused by a negative regulation of the expression of transcription factors such as ERF1, that positively regulate these genes (Boter *et al.*, 2004; Dombrecht *et al.*, 2007; Lorenzo *et al.*, 2004; Reymond *et al.*, 2004). AtMYC2 is also important for ISR-associated priming for enhanced JA-responsive gene expression upon pathogen or insect attack (Pozo *et al.*, 2008).

D. BZIP TRANSCRIPTION FACTORS

bZIP transcription factors are characterized by their basic leucine zipper (bZIP) domain. 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).

Two of the 10 groups of bZIP transcription factors (Jakoby *et al.*, 2002) in *Arabidopsis* have been implicated to play a role in plant innate immunity. AtbZIP10, a member of group C, is a positive regulator of basal defense responses, *R* gene-mediated hypersensitivity, and reactive oxygen-induced cell death. AtbZIP10 is controlled by Lesions simulating disease resistance 1 (LSD1), a plant-specific zinc-finger protein that negatively regulates cell death by inhibiting nuclear translocation of AtbZIP10 (Kaminaka *et al.*, 2006). Likewise, the tobacco bZIP transcription factor BZI-1, which is related to *Arabidopsis* group C transcription factors, regulates cell death. Again, nuclear localization is regulated, in this case through the interacting ankyrin-repeat protein ANK1. In addition, *BZI-1* transcription is upregulated in response to pathogen attack and pathogen-induced phosphorylation of BZI-1-related proteins has been described (Kuhlmann *et al.*, 2003).

The second group of *Arabidopsis* bZIP transcription factors involved in innate immunity is group D, which harbors the 10 members of the TGA family of transcription factors. So far, six of them, TGA1, TGA2, TGA3, TGA4, TGA5, and TGA6, have been shown to be involved in defense responses against pathogen attack (Kesarwani *et al.*, 2007; Zhang *et al.*, 2003). The first TGA factor to be identified was TGA1a from tobacco, 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 was established (Després *et al.*, 2003; Zhang *et al.*, 1999).

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 uninduced 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 uninduced 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. However, more information is needed to unravel the in vivo function of TGA1 and TGA4 with respect to the regulation of SA-inducible genes.

The so-called class II TGA factors TGA2, TGA5, and TGA6 are not only known to activate gene expression in the presence of enhanced levels of SA, but they are also necessary for the negative cross-talk that is exerted by SA on the JA/ET pathway. A yeast two-hybrid screen of an *Arabidopsis* library with tobacco TGA2.2 as a bait identified Glutaredoxin 480 (GRX480) as an interactor of TGA factors. Overexpression of *GRX480* interfered with the induction of *PDF1.2* (Ndamukong *et al.*, 2007), indicating that this interaction is functional with respect to SA/JA/ET signaling.

Furthermore, TGA4 was found to interact with ERF72/AtEBP (see above). Recently, we 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 (M. C. van Verk and H. J. M. Linthorst, unpublished data). More details are described in the next section.

E. 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, and 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 in 1994 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 belong 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 coregulated 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, we identified tobacco NtWRKY12 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 *ISOAMYLASE 1* (*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 the 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 MAPK cascade (MEKK1–MEK1/MKK2–MAPK4), induced by challenge inoculation with *Ps. syringae* or treatment with flg22 leads to phosphorylation ofMap kinase substrate 1 (MKS1), through which WRKY33 and possibly WRKY25 are bound to MAPK4. Upon phosphorylation 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). We have found that transient overexpression of WRKY28 in *Arabidopsis* protoplasts leads to induction of a *β-GLUCURONIDASE* (*GUS*) reporter gene under control of the 1 kb *ICS1* upstream promoter region as well as elevated levels of endogenous *ICS1* mRNA. This may indicate a link between PAMP signaling and the biosynthesis of SA. From public databases it appears that WRKY28 is the only WRKY protein of which the expression is suppressed by both JA and ET. As the 1 kb *ICS1* promoter lacks a consensus W-box, WRKY28 probably exerts its effect through binding to a different sequence motif. A *WK-like box* (TTTTCCA) is present in the 1 kb upstream

region and this might function as the WRKY28 binding site (M. C. van Verk and H. J. M. Linthorst, unpublished data).

The *PBS3* gene is induced by avirulent strains of *Ps. syringae* and has an important role in SA accumulation (Nobuta *et al.*, 2007). By a similar approach as described above, we found that the 1 kb *PBS3* promoter directs reporter gene expression in *Arabidopsis* protoplasts upon transient expression of WRKY46. 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; M. C. van Verk and H. J. M. Linthorst, unpublished data).

A more direct link between defense responses and WRKY proteins is supported by the structure of WRKY52/Resistance to *Ralstonia solanacearum* 1 (RRS1). In addition to a WRKY domain, this protein contains a domain that is characteristic of TIR-NBS-LRR R proteins. In the nucleus, WRKY52 interacts with the *R. solanacearum* effector PopP2. Plants challenged with strains of *R. solanacearum* that lack the *popP2* gene are highly susceptible to the pathogen, indicating the importance of WRKY52 in resistance against this pathogen (Deslandes *et al.*, 2002, 2003). The barley R protein Mildew A (MLA) appears to interfere with the PAMP-inducible repressors of basal resistance HvWRKY1 and HvWRKY2. In this manner the repressor effect of the PAMP-induced WRKY genes is derepressed, thereby triggering basal defense responses (Shen *et al.*, 2007).

The important function of NPR1 in defense pathways is evident by the requirement of this cofactor for the development of SAR, ISR, and defense-related *PR* gene expression. Eight WRKY genes (*WRKY18*, *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 are known to have a function in the regulation of *PR* genes and in SAR. Negative regulators are WRKY58, a direct negative regulator of SAR, and WRKY38, which, similar to WRKY62, can activate repressors of *PR-1*. WRKY62 also acts in the cross-talk between SA and JA signaling by repressing downstream JA targets such as *LOX2* and *VSP2* (Kim *et al.*, 2008; 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). Together with 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 *Ps. syringae*, co-expression of WRKY40 or WRKY60 renders plants more susceptible to this pathogen (Xu *et al.*, 2006). WRKY70 and the 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, probably 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 (M. C. van Verk *et al.*, 2008). This is supported by our finding that in *npr1-1* mutant protoplasts NtWRKY12-induced *PR-1a* expression is still fully operative (M. C. van Verk and H. J. M. Linthorst, unpublished data). 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. We found that NtWRKY12 can specifically interact with tobacco TGA2.2 both *in vitro* and *in vivo*. This suggests a role of TGA2.2 in *PR-1a* expression as a recruiter of NtWRKY12 to the promoter or to stabilize its binding. A graphical summary of the various pathways from pathogen perception to transcriptional activation of defense gene expression as described in this chapter is presented in Fig. 3.

IV. REGULATION OF PLANT DEFENSES AT THE CHROMOSOMAL LEVEL

A. CHROMATIN MODIFICATIONS AND GENE EXPRESSION

Considering the large-scale transcriptional reprogramming events that occur in plants upon pathogen infection, a relevant question is whether chromatin structure is altered either as a means to regulate transcription or as a consequence of ongoing transcription.

Chromatin is the complex combination of DNA, RNA, and protein that makes up chromosomes. One of the functions of chromatin is to compact the DNA, but it also provides mechanisms to control gene expression. In the basic "beads on a string" structure, the DNA is wrapped around the nucleosomes, histone octamers consisting of two copies each of the core histones H2A, H2B, H3, and H4. Linker histones such as H1 and its isoforms are involved in chromatin compaction, as found in heterochromatin, which contains primarily nontranscribed DNA. Noncondensed nucleosomes without the linker histone resemble "beads on a string of DNA" and are typical for euchromatin, which contains regions with actively transcribed genes (Li *et al.*, 2007).

Chromatin structure is influenced by a number of different mechanisms including: methylation of cytosine residues residing in CpG sequences of the DNA; acetylation, phosphorylation, methylation, ubiquitination, and ADP-ribosylation of histones; incorporation of histone variants; histone eviction; and chromatin remodeling, which utilizes ATP hydrolysis to alter histone-DNA contacts (Li *et al.*, 2007). The combination of histone modifications at a promoter is called "histone code" (Strahl and Allis, 2000). The histone code is likely to have at least two roles: to provide heritable epigenetic marks and to facilitate reversible control over events on chromatin in real time. Some of these modifications are known to act as sites for recruitment of regulatory proteins and enzymes (code readers), that can

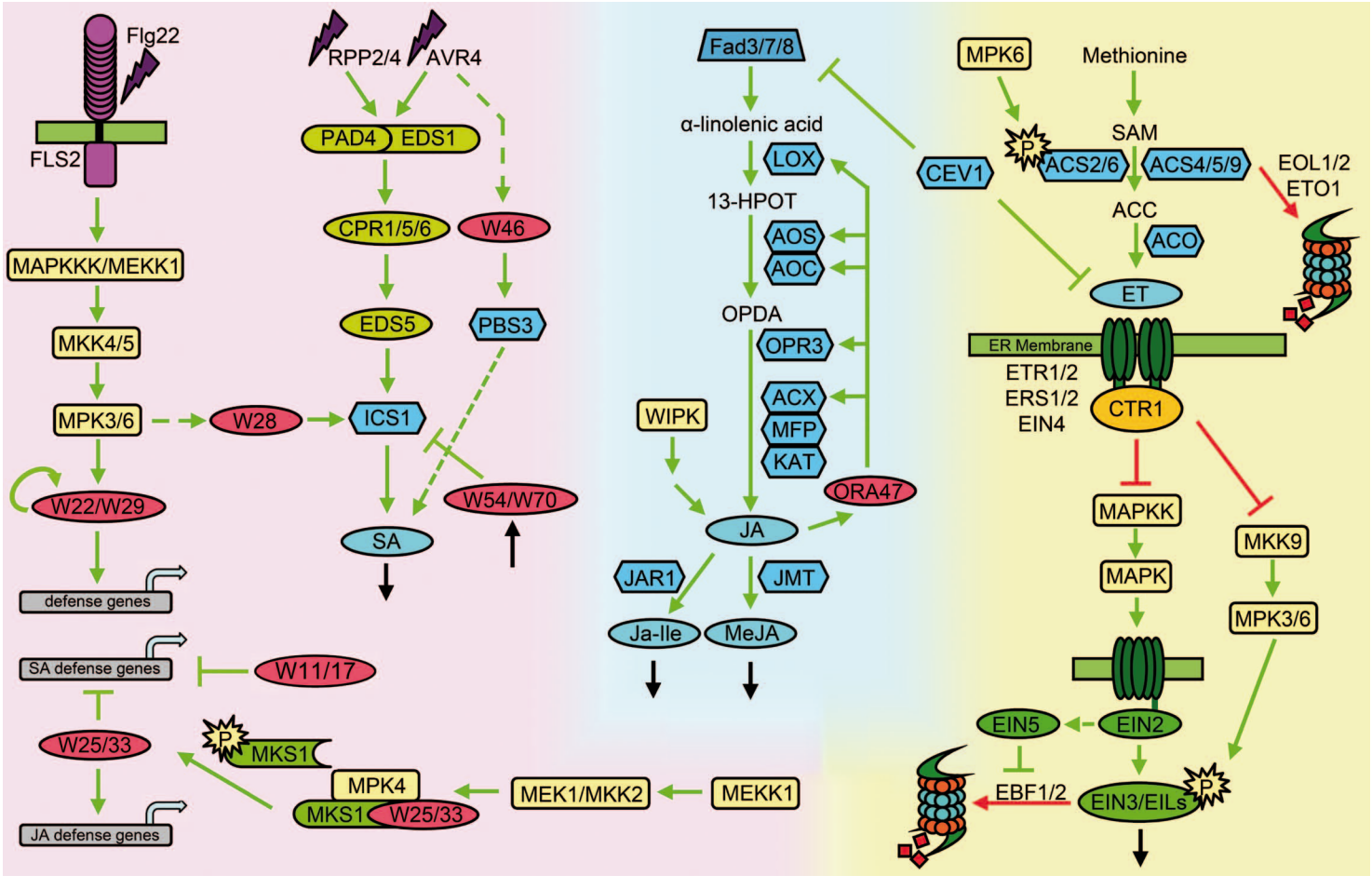
either repress or activate transcription (Seet *et al*., 2006).

The histone code is established mainly by the posttranslational modification pattern of the flexible N-terminal domains of histones H3 and H4 which protrude from the globular nucleosome. In general, transcriptionally inactive heterochromatin as well as silenced promoter regions are characterized by hypoacetylation of lysine residues in histones H3 and H4 and by methylation of lysines 9 and 27 at histone H3, and lysine 20 at histone H4 (Table I). Active genes are associated with hyperacetylation of lysine residues aswell as methylation of lysines 4, 36, and 79 on histone H3. Often, hyperacetylation of histones at promoter regions is necessary but not sufficient for transcriptional activation (Berger, 2002;Cosma, 2002;Narlikar *et al*., 2002;Ng *et al*., 2006). Modifications can be detected by chromatin immunoprecipitation experiments using commercially available antibodies directed against specifically modified histones.

Enzymes responsible for establishing/maintaining the steady-state balance of histone acetylation are histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Strahl and Allis, 2000). Histone methyl transferases (HMTs) and histone demethylases are responsible for reversible histone methylation (Mellor, 2006). A crucial question is how these enzymes are recruited to the promoters depending on the developmental stage or environmental conditions. Most likely, regulatory *trans* factors binding to specific *cis* elements function either directly or indirectly as anchor proteins. The resulting changes in chromatin structure can again be the prerequisite for binding of other transcription factors: For example, the yeast protein General
control nonrepressed protein 5 (GCN5), which exhibits histone acetyl transferase activity, is targeted to specific promoter regions by the transcriptional activator GCN4 (Kuo *et al*., 2000) under conditions of amino acid starvation. Subsequently, the relaxation of the chromatin structure allows recruitment of general transcription factors (Narlikar *et al*., 2002).

B. CHROMATIN MODIFICATIONS IN PLANTS

In plants, histone modifications have been demonstrated to be involved in the control of various developmental processes. Classical and well-studied examples are the chromatin modifications at the *FLOWERING LOCUS C* (*FLC*) in *Arabidopsis* and at the *β-PHASEOLIN* (*PHAS*) promoter in bean (*Phaseolus vulgaris*). Histone H3 and H4 acetylation as well as trimethylation of K4 of histone H3 at the *FLC* locus are associated with active *FLC* expression, whereas histone deacetylation and dimethylation at lysines K9 and K27 are involved in *FLC* repression after vernalization (Bastow *et al*., 2004; He and Amasino, 2005; He *et al*., 2004). Vernalization-induced changes in chromatin structure are "remembered" through subsequent rounds of replication of the meristem allowing *FLC* repression at higher temperatures following the cold period. Thus, histone modifications at the *FLC* locus constitute a heritable epigenetic mark. At the embryo-specific PHAS promoter, dimethylation of histone H4 at lysine K20 is suggested to contribute to the establishment of the heterochromatic *PHAS* chromatin. The transcription factor ABI3-like factor (ALF), which is only expressed in the embryo, may function as an anchor protein that promotes recruitment of histone acetyl transferases


Flg22
FLS2
MAPKKK/MEKK1
MKK4/5
MPK3/6
W22/W29
defense genes
SA defense genes
W11/17
W25/33
JA defense genes
RPP2/4
AVR4
PAD4
EDS1
CPR1/5/6
W46
EDS5
PBS3
W28
ICS1
SA
W54/W70
P
MKS1
MPK4
MKS1
W25/33
MEK1/MKK2
MEKK1
Fad3/7/8
α-linolenic acid
LOX
13-HPOT
AOS
AOC
OPDA
OPR3
ACX
MFP
KAT
ORA47
WIPK
JA
JAR1
JMT
Ja-Ile
MeJA
MPK6
Methionine
SAM
P
ACS2/6
ACS4/5/9
CEV1
EOL1/2
ETO1
ACC
ACO
ET
ER Membrane
ETR1/2
ERS1/2
EIN4
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MAPKK
MKK9
MAPK
MPK3/6
EIN5
EIN2
EBF1/2
EIN3/EILs
P

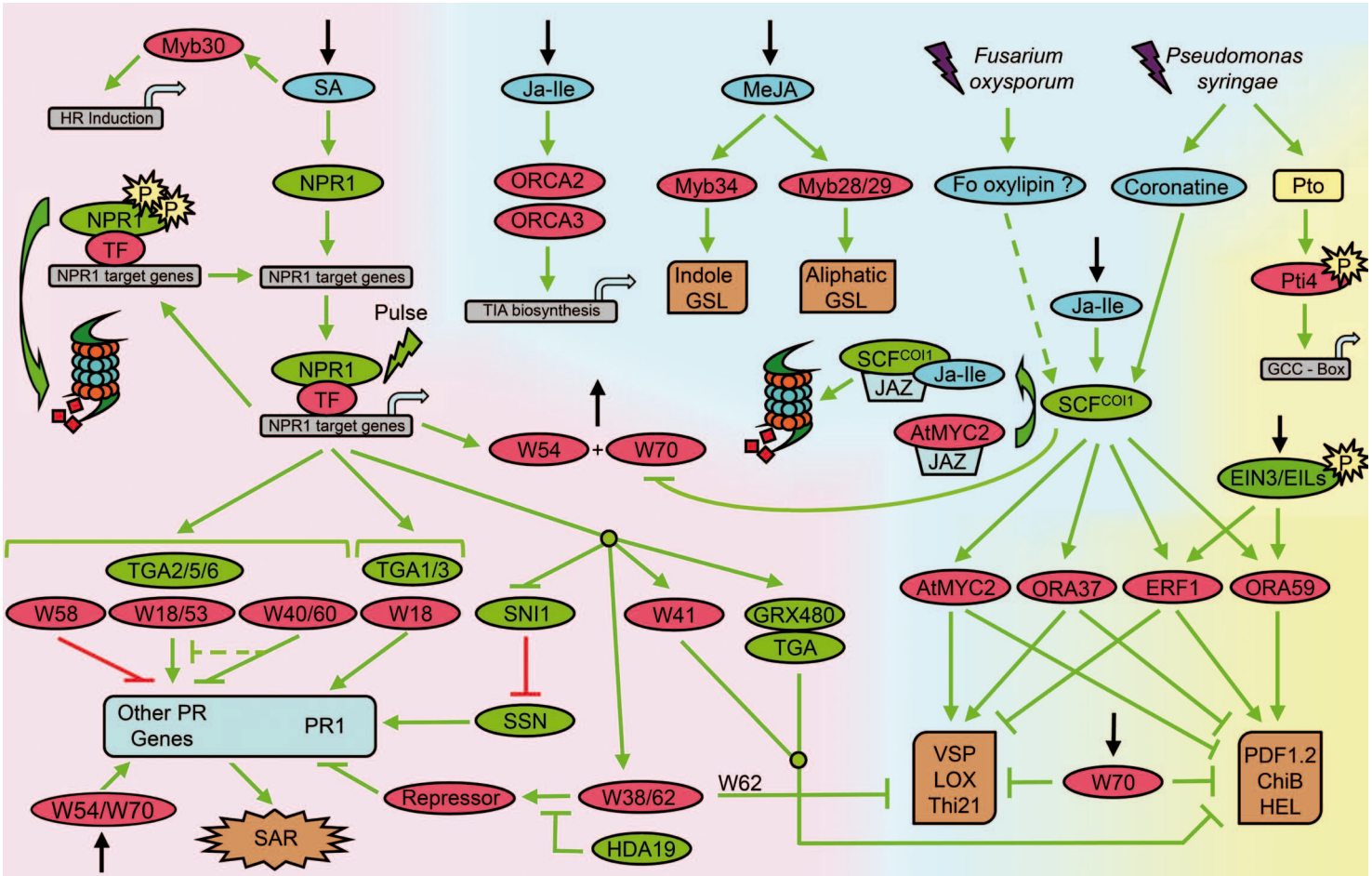

Fig. 3. Graphical summary of the pathways from pathogen perception to transcriptional activation of defense gene expression described in this chapter.

that acetylate H3-K9 and H4-K12 residues. However, this modification is not sufficient for transcription. Instead, it renders the *PHAS* promoter more accessible for the assembly of other factors after activation of the ABA signal transduction cascade leading to the activation of transcription (Ng *et al.*, 2006). In this situation, the histone code serves to "prime" promoters for rapid transcriptional activation.

C. CHROMATIN MODIFICATIONS AT PROMOTERS INVOLVED IN INNATE IMMUNITY

1. The SA pathway

As described in Section III.D, one of the best studied promoters that is being analyzed as a paradigm for understanding regulatory events of the innate immune response is the SA-inducible *PR-1* promoter from *Arabidopsis* (Lebel *et al.*, 1998). Increased methylation at lysine K4 and acetylation at lysines K9 and K14 of histone H3 are observed in the *sni1* mutant, which indicates that SNI1 is required for histone modifications related to transcriptional repression (Mosher *et al.*, 2006). In wild-type plants, K4 methylation and K9K13 acetylations can be induced after 48 h of treatment with the SA-analog benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH; synonym: acibenzolar *S*-methyl, ASM). However, using the same antibody (anti-dimethyl-histone H2 (Lys4), Alvarez-Venegas *et al.* (2007) were not able to detect significant changes in chromatin marks at the *PR-1* promoter after 24 h of SA treatment. Likewise, in our hands SA-induced changes on histone acetylation were not detected after 3 and 12 h of SA treatment when deploying a polyclonal antiserum against hyperacetylated lysines 5, 8, 12, and 16. Thus, chromatin modifications at the *PR-1* promoter might depend on the growth conditions and might not be essential for appropriate regulation.

Still, *PR-1* seems to be under the control of at least indirect effects on chromatin structure. For instance, its expression is reduced in *atx1* plants, which have a disruption at the *ARABIDOPSIS HOMOLOG OF TRITHORAX* locus (Alvarez-Venegas *et al.*, 2007). ATX1 carries a highly conserved SET domain. SET domain peptides (named after the three *Drosophila* proteins SUPPRESSOR OF VARIEGATION 3-9 [SU(VAR) 3-9], ENHANCER OF ZESTE [E(Z)] and TRITHORAX (TRX)) of the Trithorax-family can methylate K4 of histone H3 (Rea *et al.*, 2000) and functional analysis of the *atx1* mutant supports the notion that ATX1 is involved

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See text for details. Pointed arrows indicate positive regulation, blunted arrows indicate negative regulation. Green arrows: regulation under inducing conditions. Red arrows: Regulation under noninducing conditions. Dashed arrows: hypothetical regulation. Black arrow: pointing away—signal continues elsewhere in the figure, pointing towards—continued signal from elsewhere in the figure. Lightning arrow: elicitor. Blue hexagon: enzyme. Yellow rectangle: MAP kinase. Brown rectangle/star: downstream target. Yellow star with a P: phosphorylation. Blue ellipse: hormone/signaling molecule. Pink ellipse: transcription factor (TF, transcription factor; W, WRKY). Green ellipse: modulator of transcriptional activity.

in methylation of histones at specific promoters (Alvarez-Venegas *et al.*, 2007). *Ps. syringae* pv. *tomato*-induced expression of the *WRKY70* gene is reduced in the *atx1* mutant, which correlates with a decreased level of trimethylated H3-K4. As increased levels of WRKY70 lead to the constitutive accumulation of an abnormal *PR-1* transcript (Li *et al.*, 2004), the authors concluded that decreased WRKY70 expression in the *atx1* mutant leads to decreased *PR-1* expression. However, as the *wrky70* mutant is not affected in SA-induced *PR-1* transcription (Ren *et al.*, 2008), unknown effects of ATX1 on proteins regulating *PR-1* expression have to be postulated.

Ps. syringae pv. *tomato*-induced *PR-1* transcription is also reduced in the *hda19* mutant, which is deficient in a HDAC that physically interacts with WRKY38 and WRKY62 (Kim *et al.*, 2008). It was hypothesized that WRKY38 and WRKY62, which presumably activate transcription of negative regulators of *PR-1* expression, are inhibited by HDA19. The notion that increased histone acetylation (as mimicked by a mutation in a *HDAC* gene) leads to decreased *PR-1* expression is supported by pharmacological studies: treatment of plants with trochostatin A (TSA), a chemical that blocks histone deacetylation, also leads to reduced basal levels of *PR-1* expression (Chang and Pikaard, 2005). The target genes of WRKY38 and WRKY62 are likely candidates to be acetylated in hda19 mutants or in TSA-treated plants. When acetylated, their expression might be increased, which in turn would inhibit *PR-1* expression.

PR-1 expression has been analyzed in the *splayed* (*syd*) mutant, which has a defect in the SWI/SNF class chromatin remodeling ATPase SYD (Walley *et al.*, 2008). Chromatin remodeling complexes use the energy of ATP hydrolysis to move, destabilize, eject, or restructure nucleosomes and are thus of major importance to facilitate transcription (Clapier and Cairns, 2009). In contrast to wild-type plants, *syd* mutant plants show a strong induction of *PR-1* expression after infection with the necrotrophic fungus *B. cinerea*. As many JA-dependent responses, such as the expression of *PDF1.2* and *VSP2*, are compromised in the *syd* mutant, it is hypothesized that the negative cross-talk between JA and SA is affected by a SYD-dependent mechanism.

In addition to the *Arabidopsis PR-1* gene, the tobacco *PR-1a* gene has been studied by different groups (Buchel *et al.*, 1999; Grüner and Pfitzner, 1994; Grüner *et al.*, 2003; Uknes *et al.*, 1993). Like the *Arabidopsis PR-1* promoter, the tobacco *PR-1a* promoter contains a functionally important

TABLE I
Simplified Overview of the Histone Code

	Transcriptionally inactive heterochromatin	Transcriptionally active euchromatin
Acetylation	Low	High
Methylation	Dimethylation of H3-K9, H3-K27, H4-K20	Di- and trimethylation of H3-K4

TGA binding site (Strompen *et al.*, 1998) and its expression depends on TGA (Thurow *et al.*, 2005) and WRKY transcription factors (Van Verk *et al.*, 2008). Chromatin immunoprecipitation experiments unraveled increased histone H4 hyperacetylation at the *PR-1a* promoter at 3 h and even more at 12 h after SA treatment, which correlates with the kinetics of transcript accumulation (Butterbrodt *et al.*, 2006). In RNAi plants with reduced levels of TGA2.2, histone acetylation also increased after SA treatment, although the promoter was not activated. This indicates that histone acetylation is not sufficient for transcription. In addition, this histone hyperacetylation does not seem to be triggered by TGA2.2 binding. Indeed, it might even be the prerequisite for binding of TGA2.2 to the promoter, as TGA2.2 is recruited to the *PR-1a* promoter only after SA treatment. In contrast, constitutive histone acetylation and constitutive binding of TGA2.2 were observed at the truncated CaMV*35S* promoter that contains the TGA2.2 binding element *as-1* as the only regulatory *cis* element. The truncated CaMV*35S* promoter, which can be activated by SA independently from NPR1, is activated with faster kinetics (maximum at 3 h after SA treatment) than the *PR-1a* promoter (maximum after 12 h of SA treatment), which might be due to the fact that the chromatin of the truncated CaMV*35S* promoter is already in an "open (potentiated)" chromatin configuration, allowing constitutive binding of TGA2.2 and subsequent fast activation by an as yet unknown activation mechanism (Fig. 4).

2. The JA pathway

Few data are available with respect to the analysis of the histone code at JA-dependent promoters. No significant changes in the amount of acetyl groups were detected at the *PDF1.2* promoter when performing chromatin immunoprecipitation experiments with antibodies directed against diacetylated histone H3 (Koornneef *et al.*, 2008). However, chromatin remodeling seems to play a role in the positive regulation of JA-dependent genes. In the *syd* mutant background, *PDF1.2* expression is severely compromised after *B. cinerea* infection (Walley *et al.*, 2008), but recruitment of SYD to this promoter was not detected. Induction of the transcriptional activator *AtMYC2/JIN1* and its downstream gene *VSP2* was reduced in the *syd* mutant after wounding. Chromatin immunoprecipitation experiments suggest that these promoters are direct targets of SYD.

The *atx1* mutant, which is deficient in a putative HMT, reveals constitutive *THI1.2* expression, which is most likely due to the reduced expression of *WRKY70* and other genes that are direct targets of ATX1 (Alvarez-Venegas *et al.*, 2007). Interestingly, ectopic expression of the HDAC gene *AtHDAC19*, which is induced by JA, leads to increased expression of *ERF1* and its target genes (Zhou *et al.*, 2005). This suggests a similar indirect positive effect of decreased histone acetylation on defense gene expression as already observed for *PR-1*. Likewise, loss of function analysis of plants deficient in AtHDAC6 leads to reduced expression of JA-responsive genes such as *JIN1*, *ERF1*, *PDF1.2*, and *VSP2* (Wu *et al.*, 2008). As speculated for the *PR-1* promoter, hyperacetylation of a negative regulator might be responsible for the compromised JA response. AtHDAC6 interacts with the F-box protein COI1 which is of critical importance for all JA-mediated responses (Devoto *et al.*, 2002). A plausible model would be that COI1 activates AtHDAC6 by forming a complex which would lead to the

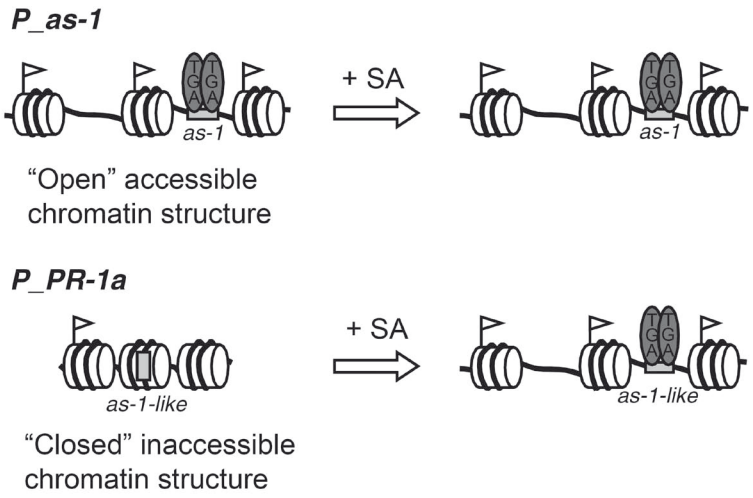

Fig. 4. Proposed model of the histone acetylation status and the status of TGA binding at the "immediate early" truncated CaMV *35S* (P-as-1) and "late" *PR-1a* promoter in tobacco as suggested by results of ChIP experiments. Histones at promoters that respond with a fast kinetics (within 3 hours) are constitutively acetylated, thus allowing binding of TGA2.2. At the same time, hypoacetylation of promoters which respond with slower kinetics to SA makes the DNA inaccessible to TGA2.2. After induction with SA, the histones at the *PR-1a* promoter get acetylated, leading to a decondensation of the chromatin, allowing binding of TGA2.2. The cylinders represent histone octamers, flags stand for acetyl groups and TGA binding sites are indicated by grey boxes. As hyperacetylation of histones often leads to a loss of histones (Reinke and Horz, 2003), the decondensed state of the chromatin in the presence of SA is schematically represented by three instead of four nucleosomes.

silencing of a general negative regulator of the JA response.

In conclusion, the increased susceptibility of *hda19* (Kim *et al.*, 2008), *atx1* (Alvarez-Venegas *et al.*, 2007), and *syd* (Walley *et al.*, 2008) plants indicates that chromatin structure plays a crucial role in pathogen responses. However, it seems that transcription of key regulators (e.g., WRKY70) is under direct control of chromatin modifications, whereas downstream genes like *PR-1* and *PDF1.2* are misregulated as a consequence of these primary events. The identification of promoters which show strong and robust changes in epigenetic marks under inducing conditions is a major goal that will help our understanding of the role of chromatin structure for innate immunity.

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2

A Novel WRKY Transcription Factor Is Required for Induction of *PR-1a* Gene Expression by Salicylic Acid and Bacterial Elicitors

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ABSTRACT

PR-1a is a salicylic acid-inducible defense gene of tobacco (*Nicotiana tabacum*). One-hybrid screens identified a novel tobacco WRKY transcription factor (NtWRKY12) with specific binding sites in the *PR-1a* promoter at positions -564 (box WK_1) and -859 (box WK_2). NtWRKY12 belongs to the class of transcription factors in which the WRKY sequence is followed by a GKK rather than a GQK sequence. The binding sequence of NtWRKY12 (WK box TTTTCCAC) deviated significantly from the consensus sequence (W box TTGAC[C/T]) shown to be recognized by WRKY factors with the GQK sequence. Mutation of the GKK sequence in NtWRKY12 into GQK or GEK abolished binding to the WK box. The WK_1 box is in close proximity to binding sites in the *PR-1a* promoter for transcription factors TGA1a (*as-1* box) and Myb1 (MBSII box). Expression studies with *PR-1a promoter::β-glucuronidase* (*GUS*) genes in stably and transiently transformed tobacco indicated that NtWRKY12 and TGA1a act synergistically in *PR-1a* expression induced by salicylic acid and bacterial elicitors. Cotransfection of *Arabidopsis thaliana* protoplasts with *35S::NtWRKY12* and *PR-1a::GUS* promoter fusions showed that overexpression of NtWRKY12 resulted in a strong increase in GUS expression, which required functional WK boxes in the *PR-1a* promoter.

INTRODUCTION

R-gene-mediated recognition of pathogens by plants typically results in a hypersensitive response (HR) mediated by generation of reactive oxygen species and the increased production of salicylic acid (SA). The HR is accompanied by the induction of local and systemic expression of numerous genes involved in defense. The *N*-gene-mediated resistance of tobacco (*Nicotiana tabacum*) to infection with *Tobacco mosaic virus* (TMV) represents a classical model to study expression of pathogenesis-related (PR) proteins and development of systemic acquired resistance (SAR) in plant-pathogen interactions (van Loon and van Strien, 1999). Tobacco PR proteins of classes 1 to 5 are subdivided into acidic, extracellular proteins and basic, vacuolar proteins. Generally, TMV-induced expression of acidic PR proteins is mediated by SA, whereas expression of basic PR proteins is mediated by ethylene (Bol *et al.*, 1990; Brederode *et al.*, 1991; Linthorst, 1991). Although the function of tobacco PR-1 proteins is not clear, these proteins are highly conserved in the plant kingdom and are widely used as markers in studies of signal transduction processes involved in plant pathogenesis and induced resistance.

Studies on expression of PR genes in Arabidopsis (*Arabidopsis thaliana*) and tobacco revealed the central role of protein NONEXPRESSER OF PR GENES1 (NPR1). NPR1 also mediates cross talk between the SA signaling pathway and the jasmonic acid and ethylene signaling pathways, and interacts with members of the TGA family of transcription factors that bind to *activator sequence-1* (*as-1*) or *as-1*-like elements that have been identified in promoters of *PR-1* genes (Durrant and Dong, 2004). Two *as-1*-like elements in the Arabidopsis *PR-1* promoter were shown to bind several of

the 10 TGA factors in Arabidopsis with different affinity (Lebel *et al.*, 1998; Johnson *et al.*, 2003). The two *as-1*-like elements in the promoter of the tobacco gene encoding the acidic PR-1a protein bind TGA1a. Mutation of these elements affected SA-induced expression of a GUS reporter gene in transgenic plants (Strompen *et al.*, 1998; Grüner *et al.*, 2003). In addition to TGA1a, the Myb1 protein has been shown to bind to the *PR-1a* promoter in tobacco. Expression of the *Myb1* gene was enhanced by TMV infection and application of exogenous SA, and the Myb1 protein preferentially bound to the MBSII sequence in the *PR-1a* promoter (Yang and Klessig, 1996). Silencing of Myb1 gene expression attenuated *N*-gene-mediated resistance to TMV (Liu *et al.*, 2004).

