A Genomic Approach to Identify Regulatory Nodes in the Transcriptional Network of Systemic Acquired Resistance in Plants

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Many biological processes are controlled by intricate networks of transcriptional regulators. With the development of microarray technology, transcriptional changes can be examined at the whole-genome level. However, such analysis often lacks information on the hierarchical relationship between components of a given system. Systemic acquired resistance (SAR) is an inducible plant defense response involving a cascade of transcriptional events induced by salicylic acid through the transcription cofactor NPR1. To identify additional regulatory nodes in the SAR network, we performed microarray analysis on *Arabidopsis* plants expressing the NPR1-GR (glucocorticoid receptor) fusion protein. Since nuclear translocation of NPR1-GR requires dexamethasone, we were able to control NPR1-dependent transcription and identify direct transcriptional targets of NPR1. We show that NPR1 directly upregulates the expression of eight *WRKY* transcription factor genes. This large family of 74 transcription factors has been implicated in various defense responses, but no specific WRKY factor has been placed in the SAR network. Identification of NPR1-regulated WRKY factors allowed us to perform in-depth genetic analysis on a small number of WRKY factors and test well-defined phenotypes of single and double mutants associated with NPR1. Among these WRKY factors we found both positive and negative regulators of SAR. This genomics-directed approach unambiguously positioned five WRKY factors in the complex transcriptional regulatory network of SAR. Our work not only discovered new transcription regulatory components in the signaling network of SAR but also demonstrated that functional studies of large gene families have to take into consideration sequence similarity as well as the expression patterns of the candidates.

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Introduction

Systemic acquired resistance (SAR) is an inducible plant defense response against pathogens. In *Arabidopsis*, the onset of SAR is preceded by an accumulation of the signaling molecule salicylic acid (SA). SA induces nuclear translocation of the transcription cofactor NPR1 to activate many genes required for disease resistance [1]. NPR1 also negatively feedback-regulates SA synthesis to mitigate its cytotoxic effect [2]. It is known that NPR1 controls the expression of antimicrobial pathogenesis-related genes (*PR* genes) by interacting with TGA transcription factors [3–5]. A microarray experiment showed that NPR1 also directly upregulates the protein secretory pathway. This is essential for SAR since disrupting this pathway diminished the secretion of PR proteins and resulted in reduced resistance [6]. NPR1 likely regulates these secretion-related genes through a novel transcription factor [6]. In addition to this unknown transcription factor and the TGAs, WRKY transcription factors have also been implicated in regulating the response against pathogen infection. Many *WRKY* genes are rapidly induced after treatment with elicitors associated with infection [7–9]. Moreover, genes induced during defense responses often contain WRKY transcription factor–binding sites, W boxes, in their promoter regions. For example, the promoter of an SA biosynthesis gene is enriched with W boxes [10]. The expression of *NPR1* itself has been shown to be under the regulation of WRKY factors [11]. In a transcriptional profiling study, Maleck et al. discovered that W boxes are over-represented in a cluster of genes sharing the induction pattern of *PR-1*, suggesting a role for WRKY factors in SAR [12]. Ectopic expression studies have shed some light on the functions of WRKY genes. Plants overexpressing *WRKY70* have heightened resistance toward two bacterial pathogens [13]. Likewise, overexpressing *WRKY18* resulted in gain of *PR* gene expression and resistance in a developmentally regulated manner [14]. Transiently overexpressed *WRKY29*, a target of a MAPK cascade activated by bacterial flagellin, also led to stronger resistance [15]. However, data from overexpression studies need to be interpreted with caution. For example, since ectopically expressing several WRKY genes all resulted in a similar range of phenotypes, it is difficult to conclude functional specificity from these studies.

Typical of large gene families, phenotypic analysis of loss-

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Abbreviations: ANOVA, analysis of variance; BTH, benzothiadiazole S-methylester; dpi, days post-inoculation; EDS, enhanced disease symptoms; GR, glucocorticoid receptor; PR, pathogenesis-related; *Psm, Pseudomonas syringae* pv. *Maculicola;* SA, salicylic acid, SAR, systemic acquired resistance

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Synopsis

Many biological processes are controlled by intricate regulatory networks of gene expression. Identifying the regulatory nodes in these networks and understanding the hierarchical relationship between them are vital to our understanding of biological systems. However, this task is frequently hampered by the intrinsic complexity of these processes. Here, the authors used a controlled transcriptional profiling strategy to a plant immune response called systemic acquired resistance to study the transcriptional events one at a time. Systemic acquired resistance is activated through the induction of thousands of genes by the transcriptional regulator protein NPR1. The authors found that downstream of NPR1 are several regulatory nodes comprised of members from a large family of transcriptional factors. Disrupting these regulatory nodes compromised various functions assigned to NPR1, providing the information needed to construct a gene regulation network.

of-function WRKY mutants has been hampered by functional redundancy. It has been reported that in a collection of more than 40 *wrky* knockout mutants, phenotypes were rarely observed [16]. This difficulty is further exacerbated by the wide range of defense responses in which WRKY factors participate. Therefore, to elucidate the function of specific WRKY genes, it is critical to identify a small number of candidates within a well-defined biological process.

In this study, we used a genomics-directed approach to identify those *WRKY* genes whose expression is directly regulated by NPR1. The small number of candidate genes allowed more informed construction of double mutants and focused examination of the mutants on NPR1-associated phenotypes. As a result, we were able to find new regulatory nodes (i.e., WRKY factors) in the complex transcriptional regulatory network of SAR.

