Vascular mobile signals prime plant immune responses through chromatin manipulation

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Abstract

Globally, disease is responsible for reducing crop yield by over 20% every year. Mitigating these losses will drastically improve global food security, aiding efforts to feed the growing human population. Traditionally, pesticides and breeding are used to reduce disease in plants, but breeding proves too slow to combat rapidly emerging pathogens, pesticides are increasingly regulated due to toxic side effects, and both are losing efficacy as established pathogens evolve. Initial infection can immunise plants to establish systemic acquired resistance (SAR) against future pathogen attacks. SAR is associated with the priming of immune responses, leading to faster and stronger immune activation against a wide range of pathogens. Vascular mobile signals that travel through the phloem are vital for the establishment of SAR throughout the entire plant. The aim of this study is to investigate the roles of mobile signals in immune priming, as these pose a promising avenue for harnessing the plant's own immune system to improve disease resistance.

Phloem mobile phytohormones have been identified as the signals that establish the primed state, but how they induce priming remains unknown. Here, I focus on the interactions between mobile signals and the immune hormone salicylic acid (SA), a key component of SAR. In Chapter 3, I assessed interplay between the mobile signal azelaic acid (AzA) and SA in terms of SA-responsive gene expression and SA-mediated immunity. Using sequential AzA and SA treatments I show that AzA pre-treatment modulates the expression of 74.1% of SA-dependent transcripts, primarily by reducing their SA-responsiveness. GO term analysis revealed that many of the genes targeted by AzA are involved in hypersensitive cell death and suppression of photosynthesis, suggesting AzA acts to balance investment in growth and hypersensitive defence during activation of a primed immune response. Accordingly, AzA was unable to enhance SA-responsiveness of immune responses against *Pseudomonas syringae*, suggesting other signals act to boost immunity, while AzA may balance energy investment.

N-hydroxy-pipecolic acid (NHP) is a phloem mobile signal that has been shown to directly prime plant immunity. In Chapter 4, I investigated the interaction between NHP and SA in establishing immunity. I discovered that NHP affects 75.2% of SA-responsive transcriptome, primarily by priming or cumulatively increasing their expression. GO term analysis showed that NHP primed immune-responsive genes but not those linked to programmed cell death, showing little overlap with AzA targets. I also show that NHP primed SA-mediated immunity against pathogen infection such that in presence of NHP, previously inert concentrations of SA induced resistance against *P. syringae*. Thus, NHP-mediated priming of the SA-responsive transcriptome enhances immune responsiveness. In search of an NHP regulator, I found that NHP stabilised the levels of SA-induced NPR1 protein, a master transcription coactivator of SAR, possibly through post-translational modification. Overall, these data show that NHP primes SA-responsiveness to increase the strength of immune responses, possibly through manipulation of transcriptional regulators such as NPR1.

To understand how NHP primes SA-responsive genes, I then searched for binding motifs in NHP-primed genes. Strikingly, NHP-primed genes were enriched with WRKY family transcription factor binding sites, and after testing several mutants I found that *WRKY38* and *WRKY62* are indispensable for priming. Through RNA sequencing (RNA-seq) I demonstrate that in *wrky38/62* double mutants the majority of NHP responses are dysregulated and immune priming by NHP is lost. Because WRKY38/62 may be involved in chromatin remodelling, I investigated the impact of NHP on chromatin accessibility through Assay for Transposase-Accessible Chromatin sequencing (ATAC-seq). Here, I found that NHP dramatically reorganises chromatin accessibility, but this effect is largely abolished in absence of *WRKY38/62*. Comparison between NHP-primed genes and NHP-dependent chromatin reorganisation showed that NHP mediates priming primarily through WRKY38/62-dependent chromatin remodelling.

In summary, I demonstrate phloem mobile signals have previously unrecognised roles in SAR and suggest that several phytohormones work in tandem to optimise and regulate the process of immune priming. By identifying the key priming regulators WRKY38 and WRKY62, I propose targets for either chemically-induced or gene-edited induction of priming, which could be used to protect a variety of crop species without limiting yield or damaging the environment.

Lay summary

Disease destroys over 20% of crops every year, threatening food security for the ever-growing human population. Traditional disease control measures such as pesticides and breeding are losing efficacy, so new approaches are needed to protect food production. In plants, a process called systemic acquired resistance (SAR) is activated after infection in which the plant enters a state of heightened disease resistance. SAR is controlled by mobile signals that travel in the plant vascular system. These signals could be harnessed to activate SAR and protect plants without prior exposure to disease. The aim of this study is to investigate the roles of mobile signals in SAR to assess their viability as crop protective agents. My results suggest that the mobile signal N-hydroxy-pipecolic acid (NHP) enhances disease resistance, while the mobile signal azelaic acid (AzA) reduces the side effects of enhanced resistance. AzA reduces gene expression in immune pathways linked to cell death and suppression of growth, while NHP enhances expression in distinct pathways linked to immunity and stress responses. NHP was found to stabilise key immune regulators, suggesting it could prime immunity by promoting accumulation of inactive signalling components, poised to respond in future. I also identify a mutant that displays abolished NHP signalling. The mutation was in a pair of transcription factors (TFs), that regulate gene expression by interacting with DNA. Previous research suggests these TFs are linked to regulation of DNA at the epigenetic level, which considers change to DNA storage and regulation without altering DNA sequences. Indeed, NHP was found to alter DNA accessibility across the genome, and many ‘peaks’ of accessibility correlate with genes enhanced by NHP. This process is lost in the mutants, suggesting that the loss of NHP-responsive gene expression in these mutants is because they lose NHP-responsive epigenetic modification. Overall, this study shows that different mobile SAR signals have different effects on plant immunity and identifies a pathway to explain how they prime immune responses.

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Key abbreviations and acronyms

ABA	abscisic acid
ATAC	assay for transposase-accessible chromatin
AzA	azelaic acid
BABA	β-aminobutyric acid
BTH	benzothiadiazole
CFU	colony forming units
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
DA	dehydroabietinal
DAMP	damage associated molecular pattern
DAP	DNA affinity purity
DDT	dichlorodiphenyltrichloroethane
DTT	dithiothreitol
ET	ethylene
ETI	effector triggered immunity
FDR	false discovery rate
G3P	glycerol-3-phosphate
GE	gene edited
GFP	green fluorescent protein
GM	genetically modified
GO	gene ontology
H_2O_2	hydrogen peroxide
HR	hypersensitive response
INA	2,6-dichloroisonicotinc acid
ISR	induced systemic resistance
JA	jasmonic acid
LPS	lipopolysaccharide

MES	2-(N-morpholino)ethane-sulfonic acid
MeSA	methyl salicylate
NGT	new genomic techniques
NHP	n-hydroxy pipecolic acid
NLR	nucleotide binding leucine rich repeat receptor
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered solution
PCD	programmed cell death
PGPM	plant growth promoting microbe
Pip	pipecolic acid
PRR	pattern recognition receptor
PSII	photosystem II
Psm	*Pseudomonas syringae pv. maculicola*
PTI	pattern triggered immunity
PTM	post translational modification
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
SINC	salicylic acid-induced NPR1 condensate
TES	transcription end site
TF	transcription factor
TSS	transcription start site
TUBE	tandem ubiquitin binding entity
Ub	ubiquitin
WT	wild type

Chapter 1 – General Introduction

1.1 – The current state of crop protection

With the global population forecast to reach ~10 billion by 2050, food production needs to improve by 50-60% to meet increasing demand (Ray *et al.*, 2013; Van Dijk *et al.*, 2021). Furthermore, population and yield models struggle to consider the impact of fluctuating events such as conflicts, pandemics, economic challenges and climate change, so in reality, the improvements may need to be even larger (Van Dijk *et al.*, 2021). While crop yields are still increasing globally, they are stagnating in most major crop producing countries, meaning staple crop yields are only forecast to increase 40% by 2050 (Ray *et al.*, 2013). When considering climate change and other events, this 40% may be a large overestimate, and it appears unlikely that yields will improve significantly if no further advances in crop growth are made.

A significant contributor to stagnating crop yield is damage from pests and pathogens. In major staple crops, 20-30% of potential yield is lost to pests and pathogens, causing around $220 billion damage annually (Savary *et al.*, 2019; Singh *et al.*, 2023). Another major threat is climate change, which is predicted to directly impact yield and offset increases coming from recent advances (Singh *et al.*, 2023). Climate change and disease are inherently linked, as increased temperatures and water fluctuations will both weaken plant defences and create more favourable habitats for pathogen spread. For example, *Pseudomonas syringae* favours high humidity and mild temperatures, so warmer winters and wetter springs are increasing survival and pathogenicity of *P. syringae* in the UK (Xin *et al.*, 2018). Furthermore, emerging pathogens are becoming a serious threat as conditions change in their favour, including the fungal leaf spot pathogen *Ramularia collo-cygni*, which is increasingly becoming a major problem for barley production in the British Isles (Dussart *et al.*, 2020; Delpont *et al.*, 2025). Alterations in climate conditions also promote pathogen range shifts, which may prove devastating as farmed crops have no evolved defences against migrating pathogens. Pathogens such as *Xylella fastidiosa*, an olive infecting bacterium, and *Phytophthora ramorum*, the cause of ash dieback, have spread from southern Europe through trade and are taking foothold in Britain as summers get warmer (Castro *et al.*, 2021; Green *et al.*, 2021).

Contributing to the complexity of biotic threats, not all pests behave the same. Broadly, plant pests can be separated into biotrophs, necrotrophs and herbivores, all with distinct attack strategies. Biotrophic pathogens colonise living tissues to parasitise host resources. Depending on the species of biotroph, this can be through the development of feeding structures such as haustoria, release of cell wall degrading enzymes or use of type III secretion systems to manipulate host defence and metabolism (Fei and Liu, 2023). Conversely, necrotrophic pathogens directly kill host cells to harvest nutrients. Necrotrophs are also diverse, as some species directly release toxins to damage host tissues and others use intracellular signals to trigger host programmed cell death and autophagy responses (Mengiste, 2012). As is often the case, the distinction between biotrophs and necrotrophs is not clear cut. Hemi-biotrophs are a class of pathogens that colonise the plant as biotrophs but switch to necrotrophic infection strategies as they mature and spread. This is very common in bacterial pathogens and is increasingly found in fungi (Kraepiel and Barny, 2016; Sanchez-Vallet *et al.*, 2015). Furthermore, some pathogens switch between noninfectious and pathogenic states. *Ramularia collo-cygni* acts initially as a barley endophyte, growing within the plant without consequence, but life cycle changes in the host trigger a switch to necrotrophy, which results in rapid disease symptoms in the host plant (Dussart *et al.*, 2020). Distinct from microbial pathogens, herbivores are macroscopic plant pests, feeding on plant leaves and roots by chewing or harvesting sap from the vasculature (Savary *et al.*, 2019). While herbivores are distinct from pathogens, the plant response to herbivores is often similar to that of necrotrophs, as both pests cause damage to tissues.

Farmers have deployed several responses to combat the rising spread of pathogens and maintain high yields. One commonly used crop protection measure is the application of fungicides and other antimicrobial chemicals. Without pesticides, yields would reduce by 78% in fruit, 54% in vegetables and 32% in cereals (Tudi *et al.*, 2021). As a major contributor to the green revolution, pesticide usage rapidly rose since the 1950s, increasing from 0.2 million tons produced per year to over 6 million by 2005 (Carvalho, 2017). Such extensive use comes with drawbacks. Recent studies suggest that only 1% of applied fungicide or pesticide effectively combats pathogens, with 2% - 25% drifting off target by wind or other adverse weather conditions, and the rest soaking into the soil (Tudi *et al.*, 2021). Due to the high rate of use, fungicides and pesticides are also decreasing in efficacy as repeat exposure promotes proliferation of resistant pathogen strains (Hawkins *et al.*, 2018). Both of these factors result in application of increased volumes and strengths of these chemicals, which negatively effects local ecology and the environment. Due to the diversity of pest species and infection strategies, farmers often have to apply several classes of chemicals at once. Some chemicals protect the external plant tissues from microbial colonisation and some act as plant activators by being taken up into the plant to protect from within, and both types must be added several times during the growing season to maximise protection from all threats. The production of fungicides and pesticides is energetically costly (Zhang, 2018), so as demand for single and mixed pesticides rises, so does chemical and environmental pollution. Furthermore, several high-profile pesticides have been banned due to toxic side effects on humans and local wildlife. Dichlorodiphenyltrichloroethane (DDT) was famously banned in the 1970s (Grier, 1982) as it built up to dangerous concentrations in the soil, while neonicotinoids were recently banned in the UK as they were proven toxic to many pollinators (Matsuda *et al.*, 2020). Damage to local wildlife negatively impacts yield, as insects are often required to promote pollination and antimicrobials can limit the growth and colonisation by beneficial microbes, such as mycorrhizas, which reduce yield in the long term.

This has created a vicious cycle. Evolving pests require increased pesticide application, which damages local wildlife and promotes faster pest evolution, which in turn further damages yield. Although they remain helpful measures, fungicide and pesticide usage are becoming increasingly inefficient and unsustainable, so new approaches to crop protection are urgently needed to sustain and develop global crop yields. One possible approach is to harness and boost the plants own immune system, as enhancing pathogen-specific resistance will limit off-target effects on other species and function in a system that will evolve with the pathogen. To exploit and enhance the plant’s own defences against pathogens, we must first understand how the plant immune system functions.

1.2 – The plant immune system

1.2.1 – Pattern- and effector-triggered immunity

Early plant immune signalling can be separated into two broad categories: pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI is activated by the recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Jones and Dangl 2006). PAMPs include microbial proteins, such as flagellin, chitin and elongation factor Tu, while DAMPs include intracellular molecules, such as ATP, systemin and elicitor peptides (Choi and Klessig 2016). PAMPs and DAMPs are detected at the cell surface by transmembrane pattern recognition receptors (PRRs). Upon ligand recognition, PRRs recruit co-receptors to form a complex that activates downstream signalling through receptor-like cytoplasmic kinases (Sun *et al.*, 2013). Activation of PTI results in several downstream responses, including calcium (Ca_{2+}) signalling, reactive oxygen species (ROS) bursts and kinase signalling (Peng *et al.*, 2018). Together, these signalling pathways promote transcriptional reprogramming to induce the expression of immune genes, including those encoding antimicrobial proteins, and physiological responses, such as stomatal closure and callose deposition in cell walls to prevent pathogen spread (Yuan *et al.*, 2021).

Under the high selective pressure of PTI, plant pathogens have evolved intracellular ‘effector’ proteins that are injected into the plant cell via a type III secretion system, and target PTI signalling hubs to inhibit immune responses. In the so called ‘zig-zag’ model of immunity (Jones and Dangl, 2006), ETI evolved in plants as a measure of combating pathogen effectors. ETI is activated by intracellular nucleotide binding leucine-rich repeat (NLR) receptors. The number of effector proteins far outnumbers the number of identified NLRs (DeYoung and Innes, 2006), so it is unlikely that each NLR directly targets an effector. Instead, NLRs detect effector action on effector targets as several distinct effectors target conserved signalling hubs (Macho and Zipfel, 2015). For example, RPM1 is an NLR that activates immune signalling when the PTI component RPM1-INTERACTING PROTEIN 4 (RIN4) is phosphorylated by any effector (Chung *et al.*, 2011). ETI promotes accumulation of PTI signalling components, but also often triggers programmed cell death in a process called the hypersensitive response (HR) (Ngou *et al.*, 2020; Yuan *et al.*, 2021). With these results in mind, it has been proposed that ETI signalling potentiates PTI signalling to more effectively respond upon pathogen detection (Ngou *et al.*, 2020).

1.2.2 – Salicylic acid- and NPR1-mediated immunity

A shared aspect of PTI and ETI is the biosynthesis of salicylic acid (SA). SA signalling has been associated with abiotic stresses such as heavy metal toxicity and drought tolerance, but is primarily a driver of responses to biotrophic pathogens (Rivas-San Vicente and Plasencia 2011). In plants, SA is produced through one of two pathways: the phenylalanine ammonia lyase (PAL) and the isochorismate pathways (Lefevere *et al.*, 2020; Figure 1.1). In the PAL pathway, phenylalanine is converted to SA through the key enzyme PHENYLALANINE AMMONIA LYASE 1 (PAL1) (Klessig *et al.*, 2018). In the isochorismate pathway, chorismate is converted to SA through the intermediary isochorismate and the key enzyme ISOCHORISMATE SYNTHETASE 1 (ICS1) (Wildermuth *et al.*, 2001). The relative importance of each pathway varies depending on the species, with the isochorismate pathway the most important during infection in *Arabidopsis*

(Klessig *et al.*, 2018; Duan *et al.*, 2014). SA biosynthesis is regulated at several molecular layers. The transcription factors SAR DEFICIENT (SARD1) and CALMODULIN-BINDING PROTEIN 60g (CBP60g) target the *ICS1* promoter to promote SA biosynthesis. Despite their name, the CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR (CAMTA) family of transcription factors inhibit SA biosynthesis by blocking *SARD1*/*CBP60g* expression. Another important regulator is CCA1 HIKING EXPEDITION (CHE), which controls circadian regulation of SA biosynthesis by promoting *ICS1* expression in anticipation of sunrise (Zheng *et al.*, 2015). Several other transcription factors are involved in promoting or suppressing SA biosynthesis, including members of the WRKY, NAC, and Ethylene responsive (ERF) families (Spoel and Dong, 2024).