Accumulating evidence indicates that WRKY proteins are involved in differential responses to biotic stresses, either as transcriptional activators or as repressors in Arabidopsis (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) and other plants (for review, see Ülker and Somssich, 2004). For instance, silencing of the *Nicotiana benthamiana* homologs of the tobacco WRKY factors NtWRKY1, NtWRKY2, and NtWRKY3 compromised N resistance. These WRKY proteins share highest similarity at the amino acid level with Arabidopsis WRKY20, WRKY4, and WRKY70, and particularly expression of NtWRKY3 is rapidly induced upon infection with TMV (Liu *et al.*, 2004). WRKY proteins bind to the W box (TTGAC[C/T]) in promoters of various pathogen-responsive genes, including genes encoding the basic, ethylene-responsive tobacco PR-1, PR-2, PR-3, and PR-5 proteins (Eulgem *et al.*, 2000; Kim and Zhang, 2004; Yamamoto *et al.*, 2004).

We have shown that a fragment of 902 bp upstream of the transcription start site of the tobacco *PR-1a* gene confers inducibility to the *GUS* reporter gene by TMV infection and SA treatment. This inducibility involved multiple elements in the promoter fragment (van de Rhee and Bol, 1993). The *PR-1a* promoter was found to contain a number of sites that bind GT-1-like factors with different affinity. The observation that the level of GT-1 decreased after infection of tobacco with TMV suggested a negative role of GT-1 in regulation of *PR-1a* expression. However, mutation of the GT-1 binding sites did not affect promoter activity (Buchel *et al.*, 1996). In this article, we used the yeast one-hybrid system to identify tobacco proteins interacting with fragments of the *PR-1a* promoter. One of the proteins obtained turned out to be a novel WRKY protein, named NtWRKY12. Similar to *PR-1a*, expression of the *NtWRKY12* gene was strongly induced by TMV infection, SA treatment, or infiltration of tobacco leaves with a suspension of *Agrobacterium tumefaciens*. Two binding sites for NtWRKY12 were identified in the *PR-1a* promoter with a surprisingly low similarity to the consensus W box sequence.Wild-type and mutant *PR-1a* promoter sequences were fused to the *GUS* reporter gene and these fusions were expressed in transgenic tobacco to assay induction by SA and expressed from a T-DNA vector in agroinfiltrated leaves to assay induction by bacterial elicitors. The results indicated that NtWRKY12 acts synergistically with TGA1a in the SA-mediated and pathogen-associated molecular pattern (PAMP)-mediated expression of the *PR-1a* gene. In addition, transactivation assays in Arabidopsis protoplasts provided evidence that NtWRKY12 is a transcriptional activator of *PR-1a* gene expression.

RESULTS

A Novel WRKY Factor Binds to the *PR-1a* Promoter

Previous studies have indicated that elements in the 902-bp tobacco *PR-1a* promoter are important for SA and TMV-induced expression (van de Rhee *et al*., 1990; van de Rhee and Bol, 1993; Strompen *et al*., 1998). Here, we used the yeast one-hybrid system to identify transcription factors binding to the *PR-1a* promoter. Tetramers of various fragments of the 902-bp promoter sequence were inserted in front of the yeast (*Saccharomyces cerevisiae*) *His* reporter gene and integrated into the genome of his^- yeast strain Y187. TMV-infected tobacco was used as a source for construction of a library of cDNAs fused to the GAL4 activation domain in vector pACT. This library was used to transform yeast strains harboring the various *PR-1a* promoter fragments. Screening of the cDNA library with fragment IV (bp -605 to -513 of the *PR-1a* promoter in yeast strain Y187-IV) yielded 37 independent transformants growing on His-free medium (pACT/IV clones). Of the cDNA inserts in these clones, 22 cross-hybridized with each other. Clone pACT/IV-80 was selected for further analysis.

Sequencing of the cDNA insert of pACT/IV-80 revealed that it corresponded to the 610 3'-terminal nucleotides of a mRNA, excluding a poly(A) track of 54 residues probably representing the 3'-terminal poly(A) tail. The cDNA corresponding to the missing 5'-part of the mRNA was obtained using RACE on total RNA from TMV-infected tobacco plants. This resulted in a stretch of 415 additional nucleotides at the 5'-end of the mRNA. The combined 5'- and 3'-sequences revealed an open reading frame for a protein of 220 amino acid residues. The insert in pACT/IV-80 encoded the C-terminal 107 amino acids of this protein. The presence of WRKY and zinc (Zn)-finger domains in the C-terminal half indicates that the protein is a member of the large group of DNA-binding WRKY proteins. Upstream of the WRKY domain, the amino acid sequence contains a stretch of basic residues, reminiscent of nuclear targeting signals. The N-terminal region is relatively rich in acidic residues and has low similarity to WRKY51 from Arabidopsis. Based on the criteria described by Eulgem *et al*. (2000), the novel tobacco WRKY protein appears to be a member of subgroup 2c of the WRKY superfamily of plant transcription factors. Currently, 11 different tobacco WRKY genes are described in the EMBL/GenBank database. In line with the tobacco WRKY nomenclature, the novel protein identified in our study was named NtWRKY12. The accession number of the full-length cDNA is DQ460475. DNA-blot analyses of restriction enzyme digests of genomic DNA using a probe corresponding to the cDNA insert from pACT/IV-80 showed that the amphidiploid tobacco varieties Samsun NN and Samsun nn contain two to four NtWRKY12-related genes (Fig. 1).

Expression of the full-length NtWRKY12 protein in yeast strain Y187-IV rendered the strain independent of exogenous His (data not shown). This indicates that NtWRKY12 contains an activation domain that is able to replace the GAL4 activation domain fused to the DNA binding region of NtWRKY12 in pACT/IV-80 and to activate transcription of the *His* reporter gene in yeast.

This strongly supports a role for NtWRKY12 as a transcription factor in tobacco. Expression of an NtWRKY12/GFP fusion construct using an alfalfa mosaic virus-based expression system (Sánchez-Navarro *et al.*, 2001) resulted in specific fluorescence of tobacco nuclei. Similar expression of non-fused GFP showed a more diffuse fluorescence of the cytoplasm and nuclei (Fig. 2). This indicates that the NtWRKY12 sequence contains a nuclear localization signal, which targets the fusion protein to the nucleus. The reverse transcription (RT)-PCR results shown in Figure 3 indicate that, like *PR-1a*, expression of the *NtWRKY12* gene was induced in tobacco leaves by salicylate treatment and by infiltration of leaf tissue with *A. tumefaciens* strain LBA4404. The last type of induction probably corresponds to a PAMP-type response, similar to responses triggered by peptide patterns of conserved elicitors like bacterial flagellins or elongation factor (EF)-Tu (Felix *et al.*, 1999; Kunze *et al.*, 2004). Indeed, also infiltration with *Escherichia coli* resulted in induced expression of *NtWRKY12* and *PR-1a* (data not shown).

The time course of expression of the *NtWRKY12* gene was studied in TMV-infected Samsun NN tobacco plants. Figure 4 shows northern blots with RNA isolated at various time points after inoculation. It is evident that in noninfected plants the gene was expressed at relatively low levels (top, lane 0). After infection with TMV, expression increased in the inoculated leaves (local) and reached a transient maximum after 1 h. At 2 h postinoculation (hpi), expression was back to the low basal level and remained low until 8 hpi. Subsequently, *NtWRKY12* mRNA accumulation was slightly increased at 12 and 24 hpi and became very high at 48 h and later. The strong increase in *NtWRKY12* expression coincided with the development of local lesions that first appeared at 36 hpi. Also, in the noninoculated leaves, expression increased, although with some delay and to lower levels. We have not investigated mRNA accumulation in the systemic tissues at later time points. The second image shows the TMV-induced expression pattern of the gene encoding transcription factor Myb1 (Yang and Klessig, 1996). Like *NtWRKY12*, the *Myb1* gene is transiently expressed until 1 hpi and at high levels at 48 and 72 hpi. The timing of *NtWRKY12* and *Myb1* expression corresponded to that of the *PR-1a* gene (middle), although *PR-1a* was not transiently expressed immediately after inoculation.

Characterization of NtWRKY12 Binding Sites in the *PR-1a* Promoter

The results of the yeast one-hybrid screening indicated that NtWRKY12 specifically bound to a *PR-1a* promoter sequence ranging from positions -605 to -513 upstream of the transcription start site. To delineate the binding site in the DNA, this region was further divided into four overlapping subfragments A to D (Fig. 5). With a similar approach as was used above, tetramerized versions of subfragments B and C were able to confer His independence in the yeast one-hybrid system, whereas fragments A and D were not (data not shown). This suggested that the overlap region of fragments B and C contains the NtWRKY12 binding site. This was confirmed in the one-hybrid system with mutants of subfragments B and C of which either the left halves (mutants Blm and Clm) or the right halves (mutants Brm and Crm) were mutated by changing each G to A, A to G, C to T, and T to C (e.g. compare the sequences of B and Blm in

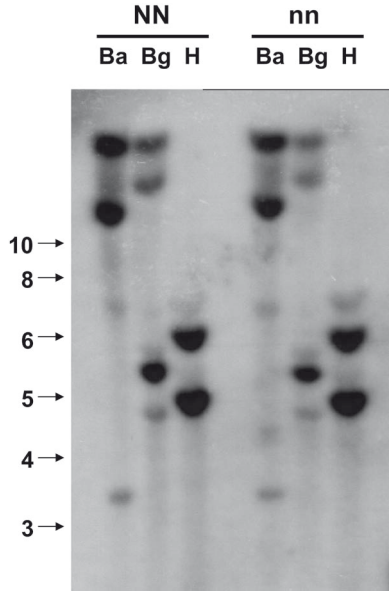

Figure 1. Analysis of *NtWRKY12* in the tobacco genome by Southern blot hybridization. Genomic DNA from tobacco with (NN) or without (nn) the TMV resistance gene *N* was digested with restriction enzymes *Bam*HI (Ba), *Bgl*II (Bg) or *Hind*III (H), electrophorezed, blotted and hybridized to the radioactively labeled cDNA insert of *NtWRKY12* clone pACT/IV-80. The positions of the DNA size markers (bp x 10^3) are indicated to the left.

Fig. 5).

The results of the yeast one-hybrid assays were confirmed in vitro using electrophoretic mobility shift assays (EMSAs) with complementary oligonucleotides corresponding to regions C and B and a glutathione S-transferase (GST)/NtWRKY12-binding domain (BD) fusion protein expressed in *E. coli*. This fusion protein contained the C-terminal 111 amino acids of NtWRKY12 and was purified by glutathione-Sepharose 4B column chromatography. In the EMSA, the complementary oligonucleotides could anneal to double-stranded structures. Figure 6A, lane 1, shows a band corresponding to the labeled fragment C probe. After incubation with the GST/NtWRKY12-BD protein, part of the probe is shifted to a higher position in the gel (Fig. 6A, lane 2). When only GST protein is used, no band shift is observed (Fig. 6A, lane 3). This indicates that the NtWRKY12-BD is able to form a protein-DNA complex with fragment C. Similarly, lane 5 shows the formation of a complex of GST/NtWRKY12-BD with fragment Blm, but not with Brm (Fig. 6A, lane 7). To determine the exact location of the NtWRKY12 binding site in subfragment Blm, a scanning analysis was performed with a series of complementary oligonucleotides based on Blm, in which two adjacent base pair were changed (Fig. 5; Blm-m1–Blm-m9). The results of EMSAs with these fragments

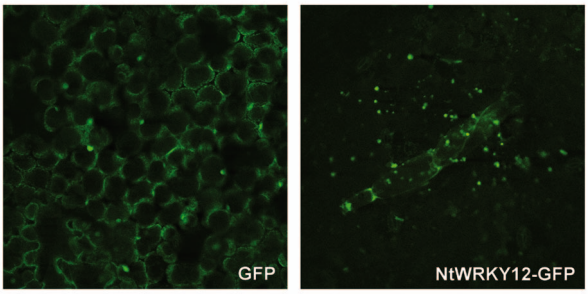

Figure 2. Nuclear localization of NtWRKY12. Confocal laser scanning microscopic images of transgenic P12 tobacco leaf cells expressing alfalfa mosaic virus (AMV) replicase genes, infected with an AMV-RNA 3 transcript containing the GFP gene, or the *NtWRKY12* coding region fused to GFP, as indicated.

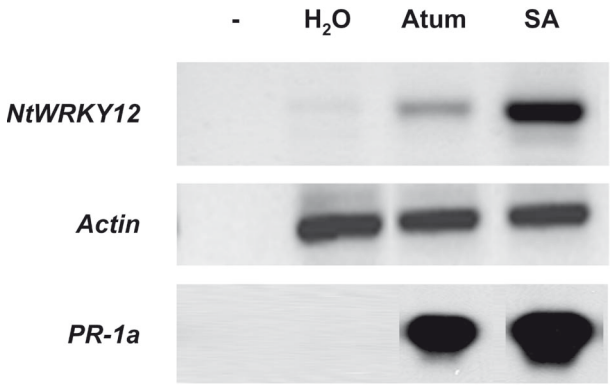

Figure 3. Induction of expression of *NtWRKY12* and *PR-1a* by *A. tumefaciens* elicitors and SA treatment of tobacco. Leaves were infiltrated with water (H_2O), a suspension of *A. tumefaciens* (Atum), or a solution of salicylic acid (SA), and RNA was extracted 2 d after infiltration. RNA from infiltrated leaves was analyzed by RT-PCR with primers corresponding to *NtWRKY12*, *Actin*, or *PR-1a*. As a control for genomic DNA contamination, the first lane (-) was loaded with the product of a PCR on RNA from untreated leaf without initial RT reaction. The PCR products were electrophoresed and stained with ethidium bromide.

are shown in Figure 6A, lanes 8 to 27. It is evident that the lanes with mutants Blm-m3 to Blm-m6 lack a band shift and neither did the single mutants Blm-m3' and Blm-m6' (Figs. 5 and 6, lanes 29–33). This suggests that the corresponding sequence TTTTCCAC is essential for binding to the NtWRKY12-BD. Complementary oligonucleotides corresponding to fragment Blm-m1, but with the central TTTCCA sequence of the binding site changed into the consensus WRKY box TTGACC (Fig. 5; Blm-m10), were not able to compete with fragment Blm-m1 for binding of GST/NtWRKY12-BD in EMSAs (Fig. 6B, lanes 5 and 6), whereas fragment Blm-m10 alone showed

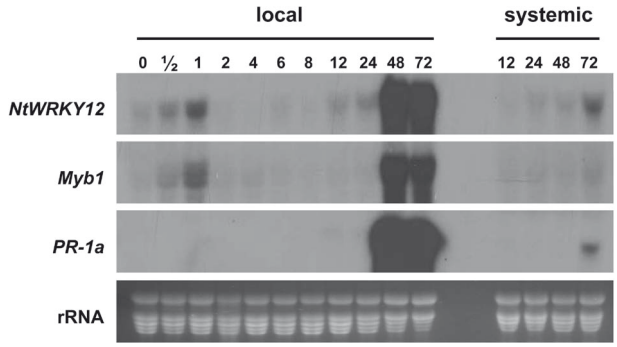

Figure 4. Time course of the expression of *NtWRKY12*, *Myb1*, and *PR-1a* genes after inoculation of Samsun NN tobacco with TMV. Total RNA was extracted from inoculated (local) leaves and uninoculated (systemic) upper leaves at the indicated time points (hpi). After electrophoresis and blotting, the membranes were hybridized to cDNA probes corresponding to *NtWRKY12*, *Myb1*, and *PR-1a* as indicated in the left margin. A photograph of an ethidium bromide-stained gel is included as a loading control (rRNA).

no binding to GST/NtWRKY12-BD (Fig. 6B, lane 8). This indicates that NtWRKY12 does not bind to the consensus WRKY binding site. As discussed in more detail below (see "Discussion"), in NtWRKY12 the WRKY sequence is followed by the sequence GKK rather than by the sequence GQK found in WRKY factors that have been shown to bind to the consensus W box. We have expressed GST/NtWRKY12-BD with the GKK sequence mutated into GQK or GEK in *E. coli* (Fig. 6F), but the purified mutant proteins showed no binding in band shift assays to either the WK box in the PR-1a promoter or the consensus W box sequence (Fig. 6, C and D). Apparently, the central Lys in the GKK sequence is essential for binding of NtWRKY12 to the WK box sequence.

We have investigated whether binding to the WK box is a general feature of WRKY proteins with a GKK sequence. Therefore, the full-length GKK-containing AtWRKY51 coding sequence was expressed as a GST fusion protein in *E. coli* (Fig. 6G). However, this Arabidopsis WRKY was not able to bind to either the WK or the W box sequence (Fig. 6E). The faint bands visible at higher positions in the gel (Fig. 6E, lanes 2–6 and 8–12) are the result of aspecific binding because they cannot be competed by an excess of either unlabeled WK or W box. The same results were obtained with a full-length GST/AtWRKY59 fusion protein (data not shown). These results suggest that the WK box is not a general consensus binding site for GKK WRKYs.

The synthetic oligonucleotides that were used for the above band shift assays contained nonpaired GTAC extensions at the 5' termini. These sticky ends allowed transient base pairing and formation of multimerized fragments, which greatly facilitated DNA-protein interaction during incubation. Annealed oligonucleotides that did not contain sticky ends at best showed only weak band shifts. Apparently, the multimers remained at least partly intact during electrophoresis and are visible as faint bands above the positions of the monomeric free probes. In several lanes, these oligomers were

Figure 5. Wild-type (WT) and mutant *PR-1a* promoter fragments analyzed in yeast one-hybrid assays and EMSAs. Promoter fragment IV (nucleotides -603 to -513) is subdivided into overlapping fragments A, B, C, and D. In Blm, Brm, Clm, and Crm, either the left or the right halves of fragments B and C were mutated (*). Plus and minus signs indicate binding and no binding in the yeast one-hybrid assays and EMSAs, respectively. Mutants Blm-m1 to Blm-m10 contain additional mutations in the right half of mutant Blm. In the wild-type sequence, the position of the *as-1*, WK_1, and MBSII boxes are italic and underlined.

apparently stable enough to produce stable multiple free probe bands (Fig. 6A, lanes 14/15, 26) and even double band shifts (Fig. 6A, lane 27).

The sequence TTTTCCAC also occurs at the far upstream position -859 in the *PR-1a* promoter (Fig. 7). EMSAs with annealed oligonucleotides corresponding to the region -871 to -839 confirmed also that this region of the promoter is able to bind NtWRKY12 (data not shown). The two TTTTCCAC sequences in the *PR-1a* promoter were named box WK_1 (-564) and box WK_2 (-859).

Role of NtWRKY12 and TGA1a in SA-Induced *PR-1a* Expression

To determine whether the NtWRKY12 binding site has functional significance for SA-induced expression of *PR-1a*, stably transformed transgenic tobacco plants were made containing a series of mutant *PR-1a* promoter::*GUS* constructs. In close proximity to the WK_1 box (-564 to -558), the *PR-1a* promoter contains binding sites for transcription factors TGA1a (box *as-1*, -592 to -577)

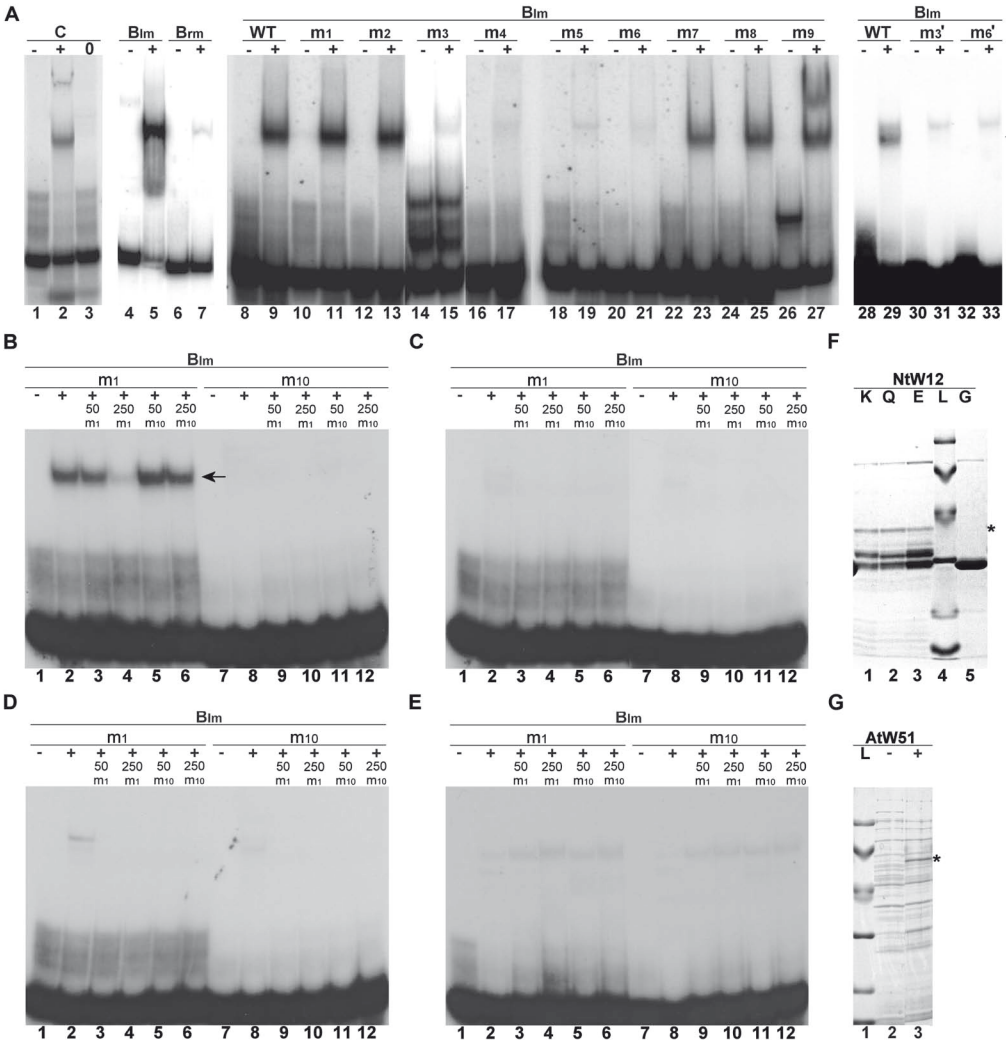

Figure 6. Binding of NtWRKY12 to wild-type (WT) and mutant *PR-1a* promoter fragments. EMSAs were done with the promoter fragments shown in Figure 5: wild-type fragment C (A, lanes 1, 2, and 3), Blm (A, lanes 4, 5, 8, 9, 28, and 29), Brm (A, lanes 6 and 7), and the indicated Blm mutants (A, lanes 10–27 and 30–33; B, C, D, and E, lanes 1–12). In A, B, C, D, and E, plus signs indicate binding mixtures containing 0.5 µg recombinant GST fusion protein purified from *E. coli* transformed using a pGEX-KG vector with wild-type NtWRKY12-BD (A and B), mutant NtWRKY12-BD with the amino acids GKK mutated to GQK (C), mutant NtWRKY12-BD with the amino acids GKK mutated to GEK (D), and AtWRKY51 (E). In these sections, minus signs above the lanes indicate binding mixtures without recombinant protein. In A and B, the position of the protein-DNA complexes is indicated by an arrow. In B, C, D, and E, lanes 3 to 6 and 9 to 12, a 50- or 250-fold excess of unlabeled fragment Blm-m1 (m1) or Blm-m10 (m10) was added as competitor to the EMSA incubation mixtures. Zero sign (A, lane 3), Control with recombinant GST protein purified from *E. coli*. F, SDS-PAGE gel containing purified GST fusion proteins of wild-type NtWRKY12-BD withWRKY-GKK (K, lane 1) and mutants NtWRKY12-BDs with WRKYGQK (Q, lane 2)

and Myb1 (box MBSII, -520 to -514; Fig. 7; Yang and Klessig, 1996; Strompen *et al.*, 1998; Grüner *et al.*, 2003). Mutations affecting single transcription factor binding sites in mutants WK_2, *as-1*, and WK_1 are shown in Figure 7. The double mutant WK_2/WK_1 contains both the WK_2 and WK_1 mutations. In addition, promoter deletions of 85 bp (mutant Δ85) and 32 bp (mutant Δ32) were made. As outlined in Figure 7, the 85-bp deletion removed binding sites *as-1*,WK_1, and MBSII, whereas the 32-bp deletion removed the WK_1 binding site only. The double mutant WK_2/Δ85 contained both the WK_2 and the Δ85 mutations.

The number of independent, phenotypically normal transformants obtained with wild-type and mutant *PR-1a* promoter::*GUS* constructs ranged between 2 and 16. Primary transformants were analyzed at the six- to eight-leaf stage for noninduced GUS expression and for GUS activity after floating leaf discs on water and SA. The results are presented in Figure 8. As expected, the reporter gene was constitutively expressed in *35S::GUS* plants, whereas the wild-type *PR-1a* promoter conferred strong SA-inducibility to the GUS gene. In agreement with the results of Strompen *et al.*, (1998), we noticed that mutation of the *as-1* box resulted in a modest reduction of SA inducibility of the *PR-1a* promoter. Mutation of the upstream binding site for NtWRKY12 (mutant WK_2) did not reduce SA inducible reporter gene expression. However, mutation (mutant WK_1) or deletion (mutant Δ32) of the downstream NtWRKY12 binding site reduced the SA inducibility of the *PR-1a* promoter by approximately 60% to 70%. Although the number of transgenic lines with the WK_1 mutation (two lines) or the Δ32 mutation (three lines) were relatively low, the results with these two mutants demonstrate that mutation or deletion of the WK_1 box only partially affects *PR-1a* promoter activity. The combined mutation of both the WK_2 and the WK_1 binding site (mutant WK_2/WK_1; six lines) further reduced expression. Mutant Δ85 (16 lines) lacks the WK_1, *as-1*, and MBSII boxes and showed no significant SA-inducible expression. Probably, NtWRKY12 is able to bind to the WK_2 box of mutant Δ85, but this binding is not sufficient for SA-inducible expression. Mutation of the WK_2 box in mutant Δ85 (mutant WK_2/Δ85; seven lines) did not affect the phenotype of mutant Δ85. Binding of TGA1a and/or Myb1 factors to the *PR-1a* promoter may be responsible for the approximately 20% level of SA inducibility observed with mutantWK_2/WK_1. Together, the results indicate that full SA inducibility of the *PR-1a* promoter requires synergistic interactions between NtWRKY12 and TGA1a or Myb1 factors.

Role of NtWRKY12, TGA1a, and Myb1 Factors in Elicitor-Induced *PR-1a* Expression

As shown in Figure 3, infiltration of tobacco leaves with *A. tumefaciens* results in induction of *NtWRKY12* and *PR-1a* gene expression. Probably this expression is induced by bacterial elicitors

and WRKYGEK (E, lane 3), respectively, which were used in the EMSAs of A to E. Lane 5 of F was loaded with a purified extract from empty GST expression vector (G). G, SDS-PAGE gel of extract from uninduced (minus sign, lane 2) or induced (plus sign, lane 3) *E. coli* containing pGEX-KG vector with AtWRKY51-GST fusion protein. In F and G, the position of the full-length induced fusion proteins is indicated by asterisks, whereas lanes labeled L were loaded with size markers of 94, 67, 43, 30, 20, and 14 kD. NtW12, NtWRKY12; AtW51, AtWRKY51.

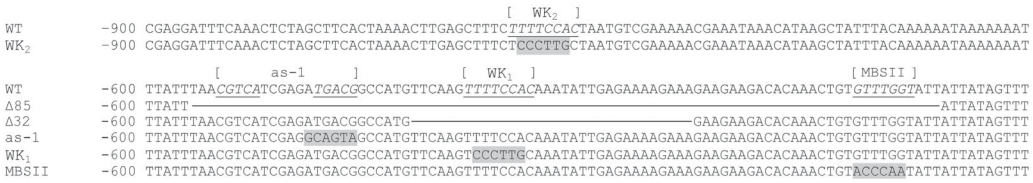

Figure 7. Partial sequence of the wild-type (WT) and mutant *PR-1a* promoters. The sequence of the wild-type *PR-1a* promoter is shown from nucleotides -900 to -801 (first row) and from -600 to -501 (second row), taking the transcription start site as +1. Binding sites for transcription factors NtWRKY12 (WK_2 and WK_1), TGA1a (*as-1*), and Myb1 (MBSII) are underlined and mutant sequences (blocked) are shown below the wild-type sequence. The lines in mutants Δ32 and Δ85 represent deleted nucleotides.

(see "Discussion"). To study a possible role of NtWRKY12 in elicitor-induced expression of the *PR-1a* gene, tobacco leaves were agroinfiltrated with *A. tumefaciens* suspensions harboring *PR-1a* promoter::*GUS* fusions in the T-DNA vector. For these experiments, the collection of promoter mutants used in the plant transformation experiments was extended with a mutant containing an altered Myb1 binding site (mutant MBSII; see Fig. 7) and a series of double and triple mutants. In double mutants *as-1*/WK_1, *as-1*/MBSII, and WK_1/MBSII, two of the boxes *as-1*, WK_1, and MBSII contain the point mutations specified in Figure 7. In the triple mutant *as-1*/WK_1/MBSII, all three boxes are mutated.

The results are shown in Figure 9, A and B. The relatively low GUS expression of the *35S::GUS* constructs can be ascribed to the much lower density of the *35S::GUS Agrobacterium* inoculum obtained in comparison to that of the *PR-1a::GUS* strains (approximately A_{600} = 0.2 versus A_{600} = 1, respectively). To enable a comparison of different experiments, GUS activity in leaves expressing the wild-type *PR-1a::GUS* construct was taken as 100%. The effects of mutations WK_2, WK_1, WK_2/WK_1, Δ32, Δ85, and WK_2/Δ85 on elicitor-mediated GUS expression in tobacco plants (Fig. 9A) were largely similar to their effects on SA-mediated expression of GUS in plants with *PR-1a::GUS* transgenes (Fig. 8). Expression of mutants WK_1, WK_2/WK_1, and Δ32 was reduced by 40% to 60%, whereas mutants Δ85 and WK_2/Δ85 did not support significant levels of elicitor-mediated GUS expression (Fig. 9A).

In mutant Δ85, the *as-1*, WK_1, and MBSII boxes are deleted. To analyze the role of these boxes in the Δ85 phenotype, we made mutants with two or all three boxes mutated. Figure 9B shows that elicitor-mediated expression of the double mutant *as-1*/WK_1 is as low as that of the Δ85 mutant. The effect of the double mutation in mutant *as-1*/WK_1 (<5% of wild-type induction) is much stronger than the combined effects of the two single mutations *as-1* (no significant reduction of wild-type induction; Fig. 9A) and WK_1 (40%–60% of wild-type induction; Fig. 9, A and B). This demonstrates that a synergistic action of factors binding to the *as-1* and WK_1 boxes is essential for elicitor-induced *PR-1a* promoter activity. The additional mutation of the MBSII box in triple mutant

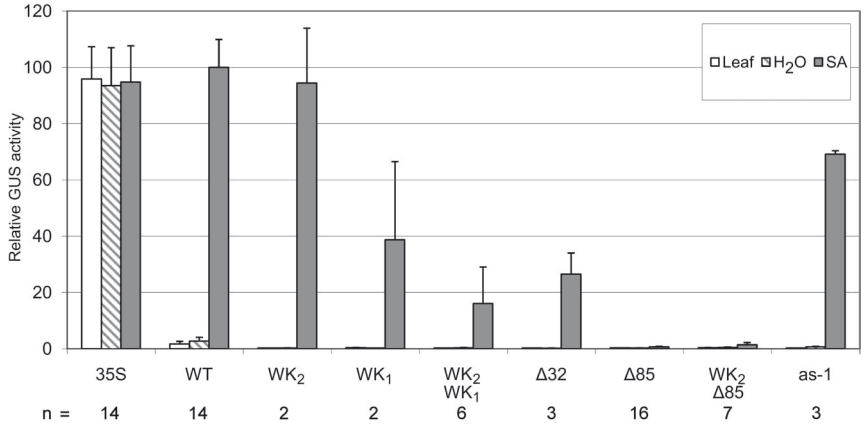

Figure 8. SA-induced expression of *PR-1a::GUS* fusions in transgenic tobacco. Plants were transformed with constructs encoding a CaMV *35S* promoter::*GUS* fusion (35S), a wild-type (WT) 902-bp *PR-1a* promoter::*GUS* fusion, and 902-bp *PR-1a* promoter::*GUS* fusions containing the mutations shown in Figure 7. Four leaf discs from each plant were floated on water (H_2O) or 1 mM SA for 48 h before GUS activity was measured, or four leaf discs were taken from the untreated transgenic plants (Leaf). The number of transgenic lines used is indicated (n). The bars represent the GUS activity of all transgenic plants per construct relative to that of wild type treated with 1 mM SA (100%). Error bars represent the SEM.

as-1/WK_1/MBSII did not alter the phenotype of the *as-1*/WK_1 mutant.

Elicitor-mediated induction of the double mutants *as-1*/MBSII and WK_1/MBSII is about 40% of the induction driven by the wild-type PR-1a promoter (Fig. 9B). The observation that expression by these double mutants is modestly reduced when compared to the single mutants *as-1*, WK_1, and MBSII indicates that MBSII contributes to some extent to the expression driven jointly by the *as-1* and WK_1 boxes.

NtWRKY12 Activates *PR-1a::GUS* Gene Expression in Arabidopsis Protoplasts

The above results indicate that NtWRKY12 plays a role in inducible *PR-1a* gene expression. To more directly demonstrate that NtWRKY12 functions as a positive transcriptional activator of *PR-1a* gene expression, Arabidopsis protoplasts were cotransfected with a plasmid containing the NtWRKY12 coding region under the control of the cauliflower mosaic virus (CaMV) 35S promoter together with a plasmid containing the GUS reporter gene cloned either behind the full-length (902 bp) wild-type *PR-1a* promoter or behind *PR-1a* promoters with mutations in the WK_1 box or in both the WK_1 and WK_2 boxes. Similar cotransfections with a plasmid lacking the NtWRKY12 coding sequence were performed as controls. The results of these transactivation assays are shown in Figure 10. In the presence of the NtWRKY12 plasmid, *PR-1a* promoter-directed

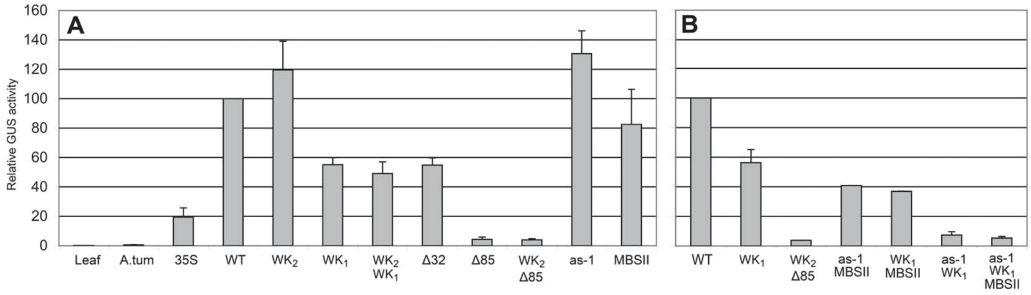

Figure 9. Transient expression of *PR-1a::GUS* fusions, induced by bacterial elicitors in agroinfiltrated tobacco leaves. Leaves were infiltrated with suspensions of *A. tumefaciens* harboring T-DNA vectors with promoter::*GUS* fusions. 35S, Constitutive promoter from CaMV; WT, *PR-1a* promoter from base -1 to -902. Mutations shown in Figure 7 were engineered in the WT promoter. A.tum, *A. tumefaciens* with a promoterless *GUS* gene in the T-DNA vector. Leaf, Leaf material collected before agroinfiltration. The first series of experiments covered single mutations or combinations with WK_2 (A). Additional agroinfiltration experiments of B show GUS expression levels of double and triple mutants in the Δ85 region. For each construct, GUS activity was determined in homogenates of 10 leaf discs from five infiltrated plants. The bars represent the GUS activity per construct relative to that of the wild type (100%). The SEM was calculated for four (A) and two to four (B) replicates of independent experiments.