Results

Identification of Eight *WRKY* Genes as Direct Transcriptional Targets of NPR1

To dissect the transcriptional cascade leading to SAR, we performed a microarray experiment with the Affymetrix ATH1 GeneChip (24,000 genes) to identify direct transcriptional targets of NPR1 using a previously described strategy [6] (Figure 1A). Two biological replicates were used (the full datasets can be found at the Integrated Microarray Database System (http://ausubellab.mgh.harvard.edu/imds) and NASCArrays (http://affymetrix.arabidopsis.info/donating.html). We performed a Bayesian *t* test (http://visitor.ics.uci.edu/genex/cybert) [17] to compute the *p*-values. Assuming the expression measurements of a gene have a normal distribution, the Bayesian *t* test models the variance as a function of the mean. For experiments with few replicates, the Bayesian *t* test shows better performance than the basic *t* test in simulated and biological datasets [17,18]. In our analysis, the confidence value was set to six, and window size was 100. Using $p < 0.001$ as a cutoff, we found 64 genes differentially expressed between *NPR1-GR* (glucocorticoid receptor; in *npr1–3*) and *npr1–3* (Table S1). Among the 64 genes, we found that the expression of *WRKY54, WRKY38, WRKY59, WRKY18, WRKY70, WRKY66,* and *WRKY53* was reproducibly induced in two biological replicates. We also carried out a mixed-model analysis of variance (ANOVA) [19] of the data. Using $p < 0.05$ as a cutoff, *WRKY38, WRKY54, WRKY66,* and *WRKY58* were the top candidates with the most fold changes. We therefore added *WRKY58* to the list. We then confirmed the inducibility of six of these genes, *WRKY18, WRKY38, WRKY53, WRKY54, WRKY58,* and *WRKY70,* in wild-type (WT) plants in response to benzothiadiazole S-methylester (BTH; a functional analog of SA) using reverse transcription followed by quantitative PCR (RT-qPCR) (unpublished data) and by additional microarray analysis (Figure 2). Induction of these genes was either abolished or markedly reduced in the *npr1–1* mutant, confirming that they are transcriptional targets of NPR1. Furthermore, activation of these genes was similarly affected in the *tga2 tga3 tga5 tga6* quadruple mutant, suggesting that TGA transcription factors are also required for their induction (unpublished data). Induction of the other two *WRKY* genes, *WRKY59* and *WRKY66,* also appeared to be abolished in *npr1–1* and *tga2 tga3 tga5 tga6,* but the background expression for these genes was too low in WT to calculate the fold induction (unpublished data).

Several of these *WRKY* genes have been studied previously for their roles in disease resistance and related processes. Plants overexpressing *WRKY18* exhibited heightened resistance against two bacterial pathogens [14]. Constitutive expression of *WRKY70* enhanced SA-mediated resistance but compromised resistance mediated by jasmonic acid [20]. WRKY53 was found to be involved in leaf senescence [21]. However, there has been no concrete genetic evidence to place specific WRKY factors in the SAR signaling network. By focusing on the first transcriptional step downstream of NPR1, we were able to identify a small number of WRKY factors sharing not only sequence homology but also similar expression patterns. Therefore, either individually or in combination, these WRKY factors are promising candidates for transcriptional regulators required for NPR1 function.

WRKY18 Is a Positive Transcription Factor Required for Full Induction of SAR

We isolated T-DNA or transposon insertion lines in each of the NPR1-inducible *WRKY* genes, most of which disrupted the expression of their corresponding genes, as assayed by RT-qPCR (Table S2). The only insertion mutation available for *WRKY38* reduced its expression by 67% and is not a knockout mutation.

We first examined individual *wrky* mutants for effects on BTH-induced resistance. Because mutating a single transcription factor would likely cause only a partial loss of resistance, we applied a moderate concentration of BTH (60 μM) followed by inoculation of a bacterial pathogen, *Pseudomonas syringae* pv. *maculicola (Psm)* ES4326. All of the *wrky* mutants exhibited near WT-level resistance (unpublished data) except *wrky18,* which was partially impaired in BTH-induced resistance to this pathogen (Figure 3A). We then performed biological induction of SAR using *P. syringae* pv. *phaseolicola* carrying the *avrB* gene. As shown in Figure 3B, recognition of avrB led to enhanced resistance to subsequent challenge by *Psm* ES4326 in systemic leaves of WT plants. This induced resistance was absent in *wrky18.* Since the *npr1* mutant is impaired not only in SAR but also in basal resistance, we examined *wrky18* for an enhanced disease symptoms (EDS) phenotype. Indeed, when we inoculated *wrky18* with a low level of *Psm* ES4326 ($OD_{600} = 0.0001$), the EDS phenotype was also evident (Figure 3C and 3D). In all

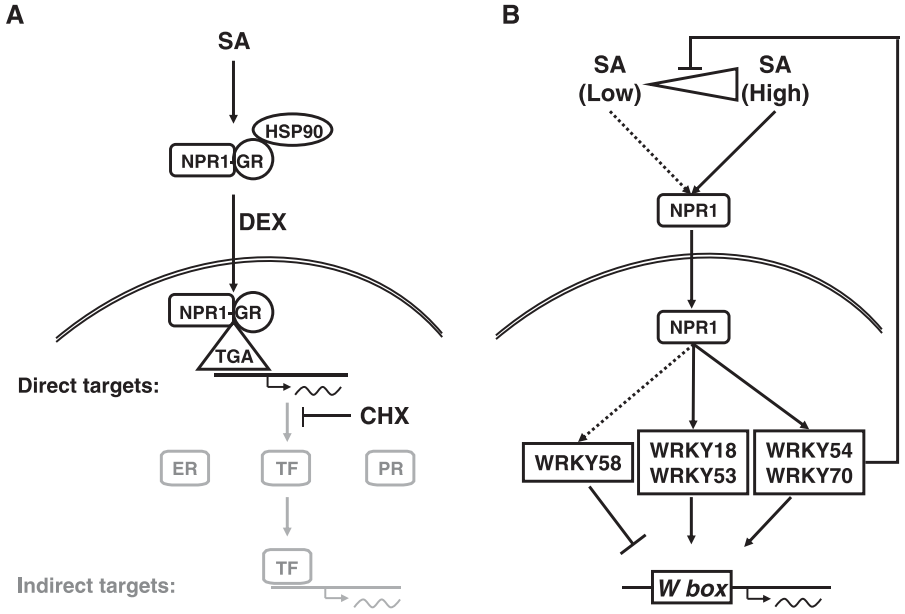

Figure 1. Differential Regulation of SAR by NPR1-Induced WRKY Factors

(A) A schematic representation of the strategy to identify NPR1 direct targets. SA treatment activates components of the SAR pathway upstream or independent of NPR1. Subsequent application of dexamethasone (DEX) triggers nuclear translocation of the NPR1-GR fusion protein to activate existing TGA transcription factors. Direct target genes of NPR1 are transcribed but not translated in the presence of the inhibitor cycloheximide (CHX) to prevent transcription of indirect target genes. ER, endoplasmic reticulum–resident proteins; TF, transcription factors; PR, pathogenesis-related proteins.