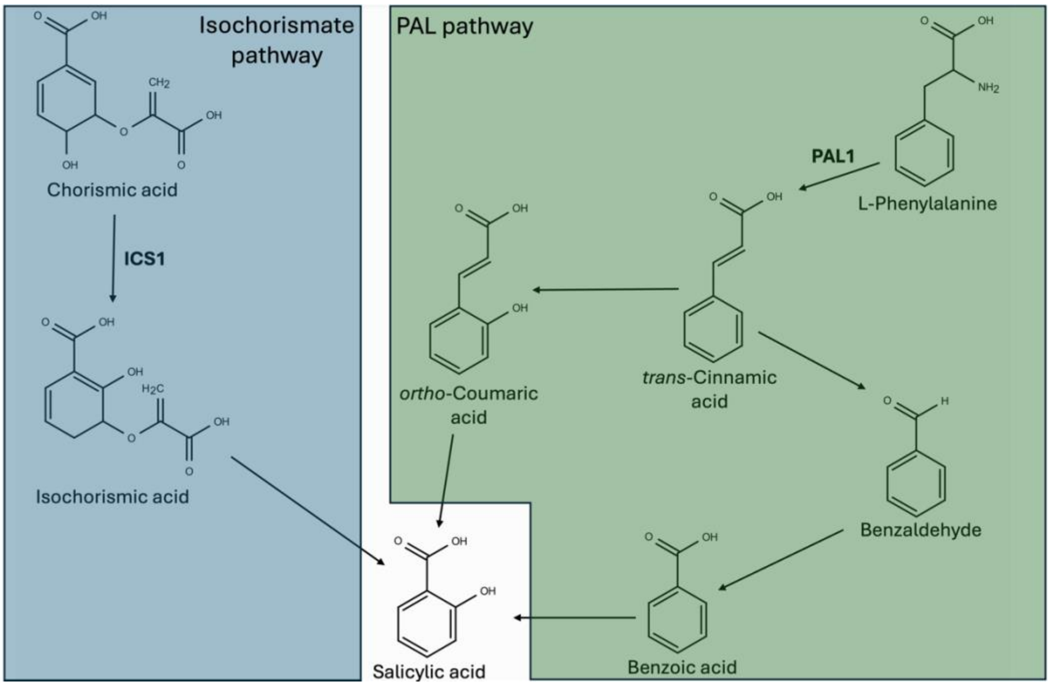


Figure 1.1 – Biosynthesis of salicylic acid in plants. *The isochorismate and PAL pathways are highlighted. Steps catalysed by ICS1 and PAL1 are highlighted in bold. Adapted from Klessig et al. (2018).*

Several potential receptors of SA have been identified, including over 100 SA-binding proteins (SABPs) (Manohar *et al.*, 2015). CATALASE 2 is a key SA target, as SA binding inhibits redox scavenging and triggers ROS accumulation during the pathogen-induced ROS burst (Chen *et al.*, 1993; Conrath *et al.*, 1995). Similarly, SA binds the thioredoxin protein TRX1, which may also promote redox signalling (Manohar *et al.*, 2015). Accumulation of SA alters the expression of approximately 20% of the *Arabidopsis* genome (Wang *et al.*, 2006), suggesting transcriptional regulators are also important SA signal-transducers. Indeed, a key group of SA signalling hubs are the transcriptional co-activator NONEXPRESSOR OF PATHOGENESIS RELATED 1 (NPR1) and the SA-binding transcriptional regulators NPR3 and NPR4.

NPR1 is composed of a central BTB domain (Broad-complex, Tramtrack and Bric-à-brac), a kelch helix bundle, four ankyrin repeats and a disordered SA-binding domain (Kumar *et al.*, 2022). In inactive cells, NPR1 oligomerises in the cytosol in an inactive configuration covalently held together by disulfide bonds between conserved cysteine residues (Tada *et al.*, 2008). SA-mediated cellular reduction together with TRX-h3 and TRX-h5 reduce these cysteine bonds to release NPR1 dimers, which translocate into the nucleus (Mou *et al.*, 2003). As NPR1 has no DNA binding domain, it must act in tandem with transcription factors to promote gene expression. The recent discovery that NPR1 acts as a dimer suggests that NPR1 may bridge multiple transcription factors to promote and enhance target expression. NPR1 primarily interacts with TGA family transcription factors (TFs), but also binds WRKY and MYC family TFs (Zhou *et al.*, 2000; Saleh *et al.*, 2015; Nomoto *et al.*, 2021; Powers *et al.*, 2024), demonstrating that it can regulate several response pathways.

The activity of nuclear localised NPR1 is tightly controlled by post-translational modifications (Figure 1.2). In addition to NPR1, it has been conclusively proven that SA can bind the NPR1 homologs, NPR3 and NPR4 (Fu *et al.*, 2012). NPR3/4 act as substrate adapters for ubiquitin CULLIN-RING LIGASE 3 (CUL3), promoting ubiquitination of NPR1. Prior to its ubiquitination, nuclear NPR1 is sequentially phosphorylated, SUMOylated and dephosphorylated at diverse residues (Spoel *et al.*, 2009; Saleh *et al.*, 2015). This recruits CUL3 to add short ubiquitin chains on NPR1, which enhances its transcription activator activity (Spoel *et al.*, 2009). To curtail NPR1's activity and associated immune responses, short ubiquitin chains added by CUL3 are recognised and extended by the E4 ligase UBE4 (also known as MUSE3), which promotes inactivation and degradation of NPR1 (Skelly *et al.*, 2019). This process is dynamic, as the deubiquitinases UBIQUITIN SPECIFIC PROTEASE 6 and 7 (UBP6/7) can shorten these ubiquitin chains and maintain NPR1 in the transcriptionally active state (Skelly *et al.*, 2019). The proteasome-associated ubiquitin ligases, UPL3 and UPL4, are also required for NPR1 regulation, further extending UBE4-generated chains on NPR1 to promote proteasome processing and subsequent degradation (Wang *et al.*, 2022). Interestingly, the immune response is perturbed when degradation of NPR1 is inhibited (Spoel *et al.*, 2009; Skelly *et al.*, 2019; Wang *et al.*, 2022), suggesting that this tight activation and degradation of NPR1 is indispensable for effective immune signalling.

Beyond transcriptional activation, NPR1 also promotes immunity by regulating protein stability. Sustained high SA levels induce the formation of cytoplasmic condensates, known as SINCs (SA-induced NPR1 condensates). Cytoplasmic SINCs (cSINCs) are enriched with NPR1, ubiquitination machinery and stress signalling proteins (Zavaliev *et al.*, 2020). In particular, NPR1 recruits CUL3 to cSINCs to promote ubiquitin-mediated degradation of the proteins sequestered within. Overall, these results demonstrate that NPR1 is also an important mediator of protein stability and degradation as well as transcriptional regulation during SA signalling. The role of NPR1-mediated degradation of stress proteins may be to promote cell survival during ETI by removing HR regulators, such as ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and PHYTOALEXIN DEFICIENT 4 (PAD4) (Zavaliev *et al.*, 2020). Further research into SINCs also uncovered nuclear NPR1 condensates, enriched with transcriptional regulators and chromatin remodelling proteins (Powers *et al.*, 2024). Nuclear SINCs are likely contributing to NPR1-mediated transcriptional reprogramming, but the presence of histone modifying proteins within the SINCs reveals another level of NPR1 regulation. NPR1 simultaneously interacts with histone acetyltransferases (Jin *et al.*, 2018), suggesting that NPR1 also modifies the epigenetic landscape of the cell.

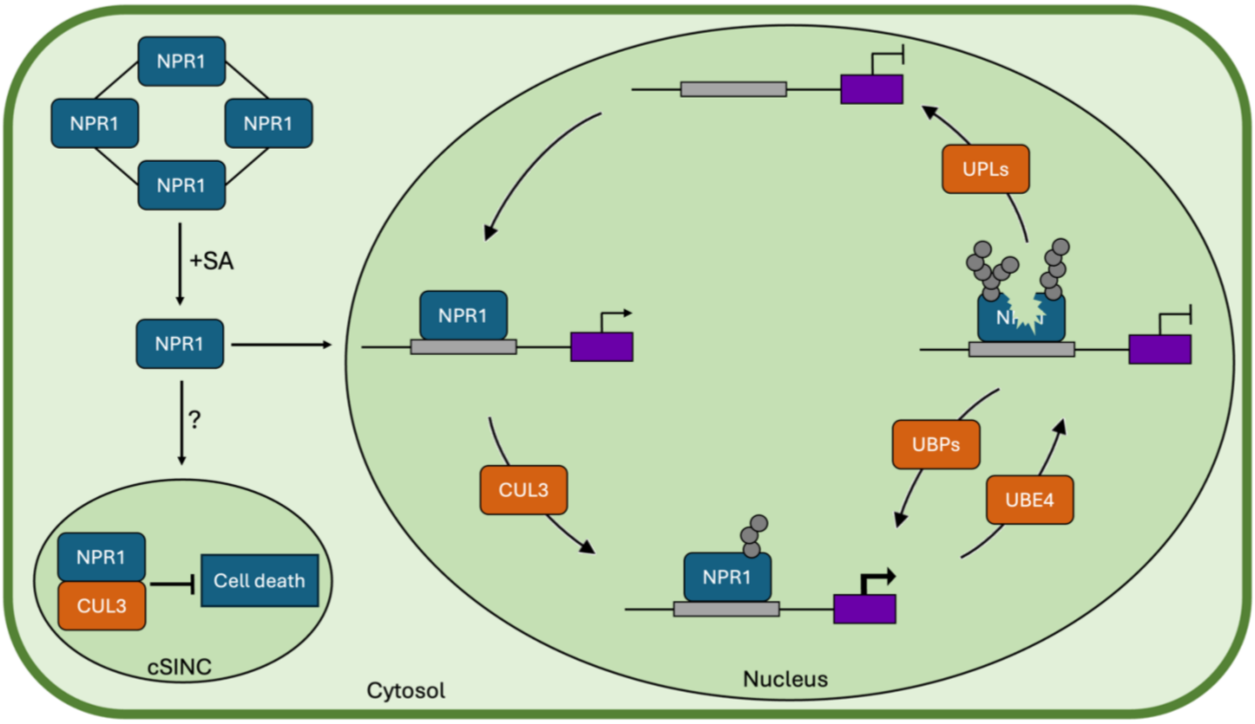


Figure 1.2 – NPR1 signalling and regulation. *NPR1 is released from the oligomer after SA accumulation and translocates to the nucleus. In the nucleus, gene expression is enhanced by CUL3-mediated ubiquitination, then gene expression is suppressed by ubiquitin chain elongation by UBE4. UBP6/7 (UBPs) act against UBE4 and prolong the time NPR1 is in the 'active' state. NPR1 ubiquitinated by UBE4 is marked for proteasome degradation, which is enabled by further ubiquitination by UPL3/4 (UPLs). Some cytosolic NPR1 may translocate into cSINCs, where it acts*

in tandem with CUL3 to suppress cell death signalling. For simplicity of illustration, one 'NPR1' node denotes an NPR1 dimer.

1.3 – Systemic immunity and immune memory

1.3.1 – Systemic acquired resistance

Plant immune signalling is not restricted to just the infected cell or tissue. In 1901 it was observed that previously infected plants displayed enhanced disease resistance (Beauverie, 1901; Ray, 1901), demonstrating that plants contain a mechanism of immune memory. The next breakthrough came in 1961, when it was found that areas of a plant not exposed to infection were still immunised against future pathogen attack (Ross *et al.*, 1961). This immune response is now known as systemic acquired resistance (SAR), where upon pathogen challenge, unchallenged systemic tissues develop resistance to future infections (Ryals *et al.*, 1996). SAR is predominantly established in response to biotrophic pathogens, so does not usually protect against necrotrophs or herbivores. However, at a species level, it provides broad-spectrum resistance (Ross *et al.*, 1961) in that infection by a biotrophic bacterium induces resistance against biotrophic bacteria, fungi and viruses. SA is indispensable for SAR, as SA biosynthesis mutants and grafted plants expressing the SA hydroxylase *NAHG* in systemic tissues, display impaired SAR (Wildermuth *et al.*, 2001; Vernooij *et al.*, 1994). This demonstrates SA is required for SAR specifically in systemic tissues. In addition to SA, NPR1 is also required for launching and establishing SAR (Dong, 2004; Yildiz *et al.*, 2021).

The core feature of SAR is a process called priming. Primed plants have sensitised immune responses, meaning they respond faster and stronger to lower levels of immune stimuli. The duration of priming is variable. Enhanced immunity often persists for a matter of days or weeks in lab conditions, but depending on species, this state can last for months or for a whole growth season (Conrath, 2006). While SAR may eventually fade within an individual, there is evidence that it is also heritable. In *Arabidopsis*, the progeny of immune stressed plants inherit a primed state, displaying increased immune responsiveness despite not having experienced prior pathogen exposure (Luna *et al.*, 2012). Research into priming has revealed it is primarily established through epigenetic modifications and accumulation of inactive signalling components (Conrath *et al.*, 2015; Harris *et al.*, 2023).

Epigenetic modifications such as DNA methylation and histone modification are established in response to environmental stimuli and are often heritable, making them ideal propagators of rapid adaptation to the environment. DNA methylation promotes heterochromatin formation, suppressing gene expression at key points across the genome such as transposons and centromeres (Fedoroff, 2012). Histone modifications are considerably more dynamic. Histone subunits contain exposed ‘tails’, which can be post translationally modified to increase or decrease DNA accessibility, modulate histone stability, and regulate protein recruitment to the DNA (Jenuwein and Allis, 2001). The mammalian innate immune system displays a similar priming response to plants, wherein frequent pathogen exposure ‘trains’ immune cells to increase responsiveness over a period of time (Conrath, 2025). Epigenetic modifications are a well characterised component of immune training (Van der Heijden *et al.*, 2018), giving precedent for the role of epigenetics in priming. Indeed, plants displaying transgenerational immune priming contain high levels of differential DNA methylation, with demethylation inducing a dose-dependent effect on immunity (Stassen *et al.*, 2018). Histone modifications are also associated with priming, as specific histone methylation marks correlate with increased pathogen-responsive gene expression (Jaskiewicz *et al.* 2010).

Another important mechanism of priming is accumulation of inactive signalling molecules. This is a broad category, covering proteins and amino acids that contribute to immune signalling but do not directly regulate the response. For example, SAR activation triggers accumulation of the MAP kinases MPK3 and MPK6 (Beckers *et al.*, 2009), which promotes strong PTI signalling cascades when activated by bacterial flagellin receptor FLAGELLIN SENSITIVE 2 (FLS2) (Asai *et al.*, 2002). Therefore, accumulation of MPK3/6 prior to infection will poise immune signalling, so in the next instance of pathogen perception a much larger pool of MPKs will be activated by FLS2, increasing the intensity of the response. Inactive signalling components also act to increase the rate of pathogen perception, and thus the speed of a response. The PRRs FLS2, BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) and CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) all accumulate after SA treatment, which increases both the breadth of surveillance and the speed of pathogen recognition (Tateda *et al.*, 2014).

1.3.2 – Induced systemic resistance

While SAR primes immune responses against biotrophic pathogens, immune responses against necrotrophic pathogens are primed in a response known as induced systemic resistance (ISR). ISR is induced by colonisation of plant roots by plant growth promoting microbes (PGPM) and provides lasting priming against hemi-biotrophic and necrotrophic pathogens, as well as herbivorous insects. ISR was independently discovered by three groups in 1991, with each group demonstrating that a different species of PGPM enhanced immunity against pathogen attack (Alstrom *et al.*, 1991; Wei *et al.*, 1991; Van peer *et al.*, 1991). Several bacterial and fungal species have been linked to ISR, including *Rhizobium* species, *Pseudomonas fluorescens*, *Bacillus cereus*, *Trichoderma harzianum* and arbuscular mycorrhizas (Yu *et al.*, 2022). While the precise mechanisms of priming involved in ISR have not yet been identified, ISR primes jasmonic acid (JA) and ethylene (ET) signalling to boost immunity to necrotrophic pathogens and herbivores (Pieterse *et al.*, 2014; Flors *et al.*, 2024).

ISR is established by soilborne PGPMs when they colonise or interact with plant roots. Specialist plant symbiotes such as *Rhizobium* and mycorrhizas form nodules and arbuscules respectively in host roots through well studied signalling pathways (Long, 1996; Helgason and Fitter, 2009), while other PGPMs often form biofilms between root cells (Rudrappa *et al.*, 2008). Following PGPM recognition, the signalling mechanisms activated in systemic tissues vary between colonising species. For example, *T. harzianum* induces JA signalling driven by transcription factors WRKY8 and WRKY33, *P. fluorescens* triggers Ca_{2+} signalling, and *B. cereus* induces expression of both SA and JA/ET marker genes (Yu *et al.*, 2022). As SA signalling is activated by some PGPMs, there is some debate as to whether ISR is an extension of SAR or its own process. Unlike SAR, however, JA signalling transcription factors MYB72 and MYC2 are indispensable for establishing ISR (Van der Ent *et al.*, 2008; Kazan and Manners, 2013). Moreover, ISR is also independent of SA and at least in some cases appears to require cytosolic NPR1 signalling rather than nuclear (Stein *et al.*, 2008), suggesting that ISR is a distinct signalling pathway from SAR. Interestingly, both NPR1 and WRKY70 contribute to ISR regulation (Jiang *et al.*, 2016), suggesting extensive interplay between SA and JA signalling during systemic immunity.

1.3.3 – Mobile immune signals

A wide variety of mobile signals have been proposed as regulators of systemic immunity and immune memory. In contrast to ISR, the mobile signals driving SAR are much better understood (Figure 1.3). ROS, such as hydrogen peroxide (H_2O_2) are a component of cell-to-cell immune responses. H_2O_2 is produced and released into the apoplast by RESPIRATORY BURST HOMOLOG D (RBOHD), where it then activates RBOHD in neighbouring cells to propagate immune signalling (Miller *et al.*, 2009). Recently it has been shown that H_2O_2 from neighbouring cells promotes CHE-mediated *ICS1* expression (Cao *et al.*, 2023), so ROS promotes the spread of SA biosynthesis throughout the plant.

Several signals that drive local immune responses are also linked to systemic immunity. As SA accumulates in both the phloem and systemic tissues during SAR, it was proposed as a mobile signal. However, despite being transported, grafting experiments demonstrated that mobile SA is dispensable for the establishment of SAR (Vernooij *et al.*, 1994). It was then proposed that SA is transported in the form of methyl-salicylate (MeSA), with MeSA converted back to SA in systemic tissues by SA-BINDING PROTEIN 2 (SABP2) (Park *et al.*, 2007). However, these conclusions are disputed, as most MeSA was found to be released as a volatile rather than transported in the phloem (Attaran *et al.*, 2009). Instead, MeSA has been proposed as an inter-plant volatile signal, but this has also been disputed (Shulaev *et al.*, 1997; Attaran *et al.*, 2009), so the role of MeSA in SAR remains contentious. JA has recently been linked to phloem mobile signalling, as SAR signal generation and perception are dependent on jasmonate biosynthesis (Gaikwad *et al.*, 2023). This finding provides further evidence that SA and JA both contribute to systemic signalling pathways.