GUS expression was increased approximately 4-fold in comparison to the basal level obtained in protoplasts cotransfected with the empty vector. Apparently, NtWRKY12 produced in the protoplasts activates transcription of the GUS reporter gene by the Arabidopsis transcriptional machinery. Obviously, NtWRKY12 does so, at least partly, by binding to the WK_1 box because mutation of the WK_1 box resulted in a reduction of GUS activity to approximately 45% of that directed by the wild-type promoter. Upon mutation of both the WK_1 and the WK_2 box, NtWRKY12 no longer activated reporter gene expression.

DISCUSSION

DNA Binding Site of NtWRKY12

Among the first WRKY-type DNA binding proteins that were identifiedwas a parsley *(Petroselinum crispum*) transcription factor involved in expression of the *Phytophthora megasperma*-induced gene encoding protein PR1 (Rushton *et al*., 1996; Eulgem*et al*., 1999). As a PR protein of class 10, parsley PR1 is not related to the classical PR-1 proteins originally characterized in tobacco and conserved in many other plant species. Induction of parsley PR1 is not mediated by SA and the protein accumulates in the cytoplasm as opposed to the classical PRs that accumulate either extracellularly or in the vacuole.

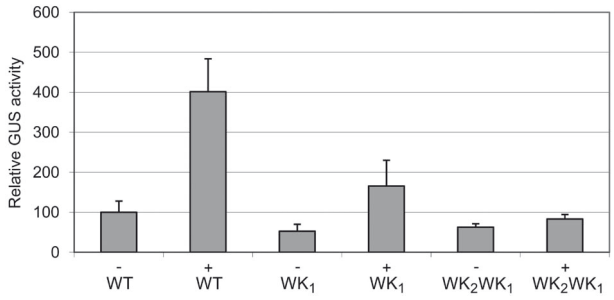

Figure 10. Transactivation of *PR-1a::GUS* gene expression by NtWRKY12 in Arabidopsis protoplasts. Protoplasts were transfected with 2 µg of wild-type *PR-1a* promoter::*GUS* (WT) construct or with *PR-1a* promoter::*GUS* constructs containing the WK_1 mutation (WK_1) or the WK_2/WK_1 double mutant (WK_2WK_1) as shown in Figure 7. Plus signs, Cotransfection with 6 µg of expression vector pRT101 containing *35S::NtWRKY12*. Minus signs, Cotransfection with 6 µg of empty expression vector. The bars represent the percentage of GUS activity from triplicate experiments relative to that of the protoplasts cotransfected with the wild-type *PR-1a::GUS* construct and the empty expression vector, which was set to 100%. Error bars represent the SEM.

A number of recent 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). In a screen of genes coexpressed with the Arabidopsis *PR-1* gene under SAR-inducing conditions, Maleck *et al.* (2000) found the consensus WRKY binding site TTGAC(C/T) to be present in the promoters at twice the statistically expected frequency, whereas the *as-1* element TGACG, the consensus binding site of TGA transcription factors, occurred only at one-half the statistically expected frequency.

In this article, we have identified NtWRKY12 as a WRKY-type DNA binding protein that specifically recognizes the sequence TTTTCCAC. This DNA element is located at two positions in the upstream region of the tobacco *PR-1a* promoter that was previously found to be important for inducible gene expression (van de Rhee *et al.*, 1990; van de Rhee and Bol, 1993; Grüner and Pfitzner, 1994; Strompen *et al.*, 1998). The NtWRKY12 binding box at position -564 is located between binding sites for transcription factors TGA1a (-592) and Myb1 (-520), which have been implicated in SA- and TMV-induced gene expression (Yang and Klessig, 1996; Strompen *et al.*, 1998).

NtWRKY12 Contains a Variant WRKY Domain

NtWRKY12 is the first WRKY protein to be identified that interacts with a DNA binding site different from the consensus WRKY binding site TTGAC(C/T). As far as the sequence of the conserved

WRKY domain is concerned, NtWRKY12 is different from most other WRKY proteins in that it contains a Lys (K) residue instead of a Gln (Q) in the conserved domain (WRKYG[Q/K]K). This variation of the WRKY domain is conserved among other plant species. A BLASTP (http://www.ncbi.nlm.nih.gov/BLAST) search of all 796 eukaryote proteins containing one or two WRKY domains of which sequence data were present in the National Center for Biotechnology Information databases resulted in 131 sequences with high protein-protein similarity to NtWRKY12. Of these, the 28 proteins with highest similarity to NtWRKY12 all contained the WRKYGKK variant domain. The 10 most similar WRKYGKK proteins (61%–86% similarity) were from both dicotyledonous (*Vitis vinifera*, *Brassica rapa*, *Glycine max*) and monocotyledonous (rice [*Oryza sativa*]) plants. All WRKY factors shown to bind the W box element contain the GQK sequence.

Of all 72 Arabidopsis WRKY genes, the three closest homologs of NtWRKY12 are AtWRKY50, AtWRKY51, and AtWRKY59 (68%, 64%, and 59% similarity, respectively; Supplemental Fig. 11). Although the similarity between NtWRKY12 and these Arabidopsis WRKYs is mainly limited to the C-terminal halves of the proteins, they share the variant WRKYGKK domain, have approximately similar sizes, and are all induced by SA and pathogenesis (Dong *et al.*, 2003). It was suggested that AtWRKY59's lack of W box binding activity might be due to the Q to K change (Dong *et al.*, 2003). Although an Ala scanning study showed that mutation of the Q residue had only a minor effect on binding of NtWRKY9 to the consensus W box (Maeo *et al.*, 2001), NMR spectroscopy measurements have revealed that the Q residue is one of the four amino acids in the WRKYGQK sequence of AtWRKY4 that contacts the bases in the major groove of the DNA and therefore is highly significant for sequence-specific recognition (Yamasaki *et al.*, 2005). Recently, an extensive mutational analysis of the region containing the C-terminal WRKY domain of AtWRKY1 confirmed that the Q to K mutation affected its binding to the consensus Wbox (Duan *et al.*, 2007). NtWRKY12 mutant proteins in which the GKK sequence was changed to GQK or GEK (another WRKY domain sequence variation occurring, for example, in WRKY proteins of rice) were not able to bind to either the WK box-containing Blm-m1 probe or the Blm-m10 probe with the consensus W box (Fig. 6B). This suggests that, in addition to the WRKYG[Q/K]K domain, other regions in the WRKY proteins are probably also involved in the specificity of DNA binding.

Role of NtWRKY12 in *PR-1a* Gene Expression

PAMPs are universally conserved in a class of microbes. As in animals, plants recognize elicitors derived from pathogens, such as viruses, bacteria, fungi, or oomycetes. Well-characterized elicitors that induce defense responses in plants are represented by bacterial flagellin and EF-Tu or peptides from these proteins. In Arabidopsis, the flagellin-derived peptide flg22 binds to a Leu-rich repeat-type receptor-like kinase (FLS2), which activates a mitogen-activated protein kinase (MAPK) pathway and expression of WRKY transcription factors (Gómez-Gómez, 2004; Boller, 2005). We observed that agroinfiltration of tobacco leaves with a suspension of *A. tumefaciens* induced the expression of *NtWRKY12* and *PR-1a*. The components of *A. tumefaciens* responsible for

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NtWRKY12        MFGSSTFQETINISHQTINPNFAFSPLINMNQDHCHNMYQDFDTSFIDQLINDSEEYSNS 60
AtWRKY51        ----------MNIS-QNPSPNFTY-----FSDENFINPFMD-NNDFSNLMFFDIDEGGN- 42
AtWRKY50        ----------MNDADTNLGSSFSDD-----THSVFEFPELDLSDEWMDDDLVSAVSGMNQ 45
AtWRKY59        ----------MNYP-SNPNP----------SSTDFTEFFKFDDFDDTFEKIMEEIGREDH 39
                          :* .  . ..          .           . .    : .    :

NtWRKY12        NNSFNTSLYSQNPFMQQEISTSTYSGSCSSASSFDATPTNIHMNENSSKGIEKEKKAEKH 120
AtWRKY51        NGLIEEEISSPTSIVSSETFTGESGGSGSATTLSKKESTNRGSKESD------QTKETGH 96
AtWRKY50        SYGYQTSDVAG-ALFSGSSSCFSHPESPSTKTYVAATATASADNQNK-----KEKKKIKG 99
AtWRKY59        SSSPTLSWSSSEKLVAAEITSPLQTSLATSPMSFEIGDKDEIKKRKR-------HKEDPI 92
                .     .  :   :.  .          ::        .    :..         *

NtWRKY12        AIAFRTKT---ELEILDDGYKWRKYGKKKVKSNTNPRNYYKCSSGCCKVKKKVERDGNDS 177
AtWRKY51        RVAFRTRS---KIDVMDDGFKWRKYGKKSVKNNINKRNYYKCSSEGCSVKKRVERDGDDA 153
AtWRKY50        RVAFKTRS---EVEVLDDGFKWRKYGKKMVKNSPHPRNYYKCSVDGCPVKKRVERDRDDP 156
AtWRKY59        IHVFKTKSSIDEKVALDDGYKWRKYGKKPITGSPFPRHYHKCSSPDCNVKKKIERDTNNP 152
                  .*:*::   :   :***:******** :...   *:*:***   * ***::*** ::.

NtWRKY12        SYLITTYEGKHNHESPFIIYCQEKSTVSFHNDQWSLQ-LADSLW------ 220
AtWRKY51        AYVITTYEGVHNHESLSNVYYN-EMVLSYDHDNWNQHSLLRS-------- 194
AtWRKY50        SFVITTYEGSHNHSSMN------------------------------- 173
AtWRKY59        DYILTTYEGRHNHPSPSVVYCDSDDFDLNSLNNWSFQTANTYSFSHSAPY 202
                 :::***** *** *
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Figure 11. ClustalW amino acid alignment of NtWRKY12 and WRKYGKK variants of Arabidopsis (AtWRKY50, AtWRKY51, AtWRKY59). Asterisks indicate residues identical in all sequences. Colons indicate amino acids with conserved substitutions. Dots indicate amino acids with semi-conserved substitutions.

this induction have not yet been identified (Felix *et al.*, 1999; Kunze *et al.*, 2004), although recent results by Djamei *et al.* (2007) show that *PR-1* gene expression is indirectly regulated by *Agrobacterium*-induced MAPK MPK3.

SA plays an essential role in the expression of extracellular acidic PR proteins and development of SAR after TMV infection of NN tobacco. To analyze the role of the NtWRKY12 binding sites in SA- or PAMP mediated expression, we investigated *PR-1a::GUS* expression in stably or transiently transformed tobacco plants. Although mutation of the upstream WK box (WK_2) had little effect on SA- or PAMP-induced GUS expression, mutation or deletion of the downstream WK box (WK_1) reduced GUS expression by 50% to 60% (Figs. 8 and 9). This clearly demonstrates the important role of the NtWRKY12 binding site in SA- and PAMP-mediated *PR-1a* expression. The consensus WRKY binding site TTGAC(C/T) has been found in promoters of genes encoding basic vacuolar PR proteins from classes PR-1, PR-2, PR-3, and PR-5 of tobacco, but has not been identified in the *PR-1a* promoter. Overexpression of the tobacco MAPK kinase NtMEK2 resulted in the expression of WRKY factors binding to the consensus WRKY binding site and expression of defense genes, including those encoding basic PR proteins (Kim and Zhang, 2004).

PR-1a promoter fragment IV, which was used to select NtWRKY12 in the one-hybrid screen, also contains binding sites for TGA1a and Myb1 factors (Yang and Klessig, 1996; Strompen *et al.*,

1998). Our *as-1* mutant was impaired in SA-mediated expression of a reporter gene (Fig. 8), but the effect was less pronounced than that observed by Strompen *et al*. (1998) and Grüner *et al*. (2003). The PAMP-mediated expression of the *as-1* mutant showed no repression in comparison to the wild-type promoter. This may be due to differences in mutations that were engineered in the *as-1* box. Our observation that single mutations in the *as-1* or WK_1 box are insufficient to completely knock out SA-induced expression driven by the *PR-1a* promoter indicates that multiple factors are required for promoter activity. A complete knock out (<5% of the wild-type activity) was obtained with the Δ85 promoter deletion, which removes the *as-1*, WK_1, and MBSII boxes. This mutational analysis of the *PR-1a* promoter revealed that similar elements are involved in SA-mediated and elicitor-mediated expression of the reporter gene (Figs. 8 and 9).

The finding that point mutations in the *as-1* and WK_1 boxes in double mutant *as-1*/WK_1 fully knocked out elicitor-mediated expression (Fig. 9B) demonstrates that TGA1a and NtWRKY12 are the major players in the regulation of *PR-1a* promoter activity. A comparison of the activity of this double mutant with the single mutants *as-1* and WK_1 revealed that TGA1a and NtWRKY12-like factors act synergistically in *PR-1a* gene expression. In contrast to mutant *as-1*/WK_1, the double mutants *as-1*/MBSII and WK_1/MBSII showed significant levels of elicitor-mediated *PR-1a* promoter activity (Fig. 9B). A comparison of this activity with that of the single mutants *as-1*, WK_1, and MBSII indicates that, in addition to the major effectors TGA1a and NtWRKY12, Myb1 plays a modest role in expression of the *PR-1a* gene. Recently, it was shown that several structurally related WRKY proteins are able to physically interact to form homologous and heterologous complexes (Xu *et al*., 2006). The synergistic effect of NtWRKY12 and TGA1a on *PR-1a* gene expression provokes a study of their possible direct or indirect interaction.

NtWRKY12 Is a Transcriptional Activator of *PR-1a* Gene Expression

The effect of NtWRKY12 overexpression on *PR-1a* promoter activity was studied by transactivation experiments in Arabidopsis protoplasts. These clearly demonstrated that NtWRKY12 acts as a transcriptional activator of *PR-1a* gene expression in vivo. GUS activity resulting from the expression of the wild-type *PR-1a* promoter::*GUS* gene was greatly enhanced in the presence of NtWRKY12 (Fig. 10). When the WK_1 box in the promoter was mutated, GUS expression was reduced, albeit still higher than in the absence of NtWRKY12. The results presented in Figures 8 and 9 indicated that the WK_2 box was less important for induction of the *PR-1a* promoter than the WK_1 box. However, in the transactivation assay (Fig. 10), the difference in GUS expression obtained with the WK_1 and WK_2/WK_1 mutants clearly points to a role of WK_2 in NtWRKY12-mediated expression.

In nonstressed tobacco, the *PR-1a* gene is not expressed (Figs. 3 and 4). The basal level of GUS expressed in the absence of NtWRKY12 in transfected Arabidopsis protoplasts (Fig. 10) indicates that the tobacco *PR-1a* promoter is recognized by the Arabidopsis transcriptional machinery. It is arguable that protoplast preparation and transfection result in a stress response that triggers a certain level of expression of stress-inducible genes, including the transfected tobacco *PR-1a::GUS* gene.

The observation that mutation of the WK_1 box results in reduced GUS expression in the absence of NtWRKY12 (Fig. 10) suggests that the WK_1 box is also involved in stress-induced expression by Arabidopsis transcription factors. Whether in Arabidopsis protoplasts NtWRKY12 activates expression of the tobacco *PR-1a* gene alone or in combination with Arabidopsis TGA, Myb, or other transcription factors is presently unknown. Experiments are under way to further investigate this.

Occurrence of the WK Box in Other Promoters

The NtWRKY12 binding site TTTTCCAC is remarkably similar to that of the *E. coli* protein DnaA (TTTTCCACA; Weigel *et al*., 1997). DnaA is involved in DNA replication and binds to single-stranded DNA. Our band shift results were not caused by contaminating DnaA from the *E. coli* extract because no band shift was produced with a similarly isolated unfused GST protein preparation from *E. coli* (Fig. 6A, lane 3).

In tobacco, a TTTTCCAC box is also found 249 bp upstream of the transcription start site in the SA-inducible *PR-2d* gene (EMBL/GenBank accession no. X69794) and 1,012 bp upstream of the initiation codon in *Sar8.2b* (U64816). We have checked the occurrence of the WK box in the Arabidopsis genome. Whereas Maleck *et al*. (2000) found the W box to be overrepresented at 2.5-fold the statistically expected level in the promoters of a set of 25 *PR-1* coregulated genes, we found the WK box to be overrepresented 3.3-fold in this set. Moreover, in the 1,000-bp upstream promoter regions of a set of 372 BTH-induced genes (Bülow *et al*., 2007), the WK box is found at twice the expected level, whereas the W box is present at 1.4-fold. Interestingly, in both sets the *as-1* element is present at exactly the statistically expected level.

Recently, Sun *et al*. (2003) characterized the region of the promoter of the sugar-responsive *iso1* gene from barley (*Hordeum vulgare*) that bound to barley transcription factor SUSIBA2. The 573-amino acid protein SUSIBA2 contains two WRKY and Zn-finger domains, which classifies it as a member of group 1 of the WRKY superfamily. Interestingly, SUSIBA2 bound to a region of the *iso1* promoter lacking the consensus TTGAC(C/T) W box. Although the authors have not further delineated the exact SUSIBA2 binding box, we noticed that the region contains the sequence TTTTCCA and that mutations in this sequence affected the formation of band shifts with SUSIBA2 protein. Our results with NtWRKY12 suggest that it could be this sequence that determines the SUSIBA2 binding site. If so, the occurrence of two such similar WRKY binding sequences in promoters of genes involved in different physiological processes and in different plant species would indicate that the consensus TTGAC(C/T) WRKY box is not the only conserved cis-element involved in binding-WRKY transcription factors. However, it must be noted that neither of SUSIBA2's WRKY domains contains the WRKYGKK sequence present in NtWRKY12.

CONCLUSION

In WRKY transcription factors, the WRKY consensus sequence is followed by the amino acid sequences GQK, GKK, or GEK. Factors of the GQK type have been shown to bind to the W box element (TTGAC[C/T]). We identified a tobacco WRKY factor (NtWRKY12) of the GKK type, which specifically recognized two WK boxes (WK_1 and WK_2; TTTTCCAC) in the promoter of the SA-inducible tobacco *PR-1a* gene, but failed to bind to the W box element. The central K residue in the GKK sequence was crucial for binding of NtWRKY12 to the WK box. Overexpression of NtWRKY12 in protoplasts strongly stimulated *PR-1a* promoter activity via functional WK_1 and WK_2 boxes. Synergistic interactions between NtWRKY12 and other transcription factors, particularly TGA1a, appeared to be required for maximal induction of the *PR-1a* promoter in planta by SA or bacterial elicitors.

MATERIALS AND METHODS

Plants and Plant Treatments

Tobacco (*Nicotiana tabacum* 'Samsun NN') plants were grown in growth chambers at 25°C, 60% relative humidity, with a 16/8-h photoperiod.

For cDNA library cloning, 8-week-old plants were inoculated with 0.1 mL per leaf of an inoculum of 18 ng TMV/mL by rubbing the inoculum on three lightly carborundum-dusted leaves per plant after which the plants were immediately placed in a growth room at 33°C with a 16-h day/8-h night regime. After 2 d, the plants were returned to the 25°C growth room and inoculated leaves were collected after 5 h. For gene expression studies, three leaves of 8-week-old tobacco plants were inoculated with 3 ng TMV/mL and kept at 25°C. Inoculated and noninoculated leaves were sampled at different time points and immediately frozen in liquid nitrogen and stored at -80°C.

Discs of 24 mm were punched out of new, fully expanded leaves of wildtype and transgenic plants and floated on water or on 1 mM sodium salicylate, pH 6.8. After 2 d, the discs were blotted dry and four 12-mm discs were punched out, transferred to Eppendorf tubes, frozen in liquid nitrogen, and stored at -80°C.

Transgenic tobacco plants containing *35S::GUS* and *PR-1a::GUS* reporter genes were obtained through *Agrobacterium tumefaciens*-mediated leaf disc transformation with transgene constructs cloned into the pMOG800 transformation vector and regeneration of kanamycin-resistant shoots (Linthorst *et al.*,1989). The number of transgenic plants obtained were 14 (35S), 14 (wild type), two (WK_2), two (WK_1), six (WK_2/WK_1), three (Δ32), 16 (Δ85), seven (WK_2/Δ85), and three (*as-1*).

Tiny punctures were made with a scalpel in the bottom epidermis of new, fully expanded leaves of 8-week-old tobacco plants, through which *Agrobacterium* infiltration mixtures (A_{600} = 1 for the *PR-1a::GUS* strains and A_{600} = 0.2 for the *35S::GUS* strain) were supplied to the intercellular spaces by gentle pressure using a syringe without needle. After 2 d, 12-mm leaf discs were sampled from fully infiltrated areas adjacent to the puncture hole, transferred to Eppendorf tubes, frozen in liquid nitrogen, and stored at -80°C.

One-Hybrid Screening

mRNA was isolated from TMV-infected tobacco 5 h after the plants were transferred from 33°C to 25°C using the PolyAtract mRNA isolation system (Promega). First-strand cDNA was synthe-

sized on 5 µg poly(A) RNA using an *Xho*I-oligo(dT) linker primer and M-MLV reverse transcriptase (Promega), after which the second strand was synthesized using RNaseH and Pfu DNA polymerase (Stratagene). After ligation of EcoRI adapters and digestion with *Xho*I, 150 ng of the sized cDNA fraction longer than 500 bp was ligated into *Xho*I/*Eco*RI double-digested λACTII arms. After packaging, the λACTII cDNA library (1.4 x 10^6 independent transformants) was amplified in *Escherichia coli* XL-1 Blue MRF$^-$. The phage library was subsequently obtained as a plasmid expression library in pACTII by in vivo excision using *E. coli* BNN132.

Fragments of the tobacco *PR-1a* promoter corresponding to the regions -701 to -612 (region III) and -605 to -513 (region IV) relative to the transcription start site and various mutants of region IV were obtained by PCR using forward primers extended with *Bam*HI and reverse primers extended with *Bgl*II restriction sites. This allowed convenient cloning and concatamerization of the fragments in plasmid pIC19H. Collinear tetramers were cloned in front of the *His-3* gene of plasmid pHIS3N/X and subsequently the *PR-1a* promoter tetramer/His-3 bait constructs were cloned into pINT1 for integration into the genome of yeast (*Saccharomyces cerevisiae*) strain Y187 containing an auxotrophic *his3* mutation (Ouwerkerk and Meijer, 2001). This resulted in strains Y187-III and Y187-IV, respectively. Leakiness of the *PR-1a* promoter tetramer/*His-3* genes of the respective strains was virtually absent.

Screening of the cDNA library in the yeast one-hybrid system was performed essentially as described by Ouwerkerk and Meijer (2001). His-independent clones resulting from the transformations with the pACTII cDNA library were named pACT/IV-n.

Tetramerized subfragments and mutations thereof of promoter fragment IV were analyzed in the one-hybrid system for their ability to confer His independent growth in one-hybrid assays with the NtWRKY12 DNA-BD of pACT/IV-80.

RACE

The cDNA region matching the 5'-part of the mRNA corresponding to the insert of pACT/IV-80 was obtained using RACE (Boehringer) on total RNA from TMV-infected tobacco plants using primer 5'-CCTTCATATGTTGTTATCAAATAGCTGG, which is complementary to an internal region starting at position 271 of the insert of clone pACT/IV-80. Resulting clones were characterized and the clone containing the longest insert was sequenced to confirm that it corresponded to pACT/IV-80. The insert was subsequently fused to the insert of pACT/IV-80 using a common *Bgl*II site to result in clone pNtWRKY12 containing the full-length coding region of NtWRK12.

Bacterial Expression of NtWRKY12 Fusion Proteins

The C-terminal partial open reading frame of pACT/IV-80 (NtWRKY12-BD), mutants in which the GKK sequence was changed into GQK or GEK, and the full-length coding sequence of AtWRKY51 and AtWRKY59 were 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 20°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.

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, after which unincorporated label was removed by Autoseq G-50 column chromatography (Amersham-Pharmacia Biotech).

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 20 min at room temperature. 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, autoradiographed, and analyzed using a Bio-Rad Phosphoimager.

RT-PCR and RNA-Blot Analysis

Total RNA was isolated from pulverized frozen tobacco leaf tissue by phenol extraction and LiCl precipitation. Oligo(dT)-primed cDNA for PCR was obtained using M-MLV reverse transcriptase. Subsequently, PCR was performed during 25 cycles with primers corresponding to NtWRKY12 (AACACAGTTTAATCCTTAAACG, AGAACAAAGACCGAGCTTGAGATC), PR-1a (ATCCTCCATTGTTACACTGAAC, GCTTCCCAATTGGCTGCAG), and tobacco actin (TGCTAGGAGCCAGTGCAGTA, GTGATGGTGTCAGCCACACT). The products were analyzed on agarose gel.

For RNA-blot analysis, total RNA was denatured using formamide/ formaldehyde, electrophoresed in 1.5% agarose gel, blotted to Hybond1(Amersham), and hybridized to ^{32}P-labeled cDNA probes as described previously (Brederode *et al*., 1991). After hybridization, the blots were washed at high stringency with a final wash step in 30 mM NaCl, 3 mM sodium citrate, 0.1% SDS at 50°C for 20 min.

Transactivation Experiments

Protoplasts were prepared from Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 cell suspensions according to Axelos *et al*. (1992), with some modifications. A 5-d-old cell suspension culture was diluted 5-fold in 50 mL medium (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°C at 250 rpm. Cells were harvested and cell walls digested with 20 mL of enzyme mix (0.4% macerozyme R-10 [Yakult], 1.5% cellulose Onozuka R-10 [Yakult], 12% sorbitol, pH 5.8) for 3 h at 28°C. The protoplasts were filtered through a 63-µm steel sieve and washed twice in 50 mL of protomedium (Gamborg B5 basalmedium, 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-1a* promoter::*GUS* constructs (wild type, WK_1, WK_2WK_1) and 6 µg of effector plasmid pRT101 (Töpfer *et al*., 1987) carrying *35S::NtWRKY12*. As a control, cotransformation of *PR-1a* 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 16 h after transformation and frozen in liquid nitrogen.

Fluorometric GUS Assays

When the transgenic plants had reached a size of 15 to 20 cm for each transgenic plant for each treatment (untreated, water, and SA), four leaf discs were separately assayed for GUS activity, with each data point being the average of duplicate measurements. Each leaf disc was homogenized in 0.5 mL GUS extraction buffer (Jefferson, 1987), supplied with 20% methanol (Kosugu *et al*., 1990). After centrifugation for 5 min at 8,000g duplicate samples of 10 µL supernatant were incubated with 90

µL 1 mM 4-methylumbelliferyl-β-D-glucuronide at 37°C for 20 h. The reaction was terminated by adding 300 µL 0.2 M sodium carbonate and 460-nm fluorescence was measured using a Fluoroscan II (Titertek) at 355-nm excitation.

For transient GUS expression measurements, homogenates were made of 10 pooled 12-mm discs from infiltrated areas of leaves of five independently infiltrated plants. GUS activity, normalized against protein concentration, was determined from the average of duplicate measurements per sample.

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.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number DQ460475.

3

Tobacco Transcription Factors NtWRKY12 and TGA2.2 Interact *in vitro* and *in vivo* and Activate *PR-1a* Gene Expression

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Manuscript in Preparation

ABSTRACT

The promoter of the salicylic acid-inducible *PR-1a* gene of *Nicotiana tabacum* contains binding sites for transcription factor NtWRKY12 (WK box at position -564) and TGA factors (*as-1*-like element at position -592). Transactivation experiments in Arabidopsis protoplasts derived from wild type, *npr1-1*, and triple and quadruple *tga* mutant plants revealed that NtWRKY12 alone was able to induce a *PR-1a::β-glucuronidase* (GUS) reporter gene to high levels, independent of co-expressed tobacco or endogenous Arabidopsis NPR1 or TGAs. In protoplasts derived from Arabidopsis cell cultures transient expression of NtWRKY12 or TGA2.2 each activated expression of the *PR-1a::GUS* gene at similar levels, whereas expression of TGA2.1 resulted in only low levels of activation. Expression of a combination of NtWRKY12 and TGA2.2 activated expression to levels similar to the sum of the levels obtained with the separate transcription factors. By in vitro pull-down assays with GST and Strep fusion proteins and by Fluorescence Resonance Energy Transfer assays with protein-CFP and protein-YFP fusions in transfected protoplasts, it was shown that NtWRKY12 and TGA2.2 could interact in vitro and in vivo. A possible interaction of NtWRKY12 with TGA1a or TGA2.1 was not detectable by these techniques. Mutations were engineered in the *PR-1a* promoter to analyze the role of the WK box (-564) and *as-1*-like element (-592) in the activation of promoter activity by NtWRKY12 and TGA2.2. Although it cannot be excluded that these two factors activate *PR-1a* expression in an additive way, several findings point to a functional interaction between NtWRKY12 and TGA2.2 in this process..

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-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.

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 the basic leucine zipper (bZIP) transcription factor TGA1a (Katagiri *et al.,* 1989; Qin *et al.,* 1994). More recently, the structurally related TGA2.2 was identified as the major DNA-binding component of ASF-1, while homolog TGA2.1 was present

at lower amounts (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 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 knock-out 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).

The ankyrin repeat protein NPR1 plays a central role in defense reponses 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). Upon pathogen induced accumulation of SA, the redox state of the cell changes, resulting in release of reduced NPR1 monomers from cytoplasmic complexes and subsequent translocalization to the nucleus where it interacts 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).

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 non-biotic stress. Of the 73 WRKY genes in Arabidopsis, 49 were found to be 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 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). The tobacco *PR-1a* promoter does not harbour a consensus W-box, however, NtWRKY12, a WRKY protein with a variant DNA binding domain, was found to bind to WK-boxes (TTTTCCAC) in the *PR-1a* promoter. Mutations in the WK box at position -564 of the *PR-1a* promoter reduced SA-mediated *PR-1a::GUS* expression in transgenic tobacco or bacterial elicitor-mediated expression in agroinfiltrated leaves by 50 to 60%. In these assays, mutations in the *as-1*-like element at position -592 to -577 of the *PR-1a* promoter had little or no effect on *PR-1a::GUS* expression. However,

combined mutation of the WK and *as-1*-like elements completely abolished inducible expression, suggesting that NtWRKY12 and TGA transcription factors interact in the regulation of *PR-1a* promoter activity (van Verk *et al.*, 2008).

In this study we used pull down assays and Fluorescence Resonance Energy Transfer (FRET) analysis to identify protein-protein interactions between NtWRKY12 and TGA factors in vitro and in vivo, respectively. In addition, we performed transactivation experiments in Arabidopsis protoplasts to study the effect of combinations of NtWRKY12 and TGAs on *PR-1a* gene expression. Our findings revealed that NtWRKY12 alone was able to induce *PR-1a* expression to high levels independent, of co-expressed tobacco or endogenous Arabidopsis NPR1 or TGAs. Furthermore, TGA2.2 was shown to specifically interact with NtWRKY12 and enhance *PR-1a::GUS* expression. The role of the WK box and *as-1*-like element in *PR-1a* promoter activity was analyzed.

RESULTS

Protein-Protein Interactions Between NtWRKY12, TGAs and NPR1

Our previous work pointed to a cooperation between NtWRKY12 and TGA transcription factors in the activation of the *PR-1a* promoter. To analyze a possible protein-protein interaction between NtWRKY12 and tobacco TGA factors in vivo and in vitro, we used FRET analysis and in vitro pull-down assays, respectively.

To elaborate the cellular localization of NtWRKY12, TGA1a, TGA2.1, TGA2.2 and NtNPR1 for the FRET analyses we transfected Arabidopsis protoplasts with plasmids in which the corresponding cDNAs were cloned upstream of the YFP or CFP coding sequence. Examples of imaging of the fusion proteins in living protoplasts by confocal laser scanning microscopy are shown in Figure 1. Whereas fluorescence of unfused CFP and YFP was dispersed throughout the cytoplasm and nucleus, NtWRKY12:CFP, TGA2.1:YFP and TGA2.2:YFP fluorescence localized mainly in the nucleus. The same results were obtained when the proteins were fused to the other chromophore (data not shown). Interestingly, the signals of both NtNPR1:CFP and NtNPR1:YFP were always concentrated in small nuclear spots (data not shown). Furthermore, it is noteworthy that we never detected fluorescence in protoplasts transformed with constructs containing TGA1a fused to either CFP or YFP. These results show that tobacco TGAs 2.1 and 2.2 localize to the nucleus, similar to what has previously been reported for the Arabidopsis homologs (Pontier *et al.,* 2002; Johnson *et al.,* 2003). Due to the extreme brightness of the uneven distributed small nuclear spots of the NtNPR1 chromophore fusions, these could not be used for FRET analysis.

FRET analysis is based on overlapping emission/excitation spectra of donor fluorophore CFP and acceptor fluorophore YFP. Emitted fluorescence from CFP can only excite YFP when both fluorophores are in close (less than 10 nm apart) spatial proximity (Wu and Brand, 1994). Thus, a close association of two proteins with fusions to the respective fluorophores would result in an increase of acceptor fluorescence and quenching of the donor fluorescence. As a positive control for

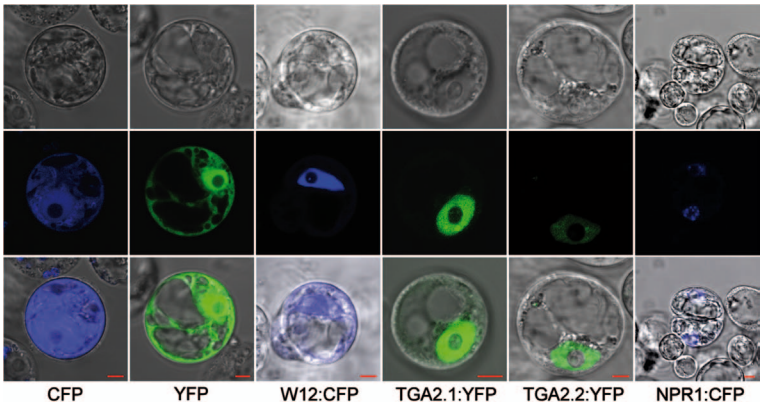

Figure 1. Nuclear localization of NtWRKY12 and TGAs. The panels on the middle row show confocal laser scanning microscopy images obtained of Arabidopsis protoplasts transfected with expression plasmids encoding unfused CFP and YFP, and fusion proteins NtWRKY12:CFP (W12:CFP), TGA2.1:YFP, TGA2.2:YFP, and NPR1:CFP. Panels on the bottom row show overlays with bright-field images (top row) of the same protoplasts. Localizations were visualized with a 63x objective. The red rulers indicate 5 µm.

FRET, Arabidopsis protoplasts were transfected with an expression plasmid encoding a YFP:CFP tandem fusion, while co-transfection with uncoupled CFP- and YFP-encoding plasmids was used as negative control. The protoplasts were incubated for 24h, after which FRET measurements were performed. The result is shown in Figure 2A. For the negative control, protoplasts were selected that showed both CFP (475nm) and YFP (527nm) emission after excitation of the respective fluorophores to confirm transfection with both CFP and YFP plasmids. Excitation of CFP with 457nm UV light in these protoplasts resulted in an emission spectrum with a maximum at 475nm and a certain level of bleeding at 527nm. CFP excitation of the YFP:CFP fusion protein in the positive control protoplasts resulted in quenched emission at 475nm, as part of the emission energy was used to excite the YFP fluorophore of the fusion protein, which was subsequently emitted at 527nm. Thus, the slope of the line connecting the normalized emission intensities at 475nm and 527nm is a measure of the amount of FRET. Similarly, FRET assays were performed on protoplasts cotransfected with combinations of plasmids encoding NtWRKY12 and TGA chromophore fusion proteins.

