WRKY transcription factors involved in salicylic acid-induced defense gene expression

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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

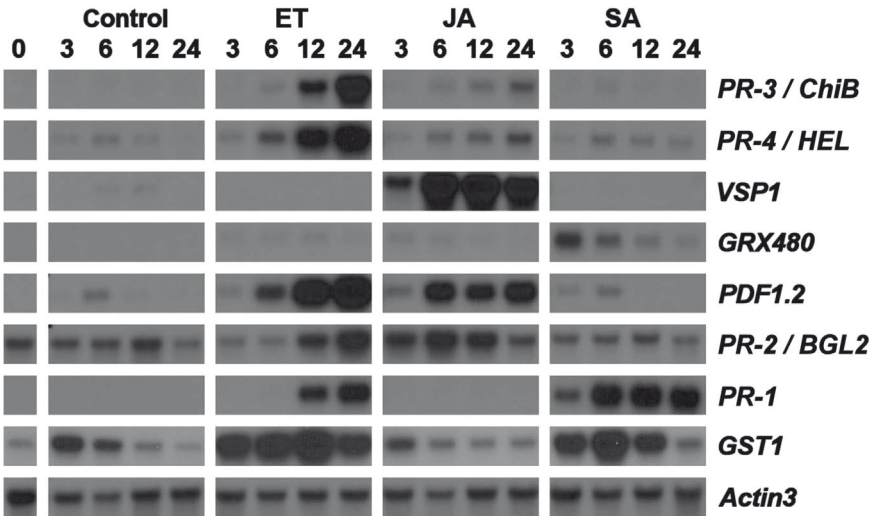


Fig. 1. Time course showing expression of ethylene-, jasmonic acid-, and salicylic acid-inducible defense-related genes in *Arabidopsis thaliana*. A total of five plants per time point per treatment were mock treated, or sprayed with 10 mM ethylene releasing ethephon (ET), 250 µm jasmonic acid (JA), 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.

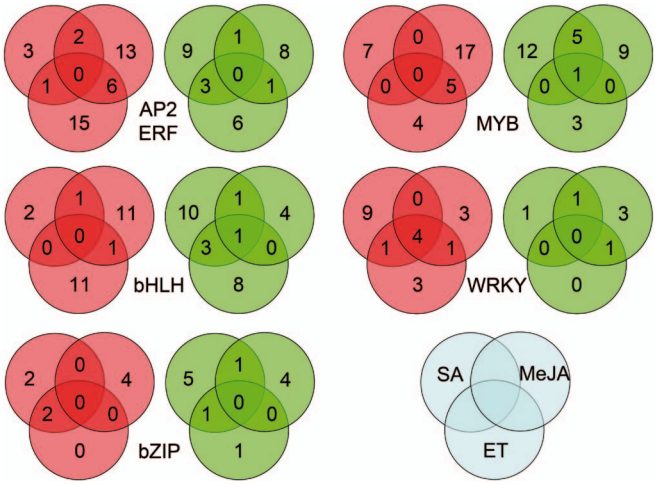


Fig. 2. Venn diagrams of transcription factor-encoding genes in *Arabidopsis thaliana* responsive to treatment of the plants with salicylic acid (SA), methyl jasmonate (MeJA), or ethylene (ET). Separate Venn diagrams for AP2/ERF-, bHLH-, bZIP-, MYB-, and WRKY-encoding genes were generated. Transcription 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