On the other hand, some proposed SAR-propagating signals have no known function in local immunity. Azelaic acid (AzA) is a 9-carbon fatty acid found to accumulate in the phloem of infected plants. AzA treatment primes pathogen-induced SA biosynthesis and SA-responsive gene expression (Jung *et al.*, 2009). While AzA is undoubtably a component of SAR, whether it primes immunity unaided is unclear. Consensus on whether AzA can or cannot prime plant immune responses has not been reached, with multiple studies supporting each side (Jung *et al.*, 2009; Cecchini *et al.*, 2018; Zoeller *et al.*, 2012; Nagy *et al.*, 2016). Interestingly, AzA has also shown capacity to prime immunity against necrotrophic pathogens (Haghpanah *et al.*, 2024), so could potentially contribute to ISR signalling. Another lipid derived signal is glycerol-3-phosphate (G3P), which accumulates in petiole extracts of infected leaves and is translocated to systemic tissues (Chanda *et al.*, 2011). SAR is impaired in G3P-deficient mutants, and G3P application boosts resistance, suggesting it is another mobile SAR signal. However, G3P is only capable of inducing SAR if combined with immune active phloem sap (Chanda *et al.*, 2011), indicating it requires another phloem mobile cofactor to effectively induce immunity.

AzA and G3P are closely linked, so could both be required for SAR induction. AzA induces the expression of GLYCEROL INSENSITIVE 1 (GLI1) and G3P DEHYDROGENASE 1 (GLY1), both of which promote G3P biosynthesis (Yu *et al.*, 2013). Furthermore, the homologous lipid transfer proteins AZELAIC ACID INDUCED 1 (AZI1) and DEFECTIVE IN

INDUCED RESISTANCE 1 (DIR1) are both required for AzA- and G3P-induced SAR. G3P is transported in tandem with DIR1, suggesting that AzA, G3P and DIR1/AZI1 are all transported together, which may explain why each signal alone is often insufficient to consistently prime immunity. Furthermore, dehydroabietinal (DA) is an abietane diterpenoid found in phloem sap after infection. DA induces the accumulation of SA in systemic tissues, dependent on DIR1 and enhanced by AzA (Chaturvedi *et al.*, 2012), suggesting DA further contributes to the multi-signal AzA-G3P ‘cocktail’.

The best studied mobile SAR signal is N-hydroxy pipecolic acid (NHP). The NHP precursor, pipecolic acid (Pip), was found to accumulate in systemic tissues after infection, with NHP later discovered as the bioactive form (Navarova *et al.*, 2012; Hartmann *et al.*, 2018). NHP is sequentially synthesised from L-lysine by AGD2-LIKE DEFENCE PROTEIN 1 (ALD1), SAR DEFICIENT 4 (SARD4) and FLAVIN MONOOXYGENASE 1 (FMO1) (Hartmann *et al.*, 2017; Ding *et al.*, 2016; Hartmann *et al.*, 2018). Mutations in any of these biosynthetic enzymes abolish SAR, demonstrating that NHP is a vital component of systemic signalling. Interestingly, NHP signalling in systemic tissues is partially SA-dependent and entirely NPR1 dependent (Bernsdorff *et al.*, 2016), suggesting that NHP primarily activates SA signalling but may also contribute to SA-independent, NPR1-mediated signalling pathways. NHP induces a strong transcriptional response in systemic tissues, which primes SA biosynthesis, antimicrobial metabolite production, and pathogen- and SA-responsive gene expression (Yildiz *et al.*, 2021; Lowe *et al.* 2023). Accordingly, and unlike the other mobile signal candidates, NHP consistently increased pathogen resistance across studies (Yildiz *et al.*, 2021; Nair *et al.* 2021; Foret *et al.*, 2025), suggesting that NHP alone is sufficient to induce SAR in plants.

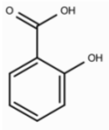
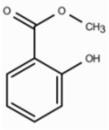
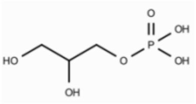
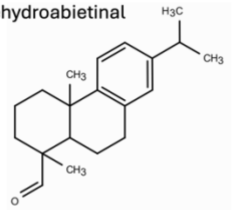
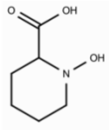
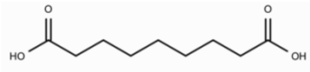
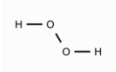
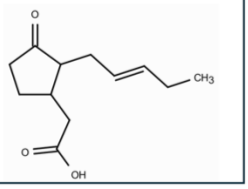
Not a phloem mobile signal	Salicylic Acid 
Possible phloem mobile signal	Methyl – Salicylate  Glycerol-3-Phosphate  Dehydroabietinal 
Confirmed phloem mobile signals	N-Hydroxy-Pipecolic Acid  Azelaic Acid  Hydrogen peroxide  Jasmonic acid 

Figure 1.3 – Structures of proposed phloem mobile signals. *Each signal is grouped by whether they are confirmed as a mobile signal, may be a mobile signal or are confirmed to not be a mobile signal.*

1.4 – Harnessing the plant immune system for crop protection

1.4.1 – Immune elicitors

The idea of harnessing plant immune systems for crop protection is not new. Since the discovery of SAR and the role of SA (White, 1979), efforts have been made to induce resistance in crops without the need for prior infection. Several synthetic SA analogues have been produced and used to induce SAR, such as 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) (Conrath *et al.*, 1995; Friedrich *et al.*, 1996). Another promising plant protective compound is β-aminobutyric acid (BABA). BABA naturally accumulates in plants in response to stress, and triggers induced resistance against necrotrophic pathogens (Thevenet *et al.*, 2017). Derivatives of these compounds and common pathogen markers, such as chitin, have been developed into commercial products such as Bion, Actiguard and Chitosan. However, despite their effectiveness in protecting plants, commercial elicitors have struggled to break into the agrochemical market (Yassin *et al.*, 2021).

The main challenge for many commercial elicitors is that they are derived from SA, and so while they trigger SAR, they also induce local immune responses upon application. In plants, resources are balanced between development and immunity in the so called ‘growth/defence-tradeoff’ (He *et al.*, 2022). Consequently, application of most elicitors was shown to reduce yield as they directly triggered immune responses (Heil *et al.*, 2000) and thus are not desirable in a commercial setting. These effects were more pronounced in crops with nutrient deficiencies (Heil *et al.*, 2000), resulting in the use of elicitors only when a pathogen challenge is already encountered, rather than for priming immunity. Some elicitors induce weak resistance without impacting yield if applied at low concentrations, and using low doses of multiple agents appears to induce resistance without trade-off (Yassin *et al.*, 2021). However, some elicitors vary in efficacy across crop cultivars and pathogen strains, meaning higher doses are required to induce resistance in certain conditions (Walters *et al.*, 2011). Overall, to ensure a given cultivar is appropriately induced, a field will need multiple applications of multiple elicitors across the growing season, which is energetically inefficient and may harm yield. Furthermore, some elicitors have shown toxic side effects on host plants. While BABA induces effective resistance against pathogens, excessive application perturbs amino acid homeostasis by inhibiting an aspartyle-tRNA synthetase (Luna *et al.*, 2014). Efforts have been made to identify BABA analogues that activate induced resistance without side effects by modelling potential interactors of the BABA receptor IMPAIRED

IN BABA-INDUCED IMMUNITY 1 (IBI1) (Buswell *et al.*, 2018). Here, (*R*)-β-homoserine (RBH) was identified as an elicitor that effectively primes immunity without growth trade-off.

With the discovery of phloem mobile signals that do not appear to influence local resistance, more potential elicitors have emerged. While the receptors of NHP, AzA, G3P and DA have not yet been identified (Vlot *et al.*, 2021), no growth trade-off has been seen in model organisms treated with these phloem mobile SAR inducers. Therefore, the confirmed phloem mobile signals (Figure 1.3) all remain promising candidates for commercial application as immune elicitors if their effects remain robust in field conditions.

1.4.2 – Genetic engineering

While phloem mobile signals are promising elicitor candidates, there is limited research into their efficacy in different crop cultivars and there have been no efforts yet to assess their robustness in field conditions. As knowledge of mobile signalling accumulates, especially for NHP, the biological mechanisms of priming are becoming more apparent (Yildiz *et al.*, 2023; Lowe *et al.*, 2023). If certain genes are identified as priming-specific regulators, they pose promising candidates for genetic engineering to establish priming without the need for elicitor treatment or pathogen exposure. This approach has the potential to overcome the key drawback of elicitor treatment, as precise engineering of specific priming pathways will not trigger local immune responses and not interfere with plant growth.

The uptake of genetic engineering is slowly increasing across the globe. Genetic engineering has been reclassified into the separate categories of gene editing (GE) or new genomic techniques (NGT), and genetic modification (GM) (Ahmad *et al.*, 2023). GE plants contain mutations and insertions that could occur naturally, while GM plants contain unnatural modifications such as cross-species gene transfers. In 2023 GE and GM crops accounted for 13.4% of farmland, primarily in the United States, Brazil and Argentina (Cheng *et al.*, 2023). Globally, the adoption of GM corn has increased yields by up to 24%, increasing farmer profits over 60% (Pellegrino *et al.*, 2018; Klumper and Qaim, 2014), demonstrating the value that can be gained from effective genetic modification. The majority of GM and GE crops are grown in the Americas, but legislation and adoption are now slowly advancing in Asia and Europe (Cheng *et al.*, 2023). Especially given the distinction between GM and GE crops, opinion is improving in Europe (Bearth *et al.,* 2024), so gene editing is becoming a viable route forward for crop protection.

As knowledge of phloem mobile signals develops, novel regulators of SAR and ISR may be uncovered. Given the link between epigenetic modification and priming (Jaskiewicz *et al.* 2010; Luna *et al.*, 2012), if epigenetic regulators of priming can be discovered, they could be ideal candidates for GE to increase immune responsiveness without growth tradeoffs. As systemic priming appears to signal through several immune pathways, including SA, JA and ET, study of specific interactions between mobile signals and specific immune pathways may uncover regulators that prime certain arms of immunity. These regulators would be ideal candidates as, with GE, specific responses to specific pathogens could be primed, ensuring there are no off-target effects and no yield trade-offs.

1.5 – Aims and objectives

While several phloem mobile signals have been discovered in systemic tissues, there is little information as to how they interact with local immune signalling pathways. Therefore, this study aims to explore the relationship between a selection of systemic mobile signals and local SA signalling. NHP and AzA were prioritised for this study as they are arguably the best established mobile signals in SAR. A sequential treatment assay was developed for *Arabidopsis thaliana* seedlings, in which plants were first treated with a mobile signal to mimic establishment of SAR, and then with SA to mimic secondary pathogen challenge. This enabled analysis of both the direct effects of each mobile signal and how each signal modulates SA signalling. The hypotheses and aims of each chapter are detailed below:

Chapter 3 – Can AzA modulate SA signalling and enhance SA-mediated immunity?

- What is the relationship between AzA- and SA- mediated gene expression?
- Can AzA modulate SA signalling sufficiently to enhance disease resistance?

Chapter 4 – Can NHP modulate SA signalling and enhance SA-mediated immunity?

- What is the relationship between NHP- and SA- mediated gene expression?
- Can NHP modulate SA signalling sufficiently to enhance disease resistance?
- How do NHP and NPR1 interact during a priming response?
- Does NHP modulate posttranslational modifications during a priming response?

Chapter 5 – What signalling pathways does NHP employ to promote priming?

- Are there transcription factors that specifically regulate priming and not immune activation?

- Does NHP induce epigenetic changes across the genome?
- Is NHP-responsive chromatin modification dependent on priming specific transcription factors?

Overall, this study shows that multiple phloem mobile signals modulate SA signalling, but each appear to have distinct roles in inducing SAR. As NHP enhances plant immunity, I provide further evidence to suggest that NHP or NHP derivatives could be employed as elicitors for crop protection. With the discovery of transcription factors WRKY38/62 as epigenetic regulators of SAR, I also uncover excellent candidates for gene editing to produce pre-primed crop varieties. Epigenetic modifications are long lasting and possibly heritable (Boyko and Kovalchuk, 2011), meaning alteration of WRKY38/62 expression may be sufficient to enhance plant immunity without yield loss and without the need for chemical application.

Chapter 2 – Materials and methods

2.1 – Plant materials and growth conditions

All plants used were *Arabidopsis thaliana* of the Columbia-0 (Col-0) ecotype. Seeds were sterilised by washing with 96% ethanol, followed by a 15 min incubation in a 50% bleach, 0.1% Triton solution. Seeds were then washed 5 times with sterilised water and stratified for 2-4 days in darkness at 4°C. For seedling experiments, seeds were plated on Murashige and Skoog agar (Murashige and Skoog, 1962) and grown at 22°C under long-day conditions (16h photoperiod; 06:00am to 22:00pm) with lighting intensity of 70-100 μmol m^{-2} sec^{-1} for 10-14 days. For experiments on adult plants (28 days old), seeds were germinated at high humidity on an autoclaved soil mix of peat moss, vermiculite and sand at a 4:1:1 ratio. Subsequently, 10–12-day-old seedlings were transplanted to larger pots and grown in the same soil until the appropriate age.

The following genotypes were used in this thesis: *sid2* (Wildermuth *et al.*, 2001), *pal1* (SALK_022804), *35S:NPR1-GFP* (in *npr1-1*) (Kinkema *et al.*, 2000), *npr3/npr4* (SALK_043055/ SALK_098460), *cul3a/3b* (SALK_046638/SALK_098014), *ube4* (AIL_713_A12), *wrky18* (SALK_093916), *wrky54/wrky70* (SALK_111964/SALK_025198), *wrky38/wrky62* (SAIL_749_B02/SM_3_38820), *hda19* (SALK_139445) and *jmj14* (SALK_135712).

The primers used in this study are presented in Appendix A. For SALK/SAIL/SM genotyping the recommended primers were selected from http://signal.salk.edu/tdnaprimers.2.html.

2.2 – Seedling chemical treatments

For AzA treatments, seedlings were immersed in 10 ml of 5 mM MES (pH 5.6) alone or 5 mM MES supplemented with 1 mM AzA for 18 hours (16:00pm to 10:00am), then moved into 10 ml water or 0.5 mM SA for 6 hours (10:00am to 16:00pm). For NHP treatments, seedlings were immersed in 10 ml of water or 1 mM NHP for 18 hours (16:00pm to 10:00am), then moved into 10 ml water or 0.5 mM SA for 6 hours (10:00am to 16:00pm). For Pip treatments, seedlings were immersed in 10 ml of water or 1 mM Pip for 18 hours (16:00pm to 10:00am), then moved into 10 ml water or 0.5 mM SA for 6 hours (10:00am to 16:00pm).

For cycloheximide (CHX) assays, seedlings were treated as above, but 4 hours after moving to SA, they were supplemented with 100 μM CHX. Samples were taken immediately after adding CHX (14:00pm) and thereafter at 2 hour (16:00pm), 4 hour (18:00pm) and 6 hour (20:00pm) timepoints. For MG132 assays, seedlings were treated as above, but 2 hours after moving to NHP or to SA they were supplemented with 100 µM MG132. Samples were taken after 6 hours of SA treatment as before.

2.3 – DNA extraction and genotyping

For DNA extraction, small sections of leaf were collected and placed in PCR strip tubes with a 3mm ball bearing. 50 µl one-step DNA extraction buffer was added (20 mM Tris-HCl pH 7.5, 25 mM NaCl, 2.5 mM EDTA, 0.05% SDS) and the tissue ground in a Tissuelyser for 3 x 30s of 18 beats/s. The samples were briefly centrifuged to collect debris and 0.5 µl of the resulting supernatant used in PCR amplification.

For SALK/SAIL/SM line genotyping. DNA was extracted from a single leaf on each plant and amplified using GoTaq DNA polymerase following the manufacturer's instructions. For SALK lines the LBb1.3 primer was used to target the t-DNA insertion, for SAIL lines the LB1 primer was used, and for SM lines the Spm32 primer was used (Appendix A).

2.4 – RNA extraction, cDNA synthesis and RT-qPCR

For RNA extractions, seedlings were grown for 10-14 days on MS plates and treated as described above. To extract RNA, seedlings were frozen in liquid nitrogen and ground into a fine powder. Equal volumes of RNA extraction buffer (100 mM LiCl, 100 mM Tris (pH 8), 10 mM EDTA, 1% SDS) and phenol:chloroform:isoamlyalcohol (25:24:1) were added and the samples vortexed. The samples were then centrifuged at 13,300 rpm at 4°C for 5 mins before the aqueous phase was added to an equal volume of chloroform:isoamylalcohol (24:1) and briefly vortexed. This step was repeated, and the aqueous phase incubated overnight with 1/3 volume 8M LiCl at 4°C. The precipitate was pelleted by centrifugation at 13,300 rpm at 4°C for 15 mins and the pellet washed with ice-cold 70% ethanol before rehydrating by resuspension in 400 µl H_20. The RNA was then precipitated by adding 1 ml ice-cold 96% ethanol and 40 µl 3M NaAc (pH 5.2), and incubating for 1 hours at -20°C. Finally, purified RNA was pelleted by centrifugation at 13,300 rpm at 4°C for 15 mins, washed with ice-cold 70% ethanol, briefly air-dried, and resuspended in 25 µl sterilised water.

RNA concentrations were quantified using a NanoDrop and 2.5 mg used to synthesise cDNA with SuperScript II reverse transcriptase according to the manufacturer's instructions. cDNA was diluted 20-fold, then 4 µl dilute cDNA was mixed with 5 µl SYBR green master mix and 0.5 µl of each 10 µM primer for qPCR analysis. The samples were run in a qPCR thermocycler with the following conditions: 95°C for 10 min, (95°C for 15 s, 60°C for 1 min) x 40. The data were analysed using the ΔΔCt method (Livak *et al.*, 2001).

2.5 – RNA sequencing and analysis

For RNA-seq analysis, seedlings were grown and treated with AzA or NHP followed by treatment with SA as described above, then total RNA was extracted as described above. RNA integrity was confirmed by Bioanalyzer and treatment efficacy was confirmed by qPCR for reporter genes as described above. High quality samples were then submitted to BGI Genomics (Hong Kong) for sequencing. Transcripts were quantified from raw reads using Kallisto (Bray *et al.*, 2016) following the authors instructions. Differentially expressed genes were identified from transcripts per million values using Sleuth (Pimentel *et al.*, 2017) following the authors instructions. SA expressed genes were identified through linear modelling (Expression ~ SA treatment, $p \leq 0.05$), after which NHP primed behaviour was identified by hierarchical clustering with hclust and visualisation with ggplot2 (Wickham, 2016), ggdendro (De Vries and Ripley, 2022) and ComplexHeatmap (Gu, 2022). Some clusters did not resolve as well as others, so were run through hclust again for better resolution. Cluster behaviour was scored through cutoffs when expression was scaled from 0 to 1, *i.e.* "NHP primed" genes were identified with “NW ≤ 0.2 and WS ≤ 0.8” to select genes where NHP-primed SA expression is higher than the sum of NHP- and SA-responsive expression alone. Clustering reliability was confirmed with heatmaps. When all groups had been assigned for the wild type and *wrky38/62*, Sankey diagrams were plotted with ggsankey (Sjoberg, 2024). GO term plots were produced using the “panther_go” function from the coriell package (Calendo *et al.*, 2024).

