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Research Article

Transcriptome analysis reveals role of transcription factor WRKY70 in early *N*-hydroxy-pipecolic acid signaling

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Abstract

N-Hydroxy-pipecolic acid (NHP) is a mobile metabolite essential for inducing and amplifying systemic acquired resistance (SAR) following a pathogen attack. Early phases of NHP signaling leading to immunity have remained elusive. Here, we report the early transcriptional changes mediated by NHP and the role salicylic acid (SA) plays during this response in *Arabidopsis* (*Arabidopsis thaliana*). We show that distinct waves of expression within minutes to hours of NHP treatment include increased expression of WRKY transcription factor genes as the primary transcriptional response, followed by the induction of WRKY-regulated defense genes as the secondary response. Most genes induced by NHP within minutes were SA dependent, whereas those induced within hours were SA independent. These data suggest that NHP induces the primary transcriptional response under basal levels of SA and that new SA biosynthesis via ISOCHORISMATE SYNTHASE 1/SA-INDUCTION DEFICIENT 2 is dispensable for inducing the secondary transcriptional response. We demonstrate that WRKY70 is required for the induced expression of a set of genes defining some of the secondary transcriptional response, SAR protection, and NHP-dependent enhancement of reactive oxygen species production in response to flagellin treatment. Our study highlights the key genes and pathways defining early NHP responses and the role of WRKY70 in regulating NHP-dependent transcription.

Introduction

Systemic acquired resistance (SAR) is a whole-plant defense response triggered by an initial pathogen attack. During the establishment of SAR, mobile signals are sent from infected tissue to uninfected tissue, initiating the priming of immune responses and enhancing immunity against secondary pathogen infections (Fu and Dong 2013; Tian and Zhang 2019; Kachroo and Kachroo 2020). To achieve a primed state, plants execute substantial transcriptional reprogramming of several hundred to a few thousand genes, resulting in the increased accumulation of defense hormone salicylic acid (SA), sensing and signaling components for pathogen detection, and production of antimicrobial compounds (Jaskiewicz et al. 2011; Conrath et al. 2015; Bernsdorff et al. 2016). In addition, several small molecules produced by plants during infection help to orchestrate this defense priming, including SA and the bioactive metabolite *N*-hydroxy-pipecolic acid (NHP) (Chen et al. 2018; Hartmann et al. 2018; Klessig et al. 2018).

NHP is a mobile metabolite that can move from one leaf to another to mediate long-distance cellular communication (Chen et al. 2018; Mohnike et al. 2021; Yildiz et al. 2021). In *Arabidopsis* (*Arabidopsis thaliana*), the enzyme FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1) catalyzes the *N*-hydroxylation of lysine-derived pipecolic acid (Pip) in response to pathogen infection to produce NHP (Chen et al. 2018; Hartmann et al. 2018). Notably, *fmo1* null mutants are impaired in local disease resistance during pathogen infection and fail to establish SAR (Bartsch et al. 2006; Mishina and Zeier 2006). Treatment of *fmo1* mutant plants with exogenous NHP is sufficient to induce systemic resistance against bacterial and oomycete pathogens (Chen et al. 2018; Hartmann et al. 2018). Furthermore, NHP treatment of a single leaf is sufficient to initiate and amplify defense signaling throughout the plant, resulting in the transcriptional activation of a large subset of SAR-associated genes within 24 h of treatment (Yildiz et al. 2021, 2023), including genes encoding the SA biosynthetic enzyme ISOCHORISMATE SYNTHASE 1/SA-INDUCTION DEFICIENT 2 (ICS1/SID2), the Pip biosynthetic enzymes AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1) and SAR-DEFICIENT 4 (SARD4), as well as its own biosynthetic enzyme FMO1 (Chen et al. 2018). Collectively, these findings highlight the importance of NHP in initiating immune signaling to establish local and systemic plant defenses.

Induction of *ICS1/SID2* transcription by NHP signaling results in the accumulation of SA in both local and systemic tissues (Chen et al. 2018; Hartmann et al. 2018). Elevation of SA in turn amplifies SAR-associated transcriptional changes (Bernsdorff et al. 2016). Signaling downstream of SA accumulation is largely driven by the action of the SA receptor and transcriptional coactivator NONEXPRESSER OF PR GENES 1 (NPR1), which interacts with a number of TGA transcription factors (TFs) from the basic leucine zipper (bZIP) family to alter gene expression (Zhang et al. 1999; Wang et al. 2006; Wu et al. 2012). Members of the biotic and abiotic

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stress response WRKY TF family are among the known targets of the NPR1/TGA interaction (Thibaud-Nissen et al. 2006; Wang et al. 2006). WRKY TFs have been identified as key regulators of NPR1-dependent and NPR1-independent SAR responses. Additionally, some WRKY members play important roles in feedback regulation of transcription (Pandey and Somssich 2009; Fu and Dong 2013). For example, WRKY54 and WRKY70 modulate SA biosynthesis through repression of *ICS1/SID2* expression (Wang et al. 2006).

The interplay between SA and NHP appears to be interdependent and synergistic with both metabolites being able to upregulate one another's biosynthetic pathways (Chen et al. 2018; Hartmann et al. 2018; Sun et al. 2020). Notably, elevated endogenous levels of Pip, the precursor of NHP, primes the expression of both NHP and SA biosynthetic genes under low concentrations of SA by stabilizing NPR1 levels (Kim et al. 2020). This points to an intriguing dynamic relationship between SA and NHP over the course of SAR activation. Evidence suggests NHP accumulates to detectable levels in systemic tissues roughly 24 h before SA accumulation is detected (Hartmann and Zeier 2019). In such a case, NHP would need to drive systemic signaling for a substantial period of time before SA could further amplify SAR responses. It is currently unclear how the levels of SA in leaves impact early NHP signaling. Furthermore, little is known about the very early phase of NHP signaling in the tissues where it is first produced or in the distal tissues once NHP arrives there. Key players of NHP signaling and the progression of NHP-dependent transcriptional reprogramming require further investigation.

Presently, the majority of genome-wide transcriptomic studies of SAR have been carried out on the timescale of 1 to 2 d postinduction; this includes pharmacological studies using Pip and NHP treatment (Bernsdorff et al. 2016; Hartmann et al. 2018; Yildiz et al. 2021, 2023). These data have revealed that NHP ultimately activates SAR in untreated tissue but do not shed light on early signaling events that lead to the observed SAR phenotype. The immediate consequences of NHP accumulation on gene expression have not been described. Studying very early transcriptional changes, within minutes to hours of elicitor treatment, has been an effective strategy for elucidating key components of signaling pathways for SA, jasmonic acid (JA), and brassinosteroid (Friedrichsen et al. 2002; Chini et al. 2007; Ding et al. 2018). Thus, in an effort to better understand NHP's primary action in signal transduction, we determined the genome-wide transcriptional responses in *Arabidopsis* from 15 min up to 6 h after treatment with exogenous NHP.

Here, we report the early NHP transcriptional profile and the role SA plays during this response. We present evidence indicating that increased expression of WRKY TFs defines the primary transcriptional responses mediated by NHP within minutes (i.e. 15 to 30 min) posttreatment, and WRKY TFs act as executors of the secondary transcriptional responses within hours (i.e. 3 to 6 h). The majority of genes induced by NHP within minutes were SA dependent, whereas those induced within hours were SA independent. These data suggest that NHP induces the primary transcriptional response at basal levels of SA and new SA biosynthesis via ICS1/SID2 is dispensable for induction of the secondary transcriptional response. We also show that WRKY70 is required for NHP-induced transcription of a set of genes defining secondary transcriptional changes as well as NHP enhancement of reactive oxygen species (ROS) and regulation of SAR. Taken together, our study highlights the key genes and pathways defining early NHP responses and a role for WRKY70 in the regulation of NHP-dependent transcription.

Results

NHP induces expression of defense-associated genes within minutes

Previous studies in *Arabidopsis* have demonstrated that exogenous application of NHP to a leaf (defined as "local tissue") is sufficient to initiate and amplify defense signaling in untreated leaves (defined as "distal tissue"), including the transcriptional activation of SAR marker genes (Chen et al. 2018; Hartmann et al. 2018; Nair et al. 2021; Yildiz et al. 2021). Notably, these studies focused on gene expression changes in distal tissues at late time points (24 to 48 h) following NHP elicitation. However, it was unknown which gene expression changes occur immediately in response to NHP treatment. We hypothesized that once present in the tissue, either via endogenous synthesis or exogenous application, NHP initiates signaling that leads to transcriptional changes on a timescale of minutes to several hours, which are essential for the execution of NHP-dependent immune responses.

In order to test this hypothesis, we set out to identify NHP-responsive transcriptional markers and determine the earliest time frame when NHP could induce these genes. We first questioned if NHP could induce the expression of classical SAR-associated marker genes (i.e. *FMO1*, *ICS1*/SID2, and *PATHOGENESIS-RELATED PROTEIN 1* [*PR1*]) within several hours of treatment. Studies have shown *FMO1*, *ICS1/SID2*, and *PR1* are induced between 8 and 48 h following NHP treatment (Chen et al. 2018; Nair et al. 2021). Therefore, we began by testing the response to NHP within 24 h of treatment. The leaves of 4.5-wk-old *Arabidopsis* Col-0 (wild type) plants were infiltrated with 1 mM NHP, and then treated leaves were collected for mRNA isolation at 2, 6, and 24 h. Reverse transcription quantitative PCR (RT-qPCR) analysis showed significantly increased transcript abundance of all 3 genes in NHP-treated leaves at 24 h but not at 2 and 6 h compared to water (mock)-treated leaves (Fig. 1A). These data indicate that *FMO1*, *ICS1/SID2*, and *PR1* are responsive to NHP treatment but are likely not primary targets of NHP transcriptional activation.

Next, we tested the ability of NHP to induce expression of *WRKY38* and UDP-GLYCOSYLTRANFERASE 76 B1 (*UGT76B1*), 2 genes known to respond to SA within 2 h of treatment (Blanco et al. 2009). *WRKY38* encodes a defense-associated TF, and *UGT76B1* encodes a UDP-dependent glycosyltransferase shown to glycosylate SA and NHP (Eulgem et al. 2000; Bauer et al. 2021; Cai et al. 2021; Holmes et al. 2021; Mohnike et al. 2021). We found a significant increase of mRNA abundance for both *WRKY38* and *UGT76B1* in NHP-treated leaves at 2 h compared to mock-treated leaves (Fig. 1A). *WRKY38* transcript levels were also elevated at 6 and 24 h after treatment with NHP (Fig. 1A). These data indicate that both *WRKY38* and *UGT76B1* are early NHP-responsive transcripts.

We used the *WRKY38* gene as a marker to define the earliest time when NHP-induced transcriptional changes could be measured. To this end, we implemented a hydroponically grown seedling assay to synchronize chemical treatment and increase the number of plants sampled, while minimizing mechanical stress. Wild-type seedlings were grown in liquid medium in 6-well plates with 15 to 18 seeds per well. The seedlings were treated with mock, 1 mM Pip, or 1 mM NHP at 10-day postgermination and then collected at 15 min, 30 min, 3 h, and 6 h posttreatment to isolate mRNA (Fig. 1B). *WRKY38* transcript abundance was significantly elevated in *Arabidopsis* seedlings treated with NHP as early as 15 min, peaking in abundance at 30 min, when compared to those treated with mock or Pip (Fig. 1C). This trend continued