(B) Mode of action for the NPR1-target WRKY factors. In WT, SA accumulation triggers nuclear localization of NPR1, which directly induces several *WRKY* genes. When SA levels are low, WRKY58 functions to prevent (blocked arrow) spurious activation of SAR (dotted lines). When SA levels are high, signaling through positive WRKY factors overcomes the negative effect of WRKY58 to activate (arrow) downstream gene transcription (solid lines). In addition, WRKY70 and WRKY54 prevent excessive SA accumulation (blocked arrow).

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three cases, however, the phenotype of *wrky18* was less severe than that of *npr1–1,* which is consistent with our hypothesis that WRKY18 is a downstream component of SAR that mediates a subset of NPR1 functions.

We then performed another microarray experiment to determine the defect in *wrky18* on gene expression during SAR. We treated WT, *wrky18,* and *npr1* plants with 60 μM BTH, and harvested leaf tissue at 0, 8, and 24 h after induction. Three biological replicates were collected for each timepoint/genotype combination. BTH treatment in WT triggered a robust change in the expression of thousands of genes. Using ANOVA, the expression of 6,525 genes was found to be altered in WT following BTH treatment ($p < 0.05$) (after multiple testing correction using the method proposed by Benjamini and Hochberg to assess false discovery rate [22]). After applying a two-fold change cutoff to these genes, the list was reduced to 2,280 genes, among which 1,147 were induced and 1,133 were repressed (Table S3). From this list, we applied a two-way ANOVA between WT and *npr1* to identify NPR1-dependent genes. Interestingly, almost all BTH-responsive genes were NPR1 dependent (2,248/2,280; 99%) (Figure 4A), highlighting the crucial role of NPR1 in BTH-mediated transcriptional reprogramming. We used the same analysis to compare WT and *wrky18.* The effect of disrupting *WRKY18* is moderate: the expression of 451 BTH-responsive genes (~19.8%) was altered in this mutant (Table S4 and Figure 4A). Furthermore, while differences in gene expression were drastic between WT and *npr1* (Figure 4B), the changes between WT and *wrky18* were mostly in the amplitude, with the degree of induction or repression less dramatic in *wrky18* than in WT (204 and 152 genes, respectively; Figure 4C). Gene Ontology searches using the DAVID software [23] revealed six functional categories overrepresented in this WRKY18 cluster ($p < 0.01$; Table S5). As expected, the most prominent functional group in the cluster was involved in responding to biotic stimuli ($p = 1.19 \times 10^{-5}$). WRKY factors are known to recognize W boxes in the promoters of their target genes [16]. The W box sequence was significantly overrepresented in the promoters of those genes for which the effect of BTH was diminished in *wrky18*: 2.3 copies per gene versus 1.7 copies at background level ($p < 2.4 \times 10^{-115}$) [24]. These data clearly show that WRKY18 plays a positive role in SAR as an auxiliary transcription factor for a subset of NPR1-dependent genes.

In an effort toward elucidating SAR transcriptional controls further downstream of NPR1, we generated plants carrying the *WRKY18-GR* construct. Upon DEX treatment, *WRKY18-GR* complemented the EDS phenotype of the parental *wrky18* mutant (Figure S1). Therefore, the GR fusion strategy can be applied again to identify direct transcriptional targets of WRKY18 and to dissect the complex SAR transcriptional network.

WRKY58 Is a Negative Transcription Factor to Prevent Spurious Induction of SAR

We also found a negative regulator of defense responses among our collection of *wrky* mutants: *wrky58* displayed several morphological phenotypes, including curly and pointed leaves with rough texture and a smaller rosette size, features that are reminiscent of a mutant with constitutive resistance, *snc1* [25]. This suggests that the WRKY58 protein

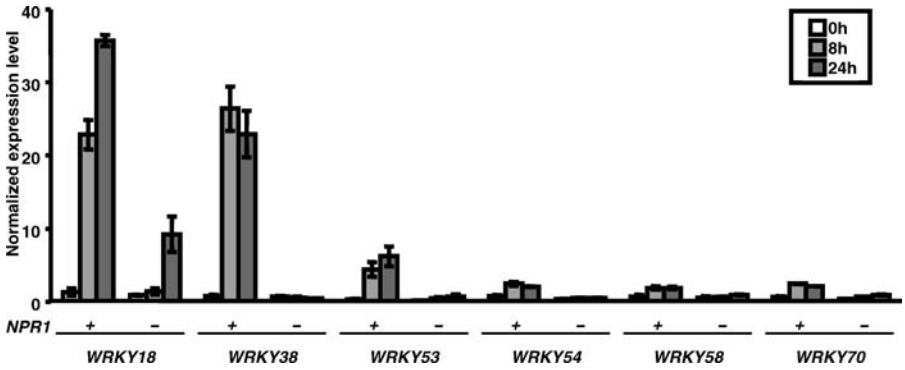

Figure 2. Induction of *WRKY* Genes by SAR Inducers and NPR1

Plotted here are $\log_2$-transformed microarray data normalized by the GeneSpring package, showing the expression levels of six WRKY genes 0, 8, and 24 h after BTH treatment in WT (*NPR1* +) and *npr1* mutant (*NPR1* –). The expression levels of *WRKY59* and *WRKY66* were too low to be detected under these conditions. Error bars represent standard deviations (SDs).