2.6 – Disease assays

For disease assays, adult plants were grown on soil until 4 weeks old. They were then treated by spraying with 1 mM AzA or 1 mM DL-pipecolic acid 48 hours before infection, followed by 0.5 mM or 0.05 mM SA 24 hours before infection. At 72 hours before infection, *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326 stocks stored at -80°C were streaked onto LB agar containing 10 mM $MgCl_2$ and streptomycin (100 µg/ml). From a single colony, a liquid culture of LB containing 10 mM $MgCl_2$ and streptomycin (100 µg/ml) was grown overnight. Bacterial cells were collected by centrifugation and resuspended in 10 mM $MgCl_2$ to 5×10^6 colony forming units/ml. Between 11:00am and 13:00pm, bacteria were pressure-infiltrated into leaves with a maximum of 2 infiltrated leaves per plant. The plants were harvested at 3 or 4 days after infection, when chlorosis symptoms were visible. Eight representative leaves per sample were photographed before circular leaf discs of 5 mm diameter circular leaf discs were cut out and ground fresh in 10 mM $MgCl_2$. The resulting suspensions were serial diluted and streaked on LB agar plates containing 10 mM $MgCl_2$ and streptomycin (100 µg/ml).

Plates were incubated at room temperature and colony-forming units counted after 2-3 days. Three biological repeats of each assay were combined for analyses and graphing.

2.7 – Protein extraction and western blotting

For NPR1 protein accumulation and CHX assays, seedlings were grown for 10-14 days on MS plates and treated as described above. To extract total protein, seedlings were frozen in liquid nitrogen and ground into a fine powder. Next, 2X (w/v) protein extraction buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.2% Nonidet P-40, 1x protease inhibitor cocktail (PIC) (142 nM TPCK, 135.5 nM TLCK, 0.5 nM PMSF)) was added and samples vortexed. Cell debris was pelleted by centrifugation at 13,300 rpm at 4°C for 15 mins, after which the supernatant was diluted into 1X protein sample buffer (40% glycerol, 240 mM Tris(pH 6.8), 8% SDS, 0.04% Bromophenol blue) supplemented with 50 mM DTT and heated at 70°C for 10 mins.

Samples were run on a 10% SDS-PAGE gel and transferred onto nitrocellulose membrane at 20V overnight. Membranes were blocked in 1X phosphate-buffered saline (PBS) with 5% non-fat milk powder and 0.1% TWEEN. Proteins were detected using α-GFP (1:5,000 dilution), α-S2 (1:2,000 dilution) and α-RPN10 (1:2,000 dilution) primary antibodies, followed by either anti-mouse (1:2,000 dilution) or anti-rabbit (1:2,000 dilution) secondary antibodies. Blots were visualised on a LICOR Odyssey Fc imaging system, using SuperSignal West Pico PLUS chemiluminescent substrate.

For NPR1 monomerisation assays, the seedlings were grown and treated as described above. Total protein (+DTT) samples were extracted as described above.

NPR1 monomer samples (-DTT) were extracted as described above, but the protein supernatant was diluted in 1x sample buffer supplemented with water instead of 50 mM DTT. All samples were run on an 8% SDS-PAGE gel and NPR1-GFP detected as described above.

For total ubiquitin assays, the seedlings were grown as described above and treated supplemented with MG132 2 hours after SA application. Total protein was extracted as above but run on a 7% SDS-PAGE gel and total ubiquitin detected with α-P4D1 (1:2,000 dilution) and anti-mouse (1:2,000 dilution) antibodies.

2.8 – TUBE expression and pulldowns

Fresh GST-tagged TUBE (GST-TUBE) was produced for each pulldown using the pGEX-6P-1 expression vector in BL21 strain *E.coli*. A total of 50 µl BL21 was mixed with 2 µl plasmid, incubated on ice for 20 mins, then heat shocked in a 42°C water bath for 45 seconds. The bacteria were cooled on ice for 1 min, then supplemented with 200 µl LB and recovered for 60 mins at 37°C at 200rpm. The bacteria were then streaked on LB agar with 100µg/ml ampicillin (+Amp) and incubated at 37°C overnight. A single colony was picked and used to grow a 20 ml LB culture +Amp overnight. A 10 ml liquid culture was added to 500 ml LB +Amp and diluted to OD_{600} 0.2, then cultured at 37°C and shaken at 200 rpm until it reached OD_{600} 0.6. The culture was briefly cooled on ice, then supplemented with 0.1 mM IPTG before incubation overnight at 16°C and 200rpm. The next day the culture was centrifuged at 6,500 rpm for 20 mins and the supernatant discarded. Pellets were stored at -20°C overnight. Frozen pellets were resuspended in 25 ml fresh lysis buffer (1x PBS, 0.1% Triton X-100, 1 mg/ml lysozyme, 1x PIC and 12.5

U/ml benzonase nuclease) and incubated with rotation for 30 mins. The cell debris was pelleted by centrifugation at 13,000 rpm at 4°C for 20 mins and the pellets discarded. The supernatant was further filtered to remove all cellular debris. 750 µl glutathione Sepharose beads were washed with 10 ml 1x PBS and added to the lysate before incubation overnight at 4°C with rotation. The next day the lysate was added to a 2 ml filtration column until all liquid had drained, then washed with 10 ml 1x PBS. Then 6 ml elution buffer (50 mM Tris-HCl (pH 8), 10 mM reduced L-glutathione) was then added to the column, and the flow through collected in 0.5 ml fractions. The protein content of each fraction was visually estimated with Bradford protein dye and the highest 2-3 fractions combined. The protein was then dialysed twice against 2 litres of 1x PBS, first for 2 hours, then overnight at 4°C with gentle agitation. The next day the dialysed protein was quantified by Bradford assay and diluted in glycerol and 1x PBS to give 2mg/ml GST-TUBE + 10% glycerol. Aliquots were stored at -80°C until use.

For TUBE pulldowns, seedlings were grown for 10-14 days on MS plates and treated supplemented with MG132 as described above. Plant tissue was frozen in liquid nitrogen and ground into a fine powder. Two volumes of polyubiquitin pulldown buffer (1x PBS, 1% Triton X-100, 10 mM NEM, 40 µM MG132, 1x PPI3, 1x PIC, 200ug/ml GST-TUBE) was added, then samples centrifuged at 13,000 rpm at 4°C for 20 mins. The supernatant was collected and centrifuged again for 10 mins with the same parameters to remove all cell debris. Next, 20 µl of each sample was removed for a control (input) sample and stored at -20°C overnight. For each sample, 20 µl of packed glutathione agarose beads were added to a fresh tube and washed with 1x PBS + 1% Triton X-100. The supernatants were added to their respective tube of beads and incubated overnight with rotation at 4°C. The next day the beads were collected by centrifugation at

5,000rpm for 30 seconds at 4°C, then washed 4 times with 1x PBS + 1% Triton X-100. The beads were eluted by adding 50 µl reducing sample buffer (40% glycerol, 240 mM Tris (pH 6.8), 8% SDS, 0.04% Bromophenol blue, 50 mM DTT) and incubating at 70°C for 15 mins. Samples were thoroughly vortexed, then centrifuged at 13,000 rpm for 1 min. The input samples taken previously were prepared by addition of 7.5 µl 4x strength reducing sample buffer and incubation at 80°C for 10 mins.

Samples were run on 8% SDS-PAGE gels and transferred onto nitrocellulose membranes at 20V overnight. Membranes were blocked, probed and imaged as described above using α-GFP (1:5,000 dilution) and anti-mouse (1:2,000 dilution) for samples and inputs. Membranes were then stripped with stripping buffer (1x PBS, 1% SDS, 7 µl/ml β-mercaptoethanol) for 20 mins, washed 3 times for 5 mins with 1x PBS, then re-blocked with blocking buffer overnight. The next day the sample blot was probed with α-FK2 (1:2,000 dilution) and the input blot probed with α-S2 (1:2,000 dilution) and imaged as described above with anti-rabbit (1:2,000 dilution) secondary antibody (1:2,000 dilution).

2.9 – ATAC sequencing and analysis

For ATAC-seq, a protocol was adapted from an *Arabidopsis* nuclei extraction protocol and an ATAC-seq analysis protocol (Wang *et al.*, 2021; Grandi *et al.*, 2022). Approximately 200 mg of 10-14 day old seedlings were grown per treatment in triplicate and treated as described above. Treated seedlings were transferred to a square dish and finely chopped with a razor blade in 500 µl pre-chilled lysis buffer (15 mM Tris-HCl (pH 7.5), 20 mM NaCl, 80 mM KCl, 0.2% Triton X-100, 5 mM β-mercaptoethanol, 1X PIC, 0.5

mM spermine). The solution was filtered through miracloth (25 µm) into a collection tube, and the dish rinsed with 1.5 ml lysis buffer into the collection tube. The nuclei suspension was stored overnight at 4°C. Then, 20 mins prior to flow cytometry, 1 µl of 1mg/ml DAPI was added to 1 ml of nuclei suspension. 50,000 nuclei were sorted into 500 µl pre-chilled lysis buffer using the following parameters: 85 µM nozzle, 45 psi pressure, sorting speed 2, V450 > 200, selecting 2N and 4N nuclei.

The nuclei were collected by centrifugation at 1,000g for 10 mins at 4°C. Next, 450 µl supernatant was carefully removed and discarded, then the nuclei washed in 1 ml wash buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% Tween). Nuclei were pelleted again using the same parameters, then the supernatant carefully removed, leaving ~10 µl nuclei suspension. Tagmentation was then performed using the Illumina TDE1 enzyme kit. A 50 µl reaction containing 1X TD buffer, 1X PBS, 2.5 µl TDE1 enzyme and 10 µl nuclei was prepared and incubated in a thermomixer at 37°C for 30 mins at 1,000 rpm. The reaction was terminated by addition of 250 µl Zymo DNA clean and concentrator DNA binding buffer. Samples were loaded onto Zymo spin columns, and centrifuged at 10,000g for 30 seconds. The columns were washed twice with 200 µl DNA wash buffer and centrifugation 10,000g for 30 seconds. The columns were centrifuged at 13,000g for 1 min to remove any excess buffers. Finally, the column was placed in a 1.5 ml LoBind tube and 21 µl DNA elution buffer added to the membrane for 1 min. The column was centrifuged for 1 min at 13,000g, giving 20 µl tagmented DNA that was stored at -80°C until sequencing.

The tagmented DNA was submitted to GENEWIZ (United States) for quality assessment and sequencing. DNA concentration and quality was assessed by Qubit, then barcodes for sequencing were added to high quality samples. Analysis was performed using the nfcore/ATAC-seq Nextflow analysis pipeline. Fastq files and *Arabidopsis* genome data from ensemble genomes (TAIR10) release 60 were used as inputs. The pipeline performed sample quality control (QC), genome alignment, alignment QC, enrichment analysis, and peak calling for each sample. Profile plots were produced for each sample from genome alignment tracks using Deeptools (Ramirez *et al.*, 2016). The genomic feature of each peak was recorded by MACS2 (Zhang *et al.*, 2008) in the Nextflow pipeline and used for constructing pie charts.

For analysis across samples, peaks with a $\log_2$(fold enrichment) ≥ 2 were selected as significantly enriched peaks. Every significant peak was compared, and peaks within 100 bp of each other in different samples were considered the same peak to compare the effects of genotype and treatment. The fold change between conditions was calculated, and a peak considered 'opened' or 'closed' if the log2(fold change) was above or equal to 0.5. The overlap of NHP-opened and NHP-closed peaks was compared between wild type and *wrky38/62* peaks Venn diagrams. Motif enrichment was determined using MEME-suite (Bailey *et al.*, 2009), inputting 500 bp upstream sequences of every gene associated with a set of peaks. The genomic location of each peak in each sample was visualised using pie charts and Sankey diagrams.

To compare RNA-seq and ATAC-seq data, SA upregulated genes that were NHP primed, NHP reverse primed, or NHP unresponsive were selected. Profile plots of the ATAC-seq signal 3 kb up- and downstream of the TSS in water and NHP treatment conditions were plotted for genes in each category using Deeptools. The genes showing the highest SA-responsive expression in each category were selected, and their signals visualised with Integrated Genome Viewer (IGV). This analysis was then repeated to show the changes in NHP-responsive ATAC-seq signal in wild-type and *wrky38/62* plants for all genes and the highly expressed examples in each category.

To compare the overlap between NHP-mediated chromatin accessibility and NHP-mediated priming, the behaviour of each peak and its associated gene was assessed. Peaks were classed as NHP-responsive and *WRKY38/62*-dependent or -independent using fold change cutoffs mentioned above. This was then compared with the NHP-responsive clusters assigned in the RNA-seq as mentioned above. Peaks that did not respond to NHP were classed as "No NHP response", and genes in NHP-unresponsive clusters or not identified in the RNA-seq were classed as "NHP independent"

Chapter 3 – Azelaic acid inhibits specific components of salicylic acid signalling

3.1 – Introduction

Upon pathogen perception, plants do not only launch local immune responses, but also produce phloem-mobile signals that travel from the site of infection to induce systemic acquired resistance (SAR). A major hallmark of SAR is immune 'priming', wherein cells are sensitised to respond faster and stronger to future infections. Azelaic acid (AzA) is a 9-carbon dicarboxylic acid that accumulates in the phloem of infected plants and is transported to systemic tissues (Jung *et al.*, 2009), suggesting it is a mobile signal. Although there are conflicting reports of the effectiveness of AzA (Zoeller *et al.*, 2012; Vicente *et al.*, 2012; Nagy *et al.*, 2016), application of AzA is thought to enhance the strength of immune responses (Jung *et al.*, 2009; Haghpanah *et al.*, 2024). AzA application alone does not induce expression of key immune responsive genes such as *PR1* (Yu, K. *et al.*, 2013), suggesting that its role is in priming rather than driving immunity.

AzA is produced by the peroxidation of depolarised 18 carbon lipids in the chloroplast (Zoeller *et al.*, 2012). This can occur through two pathways, either by the enzymatic action of lipoxygenases or through oxidation by reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$). AzA production is unperturbed in immune-linked lipoxygenase mutants, such as *lox1/5*, so the primary source of AzA is likely derived from oxidation by $^1O_2$ (Zoeller *et al.*, 2012). AzA biogenesis occurs in plastids during the second burst of ROS that occurs 5-24 hours after pathogen perception (Lamb and Dixon, 1997). In particular, increased accumulation of $^1O_2$ is triggered by the salicylic acid (SA)-mediated downregulation of photosynthesis via suppression of photosystem II. Subsequent lipid peroxidation fragments C18 and C16 fatty acids, which are spontaneously oxidised into AzA and other carboxylic acids. This process releases further ROS molecules, creating a feedback loop that causes AzA to rise linearly with pathogen growth (Zoeller *et al.*, 2012).

To enhance immunity in *Arabidopsis*, AzA may require functional AZELAIC ACID INDUCED 1 (AZI1) and its paralogue EARLY ARABIDOPIS ALUMINIUM INDUCED1 (EARLI1) (Jung *et al.*, 2009). AZI1/EARLI1 are lipid transfer proteins required for local signal production but not systemic signal perception, suggesting that they may facilitate AzA transport to systemic tissues (Jung *et al.*, 2009). AZI1/EARLI1 accumulate in chloroplasts in response to both exogenous AzA application and endogenous AzA production (Cecchini *et al.*, 2021), suggesting AzA and AZI1/EARLI1 colocalise during immune activation. Colocalisation could suggest interaction between AzA and AZI1/EARLI1, and interaction could facilitate AzA transport, although this hypothesis is yet to be tested. Both AZI1/EARLI1-dependent AzA signalling and chloroplast accumulation of AZI1/EARLI1 is reliant on the MAP kinases MPK3 and MPK6 (Cecchini *et al.*, 2018; Cecchini *et al.*, 2021). MPK3/6 are involved in a range of biotic and abiotic stress signalling, including SAR signal generation (Beckers *et al.*, 2009; Galletti *et al.*, 2011; Li *et al.*, 2017; Wang *et al.*, 2018), suggesting that AzA transport may be driven by MPK3/6 and AZI1/EARLI1. AZI1/EARLI1 are also implicated in abiotic stress responses, as *azi1* mutants show salt hypersensitivity and *AZI1* overexpressors are salt resistant (Pitzschke *et al.* 2014). As AzA is produced by ROS and many abiotic stresses are associated with dysregulation of redox homeostasis, it is plausible that AzA may not just play a role in immune signalling but may be a ROS-responsive signal that feeds into wider systemic signalling pathways.

AzA accumulation in local tissues promotes accumulation of glycerol-3-phosphate (G3P) through GLYCEROL KINASE (GLI1) and G3P DEHYDROGENASE (GLY1). AzA signalling is perturbed in *gli1/gly1* mutants, suggesting that AzA acts through or in tandem with G3P (Yu, K. *et al.*, 2013). G3P is another potential mobile priming signal, shown to be phloem mobile and to potentiate immune activity (Chanda *et al.*, 2011). However, G3P treatment alone is insufficient to rescue SAR in SAR-defective mutants, and G3P can only rescue systemic signalling when supplemented with phloem sap from immune active plants (Chanda *et al.*, 2011). Therefore, while G3P acts downstream of AzA, it requires partnering signals to induce a response in *Arabidopsis*. This suggests that G3P may act in one of several AzA signalling pathways, all of which are required for a full AzA-inducible SAR response. Therefore, in this chapter I focused on AzA signalling as the role of AzA in immunity has been established and it is clearly an upstream branching point in immune signalling.

To elucidate the role of AzA in immunity I investigated how AzA interacts with salicylic acid (SA) signalling. SA is a core component of local immune signalling, driving ROS signalling and transcriptional reprogramming to combat infection (Spoel and Dong, 2024). SA is also required for SAR, accumulating in local and systemic tissues after infection. While systemically transported SA does not act as a mobile signal (Vernooij *et al.*, 1994), methyl-salicylate is transported with unknown function (Park *et al.*, 2007; Attaran *et al.*, 2009) and *de novo* SA is required in systemic tissues to establish a primed state (Dempsey *et al.*, 1999). The interaction between AzA and SA has not been studied, so it is unknown whether AzA plays a role in systemic SA accumulation or a role in priming SA responses.