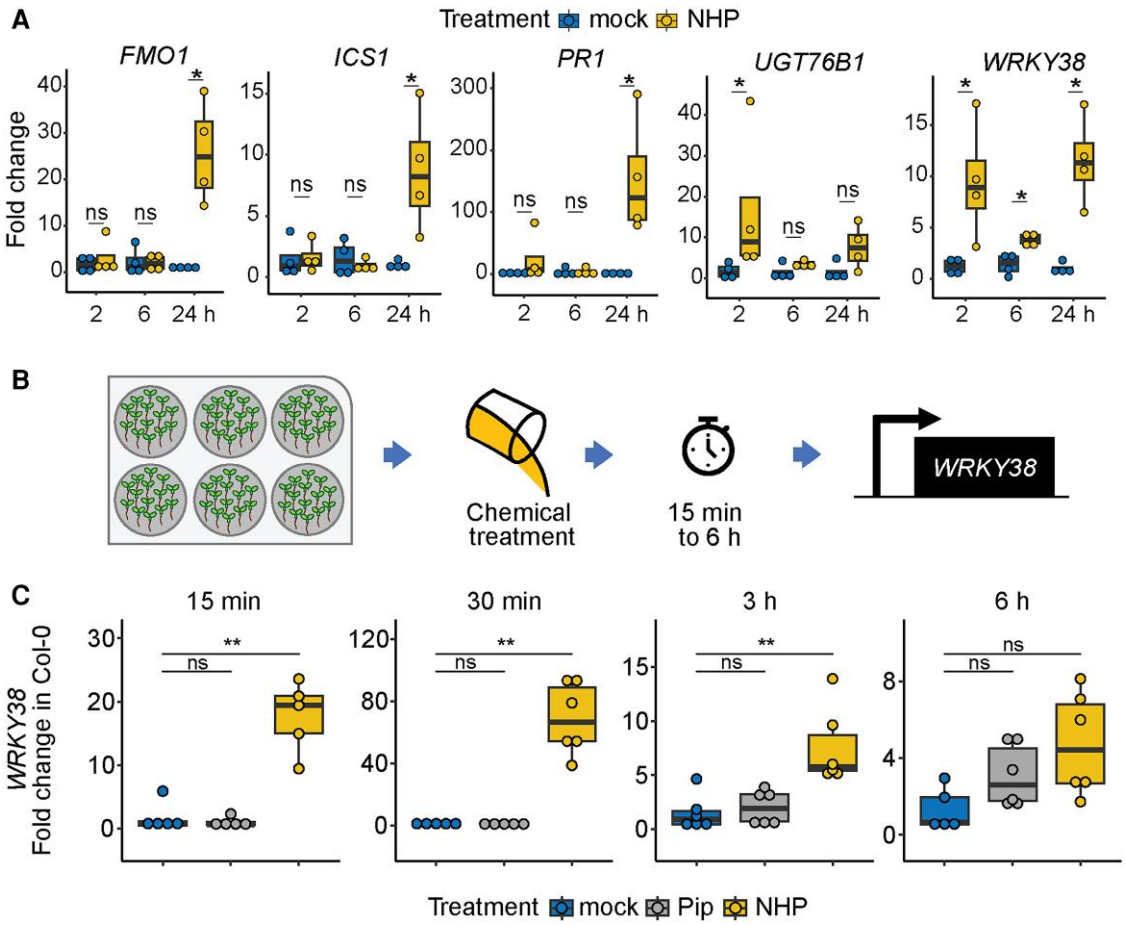

Figure 1. NHP induces *WRKY38* expression at early time points. **A)** Transcript abundance of SAR marker genes *FMO1*, *ICS1*/*SID2*, and *PR1* and early SA-responsive marker genes *WRKY38* and *UGT76B1* in 4.5-wk-old wild-type (Col-0) *Arabidopsis* plants. Three leaves were infiltrated with sterile water (mock) or 1 mM NHP and collected after 2, 6, and 24 h for mRNA isolation. Transcript abundance was measured via RT-qPCR and normalized relative to *UBC21* (AT5G25760). FC was determined relative to mock ($2^{-\Delta\Delta Ct}$). Asterisks indicate a significant difference between mock and NHP per time point ($n = 4$; Mann–Whitney *U* test; $^{*}P < 0.05$, ns = not significant). **B)** Design of seedling-based assay. *Arabidopsis* seedlings (15 to 18/well) were hydroponically grown, treated with MS medium (mock), 1 mM Pip, or 1 mM NHP, and then collected at 15 min, 30 min, 3 h, and 6 h for RT-qPCR analysis of *WRKY38* transcript abundance in wild type. **C)** Expression of *WRKY38* in seedlings from the experiment described in **B)**. Transcript abundance was normalized relative to *UBC21* and mock-treated samples for each condition ($2^{-\Delta\Delta Ct}$). Box and whisker dot plots represent the median (center line), first and third quartiles (box edges), and the minimum and maximum values (whiskers). Asterisks indicate a significant difference between mock and Pip or NHP at each time point ($n = 4$ to 6; Mann–Whitney *U* test; $^{**}P < 0.01$, ns, not significant).

through 3h following NHP treatment. The NHP precursor Pip did not elicit an increase in *WRKY38* mRNA within 3 h but showed a slight trend of increased abundance at the 6 h time point (Fig. 1C). These results indicate that exogenous NHP, but not Pip, is sufficient to elevate *WRKY38* transcript abundance within 15 min of application.

Since *WRKY38* expression is known to be induced by SA treatment (Blanco et al. 2009), we questioned if NHP-induced expression of *WRKY38* is dependent on SA derived from the ICS1/SID2 biosynthetic pathway by utilizing the well-characterized *sid2-2* mutant that does not accumulate SA upon pathogen infection (Wildermuth et al. 2001). Wild-type and *sid2-2* seedlings were grown hydroponically, treated with mock, 1 mM Pip, or 1 mM NHP, and then collected 3 h after treatment. NHP treatment increased *WRKY38* mRNA abundance in the *sid2-2* mutant relative to mock treatment, similar to wild-type seedlings (Supplementary Fig. S1). These findings indicate that NHP is sufficient to alter *WRKY38* transcript abundance in the absence of SA accumulation, defining *WRKY38* as an early NHP-induced, SA-independent transcript.

The early NHP-responsive transcriptome reveals distinct waves of expression

We next questioned how the presence of NHP impacts whole-genome transcriptional changes within minutes to hours of treatment in a SA-dependent and SA-independent manner. To address this, *Arabidopsis* wild-type and *sid2-2* mutant seedlings were grown in the same hydroponic design as previously described for RT-qPCR experiments (Fig. 1B). The *sid2-2* mutant was analyzed in order to identify transcriptional changes dependent on NHP-induced SA biosynthesis (Chen et al. 2018). Each pool of seedlings was treated with mock or 0.5 mM NHP for 15 min, 30 min, 3 h, and 6 h. A reduced concentration of NHP was used as we found that 0.5 mM NHP induced similar *WRKY38* mRNA accumulation as 1 mM NHP (Supplementary Fig. S2). The abundance of mRNA in the treated seedlings was assessed for each condition via RNA sequencing (RNA-seq). After general quality control and mapping to the *Arabidopsis* TAIR10 genome, genes differentially expressed by NHP were determined relative to mock treatment for each time point and genotype using DESeq2 (Love et al. 2014). Genes

were considered differentially expressed if they returned an adjusted P-value below 0.05 ($P_{adj} < 0.05$).

In wild-type seedlings, a total of 2,079 genes were differentially expressed in response to NHP for at least one of the measured time points. To explore the set of early NHP-responsive genes, we first selected for genes showing a robust fold change (FC) over mock by applying the cutoff of $\log_2(FC) > 1$ for upregulated genes and $\log_2(FC) < -1$ for downregulated genes. This identified 352 genes that were differentially expressed in response to NHP for at least 1 time point, with 163 genes up and 189 genes down (Supplementary Table S1). Next, the upregulated genes were hierarchically clustered based on their $\log_2$(FC) values over the indicated time points, and 4 well-defined gene clusters were identified (Fig. 2A). For each group of genes in the defined clusters, the $\log_2$(FC) values were then averaged by time point, revealing dynamic patterns of expression. Two of the clusters were upregulated within minutes (designated as "early transient" and "early strong") and 2 were upregulated by several hours (designated as "late weak" and "late strong") (Fig. 2B; Tables 1 and 2; Supplementary Table S2).

The "early transient" cluster includes 42 genes and is defined by elevated transcript abundance 15 to 30 min after NHP treatment that did not persist by 3 h. Gene ontology (GO) analysis for this cluster showed overrepresentation for biological processes associated with responses to JA, wounding, and oxidative stress. Some of these genes are associated with the regulation of defense as well as defense responses to fungi and bacteria (Fig. 2C). Among the most highly expressed genes in this cluster are JA-responsive genes, which play key roles in the regulation of wounding responses, including *CML39*, which encodes a calmodulin-like protein, *JAZ10*, which encodes a jasmonate-zim-domain protein, and *MYC2*, which encodes a jasmonate-inducible TF (Tables 1 and 2). Taken together, the early yet unsustained induction of these genes within 30 min suggests a burst of JA- and wound-responsive gene expression that acts as a primary wave of NHP signaling.

The "early strong" cluster includes 10 genes and is defined by a high average $\log_2$(FC) 15 min after NHP treatment that was maintained for all subsequent time points. Of the 10 genes, 2 (AT1G06475 and AT5G40980) were strongly upregulated by NHP at 30 min and no other time points. These 2 genes were removed from the average $\log_2$(FC) analysis and subsequent analyses because they did not behave like the other genes in this cluster (Fig. 2B). The early strong cluster is notably overrepresented in biological processes involved in the regulation of transcription, with over half the genes belonging to the WRKY TF family, including 4 Group III *WRKY* genes (*WRKY38*, *WRKY54*, *WRKY62*, and *WRKY70*) and 2 Group II-c genes (*WRKY51* and *WRKY59*) (Eulgem et al. 2000; Kalde et al. 2003) (Tables 1 and 2; Supplementary Table S2). Additional GO terms overrepresented in the early strong cluster include regulation of defense and regulation of hormonal signaling pathways such as SA, JA, brassinosteroid, and ethylene (Fig. 2C). The early and sustained induction of these genes, particularly the *WRKY*s, suggests they are primary transcriptional target genes of NHP signaling.

The "late weak" cluster includes the largest number of genes (96) averaging a $\log_2$(FC) around 1 (Fig. 2B). GO term analysis of the cluster revealed significant overrepresentation of biological processes involved in response to bacteria and oomycetes, as well as SA-mediated signaling and regulation of SAR (Fig. 2C). This indicates NHP activation of SAR and pathogen defense genes can be detected as early as 3 to 6 h in treated tissues. Notably, genes involved in the regulation and biosynthesis of NHP were observed in this wave of transcription, including *SAR-DEFICIENT 1* (*SARD1*), *NIM-INTERACTING 1* (*NIMIN-1*), *ALD1*, and *UGT76B1* (Tables 1 and 2; Supplementary Table S2) (Song et al. 2004; Zhang, Xu, et al. 2010; Holmes et al. 2021).

The "late strong" cluster consists of 15 genes with $\log_2$(FC) values averaging between 3.5 and 4 (Fig. 2B). Response to SA was the only significantly overrepresented GO term for this cluster, which included genes such as *BDA1* (*bian da; "becoming big" in Chinese*), *PATHOGEN AND CIRCADIAN CONTROLLED 1* (*PCC1*), and *DMR6-LIKE OXYGENASE 1* (*DLO1*) (Fig. 2C; Tables 1 and 2). Despite having a role in the response to SA, the majority of genes classified as late strong were upregulated by NHP in both wild type and *sid2-2* (Fig. 3A; Table 1), indicating that accumulation of these transcripts was at least partially SA independent. Of the 14 SA-independent genes identified in this cluster, only 5 have been found to be upregulated 24 h after NHP treatment in a SA-independent manner (Yildiz et al. 2021). Thus, our analysis has uncovered additional NHP-responsive genes that operate independently of SA as early as 6 h post-NHP signaling.

We also performed a similar clustering analysis on the 189 NHP-downregulated transcripts and identified 3 gene clusters (Supplementary Fig. S3, A and B). In contrast to the upregulated gene clusters, the downregulated gene clusters had less distinct patterns of expression and averaged $\log_2$(FC) values around −1 to −1.5. Two clusters were downregulated within 15 to 30 min and persisted 3 to 6 h after NHP treatment. One of these showed weaker downregulation and was classified as an "early weak" cluster. The other showed stronger downregulation and was classified as an "early strong" cluster. The third did not show decreased transcript abundance until 3 to 6 h after treatment and thus was labeled as a "late" cluster (Supplementary Fig. S3B). All 3 clusters were overrepresented for biological processes involving growth and development such as root morphogenesis, plant epidermis development, plant-type cell wall organization, and root hair cell differentiation (Supplementary Fig. S3C). Given the NHP precursor Pip is known to inhibit root growth of seedlings (Wang et al. 2018), it is likely that NHP also inhibits root growth. These clusters shed light on the genes that may be associated with altered plant growth and development in response to elevated levels of NHP.

SA biosynthesis is dispensable for the early induction of a majority of NHP-upregulated genes

Interrogation into the late strong cluster suggested NHP may be sufficient to activate a subset of SA-responsive genes in the absence of NHP-induced SA biosynthesis (Figs. 2C and 3A, "late strong"). This was an intriguing possibility given NHP likely accumulates in distal tissues prior to SA accumulation following a local tissue infection (Bernsdorff et al. 2016; Hartmann et al. 2018; Hartmann and Zeier 2019). We therefore hypothesized that NHP would need to drive transcriptional reprogramming as SA levels rise from low to high. To investigate this, we assessed the impact of SA biosynthesis on NHP-induced gene expression from 15 min to 6 h.

For each time point of the wild-type gene clusters described in Fig. 2, the percentage of genes upregulated in wild type and the *sid2-2* mutant was determined. Genes upregulated in both wild type and *sid2-2* were defined as "SA independent," and genes upregulated only in wild type but not in *sid2-2* were conversely defined as "SA dependent." Notably, the majority of genes upregulated by NHP at 15 min in the early transient cluster were SA dependent. Specifically, 87% of the genes (27 of 31) were upregulated at 15 min in wild type but not in *sid2-2* seedlings, and similar trends

