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Networks of WRKY transcription factors in defense signaling

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Members of the complex family of WRKY transcription factors have been implicated in the regulation of transcriptional reprogramming associated with plant immune responses. Recently genetic evidence directly proving their significance as positive and negative regulators of disease resistance has accumulated. *WRKY* genes were shown to be functionally connected forming a transcriptional network composed of positive and negative feedback loops and feed-forward modules. Within a web of partially redundant elements some WRKY factors hold central positions mediating fast and efficient activation of defense programs. A key mechanism triggering strong immune responses appears to be based on the inactivation of defense-suppressing WRKY proteins.

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Abbreviations

ETI	effector-triggered immunity
ICS1	isochorismate synthase 1
JA	jasmonic acid
MAP kinase	mitogen-activated protein kinase
MPK4	MAP protein kinase 4
NPR1	nonexpressor of PR1, an ankyrin-type protein
PAMP	pathogen associated molecular pattern
PTI	PAMP-triggered immunity
SA	salicylic acid
SAR	systemic acquired resistance
SIPK	salicylic acid induced protein kinase
TF	transcription factor

Introduction

The plant's innate immune system consists of two interconnected branches termed PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) [1] that initiate massive transcriptional reprogramming [2,3]. PTI is elicited by pathogen/microbe-associated molecular patterns (PAMPs/MAMPs), molecular signatures ubiquitously decorating certain types of pathogens. PAMP perception activates distinct MAP-kinase cascades [4–6]. Multiple microorganisms secrete effector proteins into host cells that intercept PAMP-triggered defense signals and thereby attenuate PTI [7]. The remaining weak immune response, termed basal defense, is insufficient to prevent disease. Co-evolution of virulent pathogens with their hosts resulted in the establishment of ETI, a manifestation of gene-for-gene resistance [1]. ETI is triggered by plant disease resistance (R) proteins that activate highly efficient defense reactions upon specific recognition of pathogen effectors. Besides local immune responses, PTI and ETI activate long-distance defense reactions, such as systemic acquired resistance (SAR) [8]. In *Arabidopsis thaliana* (Arabidopsis) and other higher plants, local and systemic defense responses are controlled by the balanced action of distinct, but partially interconnected pathways involving the hormones salicylic acid (SA) and jasmonic acid (JA) [9].

Global expression profiling revealed that the major differences between PTI, ETI, basal defense, or SAR are quantitative and/or temporal rather than qualitative [3]. This suggests that most pathogens trigger a common/interconnected plant signaling network. The graded transcriptional responses associated with immunity clearly indicate the existence of a complex regulatory circuitry comprising transcriptional activators and repressors fine-tuning the expression of defense genes [2]. Members of several transcription factor (TF) families modulate the defense transcriptome [2,10]. In particular, the presence of WRKY TF binding sites (C/TTGACC/T, W boxes) in numerous co-regulated Arabidopsis defense gene promoters provided circumstantial evidence that zinc-finger-type WRKY factors play a broad and pivotal role in regulating defenses [10].

The role of WRKY factors in plant defense

Functional redundancy among certain family members has hampered attempts to causally link specific WRKY TFs to plant defense [11]. In Arabidopsis, there are 72 expressed *WRKY* genes (http://www.arabidopsis.org/browse/genefamily/WRKY.jsp). However, recent publications have provided conclusive genetic proof that Arabidopsis WRKY factors are crucial regulators of the defense transcriptome and disease resistance. *AtWRKY52/RRS1* was shown to confer resistance toward the bacterium *Ralstonia solanacearum*, but the encoded protein is quite exceptional and appears to act as an R protein (see below) [12].

Several groups have reported on the importance of *AtWRKY70*, which appears to affect the balance between

signaling branches promoting SA-dependent and suppressing JA-dependent responses [13,14•]. Loss-of-*At*WRKY70 function rendered plants susceptible to the bacteria *Erwinia carotovora* and *Pseudomonas syringae* as well as the fungi *Erysiphe cichoracearum* and *Botrytis cinerea* [13,15,16••]. Moreover, *AtWRKY70* is required for both basal defense and full *R*-gene (*RPP4*)-mediated disease resistance against the oomycete *Hyaloperonospora parasitica* [17•] Similarly, mutants compromised in *AtWRKY33* were more susceptible to infection by *B. cinerea* and *Alternaria brassicicola* [18]. Several WRKY factors act as negative regulators of resistance. For instance, basal plant resistance triggered by a virulent *P. syringae* strain was enhanced in *Atwrky7* and *Atwrky11/Atwrky17* insertional mutants [19•,20] thereby also revealing partly redundant functions for these closely related TFs.