The control experiments with combinations of NtWRKY12:YFP, TGA2.1:YFP or TGA2.2:YFP with unfused CFP did not result in increased 527nm emission (dashed lines in Fig. 2B, 2C and 2D, respectively), showing that neither NtWRKY12 nor the TGAs interacted with the CFP chromophore, which would preclude the use of FRET for analyzing interactions between these proteins. The angles of the solid lines in Fig. 2B, 2C and 2D indicate the amount of FRET obtained between the various YFP and CFP fusion proteins. In addition to providing the control that the YFP chromophore does not interact with NtWRKY12, the lack of raised 527nm emission with the combination of NtWRKY12:YFP / NtWRKY12:CFP indicates that NtWRKY12 is not able to form homodimers

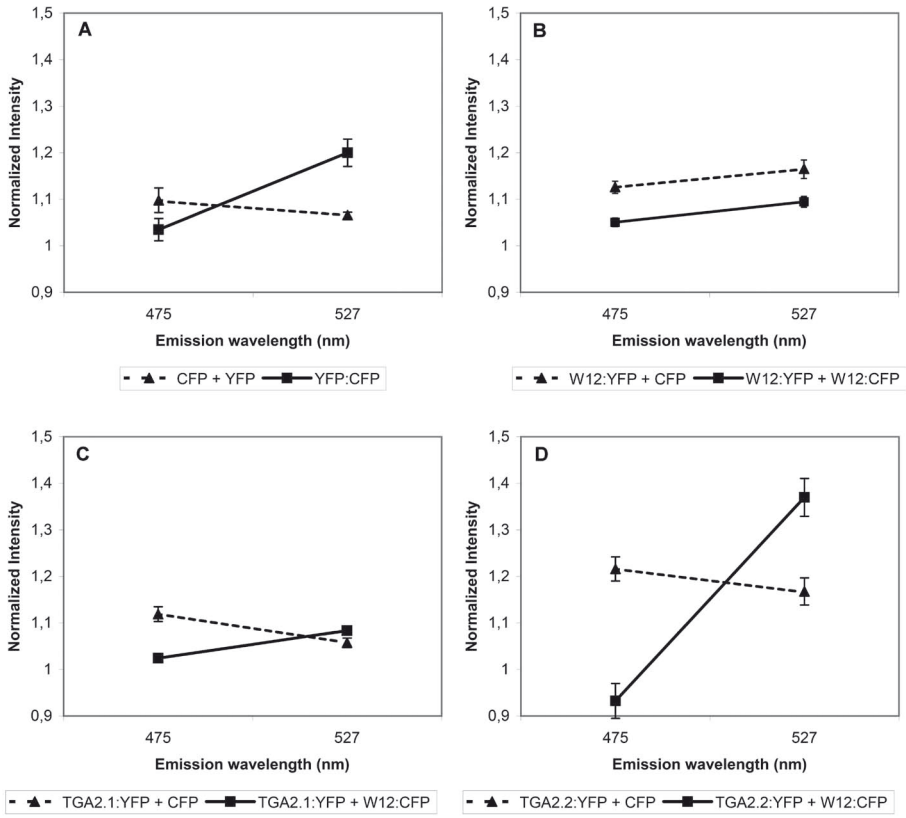

Figure 2. FRET analysis of NtWRKY12 interacting with NtWRKY12, TGA2.1 and TGA2.2. Arabidopsis protoplasts were cotransfected with the indicated expression constructs. A, Protoplasts transfected with a plasmid encoding a YFP:CFP tandem fusion (solid line) and protoplasts transfected with plasmids expressing unfused CFP and YFP (stippled line) were used as positive and negative FRET controls, respectively. B-D, FRET data from protoplasts transfected with a combination of NtWRKY12:YFP (B), TGA2.1:YFP (C) or TGA2.2:YFP (D) and NtWRKY12:CFP (solid lines), compared to unfused CFP (stippled lines). After excitation at 457nm, emission energies were measured in a total of 30, 5nm wide intervals between 468 and 587nm using confocal microscopy. Data from five protoplasts were averaged and normalized. FRET is presented by the slopes of lines connecting emission intensities at 475nm (CFP quenching) and 527nm (YFP emission). Error bars represent the SEM.

(Fig 2B). Similarly, although 475nm emission in the protoplasts transfected with the NtWRKY12:CFP / TGA2.1:YFP plasmids was quenched, 527nm emission was not significantly higher than in the control protoplasts, showing that no strong interaction occurred between TGA2.1 and NtWRKY12 (Fig. 2C). On the other hand, the large amount of FRET in the protoplasts expressing the combination NtWRKY12:CFP / TGA2.2:YFP demonstrates that NtWRKY12 strongly interacted with TGA2.2

(Fig. 2D). Although we could not detect the TGA1a:chromophore fusion proteins in our localization experiments (see above), we did perform a cotransfection of protoplasts with TGA1a:YFP and NtWRKY12:CFP. While it was not surprising to find no YFP signal in these protoplasts, what was surprising was the reproducible total absence of protoplasts showing CFP emission.

To confirm the interaction between NtWRKY12 and TGA2.2, in vitro pull-down assays were performed with *E. coli*-expressed GST and Strep/HIS fusion proteins purified using affinity chromatography. In addition to the interaction between NtWRKY12 and TGA2.2, also interactions with TGA1a, TGA2.1 and NtNPR1 were assayed.

Figure 3 shows the results of in vitro pull-down assays; the data obtained in panels A, B and C are summarized in panel D. Figure 3A shows the interactions between different TGA proteins and NtNPR1. GST:NtNPR1 was incubated with various Strep:TGA:HIS fusion proteins and with a Strep:NtNPR1:HIS fusion, after which the complexes were pulled down using Streptactin beads. The pulled-down proteins were analyzed on Western blots using anti-GST antibody conjugate. Strong NtNPR1-TGA2.2 and NtNPR1-NtNPR1 interactions were observed (Fig. 3A, lanes 3 and 5), whereas no interactions between NtNPR1 and TGA2.1 or TGA1a were detectable (Fig. 3A, lanes 2 and 4). Figure 3A, lanes 6 to 10 show the controls with single fusion proteins. The low background signal obtained with GST:NtNPR1 (Figure 4a, lane 6) was also visible in Figure 3A, lane 4. Homodimer formation as seen with the tobacco NtNPR1 has been reported for Arabidopsis NPR1 (Mou *et al.,* 2003).

In the experiments shown in Figures 3B and 3C, GST fusions of NtWRKY12 and NtNPR1 were incubated with various Strep:TGA:HIS fusions, and protein complexes were bound to Gluthatione-Sepharose 4B beads. The pulled-down proteins were analyzed on Western blots using anti-HIS antibodies. Interactions of NtWRKY12 were observed with TGA2.2 (Fig. 3B, lane 1), but not with TGA1a or TGA2.1 (Fig. 3C, lanes 1 and 5). Moreover, the conclusion from Figure 3A that NtNPR1 interacts with TGA2.2, but not with TGA1a or TGA2.1 was confirmed in this system (Fig. 3B, lane 2; Fig. 3C, lanes 2 and 6).

As a first step towards the characterization of the NtWRKY12 sequence involved in the interaction with TGA2.2, two NtWRKY12 deletion mutants were made. NtWRKY12ΔC lacks the C-terminal 87 amino acids (aa) of the 220 aa long protein; NtWRKY12BD lacks the N-terminal 113 aa. Both mutants were found to interact with TGA2.2 (Fig. 3B, lanes 6 and 7). Either the overlap between the two mutant proteins (aa 114-133) is involved in the interaction of NtWRKY12 with TGA2.2, or NtWRKY12 contains two independent binding sites for TGA2.2, possibly involved in the interaction with a TGA dimer.

Functional Domains of NtWRKY12

Previously, yeast-one-hybrid screening for tobacco proteins binding to the *PR-1a* promoter resulted in the isolation of a protein corresponding to the C-terminal 107 aa of NtWRKY12 fused to the GAL4 activation domain. This protein contained the conserved WRKY and Zn-finger domains and,

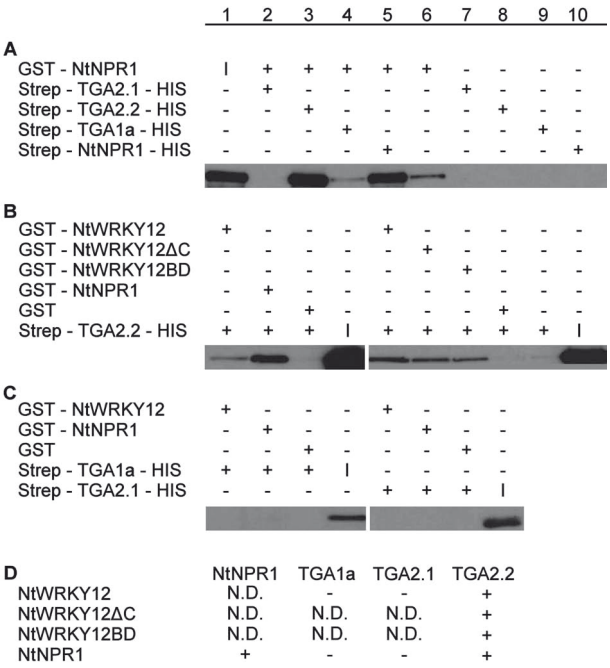

Figure 3. Pull-down assays of NtWRKY12, TGA1a, TGA2.1, TGA2.2 and NtNPR1. GST-proteins were incubated with Strep/HIS purified fusion proteins and complexes were pulled down with Streptactin-Sepharose beads (panel A) or Glutathione-Sepharose 4B beads (panels B and C). After SDS-PAGE and Western blotting fusion proteins were detected with anti-GST antibodies (panel A) or anti-HIS antibodies (panels B and C). Plus and minus signs denote the presence or absence in the incubation mixtures of the proteins indicated at the left. The input protein was loaded separately on gel and is indicated by (I). The table in panel D summarizes the results of the pull-down assays. Plus-sign, interaction, minus-sign, no interaction, N.D., not determined.

apparently, a DNA binding domain (BD) (van Verk *et al.,* 2008). Moreover, it was shown that full-length (220 aa) NtWRKY12 was able to activate *PR-1a::His* gene expression in yeast, indicating that in addition to a BD, NtWRKY12 also contains an activating domain (AD). To further characterize functional domains of NtWRKY12, deletion mutants of NtWRKY12 were assayed in the one-hybrid system in three different ways. First, the mutants were fused to the GAL4 BD and assayed for their ability to activate *GAL4* promoter::*Ade* reporter gene expression (Fig. 4; results summarized in the column with the caption "BD"). Fusions, which activated the reporter gene, were concluded to contain the NtWRKY12 AD. Second, the mutants were fused to the GAL4 AD and assayed for their ability to activate *PR-1a::HIS* gene expression (Fig. 4; results summarized in the column with the caption "AD"). Fusions which activated gene expression were concluded to contain the NtWRKY12 BD. Third, the mutants were expressed as non-fused proteins and assayed for their ability to activate *PR-1a::HIS* expression (Fig. 4; results summarized in the column with the caption "-"). Mutants which

activated gene expression were concluded to contain both the AD and BD domains of NtWRKY12.

Figure 4 (column "BD") shows that GAL4 BD fusions lacking the C-terminal 37 aa (construct 4) or N-terminal 40 aa (construct 7) of NtWRKY12, and a protein with both these deletions (construct 15) were able to activate *GAL4::Ade* expression. Apparently, the NtWRKY12 AD function is contained within the aa 41-183 region of the protein. An online search using the ExPASy (www.expasy.org) Nine Amino Acid Transactivation Domain prediction tool revealed that the region from AA 5-70 and, AA 209-217 contain six domains that have 7 out of 12 possible prediction criteria. The GAL4 AD fusion of the smallest NtWRKY12 deletion mutant that was able to activate *PR-1a::HIS* expression was construct 18 (Fig. 4, column "AD"). Thus, the NtWRKY12 BD is localized in the sequence of aa 121-201. This region encompasses both the conserved WRKY and Zn-finger domains. Apparently, aa upstream of the WRKY domain are also necessary for DNA binding, as a deletion mutant with only seven aa in front of the WRKY domain (Fig. 4, construct 13, aa 132-220) was not able to activate *HIS* gene expression. Construct 17 (aa 41-201) combined the minimal sequences with NtWRKY12 AD and BD activity (construct 15, aa 41-183, and construct 18, aa 121-201). However, the non-fused protein encoded by construct 17 was not able to activate *PR-1a::HIS* expression (Fig. 4, column "-"). To permit both AD and BD activity, the sequence of construct 17 had to be extended by either the Nterminal 40 aa of NtWRKY12 (Fig. 4, construct 2, aa 1-201) or the C-terminal 19 aa (Fig. 4, construct 7, aa 41-220). Possibly, the lack of activity of the protein encoded by construct 17 (aa 41-201) was due to instability or misfolding of the polypeptide.

PR-1a::GUS Gene Expression in Arabidopsis Protoplasts Cotransfected with Plasmids Encoding NtWRKY12, NtNPR1 and TGAs

In our previous paper we showed that cotransfection of Arabidopsis protoplasts with *35S::NtWRKY12* and *PR-1a::GUS* constructs resulted in a strong increase in *GUS* expression. To further investigate the role of NtWRKY12, TGA and NPR1 on activation of *PR-1a* driven expression, additional transactivation assays were set up in protoplasts isolated from leaves of Arabidopsis seedlings grown on MS medium. To avoid interfering effects of NtWRKY12 binding to the far upstream WK binding site (-859), this WK site in the *PR-1a::GUS* reporter gene used in these experiments was mutated (TTTTCCAC into TCCCTTGC). Fig. 5A shows the effects of overexpression of NtWRKY12, TGA2.1, TGA2.2 and NPR1 on *PR-1a::GUS* expression in wild type Arabidopsis protoplasts. Obviously, NtWRKY12 greatly enhanced beta-glucuronidase expression from the *PR-1a* promoter (6-fold over background level). Overexpression of TGA2.1, TGA2.2 or NtNPR1, or combinations of the TGAs with NtNPR1 did not result in enhanced *GUS* expression. Neither did TGA2.2, alone or in combination with NtNPR1, affect the level of NtWRKY12 enhanced *PR-1a::GUS* expression, whereas overexpression of TGA2.1, alone or together with NtNPR1, slightly reduced NtWRKY12 activated *GUS* expression.

In Arabidopsis, *PR* gene expression is dependent on NPR1 and there is accumulating evidence that NPR1 orthologs similarly effect expression of *PR* genes in other plant species (Rayapuram and

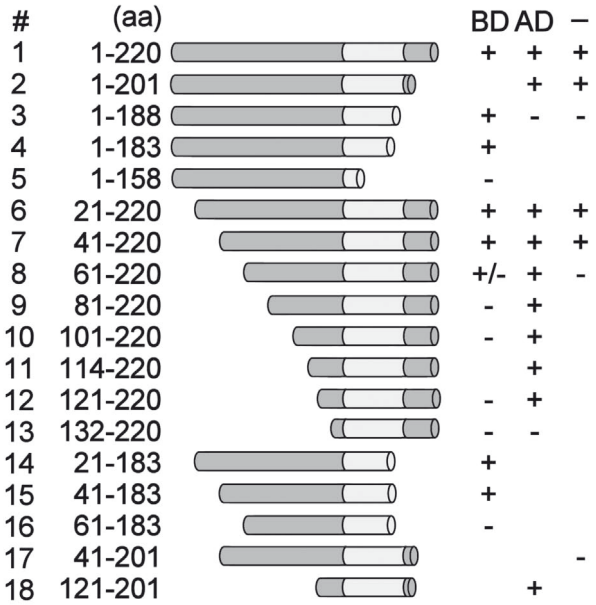

Figure 4. Mapping of the functional domains of NtWRKY12. NtWRKY12 peptides fused to the GAL4 DNA binding domain were tested for their ability to activate *GAL4::Ade* gene expression in yeast (results with these constructs are indicated in column BD). NtWRKY12 peptides fused to the GAL4 activation domain were tested for their ability to bind to *PR-1a* promoter fragment controlling the *His* marker gene (results with these constructs are indicated in column AD). Unfused NtWRKY peptides were assayed for binding to the *PR-1a* promoter and activation of the *His* gene under its control (results with these constructs are indicated in column -). Column aa shows the ranges of the amino acid residues of the respective NtWRKY12 peptides. Plus signs in columns BD, AD and - indicate growth of yeast; minus signs indicate absence of growth of yeast. The absence of plus or minus signs: not tested.

Baldwin, 2007; Anand *et al*, 2008; LeHenanff *et al*, 2009). We wondered whether the lack of effects of overexpressed NtNPR1 on *PR-1a::GUS* expression in the cotransfection experiments could be due to the presence of saturating levels of functionally equivalent Arabidopsis NPR1. However, the results of transactivation assays in protoplasts from *npr1-1* mutant plants were virtually identical to those of the wild type protoplasts (compare Figs. 5A and 5B). This implies that *PR-1a* expression in Arabidopsis protoplasts is independent of NPR1.

On the basis of sequence homology, tobacco TGA2.2 belongs to the group II TGA proteins together with Arabidopsis TGAs 2, 5 and 6 (Xiang *et al.,* 1997). To exclude the possibility that the absence of effects of overexpressed tobacco TGA on *PR-1a::GUS* expression in the Arabidopsis protoplasts was caused by functionally similar Arabidopsis TGAs, cotransfection experiments were performed in Arabidopsis protoplasts derived from *tga2-1 tga5-1 tga6-1* (*tga256*) and *tga2-1 tga3-1 tga5-1 tga6-1* (*tga2356*) mutant plants (Fig. 6). Also in these mutant backgrounds, overexpression of

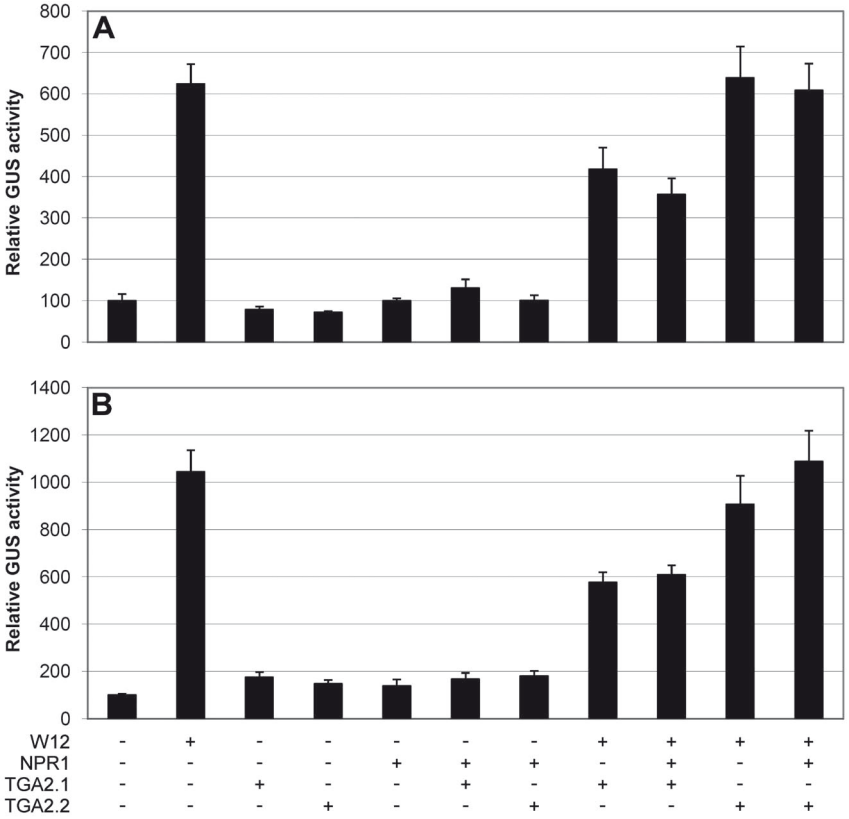

Figure 5. Activation of *PR-1a::GUS* in WT Col-0 and *npr1-1* mutant lines. Leaf protoplasts were cotransfected with *PR-1a::GUS* constructs together with expression plasmids containing *35S::NtWRKY12* (W12), *35S::NtNPR1* (NPR1), *35S::TGA2.1* (TGA2.1), *35S::TGA2.2* (TGA2.2), a combination, or with empty expression vector, as indicated by the plus and minus signs. A, Expression in protoplasts isolated from seedlings of WT Col-0 Arabidopsis, B, expression in *npr1-1* protoplasts. The bars represent the percentage of GUS activity from triplicate experiments relative to that of the protoplasts cotransfected with the corresponding *PR-1a::GUS* construct and empty vector control. Error bars represent the SEM.

NtWRKY12 led to activation of *PR-1a::GUS* expression (9-fold over background level in the triple mutant, Fig. 6A), although the enhancement in the quadruple mutant was greatly reduced (2-fold, Fig. 6B). Likely, this reduced GUS expression is the result of reduced production of NtWRKY12 from the transfected *35S::NtWRKY12* gene, similar to the reduced GUS activity from the *35S::GUS* gene in *tga2356* protoplasts compared to wild-type or *tga256* protoplasts (Fig. 6C).

Together, the results of the cotransfection assays in Arabidopsis leaf protoplasts suggest that NtWRKY12 is the main transcriptional activator of *PR-1a::GUS* expression and that TGA2.1, TGA2.2 or NtNPR1, alone or in combination, do not positively effect activation.

Role of the WK and *as-1*-like Boxes in the Activation of the *PR-1a* Promoter by NtWRKY12 and TGA2.2

Arabidopsis protoplasts isolated from leaf usually had very low levels of *PR-1a::GUS* expression in the absence of co-transfected NtWRKY12 expression plasmid, a situation comparable to that of leaves from non-induced plants that do not express the *PR-1a* gene. In contrast, protoplasts prepared from Arabidopsis cell cultures usually had much higher basal levels, suggesting that in these protoplasts the *PR-1a::GUS* reporter gene was already expressed, apparently mediated by endogenous transcription factors. We checked whether the different media in which the two types of protoplasts were incubated after cotransfection were responsible for this difference in *PR-1a* promoter activity. Therefore, protoplasts prepared from cell cultures were transfected with combinations of *PR-1a::GUS* and empty expression construct, or expression vectors for NtWRKY12 and/or TGA2.2, upon which equal numbers of protoplasts were incubated overnight in the "rich" Protomedium normally used for incubation of cell culture protoplasts, or in the minimal W5 medium used for leaf protoplasts, before GUS activity was measured. As can be seen in Fig. 7A, *GUS* expression in the absence of NtWRKY12 or TGA2.2 was 32-fold higher in the protoplasts incubated in the Protomedium than in the minimal W5 medium. While the W5 protoplasts were more sensitive to NtWRKY12, resulting in 17.2-fold increased GUS activity, NtWRKY12 further increased *GUS* expression in the protoplasts from the Protomedium only 2.7-fold over the basal level. Intriguingly, while there was no effect of TGA2.2 in the W5 protoplasts, TGA2.2 enhanced *GUS* expression in the Protomedium protoplasts to a similar level as did NtWRKY12 (3.1-fold).

To further investigate the involvement of TGA proteins in activation of *PR-1a* expression under these experimental conditions, cell culture protoplasts were cotransfected with NtWRKY12, TGA2.1 or TGA2.2, together with the *PR-1a::GUS* reporter construct and incubated in Protomedium overnight. The results of these transactivation assays are shown in Figure 7B. Similar to the results shown in Fig. 7A, the presence of the NtWRKY12 plasmid increased *PR-1a* promoter-directed GUS expression approximately 3-fold in comparison to the basal level obtained with the empty expression vector, while again addition of plasmid expressing TGA2.2 led to enhanced GUS expression, to similar levels (2.5-fold) as by NtWRKY12. However, plasmid expressing TGA2.1 could not significantly enhance transcription of the GUS reporter gene. Co-expression of NtWRKY12 together with TGA2.1 did not result in higher GUS expression than NtWRKY12 alone, indicating that TGA2.1 is not involved in activation of *PR-1a*. The combination of NtWRKY12 and TGA2.2 led to an additive enhancement of *PR-1a::GUS* expression to a 5-fold increased level over the background. Noteworthy, while expression of TGA1a alone did not enhance *PR-1a::GUS* expression over the basal level, the combination of TGA1a and NtWRKY12 resulted in expression levels similar to those of the TGA2.2/ NtWRKY12 combination (Results not shown).

Previous studies have shown that NtWRKY12 activates *PR-1a::GUS* expression in Arabidopsis protoplasts by binding to the WK box (TTTTCCAC) at position -564 in the *PR-1a* promoter

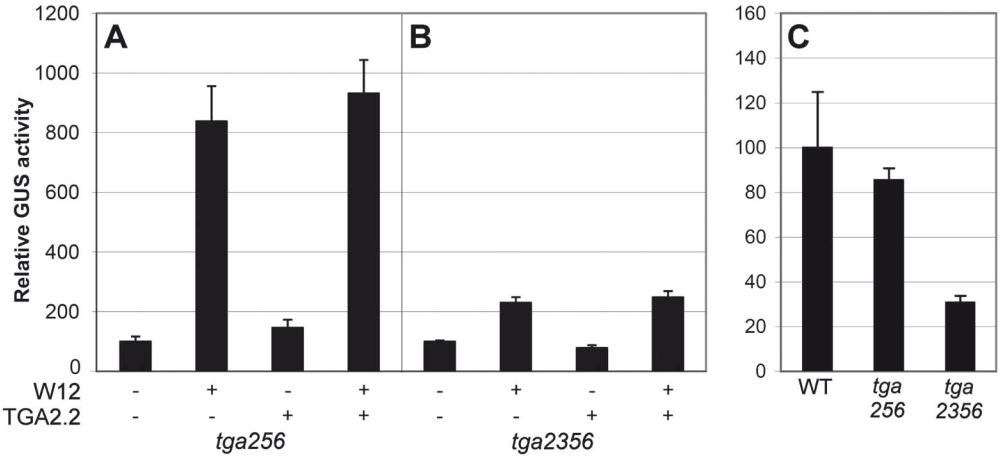

Figure 6. Activation of *PR-1a::GUS* in Arabidopsis *tga* mutant lines. Leaf protoplasts were cotransfected with *PR-1a::GUS* constructs together with expression plasmids containing *35S::NtWRKY12* (W12), *35S::TGA2.2* (TGA2.2), a combination, or with empty expression vector, as indicated by the plus and minus signs in panel A and B. Protoplasts in panel C were transfected with a *35S::GUS* construct. Protoplasts were isolated from a TGA triple mutant (*tga256*), a TGA quadruple mutant (*tga2356*) or WT Col-0 (WT) as indicated below the panels. The bars represent the percentage of GUS activity from triplicate experiments relative to that of the protoplasts cotransfected with the corresponding *promoter::GUS* construct and empty vector control incubated overnight in W5 medium. Error bars represent the SEM.

(van Verk *et al.,* 2008). The binding site involved in the induction of *PR-1a::GUS* expression by TGA2.2 has not been determined yet, but the *as-1*-like element (CGTCA[N]$_6$TGACG) at position -592 is a possible candidate for binding this TGA factor. This raises the possibility that NtWRKY12 and TGA2.2 bind to the *PR-1a* promoter in close proximity, and binding of NtWRKY12 and TGA2.2 might be stabilized by interactions between the two factors that were observed in vivo and in vitro (Figs. 2 and 3). To investigate protein-protein and protein-DNA interactions involved in the activation of the *PR-1a* promoter by NtWRKY12 and TGA2.2 in Arabidopsis protoplasts, we analyzed GUS expression driven by the *PR-1a* promoter with mutations in the WK box, the *as-1*-like element or both these boxes (Fig. 7C, D and E). The controls with the WT *PR-1a* promoter are shown in Fig. 7A (panel Protomedium) and Fig. 7B. In these experiments the protoplasts were incubated in Protomedium.

As shown before (van Verk *et al.,* 2008), mutation of the WK box abolished induction of GUS expression by NtWRKY12 (Fig. 7C). GUS expression induced by TGA2.2 was slightly reduced by the WK mutation, and co-expression of NtWRKY12 did not further enhance the expression level (Fig. 7C). This indicates that possible protein-protein interactions between TGA2.2 and NtWRKY12 do not compensate for the loss of NtWRKY12-DNA interactions. Unexpectedly, GUS expression driven by the *PR-1a* promoter with a mutated *as-1*-like element (Fig. 7D) was very similar to the

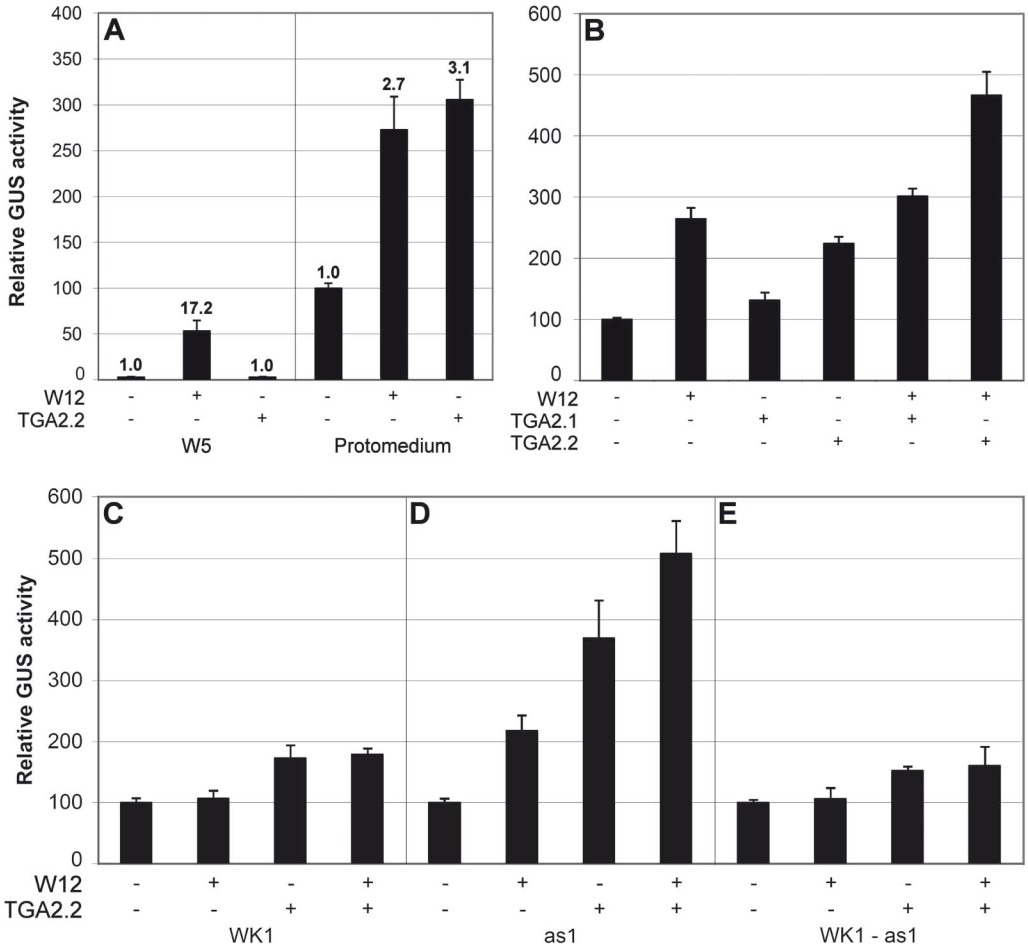

Figure 7. Transactivation of *PR-1a::GUS* gene expression by NtWRKY12 and TGA factors in Arabidopsis protoplasts. Cell culture protoplasts cotransfected with *PR-1a promoter::GUS* constructs together with expression plasmids containing *35S::NtWRKY12* (W12), *35S::TGA2.1* (TGA2.1), *35S::TGA2.2* (TGA2.2), combinations of these plasmids, or with empty expression vector, as indicated by the plus and minus signs. A, Protoplasts were incubated for 16h in either W5 medium or Protomedium, as indicated. B-E, Protoplasts were incubated for 16h in Protomedium. In A and B, the *PR-1a* promoter contained intact *as-1* and WK boxes; in C the WK box was mutated; in D the *as-1*-like element was mutated; in E both WK and *as-1*-like boxes were mutated. The bars represent the percentage of GUS activity from triplicate experiments relative to that of the protoplasts cotransfected with the corresponding *PR-1a::GUS* construct and empty vector control incubated overnight in W5 medium. Error bars represent the SEM.

expression driven by the wild-type promoter (Fig. 7A, Protomedium). Mutation of the *as-1*-like element caused no significant reduction of GUS expression by NtWRKY12, TGA2.2 or both these

factors. This indicates that the *as-1*-like element at position -592 is dispensable for TGA2.2-mediated *PR-1a* gene expression. This conclusion is further corroborated by the observation that GUS expression driven by the WK/as-1 double mutant (Fig. 7E) is similar to the expression by the WK single mutant (Fig. 7C). The role of TGA2.2 and *as-1*-like elements in activation of the *PR-1a* promoter is discussed below.

DISCUSSION

Our previous studies pointed to NtWRKY12 as the major regulator of *PR-1a* gene expression (van Verk *et al.,* 2008). Mutations in the NtWRKY12 binding site (WK box) in the *PR-1a* promoter reduced the SA-induced expression of *PR-1a::GUS* fusions in transgenic tobacco by 60%, whereas mutations in the *as-1*-like element resulted in a 30% reduction. Transient expression of *PR-1a::GUS* fusions, induced by bacterial elicitors in agroinfiltrated tobacco leaves, was not affected by mutations in the *as-1*-like element, but was reduced by 50% when mutations were made in the WK box. Interestingly, when both the WK box and *as-1*-like element were mutated, elicitor induced expression was reduced by 95%. This result pointed to synergistic interactions between factors binding to the WK box and the *as-1*-like element (van Verk *et al.,* 2008). In the present work we further analyzed the role of NtWRKY12 and TGA transcription factors in *PR-1a* gene expression.

FRET analysis of possible interactions between NtWRKY12 and the tobacco transcription factors TGA2.1 and TGA2.2 revealed a strong and specific interaction between NtWRKY12 and TGA2.2 in the nucleus of transfected Arabidopsis protoplasts (Fig. 2). This interaction was confirmed by in vitro pull-down assays. In vitro, no interaction between NtWRKY12 and TGA1a, TGA2.1 or NtNPR1 was observed (Fig. 3). Pull-down assays and studies with the yeast one-hybrid system permitted an initial localization of domains in NtWRKY12 involved in the interaction with TGA2.2, in DNA binding and in transcription activation (Figs. 3 and 4). The role of NtWRKY12 and TGA2.2 in *PR-1a* gene expression was further investigated by transactivation studies in Arabidopsis protoplasts co-transfected with one vector expressing a transcription factor and another vector containing the *PR-1a::GUS* reporter construct. The major advantage of this system over SA induced or elicitor induced gene expression in whole plants is the possibility to analyze the response induced by well-defined single transcription factors.

We noticed that results obtained with transactivation assays are affected by the medium used for incubation of the protoplasts. Previously, we showed that in protoplasts incubated in Protomedium expression of *PR-1a::GUS* was increased about 4-fold by NtWRKY12 (van Verk *et al.,* 2008). In the present study, we noticed that in protoplasts incubated in Protomedium the basal GUS expression in the absence of exogenous transcription factors is about 30-fold higher than in protoplasts incubated in W5 medium. In these W5 protoplasts, GUS expression was induced by NtWRKY12 to much higher-fold levels than in Protomedium protoplasts (Fig. 7A). Mutations in the WK box abolished NtWRKY 12 mediated GUS expression, but did not affect the basal level of GUS in Protomedium protoplasts (Fig. 7C). Similarly, this basal level was not affected by mutations in the *as-1*-like element

(Fig. 7D). Probably, Arabidopsis homologs of NtWRKY12 or factors binding to the *as-1*-like element are not responsible for the high basal level of *PR-1a* promoter activity in the Protomedium protoplasts. It could be that the presence of the synthetic auxin naphthalene acetic acid in the Protomedium and/or differences in Ca^{2+} concentration in the two media are responsible for differences in basal expression of the *PR-1a* promoter.