Therefore, I developed a sequential treatment assay, wherein plants were treated with AzA overnight, followed by SA, and then SA-responsive gene expression was measured. This showed that AzA selectively primed the SA-responsiveness of the marker gene *PR1*. However, while analyses of the full transcriptome demonstrated that a small number of SA-responsive genes were primed, the predominant role of AzA was to dampen SA-induced transcriptional reprogramming. Dampened genes were enriched for programmed cell death and photosynthesis gene ontology (GO) terms, suggesting that AzA's role is to inhibit certain components of SA signalling to tailor a primed response to systemic tissues, in line with this prediction, I show that AzA alone does not enhance immunity or increase SA-responsiveness to the pathogen *Pseudomonas syringae* in *Arabidopsis*.

3.2 – Results

3.2.1 – Azelaic acid primes salicylic acid-responsive *PR1* expression

To investigate whether AzA crosstalks with SA I first examined whether AzA can modify SA-responsive gene expression. To this end, I developed a sequential treatment assay to mimic systemic priming and elicitation of a primed response. Fourteen-day-old *Arabidopsis thaliana* seedlings were treated with 5 mM MES or 1 mM AzA (in 5 mM MES, as described in Jung *et al.* (2009)) by immersion for 18 hours to mimic SAR induction and the accumulation of AzA in systemic tissues (priming phase). The seedlings were then moved to water or 0.5 mM SA for an additional 6 hours to mimic a pathogen attack and subsequent SA accumulation (elicitation phase). Total RNA was extracted and reverse transcribed to cDNA to measure expression of a selection of SA-responsive marker genes by RT-qPCR. In line with previous reports (Jung *et al.* 2009), I found that AzA treatment alone had no effect on the expression of any of these genes, whereas SA induced their expression (Figure 3.1). However, AzA pretreatment strongly increased the SA-induced expression of *PR1*, but not *WRKY38* or *WRKY62*. These data suggest that AzA can selectively prime the expression of SA-responsive genes.

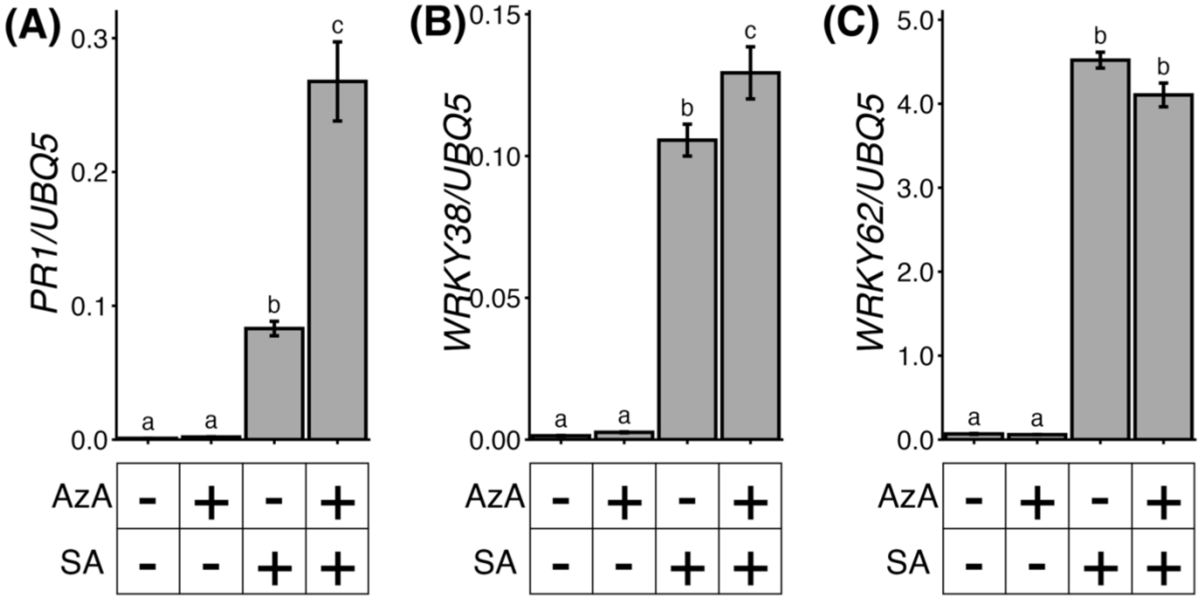


Figure 3.1 – AzA selectively primes SA-inducible gene expression. *Fourteen-day-old wild-type seedlings were treated by immersion in 5 mM MES (pH 5.6) with or without 1 mM AzA for 18 hours, followed by treatment with water or 0.5 mM SA for 6 hours. Total RNA was extracted and the expression of SA-responsive genes PR1* ***(A)****, WRKY38* ***(B)****, and WRKY62* ***(C)*** *measured by qPCR. Reporter genes were normalised against constitutively expressed UBQ5. Letters denote statistically significant differences between samples (Tukey ANOVA; α = 0.05, n = 3).*

3.2.2 – Azelaic acid dampens the SA-responsive transcriptome

To understand the extent of AzA's effects on the SA-responsive transcriptome, I performed RNA sequencing (RNA-seq) on plants treated as above. First, genes that responded to AzA but not SA were identified through linear modelling and selection of genes whose expression is significantly correlated with AzA treatment but not SA treatment. Overall, this revealed 212 AzA-upregulated transcripts and 435 AzA-downregulated transcripts. To determine whether any of these genes are linked to immune signalling or immune priming I performed gene ontology (GO) term enrichment analysis, which revealed that AzA upregulated genes are a varied group, including “defence response” and “photosynthesis” termed genes (Figure 3.2A). On the other hand, AzA downregulated genes showed only an enrichment for translation (Figure 3.2B). While this suggests that AzA does directly promote some immune signalling, the number of AzA transcripts was over 6-fold lower than the number of SA-responsive transcripts (647 vs 4,142). Therefore, AzA likely takes a priming role rather than directly regulating SA-responsive genes.

It should be noted that in this experiment gene expression is measured at the end of the elicitation phase, 6 hours after AzA has been removed. Consequently, due to transcript degradation, AzA-responsive genes may be underrepresented compared to a sample taken at the end of the priming phase where AzA is still present. Transcript stability varies a lot from gene to gene, but the average *Arabidopsis* mRNA has a half-life between 4 and 6 hours (Narsai *et al.*, 2007). While certain transcripts may no longer be detected in RNAseq, many will still be within detection thresholds even after some degradation. Therefore, the overall pattern of gene expression will be mostly intact, and this is unlikely to have had a significant impact on the GO terms enriched in the transcripts that persist 6 hours after AzA has been removed.

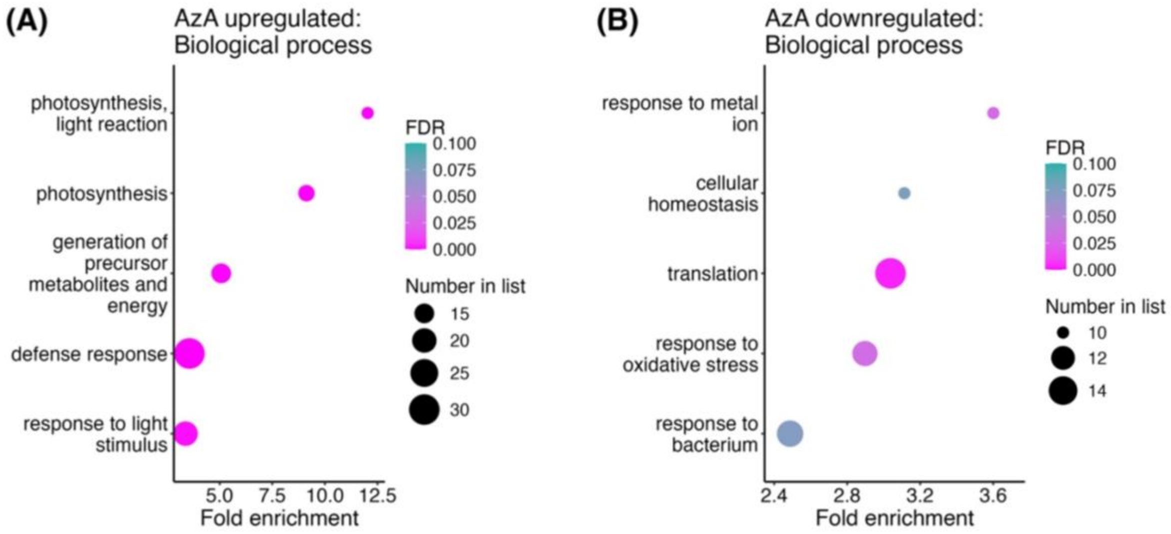


***Figure 3.2 – AzA directly induces defence and photosynthesis genes**. Top 5 most enriched GO terms in directly AzA upregulated or downregulated genes ($p \leq 0.05$, FDR ≤ 0.1).*

SA-responsive genes were identified through linear modelling and separated into SA-upregulated and SA-downregulated genes for further analysis. The AzA-primed behaviour of SA-responsive genes was then identified by hierarchical clustering. This uncovered genes showing no response to AzA, genes synergistically co-expressed by SA and AzA, and genes 'primed' by AzA (including *PR1*). I split primed expression into two categories: 'primed' genes that displayed no response to AzA alone, but after AzA treatment showed enhanced SA-responsiveness, and 'reverse primed' genes that showed no response to AzA alone but reduced SA-responsiveness after AzA treatment. Overall AzA modified the expression of 74.1% (3,070/4,142) of detected SA-responsive transcripts. AzA-mediated priming was confirmed by PCA, showing that AzA-primed SA samples are distinct from unprimed SA samples (Figure 3.3).

Among the SA-upregulated genes, AzA only primed 2.1% and synergistically co-expressed 2.9% of genes, but strikingly reverse primed 72.7% of SA-induced transcripts (Figure 3.4). To determine what components of SA signalling were primed or reverse primed, I again performed GO term enrichment analysis on each cluster of genes. This revealed that, alongside general pathogen response GO terms, the AzA reverse primed genes were enriched for terms such as “plant-type hypersensitive response” and “L-amino acid catabolic process” (Figure 3.5A). The AzA-insensitive SA-upregulated genes also included some GO terms linked to programmed cell death, but primarily were enriched with terms such as “response to salicylic acid” and “systemic acquired resistance” (Figure 3.5B). The synergistically expressed genes showed little enrichment beyond “response to biotic stimulus”, while the AzA primed cluster was too small to give any significant enrichment of GO terms (Figures 3.5C and D). These findings suggest that AzA primarily acts to suppress SA-mediated gene expression, and the priming result in *PR1* is a rare exception. Analysis of GO terms shows that the main processes reverse primed by AzA are amino acid catabolism and the hypersensitive response. The SA-upregulated processes unperturbed by AzA include SA signalling, systemic immune signalling, and the hypersensitive response.

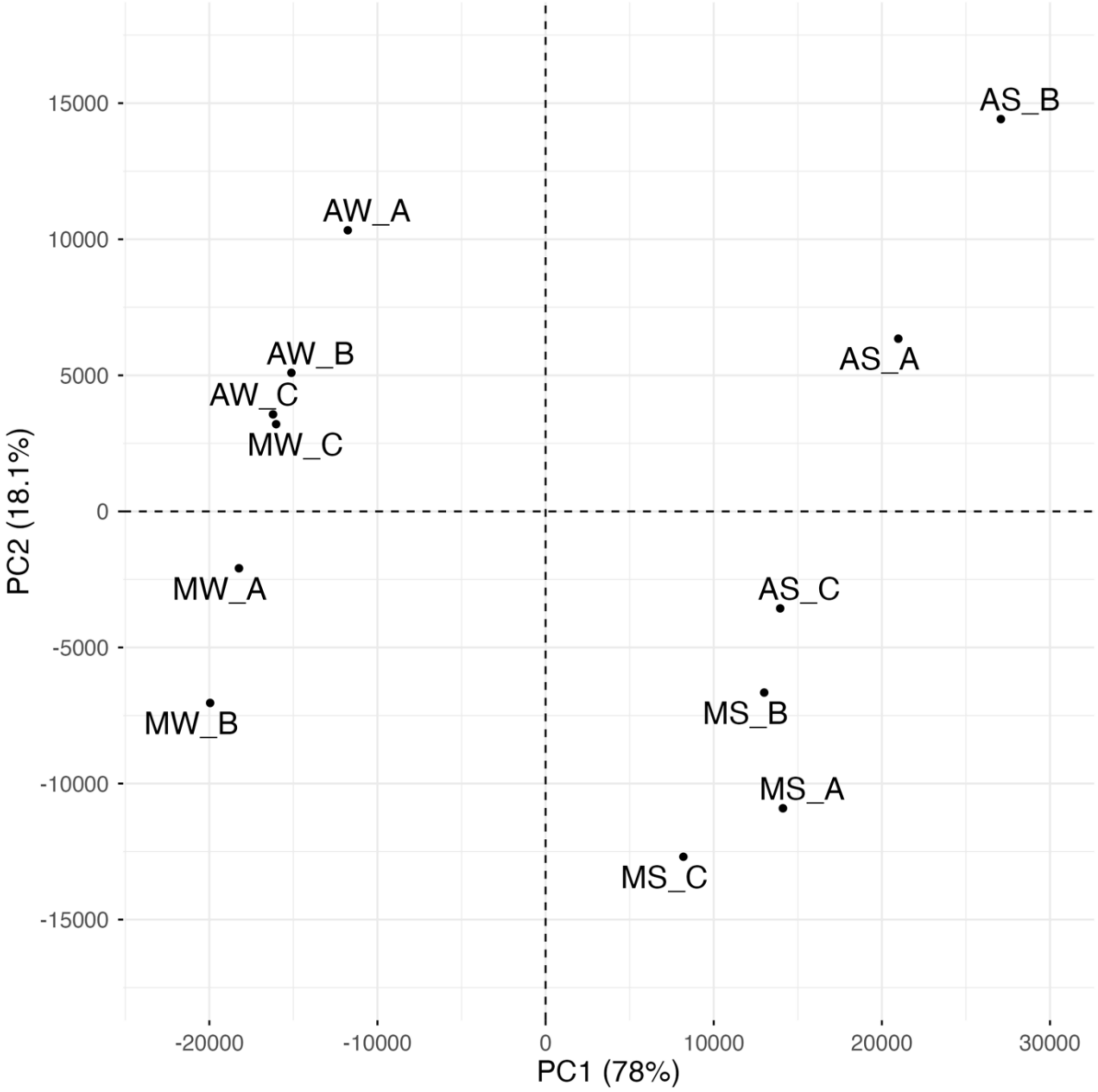


Figure 3.3 – Plants treated individually or sequentially with NHP and SA display distinct transcriptomic profiles when separated by PCA *The treatments are denoted as follows: MES + water = 'MW', AzA + water = 'AW', MES + SA = 'MS', AzA + SA = 'AS'. Each biological repeat is labelled A, B or C.*

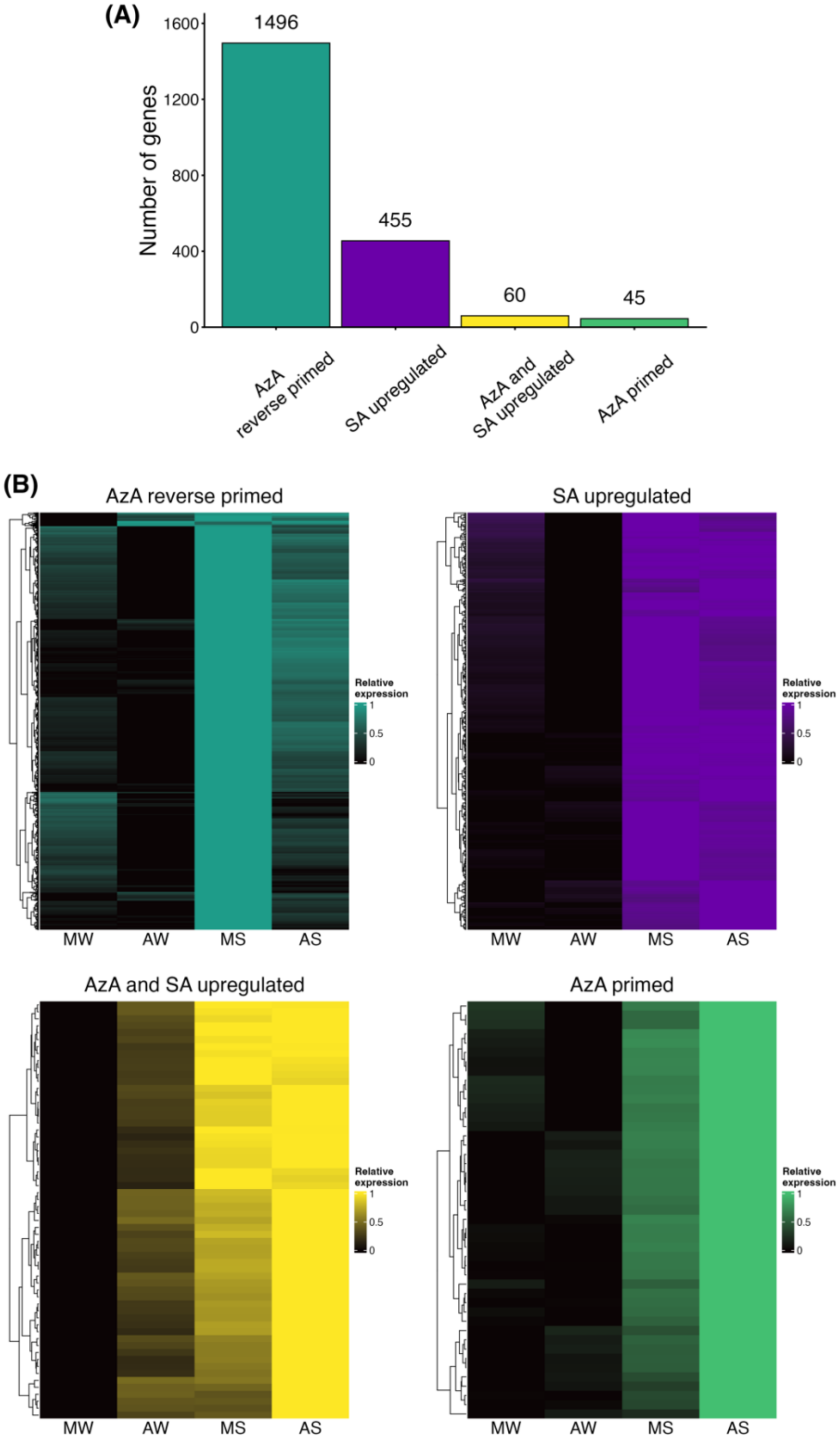


Figure 3.4 – AzA primarily dampens SA-induced transcriptional reprogramming. *Wild type seedlings were treated as described in Figure 3.1. Total RNA was extracted and SA-responsive gene expression measured by RNA-seq.* ***(A)*** *RNA-seq clusters of AzA-primed, SA-induced genes. Clusters were identified and assigned by hierarchical clustering using the hclust R package.* ***(B)*** *Heatmaps of clustered genes showing expression patterns across the four treatment*

conditions. ‘Relative expression’ is presented as transcripts per million values scaled from 0 to 1 across the four treatments. The treatments are denoted as follows: MES + water = ‘MW’, AzA + water = ‘AW’, MES + SA = ‘MS’, AzA + SA = ‘AS’.