A small clade (subgroup IIa) of WRKY genes, comprising *AtWRKY18*, *AtWRKY40*, and *AtWRKY60*, play important and partly redundant functions in regulating plant disease resistance. Xu *et al.* [21••] showed that *Atwrky18/Atwrky40* and *Atwrky18/Atwrky60* double mutants were more resistant to *P. syringae* DC3000 but more susceptible to *B. cinerea* infection. *Atwrky18/Atwrky40* double mutants were also highly resistant toward an otherwise virulent powdery mildew, *Golovinomyces orontii* [22••]. In both studies single *Atwrky* mutants behaved similar to wild-type plants. Interestingly, *At*WRKY18 was also identified as a positive regulator required for full SAR, but here *At*WRKY40 does not seem to be involved [16••]. Differences in the experimental set-ups employed by Xu *et al.* [21••] and Wang *et al.* [16••] may be responsible for the apparent discrepancy observed in the *Atwrky18* mutant when challenged by virulent *P. syringae* strains. Xu *et al.* used 10-fold higher bacterial inoculum that may have masked the effect on basal resistance caused by loss-of-*At*WRKY18 function.

In barley, two IIa WRKY members were shown to suppress basal defense to virulent *Blumeria graminis* in silencing and transient overexpression experiments [22••,23].

These results demonstrate that subgroup IIa members can have both positive and negative roles in plant defense. Consistent with this, *At*WRKY18 overexpression alone resulted in enhanced basal *P. syringae* resistance, while combined overexpression of *At*WRKY18 with other IIa WRKYs reversed this effect [21••].

Finally, two additional WRKY factors, *At*WRKY53 acting as a positive regulator and *At*WRK58 as a negative regulator, were identified as modulators of SAR [16••].

Conserved structural features may integrate WRKY TFs in the defense network

WRKY TF classification was based on phylogenetic relationships and conservation of peptide motifs [24–26]. Unfortunately, a solution structure exists only for the common zinc-finger-containing WRKY DNA-binding domain [27•] and thus no topological information regarding subgroup-specific motifs are available. Nevertheless, some of these structural hallmarks, which appear largely conserved throughout the plant kingdom, have recently been associated with defined molecular or biological functions. It is very likely that they functionally link individual WRKY molecules to each other or to additional defense signaling components. The 'D motif' of *At*WRKY25 and *At*WRKY33 that is conserved at the N-termini of multiple group I WRKY TFs [24] can be phosphorylated by MPK4, a MAP-kinase that represses SA signaling [28•]. *At*WRKY25/33 appear not to directly interact with MPK4, but rather are associated to it via the nuclear localized coupling factor MKS1 [28•]. One notable feature of D motif is a conserved pattern of 'Ser-Pro' dimers, the preferential site of MAP-kinase phosphorylation [29]. In agreement with this, the D motif-containing *Nt*WRKY1, a tobacco group I WRKY, was shown to be phosphorylated by the defense-activating MAP-kinase SIPK [5]. SIPK-mediated phosphorylation enhanced *in vitro* the W box-binding activity of *Nt*WRKY1, and co-expression of SIPK and *Nt*WRKY1 led to rapid hypersensitive response (HR)-like host cell death.

The N-terminal leucine zipper motifs of Arabidopsis IIa WRKY proteins were shown to mediate homodimerization or heterodimerization between members of this subgroup [21••]. Consistent with this, IIa representatives from rice (*Os*WRKY71) and barley (*Hv*WRKY1, *Hv*WRKY2) were found *in vivo* to engage in homomeric associations [22••,30]. The ability of IIa WRKY factors to form combinatorial dimers with potentially different functions may partly explain the conflicting data regarding a positive [16••] or a negative [21••] regulatory role of IIa WRKY TFs in basal defense of *P. syringae*. Concentration disturbances caused by environmental conditions, mutations, or overexpression could affect the balance between different IIa WRKY dimer associations, and thereby, alter the outcome of plant–pathogen interactions.