In W5 protoplasts, *PR-1a::GUS* expression was activated by co-expression of NtWRKY12, but not by co-expression of NtNPR1, TGA2.1 or TGA2.2. This expression pattern was not affected in protoplasts from *npr1-1* or *tga* Arabidopsis mutants (Figs. 5 and 6). These results indicate that NtWRKY12 activates GUS expression independently of exogenously or endogenously expressed NPR1 or TGA factors. In Protomedium protoplasts, NtWRKY12 and TGA2.2 each induced a 3-fold increase in GUS activity (Fig. 7A, right panel). The activity obtained by co-expression of both factors equaled the sum of the activities of the separate factors (Fig. 7B). As shown previously (van Verk *et al.,* 2008), mutation of the WK box in the *PR-1a* promoter abolished NtWRKY12-mediated GUS expression (Fig. 7C). Contradictory to our expectation, mutation of the *as-1*-like element in the *PR-1a* promoter did not affect TGA2.2 mediated GUS expression (Fig. 7D). Recently we observed that in EMSA experiments a 47bp fragment of the *PR-1a* promoter, harbouring the *as-1*-like element at position -592, showed a specific band-shift with TGA2.2. Mutation of the *as-1*-like element abolished this band-shift (unpublished data). This indicates that TGA2.2 binds specifically to the *as-1*-like element at position -592 in the *PR-1a* promoter. The finding that, in the absence of mutations in the WK box, mutations in this *as-1*-like element have little or no effect on *PR-1a* promoter activity in SA-treated transgenic tobacco (van Verk *et al.*, 2008), agroinfiltrated tobacco (van Verk *et al.*, 2008) or TGA2.2-transfected Arabidopsis protoplasts (this study), suggests that TGA2.2 can mediate *PR-1a* expression by binding to a second *as-1*-like element in the *PR-1a* promoter that has yet to be identified. If the *PR-1a* promoter contains indeed two *as-1*-like elements, both could be functional under in vivo conditions.

Similar to TGA2.2, TGA1a has been shown to bind to the *as-1*-like element at position -592 in the *PR-1a* promoter (Strompen *et al.*, 1998). In our experiments TGA1a did not activate expression of the *PR-1a::GUS* reporter when expressed alone, but did so when expressed together with NtWRKY12 (unpublished results). TGA1a has been shown to act as a transcriptional activator in yeast (Pascuzzi *et al.,* 1998; Niggeweg *et al.,* 2000b). Several studies have shown that TGAs are involved in *PR-1a* gene expression, either acting as positive or negative regulators (Strompen *et al.,* 1998; Niggeweg *et al.,* 2000b; Pontier *et al.,* 2001).

NPR1-mediated gene expression in Arabidopsis is largely dependent on its proteasome mediated turnover as shown by Spoel *et al.* (2009). In this same paper the authors examined whether the proteasome acitivty affects induction of direct targets of NPR1 like *WRKY18*, *WRKY38* and *WRKY62* that lack complete responsiveness in *npr1-1* mutants. The SA-mediated induction of these genes is inhibited for 50-60% by a MG115 treatment. Surprisingly the SA-induced expression of *PR-1* is only affected for 5-10% by MG115 treatment, indicating that its activation is less dependent on the proteasome. These results could also indicate that SA-mediated induction of *PR-1* is mainly achieved via other transcription factors, like Arabidopsis variants of the Tobacco NtWRKY12 that can activate

PR-1a gene expression independently of NPR1. Which most likely themselves are direct targets of NPR1, resulting in a NPR1 dependent activation of *PR-1(a)* gene expression.

In our previous paper, transient expression of NtWRKY12 in protoplasts was done in Arabidopsis protoplasts incubated in protomedium (van Verk *et al.*, 2008). In the present work, these studies were extended with transient expression of tobacco TGA factors (Fig. 7A and B). Of the factors tested, TGA2.2 was found to be most active in activation of expression of the *PR-1a::GUS* reporter. Also, TGA2.2 was the only factor that was found to interact with NtWRKY12 in FRET and/or in vitro pull-down assays. However, it is possible that binding sites involved in protein-protein interactions are masked in the fusion proteins used in these assays. Further studies are required to reveal whether or not different TGA factors use different pathways in the activation of the *PR-1a* promoter.

Separately, NtWRKY12 and TGA2.2 activated the *PR-1a* promoter to similar levels. Jointly the two factors activated the promoter rather in an additive way than synergistically. However, several observations suggest that NtWRKY12 and TGA2.2 do interact in the activation of the *PR-1a* promoter. Previously, we showed that mutation of the *as-1*-like element at position -592 had no effect on induction of *PR-1a::GUS* by bacterial elicitors but drastically reduced this induction when the as-1 mutation is made in a WK_1-mutant background (van Verk *et al.*, 2008). In the present work, a similar effect was seen on the TGA2.2-mediated expression of *PR-1a::GUS*. TGA2.2-mediated expression of the wild-type *PR-1a::GUS* construct (Fig. 7A, right panel and Fig. 7B) was little affected by mutation of the *as-1*-like element (Fig. 7D). However, TGA2.2-mediated expression was strongly reduced by mutation of the WK box, either alone (Fig. 7C) or in combination with the as-1 mutation (Fig. 7E).

This reduction suggests that TGA2.2 activity depends on the interaction of this factor with exogenously or endogenously expressed WRKY factors. Also the observation that TGA1a stimulated *PR-1a::GUS* expression when co-expressed with NtWRKY12 supports the notion that TGA and WRKY factors interact. TGA and WRKY transcription factors are known to interact with a variety of proteins. As members of the bZIP class of transcription factors, TGAs bind to DNA as homo- and heterodimers (Deppmann *et al.,* 2006). In addition to their ability to dimerize, there is accumulating evidence that TGAs are able to interact with other interaction partners involved in transcriptional processes. Previously, transcription factors of the Dof and ERF families were isolated as TGA-interacting proteins (Zhang *et al.,* 1995; Büttner and Singh, 1997). Furthermore, TGAs from tomato, tobacco and Arabidopsis were shown to interact with Arabidopsis NPR1, with Arabidopsis TGAs 2, 3, 5, 6 and 7 acting as constitutive interaction partners of NPR1, while interaction with TGAs 1 and 4 was induced by SA-mediated reduction of their intramolecular disulfide bridges (Zhang *et al.,* 1999; Niggeweg *et al.,* 2000b; Zhou *et al.,* 2000; Després *et al.,* 2003; Kesarwani *et al.,* 2007). Recently, glutaredoxin was shown to interact with Arabidopsis TGA2 and tobacco TGA2.2 (Ndamukong *et al.,* 2007), and Arabidopsis TGAs 2, 5 and 6, were found to recruit GRAS protein SCL14 to promoters of genes mediating protection to xenobiotic stress (Fode *et al.,* 2008). In the interactions with NPR1, glutaredoxin and SCL14, the TGAs are considered the DNA-binding partners, bringing the other protein to the promoter to affect transcription. WRKY transcription factors have been found to interact with other proteins involved in transcriptional regulation of stress response genes. In addition

to homo- and heterodimerization as was shown to occur with Arabidopsis WRKYs 18, 40 and 60 (Xu *et al.,* 2006), examples are Arabidopsis WRKY7 interacting with calmodulin (CaM) through a CaM binding domain in the N-terminal half of the protein, which is conserved in other members of the WRKY IId group (Park *et al.,* 2005), WRKY70 interacting with the EAR domain repressor ZAT7 (Ciftci-Yilmaz *et al.,* 2007), WRKY53 interacting with mitogen activated protein kinase kinase kinase MEKK1 (Miao *et al.,* 2007), WRKY33 interacting with mitogen activated protein kinase 4 (MAPK4; Andreasson *et al.,* 2005), and WRKYs 38 and 62 interacting with histone deacetylase19 (Kim *et al.,* 2008). Together, the increasing data on protein-protein interactions between different transcription factors fits well in the concept of evolution of transcription circuits as laid out by Tuch *et al*. (2008). Further studies on the role of the interaction between NtWRK12 and TGA2.2 observed in our study, requires the identification of the TGA2.2 binding site in the *PR-1a* promoter.

MATERIALS AND METHODS

Bacterial Expression of Fusion Proteins

The open reading frames of NtWRKY12 and NtNPR1, and mutants encoding the 133 amino acids of the N-terminal half (NtWRKY12ΔC) or 107 amino acids of the C-terminal half (NtWRKY12BD) were cloned in frame behind the GST open reading frame of expression vector pGEX-KG (Guan and Dixon, 1991), expressed and purified according to van Verk *et al.,* (2008).

The full length coding sequence of *Nicotiana tabacum* TGA1a, TGA2.1, TGA2.2 and NPR1 were cloned in frame of expression vector pASK-IBA45 plus harboring a Strep and HIS tag (IBA). These plasmids were transformed into *Escherichia coli* XL1. For induction of protein expression, cultures were grown to mid-log phase at 37°C, after which tetracycline was added to a final concentration of 0.2 µg mL-1 and incubation continued for 3.5 h at 29°C. The cells were harvested by centrifugation, resuspended in 1/25th volume lysis buffer (1x PBS containing 1% (v/v) NP40, 2 mM DTT and 1/50th volume Complete (Roche) protease inhibitors) and lysed by sonication (Vibracell, Sonics en Materials inc. USA). Soluble protein fraction was collected by centrifugation, and expressed fusion proteins were analyzed using 12% (w/v) SDS-polyacrylamide gel electrophoresis.

In Vitro Pulldown

For the in vitro pull-down assay, GST-fusion proteins were mixed with Strep-fusion-HIS proteins in binding buffer (1xPBS, 1% (w/v) NP40, 2 mM DTT) and incubated on an orbital shaker for 1h at room temperature. To this mixture Glutathione Sepharose 4B beads (GE Healthcare) or Strep-Tactin Sepharose beads (IBA) in buffer W (100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA) were added, and incubation was continued for an additional hour. The beads were washed five times with PBS (with 1% (w/v) NP40 for Glutathione beads) after which the beads were collected, resuspended in Laemmli buffer, and heated at 95°C for 2 min.

The proteins bound to the beads were separated by SDS-PAGE and transferred onto Hybond P membrane (GE Healthcare). Membranes were incubated with the anti-GST antibody (GE Healthcare), or anti-HIS antibody (5 Prime) according to manufacturers instructions and exposed to X-ray film.

One-Hybrid Screening

A tetramer fragment of the tobacco *PR-1a* promoter corresponding to the region -605 to -513 relative to the transcription start site cloned in front of the *His3* gene and integrated in the *Saccharomyces cerevisiae* genome of strain Y187 (van Verk *et al.,* 2008) was used to screen for the DNA binding

domain (BD) of NtWRKY12 and presence of both an activation (AD) and BD. Deletion mutants of NtWRKY12 were cloned in pACT2 to screen for the presence of an BD or p415GPD-HA to screen for both the BD and AD. Mutants were screened for His-independent growth with addition of 3AT up to 20 mM. To locate the AD, deletion mutants were cloned into pAS2-1 and transformed in yeast strain PJ69-4A containing the Gal4 binding site in front of the *Ade* gene. Mutants were screened for adenine independent growth.

Protoplast Preparation and Transactivation Experiments

For microscopy and transactivation experiments, protoplasts were prepared from *Arabidopsis thaliana* ecotype Col-0 cell suspensions according to van Verk *et al.,* (2008).

The leaves from approximately 50 four-week-old seedlings (Col-0, *npr1-1*, *tga256*, *tga2356*) grown on sterile medium were cut in small pieces and protoplasts were prepared according to He *et al.* (2007). In total 1x105 protoplasts were transformed per transfection using polyethylene glycol (40% (w/v) PEG 4000, 0.2 M mannitol, 0.1 M CaCl2).

Protoplasts were co-transfected with 2 µg of plasmid carrying *PR-1a promoter::GUS* construct and 6 µg of *35S::effector* plasmid pRT101 (Töpfer *et al.,* 1987). As a control, cotransformation of *PR-1a::GUS* construct with the empty expression vector pRT101 was carried out. The protoplasts were harvested 16 hrs after transformation and GUS activity was determined. GUS activities from triplicate experiments were normalized against total protein level.

Microscopy and Förster Resonance Energy Transfer (FRET)

Protoplasts were cotransfected with 10 µg of plasmid carrying *protein::CFP* and 10 µg of *protein::YFP* constructs. As controls 2.5 µg of plasmid containing unfused CFP/YFP or 10 µg YFP:CFP fusion was used. Protoplasts expressing the fusion proteins were analyzed with a Leica DM IRBE confocal laser scanning microscope with a 63x water objective, digital zoom and 51% laser intensity. The fluorescence was visualized with an Argon laser for excitation at 457nm with 471-481nm emission filter for CFP and 514nm excitation with a 522-532nm filter for YFP. A transmitted light picture was used as reference. For FRET analysis Lambda scanning was performed by excitation at 457nm and by measuring emission from 468nm to 587nm in a total of 30, 5nm wide intervals using a RSP465 filter. Of every interval the intensity of the whole cell was quantified using ImageJ. The intensity of five protoplasts were averaged and normalized. The slopes between the 475nm and 527nm point were compared for differences in quenched donor emission and increased acceptor emission in comparison to the controls. Similar results were obtained for three independent transfections.

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4

Prospecting for Genes Involved in Transcriptional Regulation of Plant Defenses, a Bioinformatics Approach

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Manuscript in Preparation

ABSTRACT

In order to comprehend the mechanisms of induced plant defense, knowledge of the biosynthesis and signaling pathways mediated by salicylic acid (SA), jasmonate (JA) and ethylene (ET) is essential. Potentially many transcription factors could be involved in the regulation of these pathways, although finding them is a difficult endeavor. Here we report the use of publicly available Arabidopsis microarray datasets to generate gene co-expression networks. By selecting datasets only related to stress treatments, a co-expression network was constructed linked to the SA/JA/ET signaling and biosynthesis pathways. After determining the Pearson Correlation Coefficient cutoff that most likely would give biologically relevant co-expressed genes, the resulting network contained many genes previously reported in literature to be relevant for stress responses and connections that fit current models of stress gene regulation, indicating the validity of our approach. In addition, the network suggested new candidate genes and connections interesting for future research to further unravel their involvement in stress responses.

INTRODUCTION

Plants exposed to biotic or abiotic stress initiate appropriate defense responses mediated by one or a combination of different signal transduction pathways, like the salicylic acid (SA)-, jasmonate (JA)-, and ethylene (ET)-mediated signaling pathways. Arabidopsis contains almost 1500 genes encoding transcription factors (Czechowski *et al.*, 2004) and it is safe to assume that many are involved in regulation of these defense signaling pathways. However, the precise regulatory mechanisms and the transcription factors involved are mostly still unknown. To fine-tune the initiated defense responses the biosynthesis and signaling pathways influence each other via cross talk. This makes discovery of novel regulatory elements within these pathways even more challenging.

The signaling that leads to defense proceeds via interactions of signaling pathway components and because of this, the genes involved are often expressed under similar conditions. This makes that their expression is cooperatively regulated and their expression patterns are highly similar. Based on this concept, an analysis of co-regulated genes under a variety of conditions can give valuable information for understanding the possible regulatory mechanisms involved in defense responses. Any dataset consisting of at least two experiments can be used to perform a co-expression analysis, although for an analysis that is independent of the experimental conditions, a minimum of approximately 100 experiments is needed (Aoki *et al.*, 2007).

To investigate co-expressed genes in Arabidopsis many co-expression databases from different micro-array sources with hundreds of experimental conditions per dataset have been developed in the last couple of years, such as Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; Edgar *et al.*, 2002), ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/; Brazma *et al.*, 2003), AthCor@

CSB.DB (http://csbdb.mpimp-golm.mpg.de; Steinhauser *et al.*, 2004), Genevestigator (http://www.genevestigator.com; Zimmerman *et al.*, 2004, 2005; Hruz *et al.*, 2008), The Botany Array Resource (BAR ; http://bbc.botany.utoronto.ca; Toufighi *et al.*, 2005), Arabidopsis Co-expression Data Mining Tool (ACT; http://www.arabidopsis.leeds.ac.uk/act/; Manfield *et al.*, 2006), ATTED-II (http://atted.jp; Obayashi *et al.*, 2007, 2008, 2009), AtGenExpress/PRIME (http://prime.psc.riken.jp/; Akiyama *et al.*, 2008), and CressExpress (http://www.cressexpress.org; Srinivasasainagendra *et al.*, 2008). Many of these databases only accept single-gene queries for analysis of a correlation coefficient. To obtain full flexibility in analysis method, data selection, filtering etc. a more tailor made approach is needed. This can only be achieved after downloading the datasets and perform a manual analysis, which requires considerable computer power and knowledge about analysis methods, which is not essential for most of the available online tools.

Within the plant field there is an increasing number of publications that report the finding of biologically relevant genes involved in certain pathways via co-expression analysis. Some examples are: genes involved in root development (Birnbaum *et al.*, 2003), genes involved in mitochondrial functions (Elo *et al.*, 2003), clusters of genes involved in primary and secondary cell wall formation (Persson *et al.*, 2005), Myb transcription factors responsible for initiation of aliphatic glucosinolate biosynthesis (Hirai *et al.*, 2007), and clusters of genes in a network related to cold stress and biochemical pathways (Ma *et al.*, 2007). In all these cases co-expression analysis assisted in building a network that linked unknown regulatory elements to already described pathways and helped expand hypotheses on how the genes in the network were regulated.

Although co-expression analysis tools are powerful in lead discovery, they cannot guarantee that observed co-expression of genes is biologically relevant. Further analysis using the 'classical' genomic and/or metabolomic approaches will still be necessary to confirm the involvement of the discovered genes. Despite this, co-expression analysis has proven itself as a very powerful tool in the discovery of new targets for analysis within a pathway or network of interest, as it can much more rapidly provide insight into potentially important network genes than random gain of function or loss of function approaches, screening for phenotypes.

RESULTS AND DISCUSSION

Public Microarray Data Selection

To discover new leads in the transcriptional regulation of the SA, JA and ET biosynthesis and signaling pathways under stress conditions an analysis of multiple transcriptome co-expression profiles was setup. For a flexible setup that is not limited to predefined settings, datasets or processing of samples, a dataset was downloaded from the TAIR website (ftp.arabidopsis.org/Microarrays/analyzed_data/). This dataset consists of 1436 Affymetrix Arabidopsis 25K arrays obtained from NASCArrays and AtGenExpress. All microarrays are normalized by TAIR using the robust multi-array method (RMA).

To focus on stress-related SA, JA and ET biosynthesis and signaling pathways we performed a bi-

clustering of all WRKY transcription factors spotted on the Affymetrix array versus a selected set of microarray data obtained from a variety of stress conditions. The stress data set of 372 microarrays as listed in Table 1 was selected from the total of 1436 currently available microarrays. For comparison, a set consisting of 237 development-related microarrays and a set of all 1436 available microarrays were also analyzed. For the bi-clustering, the raw RMA normalized expression values were transformed in such a way that the mean is 0 and the standard deviation is 1. A positive value within the bi-clustering graph represents a higher expression value for the specific gene under the given experimental condition in comparison to the average of all other genes under all conditions, and vice versa for negative values. A hierarchical cluster tree was added, with complete linkage and a dendogram cutoff of 0.50, for both the experimental conditions and the WRKY genes, and visualized using different colors. The result of this bi-clustering is shown in Figure 1A. The colors of the bar below the bi-clustering matrix corresponds to the colored sets of arrays as denoted in Table 1. Similar bi-clusterings of WRKY gene expression profiles were performed with the subset of development-related microarrays and with the set containing all micro-arrays. The hierarchical cluster trees for the latter bi-clusterings is shown in Figures 1B and 1C, respectively.

It is evident that substantial differences occur in the hierarchical clustering of the WRKYs between the three sets of arrays. WRKY genes with coordinated expression patterns clustering close together under conditions of stress (Fig. 1A), appeared not necessarily also co-regulated during development (Fig. 1B). E.g., WRKYs 19 and 4 (Fig. 1A, top) were clustered close together in the same subtree when the bi-clustering was done with the set of stress microarrays, but were situated far apart in separate subtrees when the development-related arrays were used. The same is the case for WRKYs 28 and 46 (see below). To maximize the probability that only biologically relevant correlations were obtained, we chose to use the dataset of the stress-related micro arrays listed in Table 1 to investigate co-expression of additional sets of genes involved in the SA, JA and ET pathways.

Table 1. Selected microarray experiments.

Group	Treatment	Array code	Arrays
	Wounding stress	X8111 – X8724	28
	Ps. syringae pv. tomato DC3000/avrRpm1/*phaseolicola*	AtGen_A	48
	Bacterial/fungal elicitors (NPP1, HrpZ, Flg22, LPS)	AtGen_B	42
	Phytophthora infestans spores	AtGen_C	18
	Light treatments (Red, Far-red, Blue, UV-A, UV-B)	AtGen_D	48
	ABA, ACC, BL, GA3, IAA, MJ, BL, Zeatin	Goda_I	72
	Variety of chemicals (including SA)	Goda_II / III	86
	Sulfate	Maruyama	22
	ABA	Nakabayashi	8

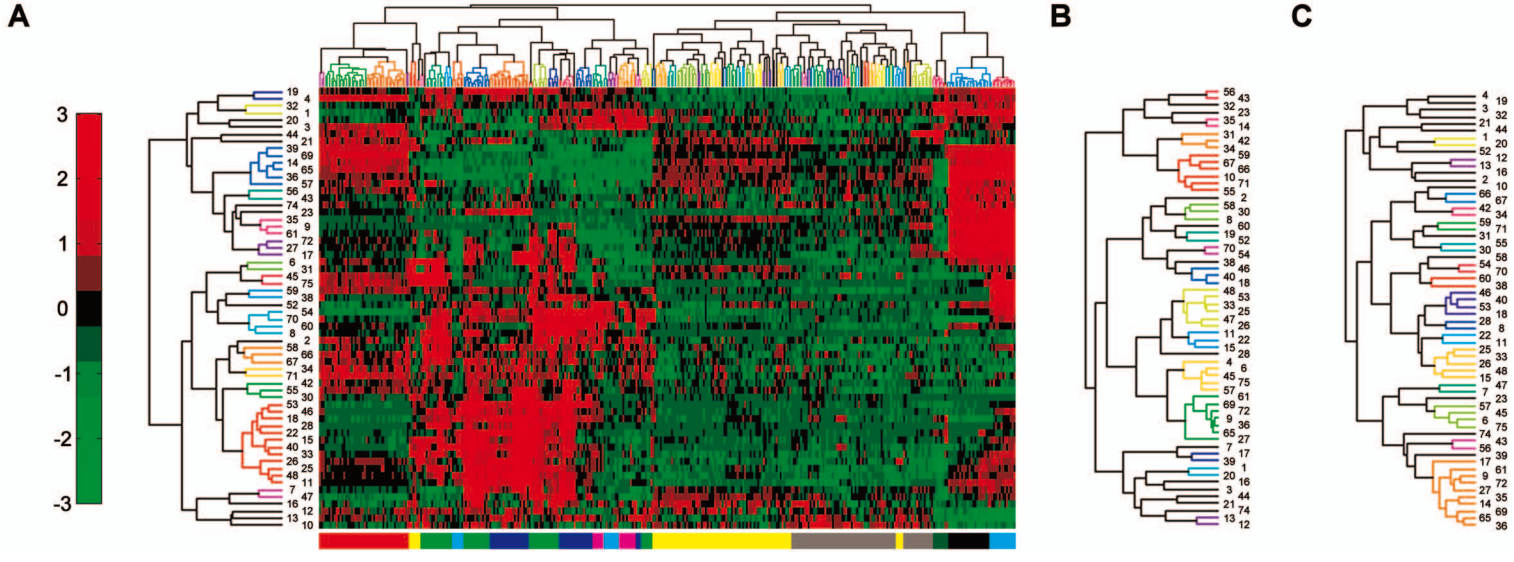

Figure 1. Bi-clustering of WRKY genes under different experimental conditions. Bi-clustering of WRKY genes under stress conditions (A), development-related processes (B), and all micro-arrays in the dataset (C). The colors in the bar underneath the bi-clustering in panel A correspond to the colored datasets of the microarrays listed in Table 1. The numbers on the left side of the bi-clustering indicate the corresponding WRKY numbers. Similarly colored branches within the dendogram represents groups with a linkage between nodes lower then 0.50. The color range in the bi-clustering matrix ranges from +3 (red, above average expression) to -3 (green, below average expression).

Target Gene Selection and Co-expression Cutoff Determination

To elucidate new transcription factors regulating SA, JA and ET biosynthesis and signaling pathways we composed a set of genes consisting of all color-coded genes indicated in Figure 2 with almost 1400 transcription factor genes according to Czechowski *et al.* (2004), supplemented with the genes for all the known JAZ repressor proteins. The best way to determine the Pearson Correlation Coefficient (PCC) cutoff for finding biologically relevant co-expressed genes and networks, would be a maximal clique approach, as reviewed by Borate *et al.* (2009). However the calculation of maximal cliques requires extensive computer power and memory. Since limited computer resources were available, we opted for the approach used by Aoki *et al.* (2007). The number of nodes (genes), edges (links between genes), the network density (a ratio of the observed number of edges to all possible edges), and the number of individual clusters obtained using the MCODE algorithm was determined for different PCC cutoffs. The results are visualized in Figure 3A-D. The total number of nodes and edges increased with a decreasing PCC threshold (Figure 3A and B). Decreasing the PCC cutoff to below 0.70 has the effect that the number of nodes that have at least one link with another node, as depicted in Figure 3A, no longer increases linearly. On the other hand, the number of edges starts to rapidly increase below this cutoff (Fig. 3B), indicating that the available nodes become more densely connected as can also be seen with the increase in network density below this cutoff (Fig. 3C). To evaluate the number of clusters of closely co-regulated genes inside the network, the MCODE algorithm was used to determine the number of clusters for decreasing PCC values between 0.9 and 0.5 at 0.01 intervals (Fig. 3D). The number of clusters increases steadily when lowering the PCC cutoff from 0.90 to approximately 0.70 after which it stabilizes between 0.7 and 0.6 and at lower thresholds even decreases. This implies that biologically significant modules are most likely to be expected above the 0.70 threshold.

Using the PCC threshold of 0.70 a co-expression network was constructed and visualized with Cytoscape (Figure 3E). The blue dots represent the selection of transcription factors and JAZ proteins having at least one edge (i.e. sharing at least one connection with other genes), and the colored dots represent the correspondingly colored genes from Figure 2. The total co-expression network thus obtained consists of 808 nodes that share 5951 edges.

Exploration of Co-expressed Closest Neighbor Transcription Factors of Regulatory Genes

The closest neighbors with a single edge distance from the regulatory genes shown in Figure 2 were separated in multiple sub cluster networks (Figs. 4-7). The MAP kinase pathway from Flagellin to defense genes (Fig. 2, dark green boxes) is depicted in Figure 4A, and the MAP kinase pathway leading to the suppression of SA and induction of JA defense genes (Fig. 2, purple boxes) is shown in Figure 4B. The network of genes co-expressed with the JA biosynthesis genes (Fig. 2, yellow boxes)

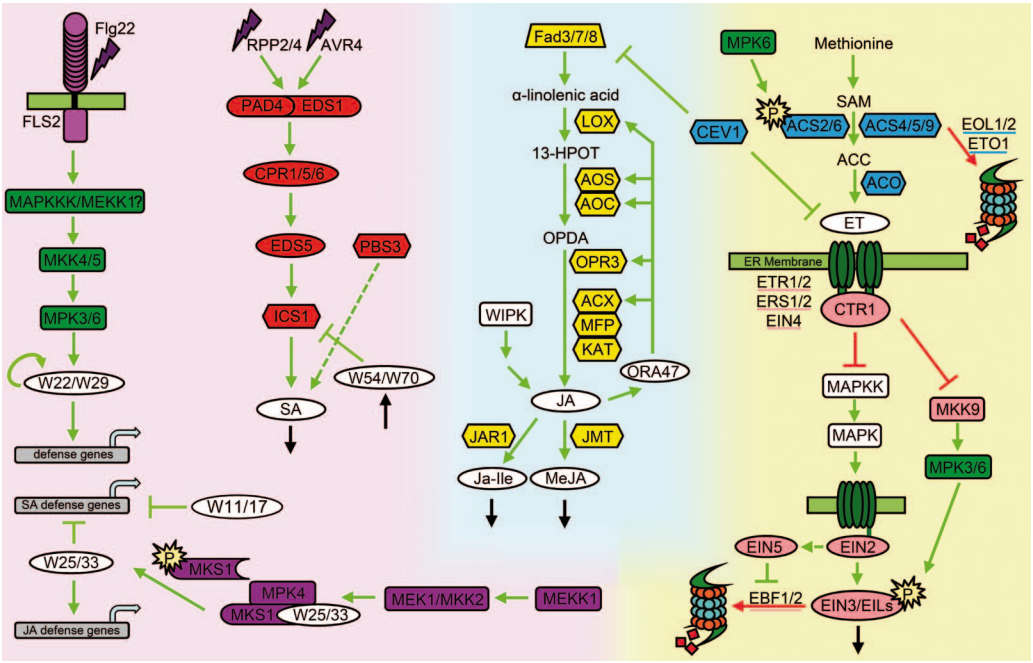

Figure 2. Visual representation of the JA/SA/ET biosynthesis and signaling pathways. Dark green boxes, MAPK kinases leading from Flagellin to defense genes; red boxes, genes within the SA biosynthesis pathway; purple boxes, MAPK kinases leading to repression of SA and induction of JA defense genes; yellow boxes, genes involved in JA biosynthesis; light blue boxes, genes involved in ET biosynthesis; pink boxes, genes involved in ET signaling.

is depicted in Figure 5. Networks of ET biosynthesis (Fig. 2, light blue boxes) and ET signaling (Fig. 2, pink genes) are shown in Figures 6A and 6B, respectively. Figure 7 shows the network of genes co-expressed with the genes leading to SA biosynthesis (Fig. 2, red boxes). A detailed description of the above networks is given in the following paragraphs.

The MAP Kinase Pathways

The response to flagellin fragment flg22 as part of the PAMP signaling pathway is mediated via a MAPK cascade (Asai *et al.*, 2002; Suarez-Rodriguez *et al.*, 2007). This signal transduction via MAPKKK/MEKK1?–MKK4/MKK5–MPK3/MPK6 leads to transcriptional activation of downstream *WRKY22* and *WRKY29* genes, which results in the induction of resistance to both bacterial and fungal pathogens (Fig. 2; Asai *et al.*, 2002). Our results show that the genes encoding the MAPK components are highly co-expressed and form a network with

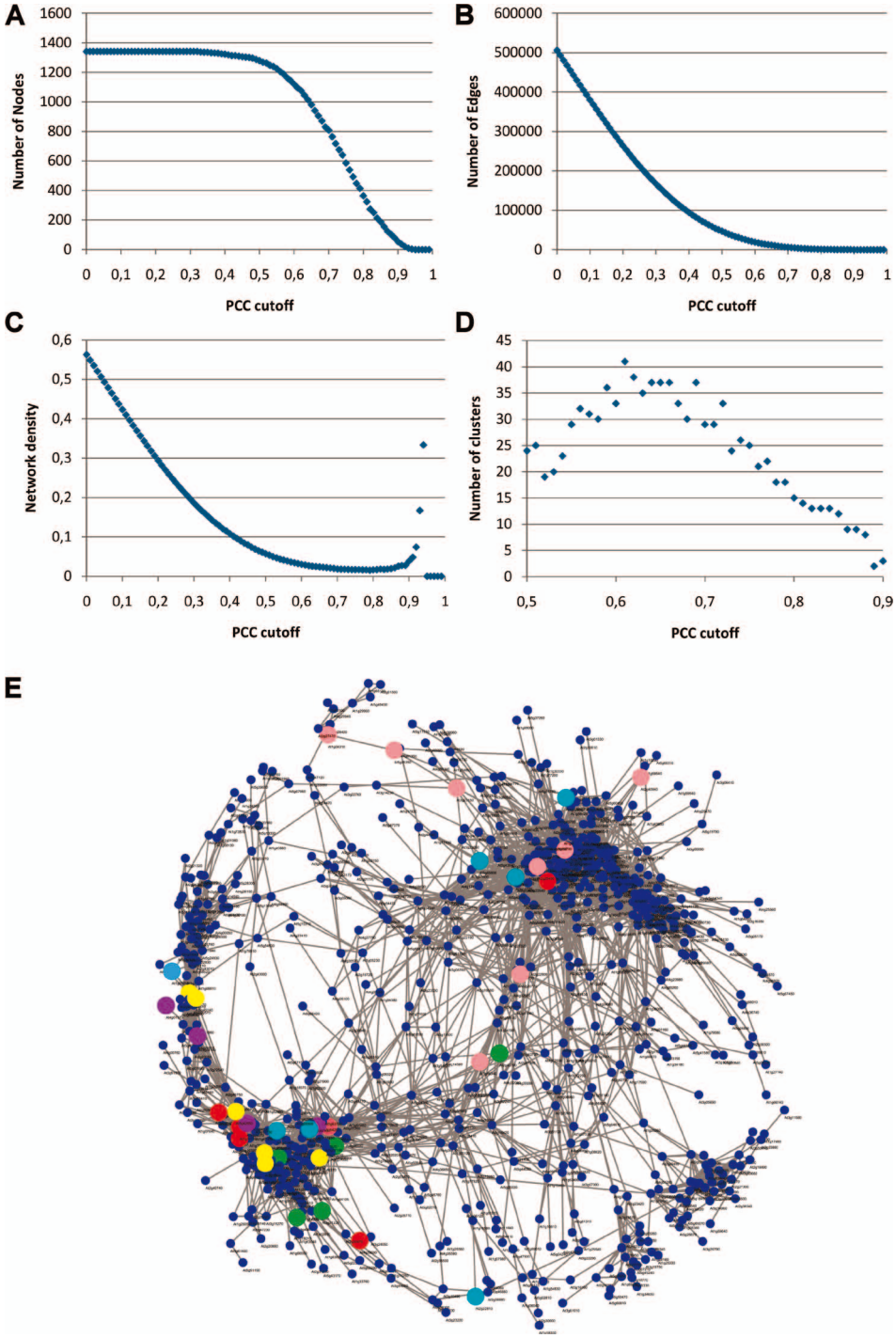

Figure 3. Pearson correlation coefficient cutoff determination and co-expression network.

a large number of co-expressed transcription factors (Fig. 4A). The known downstream target of this cascade, *WRKY22*, is connected to *MEKK1* and *MKK4*/*MKK5*. Surprisingly, *MPK6* was not linked to any of the genes in the network, but was found to be co-expressed with *EIN3* and *ETR1*, both involved in the ET signaling pathway (Fig. 4A; see below). As revealed by Mészáros *et al.* (2007), multiple different models are possible of how MPK6 could be regulated directly under MEKK1. On the other hand, MPK6 has been described as the MAP kinase substrate of MKK3 and the MKK3-MPK6 cascade is important for the JA-dependent negative regulation of *MYC2* (Takahashi *et al.*, 2007). MYC2 has the opposite effect on the MKK4/MPK3 branch. Induction of *ERF2* activates a variety of wound response/insect resistance genes in JA-treated plants and regulates JA-dependent responses. *ERF2* is positively regulated by MYC2 and in our analysis is connected to *MKK4* and *MPK3* (McGrath *et al.*, 2005; Dombrecht *et al.*, 2007). Besides this connection, *MKK4* is co-expressed with *AOS* and *OPR3* (Fig. 5) that are both important genes in the biosynthesis pathway of JA, suggesting that ERF2 might activate the MKK4/MPK3 cascade and via this route induce JA biosynthesis. With the biosynthesis of JA, in many cases also the JAZ repressor genes are positively regulated (Chini *et al.*, 2007). The connection between *MKK4* and *JAZ5* might indicate that this branch is under control of the JAZ5 repressor.