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may be a negative regulator of disease resistance. Although *wrky58* showed no consistent difference from WT without induction or when resistance was induced by 60 μM BTH, we reasoned that WRKY58 might function at a suboptimal level of the inducer. Indeed, after treatment with a lower concentration of BTH (30 μM), *wrky58* was clearly more resistant to *Psm* ES4326 than WT (Figure 5A). Furthermore, in the *wrky58 wrky18* double mutant, the EDS phenotype of *wrky18* was abolished (Figure 5B). The *wrky58* mutation had no effect on SA levels (unpublished data), consistent with it being a downstream regulator. The role of WRKY58 may be to provide a safeguard mechanism for preventing spurious activation of defense responses at suboptimal levels of SA or to turn off SAR once the pathogen challenge subsides. The enhanced resistance phenotype in *wrky58* is not due to constitutive expression of *PR* genes (unpublished data), suggesting a new mode of regulating disease resistance for this transcription factor.

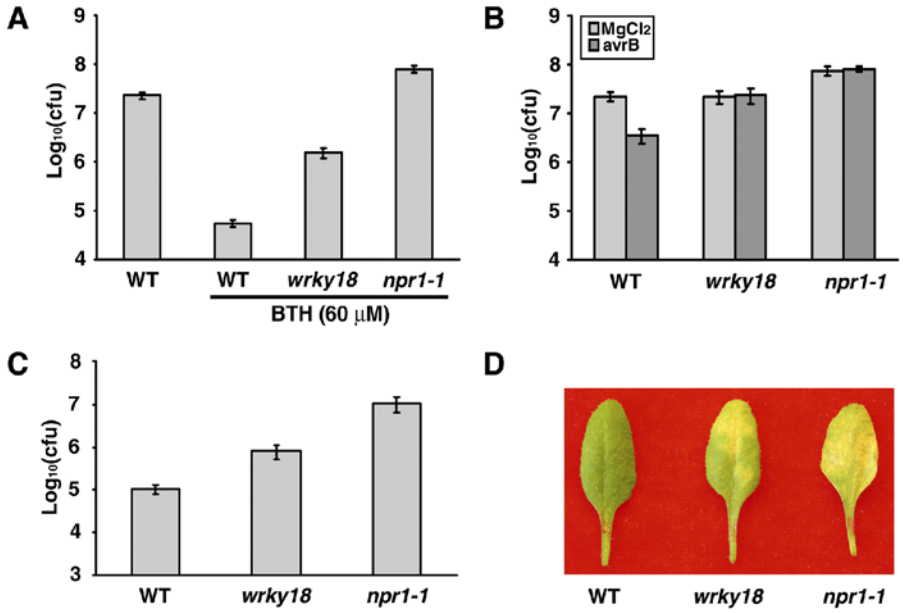

Figure 3. Resistance Defects of *wrky18*

(A) Plants were chemically induced with 60 μM BTH 24 h before inoculation with a high dose of *Psm* ES4326 ($OD_{600} = 0.001$). As a control, uninduced plants were inoculated at the same time. Bacterial growth was scored 3 dpi. Each datapoint represents the average colony-forming units (cfu) from 16 leaf disks plotted on a log scale, with error bars indicating 95% confidence intervals. This experiment was repeated more than five times with similar results.

(B) Plants were first inoculated with either *P. syringae* pv. *phaseolicola avrB* or 10 mM $MgCl_2$ on two lower leaves. Later (3 d), three upper leaves were inoculated with *Psm* ES4326 ($OD_{600} = 0.001$). Leaf disks from the second inoculation were collected 3 dpi to measure bacterial growth. This experiment was carried out twice with similar results.

(C and D) To examine *wrky18* for an EDS phenotype, plants were inoculated with a low dose of *Psm* ES4326 ($OD_{600} = 0.0001$). Bacterial growth was measured in 3 dpi (C), and disease symptoms were recorded in (D) 3 dpi. These experiments were performed more than five times with similar results.

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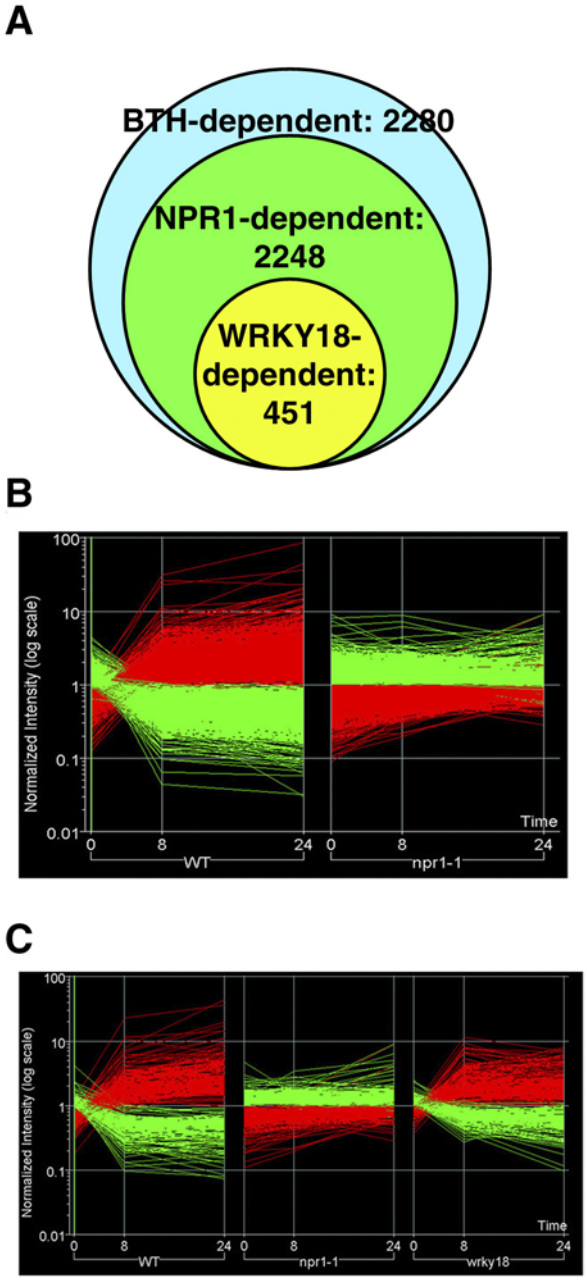