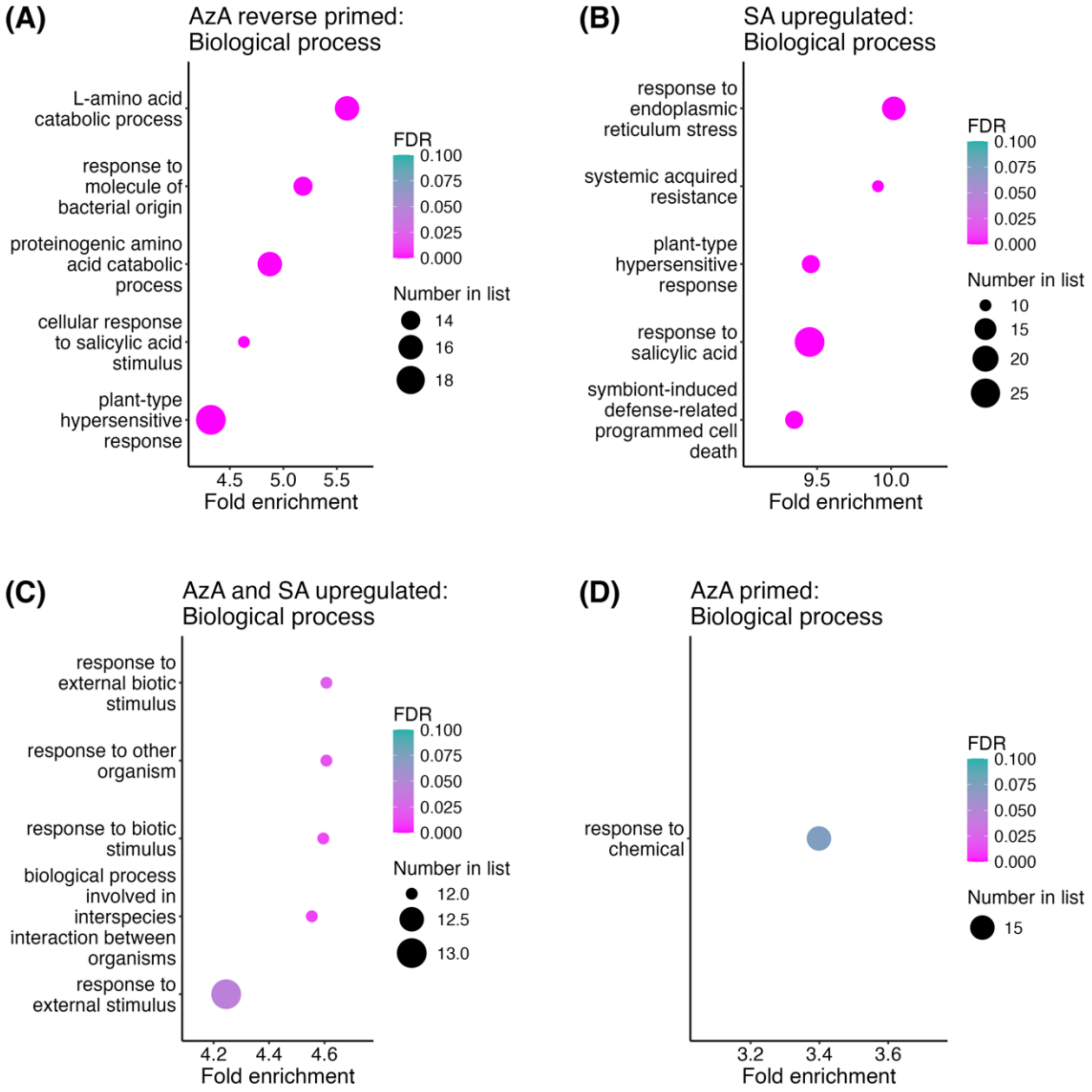


Figure 3.5 – AzA dampens SA-mediated expression of hypersensitive response and amino acid catabolism genes. *Top 5 most enriched GO terms in each cluster of SA-upregulated genes ($p \leq 0.05$, FDR ≤ 0.1).*

Amongst SA-downregulated genes, the AzA response was more diverse. Here, AzA synergistically expressed 32.2% of SA-responsive genes and antagonistically expressed 19.4% (Figure 3.6). Priming was less prevalent, with only 8.8% of genes primed and 9.9% reverse primed. Of particular interest, GO term analysis showed that both AzA and SA controlled the expression of many genes involved in photosynthesis (Figures 3.7B-C). While SA suppressed genes involved in photosynthetic processes, AzA partly countered this (Figures 3.7B-C). This may suggest that AzA directly promotes some photosynthetic processes, counteracting SA. AzA did show some synergism with SA signalling in the downregulated group, with synergistic and primed downregulation genes enriched with GO terms such as “plant type cell wall organisation” and “fatty acid metabolic process” (Figures 3.7A and E). This shows that AzA does not entirely antagonise SA, and may help promote suppression of some metabolic and homeostasis pathways. The reverse primed cluster did not contain any enrichment of GO terms with a false discovery rate (FDR) below 0.05 (Figure 3.7D).

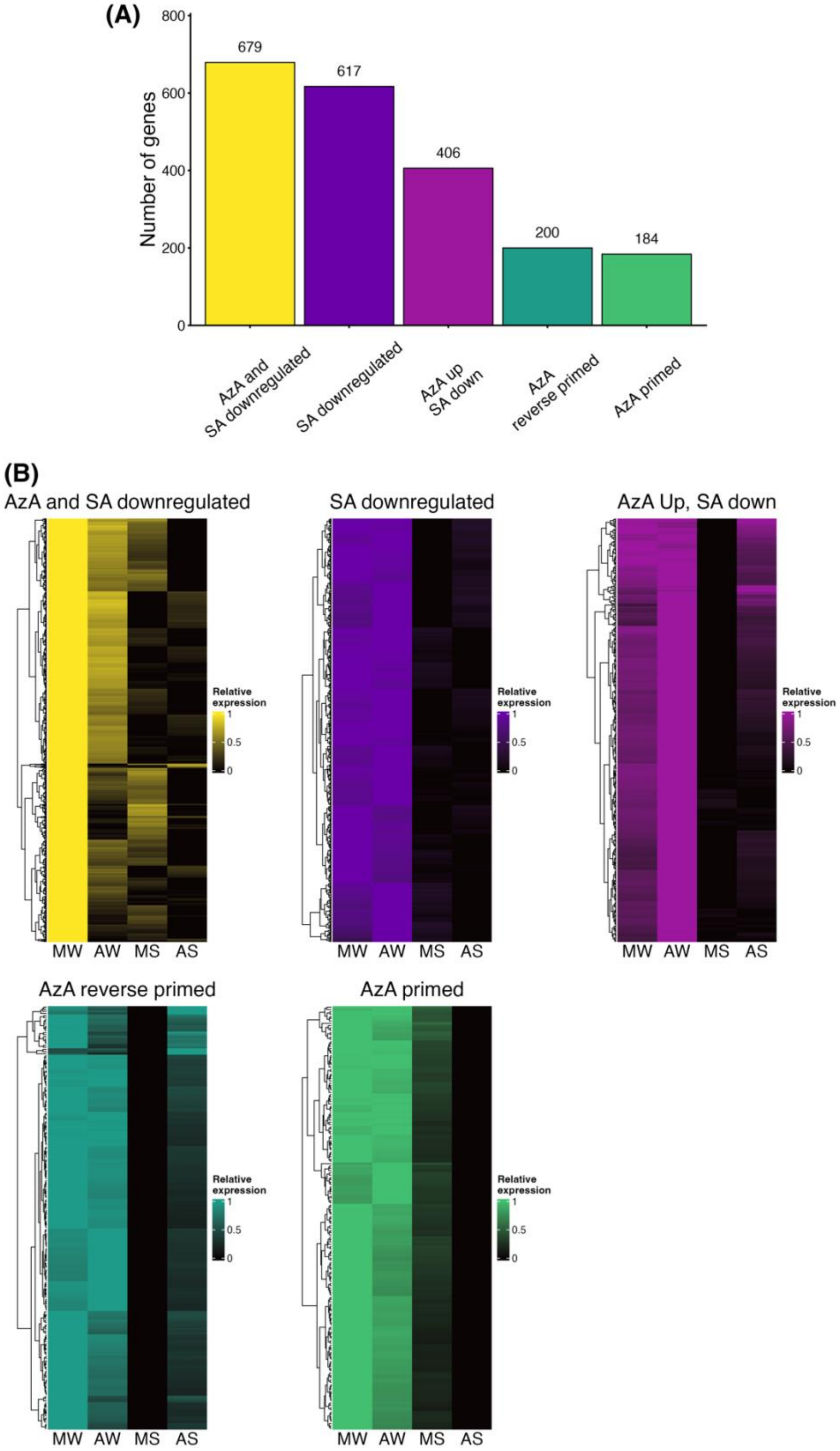


__Figure 3.6 – AzA directly antagonises SA-mediated repression.__ Wild type seedlings were treated as described in Figure 3.1. Total RNA was extracted and SA-responsive gene expression measured by RNA-seq. __(A)__ RNA-seq clusters

of AzA-primed, SA-repressed genes. Clusters were identified and assigned by hierarchical clustering using the hclust R package. ***(B)*** *Heatmaps of clustered genes showing expression patterns across the four treatment conditions. 'Relative expression' is presented as transcripts per million values scaled from 0 to 1 across the four treatments. The treatments are denoted as follows: MES + water = 'MW', AzA + water = 'AW', MES + SA = 'MS', AzA + SA = 'AS'.*

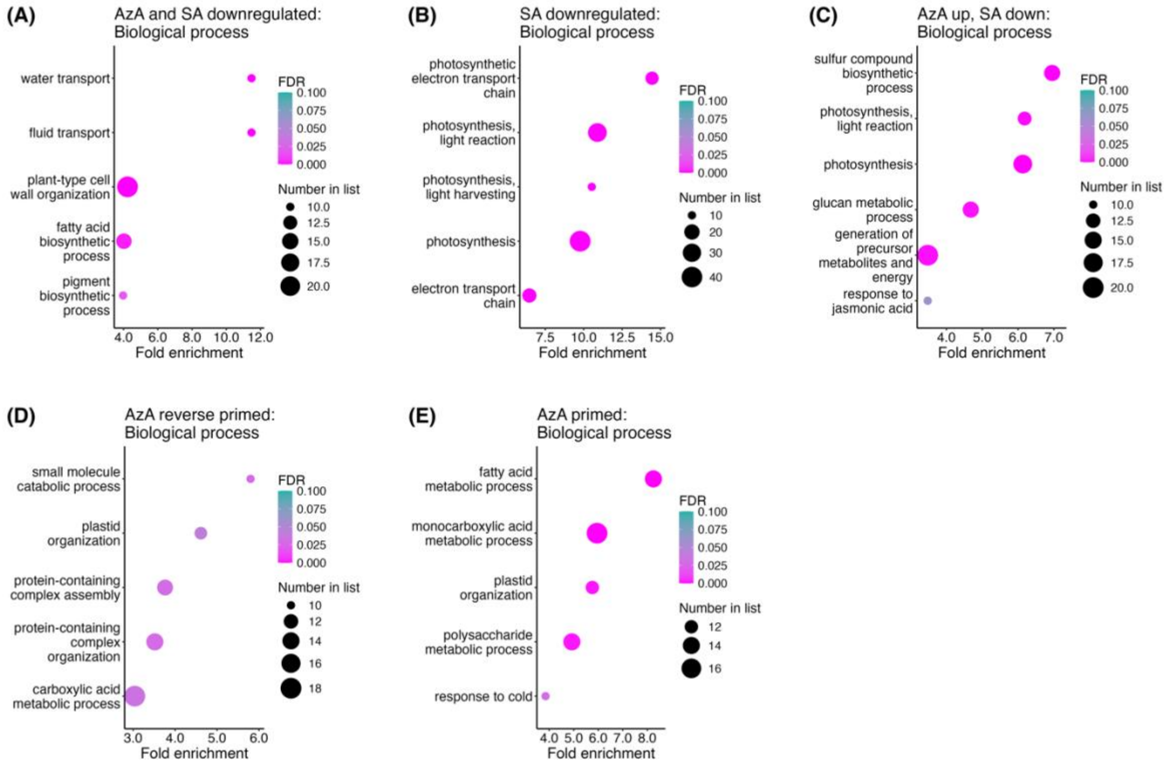
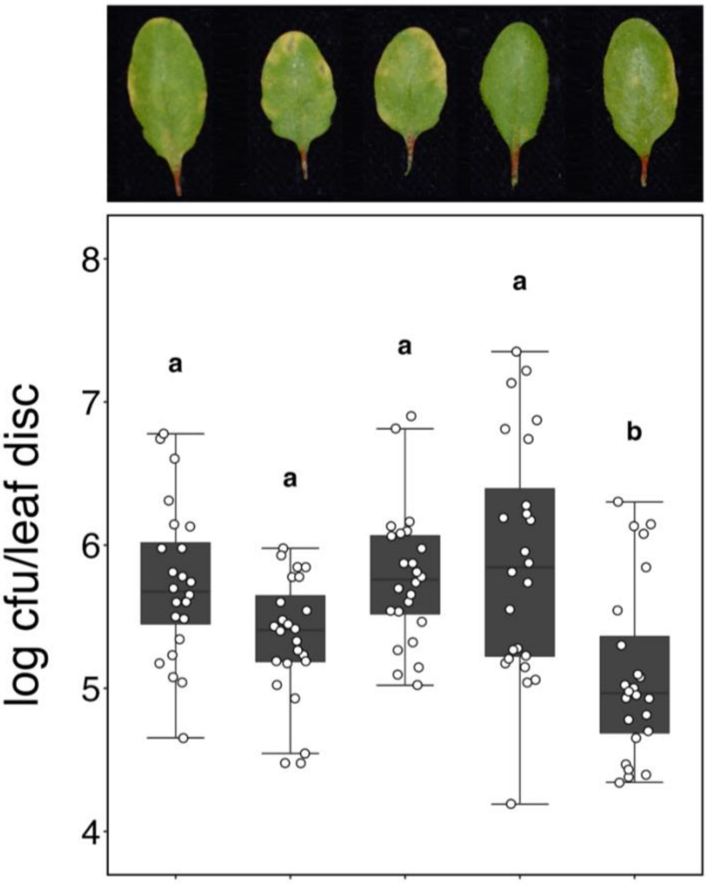


Figure 3.7 – AzA antagonises SA-mediated suppression of photosynthesis. *Top 5 most enriched GO terms in each cluster of SA-downregulated genes (p ≤ 0.05, FDR ≤ 0.1).*

All together these transcriptomic data suggest that AzA's primary role is to suppress SA signalling during SAR, but also aid SA-mediated metabolic reorganisation. AzA both reverse primes SA-upregulated genes to reduce their responsiveness, and directly upregulates many genes that are downregulated by SA. GO term analysis reveals that the main processes targeted by AzA are SA-mediated programmed cell death and SA-mediated photosynthesis suppression, both of which are detrimental to plant growth and development. As AzA showed no evidence of enhancing immunity linked genes, this suggests that other mobile signals act to enhance immunity, while AzA fine tunes SA signalling to suit the needs of a primed cell.

3.2.3 – Azelaic acid does not amplify SA-induced disease resistance

To determine whether the large-scale modulation of SA-responsive transcriptional reprogramming by AzA impacts immunity, I performed disease assays in adult plants. Here, the priming and elicitation phases were split into 24-hour periods, with the priming treatment given 48 hours before infection and the elicitation treatment given 24 hours before infection. For elicitation I treated with either 0.05 mM SA and 0.5 mM SA, using a low concentration to determine whether AzA can prime SA and a high concentration as a control for immune activation. Twenty-eight-day-old *Arabidopsis* plants were mock sprayed with 5 mM MES, or sprayed with 1 mM AzA 48 hours before infection, then sprayed with water, 0.05 mM SA or 0.5 mM SA 24 hours before infection. All plants were then infected with $OD_{0.005}$ *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326, with colonies extracted and counted 3 days after infection. I found that mock treatment or application of AzA or 0.05 mM SA alone did not induce disease resistance, but treatment with ten-fold higher 0.5 mM SA induced strong immunity (Figure 3.8). Interestingly, in this assay AzA alone failed to enhance immunity and failed to sensitise the plants to SA. Therefore, while AzA clearly has a strong influence on the SA-responsive transcriptome, these changes alone are not sufficient to enhance disease resistance as seen in a pathogen-responsive SAR response.



1mM AzA	-	-	+	+	-
0.05mM SA	-	+	-	+	-
0.5mM SA	-	-	-	-	+

Figure 3.8 – Pre-treatment with AzA does not prime SA-induced immunity against Psm ES4326. *Twenty-eight day-old wild-type plants were treated 48 hours before infection by spraying with 5 mM MES (pH 5.6) supplemented with or without 1 mM AzA, followed by treatment with water, 0.05 mM SA or 0.5 mM SA, 24 hours before infection. Leaves were then infiltrated with 5 x 10^6 colony forming units (cfu)/ml Psm ES4326. Leaf discs were analysed for bacterial growth 3 days post infection. Error bars represent interquartile range x1.5, while letters denote statistically significant differences between samples (Tukey ANOVA; α = 0.05, n = 24). Representative photos of leaves are displayed above the respective boxplots.*

3.3 – Discussion

3.3.1 – Interactions between azelaic acid and salicylic acid

Azelaic acid has been identified as a systemic immune signal, as it accumulates in the phloem in response to infection and treatment with AzA can induce disease resistance. However, how AzA communicates with other reported immune hormones to establish SAR remains unclear. In this chapter I show that AzA can enhance the responsiveness of some SA-regulated genes, but mainly acts to dampen the SA-responsive transcriptome. AzA primarily targeted SA-responsive genes involved in the hypersensitive response, photosynthesis suppression and amino acid processing, leaving immune and SAR signalling untouched. In accordance, AzA-mediated countering of specific SA signalling sectors alone was not sufficient to enhance resistance against pathogen attack.