The flagellin fragment flg22 not only affects the regulation of JA and ET pathways, but also activates the SA pathway. Many WRKY genes are co-expressed with *MEKK1* and *MKK4*. *WRKY28* is rapidly induced to very high levels upon flg22 treatment (Navarro *et al.*, 2004). Together with *WRKY28*, *WRKY46* is also co-regulated and they are both found as co-expressed genes with important genes in the SA biosynthesis pathway (Fig. 7).

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 enhances resistance against *Pseudomonas syringae* (Xu *et al.*, 2006). The link between *WRKY53* and *MEK1* can be explained via *MEKK1* (Figure 4B). MEKK1 is upstream of MEK1 and interacts with an activation domain protein that can be phosphorylated and bind to the promoter of *WRKY53* and acts as a positive regulator of *WRKY53* (Miao *et al.*, 2007). This links *WRKY18* and *WRKY53* to flg22 and the initiation of SAR mediated defense within our co-expression network.

The MAPK cascade (MEKK1–MEK1/MKK2–MPK4), induced by challenge inoculation with *Ps. syringae* or treatment with flg22, leads to phosphorylation of MAP kinase substrate 1 (MKS1), which forms a complex with MPK4 and WRKY33 and possibly WRKY25. Upon phosphorylation 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. (Andreasson *et al.*, 2005; Brodersen

(A) Graph of the number of nodes with at least one link for each PCC cutoff. (B) Graph of the number of edges between nodes for each PCC cutoff. (C) Graph of the network density for each PCC cutoff. (D) Graph of the total number of clusters determined with the MCODE algorithm for each PCC cutoff. (E) Visualization using Cytoscape of the co-expression network. Blue-dots, on microarray spotted selection of >1400 transcription factors and JAZ proteins; other colored dots represent similarly colored genes from Figure 2.

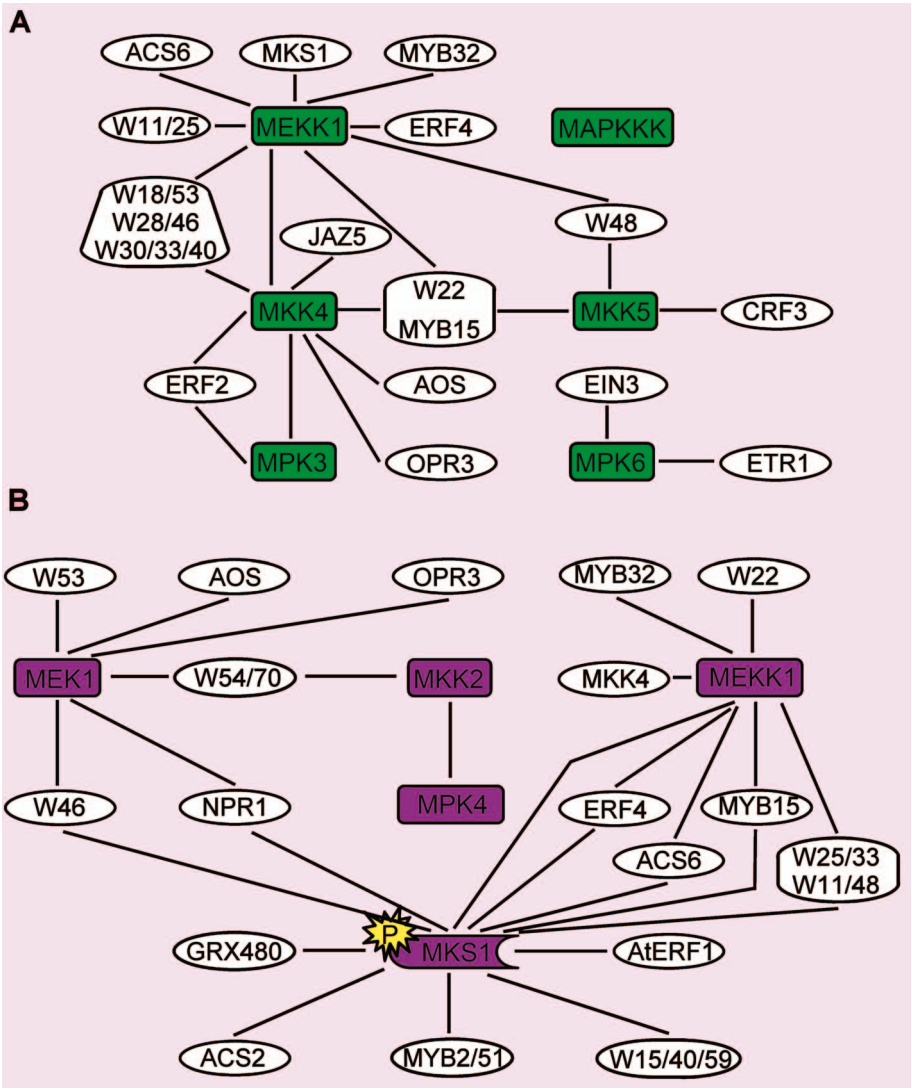

Figure 4. Co-expression network of the MAP kinase pathways. Co-expression network of MAP kinases leading to defense genes (A) and to SA defense gene repression and JA defense gene induction (B). The genes in colored boxes in the network correspond to similarly colored components of the signaling pathways indicated in Figure 2. The genes in white boxes indicate co-expressed genes with at least one edge to the kinase genes in the colored boxes.

et al., 2006; Journot-Catalino *et al.*, 2006; Qiu *et al.*, 2008). Almost all of the genes encoding these WRKYs were found interconnected in the co-expression network (Fig. 4B). *WRKY48* is also stress and pathogen inducible and acts as a transcription factor that represses plant basal defense and

PR-gene expression. When considering its location in the co-expression network, WRKY48 could function in a similar manner as WRKY11/17 and/or WRKY25/33 (Xing *et al.*, 2008).

WRKY70 and the 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. Besides this negative role, they activate other SA-regulated genes (Kalde *et al.*, 2003; Wang *et al.*, 2006). The route via which WRKY54 and WRKY70 repress SA biosynthesis is unknown. Within the co-expression network both these WRKYs link to both *MEK1* and *MKK2*, two important kinases in the cascade that leads to repression of SA defense genes. It may be that negative regulation of SA biosynthesis is brought about through activation of this MAP kinase cascade by WRKY54 and WRKY70.

The JA Biosynthesis Pathway

The JAZ repressor proteins play an important role in JA signaling. The initial JAZ repressor that is released from MYC2 to activate transcription of target genes is JAZ3 (Chini *et al.*, 2007; Thines *et al.*, 2007). *MYC2*, *JAZ1* and *JAZ3* are directly linked in the co-expression network with *OPR3*, encoding 12-oxophytodienoate reductase, an essential enzyme in JA biosynthesis (Fig. 5). Several other genes encode JAZ repressors are also connected to *OPR3* and in addition to the gene encoding *JA methyl transferase* (*JMT*), while others link to both *JMT* and the gene for *allene oxide synthase* (*AOS*). The various connections of these *JAZ* genes may hint at which levels the different JAZ repressors are operational (Fig. 5).

Surprisingly, many of the WRKY transcription factors that are involved in positive or negative regulation of *PR*-genes and SAR are also connected to the JA biosynthesis pathway (Fig. 5), like the positive regulatory combinations *WRKY18*/*53* (Fig. 4A), *WRKY54*/*70* (Fig. 4B), *WRKY28*/*46* that are possibly involved in the regulation of SA biosynthesis (Fig. 7) and *WRKY11/48* that act as negative regulators of SA defense genes.

Several members of the MYB transcription factor family were also found to be closely co-expressed with the JA biosynthesis genes *AOS*, *OPR3* and *JMT*. Most of the co-expressed MYB transcription factors have no known function. Using publicly available online co-expression analyses, a link was found between *MYB29* and the regulation of aliphatic glucosinolate biosynthesis (Hirai *et al.*, 2007). Since methyl-JA is involved in regulation of glucosinolate biosynthesis it would be expected that *MYB29* would be co-expressed at the level of *JMT* or below. However, the upstream connection of *MYB29* with *AOS* suggests that activation of the glucosinolate pathway by MYB29 is already initiated before methyl-JA is synthesized.

The ET Biosynthesis and Signaling Pathway

ET is produced from S-adenosyl-methionine in a two step reaction catalyzed by the enzymes

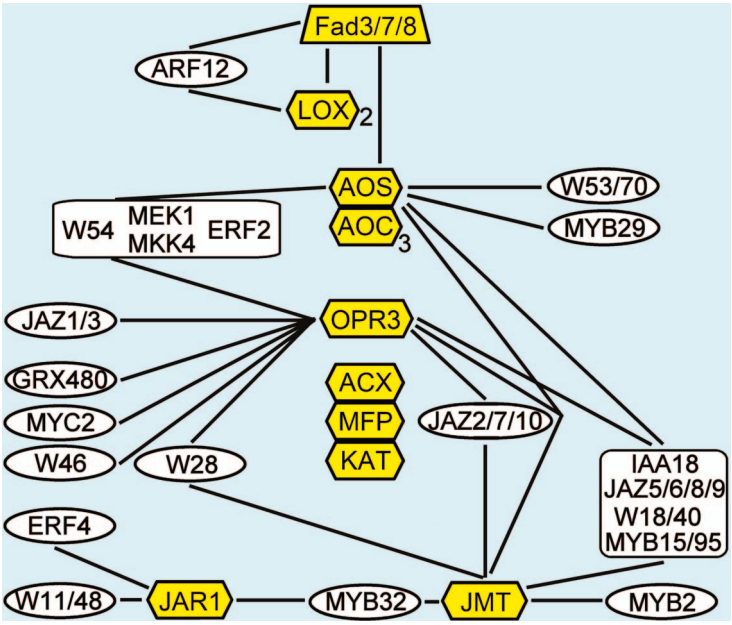

Figure 5. Co-expression network of the JA biosynthesis pathway. The genes in the yellow boxes in the network correspond to the yellow-colored components of the JA biosynthesis pathway indicated in Figure 2. The genes in white boxes indicate co-expressed genes with at least one edge to the pathway genes.

aminocyclopropane carboxylic acid (ACC)-synthase (encoded by *ACS* genes) and ACC-oxidase (encoded by *ACO*), respectively. Genes co-expressed with the ET biosynthesis genes are depicted in Figure 6A. We found a connection between *ACS2/6* and *MEKK1/MKS1* of the MAP Kinase pathway. MEKK1 has been proposed to lead to phophorylation of MPK6, although the mechanism through which this might occur has not yet been established. Different models for this regulation have been proposed by Mészáros *et al.* (2007). ACS2 and ACS6 can be phosphorylated by MPK6 (Fig. 2). This phosphorylation stabilizes the protein, which results in increased ET production (Liu and Zhang, 2004). Other genes co-expressed with the ET biosynthesis genes *ACS4*, *ACS5* and *ACO* encode a variety of Aux/IAA and ARF factors. In a review from Reed *et al.* (2001) it is proposed that targets of Aux/IAA and ARF might include genes encoding ACC synthase. Various other Aux/IAA and ARF genes were found to be closely co-expressed with a number of other regulator genes (encoding ubiquitin ligases *EOL1*, *ETO1*) involved in ET biosynthesis, indicative of a possible function in the integration of ET and auxin signaling pathways.

The MAP kinases in the ET signaling pathway (Fig. 6B) are connected to a limited number of other nodes. The link between *MPK3* and *ERF2* was discussed above. Mutant studies with the *etr1-1* gain-of-function ET-insensitive mutant placed MPK6 directly downstream of ETR1 (Chang *et al.*, 2003; Ouaked *et al.*, 2003). This is also observed within the co-expression network. In the

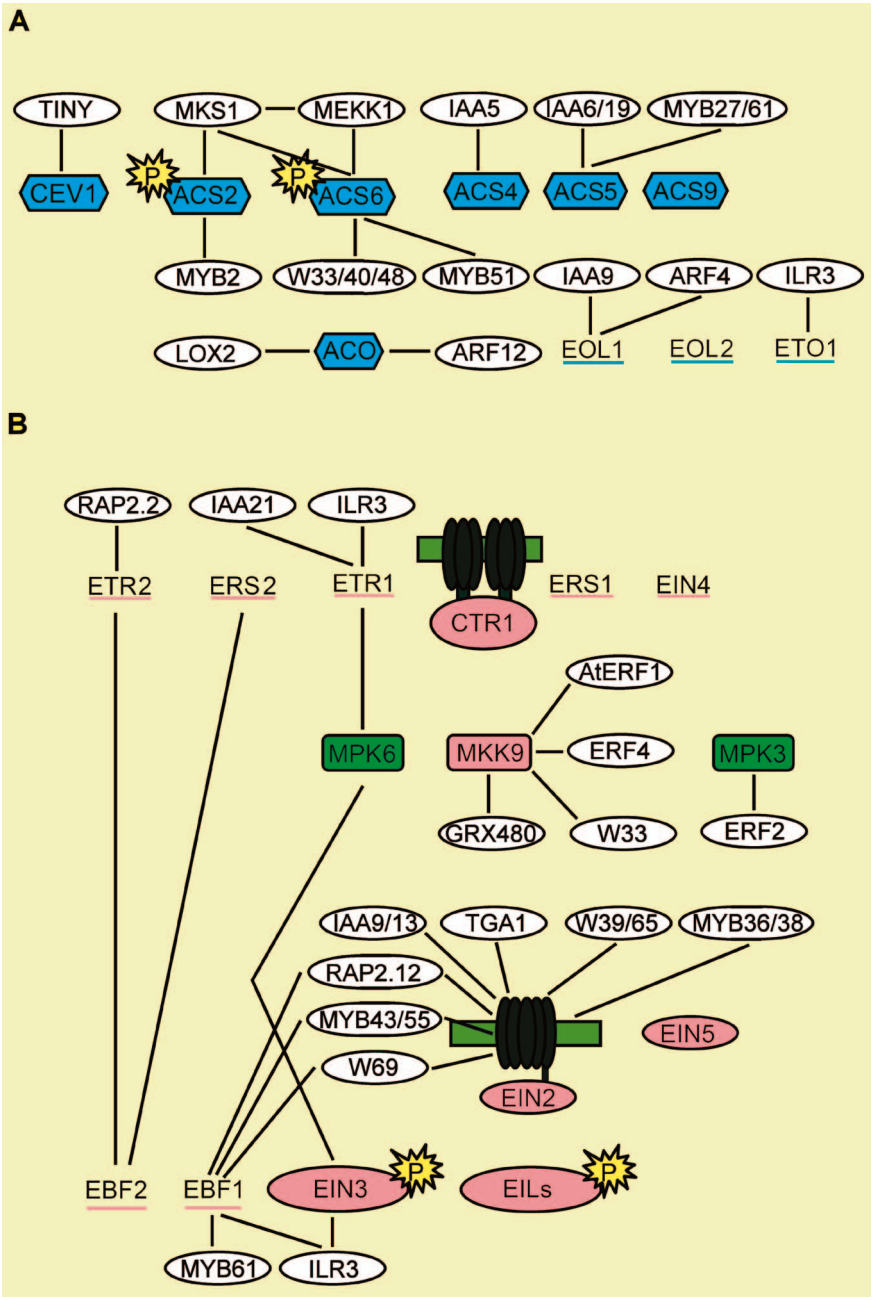

Figure 6. Co-expression network of the ET biosynthesis and signaling pathways. In panel A, the genes in the blue boxes in the network correspond to the blue-colored components of the ET biosynthesis pathway indicated in Figure 2. In panel B, The genes in coloredboxes correspond to genes in similarly colored boxes of the ET signal transduction pathway shown in Figure 2. The genes in the white boxes in both panels indicate co-expressed genes having at least one edge to the pathway genes.

network *EIN3* is also connected to *MPK6*. In the MKK9-MPK3/6 cascade it is shown that direct phosphorylation in the nucleus via this cascade stabilizes the EIN3 protein, which may be a key step in ET signaling (Fig. 2; Yoo *et al.*, 2008). Within the co-expression network depicted in Figure 3E both genes for *ETR1* and *MPK6* (represented by the pink and green dot almost in the middle of the network), are in between the super cluster with the genes encoding proteins involved in SA signaling (red dots), Flg22-initiated MAPK kinase cascade (green dots) and the JA biosynthesis genes (yellow dots), and the super cluster with several genes involved in the ET signaling pathway (pink dots). The central location of *MPK6* and *ETR1* between the super clusters with the other signaling genes might be indicative for a role of the combination of ETR1/MPK6 in crosstalk between these clusters.

Within the ethylene signaling network (Fig. 6B) we found many genes co-expressed with *EIN2*. For almost none of these genes a clear function has been described in literature so far. Recently it was found that the modulation of the NPR1 dependency of SA-JA cross-talk by ET is dependent on *EIN2* (Leon-Reyes *et al.*, 2009). Most of the genes involved in the cross-talk have not yet been assigned to a particular function. Surprisingly, in our analysis many of the genes that are co-expressed with *EIN2* (*IAA13*, *RAP2.12*, *MYB36*, *MYB43*, *WRKY39*, *WRKY69*) are also connected to *CPR5* in the SA biosynthesis pathway (see below). It is likely to assume that some of these genes are involved in the *EIN2*-dependent cross-talk with SA.

The SA Biosynthesis Pathway

Heterodimerization of EDS1 and PAD4 and their nuclear localization are important for subsequent steps in the SA signaling pathway (Feys *et al.*, 2001). Recently, it was found that *EDS1* expression is repressed by the Ca^{2+}/calmodulin-binding transcription factor AtSR1, indicating that SA levels are regulated by Ca^{2+} (Du *et al.*, 2009). We found that the gene encoding the Ca^{2+}/calmodulin-binding transcription factor MYB2, is co-expressed with *PBS3* (Yoo *et al.*, 2005; Fig. 7). If MYB2 acts like AtSR1 as a repressor of SA levels, this might indicate another point of regulation. In addition to the link to *PBS3*, *MYB2* is also connected to *JMT* in the methyl-JA synthesis pathway and to *ACS2* in the ET biosynthesis pathway, suggestive for a role for MYB2 in fine-tuning the SA, JA, and ET biosynthesis pathways. Besides the connections of *WRKY54* and *WRKY70* that are already known to have an influence on the biosynthesis of SA, we found two new WRKY genes (*WRKY28* and *WRKY46*) that are co-expressed with *isochorismate synthase 1* (*ICS1*), a key enzyme in the biosynthesis of SA. *WRKY28*, as described above, is known to be rapidly induced by flg22, while *WRKY46* is rapidly induced downstream of avirulence effectors (He *et al.*, 2006). This might indicate a direct role for these WRKYs in flagellin and avirulence effector-induced biosynthesis of SA. Another WRKY gene that we found to be co-expressed with *PBS3* is *WRKY8*. This WRKY is described in literature as one that is evolutionary highly related to WRKY28 (Yamasakia *et al.*, 2005).

To illustrate the validity of our choice to limit the co-expression analysis to the set of stress-related micro-arrays, in Figure 8 we focused on the sub network around *ICS1*/*PBS3*. In Figure 8A, all genes that were found co-expressed in the stress-related set within one edge at the PCC cutoff of 0.7

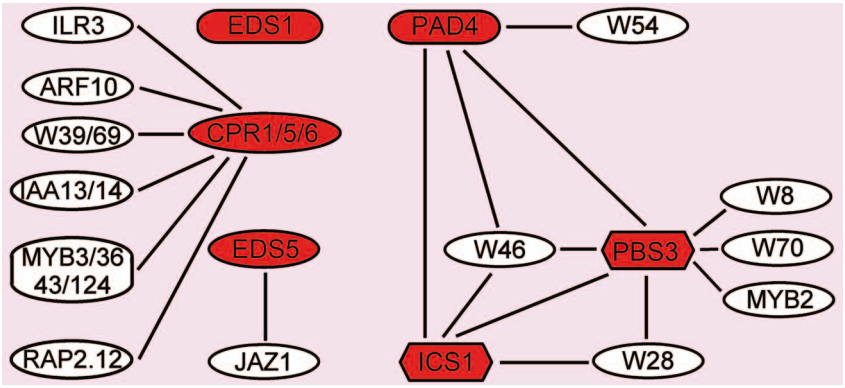

Figure 7. Co-expression network of the SA biosynthesis pathway. The genes in the red boxes in the network correspond to the red-colored components of the SA biosynthesis pathway indicated in Figure 2. The genes in white boxes indicate co-expressed genes with at least one edge to the pathway genes.

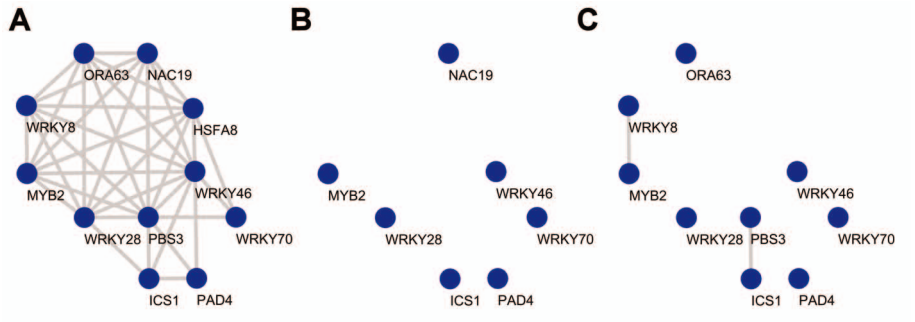

Figure 8, Co-expression subnetworks of *ICS1* and *PBS3*. The subnetwork of genes that are co-expressed within one edge of *ICS1* and *PBS3* as obtained from the data set of stress-related Arabidopsis microarrays (A), development-related microarrays (B) and all micro-arrays (C). Nodes from panel A are only shown in panels B and C if they have at least one edge within our outside of the *ICS1* and *PBS3* network.

are displayed. Among the co-expressed genes are *WRKY70* and *PAD4*, which are proven factors in the SA-signaling pathway (Wang *et al.*, 2006; Feys *et al.*, 2001). This sub network degraded when only the set of development-related genes (Fig. 8B) or when all 1436 available micro-arrays were considered (Fig. 8C).

Since our group is focused on salicylic acid related responses we decided to explore the co-expression network around *ICS1* and *PBS3* in more detail. With the nodes of *ICS1* and *PBS3* as a starting point we explored which genes were co-expressed up to two edges from *ICS1* and *PBS3*. Since this network is too dense to graphically display, the linked genes are shown in Table 2. It is

surprising that a large number of JAZ repressor proteins are closely co-regulated in the network. This may be indicative of a mechanism for negative regulation of JA-signaling by SA

Concluding Remarks

The differences between the co-expression subnetworks around two important genes in the SA-signaling pathway shown in Figure 8 indicated that the choice of the dataset is of major importance for the analysis. In our analysis, only with the biologically relevant set of stress-related micro-arrays a network was generated containing several genes that were already identified as important components of SA-signal transduction. Also, using a proper PCC cutoff is essential for a meaningful outcome. With a cutoff taken too low, a large, unworkable number of connections will be obtained of which many may not be biologically relevant, whereas a cutoff set too high could result in missing important connections. The results we obtained with our analysis corresponds well with results described in literature (e.g., the presence in the subnetwork around *ICS1* and *PBS3* of *PAD4* and *WRKY70*, established components of SA-signal transduction), which supports the notion that also other genes in the dataset may play roles in the various pathways investigated. In Figures 4-7 only co-expressed, established transcriptional regulators are depicted. A full list of all genes found to be closely co-expressed with the pathway components in Figure 2 is given in Supplementary Table 1.

Table 2. Co-expressed genes within two edges of *ICS1* and *PBS3*

At1g01480	ACS2	At1g19640	JMT	At1g76580	TF
At5g42650	AOS	At4g26070	MEK1	At5g46350	WRKY8
At2g20880	AP2	At4g29810	MKK2	At2g23320	WRKY15
At4g28140	AP2/ERF	At1g51660	MKK4	At4g31800	WRKY18
At4g32800	AP2/ERF	At3g18690	MKS1	At4g01250	WRKY22
At3g50260	AP2/ERF	At3g04030	MYB fam.	At2g30250	WRKY25
At5g61890	ORA63	At3g12730	MYB fam.	At4g18170	WRKY28
At2g22760	bHLH	At2g47190	MYB2	At2g38470	WRKY33
At2g46510	bHLH	At3g23250	MYB15	At1g80840	WRKY40
At3g53600	C2H2 ZF	At1g52890	ANAC019	At2g46400	WRKY46
At2g38250	DNA bin.	At5g64530	ANAC104	At5g49520	WRKY48
At1g28480	GRX480	At1g52880	NAM	At4g23810	WRKY53
At4g18880	HSF A4A	At1g64280	NPR1	At2g40750	WRKY54
At1g67970	HSF A8	At2g06050	OPR3	At1g66550	WRKY67
At1g19180	JAZ1	At3g52430	PAD4	At3g56400	WRKY70
At1g17380	JAZ5	At2g22540	SVP	At1g28050	ZF
At2g34600	JAZ7	At1g22070	TGA3	At3g55980	SZF1
At1g30135	JAZ8	At5g67180	TOE3	At1g27730	ZAT10
At1g70700	JAZ9	At1g30810	TF	At2g37430	ZAT11
At5g13220	JAZ10			At5g59820	ZAT12

MATERIALS AND METHODS

Microarray Dataset

The dataset of 1436 Affymetrix Arabidopsis 25K arrays obtained from NASCArrays and AtGenExpress was downloaded from ftp.arabidopsis.org. This dataset has already been normalized using the robust multi-array method (RMA). For tracking down the experimental conditions of the different arrays we used the mapping file provided and with assistance from the curators of TAIR the codes were converted into matching experimental conditions that can be found on the website. Based on these experimental conditions a selection was made of stress- and development-related datasets that were used in our experiments.

Bi-clustering, Pearson Cutoff Determination and Co-expression Analysis

The raw RMA normalized expression values were transformed such, to obtain mean expression values of 0 and a standard deviation of 1. The data was clustered using the following parameters: the distance between objects in the data matrix was one minus the sample correlation between points (treated as sequences of values), linkage was set to complete (furthest distance), and the cutoff within the dendogram for the hierarchical cluster tree was set to 0.50. All values below this cutoff were given a different color for both the experimental conditions as the genes.

To determine a biological relevant Pearson correlation cutoff, the number of nodes, edges and network density were determined for different PCC cutoffs ranging from 0 to 1 at 0.01 intervals per data point using the 372 microarrays from the selected set of stress-related micro-arrays. The total number of clusters was determined using the MCODE algorithm within Cytoscape for PCC cutoffs from 0.5 to 0.9 at 0.01 intervals using the following settings: Loops not included, degree cutoff = 2, Haircut on, fluff off, node score cutoff = 0.2, K-score = 2, Max depth = 100.

The co-expression network was built using a PCC cutoff of 0.70 for the stress dataset and was visualized using Cytoscape using standard settings.

ACKNOWLEDGEMENTS

We would like to thank the curators of The Arabidopsis Information Resource (TAIR) for helpful suggestions for tracking the experimental conditions of most of the micro-arrays in the dataset.

Supplementary Table 1. Genes encoding transcriptional regulators closely co-expressed with signaling pathway genes.

At1g51660 MKK4		At1g51660	MKK4	At2g43790 MPK6		At3g19290	ABF4
		At1g64105	ANAC027			At3g24120	MYB
At1g17380	JAZ5	At2g03470	MYB	At1g34190	ANAC017	At3g29035	ANAC059
At1g27730	STZ	At2g30250	WRKY25	At1g34370	STOP1	At3g51910	HSF A7A
At1g67970	HSFA8	At2g38470	WRKY33	At1g66340	ETR1	At3g55770	LIM domain
At1g80840	WRKY40	At3g10800	bZIP28	At2g01650	PUX2	At3g58710	WRKY69
At2g06050	OPR3	At3g15210	ERF4	At3g20770	EIN3	At3g60030	SPL12
At2g37430	C2H2 zincfinger	At3g18690	MKS1	At3g54620	bZIP25	At4g01120	GBF2
At2g38470	WRKY33	At3g23250	MYB15	At3g62240	C2H2 zincfinger	At4g14550	IAA14
At2g46400	WRKY46	At3g25990	DNA binding	At4g32040	KNAT5	At4g16110	ARR2
At3g23250	MYB15	At3g49530	ANAC062	At3g52430 PAD4		At4g34000	ABF3
At3g45640	MPK3	At3g55980	SZF1			At4g34680	GATA3
At3g55980	SZF1	At4g01250	WRKY22	At1g74710	ICS1	At5g02840	LCL1
At4g01250	WRKY22	At4g11280	ACS6	At2g40750	WRKY54	At5g06800	MYB
At4g08500	MEKK1	At4g18880	HSF A4A	At2g46400	WRKY46	At5g11260	HY5
At4g18170	WRKY28	At4g31550	WRKY11	At5g13320	PBS3	At5g16600	MYB43
At4g23810	WRKY53	At4g31800	WRKY18	At5g64930 CPR5		At5g26210	AL4
At4g31800	WRKY18	At4g34990	MYB32			At5g39660	CDF2
At4g32800	AP2/ERF DREB	At5g04340	ZAT6	At1g10480	ZFP5	At5g49450	bZIP1
At5g24110	WRKY30	At5g04760	MYB	At1g14350	FLP	At5g54680	ILR3
At5g42650	AOS	At5g08190	NF-YB12	At1g22640	MYB3	At5g57620	MYB36
At5g47220	ERF2	At5g49520	WRKY48	At1g53910	RAP2.12	At4g39030 EDS5	
At3g45640 MPK3		At5g59820	RHL41	At1g62990	KNAT7		
		At5g66730	C2H2 zincfinger	At1g74840	MYB	At1g19180	JAZ1
At1g51660	MKK4	At3g21220 MKK5		At2g02470	AL6	At5g13320 PBS3	
At5g47220	ERF2			At2g28350	ARF10		
At4g08500 MEKK1		At3g23250	MYB15	At2g33310	IAA13	At1g52890	ANAC019
		At4g01250	WRKY22	At2g47900	AtTLP3	At1g67970	HSF A8
At1g27730	STZ	At5g49520	WRKY48	At3g04670	WRKY39	At1g74710	ICS1
At1g30810	Transcription F.	At5g53290	AP2/ERF CRF3	At3g06380	AtTLP9	At2g46400	WRKY46
At1g49950	TRB1			At3g16857	ARR1	At2g47190	MYB2

At3g52430	PAD4	At3g25990	DNA binding	At4g23810	WRKY53	At2g20570	GPRI1
At3g56400	WRKY70	At3g49530	ANAC062	At4g31820	ENP	At2g24790	COL3
At4g18170	WRKY28	At3g55980	SZF1	At5g42650	AOS	At2g41940	ZFP8
At5g46350	WRKY8	At3g57480	C2H2 zincfinger	At4g29810 MKK2		At3g02830	ZFN1
At5g61890	AP2/ERF	At4g08500	MEKK1			At3g11170	FAD7
At1g74710 ICS1		At4g11280	ACS6	At2g40750	WRKY54	At3g45260	C2H2 zincfinger
		At4g17500	AtERF1	At3g56400	WRKY70	At5g02030	RPL
At2g46400	WRKY46	At4g18880	HSF A4A	At4g01370	MPK4	At5g11060	KNAT4
At3g52430	PAD4	At4g31550	WRKY11	At3g11170 FAD7		At5g42650	AOS
At4g18170	WRKY28	At5g04340	ZAT6			At5g42650 AOS	
At5g13320	PBS3	At5g04760	MYB	At1g22590	AGL87		
At3g18690 MKS1		At5g49520	WRKY48	At1g34310	ARF12	At1g17380	JAZ5
		At5g59820	RHL41	At1g68520	Zinc finger	At1g19640	JMT
At1g01480	ACS2	At5g62020	HSF B2A	At2g03710	SEP4	At1g30135	JAZ8
At1g18570	MYB51	At5g63790	ANAC102	At2g20570	GPRI1	At1g51660	MKK4
At1g27730	STZ	At4g01370 MPK4		At2g24790	COL3	At1g51950	IAA18
At1g28480	GRX480			At2g41940	ZFP8	At1g52890	ANAC019
At1g30810	Transcription F.	At4g29810	MKK2	At3g02830	ZFN1	At1g53160	SPL4
At1g42990	AtbZIP60	At4g26070 MEK1/MKK1		At3g45140	LOX2	At1g67970	HSF A8
At1g64280	NPR1			At3g45260	C2H2 zincfinger	At1g70700	JAZ9
At1g80840	WRKY40	At1g07640	OBP2	At3g57600	AP2/ERF DREB	At1g72450	JAZ6
At2g21900	WRKY59	At1g52880	NAM	At3g59060	PIL6	At1g74430	MYB95
At2g23320	WRKY15	At1g64280	NPR1	At5g11060	KNAT4	At1g80840	WRKY40
At2g30250	WRKY25	At1g67970	HSF A8	At5g42650	AOS	At2g06050	OPR3
At2g37430	C2H2 zincfinger	At2g06050	OPR3	At3g45140 LOX2		At2g20570	GPRI1
At2g38470	WRKY33	At2g40750	WRKY54			At2g23760	BLH4
At2g46400	WRKY46	At2g46400	WRKY46	At1g05010	ACO	At2g24790	COL3
At2g47190	MYB2	At3g04030	MYB	At1g22590	AGL87	At2g33810	SPL3
At3g10800	bZIP28	At3g12730	MYB	At1g34310	ARF12	At2g38250	DNA binding
At3g15210	ERF4	At3g56400	WRKY70	At1g68520	Zinc finger	At2g40750	WRKY54
At3g23250	MYB15	At4g18880	HSF A4A	At2g03710	SEP4	At2g46510	AtAIB

At3g11170	FAD7	At1g76580	Transcription F.	At4g34990	AtMYB32	At5g42650	AOS
At3g23250	MYB15	At1g79430	APL	At5g01380	Transcription F.	At5g50570	Squamosa prom.
At3g24140	FMA	At1g80840	WRKY40	At5g06710	HAT14	At5g67180	TOE3
At3g45140	LOX2	At2g34600	JAZ7	At5g24780	VSP1	At5g05170 CEV1	
At3g45260	C2H2 zincfinger	At2g38250	DNA binding	At5g49520	WRKY48		
At3g56400	WRKY70	At2g40750	WRKY54	At1g19640 JMT		At5g25810	TINY
At4g23810	WRKY53	At2g46400	WRKY46			At1g01480 ACS2	
At4g26070	MEK1/MKK1	At2g46510	AtAIB	At1g17380	JAZ5		
At4g31800	WRKY18	At3g17860	JAZ3	At1g30135	JAZ8	At1g42990	AtbZIP60
At4g32800	AP2/ERF DREB	At3g23250	MYB15	At1g51950	IAA18	At2g22760	bHLH
At5g07690	MYB29	At3g50260	CEJ1	At1g52880	NAM	At2g47190	MYB2
At5g11060	KNAT4	At4g18170	WRKY28	At1g52890	ANAC019	At3g18690	MKS1
At5g47220	ERF2	At4g23810	WRKY53	At1g70700	JAZ9	At4g11280 ACS6	
At5g67180	TOE3	At4g26070	MEK1/MKK1	At1g72450	JAZ6		
At2g06050 OPR3		At4g31800	WRKY18	At1g74430	MYB95	At1g18570	MYB51
		At4g31820	ENP	At1g74950	JAZ2	At1g27730	STZ
At1g17380	JAZ5	At4g32800	AP2/ERF DREB	At1g76580	Transcription F.	At1g42990	AtbZIP60
At1g19180	JAZ1	At5g01380	Transcription F.	At1g80840	WRKY40	At1g80840	WRKY40
At1g19640	JMT	At5g13220	JAZ10	At2g06050	OPR3	At2g38470	WRKY33
At1g27730	STZ	At5g24780	VSP1	At2g25820	AP2/ERF DREB	At3g18690	MKS1
At1g28480	GRX480	At5g42650	AOS	At2g34600	JAZ7	At3g49530	ANAC062
At1g30135	JAZ8	At5g47220	ERF2	At2g38250	DNA binding	At3g55980	SZF1
At1g32640	MYC2	At5g50570	Squamosa prom.	At2g46510	AtAIB	At4g08500	MEKK1
At1g51660	MKK4	At5g67180	TOE3	At2g47190	MYB2	At4g18880	HSF A4A
At1g51950	IAA18	At2g46370 JAR1		At3g23250	MYB15	At5g04340	ZAT6
At1g52890	ANAC019			At4g18170	WRKY28	At5g49520	WRKY48
At1g67970	HSF A8	At1g64105	ANAC027	At4g31800	WRKY18	At5g59820	RHL41
At1g70700	JAZ9	At1g78080	RAP2.4	At4g32800	AP2/ERF DREB	At2g22810 ACS4	
At1g72450	JAZ6	At2g23340	DEAR3	At4g34990	AtMYB32		
At1g74430	MYB95	At3g15210	ERF4	At5g01380	Transcription F.	At1g15580	IAA5
At1g74950	JAZ2	At4g31550	WRKY11	At5g13220	JAZ10		