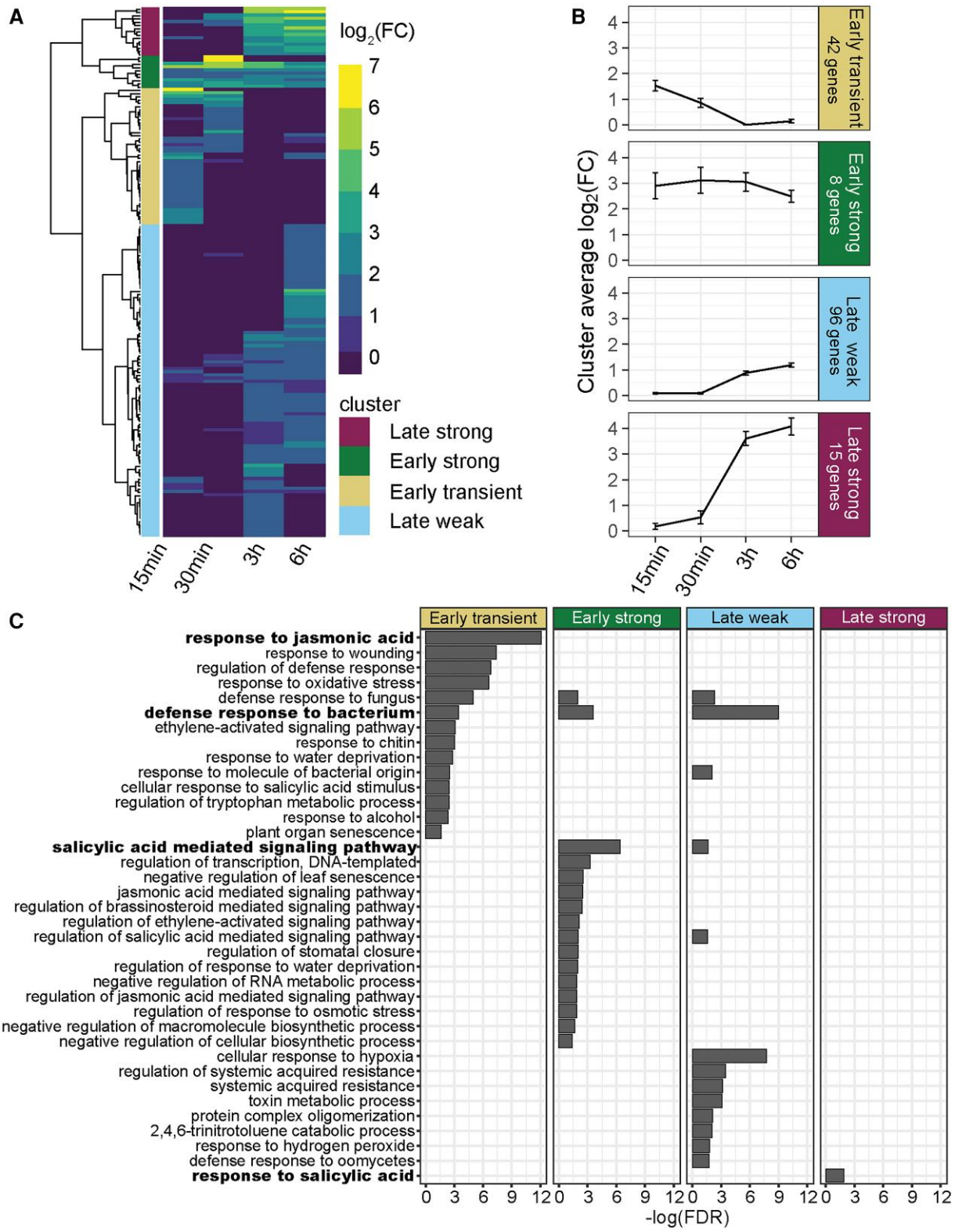

Figure 2. Profile of early NHP-upregulated genes in wild-type seedlings. **A)** Heatmap of genes upregulated ($log_2(FC) > 1$; $P_{adj} < 0.05$) in response to NHP treatment in wild-type seedlings. One biological replicate consists of 15 pooled seedlings per condition ($n = 3$). Euclidean distances determined from the $log_2(FC)$ values were hierarchically clustered across the indicated time points (15 min, 30 min, 3 h and 6 h), resulting in 4 distinct clusters of gene expression. **B)** Average $log_2(FC)$ of all genes within each cluster defined in **A)** showing the expression trends across the 4 time points. Note: the 2 genes expressed only at 30 min, and no other time, were removed from the "early strong" cluster average. Total number of genes in each cluster is indicated, and error bars represent the SEM. **C)** Biological processes of significantly overrepresented (FDR < 0.05) GO terms for the genes within each cluster are shown. Bars represent the –log(FDR) of each significant GO term. Bars were omitted if the GO term was not significant for the defined cluster. The most significantly overrepresented GO term for each cluster is indicated in bold.

Table 1. SA-independent/partially independent NHP-upregulated genes by cluster

			Log_2(FC)							
			Col-0				*sid2-2*			
AGI	Name	Annotation	15 m	30 m	3 h	6 h	15 m	30 m	3 h	6 h
Early transient										
AT3G44870	FAMT-L	FARNESOIC ACID METHYL TRANSFERASE-LIKE	4.3	4.4	…	…	2.3	2.7	…	…
AT2G44840	ERF13	Ethylene-responsive transcription factor	3.3	2.1	…	…	1.4	-	…	…
AT1G76640	CML39	CALMODULIN LIKE 39	2.7	2.7	…	…	1.7	…	…	…
AT5G13220	JAZ10	JASMONATE-ZIM-DOMAIN PROTEIN 10	1.6	1.1	…	0.6	1.2	1.0	…	…
AT1G80840	WRKY40	WRKY transcription factor; Group II-a	1.8	1.1	…	0.5	…	1.4	1.4	0.7
AT3G17690	CNGC19	Cyclic nucleotide-gated ion channel	1.6	1.6	…	…	…	1.2	…	…
AT4G21840	MSRB8	Methionine sulfoxide reductase B8	3.1	…	…	1.5		2.2	…	…
Early strong										
AT5G22570	WRKY38	WRKY transcription factor; Group III	5.8	5.2	4.7	3.6	3.9	6.2	5.8	2.9
AT5G01900	WRKY62	WRKY transcription factor; Group III	3.9	5.0	4.4	2.6	…	5.8	4.1	4.4
AT1G28480	GRXC9	GRX480/ROXY19, glutaredoxin family	3.2	2.2	2.7	1.8	3.2	3.1	2.3	2.1
AT2G40750	WRKY54	WRKY transcription factor; Group III	2.7	3.7	2.8	2.9	3.0	3.6	3.5	3.0
AT5G64810	WRKY51	WRKY transcription factor; Group II-c	2.6	3.4	3.3	3.0	1.7	3.2	3.2	3.5
AT3G56400	WRKY70	WRKY transcription factor; Group III	1.6	1.6	2.4	2.1	…	1.3	2.3	1.8
AT3G25882	NIMIN-2	NIM1-INTERACTING 2	1.4	1.5	1.8	1.6	1.2	1.1	1.0	1.6
Late weak										
AT3G60470	…	Transmembrane protein, putative (DUF247)	…	…	…	3.3	…	…	…	5.1
AT2G13810	ALD1	AGD2-LIKE DEFENSE RESPONSE PROTEIN 1	…	…	…	2.3	…	…	…	6.6
AT1G01680	PUB54	Plant U-box type E3 ubiquitin ligase	…	…	…	2.2	…	…	…	1.4
AT3G12220	SCPL16	Serine carboxypeptidase-like	…	…	1.1	2.0	…	…	…	2.0
AT1G78340	GSTU22	Glutathione S-transferase U22	…	…	2.0	2.0	…	…	1.6	1.7
AT5G03350	LLP	Lectin-like protein	…	…	2.1	1.8	…	…	2.1	1.9
AT5G24530	DMR6	DOWNY MILDEW RESISTANCE 6	0.7	1.2	1.6	1.7	0.5	1.6	2.0	1.7
AT2G41090	CML10	Calmodulin-like protein	…	…	1.1	1.7	…	…	0.3	1.2
AT4G14400	ACD6	ACCELERATED CELL DEATH 6	…	…	2.1	1.6	…	…	1.4	1.4
AT2G15490	UGT73B4	UDP-DEPENDENT GLYCOSYLTRANSFERASE 73B4	…	0.7	1.2	1.5	…	0.9	2.7	1.5
AT4G08040	ACS11	1-Aminocyclopropane-1-carboxylate synthase 11	…	1.0	1.5	1.5	…	…	1.0	1.2
AT1G21250	WAK1	CELL WALL-ASSOCIATED KINASE 1	0.7	…	1.8	1.5	…	…	1.7	2.0
AT1G17170	GSTU24	Glutathione S-transferase U24	…	…	…	1.4	…	…	1.6	1.5
AT3G11340	UGT76B1	UDP-DEPENDENT GLYCOSYLTRANSFERASE 76B1	…	1.0	0.9	1.1	…	0.7	1.1	1.0
AT1G73805	SARD1	SAR-DEFICIENT 1	1.0	0.7	1.5	1.0	…	…	1.3	1.3
Late strong										
AT3G12230	SCPL14	Serine carboxypeptidase-like	…	…	5.9	6.9	…	…	5.4	4.6
AT1G15610	…	Transmembrane protein	…	…	5.5	5.9	…	…	…	5.1
AT3G28510	…	AAA-type ATPase family protein	…	…	3.2	5.6	…	…	…	4.9
AT2G26400	ARD	ACIREDUCTONE DIOXYGENASE 3	…	2.2	4.4	5.0	…	…	5.1	5.8
AT4G19750	…	Glycosyl hydrolase family with chitinase insertion domain	…	…	3.9	4.8	…	…	6.7	5.3
AT1G19960	…	Putative uncharacterized protein	1.2	1.6	4.0	4.0	…	2.9	5.5	4.4
AT4G10500	DLO1	DMR6-LIKE OXYGENASE 1	…	1.4	3.1	3.8	…	…	3.4	4.2
AT5G41280	CRRSP57	Receptor-like protein kinase-related family protein	…	…	2.6	3.2	…	…	3.2	2.2
AT2G14560	LURP1	Late upregulated in response to *Hyaloperonospora parasitica*	…	…	3.0	3.1	…	…	…	4.5
AT1G02230	NAC004	NAC DOMAIN CONTAINING PROTEIN 4	…	2.8	3.8	3.0	…	2.5	3.5	4.3
AT5G54610	BDA1	Ankyrin-repeat protein family	…	…	2.2	3.0	…	…	3.0	6.4

Selection of genes upregulated by NHP in Col-0 and *sid2-2* seedlings. Clusters defined for wild-type expression are noted (i.e. early transient, early strong, late weak, and late strong). Indicated log_2(FC) values determined relative to mock treatment for each genotype and time point. Genes are categorized as SA independent/partially independent if significant upregulation was observed in *sid2-2* within 15 to 30 min for the early clusters and within 3 to 6 h for the late clusters. Not all NHP-upregulated genes are shown in this table, and the complete list can be found in Supplementary Table S1.

were seen at 30 min (Fig. 3A). By contrast, 100% of the genes (8 of 8) upregulated in the early strong cluster 3 to 6 h after NHP treatment were SA independent (Fig. 3A). Likewise, in the late weak cluster, 69% of the genes (36 of 52) were SA independent at 3 h, dropping to 53% (37 of 70) at 6 h (Fig. 3A). In the late strong cluster, 73% of the genes (11 of 14) at 3 h and 93% (14 of 15) at 6 h were SA independent (Fig. 3A).

Without clustering, we observed similar gene expression patterns as the majority of genes induced by NHP between 15 and 30 min were SA dependent (i.e. 36 of 45 at 15 min and 23 of 36 at 30 min), and the majority of genes induced by NHP between 3 and 6 h were SA independent (i.e. 55 of 75 at 3 h and 60 of 96 at 6 h) (Fig. 3B). Our data suggest that either new SA biosynthesis or an unknown basal level of SA is required for the induction of a subset of NHP-responsive genes within minutes of exposure to NHP. Furthermore, SA biosynthesis via ICS1/SID2 is dispensable for a large portion of NHP-induced expression several hours later. Taken together, our analysis highlights an important temporal role of SA on NHP-induced transcription.

Unlike the early transient subset of upregulated genes, which was largely SA dependent, we found the early strong cluster was largely SA independent (Fig. 3A). Given the importance of NPR1 in relaying SA-mediated responses (Wang et al. 2006), we asked if the expression of select genes in the early strong cluster was dependent on NPR1. We measured transcript abundance of *WRKY38*, *WRKY54*, and *WRKY70* in hydroponically grown wild-type, *sid2-2*, and *npr1-3* seedlings 30 min and 3 h after NHP treatment. NHP significantly increased transcript abundance of *WRKY38* and *WRKY70* after 30 min and increased *WRKY38*, *WRKY54*, and *WRKY70* after 3 h in *npr1-3* seedlings. Additionally, NHP increased transcript abundance of all 3 genes in *sid2-2* seedlings (Supplementary Fig. S4, A to C). Together, these data indicate

Table 2. SA-dependent NHP-upregulated genes by cluster

			Log_2(FC)							
			Col-0				*sid2-2*			
AGI	Name	Annotation	15 m	30 m	3 h	6 h	15 m	30 m	3 h	6 h
Early transient										
AT1G32970	SBT3.2	Subtilisin-like protease	2.8	3.2	…	…	…	…	…	…
AT1G21240	WAK3	Wall-associated receptor kinase	2.5	…	…	1.4	…	…	…	2.0
AT5G44430	PDF1.2C	PLANT DEFENSIN 1.2C	2.4	…	…	…	…	…	…	…
AT2G14610	PR1	PATHOGENESIS-RELATED GENE 1	2.3	…	…	…	…	…	…	…
AT4G34410	ERF109	Ethylene-responsive transcription factor	2.1	…	…	…	…	…	…	…
AT2G32140	…	Transmembrane receptor	2.1	…	…	…	…	…	2.0	…
AT5G44420	PDF1.2A	PLANT DEFENSIN 1.2	2.0	…	…	…	…	…	…	…
AT3G14260	…	LURP-one-like protein (DUF567)	1.8	…	…	…	…	…	…	…
AT2G26020	PDF1.2B	PLANT DEFENSIN 1.2B	1.6	…	…	…	…	…	…	…
AT5G64905	PROPEP3	ELICITOR PEPTIDE 3 PRECURSOR	1.5	…	…	…	…	…	…	…
AT1G32640	MYC2	JASMONATE INSENSITIVE 1	1.4	…	…	…	…	…	…	…
AT2G18660	EGC2	PLANT NATRIURETIC PEPTIDE A	1.4	…	…	…	…	…	…	…
Early strong										
AT2G21900	WRKY59	WRKY transcription factor; Group II-c	1.9	2.4	2.3	2.3	…	…	2.2	2.7
Late weak										
AT2G28850	CYP710A3	Cytochrome P450 710A3	…	…	2.4	2.1	…	…	…	…
AT1G15630	…	Transmembrane protein	…	…	1.9	1.4	…	…	…	…
AT5G44568	PROSCOOP4	SERINE-RICH ENDOGENOUS PEPTIDE	…	…	1.3	1.3	…	…	0.7	1.0
AT1G18830	SEC31A	Protein transport protein SEC31 homolog A	…	…	…	2.8	…	…	…	…
AT2G45550	CYP76C4	Cytochrome P450 76C4	…	…	…	2.6	…	…	…	…
AT1G10155	ATPP2-A10	PHLOEM PROTEIN 2-A10	…	…	…	2.3	…	…	…	…
AT1G02450	NIMIN-1	NIM1-INTERACTING 1	…	…	…	2.3	…	…	…	…
AT3G45860	CRK4	Cysteine-rich receptor-like protein kinase	…	…	…	2.1	…	…	…	…
AT5G51500	PME60	Pectin methylesterase inhibitor	…	…	…	1.7	…	…	…	…
AT2G29350	SAG13	SENESCENCE-ASSOCIATED GENE 13	…	…	…	1.3	…	…	…	…
Late strong										
AT3G22231	PCC1	PATHOGEN AND CIRCADIAN CONTROLLED 1	…	…	3.8	3.9	…	…	…	…

Selection of genes upregulated by NHP in Col-0 and *sid2-2* seedlings. Clusters defined for wild-type (Col-0) expression are noted. Indicated log_2(FC) values were determined relative to mock treatment for each genotype and time point. Genes are categorized as SA dependent if significant upregulation was not observed in *sid2-2* within 15 to 30 min for the early clusters and within 3 to 6 h for the late clusters. Not all NHP-upregulated genes are shown in this table, and the complete list can be found in Supplementary Table S1.

that some WRKY TFs play a role in a SA- and NPR1-independent branch of early NHP signaling.

