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Opinions

Dissecting the *WRKY* Web of Plant Defense Regulators

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During the past ten years, a large body of circumstantial evidence has accumulated, implicating WRKY factors in transcriptional reprogramming during plant immune responses [1–3]. Encoded by complex gene families in higher plants [4], these transcription factors share a DNA-binding domain (WRKY domain) comprising approximately 60 amino acids [5]. Additional conserved features of WRKYs are limited to separate subgroups of this family and include putative leucine zippers, nuclear localization signals, calmodulin binding sites, and several domains of unknown function [5–7]. Multiple studies have demonstrated the ability of WRKYs to bind to promoters of defense-associated genes via specific interactions of their WRKY domains with pathogen response elements termed W boxes (TTGACC/T) [8–11]. Both activating and repressing effects of WRKYs and W boxes on transcription have been observed [12–14]. It has also been shown that stable and transient overexpression of several WRKYs in the model plant *Arabidopsis thaliana (Arabidopsis)* conveys enhanced resistance to various bacterial or fungal pathogens [15–17].

The majority of the 74 *Arabidopsis WRKY* genes are transcriptionally inducible upon pathogen infection and other defense-related stimuli [18,19]. Interestingly, *WRKY* promoters are typically enriched for W boxes, thereby pointing to the existence of intricate regulatory circuits wherein functionally interconnected members of this family reside [19]. Indeed, multiple studies have revealed interactions of WRKYs with either their own promoters or those of other family members, suggesting that these transcription factors extensively engage in auto- and cross-regulation [9,12,13,20]. Such a *WRKY* web may ensure fast and efficient signal amplification. It may also allow for a tighter control in limiting the extent of defense responses via negative feedback mechanisms.

A central component of the transcriptional network activated during immune responses in *Arabidopsis* is Nonexpresser of Pathogenesis-Related genes 1 (NPR1) [21]. This transcriptional cofactor is required for several different types of plant immune responses, including basal defense and systemic acquired resistance (SAR), which are dependent on the defense hormone salicylic acid (SA) [22]. Upon induction of basal defense or SAR, NPR1 is translocated from the cytosol to the nucleus where it mediates the binding of TGA basic leucine zipper protein transcription factors to their cognate promoter elements, resulting in the upregulation of a multitude of genes [23–28]. In addition, NPR1 is functionally linked to WRKYs during plant immune responses. Intriguingly, WRKYs control NPR1 expression on the one hand, while on the other hand seem to operate downstream from it [17,29,30]. Until now, the identity of WRKYs involved in NPR1-dependent signaling branches has for the most part remained a mystery.

In contrast to WRKYs, NPR1 and TGA basic leucine zipper proteins are members of small families of only six and ten members in *Arabidopsis,* respectively. While mutant analyses have clearly proven the roles of NPR1 and several defined TGA basic leucine zipper proteins in pathogen defense [31–33], such direct genetic evidence is scarce for WRKYs. With the exception of an atypical family member *(AtWRKY52/RRS1)* [34], no WRKYs have been found to be involved in plant immune responses by mutant screens or other forward genetics approaches. In many cases, reverse genetics–based strategies to reveal biological roles of individual WRKYs using sequence-indexed *Arabidopsis* transferred DNA (T-DNA) or transposon insertion mutants have also proved to be unsuccessful in revealing defense-related phenotypes [2]. Functional redundancy among structurally related WRKY family members has been partially blamed for these experimental shortcomings [7]. In addition, in vivo roles of individual WRKYs may be limited to defined branches or nodes of the defense network. Hence, elimination of their function by mutation may result in quite specific or subtle phenotypes. Therefore, a major challenge in dissecting the *WRKY* web appears to be accurately pinpointing each WRKY's sphere of activity prior to mutant analyses. This would narrow the choice of defense phenotypes to examine and may allow for the selection of appropriate candidates for double or higher-order mutant analyses.