At5g65800 ACS5		At3g45140	LOX2	At2g38470	WRKY33	At2g28200	DNA binding
		At1g66340 ETR1		At3g15210	ERF4	At2g29660	C2H2 zincfinger
At1g02340	HFR1			At4g17500	AtERF1	At2g33310	IAA13
At1g09540	MYB61	At1g34190	ANAC017	At5g04340	ZAT6	At2g36890	RAX2
At1g52830	IAA6	At1g58100	TCP transcript. F	At5g04760	MYB	At2g37060	NF-YB8
At2g37740	ZFP10	At2g01650	PUX2	At5g59820	RHL41	At2g38090	MYB
At3g15540	IAA19	At2g43790	MPK6	At3g20770 EIN3		At2g40620	bZIP
At3g49930	C2H2 zincfinger	At3g10800	bZIP28			At2g45050	GATA2
At3g53200	AtMYB27	At3g62240	C2H2 zincfinger	At1g34190	ANAC017	At2g47900	AtTLP3
At5g53980	AtHB52	At4g16420	ADA2B	At1g53320	AtTLP7	At3g01220	AtHB20
At4g02680 EOL1		At5g20730	IAA21	At1g53910	RAP2.12	At3g01470	AtHB1
		At5g54680	ILR3	At2g43790	MPK6	At3g04670	WRKY39
At2g34710	PHB	At5g66730	C2H2 zincfinger	At3g06380	AtTLP9	At3g16857	ARR1
At3g22780	TSO1	At3g23150 ETR2		At3g60030	SPL12	At3g23690	bHLH
At3g51950	Zinc finger			At4g16110	ARR2	At3g29035	AtNAC3
At4g38130	HD1	At3g14230	RAP2.2	At5g54680	ILR3	At3g51910	HSF A7A
At5g18830	SPL7	At5g14000	ANAC084	At5g03280 EIN2		At3g54990	SMZ
At5g60450	ARF4	At5g25350	EBF2			At3g55770	LIM domain
At5g65670	IAA9	At5g65640	bHLH093	At1g10480	ZFP5	At3g56850	AREB3
At3g51770 ETO1		At2g40940 ERS1		At1g23380	KNAT6	At3g58710	WRKY69
				At1g29280	WRKY65	At3g60530	GATA4
At1g06850	AtbZIP52	At1g10200	WLIM1	At1g53910	RAP2.12	At3g61850	DAG1
At1g53910	RAP2.12	At1g75390	AtbZIP44	At1g56010	NAC1	At4g01680	MYB55
At2g40620	bZIP	At2g45050	GATA2	At1g62990	KNAT7	At4g08150	KNAT1
At3g60030	SPL12	At1g04310 ERS2		At1g67030	ZFP6	At5g03150	JKD
At4g16110	ARR2			At1g68920	bHLH	At5g06800	MYB
At5g54680	ILR3	At5g25350	EBF2	At1g69780	AtHB13	At5g14000	ANAC084
At1g05010 ACO		At1g73500 MKK9		At1g74840	MYB	At5g15150	AtHB3
				At1g75390	AtbZIP44	At5g15830	AtbZIP3
At1g34310	ARF12	At1g27730	STZ	At1g75540	STH2	At5g16600	MYB43
At1g68520	Zinc finger	At1g28480	GRX480	At2g25180	ARR12	At5g18680	AtTLP11

At5g26210	AL4	At1g02340	HFR1	At3g10760	MYB	At5g16600	MYB43
At5g53980	AtHB52	At1g06850	AtbZIP52	At3g54990	SMZ	At5g53980	AtHB52
At5g57620	MYB36	At1g09540	MYB61	At3g55770	LIM domain	At5g54680	ILR3
At5g60120	TOE2	At1g10480	ZFP5	At3g58710	WRKY69	At5g25350 EBF2	
At5g65210	TGA1	At1g53910	RAP2.12	At3g60030	SPL12		
At5g65670	IAA9	At1g62990	KNAT7	At4g01680	MYB55	At1g04310	ERS2
At2g25490 EBF1		At1g75540	STH2	At4g05170	DNA binding	At3g23150	ETR2
		At2g37740	ZFP10	At4g16110	ARR2	At3g60530	GATA4

5

WRKY Transcription Factors Involved in Activation of SA Biosynthesis Genes

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ABSTRACT

Increased defense against a variety of pathogens in plants is achieved through activation of a mechanism known as systemic acquired resistance (SAR). The broad-spectrum resistance brought about by SAR is mediated through salicylic acid (SA). An important step in SA biosynthesis in Arabidopsis is the conversion of chorismate to isochorismate through the action of isochorismate synthase, encoded by the *ICS1* gene. Also AVR_{PPHB} *SUSCEPTIBLE 3* (*PBS3*) plays an important role in SA metabolism, as *pbs3* mutants accumulate drastically reduced levels of SA-glucoside, a putative storage form of SA. Bioinformatics analysis previously performed in our group identified WRKY28 and WRKY46 as possible regulators of *ICS1* and *PBS3*. Expression studies with *ICS1 promoter::β-glucuronidase* (*GUS*) genes in *Arabidopsis thaliana* protoplasts cotransfected with *35S::WRKY28* showed that over expression of WRKY28 resulted in a strong increase in GUS expression. Moreover, qRT-PCR analyses indicated that the endogenous *ICS1* and *PBS3* genes were highly expressed in protoplasts overexpressing WRKY28 or WRKY46, respectively. Electrophoretic shift assays indentified three potential WRKY28 binding sites in the *ICS1* promoter, positioned -445, -460 and -121 base pairs upstream of the transcription start site. Mutation of these sites in protoplast transactivation assays showed that the binding sites at -445 and -460 are functionally important for activation of the *ICS1* promoter. Chromatin immunoprecipitation assays with haemagglutinin-epitope tagged WRKY28 showed that the region of the *ICS1* promoter containing the binding sites at -445 and -460 was highly enriched in the immunoprecipitated DNA.

INTRODUCTION

Because of their sessile nature, plants have evolved very sophisticated mechanisms to actively cope with different sorts of stresses. The various defense mechanisms that can be initiated are controlled by signaling molecules like salicylic acid (SA) or jasmonic acid (JA) or by combinations of these signal compounds. SA accumulates locally in infected leaves, as well as in non-infected systemic leaves after infection with biotrophic pathogens and mediates the induced expression of defense genes, resulting in an enhanced state of defense known as systemic acquired resistance (SAR) (Métraux *et al.*, 1990; Malamy *et al.*, 1990; Dempsey *et al.*, 1999; Ryals *et al.*, 1996; Glazebrook 2005). SAR is a long-lasting broad spectrum resistance against a variety of pathogenic fungi, bacteria and viruses (Thomma *et al.*, 2001; Durrant and Dong 2004). Also exogenous application of SA results in induced expression of defense related genes (White, 1979; van Loon *et al.*, 1997). Among the genes that are induced during SAR is a set of genes collectively known as PR (pathogenesis-related) genes, with members encoding anti-fungal ß-1,3-glucanases (PR-2), chitinases (PR-3, PR-4) and PR-1, which are often used as molecular markers for SAR (Hunt *et al.*, 1996; van Loon *et al.*, 1997; Mou *et al.*, 2003; Durrant and Dong 2004).

Biosynthesis of SA can occur via two different pathways, the pathway that synthesizes SA from phenylalanine (Lee *et al.*, 1995), and the isochorismate pathway. Inhibition of the phenylalanine pathway still allows accumulation of SA (Yalpani *et al.*, 1993; Mauch-Mani *et al.*, 1996). An important step in the isochorismate pathway is the conversion of chorismate to isochorismate (ICS). Expression of a bacterial *ICS* gene in plants causes accumulation of SA, constitutive expression of *PR* genes and constitutive SAR (Verberne *et al.*, 2000), whereas the *sid2* mutant corresponding with a defective *ICS1* gene, is compromised in accumulation of SA and unable to mount SAR (Wildermuth *et al.*, 2001; Nawrath and Métraux, 1999). Expression of the *ICS1* gene is rapidly induced after infection (Wildermuth *et al.,* 2001). AVR_{PPHB} *SUSCEPTIBLE 3* (*PBS3*), of which the pathogen-induced expression is highly correlated with expression of *ICS1*, is acting downstream of SA. In the *pbs3* mutant, accumulation of SA-glucoside and expression of *PR-1* are drastically reduced. The *PBS3* gene product is a member of the auxin-responsive GH3 family of acyl-adenylate/thioester forming enzymes of which some have been shown to catalyze hormone–amino acid conjugation, like the protein encoded by the *JAR1* gene catalyzes the formation of JA-isoleucine. Although the observation that PBS3 is not active on SA, INA and Chorisamate leads to the hypothesis that PBS3 must be placed upstream of SA. (Jagadeeswaran *et al.*, 2007; Nobuta *et al.*, 2007; Okrent *et al.*, 2009).

After perception of pathogen attack by cytoplasmic TIR-NB-LRR receptors, several genes are involved in initiation of the defense response upstream of *ICS1*. One of these genes is *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*), which is probably activated after elicitor perception (Wirthmueller *et al.*, 2007). EDS1 heterodimerizes with *PHYTOALEXIN DEFICIENT 4* (*PAD4*) and their nuclear localization is important for subsequent steps in the signaling pathway (Aarts *et al.*, 1998; Feys *et al.*, 2001). Both *EDS1* and *PAD4* are induced by pathogen infection and SA application. The accumulation of SA is also regulated by Ca^{2+} via EDS1. *EDS1* expression is repressed by the Ca^{2+}/calmodulin-binding transcription factor Serine/threonine protein kinase 1 (AtSR1) that can bind to the *EDS1* promoter and repress *EDS1* gene expression (Du *et al.*, 2009). Another enhanced disease susceptibility gene (*EDS5*) that is also situated upstream of SA biosynthesis is expressed at high levels upon pathogen infection in an *EDS1*- and *PAD4*-dependent manner (Rogers and Ausubel, 1997). The *eds5* mutant plants are no longer able to accumulate high levels of SA upon pathogen infection and are unable to initiate the SAR response (Nawrath and Métraux, 1999).

Although many mutants have been reported to affect SA accumulation, no direct transcriptional regulators of genes like *ICS1* or *PBS3* have been identified. For *ICS1* the presence of many TGAC core sequences, as present in the binding sites for WRKY transcription factors, has been hypothesized to be important for transcriptional regulation of *ICS1* gene expression (Eulgem and Somssich, 2007). Here we describe two WRKY transcription factors that were previously identified in our group via a bioinformatics analysis to be closely co-expressed with *ICS1* and *PBS3*. Co-expression analyses in protoplasts showed that WRKY28 and WRKY46 positively regulated the expression of *ICS1* and *PBS3*, respectively. In addition, the binding sites for WRKY28 in the *ICS1* promoter were identified.

Our results indicate that WRKY28 and WRKY46, which themselves are both rapidly induced by pathogen elicitors (Navarro *et al.*, 2004; He *et al.*, 2006), link pathogen-triggered defense gene expression to the accumulation of SA via induction of *ICS1* and *PBS3* gene expression.

RESULTS

WRKY28 Activates *ICS1::GUS* Gene Expression in Arabidopsis Protoplasts

The co-expression analysis from Chapter 4 indicated that WRKY28 and WRKY46 could play a role in regulation of *ICS1* and *PBS3*. To verify if WRKY28 and WRKY46 can act as positive transcriptional regulators of *ICS1* and/or *PBS3* gene expression we performed transactivation assays in Arabidopsis protoplasts. Protoplasts were cotransfected with plasmids containing either the *WRKY28* or *WRKY46* coding region behind the *35S* promoter, together with a plasmid containing the GUS reporter gene cloned behind the 1kb promoter region of *ICS1* or of *PBS3*. As controls, the *promoter::GUS* fusions were cotransfected with an "empty" plasmid lacking the *WRKY28* or *WRKY46* coding region. The results of these transactivation assays are shown in Figure 1. *ICS1* promoter-directed GUS expression is increased approximately 4-fold by WRKY28 in comparison to the empty vector control. No increase is observed after cotransfection with the WRKY46 plasmid. In the case of *PBS3* promoter-directed GUS expression, neither WRKY28 nor WRKY46 positively stimulated gene expression.

To analyze the effect of WRKY28 and WRKY46 on expression of endogenous *ICS1* and *PBS3* genes, Arabidopsis protoplasts were transfected with *35S::WRKY28* or *35S::WRKY46* plasmids and incubated overnight, after which total RNA was isolated for qRT-PCR analysis of the expression of the endogenous *ICS1* and *PBS3* genes. Often, WRKYs positively regulate their own expression (Pandey *et al.*, 2009) and therefore expression of the endogenous *WRKY28* and *WRKY46* genes was also investigated. The constitutive housekeeping genes *Actin3*, *Actin7*, *Actin8* and β-*Tubelin* were used as controls. The results of the qRT-PCR analyses are shown in Figure 2. WRKY28 overexpression resulted in a 4.5 fold increase of *ICS1* mRNA. This confirms the presence of WRKY28 responsive elements in the *ICS1* promoter, at least part of which are present in the 1 kb fragment analyzed in Figure 1. WRKY28 did not increase expression of the *PBS3* gene. Apparently neither the 1 kb fragment of the *PBS3* promoter (Fig. 1) nor the full-length promoter contains WRKY28 responsive elements. Overexpression of WRKY46 had no effect on expression of the *ICS1* gene, indicating that the full-length promoter of this gene does not contain WRKY46 responsive elements. However, WRKY46 overexpression resulted in a 4-fold increase of the *PBS3* mRNA level. This indicates that the *PBS3* promoter contains WRKY46 responsive elements, located more than 1 kb upstream of the transcription start site. Obviously, there is no positive effect of WRKY28 or WRKY46 on the expression of the corresponding endogenous WRKY genes, but both WRKYs did have a slightly negative effect on the expression of the endogenous *WRKY28* gene.

Characterization of the WRKY28 Binding Sites in the *ICS1* Promoter

As a first step towards the characterization of WRKY28 binding sites in the *ICS1*

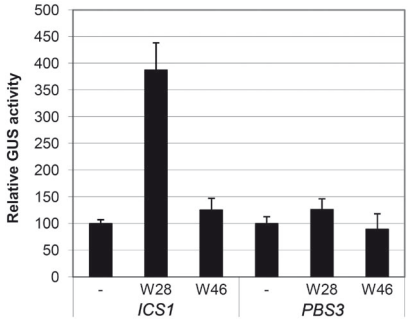

Figure 1. Transactivation of *ICS1::GUS* and *PBS3::GUS* promoter fusions by WRKY28 and WRKY46 in Arabidopsis protoplasts. The fusions contained promoter sequences of 1 kb upstream of the transcription start sites of the *ICS1* or *PBS3* genes. Protoplasts were transfected with vector pRT101 containing *35S::WRKY28* (W28) or *35S::WRKY46* (W46) inserts, or with the empty vector (minus sign). In the left three columns, the protoplasts were co-transfected with the *ICS1::GUS* fusion, in the right three columns, the protoplasts were co-transfected with the *PBS3::GUS* fusion. The columns represent the average relative GUS expression observed in four experiments. GUS expression induced in the presence of the empty pRT101 vector was taken as 100%. Error bars represent the SEM.

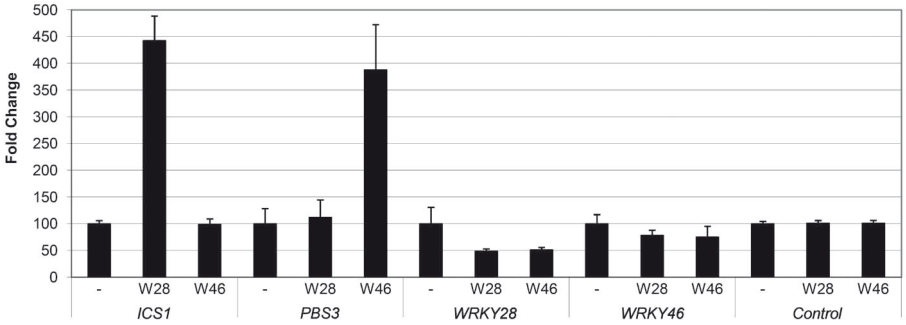

Figure 2. Effect of WRKY28 and WRKY46 on the expression of several endogenous Arabidopsis genes. Expression of ICS1, PBS3, WRKY28, WRKY46 and four household genes in Arabidopsis protoplasts was measured by qRT-PCR. Expression of each gene was measured in protoplasts transfected with the empty pRT101 vector (minus sign) or with the pRT101 vector containing *35S::WRKY28* (W28) or *35S::WRKY46* (W46) inserts. Bars represent the average fold change in mRNA levels observed in three experiments. mRNA levels in protoplasts transfected with the empty pRT101 vector were taken as 100%. The control represents the average of the data obtained with the four household genes. Error bars represent the SEM.

promoter, a region of this promoter of 960 base pairs (bp) upstream of the transcription start site was divided by PCR into six overlapping fragments. After labeling, the fragments were assayed for their ability to bind to purified glutathione S-transferase (GST)/WRKY28 fusion protein expressed in *E. coli* using electrophoretic mobility shift assays (EMSAs). The results

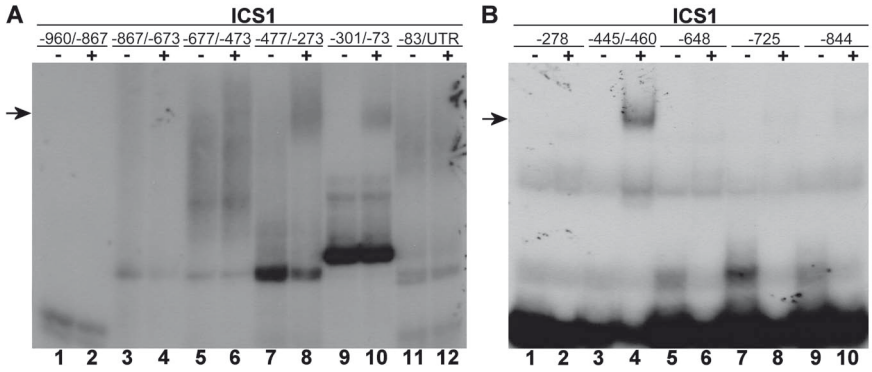

Figure 3. Binding of WRKY28 to *ICS1* promoter fragments. (A) EMSAs were done with six overlapping promoter fragments spanning the 960 bp sequence upstream of the transcription start site of the ICS1 gene. The borders of these fragments are given on top of the lanes. (B) EMSAs were done with promoter fragments of 30 bp, each containing a TGAC core sequence (positions -278, -445/-460, -648, -725) or a WK-like box (-844) in the center. The location of these sequences in the *ICS1* promoter is given on top of the lanes. The promoter fragments were incubated with recombinant GST/WRKY28 fusion protein (plus-signs) or without this protein (minus-signs). The position of protein-DNA complexes is indicated by an arrow.

are shown in Figure 3A. It is evident that in the presence of GST/WRKY28, part of the probes corresponding to fragments -477/-273 (Fig 3A, compare Lanes 7 and 8) and -301/-73 (Fig. 3A, compare Lanes 9 and 10) shifted to a higher position in the gel. No such band shifts were observed with the other promoter fragments.

WRKY proteins are generally considered to bind to the consensus W-box sequence TTGAC(C/T) (Eulgem *et al.*, 2000). The 1 kb *ICS1* promoter does not contain a true W-box, although a number of TGAC core sequences is present (positions -725, -648, -460, -445 and -278). Furthermore, a WK-like box (TTTTCCA) that resembles the WK-box TTTTCCAC identified by van Verk *et al.* (2008) is present at position -844. In addition to the six promoter fragments that spanned the 960 bp sequence, we prepared 30-bp promoter fragments that contained a TGAC core sequence or the WK-like box in the center. (The two inverted TGAC sequences at positions -445 and -460 were present in one 30-bp fragment.) The results of EMSAs with these fragments as probes are shown in Figure 3B. The shifted band in Lane 4 indicates that the 30-bp fragment containing the two cores at -445 and -460 was bound to GST/WRKY28 protein and this could explain the observed shift in the -477/-273 fragment shown in Figure 3A, Lane 8. With none of the other WK-like or W-box core sequences a shift could be observed (Fig. 3B, Lanes 2, 6, 8, 10). To verify the binding specificity of the 30-bp fragment containing the TGAC cores at positions -445 and -460, competition experiments were done with 50- and 250-fold excess unlabelled fragments (Fig. 4B). Evidently, addition of a 250-fold excess unlabelled fragment completely outcompeted the binding to the probe (Fig 4B, Lane 4), indicating that this *ICS1* promoter fragments specifically interacted with WRKY28.

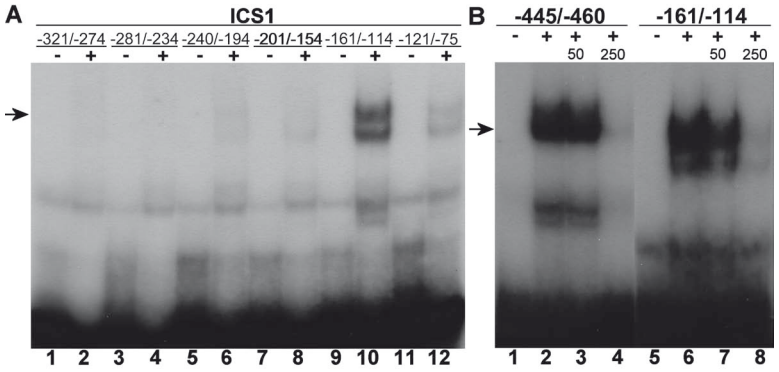

Figure 4. Binding of WRKY28 to *ICS1* promoter fragments. (A) EMSAs were done with *ICS1* promoter fragments corresponding to the positions given on top of the lanes. (B) EMSAs were done with a 30-bp fragment of the *ICS1* promoter containing TGAC core sequences at position -445 and -460 (four left lanes) and with a fragment of the *ICS1* promoter from position -161 to -114 (four right lanes. The EMSAs in panel B were done without addition of unlabeled competitor DNA, or in the presence of a 50-fold or 250-fold excess of unlabeled competitor DNA as indicated on top of the lanes. The promoter fragments were incubated with recombinant GST/WRKY28 fusion protein (plus-signs) or without this protein (minus-signs). The position of protein-DNA complexes is indicated by an arrow.

We speculated that the two TGAC core sequences at -445 and -460 could be binding sites for WRKY28 and set out to further investigate which site is responsible for the observed shift. To determine if the TGAC cores at -445 and -460 are involved in binding WRKY28, a scanning analysis was performed with a series of annealed complementary oligonucleotide probes in which the core sequences were changed to CCGG (Fig. 5C, m1, m2 and m1+2). The results of EMSAs with these fragments are shown in Figure 5A, Lanes 1 to 8. Mutation of either the core at -460 (m1) or at -445 (m2) does not abolish binding of WRKY28 to the fragment (Fig. 5A, compare Lanes 2, 4 and 6). However, mutation of both cores in mutant m1+2 disrupts binding (Fig. 5A, Lane 8). This suggests that both binding sites are equally important.

To further analyze the requirements for binding of WRKY28, pairwise mutations of the sequence around the core at -445 were scanned in an m1 background (Fig. 5C). The results are shown in Figure 5A, Lanes 9 to 24. Mutation of m2.1 and m2.4 show binding to WRKY28 (Fig. 5A, Lanes 10 and 16). As would be expected, mutations within the core sequence completely abolished binding of WRKY28 (m2.2 and m2.3, Fig. 5A, Lanes 12 and 14). Since the TGAC core at -460 has TC upstream of the core and the inverted core at -445 has a CT in this position, we checked to which extend the T or C nucleotides are important for binding. Changing CT to TC resulted in a binding of WRKY28 that was as strong as to the wild type sequence (m2.5, Fig. 5A, Lane 18). Changing CT to TT significantly lowered binding (m2.6, Fig. 5A, lane 20), suggesting that the presence of a C at either position -1 or -2 from the core is important for binding WRKY28. We further analyzed the effect of mutations at positions -3/-4 and +3/+4 from the core. Pairwise mutation of nucleotides at

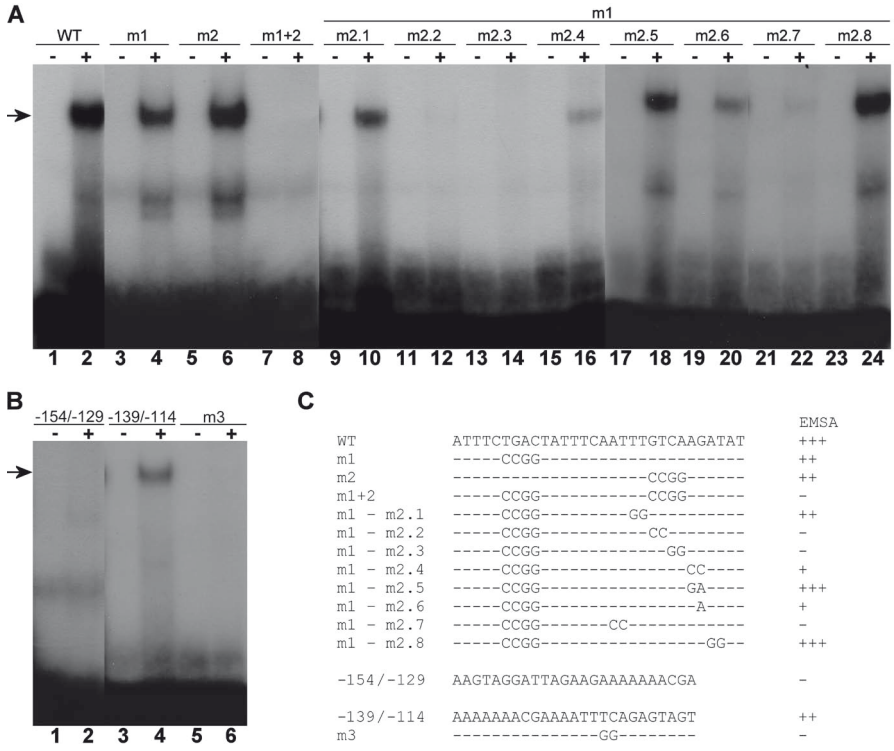

Figure 5. Binding of WRKY28 to mutated *ICS1* promoter fragments. EMSAs were done with annealed 30-bp oligonucleotides containing the *ICS1* promoter region indicated -445/-460 in the legend of Figure 3 with mutations as indicated in panel C (A). Annealed 25-bp oligonucleotides corresponding to promoter regions indicated in panel C, bottom two lines (B). Plus signs above the lanes indicate binding mixtures containing 0.5 µg recombinant GST/WRKY28. Minus signs above the lanes indicate binding mixtures without recombinant protein. The position of the protein-DNA complexes is indicated by an arrow. Plus and minus signs in panel C indicate the relative abundance of the shifted probe.

-3/-4 did not alter the binding of WRKY28 (m2.8, Fig. 5A, Lane 24), however no shift was observed when the nucleotides at +3/+4 were mutated, indicating that this flanking sequence is important for binding of WRKY28 (m2.7, Fig. 5A, Lane 22).

To more directly demonstrate that the binding sites at positions -460 and -445 are involved in WRKY28 activation of *ICS1* gene expression, mutants m1, m2 and m1+2 were introduced in the 1 kb *ICS1* promoter and their effects studied in cotransfection experiments in Arabidopsis protoplasts. While cotransfection of *35S::WRKY28* with the wild-type *ICS1 promoter::GUS* increased GUS expression approximately 3.5-fold over the background level, expression dropped significantly with promoter constructs containing the m1 or m2 mutation (Fig. 6). Combination of m1 and m2 (m1+2) did not lower GUS expression more than the single mutations (Fig. 6). This result supports the notion that WRKY28 activates *ICS1* expression through specific binding sites in the promoter at

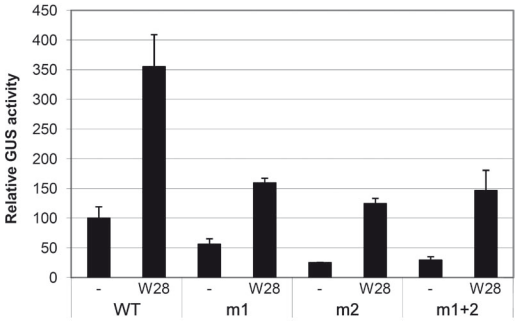

Figure 6. Transactivation of *ICS1::GUS* genes with mutations in WRKY28 binding sites. Protoplasts were transfected with 2 µg of wild-type *promoter::GUS* constructs or *promoter::GUS* constructs containing the mutations m1, m2 or m1+2 as indicated in Figure 5C. W28, cotransfection with 6 µg of expression vector pRT101 containing *35S::WRKY28*. Minus signs, cotransfection with 6 µg of empty expression vector. The bars represent the percentage of GUS activity from triple experiments relative to that of the protoplasts cotransfected with the *promoter::GUS* construct and an empty expression vector, which was set to 100%. Error bars represent the SEM.

-445 and -460 bp upstream of the transcription start site.

The EMSA experiments shown in Fig. 3A indicated that promoter fragment -301/-73 contains a binding site for WRKY28 (Fig. 3A, lane 10). Probably, this site is not located in the 30 bp sequence centered around the TGAC core sequence at position -278, because no WRKY28 binding site was detectable in the 30 bp fragment corresponding to this position (Fig. 3B, lane 2). Outside this 30 bp sequence, no sequences with similarity to known WRKY binding sites were detectable in the -301/-73 fragment. To delineate WRKY28 binding sites in the -301/-73 sequenceof the *ICS1* promoter, the entire region was divided into six overlapping fragments of approximately 50 bp. These fragments, offered as annealed complementary oligonucleotides in EMSAs, were evaluated for binding to GST/WRKY28. The results of this experiment are shown in Figure 4A. With fragments within the regions -321 to -154 and -121 to -75 bp upstream of the transcription start site no shift or only a very faint shift was observed (Fig. 4A, Lanes 1-8, and 11-12). However, a prominent shift occurred with fragment -161/-114 (Fig. 4A, Lane 10), suggesting that this region contains a WRKY28 binding site. Competition with 50- and 250-fold excess unlabeled fragment -161/-114 confirmed the specificity of the fragment for binding WRKY28 (Fig. 4B, right panel).

The region between -161 and -114 lacks a TGAC core sequence. To identify the WRKY28 binding site in this region, the left half (nucleotides -154 to -129) and the right half (nucleotides -139 to -114) were separately assayed for binding WRKY28 in EMSA (Fig. 5B, Lanes 1-4). Only with the right half a shift could be observed (Fig. 5B, Lane 4). This region harbors the sequence TTCA (-121), which in reverse orientation is somewhat similar to the W-box core TGAC. To investigate if this sequence is part of the binding site in this region, the middle two nucleotides were mutated (Fig. 5C). This mutation abolished the ability of WRKY28 to bind (m3, Fig. 5B, Lane 6), suggesting that

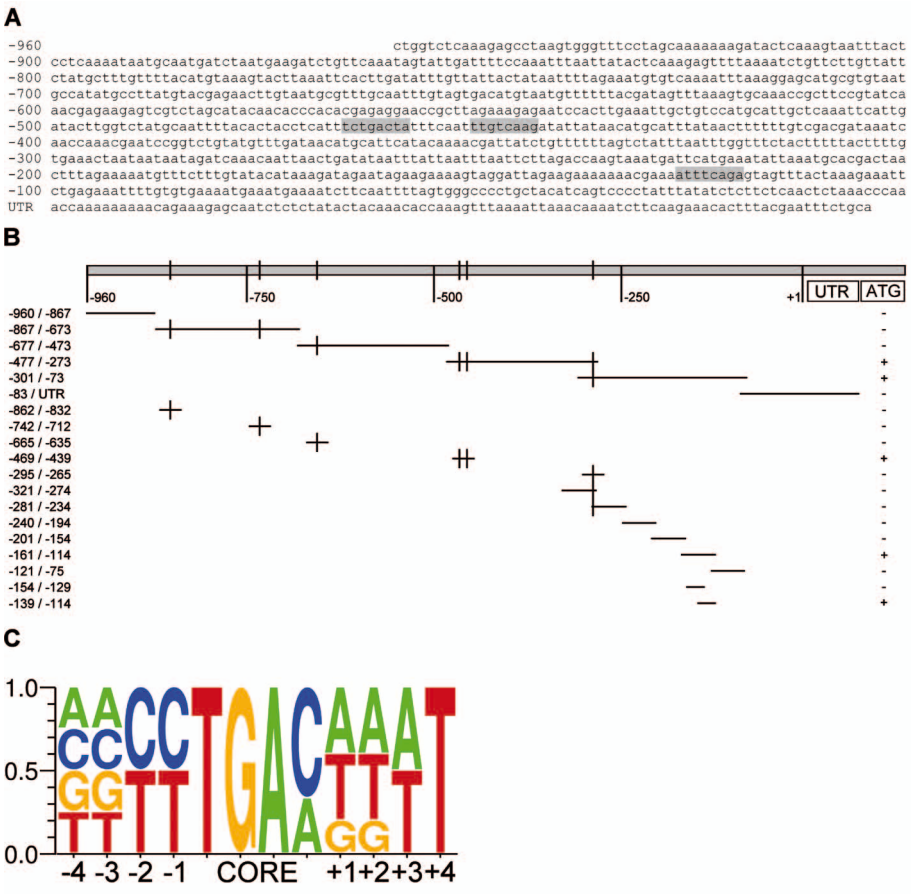

Figure 7, Summary of Electrophoretic Mobility Shift Assays. Sequence of the 960 bp *ICS1* promoter. The indentified WRKY28 binding sites are indicated against a grey background (A). Schematic representation of the *ICS1* promoter fragments analyzed by EMSA (B). Consensus WRKY28 binding sequence deduced from the EMSAs (C). In B, plus-signs in the right column indicate fragments that produced band shifts; minus-signs, fragments that did not produced a band shift. The position of the WK-like sequence or TGAC core sequences is indicated by vertical lines.

besides W-boxes with the conserved TGAC core, variants with cores like TGAA may also facilitate binding to WRKY transcription factors.

To summarize the results of the EMSAs, Figure 7A shows the 960 bp of the *ICS1* promoter with the characterized WRKY28 binding sites indicated against a grey background. A schematic representation of the fragments tested in EMSAs for binding WRKY28 is given in Figure 7B. The consensus binding sequence generated using the program WebLogo (Crooks *et al.*, 2004) by combination of the characterized binding sites and the results of the mutational analysis of the binding site at -445, is shown in Figure 7C.