Analysis of the downregulated genes found a slight trend of SA dependence following NHP treatment. For example, the early weak and late clusters defined in Supplementary Fig. S3 showed that a majority of genes are downregulated in wild-type but not the *sid2-2* mutant at each time point (Supplementary Fig. S5A). By contrast, the genes of the early strong cluster were less dependent on SA biosynthesis, such that by the 6-h time point only 15% of genes (8 of 44) were downregulated in wild-type but not in *sid2-2* seedlings (Supplementary Fig. S5A). Independent of clustering, >60% of the downregulated genes were found to be SA dependent at 15 min and 3 h, and ∼40% were SA dependent at 30 min and 6 h (Supplementary Fig. S5B). Taken together, these findings suggest a stronger dependence on SA biosynthesis for NHP-dependent downregulation of gene expression over the time frame studied.

SA biosynthesis antagonizes NHP-elicited gene expression at 3 to 6 h

Looking at the genes upregulated only in the *sid2-2* mutant and not wild type, we discovered an unexpectedly large number of transcripts that increased in abundance upon NHP treatment at 3 and 6 h posttreatment (Fig. 3B). This includes 151 genes at 3 h and 98 genes at 6 h that were significantly upregulated by NHP in the *sid2-2* mutant but not in wild type (Fig. 3B). GO term enrichment analysis of these genes showed significant enrichment for a number of biotic and abiotic stress responses, such as defense response to bacteria and fungi, SAR, response to abscisic acid, and response to hypoxia (Fig. 3C). These data suggest that SA may play an important role in modulating the transcription of a suite of genes, through an unknown mechanism, once NHP-mediated transcriptional reprogramming has been initiated.

TGA and WRKY TF CREs are enriched in the promoters of early NHP-upregulated genes

To identify TFs that might directly control the expression of early NHP-responsive genes, we analyzed the promoter regions of all genes upregulated after NHP treatment in both wild-type and *sid2-2* mutant seedlings to identify putative TF-binding sites (i.e. *cis*-regulatory elements [CREs]). A less stringent cutoff of log_2(FC) > 0 and $P_{adj} < 0.05$ was used for the upregulated genes to capture putative transcriptional regulators of all genes significantly elevated in transcript abundance, regardless of the magnitude of expression change. Following a modified workflow detailed by Bjornson et al. (2021), the NHP-upregulated genes were then separated by the time points in which they were differentially expressed. Enrichment of TF CREs was then determined for each time point using the DAPseq database (O'Malley et al. 2016) (Fig. 4A). Supplementary Figure S6 shows all the CREs found to be significantly enriched in the promoters of NHP-upregulated genes in both wild-type and *sid2-2* seedlings between 15 min and 6 h following NHP treatment.

We found that CREs for bZIP family members, specifically TGA TFs, were enriched in the promoters of genes upregulated by NHP as early as 15 min. For some TGAs (e.g. TGA2, TGA5, and TGA7), the enrichment of their TF-binding sites was observed for all

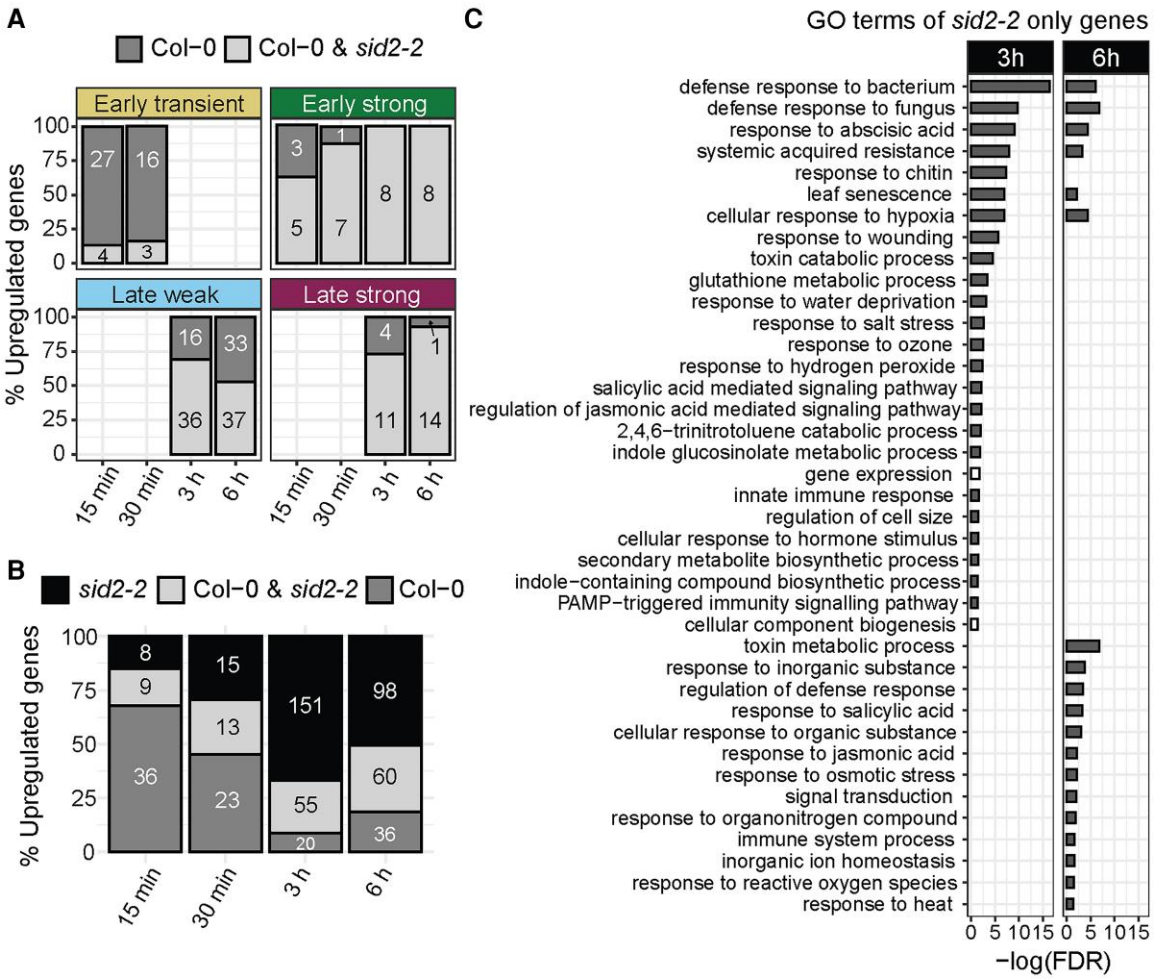

Figure 3. Comparison of NHP-upregulated genes in wild-type and *sid2-2* seedlings. **A)** Percent of NHP-upregulated genes expressed only in wild-type (Col-0, SA-dependent) and in both wild-type and the *sid2-2* mutant (SA-independent) for the wild-type gene expression clusters defined in Fig. 2. The total number of genes in each condition is indicated. **B)** Total number of NHP-upregulated genes ($\log_2(FC) > 1$; $P_{adj} < 0.05$) per time point unique to wild type (Col-0), shared between wild type and *sid2-2*, and unique to *sid2-2*. The number of upregulated genes in each grouping is indicated. **C)** Biological processes of significantly overrepresented (gray bar) or underrepresented (white bar) (FDR < 0.05) GO terms for the set of genes upregulated 3 and 6 h after NHP treatment that were upregulated only in the *sid2-2* mutant and not wild type. Bars represent the negative log(FDR) of each significantly enriched GO term; bars were omitted if the term was not significantly over- or underrepresented for the defined time point.

time points (Fig. 4B). Enrichment of TGA-binding sites in the promoters of early NHP-upregulated genes is consistent with previous work demonstrating TGA2/5/6 and TGA1/4 are required for the induction of NHP-responsive genes at 24 to 48 h post-NHP treatment (Nair et al. 2021; Yildiz et al. 2023). TGA TFs are known to interact with the transcriptional coactivator NPR1 to drive SAR- and SA-induced transcriptional changes (Zhang et al. 1999). The enrichment of TGA-binding sites in the promoters of NHP-upregulated genes as early as 15 min implicates TGAs among the primary transcriptional regulators of NHP signaling. Furthermore, enrichment of TGA CREs was not observed in *sid2-2* seedlings until 30 min and was also not significantly enriched at 3 h (Fig. 4B). These findings suggest that SA biosynthesis, and likely elevated SA levels, are required for rapid and stable induction of some TGA-regulated genes induced by NHP signaling.

Moreover, we discovered that a large number of WRKY CREs were highly enriched in the promoter regions of NHP-upregulated genes at 3 to 6 h in both wild-type and *sid2-2* seedlings (Fig. 4C). This corresponds with the majority of genes identified in the NHP-upregulated early strong cluster belonging to the WRKY TF family (Fig. 2; Table 1). This finding suggests a model where NHP induces the expression of *WRKY* genes as a primary transcriptional response, followed by WRKY-regulated gene expression changes that act as a secondary wave of transcriptional control.

It is striking that the confidence in WRKY CRE enrichment was greater (i.e. lower *P*-value) at 3 and 6 h in the promoters of genes upregulated in the *sid2-2* mutant compared to those of wild type (Fig. 4C). These data suggest that a substantial number of NHP-upregulated genes are directly regulated by WRKY TFs, and such regulation is influenced by SA levels. A similar pattern emerged for members of the NAC TF family, where NAC CREs were enriched in the promoters of NHP-upregulated genes at 3 h in *sid2-2* but not in the promoters of genes upregulated in wild type (Supplementary Fig. S6). Both WRKY and NAC TFs are involved in the regulation of a number of biotic and abiotic stress responses (Eulgem et al. 2000; Mohanta et al. 2020). Notably, a set of genes upregulated by NHP at 3 to 6 h only in the *sid2-2* mutant but not in wild type were overrepresented in biological processes associated with biotic and abiotic stress responses (Fig. 3, B and C). Taken together, these data suggest SA may antagonize the transcription of a subset of NHP-responsive genes by regulating the activities of WRKY and NAC TFs.