Figure 4. Genes Affected by BTH, *npr1*, and *wrky18* 0, 8, and 24 h after Induction

Using ANOVA, the expression of 6,525 genes was found to be altered in WT following BTH treatment ($p < 0.05$) (after multiple testing correction using the method proposed by Benjamini and Hochberg to assess false discovery rate [22]). After applying a 2-fold change cutoff to these genes, the list was reduced to 2,280 genes, among which 1,147 were induced and 1,133 were repressed. From this list, a two-way ANOVA was applied between WT and *npr1* data sets and between WT and *wrky18* data sets to identify NPR1-dependent and WRKY18-dependent genes, respectively.

(A) The Venn diagram shows that almost all BTH-responsive genes were NPR1-dependent (2,248/2,280; 99%) whereas the expression of 451 BTH-responsive genes (~19.8%) was altered in the *wrky18* mutant.

(B) The expression levels of 2,280 BTH-dependent genes normalized by GeneSpring were plotted on log scale on the *y*-axis and in time order on the *x*-axis. Genes induced and repressed in WT are colored red and green, respectively. The profile of these genes in the *npr1* mutant is also depicted.

(C) The expression levels of 451 WRKY-dependent genes in WT and in *wrky18* mutant were normalized by GeneSpring and plotted on log scale on the *y*-axis and in time order on the *x*-axis. Genes induced and repressed in WT are colored red and green, respectively. The majority of them showed either diminished induction (204 genes) or diminished repression (152 genes) in *wrky18,* in contrast to the robust response in WT and the almost complete lack of response in *npr1*.

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WRKY70 and Its Functional Homologs Play Dual Roles as Negative Regulators of SA Biosynthesis and Positive Regulators of SA-Mediated Gene Expression and Resistance

NPR1 is not only an essential transducer of the SA signal but also a negative regulator of SA synthesis. In *npr1,* SA accumulates to extremely high levels after infection [2], causing cytotoxicity in the mutant. We first investigated whether WRKY18 could be responsible for this function of NPR1. SA levels in *wrky18* were similar to WT, both with and without infection (unpublished data). We then surveyed the SA levels of the other seven *wrky* mutants and found that the *wrky70* mutant accumulates free SA to a level significantly higher than that of WT in the absence of infection (Figure 6A). A BLAST search using the amino acid sequence of WRKY70 against the *Arabidopsis* proteome identified WRKY54 as its closest homolog, which is also an NPR1 direct target (Figure 2 and Table S1). Although the single *wrky54* mutant had normal SA levels, the *wrky54 wrky70* double mutant showed a significantly higher level of free SA compared to *wrky70* (Figure 6A). This high SA level was further elevated after induction by *Psm* ES4326 carrying the *avrRpt2* gene (Figure 6A). Consistent with elevated SA levels, the SA biosynthesis gene *ICS1* (encoding isochorismate synthase [10]) was clearly upregulated in *wrky54 wrky70* (Figure 6B).

Accumulation of SA in naïve *wrky70* and *wrky54 wrky70* indicates that in WT plants, SA biosynthesis is actively repressed by basal levels of WRKY70 and WRKY54. The fact that SA levels can be further induced in *wrky54 wrky70* suggests that during the onset of SAR, a positive regulator, possibly a transcriptional activator, is recruited to initiate SA synthesis. Activated NPR1 then induces WRKY70 and WRKY54 to negatively control SA accumulation.

Surprisingly, neither *wrky54 wrky70* nor the corresponding single mutants exhibited heightened resistance to *Psm* ES4326 ($OD_{600} = 0.001$; Figure 6C). At the molecular level, we found that the SAR effector genes *PR-1, PR-2,* and *PR-5* were not constitutively expressed in *wrky54 wrky70* despite the elevated SA levels (Figure S2). This suggests that WRKY70 and WRKY54 play dual roles in repressing SA biosynthesis and transducing the SA signal. This result is consistent with a recent report showing that in the same loss-of-function *wrky70* mutant, SA-mediated resistance against the fungal pathogen *Erysiphe cichoracearum* was impaired [26].

A positive role for WRKY70 in disease resistance was further supported by characterizing the *wrky53 wrky70* double mutant. In our initial characterization of the single *wrky* mutants, *wrky53* showed a minor deficiency in resistance (unpublished data). Because the expression of *WRKY53* and *WRKY70* was highly correlated after SAR induction ($r^2 = 0.945$; Spivey et al., unpublished data), we generated the *wrky53 wrky70* double mutant. The double mutant had similar SA content to *wrky70* (unpublished data), yet exhibited an

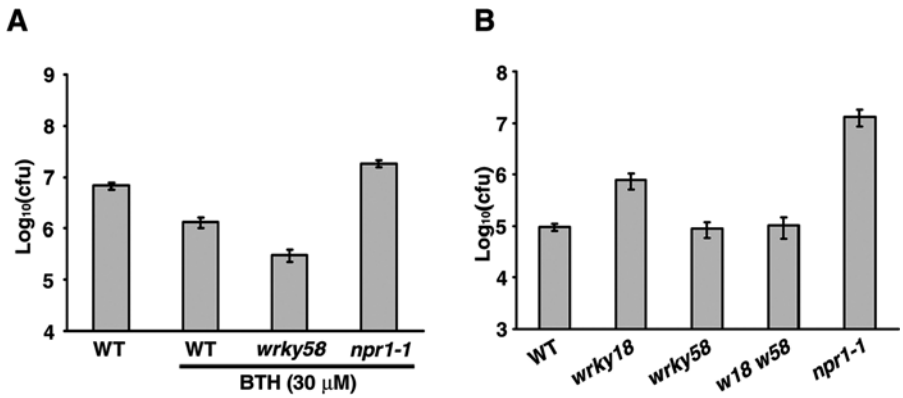

Figure 5. Resistance Defects of *wrky58*

(A) Loss of WRKY58 confers resistance when plants were weakly induced with 30 mM BTH.