The experimental design in this chapter differs from previously established approaches in AzA signalling. Most published AzA research focuses on the direct transcriptional and immune responses to AzA treatment (Jung *et al.* 2009; Nagy *et al.* 2016; Haghpanah *et al.* 2024), and not whether AzA signalling influences immune responses over longer periods of time. Here, with sequential treatment of AzA followed by SA, I pick apart the effects induced directly by AzA treatment and how previous AzA exposure modulates future SA signalling. Despite the difference in methodology, the results in this chapter do align with the consensus on AzA signalling. Several SA responses targeted by AzA have been identified as pathways of AzA signalling, and the inability of AzA to prime an immune response has been previously described (Haghpanah *et al.* 2024; Zoeller *et al.* 2012). While this approach limited the amount of information I collected on direct AzA signalling, it enabled the discovery of several novel insights in how systemic accumulation of AzA primes tissues to respond differently during SAR.

First, I found that AzA primed SA-responsive *PR1* marker gene expression (Figure 3.1). However, RNAseq analysis demonstrated that AzA directly or indirectly antagonised the expression of 50.2% of SA-responsive transcripts (Figures 3.4 and 3.6). GO term analysis of the antagonised genes showed that, among other roles, AzA reduced the expression of SA-induced genes involved in programmed cell death (PCD) and counteracted the SA-mediated suppression of photosynthetic genes (Figures 3.5 and 3.7). I also found that AzA treatment induces a relatively small subset of genes, suggesting that AzA signalling is primarily through indirect priming. These genes contained immune signalling and photosynthesis genes (Figure 3.2), supporting the hypothesis that AzA directly promotes and suppresses certain aspects of SA signalling. Overall, these data suggest that the role of AzA in systemic immunity is to remodel SA signalling to prioritise certain SA-mediated processes over others, rather than just enhancing SA signalling. The purpose of this may be to distinguish systemic, primed SA signalling from local pathogen-responsive SA signalling.

A surprising finding was that AzA did not prime SA-responsive immunity against *Psm* ES4326 infection. Previously, AzA alone was shown to enhance plant disease resistance (Jung *et al.*, 2009), but in recent years this has been disputed. In *Arabidopsis* there are reports that AzA both can (Torre *et al.*, 2023) and cannot (Zoeller *et al.*, 2012) enhance immunity. Here, I corroborate the latter hypothesis, showing that AzA application alone is not sufficient to elicit enhanced plant immune responses (Figure 3.8). This inconsistency has also been observed in research that spans a range of agriculturally important plants. Immune-responsive AzA accumulation has been found in tobacco and olive, although in both cases this did not enhance resistance (Nagy *et al.* 2016; Nicoli *et al.*, 2019). Conversely, in soybean AzA treatment reduced the symptoms of biotroph *P. pachyrhizi*, and in tomato AzA treatment reduced the growth of necrotroph *A. solani* (Rodrigues *et al.* 2023; Haghpanah *et al.* 2024). Overall, it appears that the ability of AzA to enhance resistance is yet to be consistently confirmed, and whether it does may be heavily dependent on the dose, pathogen and species involved.

Despite this conclusion, AzA priming clearly has a strong effect on the SA-responsive transcriptome. This raises the possibility that AzA may act in tandem with other previously described immune hormones as a single component of a wholistic SAR response. Where AzA targets SA-mediated cell death and photosynthetic regulation, other signals may enhance pathogen surveillance and antimicrobial responses, which will directly increase pathogen resistance. Therefore, when AzA is used for treatments or detected in infected plants, the resulting immune responsive phenotype may be regulated by other, unmeasured, mobile signals, which causes the inconsistent response across experiments and species.

3.3.2 – Interactions between azelaic acid and the hypersensitive response

One consistent factor of all the studies is that AzA accumulates to high levels in response to infection, suggesting it does have a role to play in immunity. My data support this, as I show AzA modulates the SA-responsive expression of 3,070 (74.1%) transcripts, while inducing or repressing another 647. (Figures 3.2, 3.4 and 3.6). Providing an insight into the role of AzA priming, I found that AzA reverse primed genes are enriched with GO terms for PCD (Figure 3.5). This suggests that the role of AzA in a priming response is to reconfigure SA signalling for SAR, reducing costly components of local immunity such as cell death during the hypersensitive response (HR).

Both AzA signalling and HR are induced by ROS during the immune responsive redox burst (Zoeller *et al.*, 2012; Triantaphylides *et al.*, 2009), further suggesting that they may interact. A major contributor to the redox burst is SA, as it both suppresses redox scavenging enzymes and suppresses chloroplast function to focus energy on the immune response (Sanchez-Casas and Klessig, 1994; Janda *et al.*, 2014). One of the side effects of suppressing photosystem II is increased production of singlet oxygen ($^1O_2$), as the environment becomes increasingly reductive (Triantaphylides *et al.*, 2008). AzA is produced by $^1O_2$ and then released from fragmented membranes by lipases in the chloroplast (Zoeller *et al.*, 2012). Therefore, upon pathogen perception and SA production the strong burst of ROS production, and particularly $^1O_2$,will promote the production of AzA in the chloroplast. A sustained period of immune signalling will then cause high levels of oxidative stress, which is an established trigger of cell death (Triantaphylides *et al.*, 2009).

There are two possible pathways by which cell death may occur in plant immunity (Coll *et al.*, 2011). The first is programmed cell death, wherein the plant actively undergoes cell death to limit the available resources and tissues that biotrophic pathogens require to survive within the host. The second pathway to cell death may be due to cellular damage caused by oxidative stress as a byproduct of immune activation. A core component of SA signalling is an increase in ROS production due to inhibition of catalase and other redox scavengers. While ROS are a key intra- and intercellular signal, pervasive high levels of ROS will inevitably damage proteins and DNA in the cell, which may result in cell death as key components are damaged. In either scenario it is entirely plausible that one aspect of priming is a reduction in cell death. Primed plants react faster and stronger to infection, so will either clear the infection before HR needs to be activated, or before too many ROS build up to trigger PCD. If the pathogen is quickly and effectively cleared, the primed immune response will also require less energy, so the energetic prioritisation towards immunity is less vital.

From the data in this chapter, I propose that phloem mobile AzA attenuates SA-responsiveness of some genes in systemic tissues to balance the side effects of a primed response with effective immune signalling. In support of this hypothesis, it has been shown that cell death can be uncoupled from effective immunity. The *defence no death 1* (Clough *et al.*, 2000) and *aberrant growth and death 2* (Rate and Greenberg, 2001) mutants display not just intact but enhanced immunity without induction of HR, showing it is not always required to combat infection. Furthermore, several pathogen effector receptors have been identified that induce disease resistance without HR (LaFlamme *et al.*, 2020), showing that even in a natural state, some plant immune responses do not require cell death. It would be interesting to test this hypothesis by applying AzA to plants prior to infection with an avirulent pathogen, and subsequently measuring the extent of HR lesions and the growth of bacteria to test whether AzA suppresses cell death.

3.3.3 – Interactions between azelaic acid and photosynthesis

The other main GO terms associated with AzA-primed genes were linked to photosynthesis. Unlike hypersensitive response GO terms, the photosynthetic GO terms were enriched in directly AzA induced clusters, including AzA upregulated genes and AzA up, SA down genes (Figures 3.2 and 3.7C). Photosynthetic genes were also enriched in SA downregulated genes (Figure 3.7B), suggesting that AzA directly, but only partially, counteracts SA-mediated photosynthetic suppression.

A core principle of plant immunity is the growth/defence trade-off. Plant immune activation is an energetically costly process, with a great number of genetic and metabolic pathways rewired to focus on fighting infection (He *et al.*, 2022). This results in suppression of several growth-related processes such as auxin signalling, nutrient allocation, and photosynthesis (Huot *et al.*, 2014). Here, I found that multiple SA-downregulated clusters were enriched with photosynthetic GO terms (Figure 3.7 B and C), suggesting that SA signalling directly reduces photosynthetic capacity during an immune response. Indeed, SA plays a key role in suppression of photosynthesis, inducing stomatal closure and reducing photosystem II (PSII) activity (Janda *et al.*, 2014). Stomatal closure is a common response to pathogen perception as many pathogens, including *Psm*, can infiltrate plant tissues via open stomata (Xin *et al.*, 2018), so closure prevents further pathogen ingress. However, closing stomata reduces the rate of gas exchange and thus photosynthetic efficiency, so growth must be sacrificed for adequate defence. Furthermore, SA signalling induces large changes in cellular redox state, stimulating increased electron flow to PSII (Janda *et al.*, 2014). Overreduction of PSII increases ROS production for immune signalling but reduces photosynthetic efficiency, again demonstrating SA induces immune signalling at the expense of photosynthesis.

As AzA directly upregulates photosynthetic genes, including several that are downregulated by SA, this suggests that AzA limits SA-mediated suppression of photosynthesis during systemic immune signalling. This may contribute to the previously described suppression of PCD, as limiting PSII reduction will limit the SA-inducible redox burst that triggers PCD. Limiting suppression of photosynthesis will also tilt the growth/defence trade-off back towards growth. In the case of a primed immune response, pathogen surveillance is enhanced and immune response strength and speed is increased (Conrath *et al.* 2015). With increased speed of perception and response, the plant is unlikely to require full and lengthy activation of immunity, so may take steps to limit the energy investment into immunity after priming. These data further support my hypothesis that the role of AzA is to fine-tune SA responses to best suit a systemic immune response, not directly enhancing immunity but mitigating growth suppression to distinguish local immunity from systemic. This hypothesis could be supported by applying AzA to mock- and pathogen challenged plants and measuring gas exchange rates to quantifiably measure whether AzA can enhance photosynthetic rate and mitigate immune-linked suppression of photosynthesis.

3.3.4 – Interactions between azelaic acid and necrotrophic pathogens

SAR is a response primarily activated in response to biotrophic pathogens, which colonise living tissues to parasitise resources from the plant. However, a recent study has found an interesting interaction between AzA and necrotrophic pathogens, which attack plants to harvest resources directly from dead tissues. In tomato, it was shown that during attack by *Alternaria solani*, plants primed with AzA display less ROS accumulation and reduced cell death (Haghpannah *et al.*, 2024). In particular, AzA primed plants showed increased catalase activity and reduced peroxidase activity after infection. Overall, it was proposed that the role of AzA during SAR was to reduce the damage and potential cell death caused by ROS during an immune response (Haghpannah *et al.*, 2024). Interestingly these results are seen in response to necrotrophic infection, which typically induces jasmonic acid (JA) and ethylene (ET) signalling rather than SA. The reduction in cell death and ROS production support my findings that AzA reverse primes gene expression that relates to cell death and suppression of photosynthesis, but in this case it is unlikely that the AzA is reverse priming SA signalling specifically. JA signalling is predominantly primed by root interacting microbes in a process called induced systemic resistance (ISR) (Pieterse *et al.*, 2014), but a role for JA in SAR has also been proposed (Gaikwad *et al.*, 2023). Therefore, AzA produced by local infection may also contribute to the remodelling of systemic JA signalling as well as systemic SA signalling during SAR. Necrotrophic pathogens often trigger HR to facilitate infection (Govrin and Levine, 2000), so in this case AzA-primed suppression of PCD may act as a positive priming response rather than a side-effect reducing response. To test this, HR-linked genes could be selected from the AzA reverse primed cluster for testing by RT-qPCR. Plants would be sequentially treated with AzA and JA instead of AzA and SA, then the expression of HR genes compared to determine whether JA regulates these genes and whether AzA enhances JA-mediated suppression of HR. This result could also be verified by pre-treating plants with AzA, then an avirulent necrotrophic pathogen to measure the extent of HR lesions after necrotrophic infection.

The possible link between AzA and JA poses the question: could AzA be a component of ISR as well as SAR? Consensus on the mobile signals driving ISR has not been reached (Vlot *et al.*, 2021), but AzA supplied to roots appears to enhance immunity in shoots (Cecchini *et al.*, 2018), suggesting it could contribute to ISR. Furthermore, there are points of overlap in SAR and ISR. Both responses require intact NPR1 for effective priming, and redox signalling is a key component in both (Mou *et al.* 2003; Pieterse *et al.* 1998). Root treatment with ISR inducing bacteria has been shown to prime JA-responsive and SA-responsive gene expression (Niu *et al.*, 2011), so signals may be shared between the pathways. Redox bursts are a core component of both SA and JA signalling, and especially against necrotrophs it is important not to undergo cell death, so AzA may play a role in suppressing PCD in both SAR and ISR. AzA signalling has also been implicated in abiotic stress responses (Pitzschke *et al.*, 2014), which are driven by redox imbalances, giving precedent that AzA may have multifaceted signalling roles. It is entirely possible that AzA signalling is shared between SAR and ISR to suppress cell death, while other specific signals respectively prime SA- and JA-responsive signalling pathways depending on the inducing stimulus.

3.3.5 – Conclusion

The ability for AzA to modulate SA signalling but not boost immunity suggests that systemic signalling requires multiple signals that work synergistically, each with their own role. My data paint a clear picture of AzA signalling during SAR (Figure 3.9). Here, I hypothesise that multiple signals have distinct roles in systemic signalling. While other immune signals may establish priming of SA-mediated SAR, the main role of AzA is to distinguish local immune responses from systemic immune responses by suppressing ROS-mediated cell death and limiting the growth-defence trade-off. The action of AzA will increase the energetic efficiency of SAR, as with the increased speed and intensity of signalling from other signals, fewer resources need to be diverted from growth towards immunity.

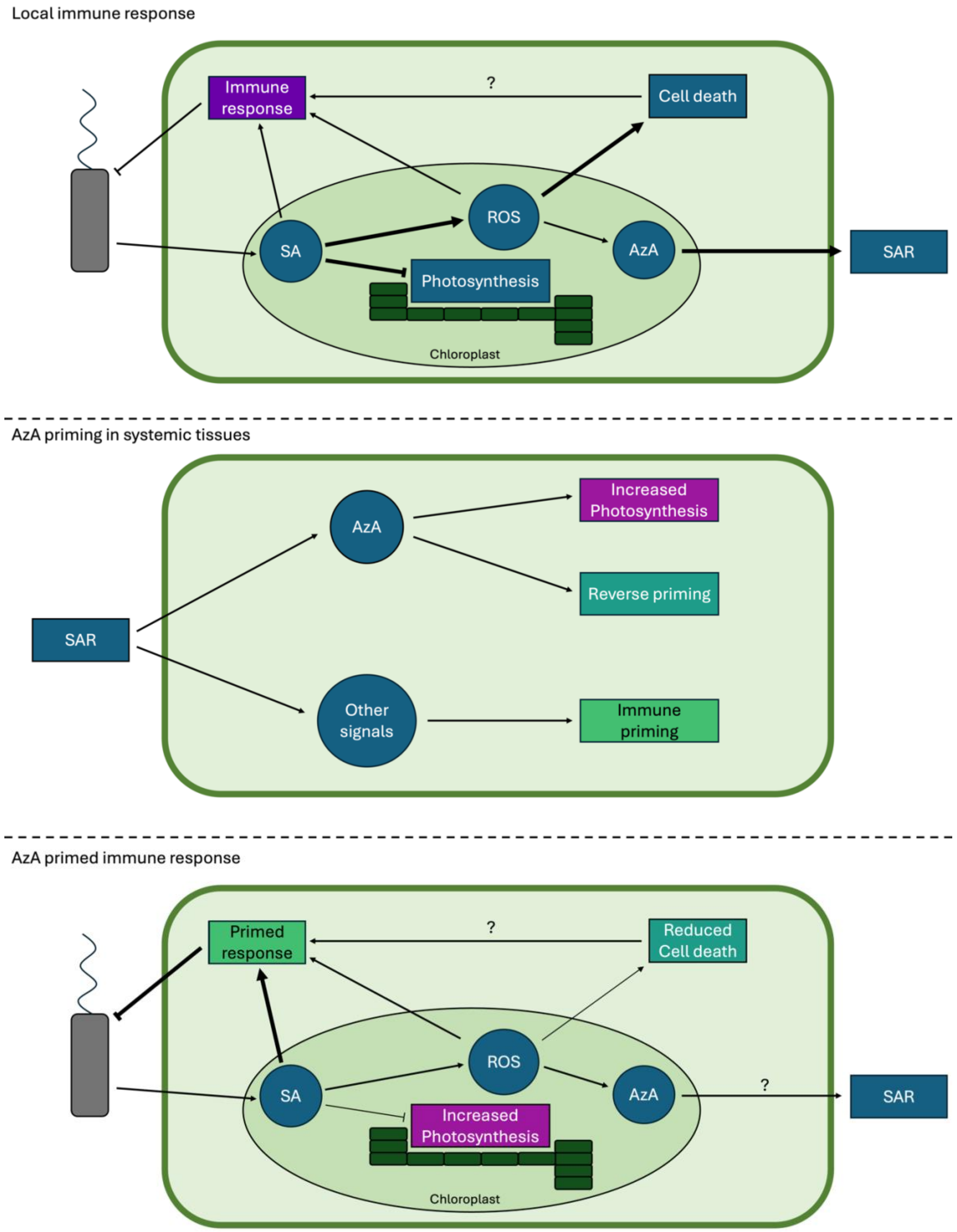


Figure 3.9 – Schematic diagram of AzA production and priming in local and systemic tissues. *In local immune responses, pathogen perception triggers SA signalling, which promotes ROS accumulation to drive an immune response. Sustained high levels of ROS produce AzA and promote cell death, which may or may not contribute to the response. AzA is then translocated to systemic tissues and primes the cells, reducing hypersensitive response gene expression and increasing photosynthetic rate. Partnering vascular mobile signals produced by the initial infection are*

also translocated to the systemic tissues, where they prime cells to potentiate immune signalling. When primed cells are challenged, a strong immune response is launched but ROS production and photosynthesis suppression is curtailed. Working together, these mobile signals potentiate the immune response while reducing the damaging side effects of sustained signalling.