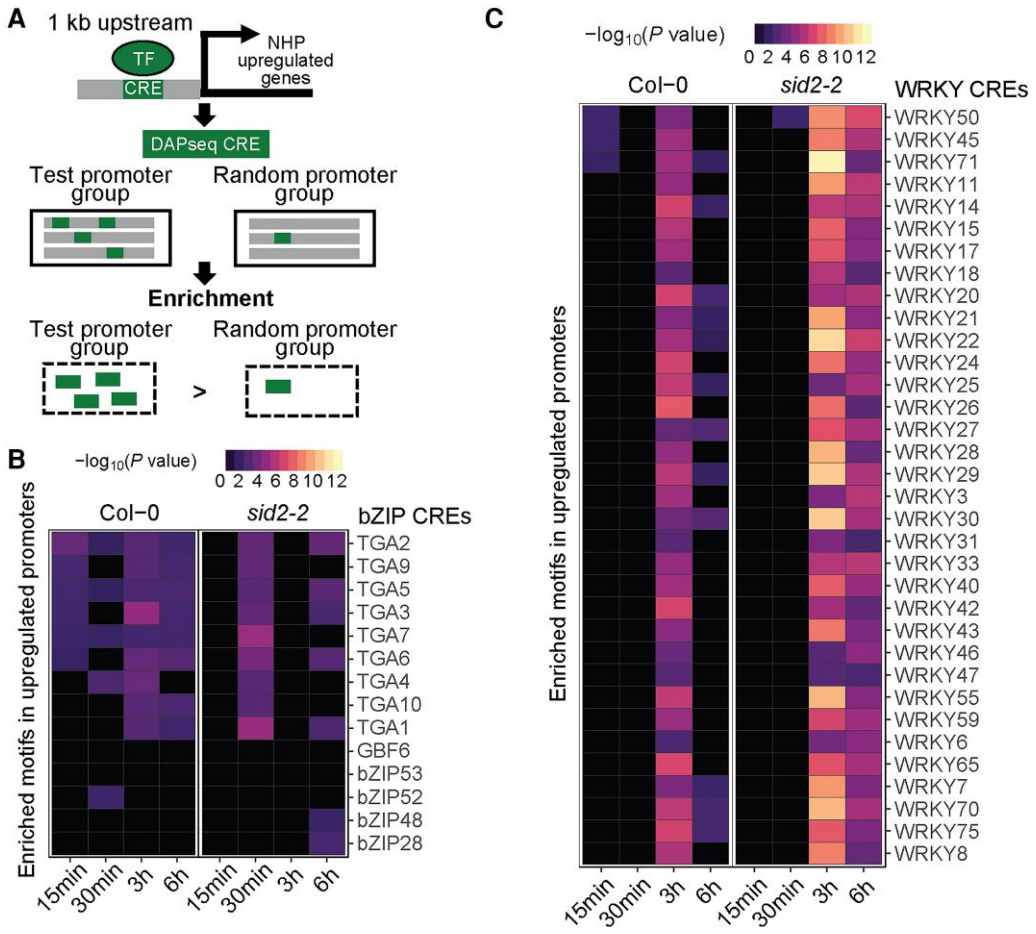

Figure 4. Presence of TGA/bZIP and WRKY TF CREs in the promoters of NHP-upregulated genes in wild-type (Col-0) and *sid2-2* seedlings. **A)** Schematic of the TF-binding motif (i.e. CRE) enrichment analysis performed. All genes with increased transcript abundance upon NHP treatment ($\log_2$(FC) > 0; P_{adj} < 0.05) were grouped by time point for the indicated genotype; these groups are represented as "test promoter group" in the diagram. CREs of known *Arabidopsis* TFs from the DAPseq database (O'Malley et al. 2016) were identified in the promoters (1-kb upstream of the transcriptional start site) of each group of promoters. Enrichment of identified CREs was determined relative to CREs found in promoters pulled from a random sampling of genes detected in this RNA-seq experiment. The schematic design is modified from Mariani et al. (2017). Enrichment analysis of TGA/bZIP **B)** and WRKY **C)** CREs. The name of the TF known to bind the enriched CRE is listed on the right, and the TF family name is indicated above (i.e. bZIP or WRKY). The $-\log_2$(*P*-value) of the enrichment analysis is indicated by the scale bar with black indicating no significant enrichment of the CRE and purple to yellow denoting an enriched CRE.

The same CRE enrichment analysis was also carried out on all NHP-downregulated genes, regardless of the magnitude of FC ($\log_2$(FC) < 0 and P_{adj} < 0.05). We found multiple WRKY CREs enriched in the promoter regions of NHP-downregulated genes at 15 min, 3 h, and 6 h, in wild-type seedlings (Supplementary Fig. S7), further supporting a role for WRKY TFs in relaying NHP-induced transcriptional changes.

Finally, we found the CREs of Homeobox gene family members to be abundant in the promoters of NHP-downregulated genes spanning from 15 min through 6 h, with the greatest confidence of enrichment found in the promoters of genes downregulated by NHP at 3 to 6 h in a SA-dependent manner (Supplementary Fig. S7). Among these was ATHB5, a homeodomain leucine zipper (HDZip) protein linked to the abscisic acid-driven repression of germination and root growth in seedlings (Johannesson et al. 2003). Given that NHP precursor Pip inhibits seedling root growth, we now hypothesize that NHP may drive the repression of root growth and development genes (Supplementary Fig. S3C) through the action of HDZip Homeobox proteins like ATHB5.

Early NHP-responsive gene *WRKY70* is required for NHP-elicited SAR

To further dissect the NHP response pathway, we carried out a reverse genetic screen to identify *Arabidopsis* mutants with compromised NHP-elicited SAR. Given WRKY CREs were the predominant regulatory elements identified in genes responsive to NHP 3 to 6 h following treatment (Fig. 4C; Supplementary Fig. S7), we selected the 6 *WRKY*s (*WRKY38*, *WRKY51*, *WRKY54*, *WRKY59*, *WRKY62*, and *WRKY70*) from the early strong NHP-upregulated cluster (Fig. 2B; Tables 1 and 2) for mutant analysis.

To measure NHP-induced SAR, we obtained homozygous *Arabidopsis* mutants and quantified bacterial growth in their leaves after treatment with exogenous NHP (Chen et al. 2018). Specifically, 3 lower leaves of 4.5-wk-old wild-type and mutant plants were infiltrated with water (mock) or 0.5 mM NHP. One day later, 1 distal upper leaf was inoculated with a 1×10^5 CFU/mL suspension of the virulent bacterium *Pseudomonas syringae* pathovar *maculicola* strain ES4326 (*Psm*). Bacterial growth of *Psm* was quantified 3 d postinfection (dpi) to determine if the mutants

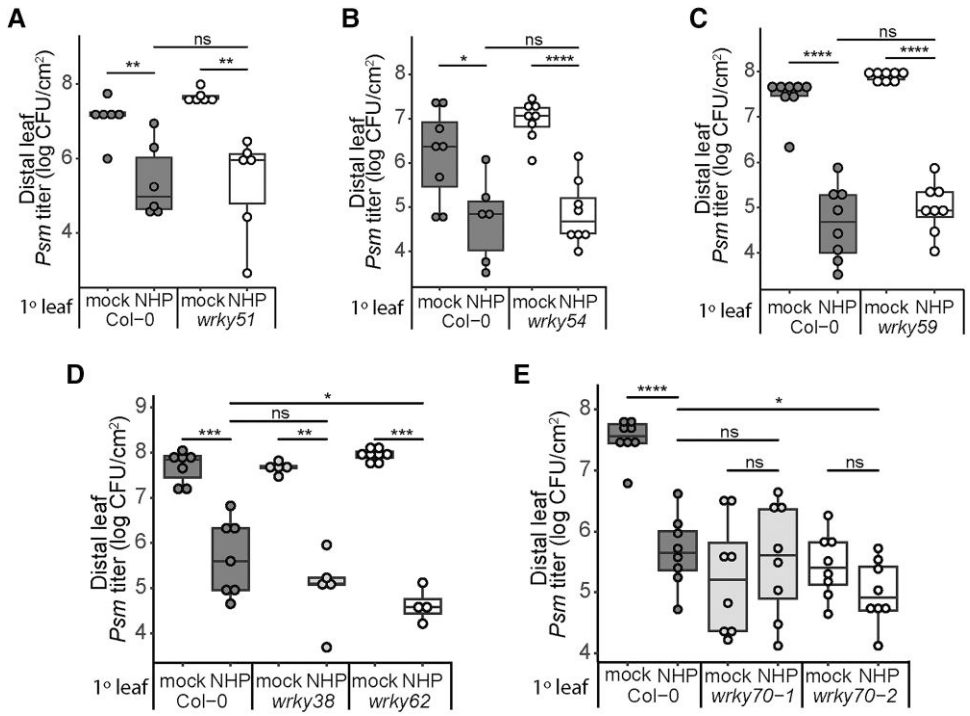

Figure 5. WRKY70 is involved in NHP-elicited SAR. Bacterial growth in the distal leaves of mock- and NHP-treated wild-type (Col-0) and *wrky* mutant plants. *WRKY* genes for mutant analysis were selected from the early stable cluster defined in Fig. 2 and include WRKY51 **A)**, WRKY54 **B)**, WRKY59 **C)**, WRKY38 and WRKY62 **D)**, and WRKY70 **E)**. Three lower (1°) leaves were infiltrated with water (mock), 0.5 mM NHP, or 1 mM NHP (*wrky59* only), and 1 d later, 1 upper, distal leaf was inoculated with a 1×10^5 CFU/mL suspension of *Psm*, followed by quantification of bacterial titer 3 dpi ($n = 4$ to 8). Box and whisker dot plots represent the median (center line), first and third quartiles (box edges), and the minimum and maximum values (whiskers). Asterisks indicate significant differences in bacterial titer (2-tailed *t*-test; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns, not significant).

were altered in their resistance to pathogen infection. Mutants were considered to exhibit normal NHP-elicited SAR if bacterial titer was significantly lower in NHP-treated plants compared to mock-treated plants.

Of the 6 *wrky* mutants screened, 5 (*wrky51*, *wrky54*, *wrky59*, *wrky38*, and *wrky62*) exhibited normal NHP-elicited SAR (Fig. 5, A to D). NHP treatment of the *wrky62* mutant resulted in enhanced resistance relative to NHP-treated wild type, suggesting WRKY62 may negatively affect NHP-elicited SAR. However, since *wrky62* was not compromised in NHP-elicited SAR, we instead focused on *wrky70-1* (SALK_025198; Li et al. 2006), which displayed compromised NHP-elicited SAR (Fig. 5E). That is, NHP treatment did not decrease bacterial growth in distal leaves of *wrky70-1* plants compared to mock treatment. We also observed that *wrky70-1* mutants treated with mock contained less bacteria than wild-type plants treated with mock, demonstrating basal resistance to *Psm* is enhanced in the *wrk70-1* mutant (Fig. 5E). Analysis of a second *wrky70* allele, *wrky70-2* (GABI_324D11; Li et al. 2006), showed similar patterns to *wrky70-1*, with compromised SAR and enhanced basal resistance (Fig. 5E). Unlike NHP-treated *wrky70-1*, NHP-treated *wrky70-2* plants exhibited enhanced resistance when compared to wild type treated with NHP. Despite this, no significant decrease in bacterial growth was seen between mock- and NHP-treated *wrky70-2*. The inability of the *wrky70* mutants to further restrict bacterial growth following NHP treatment suggests they may be insensitive to NHP or defective in NHP signaling.

We next questioned if systemic resistance to *Psm* was similarly compromised in *wrky70-1* plants primed with the avirulent pathogen *P. syringae* pathovar *tomato* (*Pst*) strain DC3000 carrying *avrRpt2* (*Pst avrRpt2*). Localized infection with *Pst avrRpt2* is known to elicit a strong immune response that leads to SAR and protection against *Psm* in distal leaves (Kohler et al. 2002). We found that the *Psm* titer in distal leaves reached similar levels for *wrk70-1* plants treated with mock or *Pst avrRpt2*, and this level was comparable to *Psm* growth in wild-type leaves of plants treated with *Pst avrRpt2* (Supplementary Fig. S8A). These data indicate that *wrky70-1* mutants exhibit a basal level of defense priming and *Pst avrRpt2* primary infection does not further enhance this resistance.

We also examined pathogen growth in local *wrky70-1* leaves. We found that the titer of *Psm* in infected *wrky70-1* leaves was lower than in *Psm*-infected wild-type leaves (Supplementary Fig. S8B). This phenotype corroborates previous work demonstrating loss of *WRKY70* expression enhances resistance to *Psm* infection (Zhou et al. 2018).

Expression of *SARD1* and *PR* genes is elevated in *wrky70-1* independent of NHP

Previous studies in *Arabidopsis* have demonstrated a role for WRKY70 in the transcriptional regulation of several genes required for SAR signaling and disease resistance, including *SARD1* and the *PATHOGENESIS-RELATED* genes *PR1*, *PR2*, and *PR5* (Li et al. 2004; Zhou et al. 2018; Liu et al. 2021). These genes were also shown to be upregulated in wild-type *Arabidopsis* plants 24 h after treatment with NHP (Yildiz et al. 2021). To determine if WRKY70 is required for NHP-induced expression of *SARD1*, *PR1*, *PR2*, and *PR5* at 24 h, we quantified transcript abundance in wild-type and *wrky70-1* plants treated with water or 1 mM NHP. As expected, RT-qPCR showed increased abundance of *SARD1*, *PR1*, *PR2*, and *PR5* mRNA in wild-type plants treated with NHP compared to water (Supplementary Fig. S8C). However, NHP

treatment did not further increase transcript levels in *wrky70-1* plants (Supplementary Fig. S8C). Notably, all 4 genes showed elevated transcript abundance in water-treated *wrky70-1* mutants compared to water-treated wild type. The elevated expression of *SARD1* and *PR1* in water-treated *wrky70-1* plants is consistent with previous studies demonstrating WRKY70 negatively regulates these 2 genes in the absence of a pathogen (Zhou et al. 2018; Liu et al. 2021).

WRKY70 is required for NHP induction of genes in the late weak and late strong clusters

Given the *wrky70* mutants were unable to further enhance resistance to *Psm* infection following NHP treatment (Fig. 5E), we hypothesized that a subset of early NHP-responsive genes, containing putative WRKY70 CREs in their promoters, may require WRKY70 function for full, wild-type gene expression levels following NHP treatment. To investigate this, we selected 3 genes from the late weak and late strong clusters (Fig. 2; Table 1), specifically *BDA1*, *PROSCOOP4*, and *CML10*, and examined their transcript accumulation in response to 1 mM NHP over a time course of 0 to 12 h. The promoters of these genes contain 1 or more WRKY70 CREs (i.e. W-box [TTGACY], WT-box [YGACTTTT], and WRKY70 DAPseq motif; Rushton et al. 2010; Machens et al. 2014; O'Malley et al. 2016) within 2,000-bp upstream of their respective putative transcriptional start site (Fig. 6A).