(B) To examine the function of WRKY58, the *wrky58* mutation was introduced into *wrky18,* and the effect was observed in an EDS test 3 dpi. *w18 w58* represents the *wrky18 wrky58* double mutant.

Both (A) and (B) were performed twice with similar results.

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EDS phenotype (Figure 6D and 6E). This result indicates that both WRKY53 and WRKY70 are positive regulators of defense responses, with possibly redundant functions.

All the double mutant analysis described above was carried out on multiple independent populations with similar results (Figure S3), verifying the phenotypes were linked to the mutations under consideration.

Discussion

The WRKY family of transcription factors experienced significant expansion during the evolution of land plants. Genetic redundancy within such a large family of genes makes dissecting the function of individual *WRKY* genes a daunting task. Taking advantage of the fact that many *WRKY* genes are inducible, we focused on one step of a specific signal transduction event and identified eight WRKY factors as important transcriptional regulators of SAR downstream of NPR1. This approach also allowed us to test a well-defined set of phenotypes associated with NPR1 and to assign specific functions to these individual WRKY genes. As a result, we elucidated functions for five of the eight NPR1 direct targets in the model illustrated in Figure 1B.

Our data established WRKY18 as a significant positive regulator of SAR. The partial loss of resistance in *wrky18* (Figure 3) suggests that the endogenous protein performs a subset of functions directed by NPR1. This has been clearly demonstrated by comparing the transcriptomes of *wrky18* and *npr1* with that of WT after induction with 60 μM BTH. The *wrky18* mutation affected the amplitude of gene expression triggered by BTH (Figure 4C). Because the *WRKY18* transcript levels remain high even beyond the 24-h induction period, this transcription factor may also be required to sustain SAR-related gene expression.

Recently, it was reported that WRKY18 physically interacts with two negative regulators of defense, WRKY40 and WRKY60 [27]. It is possible that during SAR, WRKY18 releases the inhibitory effects of WRKY40 and WRKY60 to induce gene expression. Unfortunately, under the experimental conditions used in this recent report, where 10-fold more pathogens were used, the WT plants developed disease symptoms, and the defect caused by *wrky18* on basal resistance was masked. SAR was not tested on *wrky18*. The same authors showed that overexpression of *WRKY18* led to enhanced resistance [14], consistent with our finding that WRKY18 alone is a positive regulator of defense.

Activation of SAR is a costly process involving dramatic induction of more than 1,000 genes (Figure 4B and Table S3; [28]). Therefore, SAR should only be activated when the benefit of resistance outweighs the costs. WRKY58 appears to be a negative regulator that functions at a suboptimal level of BTH to prevent spurious induction of SAR (Figure 1B). The stunted growth of the *wrky58* mutant plants is consistent with this hypothesis.

Characterization of both *wrky70* and the *wrky54 wrky70* mutants in our study provided new insights into another function of NPR1, namely the ability to curtail excessive SA accumulation. Hyperaccumulation of the *ICS1* transcript and SA observed in these mutants (Figure 6) suggests that in WT plants, SA biosynthesis is actively repressed by low levels of WRKY70 and WRKY54. Because WRKY70 and WRKY54 are both NPR1 targets, it is reasonable to hypothesize that they are also involved in shutting down SA biosynthesis once the pathogen challenge subsides (Figure 1B). The observation that free SA levels in the *wrky54 wrky70* double mutant were further elevated in response to pathogen infection (Figure 6A) suggests that induction of SA biosynthesis involves a positive regulator. However, this regulatory gene is unlikely to be among the NPR1 targets examined in this study since SA biosynthesis occurs prior to activation of NPR1.

In an earlier study, it was reported that reducing *WRKY70* expression by an antisense construct did not change SA levels [20]. It is possible that in addition to *WRKY70,* a related *WRKY* gene required for activating SA biosynthesis was also silenced. This suggests that this unknown positive regulator may be another WRKY factor. The presence of multiple W boxes in the promoter of the SA biosynthesis gene *ICS1* [10] is consistent with this hypothesis.

Unlike many reported SA-overaccumulating mutants, *wrky54 wrky70* did not exhibit constitutive resistance to pathogens. In fact, this double mutant may have an EDS phenotype (unpublished data). We believe that WRKY70 and