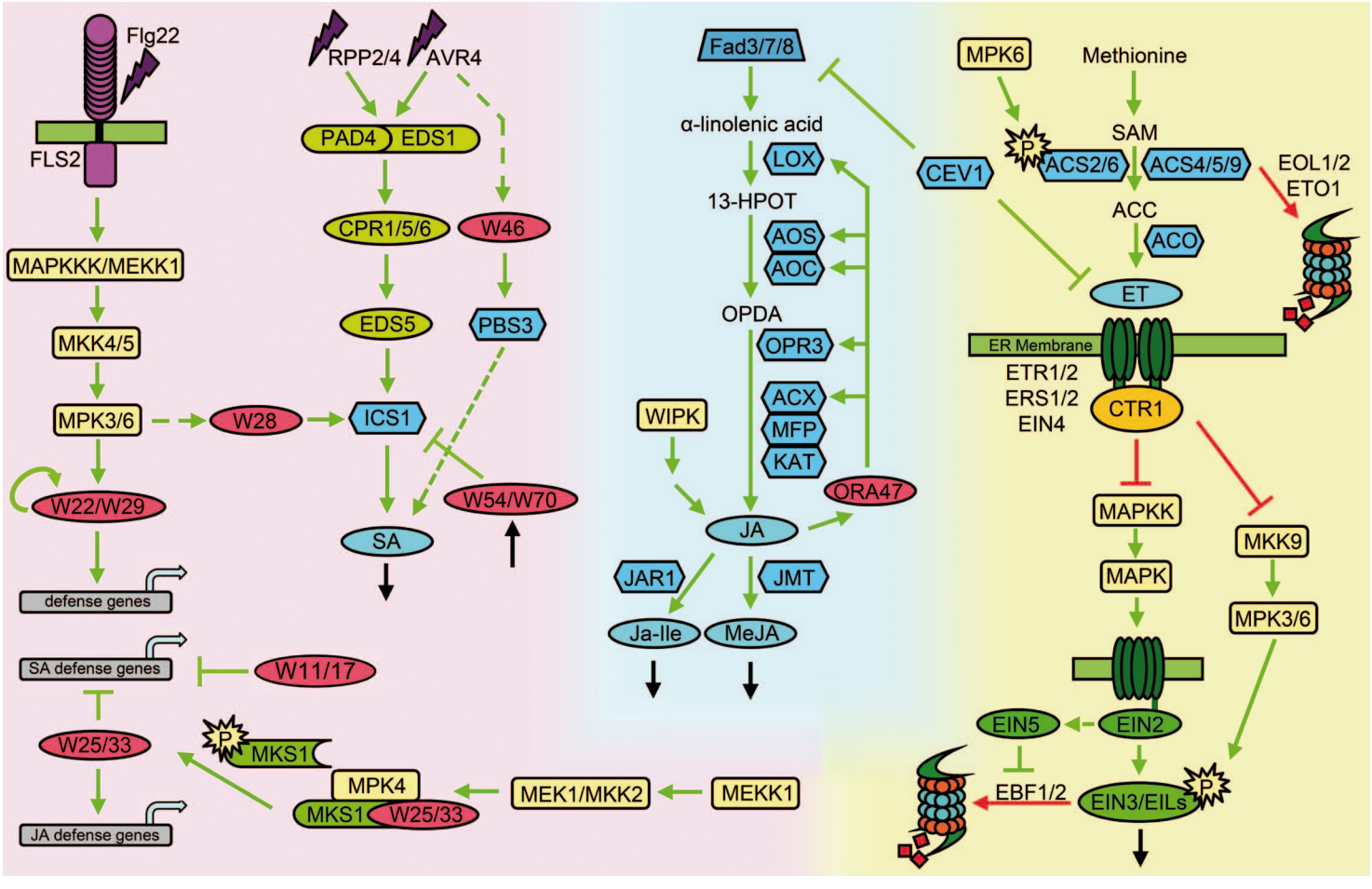
either repress or activate transcription (Seet *et al*., 2006).

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
CTR1
MAPKK
MKK9
MAPK
MPK3/6
EIN5
EIN2
EBF1/2
EIN3/EILs
P