The conserved 'C motif' present among IId WRKY members was shown to constitute a calmodulin-binding domain [31•]. Hence, like several other known defense regulators [32], IId WRKY TFs may sense and respond to pathogen-triggered fluctuations of intracellular Ca^{2+} levels.

Two other conserved sequences of unknown function are unique to IId WRKY members, namely GHARFRR and a plant specific zinc cluster directly preceding their single WRKY domains [24,33•]. Mutation of a strictly conserved residue within this zinc cluster region reduced binding of *At*WRKY11 to a W box (Ciolkowski and Somssich, unpublished), suggesting a role of this motif in enhancing DNA affinity. As described above, the IId members *At*WRKY7, *At*WRKY11 and *At*WRKY17, act as negative

defense regulators [19•,20]. How they exert this effect, either directly by repressing transcription or indirectly by activating an undefined defense-suppressor, remains unresolved. However, both *At*WRKY7 and *At*WRKY11 can act as transcriptional repressors ([20]; Ciolkowski and Somssich, unpublished).

It will be important to determine whether repression of defense and transcription is a general attribute of IId WRKY TFs and if these functions can be assigned to specific structural features of this subgroup.

The *WRKY* web

Plant immune responses are associated with the concerted modulation of a large number of different *WRKY* transcripts and proteins [15,34–36,37••]. Upon triggering of SA-dependent defenses, at least 49 *AtWRKY* genes exhibited differential regulation representing separate waves of transcript accumulation or repression [34]. Their promoters are statistically enriched for W boxes, suggesting that they are autoregulated or controlled by other WRKY proteins [34]. Consistent with this, multiple WRKY TFs interacted with the promoters of their own and other *WRKY* genes in co-transfection experiments [38–40]. Furthermore, Arabidopsis insertion mutant studies revealed that some *WRKY* genes positively or negatively influence expression of other family members [19•,35]. These observations point toward a functional linkage of many *WRKY* genes by auto-regulatory and cross-regulatory mechanisms. They form the core of a transcriptional network that along with additional signaling components controls a multitude of defense genes. This *WRKY* web appears to consist of positive and negative control elements possibly allowing for an efficient yet balanced amplification and diversification of defense signals.

Details of auto-regulation or cross-regulation by WRKY factors were provided for the parsley group I member *Pc*WRKY1 and its ortholog *At*WRKY33 [37••,39,41]. In response to PAMP treatment *PcWRKY1* transcripts accumulate rapidly and transiently [42]. *AtWRKY33* is activated with similar kinetics by defense-related stimuli [18,34,41]. This rapid response is mediated by a conserved arrangement of three synergistically acting W boxes (W_{ABC}). Chromatin immunoprecipitation (ChIP) revealed that *in vivo* these orthologous W boxes are constitutively occupied by WRKY proteins [37••,41]. PAMP treatment triggered simultaneous recruitment of *Pc*WRKY1 to W_{ABC} and to another target site, the W box-containing region of the *PcPR1* promoter. Binding of *Pc*WRKY1 to these sites coincided with the downregulation of *PcWRKY1* and upregulation of *PcPR1* transcript levels, suggesting a dual role of this factor as a repressor of its own gene and as an activator of *PcPR1*. This illustrates the wiring of two basic circuits within the *WRKY* web, the negative feedback loops and feed-forward modules both requiring an induced transcription factor to repress its own expression or to activate additional steps within a transcriptional cascade, respectively [43]. The early PAMP-triggered upregulation of *PcWRKY1* may be mediated either via rapid displacement of pre-bound WRKY repressors by activated family members or via post-translational activation of the pre-bound WRKY proteins (Figure 1).