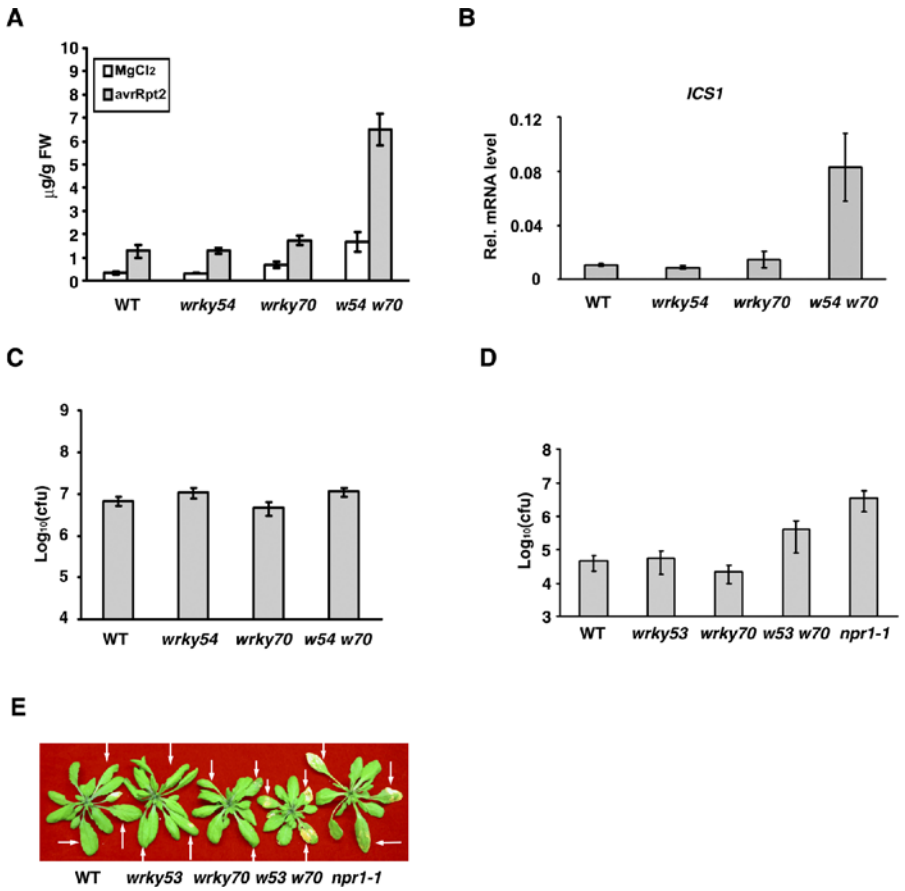

Figure 6. Defects in WRKY70 and WRKY54 Result in SA Overaccumulation

(A) Plants were dipped in either a 10 mM $MgCl_2$ solution or a suspension of *Psm* ES4326 *avrRpt2* to trigger SA production. Free SA was extracted and measured from three samples for each datapoint 3 dpi. Error bars represent SDs. This experiment was repeated twice with similar results.
(B) The SA biosynthesis gene *ICS1* is upregulated in the *wrky54 wrky70* (*w54 w70*) double mutant. Relative transcript levels were determined by RT-qPCR after normalization to ubiquitin. Error bars represent SD from three PCR runs.
(C) Lack of resistance in *w54 w70,* measured by bacterial growth 3 dpi with a high dose of *Psm* ES4326.
(D and E) The *wrky53 wrky70* (*w53 w70*) double mutant displays an EDS phenotype. Bacterial growth was measured in 3 dpi (D) and disease symptoms were recorded in 3 dpi (E).
Both (C) and (D) were done three times each with similar results.
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WRKY54 play dual roles during SAR: both as negative regulators of SA synthesis and as positive regulators of SA signaling (Figure 1B). Supporting evidence came from a report where *WRKY70*-overexpressing plants showed constitutive resistance to two bacterial pathogens [20]. If the sole function of WRKY70 were to repress SA biosynthesis, one would expect compromised resistance in plants with elevated *WRKY70* expression. Finally, our characterization of the *wrky53 wrky70* double mutant suggests that WRKY53 also plays a positive role in activating defense responses, as this double mutant showed an EDS phenotype (Figure 1B).

In conclusion, our genomics-directed genetic studies of *WRKY* genes led to unambiguous placement of specific WRKY factors in the intricate signaling network induced by SA. With this stepwise approach, we will continue to identify new regulatory nodes up and down this transcription cascade.

Materials and Methods

Plant growth and treatments. T-DNA and transposon insertion mutants described in this study were acquired from the *Arabidopsis* Biological Resource Center (http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm) and genotyped with allele-specific PCR. All of the mutants have been backcrossed and shown to breed true in the progeny. The *wrky18* has also been complemented by a WRKY18-GR construct. WT and mutant plants (all of ecotype Columbia) were grown on soil (Metro Mix 200) at 22 °C under a 16/8-h light/dark cycle. To chemically induce SAR, 4-wk-old plants were sprayed with BTH 24 h before inoculation with *Psm* ES4326 at $OD_{600} = 0.001$. Biologically induced SAR was performed by inoculating lower leaves first with *P. syringae* pv. *phaseolicola* carrying the *avrB* gene ($OD_{600} = 0.02$) 3 d before *Psm* ES4326 infection. The EDS phenotype was tested using a low titer ($OD_{600} = 0.0001$) of *Psm* ES4326. Pathogen growth was assayed 3 d after infection.

Gene expression and microarray analysis. RNA samples were prepared using a previously described protocol [6]. For real-time RT-qPCR, RNA samples were reversed transcribed into cDNA using SuperScript Reverse Transcriptase (Invitrogen, http://www.invitrogen.com). The cDNA was quantified using gene specific primers and the QuantiTect reagent (Qiagen, http://www1.qiagen.com) in a LightCycler (Roche, www.roche.com). For microarray, probes were synthesized and hybridized to the Affymetrix *Arabidopsis* ATH1 GeneChip arrays (Affymetrix, http://www.affymetrix.com) according the manufacturer's protocol. Hybridization reactions were performed by the Microarray Core Facility at the Center for Applied Genomics and Technology at Duke University. For the initial microarray to identify NPR1 direct target genes where there

were two biological replicates, we performed the Bayesian *t* test (http://visitor.ics.uci.edu/genex/cybert) [17] to compute the *p*-values. Assuming the expression measurements of a gene have a normal distribution, the Bayesian *t* test models the variance as a function of the mean. For experiments with few replicates, the Bayesian *t* test shows better performance than the basic *t* test in simulated and biological datasets [17,18]. In this analysis, the confidence value was set to six, and window size was 100. For the other microarrays, where there were three biological replicates, and data were analyzed using GeneSpring (Agilent Technologies, http://www.agilent.com). BTH-responsive genes in WT were identified based on both significance (ANOVA p-value < 0.05) and fold change (≥ 2). These genes were filtered through a two-way ANOVA considering both genotype and treatment effects. NPR1- and WRKY18-dependent genes were identified as ones that either showed genotype–treatment interaction, or as ones affected by genotype and treatment. WRKY18-dependent genes were then subjected to Gene Ontology functional annotation using the DAVID tool (http://david.niaid.nih.gov) [23]. Their promoter sequences (1 kb upstream of the start codon) were extracted from TAIR (http://www.arabidopsis.org) and analyzed by POBO (http://ekhidna.biocenter.helsinki.fi/pobo) [24] for the presence of the W box sequence (C/T)TGAC(T/C).