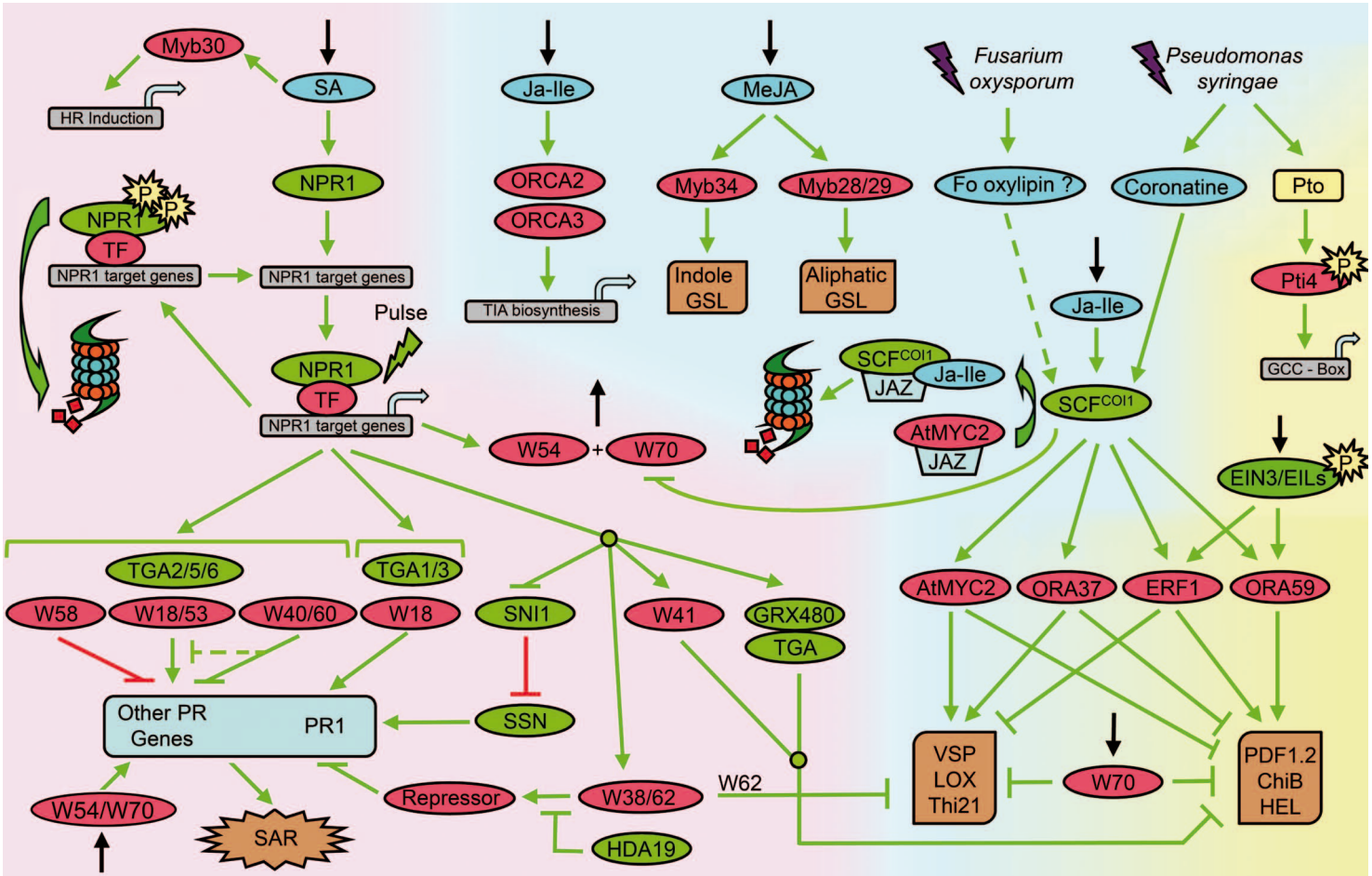


Fig. 3. Graphical summary of the pathways from pathogen perception to transcriptional activation of defense gene expression described in this chapter.

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

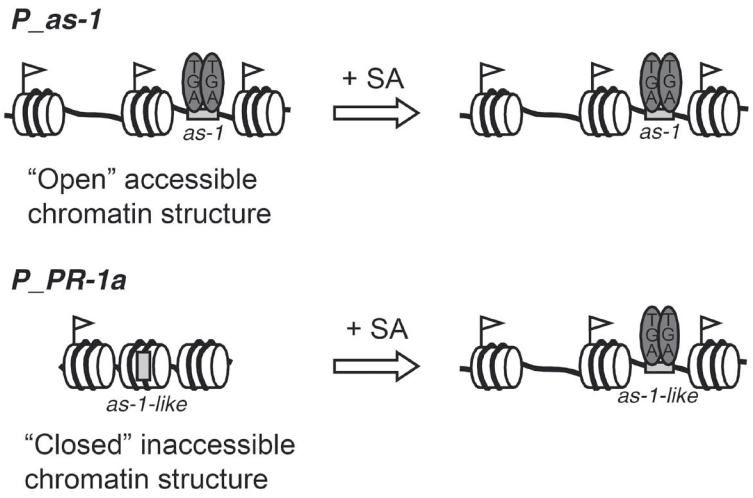

Fig. 4. Proposed model of the histone acetylation status and the status of TGA binding at the "immediate early" truncated CaMV *35S* (P-as-1) and "late" *PR-1a* promoter in tobacco as suggested by results of ChIP experiments. Histones at promoters that respond with a fast kinetics (within 3 hours) are 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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