Chromatin Immunoprecipitation Analysis of WRKY28

The transactivation experiments in protoplasts and the *in vitro* binding studies described above support a role for WRKY28 as a transcriptional activator of *ICS1*. To check if WRKY28 is able to bind to the *ICS1* promoter *in vivo*, chromatin immunoprecipitation (ChIP) assays were set up using Arabidopsis protoplasts, as described by Lee *et al.* (2007). The *WRKY28* coding sequence was fused to a haemagglutinin (HA) tag and expressed in Arabidopsis protoplasts. The resulting WRKY28-HA fusion protein was able to induce GUS expression when cotransfected with an *ICS1 promoter::GUS* construct, indicating that the HA tag did not interfere with WRKY28's functionality (Results not shown).

For ChIP analysis WRKY28-HA or unfused HA were expressed in protoplasts. After 24h incubation, chromatin complexes were cross-linked using formaldehyde. Upon shearing by sonication, the fragmented chromatin was incubated with monoclonal anti-HA antibodies overnight, after which immunoprecipitated complexes were captured using magnetic protein G beads and extensively washed. DNA eluted from the beads was analyzed by qPCR with primers corresponding to six overlapping regions of the *ICS1* promoter (Fig. 8A). qPCRs with primers corresponding to the coding region of *PR1* and the promoter region of *PDF1.2* were included as controls. The results are shown in Figure 8B. With the primer sets corresponding to *PR1* and *PDF1.2* no specific products were amplified, indicating that these sequences were absent from the immunoprecipitated chromatin. While no specific PCR products were amplified with primer sets A, B, D and F, it is evident that the region corresponding to the *ICS1* promoter bordered by primers C was highly enriched in the immunoprecipitated chromatin from the WRKY28-HA transfected protoplasts (38-fold in comparison to the control). This region contains the two WRKY28 binding sites at -445 and -460 as determined by EMSA (Fig. 5A). A similar result was obtained with a primer pair covering a smaller region containing the two binding sites (Results not shown). Surprisingly, approximately 20-fold enrichment was observed when primer set E was used with the chromatin precipitate from the WRKY28-HA transfected protoplasts (Fig. 8B). This primer set encompasses a further upstream region of the *ICS1* promoter, which was not found to bind to WRKY28 in the EMSA assays. However, while the amplification efficiencies of the qPCRs with all other primer sets was always above 70%, the qPCRs with primers E had a low efficiency of less than 30%, suggesting amplification of non-specific DNA sequences. Moreover, the atypical melting curve of the PCR product obtained with primers E indicated that the product was heterogeneous. Indeed, when checked on gel, primer set C produced a discrete band of the expected size, while the product of primers E consisted of a mixture of differently sized fragments (Results not shown). In conclusion, the ChIP assays indicated that WRKY28 specifically binds to the *ICS1* promoter *in vivo*, most probably to one or both binding sites at position -460 and -445 upstream of the transcription start site.

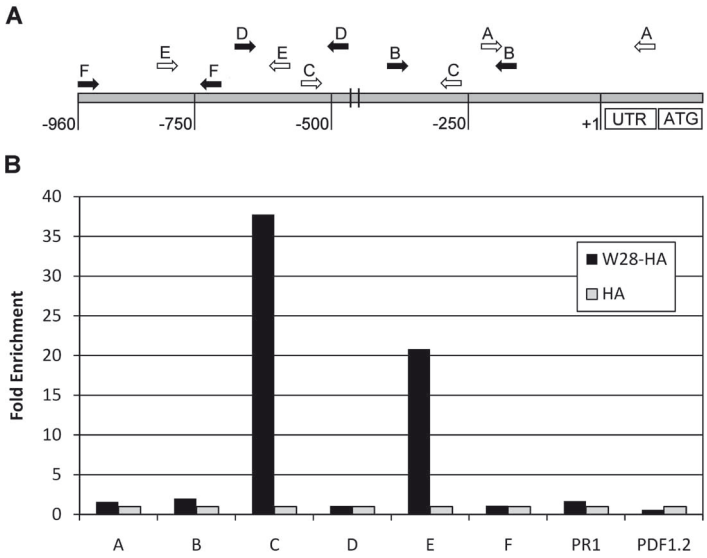

Figure 8. Chromatin Immunoprecipitation assay. Schematic representation of the location of primers corresponding to regions of the *ICS1* gene used in the ChIP assays (A). Fold enrichment of immunoprecipitated DNA from protoplasts expressing WRKY28-HA versus protoplasts expressing unfused HA (B). The position of the WRKY28 binding sites at -445 and -460 is indicated.

DISCUSSION

WRKY28 and WRKY46 Activate Expression of *ICS1* and *PBS3*, Respectively

Our *in silico* co-expression analysis of Arabidopsis transcription factor genes and genes involved in stress signaling suggested many putative new components of the signal transduction pathways (Chapter 4). Among the genes resulting from this screening were two encoding WRKY transcription factors linked to genes involved in SA metabolism. The gene encoding the type II member WRKY28 was found to be closely co-regulated with the *ICS1* gene involved in SA biosynthesis, whereas the type III *WRKY46* gene linked to *PBS3*. Based on this finding we decided to investigate the effects of these WRKYs on transcriptional activation of *ICS1* and *PBS3*. Indeed, overexpression of WRKY28 in Arabidopsis protoplasts lead to enhanced GUS activity from a co-expressed *GUS* reporter gene under control of a 1 kb *ICS1* promoter, and also expression of the endogenous *ICS1* gene was increased (Figs. 1 and 2). Likewise, overexpression of WRKY46 resulted in increased accumulation of *PBS3* mRNA, supporting the notion that WRKY46 is a transcriptional activator of *PBS3* (Fig. 2). GUS activity was not enhanced from a co-expressed 1 kb *PBS3* promoter*::GUS* gene. This indicates that WRKY46 activates the *PBS3* gene by binding at a position in the promoter further upstream than

1 kb. However, we cannot exclude the possibility that the 1 kb promoter used for the construction of the reporter construct and which was derived from curated genome sequence data by The Arabidopsis Information Resource (TAIR), is not the actual *PBS3* promoter. A detailed analysis of the region upstream of the coding sequence in the Arabidopsis genome shows that the intron of almost 1 kb suggested to be present in the 5'-UTR contains several putative binding sites for transcription factors like WRKYs and TGAs. It will be interesting to investigate if the suggested "intron" is the actual *PBS3* promoter.

DNA Binding Site of WRKY28

Several studies on DNA binding characteristics of WRKY transcription factors have led to the generally accepted consensus binding sequence TTGAC[C/T], commonly referred to as the W-box (Rushton *et al.*, 1996; de Pater *et al.*, 1996; Wang *et al.*, 1998; Eulgem *et al.*, 2000; Chen and Chen, 2000; Cormack *et al.*, 2002; Eulgem and Somssich, 2007; Ciolkowski *et al.*, 2008). Recently, we identified a variant binding site for the tobacco NtWRKY12 transcription factor (van Verk *et al.*, 2008). NtWRKY12 binds to a WK-box (TTTTCCAC), which deviates significantly from the W-box consensus sequence. Based on this finding we have suggested that the TTTTCCA sequence in EMSA probes binding to the barley transcription factor SUSIBA2 could be this WRKY's WK-like binding site (van Verk *et al.*, 2008; Sun *et al.*, 2003).

In this study we have characterized three sites in the *ICS1* promoter that have a high affinity for WRKY28. The consensus WRKY28 binding site that emerged from this analysis has some characteristics that differ from the W-box consensus (Fig. 7). We found that, unlike the consensus W-box, a C may be present at position -1 in front of the TGAC core, and although a T is also allowed at -1, a C is then required at -2. Similarly, for the sequence after the core, in two of the binding sites an A is present at +1, which in the W-box is usually either a C or a T. Remarkably, one of the WRKY28 binding sites has TGAA, instead of TGAC as core. These findings indicate that the consensus W-box is not the only WRKY binding site.

To disable binding of WRKY28 to the 30-bp EMSA probe harboring the binding sites at -460 and -445, mutation of both these sites was necessary. With only one site intact, binding was still possible (Fig. 5A, Lanes 4 and 6). Nevertheless, with the 1 kb promoter, mutation of only one of the sites had a severe effect on reporter gene expression and expression was not further reduced when both sites were mutated. Apparently, for transcriptional activation both sites are required. Possibly, activation requires that WRKY28 binds as a dimer, similar to WRKYs 18, 40 and 60, which were found to form functionally relevant homo- and heterodimers (Xu *et al.*, 2006).

The transactivation experiments also showed that mutation of the sites at -460 (m1) and -445 (m2) did not completely knock out reporter gene expression. In comparison to the GUS activity obtained with the wild type construct, approximately 20% remained. Furthermore, the reduction in basal expression levels seen with the mutant *ICS1* promoters in the absence of overexpressed WRKY28 indicates that also endogenous factors binding to the sites at -460 and -445 contribute

to the expression level. qRT-PCR has shown that the WRKY28 gene is much higher expressed in protoplasts than in suspension cells from which the protoplasts were made (Results not shown), suggesting that possibly these factors include endogenous WRKY28. Moreover, the residual GUS expression remaining with the m1, m2 and m1+2 mutant promoters could indicate that other sites in the ICS1 promoter are still able to bind WRKY28. Further analyses are required to see if the binding site identified in promoter fragment -139/-114 is a candidate for such sites.

Integrated Model for Regulation of SA Biosynthesis by WRKY28 and WRKY46

The combined results of the work described here, lead us to propose the following model for the induction of SA biosynthesis upon pathogen attack. Induction of the basal defense response starts with the detection of a pathogen-associated molecular pattern (PAMP), like in the case of flagellin, which is perceived by the FLS receptor. The activated FLS receptor triggers a MAP kinase cascade (MAPKKK/MEKK1?, MKK4/5, MPK3/6), which leads to transcriptional activation of the *WRKY28* gene (Navarro *et al.*, 2004). Transcription factor WRKY28 subsequently activates expression of the *ICS1* gene, through binding the promoter at the two binding sites at -460 and -445 and possibly at other sites, resulting in synthesis of ICS that catalyzes SA production. How the activated MAP kinase induces *WRKY28* gene expression remains a matter of speculation. The activated MAPK could activate an as of yet unknown transcription factor on stand by or release one from a repressor complex, or it may function itself as activator of *WRKY28* expression.

Less is known about the role of the product of the *PBS3* gene. It is rapidly induced in plants recognizing pathogens carrying virulence factors, like in the case of *Pseudomonas syringae* containing AVR4 (He *et al.,* 2006). A function in SA metabolism has been suggested based on its effect on SA-glucoside accumulation and its similarity to phytohormone-amino acylases (Nobuta *et al.*, 2007; Jagadeeswaran *et al.*, 2007). *PBS3* gene expression is repressed by high levels of SA, indicating that it is more likely that PBS3 functions early in the defense response before SA levels start to rise (Okrent *et al.*, 2009). Similarly, WRKY46 expression is rapidly induced upon infection and our finding that it enhances *PBS3* gene expression suggests an early role in R-gene-mediated defense. Figure 9 shows the placement of the two WRKYs in the SA-signaling pathways.

MATERIALS AND METHODS

Protoplast Preparation, Transfection and Analysis

For transactivation and qRT-PCR experiments, protoplasts were prepared from cell suspensions of *Arabidopsis thaliana* ecotype Col-0 according to van Verk *et al.*, (2008).

For transactivation experiments protoplasts were co-transfected with 2 µg of plasmid carrying *ICS1 promoter::GUS* (bp -1 to -960 relative to the transcriptional start site) or, *PBS3 promoter::GUS* (bp -1 to -1kb relative to the transcriptional start site) construct and 6 µg of *35S::effector* plasmid pRT101. As a control, cotransfection of promoter*::GUS* construct with the empty expression vector pRT101 was carried out. The protoplasts were harvested 16 hrs after transformation and GUS activity was determined (van der Fits and Memelink, 1997). GUS activities from triplicate experiments were

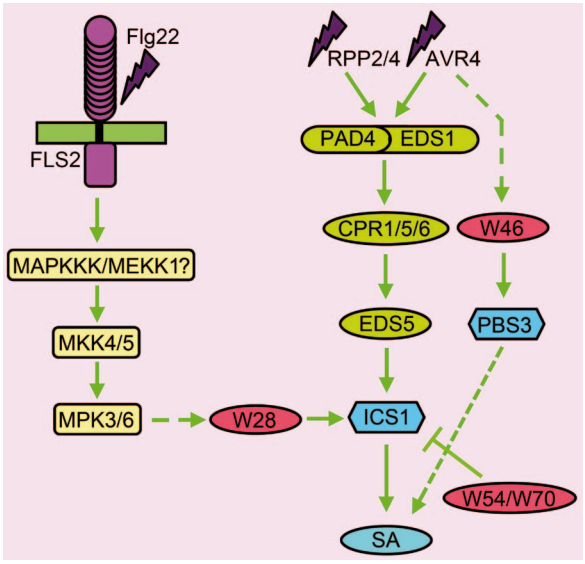

Figure 9. Model for regulation of SA biosynthesis by WRKY28 and WRKY46. Upon infection with a pathogen expressing flagellin (Flg22) or avirulence genes (RPP2/4 or AVR4), *WRKY28* or *WRKY46* are rapidly induced. Activation of FLS2 receptor by Flg22 results in activation of a MAPK cascade, which leads to induction of *WRKY28* expression, which subsequently activates *ICS1* gene expression leading to SA production. Avirulence factors like AVR4 trigger SA production through a pathway involving genes *PAD4*, *EDS1*, *CPR1/5/6*, *EDS5* and *ICS1*. WRKY46 is rapidly synthesized and either directly or indirectly positively regulates *PBS3* gene expression, having a positive influence on SA metabolism.

normalized against total protein level.

To analyze effects on expression of endogenous genes by WRKY28 and WRKY46, protoplasts were transfected with 6 µg of *35S::WRKY28* or *35S::WRKY46* expression plasmids. After 24h protoplasts were harvested and total RNA isolated. Total RNA was treated with DNAse using the Turbo DNA-free kit (Ambion) and cDNA was synthesized using the universal first strand cDNA synthesis kit (Fermentas). Expression of endogenous genes was determined by qPCR using primers listed in Table 1. qPCR was performed using a standard Phusion high fidelity polymyerase reaction (Finzymes) supplemented with 0.145µl Tween-20, 1.45µl Glycerol, 1 mM $MgCl_2$ and 1x Sybr green (Roche #70140720) per 50µl reaction and analyzed using a BioRad Chromo4 qPCR machine.

Electrophorectic Shift Assays

Protein for EMSAs was purified from *E. coli* transformed with pGEX-KG constructs containing the open reading frame of *WRKY28* in frame behind the GST open reading frame, according to van Verk *et al.*, (2008).

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 with a 5'-GGG overhangs from 95°C to room temperature or by PCR of the *ICS1* promoter fragments. Annealed oligonucleotides were subsequently end-filled and PCR fragments labeled throughout using Klenow fragment and [α-^{32}P]-dCTP, after which unincorporated label was removed by Autoseq G-50 column chromatography (Amersham-Pharmacia Biotech).

Table 1, Oligonucleotides used for qRT-PCR and ChIP qPCR analysis

qPCR-*Actin 3*	F	5'-CCTCATGCCATCCTCCGTCT-3'
	R	5'-CAGCGATACCTGAGAACATAGTGG-3'
qPCR-*Actin 7*	F	5'-AGTGGTCGTACAACCGGTATTGT-3'
	R	5'-GAGGAAGAGCATACCCCTCGTA-3'
qPCR-*Actin 8*	F	5'-AGTGGTCGTACAACCGGTATTGT-3'
	R	5'-GAGGATAGCATGTGGAAGTGAGAA-3'
qPCR-*β-Tubelin*	F	5'-GGAAGAAGCTGAGTACGAGCA-3'
	R	5'-GCAACTGGAAGTTGAGGTGTT-3'
qPCR-*ICS1*	F	5'-GGAACAGTGTCATCTGATCGTAATC-3'
	R	5'-CATTAAACTCAACCTGAGGGACTG-3'
qPCR-*PBS3*	F	5'-CGTACCGATCGTGTCATATGAAG-3'
	R	5'-CTTCACATGCTTGGTTATAACTTGC-3'
qPCR-*WRKY28*	F	5'-CAAGAGCCTTGATCGATCATTG-3'
	R	5'-GCAAGCCCAACTGTCTCATTC-3'
qPCR-*WRKY46*	F	5'-CATGAGATTGAGAACGGTGTG-3'
	R	5'-CTGCCATTAAGAGAGAGACATTACATTC-3'
ChIP-A	F	5'-GTCAAAGCTTGCACGACTAACTTTAGAAAAATG-3'
	R	5'-CAGTGGATCCTGCAGAAATTCGTAAAGTGTTTC-3'
ChIP-B	F	5'-GTCAAAGCTTCAACCAAACGAATCCGGTCTGT-3'
	R	5'-GAAGAGATCTATTTCATTTTCACACAAAATTTCTC-3'
ChIP-C	F	5'-GTCAAAGCTTCAAACGAGAAGAGTCGTCTAGC-3'
	R	5'-GGGTCAGTTAATTGTTTGATCTATTATTATTAG-3'
ChIP-D	F	5'-GTCAAAGCTTGCCATATGCCTTATGTACGAGA-3'
	R	5'-AGAAAGATCTTAGTGTAAAATTGCATAGACCAAG-3'
ChIP-E	F	5'-GTCAAAGCTTCTATGCTTTGTTTTACATGTAAAG-3'
	R	5'-GGGAAAAACATTACATGTCACTACAAATTGCAA-3'
ChIP-F	F	5'-GTCAAAGCTTCTGGTCTCAAAGAGCCTAAGTG-3'
	R	5'-GGGCTCCTTTAAATTTTGACACATTTCTAAAAT-3'
ChIP-*PR1*	F	5'-GTTCTTCCCTCGAAAGCTCAAGAT-3'
	R	5'-CACCTCACTTTGGCACATCCG-3'
ChIP-*PDF1.2*	F	5'-TATACTTGTGTAACTATGGCTTGG-3'
	R	5'-TGTTGATGGCTGGTTTCTCC-3'

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 30,000 cpm of labeled probe, representing approximately 0.01 pmol, was added and incubation was continued for 20 min at room temperature. 50- and 250-fold molar excess of unlabelled competitor was added for some reactions, representing 0.50 and 2.50 pmol respectively. The total mixture was loaded onto a 5% polyacrylamide gel in Tris-borate buffer and electrophoresed. After electrophoresis, the gel was dried, autoradiographed, and analyzed using X-ray film.

Chromatin Immunoprecipitation

For ChIP assays, protoplasts were prepared as described above and transfected with 6 µg of *35S::WRKY28-HA* or *35S::HA* constructs in plasmid pRT101. After 24h, protoplasts were harvested and ChIP assays were conducted as described by Lee *et al.* (2007), with minor modifications. After formaldehyde fixation, the chromatin of the protoplasts was isolated and extensively sheared by

sonication to obtain feagment sizes between 300-400 bp. Rat anti-HA monoclonal antibodies (clone 3F10, Roche) and Dynabeads Protein G magnetic beads (Invitrogen) were used to immunoprecipitate the genomic fragments. qPCRs were performed on the immunoprecipitated DNA using primers corresponding to six overlapping regions of the *ICS1* promoter as shown in Figure 8A. qPCRs with primers specific for the coding region of the *PR1* gene and the promoter of *PDF1.2* gene of Arabidopsis were used as controls. The primers used for the ChIP assays are listed in Table 1.

ACKNOWLEDGEMENTS

We gratefully acknowledge the group of dr. Ji Hoon Ahn for providing a detailed protocol for ChIP analysis in Arabidopsis protoplasts. Rob van Eck and Roy Baas are acknowledged for their help with some of the experiments and we are grateful to Ward de Winter for technical assistance.

6

Summary

SUMMARY

As plants are constantly challenged to cope with a broad variety of stresses, they have developed sophisticated mechanisms that control a range of defense responses. Defense programs against pathogens are regulated through three important signaling pathways, the salicylic acid (SA), jasmonate (JA) and ethylene (ET) signaling pathways. The current knowledge of SA, JA and ET biosynthesis and the transcriptional regulation of defense responses mediated through these signal molecules is discussed in **Chapter 1**.

The SA signaling pathway triggered by attack of biotrophic pathogens leads to broad spectrum resistance against a plethora of pathogenic fungi, bacteria and viruses and is known as systemic acquired resistance (SAR). One of the hallmarks of SAR is the accumulation of PR proteins and the induced expression of the *PR-1* gene is often used as a marker for SAR.

In **Chapter 2** we used a cDNA library from *tobacco mosaic virus* (TMV) infected tobacco plants to screen in a yeast one hybrid assay for proteins that can bind to the tobacco *PR-1a* promoter. This screening resulted in the identification of NtWRKY12, a protein belonging to the group of WRKY transcription factors. Detailed expression studies of the *NtWRKY12* gene revealed that induction of *NtWRKY12* coincides with the expression of *PR-1a*, suggesting a regulatory link between *NtWRKY12* and *PR-1a*. The expression of *NtWRKY12* was induced after exogenous application of SA, infection with Tobacco mosaic virus and upon leaf infiltration with *Agrobacterium tumefaciens* or *Escherichia coli*.

To elucidate the binding site of NtWRKY12 in the *PR-1a* promoter, we employed Electro mobility shift assays (EMSA) with *PR-1a* promoter fragments. Through mutational analyses the binding site was narrowed down to the sequence TTTTCCAC. This sequence differs significantly from the consensus WRKY protein binding site TTGAC[C/T] (W-box), and was designated as the "WK-box".

The WK-box occurs in close proximity of an *as-1*-like element, which is a binding site for TGA transcription factors, and an MBSII element shown to bind a Myb transcription factor. Further upstream in the *PR-1a* promoter another WK-box is present. The downstream and upstream WK-boxes are referred to as WK_1-box and WK_2-box, respectively.

The functional importance of the WK-box, *as-1* element and MBSII site was analyzed using *PR-1a promoter::GUS* reporter genes. The effects of mutations in the binding sites were studied in stably transformed tobacco plants sprayed with SA, or in leaves agroinfiltrated with *A. tumefaciens* carrying *PR-1a::GUS* constructs. Mutations in the WK_1-box resulted in a major reduction of the SA- or elicitor-induced GUS expression, whereas mutations in the WK_2-box, *as-1*-like element or MBSII box had little or no effect on the induction of GUS expression. Combined mutations in the WK-box and the *as-1*-like element completely abolished *PR-1a* promoter-induced GUS expression. A more direct proof that NtWRKY12 was necessary for induction of *PR-1a* promoter activity came from transactivation assays in Arabidopsis protoplasts using *35S::NtWRKY12* and *PR-1a promoter::GUS*

constructs.

The close proximity of the *as-1*-like element and the WK_1-box in the *PR-1a* promoter suggested that protein-protein interactions could occur between NtWRKY12 and TGA transcription factor. This possibility was further investigated in **Chapter 3**. Using *in vitro* pull down assays and *in vivo* Fluorescence Resonance Energy Transfer analyses it was shown that NtWRKY12 specifically interacts with TGA2.2. No interaction could be found between the closely related TGA2.1 and NtWRKY12.

Further analyses in Arabidopsis protoplasts confirmed that NtWRKY12 and TGA2.2 have an additive effect on *PR-1a::GUS* expression. Current models for transcriptional activation of the *PR-1* gene of Arabidopsis imply the binding of co-activator NPR1 to TGA proteins on the promoter. However, co-expressed tobacco NPR1 did not further enhance reporter gene expression, and transactivation assays in protoplasts from *npr1-1* plants demonstrated that activation of the tobacco *PR-1a* promoter is independent of endogenous NPR1. Furthermore, assays in protoplasts lacking four functional Arabidopsis TGAs revealed that NtWRKY12-mediated activation of the *PR-1a* promoter is independent of endogenous TGAs, supporting the notion that NtWRKY12 is the main transcriptional activator of *PR-1a* expression.

Accumulating genetic data indicate that WRKY transcription factors function in the regulation of defense responses acting along SA, JA and ET signaling routes. Although for most of these transcription factors their involvement in defense has been deduced from gain or loss of function mutants and no direct target genes have been indentified. The close temporal correlation of the induced expression profiles of *NtWRKY12* and *PR-1a* prompted a bioinformatics approach to prospect for other links between transcription factors and genes involved in the biosynthesis of SA, JA and ET and their signaling pathways in Arabidopsis. In **Chapter 4** we used publicly available datasets derived from micro arrays related to stress. First, the optimal Pearson Correlation Coefficient (PCC) cutoff was determined to enable identification of biologically relevant co-expression data. Using this PCC cutoff, a co-expression network was constructed comprising the genes involved in SA, JA and ET biosynthesis and signaling that were described in Chapter 1, complemented with a large set of genes encoding members from various classes of transcription factors. The co-expression data derived from the network indicated several links between transcription factors and signaling components that were previously reported in literature, underscoring the validity of the constructed network. In addition, we found many previously unknown links between genes, which may help future research to further unravel the complex pathways and regulatory mechanisms in stress responses.

One of these new links reflected the closely co-regulated expression of the genes encoding transcription factor WRKY28 and isochorismate synthase 1, a key enzyme of SA biosynthesis. A second link indicated co-regulation of *WRKY46* and *PBS3*, which encodes another enzyme involved in the biosynthesis of SA and its derivatives. In **Chapter 5**, transactivation assays in Arabidopsis protoplasts were used to study expression of promoter::*GUS* fusions with *ICS1* and *PBS3* promoter sequences of 1 kb by transiently expressed WRKY28 and WRKY46. In addition, expression of endogenous *ICS1* and *PBS3* genes by WRKY28 and WRKY46 in these protoplasts was analyzed by qRT-PCR. The results showed that WRKY28 can induce *ICS1* expression whereas WRKY46 can activate *PBS3* expression. Subsequent EMSA binding studies and chromatin immunoprecipitation

analyses revealed that WRKY28 binds to sites in the *ICS1* promoter that are only remotely similar to the consensus W-box.

7

Samenvatting

SAMENVATTING

Door de voortdurende blootstelling aan verschillende vormen van stress, hebben planten verfijnde mechanismen ontwikkeld die een verscheidenheid aan verdedigingsreacties oproepen. Afweerprogramma's ter bestrijding van ziekteverwekkers worden gereguleerd via drie belangrijke signaaltransductie-routes, de salicylzuur (SA), jasmonaat (JA) en ethyleen (ET) signaalroute. De huidige kennis van de SA, JA en ET biosynthese en de regulatie van de afweer reacties die door deze signaalmoleculen worden aangestuurd, wordt besproken in **Hoofdstuk 1**.

De SA signaaltransductie-route, geïnitieerd door een aanval van biotrofe ziekteverwekkers, leidt tot een breed-spectrum resistentie tegen een veelheid aan ziekteverwekkende schimmels, bacteriën en virussen en staat bekend als systemisch verworven resistentie (SAR). Een van de kenmerken van SAR is de ophoping van PR-eiwitten en de geïnduceerde expressie van het *PR-1* gen dat vaak wordt gebruikt als een marker voor SAR.

In **Hoofdstuk 2** is gebruik gemaakt van een cDNA-bibliotheek van tabaksmozaïekvirus (TMV) geïnfecteerde tabaksplanten om met behulp van het "yeast-one-hybrid" systeem te zoeken naar eiwitten die kunnen binden aan de *PR-1a* promotor van tabak. Deze screening heeft geleid tot de identificatie van NtWRKY12, een eiwit dat behoort tot de groep van WRKY transcriptiefactoren. Gedetailleerde expressiestudies van het *NtWRKY12* gen toonden aan dat de inductie van *NtWRKY12* parallel verloopt met de expressie van *PR-1a*, wat een verband suggereert tussen de regulering van beide genen. De expressie van *NtWRKY12* werd geïnduceerd na besproeien van planten met SA, na infectie met tabaksmozaïekvirus en na infiltratie van blad met *Agrobacterium tumefaciens* of *Escherichia coli*.

Voor het ophelderen van de bindingsplaats van NtWRKY12 in de *PR-1a* promotor hebben we gebruik gemaakt van "Electro mobility shift assays" (EMSA) met *PR-1a* promotor fragmenten. Door mutatie analyse is de basevolgorde TTTTCCAC gekarakteriseerd als de bindingsplaats. Deze sequentie wijkt sterk af van de consensus WRKY eiwitbindingsplaats TTGAC[C/T] (W-box), en daarom hebben wij deze bindingplaats 'WK-box' genoemd. De WK-box (WK_1-box) ligt dichtbij een *as-1* element dat fungeert als bindingsplaats voor TGA transcriptiefactoren, en in de buurt van een MBSII element dat wordt gebonden door een MYB transcriptiefactor. Verder stroomopwaarts in de promoter van *PR-1a* ligt een tweede WK-box (WK_2-box).

Het functionele belang van de WK-box en de *as-1* en MBSII elementen werd geëvalueerd met behulp van *PR-1a promotor::GUS* reporterconstructen. Het effect van mutaties in de bindingsplaatsen werd bestudeerd in stabiel getransformeerde tabaksplanten na besproeien met SA en na agroinfiltratie van tabaksbladeren met *A. tumefaciens*, welke in de plant een *PR-1a::GUS* construct tot expressie bracht. Uit deze experimenten is gebleken dat de WK_1-box voor een belangrijk deel bijdraagt aan de geïnduceerde *PR-1a* genexpressie, terwijl de *as-1* en MBSII elementen veel minder belangrijk waren voor de expressie. Gecombineerde mutatie van de WK-box en het *as-1* element schakelde de geïnduceerde *PR-1a* promotor GUS expressie volledig uit. Een meer direct bewijs dat NtWRKY12 noodzakelijk was voor *PR-1a* geïnduceerde genexpressie kwam uit transactiveringsexperimenten in

Arabidopsis protoplasten met behulp van *35S::NtWRKY12* en *PR-1a promotor::GUS* constructen.

De nabijheid van het *as-1* element en de WK-box in de *PR-1a* promotor suggereerde dat eiwit-eiwit interacties kunnen optreden tussen NtWRKY12 en TGA transcriptiefactoren. Deze mogelijkheid is nader onderzocht in **Hoofdstuk 3**. Met behulp van *in vitro* "pull-down" en *in vivo* "Fluorescence Resonance Energy Transfer" experimenten werd aangetoond dat NtWRKY12 specifiek een interactie aangaat met TGA2.2. Er is kon geen interactie gevonden worden tussen het nauw verwante TGA2.1 en NtWRKY12.

Verdere analyses in Arabidopsis protoplasten bevestigden dat NtWRKY12 en TGA2.2 een additief effect hebben op *PR-1a::GUS* expressie. Huidige modellen voor transcriptionele activering van het *PR-1* gen van Arabidopsis impliceren de interactie van co-activator NPR1 met TGA eiwitten op de promotor. Echter, co-expressie met tabaks NPR1 kon de reporter genexpressie niet verder verhogen, en de transactiveringsexperimenten in protoplasten uit *npr1-1* planten toonden aan dat activering van de tabaks *PR-1a* promotor onafhankelijk is van endogeen NPR1. Bovendien, uit bepalingen met protoplasten waarin vier functionele Arabidopsis TGAs ontbreken, bleek dat NtWRKY12 gestuurde activering van de *PR-1a* promotor onafhankelijk is van endogene TGAs. Dit ondersteunt het idee dat NtWRKY12 de belangrijkste transcriptionele activator is van *PR-1a* expressie.

Een toenemende hoeveelheid genetische gegevens duidt erop dat WRKY transcriptiefactoren een rol spelen in de regulatie van de verdedigingsreacties die door SA, JA en ET signaaltransductieroutes worden gestuurd. Voor de meeste van deze transcriptiefactoren is hun betrokkenheid bij de verdediging afgeleid uit "gain of function" of "loss of function" mutanten en zijn er geen directe target genen geïdentificeerd. De sterke temporale correlatie van de geïnduceerde expressie profielen van *NtWRKY12* en *PR-1a* deed ons besluiten via een bioinformatica-gerichte aanpak nieuwe verbanden te onderzoeken tussen transcriptiefactoren en genen betrokken bij de biosynthese van SA, JA en ET en hun signaaltransductieroutes in Arabidopsis. In **Hoofdstuk 4** gebruikten we publiekelijk beschikbare datasets afkomstig van "micro-arrays" gerelateerd aan stress. Allereerst werd de optimale Pearson correlatiecoëfficiënt (PCC) cutoff vastgesteld voor de identificatie van biologisch relevante co-expressie gegevens. Met behulp van deze PCC cutoff, werd een co-expressienetwerk gebouwd, bestaande uit genen die betrokken zijn bij SA, JA en ET biosynthese en signaaltransductie, zoals beschreven in Hoofdstuk 1, aangevuld met een grote set van genen die coderen voor verschillende klassen van transcriptiefactoren. De co-expressie gegevens afkomstig uit het netwerk resulteerden in een aantal links tussen transcriptiefactoren en componenten uit signaaltransductieroutes die eerder zijn gerapporteerd in de literatuur, wat de validiteit van het geconstrueerde netwerk ondersteunt. Daarnaast vonden we een groot aantal voorheen onbekende verbanden tussen genen die toekomstig onderzoek aan de complexe signaaltransductie en regulatie mechanismen in de stress respons verder kunnen helpen ontrafelen.

Een van deze nieuwe verbanden bestaat uit de gecoreguleerde genen coderend voor transcriptiefactor WRKY28 en isochorismaat synthase 1, een belangrijk enzym van de SA biosynthese. Een andere link is de co-regulering van *WRKY46* en *PBS3*, welke codeert voor een andere component van de SA biosyntheseroute. **Hoofdstuk 5** beschrijft de biochemische analyses ter bepaling van de relevantie van deze transcriptiefactoren voor de expressie van de *ICS1* en *PBS3* genen. Met behulp van

transactiveringsexperimenten in Arabidopsis protoplasten met *promotor::GUS* fusies en qRT-PCR analyses werd aangetoond dat WRKY28 en WRKY46 respectievelijk *ICS1* en *PBS3* genexpressie kunnen activeren. Uit daaropvolgende EMSA- en chromatine immunoprecipitatie experimenten bleek dat WRKY28 bindt aan sites in de *ICS1* promotor die verschillen van de consensus W-box.

8

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Curriculum Vitae

CURRICULUM VITAE

Marcel van Verk was born on November 11, 1980 in Dordrecht, The Netherlands. During his general secondary education in Dordrecht he obtained the Dutch mathematics title (Kangoeroe competition) in 1996. In September 2002, he started the study of Biotechnology at the van Hall Institute, Leeuwarden. From July 2004 until December 2004 and August 2005 until March 2006, he did a research training in plant molecular biology at the Institute of Biology, Leiden University, under the supervision of Dr. Huub J.M. Linthorst. In addition, he specialized in bioinformatics during a two-months traineeship at the Department of Oncology, VU Medical Center Amsterdam. On the 30th of March 2006 he graduated Cum Laude for his Bachelor diploma in Plant Biotechnology. In May 2006, he started as a PhD student in the group of Prof. dr. Johan Memelink under supervision of Dr. Huub J.M. Linthorst and Prof. dr. John F. Bol on a project entitled "WRKY Transcription Factors Involved in Salicylic Acid-Induced Defense Gene Expression". During his PhD studies he was involved in the organization of a workshop on microarrays and of various meetings for PhD students. Within the PhD council of the Graduate School of Experimental Plant Sciences (EPS) he was initially active as a board member and later as chairman. After his graduation he will continue as post-doc in the Molecular and Developmental Genetics Department of Prof. dr. Paul J.J. Hooykaas under supervision of Dr. Bert van der Zaal on novel zinc finger-based strategies for elucidating and controlling homologous recombination in plants.