Microarray data deposition. All of the microarray data have been deposited in public databases: The Integrated Microarray Database System (http://ausubellab.mgh.harvard.edu/imds) and NASCArrays (http://affymetrix.arabidopsis.info/donating.html).

Free SA extraction and measurement. Plants were dipped into a *Psm* ES4326 *avrRpt2* suspension ($OD_{600} = 0.02$) in 10 mM $MgCl_2$ or the saline solution alone 3 d before tissue collection. SA extraction was modified from a previously described protocol [29]. Briefly, SA was extracted from 0.2 g ground tissue twice using HPLC-grade methanol. Methanol was removed under vacuum, and the pellet was resuspended in 250 μL 5% trichloroacetic acid. SA was then extracted twice into an organic phase containing a 1:1 mixture of ethyl acetate and cyclopentane. The organic solvent was evaporated under vacuum and SA was dissolved in 20% HPLC-grade methanol. Pure SA samples were included in the same procedure to account for recovery rate (usually ~66%). SA levels were quantified on an HPLC system (Waters, http://www.waters.com) with excitation at 295 nm and emission at 405 nm. Each datapoint was derived from three independently collected samples.

Supporting Information

Figure S1. Complementation of the EDS Phenotype of *wrky18* by *P_{35S}:WRKY18-GR*

The *wrky18* mutant plants were transformed with *WRKY18* fused with the sequence encoding the hormone-binding domain of the *GR*. Expression of the fusion gene is controlled by the constitutive 35S promoter *(P_{35})*, and the nuclear translocation of the fusion protein requires dexamethasone (DEX). The progeny of the transformants were sprayed with 5 μM of DEX and inoculated with a low dose of *Psm* ES4326 ($OD_{600} = 0.0001$). Bacterial growth was scored 3 d post-inoculation (dpi). Each datapoint represents the average colony-forming units (cfu) from 16 leaf disks plotted on a log scale, with error bars indicating 95% confidence intervals. Eleven independent transformants were analyzed and eight of them showed complementation. Two are presented in this figure.

Found at doi:10.1371/journal.ppat.0020123.sg001 (1.7 MB TIF).

Figure S2. *PR* Gene Expression Profile in *wrky54 wrky70*

(A) Background expression of *PR-2* and *PR-5* in WT and *wrky54 wrky70*. (B) *PR-1* expression before *(− Psm)* and 3 d after *Psm* ES4326 ($OD_{600} = 0.001$) infection *(+ Psm)*.

Found at doi:10.1371/journal.ppat.0020123.sg002 (2.0 MB TIF).

Figure S3. The *wrky* Mutant Phenotypes Bred True in Multiple Progeny after Genetic Crosses

(A) The *wrky58* mutation suppresses the EDS phenotype in *wrky18*. Plants were inoculated with a low dose of *Psm* ES4326 ($OD_{600} = 0.0001$). Bacterial growth was scored 3 dpi. Each datapoint represents the average cfu from 16 leaf disks plotted on a log scale, with error bars indicating 95% confidence intervals. *w18 w58* represents the *wrky18 wrky58* double mutant. Two independent lines were tested with similar results.

(B) Lack of resistance in *wrky54 wrky70* (*w54 w70*), measured by bacterial growth 3 dpi with a high dose of *Psm* ES4326 ($OD_{600} = 0.001$). Three independent lines were tested with similar results.

(C) The *wrky53 wrky70* (*w53 w70*) double mutant displays an EDS phenotype. Two independent lines were tested with similar results.

Found at doi:10.1371/journal.ppat.0020123.sg003 (1.6 MB TIF).

Table S1. A Partial List of Genes Directly Regulated by NPR1

We performed the Bayesian *t* test (http://visitor.ics.uci.edu/genex/cybert) to compute the *p*-values. In this analysis, the confidence value was set to six, and window size was 100. Using $p < 0.001$ as a cutoff, 64 genes were found to be differentially expressed between NPR1-GR (in *npr1–3*) and *npr1–3*. The complete dataset for this experiment can be found at the Integrated Microarray Database System (http://ausubellab.mgh.harvard.edu/imds) and NASCArrays (http://affymetrix.arabidopsis.info/donating.html)

Found at doi:10.1371/journal.ppat.0020123.st001 (62 KB PDF).

Table S2. *WRKY* Mutants Characterized in This Study

Homozygous T-DNA or transposon insertion plants were identified and the effect on the expression of the corresponding genes was assayed by RT-qPCR. For insertions in an exon or the promoter region, qPCR primers bind downstream of the insertion. For insertions in an intron, qPCR primers bind upstream of the insertion. For *WRKY38*, the transposon inserted in an intron and likely resulted in a partial loss of function. Due to low expression levels, the effect of Salk__039436 (in *WRKY59*) and Salk__055084 (in *WRKY66*) could not be determined accurately.

Found at doi:10.1371/journal.ppat.0020123.st002 (46 KB PDF).

Table S3. Genes Affected by BTH Treatment in WT

BTH-responsive genes were identified first by ANOVA ($p < 0.05$) and then by fold change (≥2-fold). Genes were ranked according to their BTH-dependency *p*-values. The vast majority of them are also NPR1 dependent ($p < 0.05$, last column). F.C., fold change.

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Table S4. BTH-Dependent Genes Affected by *wrky18*

Genes were divided into four categories according to the effect of *wrky18:* genes whose induction is diminished (204), genes whose repression is diminished (152), genes whose induction is stronger (68), and genes whose repression is stronger (27) in *wrky18*. Shown here are fold changes at 8 and 24 h after BTH treatment in WT and *wrky18*, as well as WRKY18-dependency *p*-values.

Found at doi:10.1371/journal.ppat.0020123.st004 (808 KB DOC).

Table S5. Gene Ontology Terms of Genes Affected by *wrky18*

WRKY18-dependent genes were searched for enriched functional categories using DAVID. Only groups with $p < 0.001$ were shown.

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Author contributions. DW and XD conceived and designed the experiments. DW and NA performed the experiments. DW and XD analyzed the data and wrote the paper.

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