We found that *BDA1*, a gene encoding an ankyrin-repeat transmembrane protein (Yang et al. 2012), exhibited the strongest dependence on WRKY70 for NHP-induced expression. *BDA1* transcripts were significantly increased at 6, 9, and 12 h post-NHP treatment in wild-type leaves but not *wrk70-1* leaves (Fig. 6B). Transcript levels for *PROSCOOP4*, a gene encoding the precursor of secreted peptide SCOOP4/STMP10 (Gully et al. 2019; Hou et al. 2021), were also significantly higher at 6 and 9 h post-NHP treatment in wild-type leaves compared to that observed for *wrk70-1* plants (Fig. 6C). Unlike *BDA1*, we found that *PROSCOOP4* transcripts were higher in NHP-treated *wrk70-1* leaves at 6 and 9 h compared to the 0-h time point (Fig. 6C). These data show that *wrk70-1* plants responded to NHP treatment, suggesting that they can sense NHP but are impaired in NHP signaling. Similar trends were observed

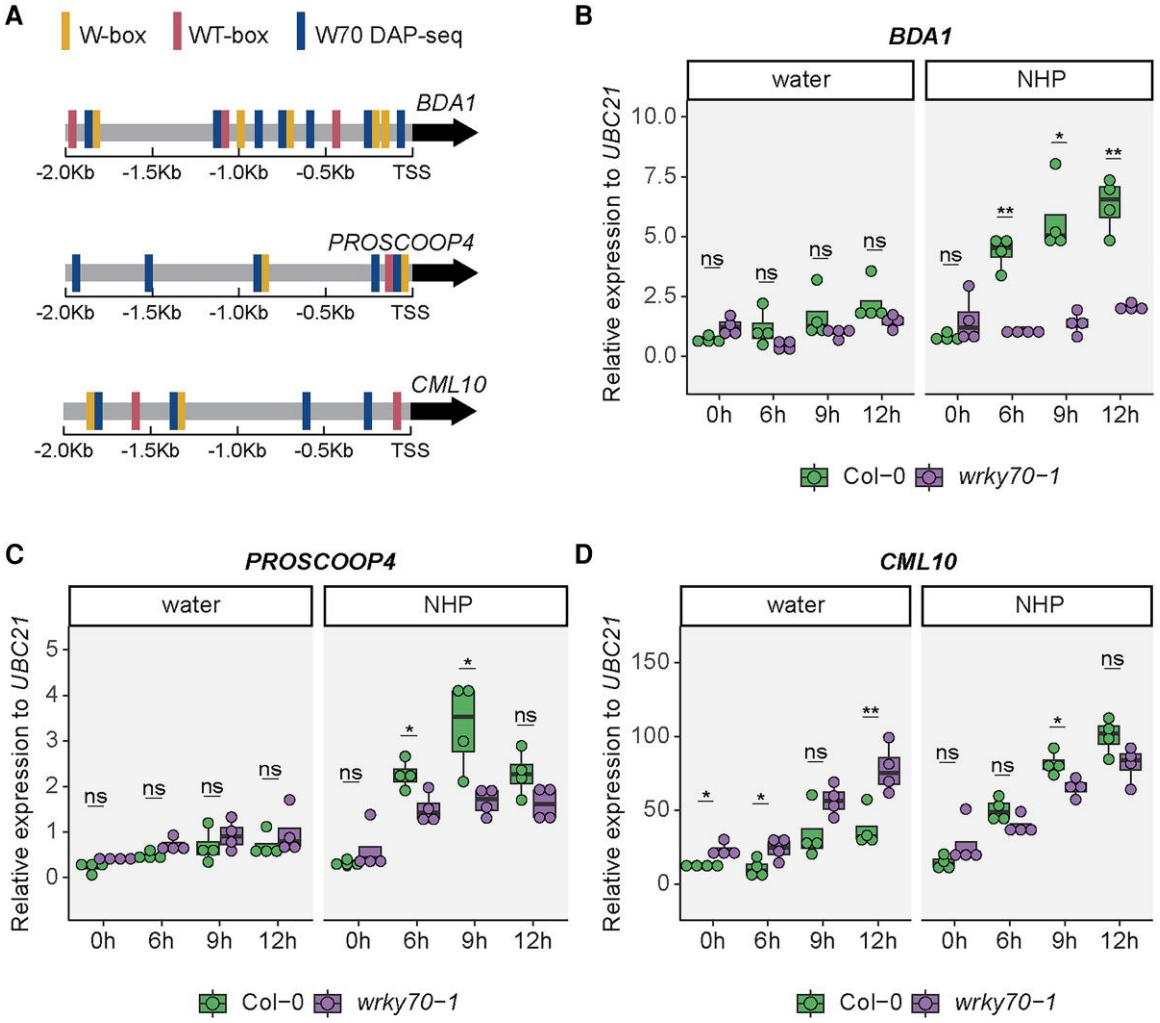

Figure 6. Loss of WRKY70 function impacts the upregulation of early NHP-responsive genes. **A)** Diagram of putative WRKY70 CREs, including W-box, WT-box, and WRKY70 (W70) DAPseq motifs in the promoters (−2-kb upstream of the TSS) of NHP-responsive genes *BDA1*, *PROSCOOP4*, and *CML10*. Note W70 DAPseq sites adjacent to a W- or WT-box overlap. Expression of *BDA1* **B)**, *PROSCOOP4* **C)**, and *CML10* **D)** in *wrky70-1* mutant plants. Three leaves of 4.5-wk-old wild-type (Col-0) and *wrky70-1* mutant plants were infiltrated with water or 1 mM NHP. Samples collected at 0 h were untreated before collection. Transcript abundance was determined relative to *UBC21* for each condition ($2^{-\Delta Ct}$). Box and whisker dot plots represent the median (center line), first and third quartiles (box edges), and the minimum and maximum values (whiskers) of 4 biological replicates. Asterisks indicate a significant difference between wild type and *wrky70-1* at each time point (2-tailed *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$, ns, not significant).

for *CML10* (*CALMODULIN-LIKE 10/CaBP22*; Luan et al. 2002) at the 9-h time point (Fig. 6D). We confirmed these results by analyzing a second *wrky70* allele, *wrky70-2* (Supplementary Fig. S9).

Taken together, our findings reveal that *WRKY70* is required for the proper expression of NHP-responsive genes from the late weak and late strong clusters, highlighting a role for WRKY70 in the transcriptional response to NHP several hours following treatment.

NHP pretreatment enhances flg22-elicited ROS production and WRKY70 contributes to the full response

We next questioned if WRKY70 regulates a specific branch of NHP defense signaling. One such branch involves the response to common molecular features of microbes (e.g. bacterial flagellin or fungal chitin) known as microbe-associated molecular patterns (MAMPs) and the response to plant-derived damage-associated molecular patterns (DAMPs), a process collectively referred to as pattern-triggered immunity (PTI). The WRKY70-dependent gene *BDA1* is known to mediate signaling in response to MAMP detection and interacts with receptor-like protein Suppressor of NPR1, Constitutive2 (SNC2) to relay MAMP-triggered defense responses (Yang et al. 2012). Production of ROS is a MAMP-triggered defense response, which can act directly or indirectly as an antimicrobial agent and serves as a secondary signal to activate further defense responses (Boller and Felix 2009; Couto and Zipfel 2016). Similarly, DAMPs, such as secreted peptides from the PROSCOOP family, are known to elicit an increase in ROS production (Gully et al. 2019; Hou et al. 2021; Rhodes et al. 2021). Incidentally, *CML10* encodes a calmodulin-like protein known to interact with phosphomannomutase in order to modulate ascorbic acid synthesis and cellular homeostasis of ROS (Cho et al. 2016).

We thus investigated how NHP alters MAMP-triggered ROS production using bacterial flagellin as the elicitor in the presence and absence of NHP pretreatment and asked if WRKY70 is required for the response. Leaf discs of wild-type and *wrky70-1* plants were floated on solutions of water, NHP, and SA. SA was included as a positive control, as it is known to enhance the MAMP-triggered ROS burst (Yi et al. 2014). After 24 h, leaf discs were treated with a 100 nM solution of flagellin peptide (flg22), and then ROS was measured using a luminol-based assay. We found ROS levels from mock-treated *wrky70-1* plants, on average, trended lower than water-treated wild type, suggesting WRKY70 is required for maximal ROS accumulation (Fig. 7, upper panel). NHP treatment increased ROS generation in both wild-type and *wrky70-1* tissue exposed to flg22 when compared to water-treated tissues. The average ROS production in NHP-treated *wrky70-1* leaf discs was lower than NHP-treated wild-type leaf discs (Fig. 7, lower panel). Similar results were found when analyzing *wrky70-2* plants (Supplementary Fig. S10). Collectively, these data indicate that NHP is able to enhance the flg22-elicited ROS burst, and this enhancement requires WRKY70 for full ROS generation.

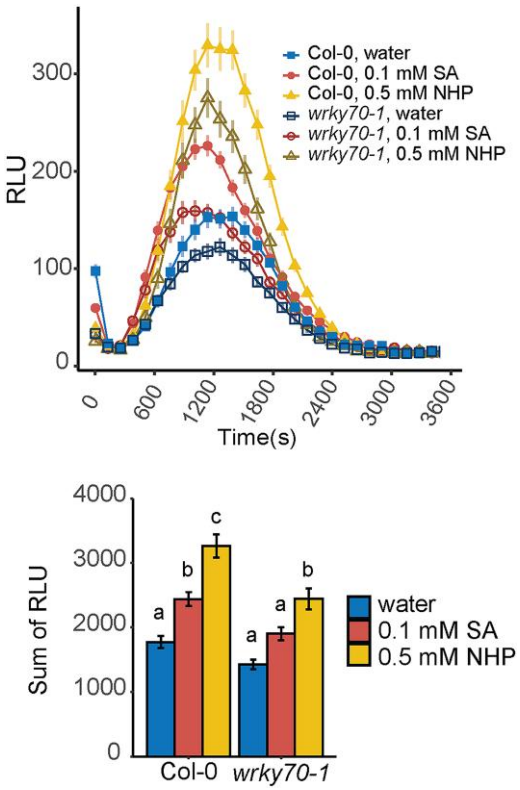

Figure 7. NHP enhances ROS production and requires WRKY70 for full ROS levels. Flg22-elicited ROS quantification in *wrky70-1* plants. Four leaf discs from 4.5 to 5-wk-old wild-type and *wrky70-1* plants were pretreated by floating leaf discs on water, 0.1 mM SA, or 0.5 mM NHP for 24 h before treating with 100 nM flg22 in horseradish peroxidase and luminol. Top: traces of the average RLUs over the indicated run time. Each point represents the average of leaf discs from 6 plants ($n = 24$) from 2 experiments, and error bars represent the SEM. Bottom: total quantification of RLUs from top plot. For each leaf disc, RLUs from all time point were summed by condition over the run time and averaged with error bars representing the SEM. Statistical analysis was performed using a 1-way ANOVA and post hoc Sidak test; different letters indicated statistical differences between means with $P < 0.05$.

Discussion

NHP has emerged as an essential signaling molecule required for the establishment and amplification of SAR in *Arabidopsis* and crop plants (Chen et al. 2018; Holmes et al. 2019), although the early signaling events mediated by NHP had yet to be described. Our study provides insights into the primary transcriptional responses to NHP, as well as the role that NHP-induced SA biosynthesis plays during this response. We also discovered that TGA and WRKY CREs are enriched in the promoters of early NHP-induced genes, providing a framework for dissecting primary and secondary transcriptional responses. Furthermore, we show that early transcriptional expression of *WRKY70*, within minutes of NHP treatment, mediates secondary transcriptional reprogramming that is required for NHP signaling, ROS production, and SAR.

Early short-lived NHP transcriptional responses associate with general stress

In the early response to NHP, we identified a primary wave of NHP signaling that was short-lived and activated transcriptional responses mirroring wounding/DAMP and JA signaling in a partially SA-dependent manner. Of the pathways activated by NHP, we found a considerable enrichment of known JA-response genes, including *MYC2* and *JAZ10* (Fig. 2C; Tables 1 and 2). Given the established antagonism between JA and SA signaling pathways (Pieterse et al. 2012), it was surprising to find the majority of genes in the early transient cluster were dependent on ICS1/SID2-derived SA for early induction (Fig. 3A). These findings suggest that part of the early NHP transcriptional response depends on synergism between JA signaling components and preinfection levels of SA. Our data support a model where general stress response signatures are activated by NHP treatment in an early

pulse of gene expression that acts synergistically with SA to modulate the activation of defense pathways (Fig. 8A).

Previous work has described a strong, systemic JA response in *Arabidopsis* leaves infected with an avirulent *Pst* strain after 4 h, suggesting JA signaling pathways are involved in SAR development (Truman et al. 2007). The JA response detected by Truman *et al.* in the systemic leaves of avirulent *Pst*-treated plants after 4 h may be reflective of the early and transient response to NHP observed in our transcriptional analysis. These JA- and early NHP-responsive marker genes may prove useful for determining optimal time points in testing the systemic translocation of NHP. Furthermore, although genetic studies contest the requirement for JA biosynthesis and signaling in SAR (Attaran et al. 2009), it remains a possibility that differences in primary pathogen treatment or secondary pathogen infection may shift the requirement for JA signaling in SAR establishment and warrant further study.

Early strong NHP responses activate a primary wave of *WRKY* transcription

We discovered another primary wave of early NHP signaling that was partially SA independent and exhibited strong induction of *WRKY* gene transcription within minutes of NHP treatment, persisting several hours after treatment (Fig. 8A). All 6 *WRKY* TF genes in the early stable upregulated gene cluster were activated by NHP treatment in *sid2-2* seedlings, and although some showed delays in the timing of induction in the *sid2-2* mutant, all 6 were strongly upregulated by 3 h (Tables 1 and 2). Furthermore, increased transcript abundance of *WRKY38*, *WRKY54*, and *WRKY70* in *npr1-3* seedlings 3 h after treatment with NHP indicates some *WRKY*s in this cluster are responsive to NHP independent of NPR1 (Supplementary Fig. S4). These findings are intriguing given SA- and SA-analog-induced expression of this group of *WRKY* TFs is known to be NPR1-dependent (Kalde et al. 2003; Wang et al. 2006). The ability of NHP to induce an early wave of *WRKY* expression independent of ICS1/SID2-derived SA and independent of NPR1 suggests NHP, not SA, is the primary signal driving this early transcriptional response, where some responses are NPR1 independent.

