WRKY transcription factors involved in salicylic acid-induced defense gene expression
Verk, M.C. van

Citation

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Note: To cite this publication please use the final published version (if applicable).
Transcriptional Regulation of Plant Defense Responses

Marcel C. van Verk, Christiane Gatz, and Huub J. M. Linthorst

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
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 \( \alpha \)-linolenic acid occurs. \( \alpha \)-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 \( \alpha \)-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 \( \beta \)-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). COII 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 COII 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 COII gene was mapped to a small region of the genome and located by complementation. COII corresponds to the JAI5 locus. The amino acid sequence of the COII 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 COII F-box protein with Cul1, Skp1, and Rbx1 was shown by co-immunoprecipitation, linking COII 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 COII is specific to the JA pathway, whereas other parts of the SCF COII complex (SGT1b/JAI4 and AXR1) are shared by other pathways.

A breakthrough in understanding how COII mediates JA signaling via the SCF COII complex came with the discovery of JA ZIM-domain (JAZ) repressor proteins. One member of this group, JAZ3
(corresponding to the \textit{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$^\text{COI}$ 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 \textit{JAZ3}, this process constitutes a direct negative regulatory loop to dampen MYC2 activity in cells with low levels of JA (Chini \textit{et al.}, 2007; Thines \textit{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. \textit{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 \textit{ETHYLENE OVERPRODUCER 1} (ETO1) and \textit{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 \textit{et al.}, 2009; Guzmán and Ecker, 1990; Wang \textit{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 \textit{et al.}, 1988; Chang \textit{et al.}, 1993; Hua \textit{et al.}, 1995, 1998; Roman \textit{et al.}, 1995; Sakai \textit{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 \textit{et al.}, 1998; Gao \textit{et al.}, 2003; Kieber \textit{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 phyloquinone (vitamin K₁), 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 phyloquinone 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 Ca²⁺/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 Ca²⁺ (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.

*AVRPPHB 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 (SNII; Li et al., 1999) allele is present. SNII 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 (GCGGCC) (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.
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 *coil-1* background, placing ORA59
downstream of COI1. Silencing of ORA59 using RNA interference (RNAi) results in reduced resistance against \textit{B. cinerea}. Besides ORA59, also ERF1 has been reported to integrate JA and ET signals and to synergistically induce \textit{PDF1.2} downstream of COI1 (Lorenzo \textit{et al.}, 2003). Although ORA59 and ERF1 appear to have similar functions, RNAi-silenced lines of \textit{ORA59} that still activate ERF1 upon application of JA or ET, nevertheless are impaired in \textit{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 \textit{VSP}, while ORA59 does not (Pré \textit{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 \textit{et al.}, 2000; Yang \textit{et al.}, 2005). AtERF14 induces \textit{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 (Onâte-Sánchez \textit{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 \textit{PRB-1b}, encoding a basic PR-1 type protein from tobacco (Büttner and Singh, 1997; Sessa \textit{et al.}, 1995). Another screen for interactors of the tomato Ser/Thr kinase Pto, the product of the \textit{R} gene recognizing the \textit{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 \textit{et al.}, 1996; Zhou \textit{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 \textit{et al.}, 2003; Gu \textit{et al.}, 2000). Overexpression of Pti4 in transgenic \textit{Arabidopsis} resulted in increased gene expression of GCC-box containing \textit{PR} genes (Wu \textit{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 \textit{et al.}, 2003).

### B. MYB TRANSCRIPTION FACTORS

The first MYB factor identified was v-MYB from \textit{Avian myeloblastosis virus}. It probably originated by capture from a vertebrate gene, which was subsequently converted into an oncogene. Vertebrates contain three \textit{Myb} genes (\textit{c-Myb}, \textit{A-Myb}, and \textit{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 \textit{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 CANNNTG, 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-
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 of Map 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 *P. 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 WRKY46 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 *P. 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 as well 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.
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 SN11 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
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 trochosatin 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

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**TABLE I**

*Simplified Overview of the Histone Code*

<table>
<thead>
<tr>
<th>Transcriptionally inactive</th>
<th>Transcriptionally active</th>
</tr>
</thead>
<tbody>
<tr>
<td>heterochromatin</td>
<td>euchromatin</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Acetylation</th>
<th>Methylation</th>
<th>Acetylation</th>
<th>Methylation</th>
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<tbody>
<tr>
<td>Low</td>
<td>Dimethylation</td>
<td>High</td>
<td>Di- and trimethylation</td>
</tr>
<tr>
<td></td>
<td>of H3-K9, H3-K27, H4-K20</td>
<td></td>
<td>of H3-K4</td>
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</table>
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 CaMV35S promoter that contains the TGA2.2 binding element _as-1_ as the only regulatory _cis_ element. The truncated CaMV35S 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 CaMV35S 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
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.

ACKNOWLEDGMENT

We gratefully acknowledge Steven Spoel for providing a copy of his latest manuscript before publication.