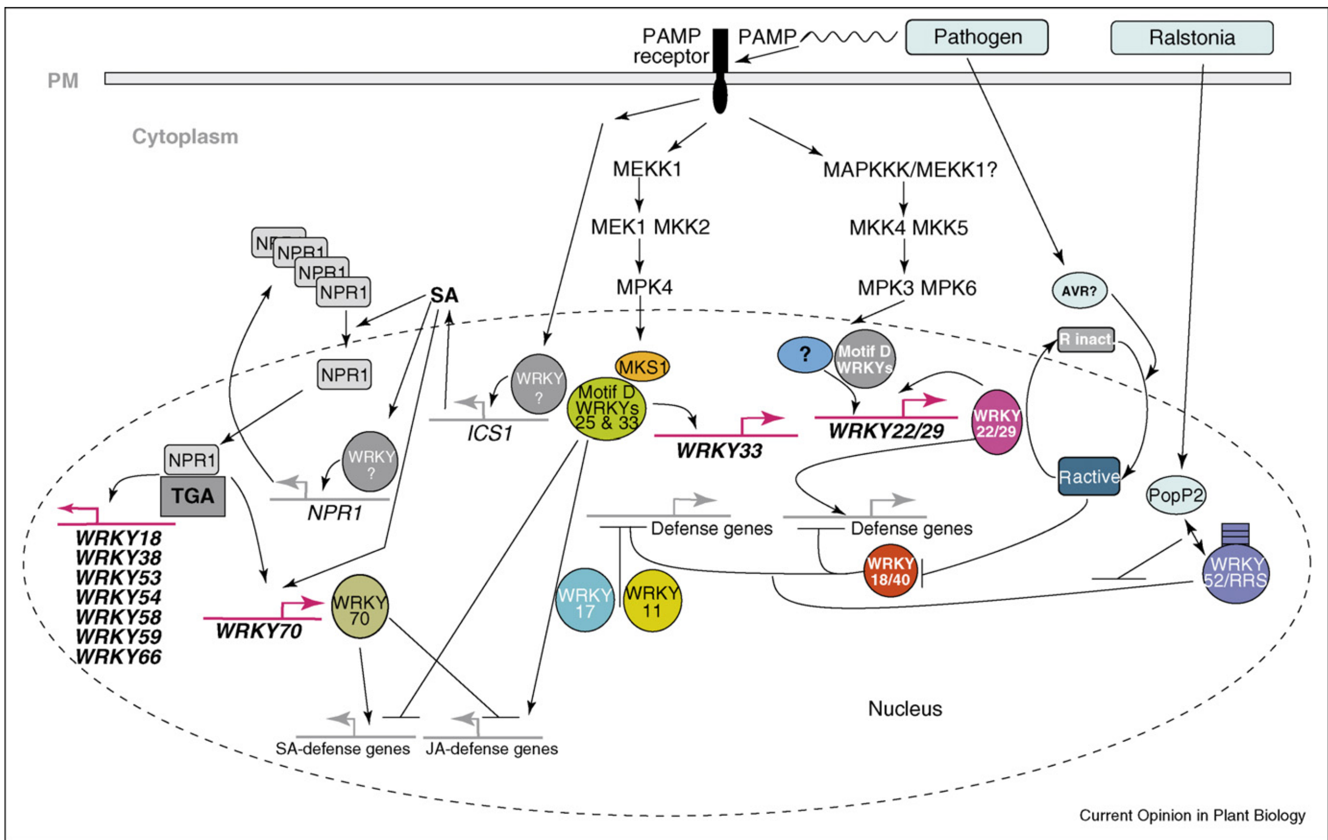
Some architectural features of the *WRKY* web are emerging. As motif D containing group I WRKY TFs can be phosphorylated by MAP-kinases, they are likely to serve as the first WRKY proteins activated in response to PAMP-triggered MAPK signaling. Their targets may include the IIe *WRKY* genes *AtWRKY22* and *AtWRKY29*, which are upregulated by a PAMP-induced MAPK cascade and contain multiple W boxes within their respective promoters [4]. Co-transfection experiments further suggested that *At*WRKY22 and *At*WRKY29 can amplify expression of their own genes via a positive feedback loop [4]. The synthesis of SA and the expression of *NPR1*, a key regulator of some PAMP-triggered responses, appear to be partly controlled by WRKY factors. *NPR1* is regulated by WRKY TFs interacting with two W box elements in its 5′UTR [44]. Defense-associated SA production is strongly dependent on pathogen-inducible expression of *ICS1* [45]. This gene is a likely target of WRKY TFs, as its promoter is enriched for W boxes. However, the identities of the specific WRKY factors controlling *ICS1* and *NPR1* are unknown.