The early and strong induction of *WRKY* transcription, including 2 sets of homologs belonging to WRKY Group III (*WRKY38* and *WRKY62* as well as *WRKY54* and *WRKY70*), suggested WRKY TFs may play a role in driving the secondary transcriptional response to NHP. Members of WRKY Group III are known to play both positive and negative regulatory roles in defense responses (Kalde et al. 2003; Pandey and Somssich 2009). Consistent with

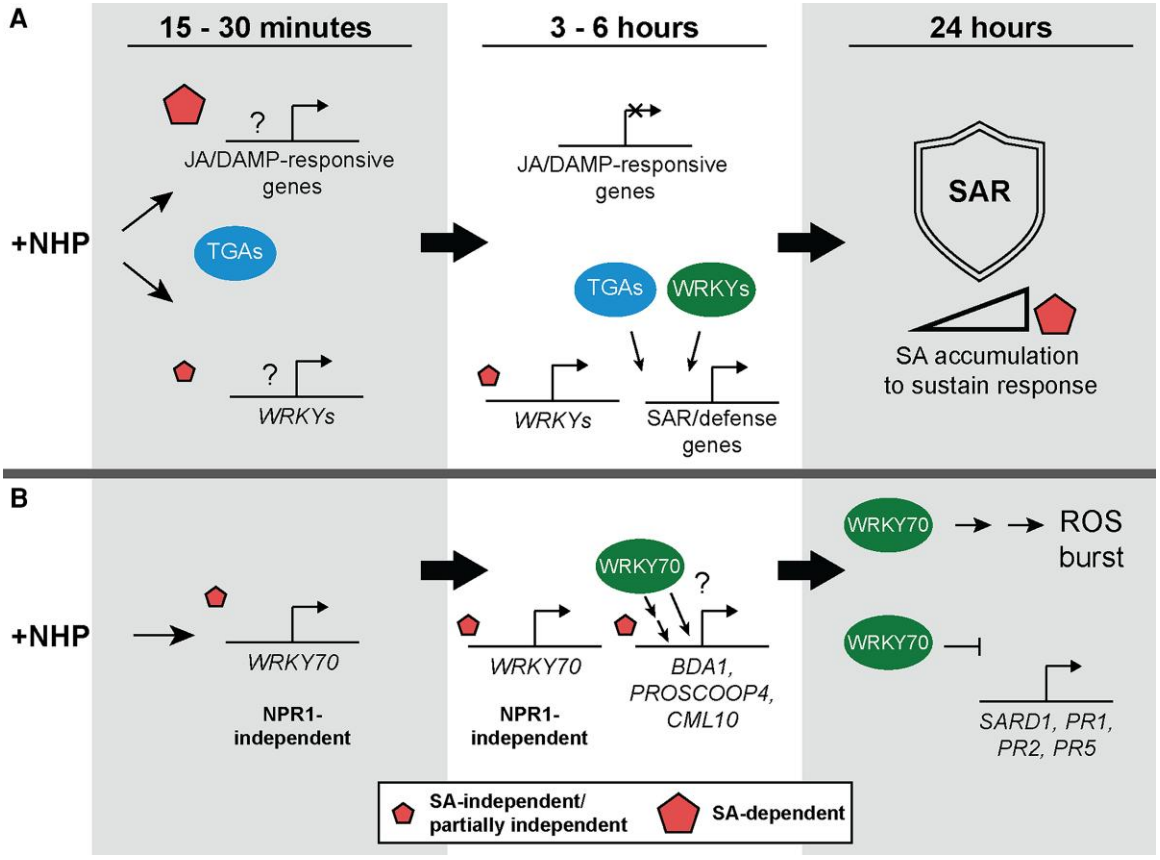

Figure 8. Working models for early transcriptional activation following NHP elicitation and the role of WRKY70 in modulating NHP responses. **A)** Presence of the NHP signal induces a primary phase of transcriptional activation within 15 to 30 min for 2 groups of genes: one with signatures of JA/DAMP-responses that is primarily dependent on ICS1/SID2-derived SA and the other consisting largely of WRKY TF genes mostly independent of SA. *WRKY* expression persists several hours later, whereas the JA/DAMP response is attenuated through an unknown regulatory mechanism. At the 3- to 6-h time point, NHP induces a secondary phase of SAR- and defense-associated gene expression with the majority activated partially independent of SA. Dependence on SA signaling increases as NHP-elicited SAR progresses by 24 h. DNA-binding motifs (i.e. CREs) of WRKY TFs are enriched in the promoters of genes expressed at 3 to 6 h, implicating WRKY TFs as executors of the secondary transcriptional phase. TGA CREs are enriched in the promoters of NHP-induced genes from 15 min to 6 h and are likely key drivers of NHP signaling. **B)** *WRKY70* expression is induced by NHP within minutes, persisting several hours later, at least in part, via SA- and NPR1-independent pathways. WRKY70 is required for full induction of a subset of NHP-induced genes via either direct or indirect regulation. It is possible these WRKY70-induced genes help in priming a NHP-driven enhanced ROS burst following flg22 elicitation. WRKY70 also serves to repress NHP-induced genes, modulating and/or attenuating the expression of key SAR genes.

this, we found CREs of WRKY TFs to be abundant in the promoters of genes, both upregulated and downregulated, several hours after NHP treatment (Fig. 4C; Supplementary Fig. S7). For example, the CRE bound by WRKY70, as defined by the DAPseq Plant Cistrome Database (O'Malley et al. 2016), was enriched in the promoters of genes upregulated by NHP at 3 and 6 h (Fig. 4C). Other canonical motifs bound by WRKY70, such as the W-box and WT-box (Rushton et al. 2010; Machens et al. 2014), were also found in the promoters of genes upregulated several hours after NHP treatment (Fig. 6A). Taken together, these findings link the early NHP-driven induction of *WRKY* expression to the subsequent upregulation and downregulation of WRKY-targeted genes 3 to 6 h after initial NHP treatment. Additionally, these WRKYs may be important in modulating or attenuating the NHP response as loss of *WRKY62* and *WRKY70* expression resulted in enhanced resistance and defense gene expression (Fig. 5, D and E; Supplementary Fig. S8, A to C). Based on these findings, we hypothesize a model of early NHP signaling wherein activation of *WRKY* transcription is a primary response to NHP signaling that is followed by a secondary wave of expression driven through WRKY regulation (Fig. 8A).

While not the focus of this study, it is noteworthy to mention that TGA TFs are also associated with the primary wave of NHP-mediated transcription. We found CREs of TGAs are abundant in the promoters of early NHP-upregulated genes across all time points sampled (Figs. 4B and 8A). These findings are in agreement with recent studies demonstrating TGAs are redundantly required for NHP induction of SAR and SAR marker genes (Nair et al. 2021; Yildiz et al. 2023). Our data provide further evidence highlighting the importance of TGAs in NHP-driven signaling (Fig. 8A) and suggest that a NPR1/TGA complex, or a key interactor of this complex, directly relays the NHP signal.

Late weak and late strong genes define the secondary wave of transcription

Finally, our RNA-seq analysis also revealed a secondary wave of NHP signaling defined by late weak and late strong upregulated genes. These genes are associated with pathogen defense, SAR, and SA responses and showed features of SA-dependent and SA-independent transcriptional induction (Figs. 2C and 3A). Upregulation of SAR-associated genes by NHP is consistent with previous studies (Bernsdorff et al. 2016; Yildiz et al. 2021, 2023). Our study reveals that transcriptional activation of these SAR-associated genes occurs as early as 3 to 6 h following detection of the NHP signal. Interestingly, 24 genes from the late weak cluster and 4 genes from the late strong cluster are SA independent within 6 h of NHP treatment but SA dependent 24 h after NHP (Yildiz et al. 2021). Among this group are key SA and NHP biosynthetic and regulatory genes including *ALD1*, *UGT76B1*, *SARD1*, and *NPR3*. Together, these data suggest NHP is sufficient to drive gene expression over a short time frame but requires SA accumulation for sustained expression (Fig. 8A), thus highlighting the importance of interrogation into early NHP transcriptional changes. Further elucidation of a timeframe for early NHP responses should prove useful in studying other aspects of NHP biology, such as the biosynthesis and translocation of NHP and the identification of NHP sensors and their respective downstream signaling pathways.

Primary NHP-responsive gene *WRKY70* mediates secondary transcriptional changes required for NHP-elicited SAR, signaling, and ROS production

We found *WRKY70* expression is induced within 15 min of NHP treatment and loss of *WRKY70* expression compromises NHP-elicited SAR (Fig. 5E). WRKY70 is known to repress many pathogen-inducible genes in the absence of infection but is also required for full activation of pathogen-inducible genes during infection (Zhou et al. 2018). We observed enhanced resistance to *Psm* infection and enhanced transcript abundance of defense genes *SARD1*, *PR1*, *PR2*, and *PR5* after mock treatment (Supplementary Fig. S8C), consistent with a repressive role for WRKY70 that likely serves to modulate or fine-tune defense responses. However, NHP treatment did not enhance resistance nor increase transcript abundance of these genes in *wrky70-1* (Fig. 5A; Supplementary Fig. S8, A to C). Furthermore, expression of the NHP-upregulated early strong gene *BDA1* was completely abolished in *wrky70* mutant lines (Fig. 6B; Supplementary Fig. S9A), demonstrating early expression of *WRKY70* is required to drive as well as modulate a subset of the secondary transcriptional responses of NHP signaling (Fig. 8B).

Evidence supports a SA- and NPR1-independent defense pathway driven by WRKY70 (Shah et al. 2001; Zhang, Yang, et al. 2010). We found that NHP was sufficient to induce expression of *WRKY70* in the *sid2-2* and *npr1-3* mutants within 30 min of treatment (Table 1; Supplementary Fig. S4C), suggesting new SA biosynthesis and the SA receptor NPR1 are dispensable for the early transcriptional activation of *WRKY70* (Fig. 8B). Likewise, WRKY70-regulated genes *BDA1* and *CML10* are induced within 3 to 6 h of NHP signaling partially independent of SA (Table 1). Notably, 24 h after NHP treatment, *BDA1* and *CML10* are also upregulated in both *sid2-1* and *npr1-3* mutants (Yildiz et al. 2021). It is therefore possible that NHP signaling activates a SA- and NPR1-independent defense pathway through the action of WRKY70.

Despite a role in SA- and NPR1-independent signaling, expression of *WRKY70* has been shown to be activated by SA and repressed by JA (AbuQamar et al. 2006). Additionally, high transcript abundance of *WRKY70* is linked to activation of SAR-related gene expression and repression of JA-responsive genes, whereas low *WRKY70* transcript levels result in the activation of JA responses over SAR-related responses (Li et al. 2004). Therefore, it is possible that elevation of *WRKY70* transcript abundance similarly acts to drive NHP-induced SAR responses while also contributing to the repression of JA-related early transient responses observed several hours (i.e. 3 to 6 h) after NHP treatment (Fig. 8A). This is consistent with wild-type expression of *WRKY70* increasing from 30 min to 3 h (Table 1; Supplementary Fig. S4C). Further work is required to determine if WRKY70 represses the expression of JA-response genes found in the early transient cluster, how SA levels impact this response, and the impact of these responses on SAR development.

Studies into the MAMP-signaling pathway functioning through the receptor-like protein SNC2 suggest a role for WRKY70 and BDA1 in PTI responses such as ROS production (Yang et al. 2012). While flg22-elicited ROS production was not significantly different between mock-treated wild-type and *wrky70* mutants, the enhanced production of flg22-elicited ROS following NHP and SA pretreatment was compromised in *wrky70* mutants (Fig. 7; Supplementary Fig. S10). Our findings indicate WRKY70 positively contributes to the NHP and SA signaling pathways that prime PTI. Furthermore, our discovery that NHP primes plants for enhanced production of flg22-elicited ROS is consistent with reports of NHP priming *Arabidopsis* for enhanced metabolic and transcriptional responses following flg22 treatment (Löwe et al. 2023). Our work links WRKY70-dependent signaling pathways to this NHP-primed PTI response.

Conclusions

Our work describes the early transcriptional response to NHP and highlights key genes and signaling pathways that may contribute to the physiological processes regulated by this important bioactive molecule. It also provides a foundation for better understanding the transcriptional regulators, particularly the role of WRKY70, and gene networks that define early NHP signal transduction.

Materials and methods

Plant materials and growth conditions

Arabidopsis (*A. thaliana*) lines used in this study were in the Col-0 ecotype (wild type) with mutant lines as follows: *sid2-2* (Wildermuth et al. 2001), *npr1-3* (Cao et al. 1997), and T-DNA insertion lines *wrky70-1* (SALK_025198) and *wrky70-2* (GABI_324D11) (Li et al. 2006), *wrky38* (WiscDsLox489-492C21), *wrky54* (SALK_017254), and *wrky59* (SALK_039436) (Wang et al. 2006), *wrky51* (SALK_022198) (Yan et al. 2018), and *wrky62* (SM_3_38820) (Kim et al. 2008). All mutant lines were obtained from the Arabidopsis Biological Resource Center. The presence of T-DNA insertions was confirmed with the genotyping primers listed in Supplementary Table S3. Plants were grown on soil in a controlled growth chamber at 80% humidity and 22 °C on a 10-h light/14-h dark cycle for gene expression and bacterial growth assays. For seedlings grown hydroponically, seeds were surface sterilized and stratified at 4 °C in darkness for 3 d. Seeds were placed into sterile 6-well plates (15 to 18 seeds/well) with half-strength MS medium, 0.5% (*w/v*) sucrose, and 0.05% (*w/v*) MES at pH 5.7. Plates were covered with a lid, sealed with micropore tape (3 M), and placed in a chamber on a 10-h light/14-h dark cycle. Liquid medium was agitated twice daily to facilitate gas exchange, and after 5 d of growth, spent medium was replaced with fresh MS.