In a study published in this issue of *PLoS Pathogens,* Wang et al. succeeded in applying such a strategic approach [35]. A cleverly designed microarray experiment uncovered several *WRKY* genes that are direct transcriptional targets of NPR1. Wang and colleagues profiled transcriptome changes in transgenic *Arabidopsis* plants overexpressing an NPR1-GR (glucocorticoid receptor) fusion protein in an *npr1* mutant background. Combined application of SA and the synthetic glucocorticoid dexamethasone activated the NPR1 pathway and triggered translocation of NPR1-GR to nuclei. By

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Abbreviations: *Arabidopsis, Arabidopsis thaliana;* GR, glucocorticoid receptor; NPR1, nonexpresser of pathogenesis-related genes 1; SA, salicylic acid; SAR, systemic acquired resistance; T-DNA, transferred DNA

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simultaneously blocking protein biosynthesis with cycloheximide, Wang and colleagues eliminated transcription of indirect NPR1 targets (see also [36]). Among transcripts that are directly upregulated by NPR1-dependent mechanisms they found eight encoding WRKYs. Subsequent analysis of known *npr1* phenotypes using single and double T-DNA mutants of most of these candidates confirmed their roles in NPR1 signaling.

These experiments established AtWRKY18 as a positive regulator of basal defense and SAR operating downstream from NPR1. Two previous studies had already implicated this family member in basal defense [7,16]. Overexpression of AtWRKY18 in both studies supported its role as a positive defense regulator. However, findings by Xu et al. using the same *wrky18* insertion allele as Wang and colleagues (SALK__093916) suggest that it represses basal defense [7]. This discrepancy may have arisen from differences in experimental conditions. Also, protein–protein interaction studies have suggested that a complex interplay between AtWRKY18 and two structurally related family members, AtWRKY40 and AtWRKY60, modulates their function [7]. These three WRKYs can form homodimers or heterodimers via N-terminal leucine zippers, a feature conserved among them. Differential formation of different types of WRKY dimers may widen the spectrum of AtWRKY18 functions and may allow this regulator to act both as an activator and repressor of defense reactions.

Additional direct targets of NPR1 examined by Wang et al. are *AtWRKY53, AtWRKY54,* and *AtWRKY70.* These three WRKYs are structurally closely related to each other and distinct from AtWRKY18. While the AtWRKY54 and AtWRKY70 proteins show the highest degree of sequence similarity, the *AtWRKY53* and *AtWRKY70* genes exhibit strongly correlated expression during SAR. None of the single mutants of these three genes examined by Wang and colleagues showed altered disease resistance. However, combined insertions in the coexpressed *AtWRKY53* and *AtWRKY70* genes resulted in a moderate but significant reduction of basal defense to virulent *Pseudomonas syringae* bacteria, suggesting partially overlapping functions of these two genes. Consistent with this finding, recent studies by Li et al. as well as Knoth et al. have demonstrated roles of *AtWRKY70* in SA-mediated basal defense to the biotrophic fungus *Erysiphe cichoracearum* and the oomycete *Hyaloperonospora parasitica,* respectively [37,38]. The study by Knoth et al. extended roles of this WRKY to SA-dependent gene-for-gene resistance [38]. In addition, *AtWRKY70* was also shown to be required for defense to the fungal necrotroph *Botrytis cinerea* [39]. Although resistance to necrotrophs is typically mediated by SA-independent signaling mechanisms, *AtWRKY70* appears to be transcriptionally upregulated by an SA-dependent pathway in response to *B. cinerea* infections. Hence, a common theme regarding the function of *AtWRKY70* seems to be its requirement for SA-mediated defenses.

Furthermore, Wang and coworkers found that AtWRKY70 contributes to a second NPR1-mediated function, which is suppression of SA accumulation [35]. In untreated *Arabidopsis* tissues, SA concentrations are low but increase to high levels after pathogen infection. NPR1 limits the extent of SA accumulation. The tested *wrky70* single mutant exhibited markedly enhanced levels of SA in untreated plants. This effect is more pronounced in a *wrky70/wrky54* double mutant, which also allowed SA to accumulate to an even higher level after pathogen infection. Hence, the structurally closely related AtWRKY70 and AtWRKY54 have overlapping roles in counteracting accumulation of SA.

Finally, Wang et al. established a role of the so far elusive AtWRKY58 as a negative regulator of SAR [35]. In summary, the strategy applied by Wang and colleagues proved to be extremely successful in providing a deeper insight into the roles of WRKYs operating directly downstream from NPR1 in SA-dependent transcriptional cascades. This strategy can be modified and reiterated for a stepwise dissection of additional layers in the plant defense signaling network. Intriguingly, members of the Xinnian Dong lab have already initiated experiments to identify direct transcriptional targets of AtWRKY18 [35]. ■

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