Eight *WRKY* genes (*AtWRKY18*, *-38*, *-53*, *-54*, *-58*, *-59*, *-66* and *-70*) were identified as direct targets of NPR1 [16••]. A nuclear-targeted NPR1-glucocotricoid receptor fusion conditionally expressed in the *npr1-1* mutant induced their transcription in the absence of protein biosynthesis [46]. Consistent with the role of NPR1 in stimulating transcription via interactions with TGA-bZIP transcription factors, expression of all eight NPR1-targeted *WRKY* genes was reduced or abolished in the *npr1-1* or *tga2/tga3/tga5/tga6* mutants. Use of T-DNA insertion mutants confirmed roles for most of these *WRKY* genes in NPR1-dependent defenses (see above).

Finally, *AtWRKY51* was identified as a potential SA-dependent downstream target of TGA2 by ChIP and whole-genome microarrays [47].

These data illustrate that WRKY TFs operate at multiple levels within complex PAMP-triggered transcriptional cascades. The activity of defense-promoting WRKY TFs is counteracted by that of PAMP-inducible WRKY factors with negative effects on defense, suggesting that feedback mechanisms limit the amplitude and duration of basal immune responses. Intriguingly, such negative feedback mechanisms seem to provide a functional interface between PTI and ETI [22••]. Upon AVR-effector recognition barley MLA resistance proteins were found to translocate to the nucleus and to physically interact with

Figure 1



Hypothetical modules of the *WRKY* web. Cellular defense signaling is triggered by recognition of pathogen-derived PAMPs via distinct plasma membrane (PM) localized receptors and transduced partly by MAP kinase cascades. Defense responses are also initiated upon detection of effector/avirulence (AVR) products of the pathogen within the host cell by major plant R proteins. In both cases, rapid alterations of gene expression ensues mediation by the action of distinct transcription factors such as WRKY TFs. ETI can be triggered by effector-mediated activation of R proteins (R inactive → R active) and subsequent inhibition of defense suppressing WRKY TFs. Pathogen-triggered SA signaling releases NPR1 from oligomer complexes resulting in the accumulation of NPR1 monomers in the nucleus and association with TGA TFs at promoter sites. A set of WRKY genes dependent on NPR1 function influence, both positively and negatively, downstream targets genes as indicated [16••]. MEKK1, MAP kinase kinase kinase; MEK1, MKK2, MKK4, MKK5, MAP kinase kinases; MPK3, MPK4, MPK6, MAP kinases. For details see text.

*Hv*WRKY1 and *Hv*WRKY2. These IIa WRKY proteins function as PAMP-inducible suppressors of basal defense. High-level expression of *Hv*WRKY2 attenuated MLA10-mediated ETI, indicating antagonistic interactions between these proteins. These observations imply that MLA-mediated effector recognition activates high-amplitude defense reactions by directly interfering with IIa WRKY TFs and thereby de-repressing PAMP-dependent basal defense. The existence of additional shortcuts in effector-triggered defense activation is supported by the unusual structure of the *AtWRKY52/RRS1 R* gene product [12]. Besides a group III-type WRKY domain, this protein contains domains characteristic for R proteins. Like barley MLAs, it interacts in the nucleus with its cognate effector, PopP2 [48]. Interestingly, a missense mutation within its WRKY domain results in conditional activation of defense responses and loss of *in vitro* binding to W boxes suggesting a negative role of this factor in defense signaling [49]. Thus, it is tempting to speculate that the interaction with PopP2 excludes *At*WRKY52/RRS1 from its proper DNA target sites and activates defenses by de-repression.

Conclusions

Transcription factors interact with other TFs as well as with additional nuclear proteins including co-activators/-repressors and components of the general transcriptional machinery to enable proper context-dependent expression of genes. As discussed above, several WRKY factors act as negative regulators of plant defense whereas others positively modulate this response implying their association with distinct regulatory complexes. Discrimination can part be determined by distinct topological features present in selected WRKY proteins.

An inherent feature of *WRKY* genes is their functional redundancy in defense programs. The existence

of redundant elements within the *WRKY* web may reflect a strong need to backup essential regulatory functions [33•] and could suggest that some WRKY TFs are manipulated by pathogen effectors to promote virulence. Multiple pathogen effectors are targeted to host nuclei and modify expression of the defense transcriptome [50]. However, except for *At*WRKY52/RRS1 and *Hv*WRKY1/2, interactions of pathogen effectors with WRKY TFs have not yet been reported. Still, on the basis of the enormous progress made within the past two years we can expect exciting novel revelations about WRKY TFs in the very near future.

Conflicts of interest

The authors declare that no conflicts of interest exits.

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