Bacterial strains and growth conditions

P. syringae pv. *maculicola* strain ES4326 (*Psm*) and *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) carrying pVSP61 + *avrRpt2* (*Pst avrRpt2*) were used in this study. Growth conditions for all bacterial strains are as previously described (Holmes et al. 2021).

Chemical stocks

Two sources of NHP were used in this study, one synthesized by Elizabeth Sattely's lab as previously described (Chen et al. 2018) and the second obtained from MedChem Express. Gene expression and NHP-elicited SAR assays showed similar results for both sources of NHP. Pipecolic acid stocks were made from L-pipecolic acid (Oakwood Chemical). SA stocks were made from sodium salicylate (Mallinckrodt). All chemical stock solutions used were dissolved in water.

Chemical treatment of plants for RT-qPCR assays

For gene expression by RT-qPCR of 4.5-wk-old, soil-grown *Arabidopsis*, 3 leaves were infiltrated with sterile water or NHP dissolved in water at the indicated concentrations. The 3 treated leaves were collected and flash frozen at the indicated time points. For the time course of gene expression in *wrky70-1* and *wrky70-2* mutants, 3 leaves were treated with sterile water or 1 mM NHP. One leaf from the same plant was sampled at 6, 9, and 12 h and pooled with a second plant of the same condition for one replicate. Samples collected at 0 h were untreated before collecting.

For gene expression in *Arabidopsis* seedling-based assays, plants were grown as described for seedling hydroponics and treated at 10-day postgermination. Before the addition of treatment, all liquid medium was removed from plate wells and replaced with fresh MS medium, and seedlings were allowed to recover under lights for at least 1 h, followed by application of 2× solutions of Pip, NHP, or mock in MS medium. The 15 to 18 seedlings within a single well were pooled and treated as a single replicate, with 3 replicates sampled per condition. Following treatment, seedlings were blotted dry and flash frozen at the indicated time points.

RNA isolation and RT-qPCR

Total RNA was isolated from leaves and seedlings using TRIsure reagent (Meridian Bioscience) according to the manufacturer's instructions. Five micrograms of RNA was used to synthesize cDNA by oligo dT (New England Biolabs) and reverse transcriptase (Thermo Fisher), followed by dilution of cDNA. RT-qPCR was performed on a MJ Opticon 2 (Bio-Rad) using Green Taq DNA polymerase (GenScript) with EvaGreen Dye (Biotium) for amplification and detection. Three technical replicates were performed per sample, and 3 to 4 biological replicates were included for each condition. Expression values were normalized to the reference gene *UBC21* (AT5G25760) for relative expression determined using $2^{-\Delta Ct}$ and normalized to both *UBC21* and mock-treated samples for FC using $2^{-\Delta\Delta Ct}$. Primers used in these experiments can be found in Supplementary Table S3.

RNA-seq treatment and library preparation

Seedlings used for mRNA isolation were grown hydroponically as described above. Six-well plates contained equal numbers of Col-0 and *sid2-2* seedlings, with 1 genotype per plate and 15 seedlings per well. At 10-day postgermination, all liquid medium was removed from each well, and fresh MS medium was added to the seedlings. Seedlings were placed back into the growth chamber for an hour to acclimate, followed by treatment with 2× NHP (final concentration 0.5 mM) or mock (MS medium). Three wells of pooled seedlings were collected for each genotype, treatment, and time (15 min, 30 min, 3 h, and 6 h; Supplementary Table S4) combinations, blotted dry, and flash frozen for mRNA isolation.

Total RNA was extracted from pooled seedlings using TRIsure reagent (Meridian Bioscience) according to the manufacturer's instructions. Total RNA was then divided with 5 μg used for cDNA synthesis and marker gene expression to assess quality of treatment, as described for RT-qPCR experiments, and 5 μg was used for cDNA library preparation. The cDNA library was prepared using NEBNext Ultra II RNA Library Prep Kit for Illumina with NEBNext Poly(A) mRNA Magnetic Isolation Module and then multiplexed via PCR amplification using NEBNext Multiplex Oligos for Illumina according to the manufacturer's instructions (New England Biolabs). Library concentration was quantified with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) followed by measurement of quality and average cDNA length using the DNA 1000 Series II chip on a 2100 Bioanalyzer (Agilent Technologies). The multiplexed cDNA libraries were then pooled (2 sets of 36 libraries) and sequenced using both lanes of the NovaSeq 6000 (Illumina) S1 flow cell with run-type 100-bp paired-end reads at the Genome Sequencing Service Center by the Stanford Center for Genomics and Personalized Medicine, supported by the grant award NIH S10OD025212.

RNA-seq data analysis

Read data were initially assessed using FastQC. CLC Genomics Workbench version 11.0.2 (QIAGEN) was used for trimming,

discarding reads trimmed below 20 bp, and then mapped to the *Arabidopsis* TAIR10 genome with the autodetect paired distances option deselected. Differential expression analysis was performed using DESeq2 (Love et al. 2014) with mock-treated samples compared to NHP-treated samples for each respective time point and genotype. Genes were considered differentially expressed if they returned an adjusted *P*-value below 0.05 ($P_{adj} < 0.05$). The complete list of early NHP-responsive transcripts is provided in Supplementary Table S1, and the raw sequencing data are deposited in the Gene Expression Omnibus under accession code GSE263276.

Exploratory analysis of differentially expressed genes

To define patterns of early expression in response to NHP treatment, differentially expressed genes were considered upregulated in wild type if they had a $\log_2(FC) > 1$ at any time point and downregulated with a $\log_2(FC) < -1$ at any time point. The sets of upregulated and downregulated genes were then hierarchically clustered by $\log_2(FC)$ over the 4 time points using the hclust function and dendextend packages in R, with Euclidean distances and Ward.D2 linkage (Galili 2015). For each defined cluster in wild type, the $\log_2(FC)$ values were averaged at each time point to visualize broad trends in expression.

The PANTHER classification system accessed via TAIR was used for GO enrichment analysis (https://www.arabidopsis.org/tools/go_term_enrichment.jsp; Mi et al. 2021). Version 17.0 of the PANTHER overrepresentation test was used (Fisher's exact test with false discovery rate [FDR] correction) and matched to the GO database 2021_03 release.

Promoter and CRE enrichment analysis

Araport11 sequences 1,000-bp upstream of the transcriptional start site (TSS) of NHP-responsive genes were downloaded from TAIR (https://www.arabidopsis.org/tools/bulk/sequences/index.jsp) for each time point and genotype grouping of the NHP-upregulated and NHP-downregulated genes. A less stringent cutoff of $\log_2(FC) > 0$ (upregulated) or $\log_2(FC) < 0$ (downregulated) with a $P_{adj} < 0.05$ was used to select for NHP-responsive genes. Motif identification and enrichment of CREs were performed using SEA, part of the MEME suite (Bailey et al. 2015; Bailey and Grant 2021) and compared to the published library of TF-binding sites found within the DAPseq database (O'Malley et al. 2016). Only CREs from the DAP-seq library were used in this study, omitting CREs from the ampDAP-seq library (where DNA modifications have been removed). Following the workflow detailed by Bjornson et al. (2021), relative enrichment was determined against the same 3 sets of randomly sampled genes detected in this RNA-seq experiment for all genotypes and time points tested. The identified motif was considered enriched if it returned a significant *P*-value (<0.05) relative to at least 2 of the 3 randomly sampled gene sets. *P*-values for significantly enriched TF-binding motifs were then averaged for visualization.

Measurement of bacterial growth in plants

Chemical treatment of leaves for NHP-elicited SAR assays was performed as described in Chen et al. (2018) with minor alterations. At 4.5 wk old, 3 lower leaves of wild type and mutant *Arabidopsis* plants were infiltrated with sterile water, 0.5 mM NHP or 1 mM NHP. After 24 h, 1 untreated distal leaf of each plant was inoculated with a 1×10^5 CFU/mL suspension of *Psm*. The distal infected leaf was always separated by 1 leaf from the original 3 treated (i.e. primary treatment in leaf no. 7 to 9 then infected leaf is no. 11). Inoculated plants were covered with a dome to increase humidity, and at 3 dpi, the titer of *Psm* in the distal leaves was quantified. Leaf discs were homogenized in 1 mL of 10 mM $MgCl_2$, plated in triplicate in a dilution series on nutrient yeast glycerol medium supplemented with 1.5% w/v agar (NYGA) with rifampicin (100 μg/mL), incubated at 28 °C for 1.5 d, and bacterial colonies counted. For each individual experiment, 4 to 12 plants were tested per condition.

Pathogen priming of leaves for SAR assays was performed as previously described (Chen et al. 2018). Three lower leaves of wild-type and mutant *Arabidopsis* plants (4.5-wk-old) were infiltrated with 10 mM $MgCl_2$ or a 5×10^6 CFU/mL suspension of *Pst avrRpt2* in 10 mM $MgCl_2$. After 48 h, 1 distal untreated leaf of each plant was inoculated with a 1×10^5 CFU/mL suspension of *Psm*, and plants were covered with a dome to increase humidity. At 3 dpi, the titer of *Psm* in the upper leaves was quantified as described above for NHP-elicited SAR. For each individual experiment, 8 plants were tested per condition, and experiments were repeated twice with similar results.

For bacterial growth measurements in unprimed leaves of *Arabidopsis* wild-type and mutant plants, a suspension of 1×10^5 CFU/mL *Psm* was infiltrated into 2 young leaves per plant. Plants were placed under a dome, and at 3 dpi, the 2 infected leaves were pooled and bacterial titer quantified for 12 plants per genotype.

Oxidative burst assay

Four leaf discs (4 mm diameter) from 5-wk-old *Arabidopsis* plants ($n = 12$) were incubated on water, 500 μM NHP, or 100 μM SA in a 96-well plate (1 leaf disc/well) for 24 h. After pretreatment, ROS was measured by the addition of flg22 (100 nM) in a solution of 20-mg/mL horseradish peroxidase and 200 μM luminol (Sigma) (Gómez-Gómez et al. 1999), followed by immediate measurement of luminescence in a Synergy H1 Microplate Reader (Biotek). Relative luminescence units (RLUs) are reported.

Statistical analyses

Two-tailed Student's *t*-tests were performed to determine statistically significant differences between the different conditions for measurements of bacterial growth using the stat_compare_means function of the ggpubr package (Kassambara 2023). *P*-values <0.05 were considered statistically significant, and all significance levels are indicated in the figure legends ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$). Statistical significance of RT-qPCR assays was determined using Mann–Whitney *U* test or 1-way ANOVA with post hoc Sidak test using the ggpubr and lsmeans R packages, respectively (Lenth 2016).

Accession numbers

Sequence data from this article can be found in TAIR (https://www.arabidopsis.org/) under accession numbers: *FMO1* (AT1G19250), *ICS1/SID2* (AT1G74710), *UGT76B1* (AT3G11340), *WRKY38* (AT5G22570), *WRKY54* (AT2G40750), *WRKY70* (AT3G56400), *SARD1* (AT1G73805), *PR1* (AT2G14610), *PR2* (AT3G57260), *PR5* (AT1G75040), *BDA1* (AT5G54610), *PROSCOOP4* (AT5G44568), *CML10* (AT2G41090), and *UBC21* (AT5G25760).

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Author contributions

J.F., J.-G.K., E.S.S., and M.B.M contributed to the study design; J.F. and J.-G.K. performed the research; J.F., J.-G.K., E.S.S., and M.B.M analyzed the data; J.F. and M.B.M. wrote the manuscript; J.F., J.-G.K., E.S.S., and M.B.M. reviewed and revised the manuscript for accuracy and final approval.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Figure S1. NHP-induced *WRKY38* expression is SA-independent.

Supplementary Figure S2. Changes in *WRKY38* mRNA abundance at 3 h in response to NHP concentrations.

Supplementary Figure S3. Profile of early NHP-downregulated genes in wild-type seedlings.

Supplementary Figure S4. Expression of select early strong cluster *WRKY* transcripts in *sid2-2* and *npr1-3* seedlings.

Supplementary Figure S5. Comparison of NHP-downregulated genes in wild type and *sid2-2*.

Supplementary Figure S6. All CREs in the promoters of NHP-upregulated genes in wild-type (Col-0) and *sid2-2* seedlings.

Supplementary Figure S7. All CREs in the promoters of NHP-downregulated genes in wild-type (Col-0) and *sid2-2* seedlings.

Supplementary Figure S8. Loss of *WRKY70* results in enhanced resistance to *Psm* infection and elevated levels of defense transcripts independent of NHP treatment.

Supplementary Figure S9. Expression of NHP-responsive transcripts in *wrky70-1* and *wrky70-2* plants.

Supplementary Figure S10. NHP-enhanced ROS production is compromised in *wrky70-2* plants.

Supplementary Table S1. Full RNA-seq data set of the early transcriptional response to exogenous NHP in Col-0 and *sid2-2*.

Supplementary Table S2. Full set of $\log_2$(FC) values grouped by upregulated and downregulated wild-type gene clusters.

Supplementary Table S3. Primer sequences used in this study.

Supplementary Table S4. Setup of early NHP RNA-seq experiment.

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Data availability

The data underlying this article are available in the article and in its online supplementary material.

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