Chapter 4 – N-hydroxy pipecolic acid selectively primes salicylic acid signalling

4.1 – Introduction

Of the phloem mobile signals involved in systemic acquired resistance (SAR), N-hydroxy-pipecolic acid (NHP) is the best studied. The NHP precursor, pipecolic acid (Pip), was first discovered accumulating in the local leaves, distal leaves, and petiole exudates of infected plants (Navarova *et al.*, 2012). Pretreatment with Pip enhances pathogen resistance and increases pathogen-responsive gene expression, while immune priming is abolished in Pip-deficient mutants (Navarova *et al.*, 2012; Bernsdorff *et al.*, 2016). Pip was then discovered to be a precursor for NHP, which accumulates to higher levels than Pip after infection and induces a stronger priming response (Hartmann *et al.*, 2018; Hartmann and Zeier, 2019). NHP treatment induces strong transcriptional responses in systemic tissues and upon infection, priming a large array of immune responses, including gene expression and metabolite biosynthesis (Yildiz *et al.*, 2021; Lowe *et al.*, 2023). The wide-ranging effects of NHP translate to the immune response phenotype, as NHP treatment induces resistance against a diverse group of pathogens, including multiple strains of the bacterium *Pseudomonas syringae* and the oomycete *Hyaloperonospora arabidopsidis* (Lowe *et al.*, 2023). The importance of NHP is exemplified in its presence and immune activity in a diverse group of plant species, including tobacco, tomato, cucumber, rice, barley and wheat (Vogel-Adgough *et al.*, 2013; Holmes *et al.*, 2019; Schnake *et al.*, 2020; Jung *et al.*, 2016; Brambilla *et al.*, 2023; Zhang *et al.*, 2021).

NHP biosynthesis begins in chloroplasts with the transamination of L-lysine into dehydropipecolic acid by AGD2-LIKE DEFENCE PROTEIN 1 (ALD1) and is subsequently reduced to L-Pip by SAR DEFICIENT 4 (SARD4) (Hartmann *et al.*, 2017; Ding *et al.*, 2016). Through unknown mechanisms, L-Pip is translocated to the cytosol, where it is hydroxylated into NHP by FLAVIN MONOOXYGENASE 1 (FMO1), which has long been known as a key node of systemic immunity (Hartmann *et al.*, 2018; Mishina and Zeier, 2006). Interestingly, the expression of all three NHP biosynthetic enzymes is regulated by SARD DEFICIENT 1 (SARD1) and CALMODULIN-BINDING PROTEIN 60g (CBP60g), a partially redundant pair of transcription factors that also promote SA biosynthesis by inducing expression of *ISOCHORISMATE SYNTHASE 1* (*ICS1*) (Sun *et al.* 2015; Sun *et al.* 2017; Wang *et al.*, 2011). The relationship between salicylic acid (SA) and NHP is complex, with multiple points of crosstalk, synergism and antagonism. Both SA and NHP individually promote the expression of SARD1/CBP60g (Sun *et al.*, 2017; Yildiz *et al.*, 2021), so each signal contributes to self-promoting and synergistic feedback loops of signal production. However, SA and NHP act antagonistically through expression of the glucosyltransferase *UDP-DEPENDENT GLYCOSYLTRANSFERASE 76B1* (*UGT76B1*) (Hartmann *et al.*, 2018; Foret *et al.*, 2025). UGT76B1 converts both SA and NHP to inactive SA-O-ß-glucoside (SAG) and NHP–N–O-glucoside (NHPG), respectively (Bauer *et al.*, 2021), which may fine tune the balance of immune activation and priming. In locally infected tissues the presence of SA promotes conversion of NHP to NHPG, while in systemic tissues the presence of NHP promotes conversion of SA to SAG (Yildiz *et al.*, 2021). Several other derivatives of NHP have also been described, although specific functions are yet to be ascribed to these molecules (Mohnike *et al.*, 2023).

Following NHP accumulation in local tissues, it is transported to the rest of the plant. While NHP exporters and systemic receptors have not yet been identified, mobility has been demonstrated by accumulation of radiolabelled NHP in the phloem and systemic tissues after infection, and NHP accumulation in systemic tissues before its precursors (Yildiz *et al.*, 2021; Hartmann and Zeier, 2019). In systemic tissues, NHP signalling relies on the transcription coactivator NONEXPRESSOR OF PR GENES 1 (NPR1) (Yildiz *et al.*, 2021). NPR1 is best known for regulating SA-mediated transcriptional reprogramming after infection (Wang *et al.*, 2006), making it another hub of NHP-SA interaction. TGA family transcription factors are also required for NHP signalling, with *tga1/4* and *tga2/5/6* mutants displaying different levels of systemic response impairment (Yildiz *et al.*, 2023). TGAs are key interaction partners of NPR1 and are expressed early in NHP signalling (Despres *et al.*, 2003; Foret *et al.*, 2025), suggesting that NHP deploys NPR1-TGA complexes for systemic immune responses. NHP also promotes rapid expression of a set of WRKY family transcription factors, including WRKY38, WRKY62 and WRKY70 (Foret *et al.*, 2025). In particular, NHP-mediated gene expression and immune responsiveness is dysregulated in *wrky70* mutants (Foret *et al.*, 2025), suggesting that WRKY family transcription factors are also important in NHP signalling.

Despite the interaction between NHP and SA biosynthesis, NHP signalling is not abolished in SA-deficient *ics1* (also known as *sid2*) mutants, although NHP-mediated priming is reduced (Bernsdorff *et al.*, 2016). It is plausible that NHP signals through NPR1 independent of SA biosynthesis, or basal SA levels in systemic tissues are sufficient to support NHP signalling. To further investigate the relationship between NHP and SA signalling, I adapted the sequential treatment protocol from Chapter 3

(Figure 3.1) to suit priming with NHP and elicitation with SA. Here, I discovered that NHP potentiates SA-responsive marker gene expression and does so independently of SA biosynthesis. Through RNA sequencing (RNA-seq), I found a more complex picture wherein NHP enhanced and diminished SA-responsive gene expression in roughly equal measures across the transcriptome. Unlike azelaic acid (AzA), NHP potentiated the SA-inducible immune response against *Pseudomonas syringae*, demonstrating that its major role is to act as an amplifier of SA-mediated immunity. In an effort to understand the molecular mechanisms by which NHP promotes SA signalling, I found that NHP stabilises NPR1 protein levels and promotes accumulation of NPR1 monomers. While accumulation of NPR1 alone was not sufficient to induce priming, this provides further evidence that NHP directly promotes SA-mediated immunity. Overall, the data in this chapter reveal that NHP selectively primes SA signalling by enhancing and suppressing SA-responsive expression of specific genes.

4.2 – Results

4.2.1 – NHP primes SA-responsive gene expression independent of SA biosynthesis

To determine whether NHP crosstalks with SA signalling, I first examined if NHP modifies SA-responsive marker gene expression. This was performed using a similar sequential treatment protocol as in Chapter 3 to mimic the role of NHP in pathogen-challenged systemic tissues. Fourteen-day-old wild-type (Columbia-0) *Arabidopsis thaliana* seedlings were treated with 1 mM NHP or water by immersion for 18 hours, then moved to 0.5 mM SA or water for an additional 6 hours. Total RNA was extracted and used in cDNA synthesis to measure expression of a selection of SA-responsive marker genes. For the SA marker genes *PR1*, *WRKY38* and *WRKY62*, NHP alone induced minor increases in transcript levels, but SA alone induced high levels of expression (Figure 4.1). However, after NHP pretreatment, SA-responsive expression was boosted well beyond the cumulative single signal effects, indicating that NHP primes the expression of SA-responsive genes.

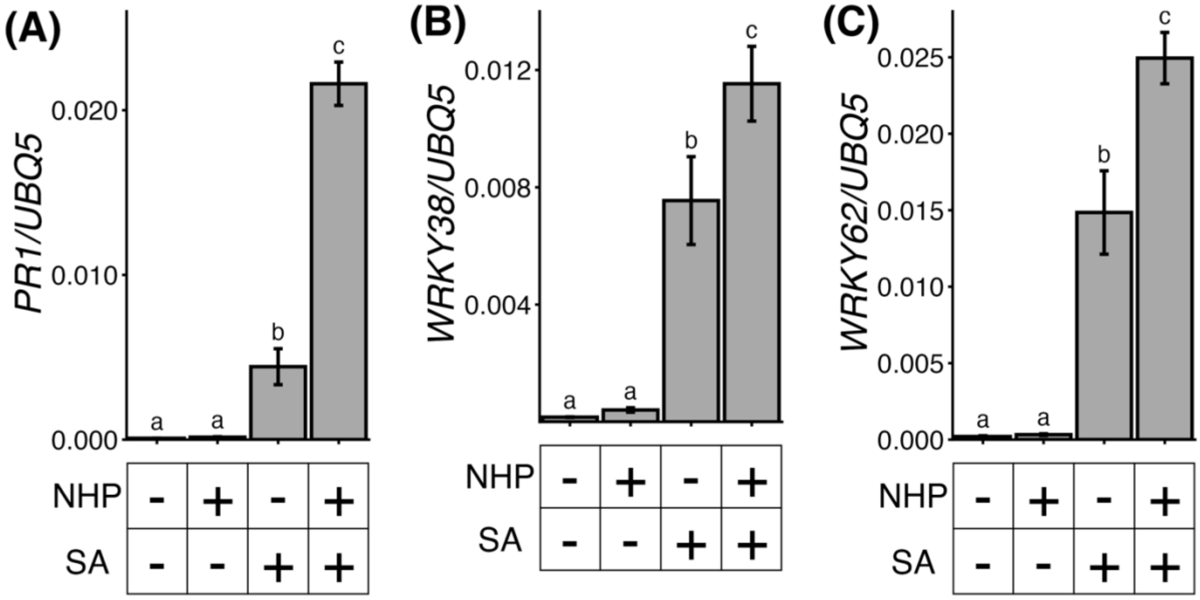


Figure 4.1 – NHP primes SA-responsive gene expression. *Fourteen-day-old wild-type seedlings were treated by immersion in water or 1 mM NHP for 18 hours, followed by treatment with water or 0.5 mM SA for 6 hours. Total RNA was extracted and the expression of SA-responsive genes PR1* ***(A)****, WRKY38* ***(B)****, and WRKY62* ***(C)*** *measured by qPCR. Reporter genes were normalised against constitutively expressed UBQ5. Letters denote statistically significant differences between samples (Tukey ANOVA; α = 0.05, n = 3).*

I then repeated the same assay on mutants with perturbed SA biosynthesis pathways to assess whether NHP-mediated priming is due to enhanced accumulation of endogenous SA. To assess both the isochorismate and phenylalanine ammonia-lyase biosynthesis pathways (Figure 1.1), I treated *ics1* (*sid2*) and *phenylalanine ammonia-*

lyase 1 (*pal1*) mutants alongside wild-type plants and measured *PR1* gene expression. In *ics1* mutants, SA signalling was not perturbed, but *pal1* mutants displayed impaired SA-responsive *PR1* expression. However, in both mutants the NHP priming effect persisted. Overall, this suggests that NHP-mediated priming is not reliant on SA-biosynthesis.

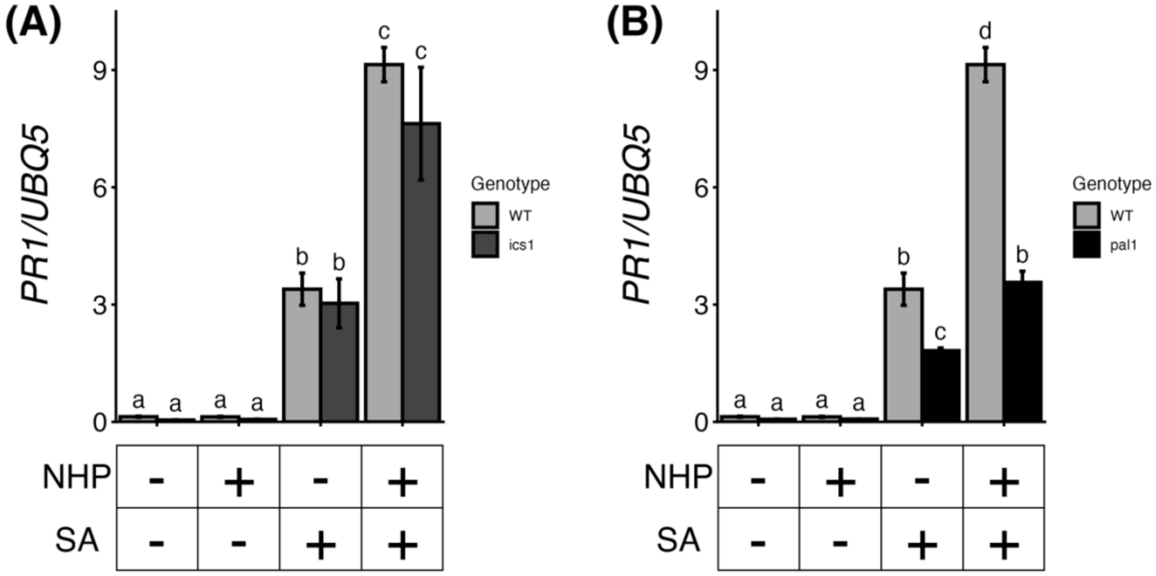


Figure 4.2 – NHP-mediated priming does not require SA biosynthesis**. Fourteen-day-old wild-type and ics1* ***(A) *or pal1* ***(B)*** *seedlings were treated by immersion in water or 1 mM NHP for 18 hours, followed by treatment with water or 0.5 mM SA for 6 hours. Total RNA was extracted and the expression PR1 measured by qPCR. Reporter genes were normalised against constitutively expressed UBQ5. Letters denote statistically significant differences between samples (Tukey ANOVA; α = 0.05, n = 3).*

4.2.2 – NHP modulates SA-responsive gene expression

To fully understand the extent by which NHP primes SA-responsive gene expression across the genome, I performed RNA sequencing on wild-type plants sequentially treated with NHP and SA as described above. First, I identified genes that were solely NHP-responsive but SA-unresponsive through linear modelling and selection of genes whose expression is significantly correlated with NHP treatment but not SA treatment.

From this I found 648 NHP-upregulated transcripts and 196 NHP-downregulated transcripts ($p \leq 0.05$), giving a total of 844 solely NHP-responsive genes. I then performed GO term enrichment analysis. While NHP downregulated genes displayed no significant GO terms, NHP upregulated genes were enriched for terms related to chlorophyll and phenylpropanoid biosynthetic or metabolic processes (Figure 4.3), suggesting signalling unique to NHP involves a wide range of secondary metabolism pathways.

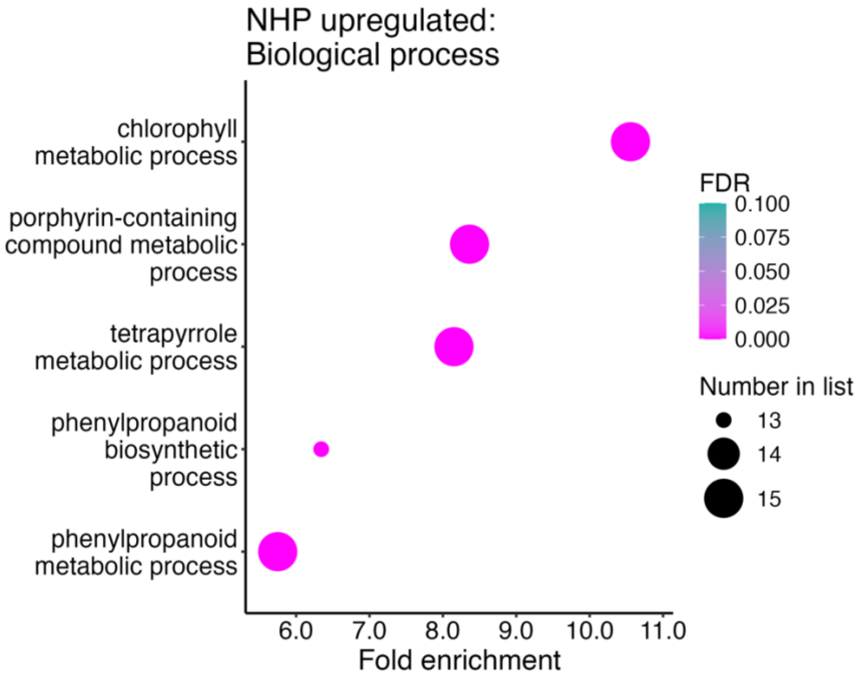


Figure 4.3 – NHP directly induces secondary metabolite processing genes. *Top 5 most enriched GO terms in solely NHP upregulated genes ($p \leq 0.05$, $FDR \leq 0.1$).*

A total of 6,626 SA-responsive genes were identified through linear modelling ($p \leq 0.05$) and separated into SA-upregulated and SA-downregulated genes for further analysis. NHP-mediated priming was confirmed by PCA, showing that NHP-primed SA samples are distinct from unprimed SA samples (Figure 4.4). The NHP-primed behaviour of SA-responsive genes was then identified by clustering. Unlike the AzA RNA-seq (Figures 3.4 and 3.6), the NHP-priming effects were more evenly spread. The same categories of priming were observed; unprimed genes, primed genes, reverse primed genes, synergistically expressed genes and antagonistically expressed genes (Figures 4.5 and 4.7). In total, NHP directly or indirectly modulated the expression of 75.2% (4,985 of 6,626) of detected SA-responsive transcripts.

Of the SA-upregulated genes, 29.4% (1,160 genes) were primed by NHP in that they showed little or no response to NHP alone, but altered SA-responsiveness after NHP pre-treatment. Of these, 601 genes exhibited primed SA-induced expression, while 559 genes were reverse primed (Figure 4.5). Interestingly, 37.5% (1,476 genes) of SA-upregulated genes were also NHP-upregulated, showing that a large proportion of genes respond cumulatively to both signals. Finally, 33.1% (1,304 genes) of SA-induced genes showed no responsiveness to NHP (Figure 4.5). To determine which SA-modulated cellular processes are also regulated by NHP, I performed GO term enrichment analysis on each cluster of genes (Figure 4.6). NHP- and SA-responsive genes were enriched for fatty acid and lipid oxidation with terms such as “lipid oxidation” and “fatty acid catabolic process” being highly enriched (Figure 4.6A). Interestingly, the most enriched GO terms in the SA-upregulated cluster included “programmed cell death” and “plant-type hypersensitive response” (Figure 4.6B), suggesting that NHP-mediated priming does not promote cell death responses. Instead, primed genes showed an abundance of hypoxia associated GO terms, such as “cellular response to hypoxia”, alongside SA-responsive GO terms (Figure 4.6C). Reverse primed genes were the most varied group, including GO terms such as “response to endoplasmic reticulum stress”, “response to light intensity” and “response to nitrogen compound” (Figure 4.6D). Overall, this suggests that NHP primarily acts to enhance SA signalling, including by priming SA-responsive genes and by synergistically regulating SA-expressed metabolic genes. It also shows that NHP does not enhance the expression of cell death-related genes and may instead fine tune some aspects of nutrient and light signalling.

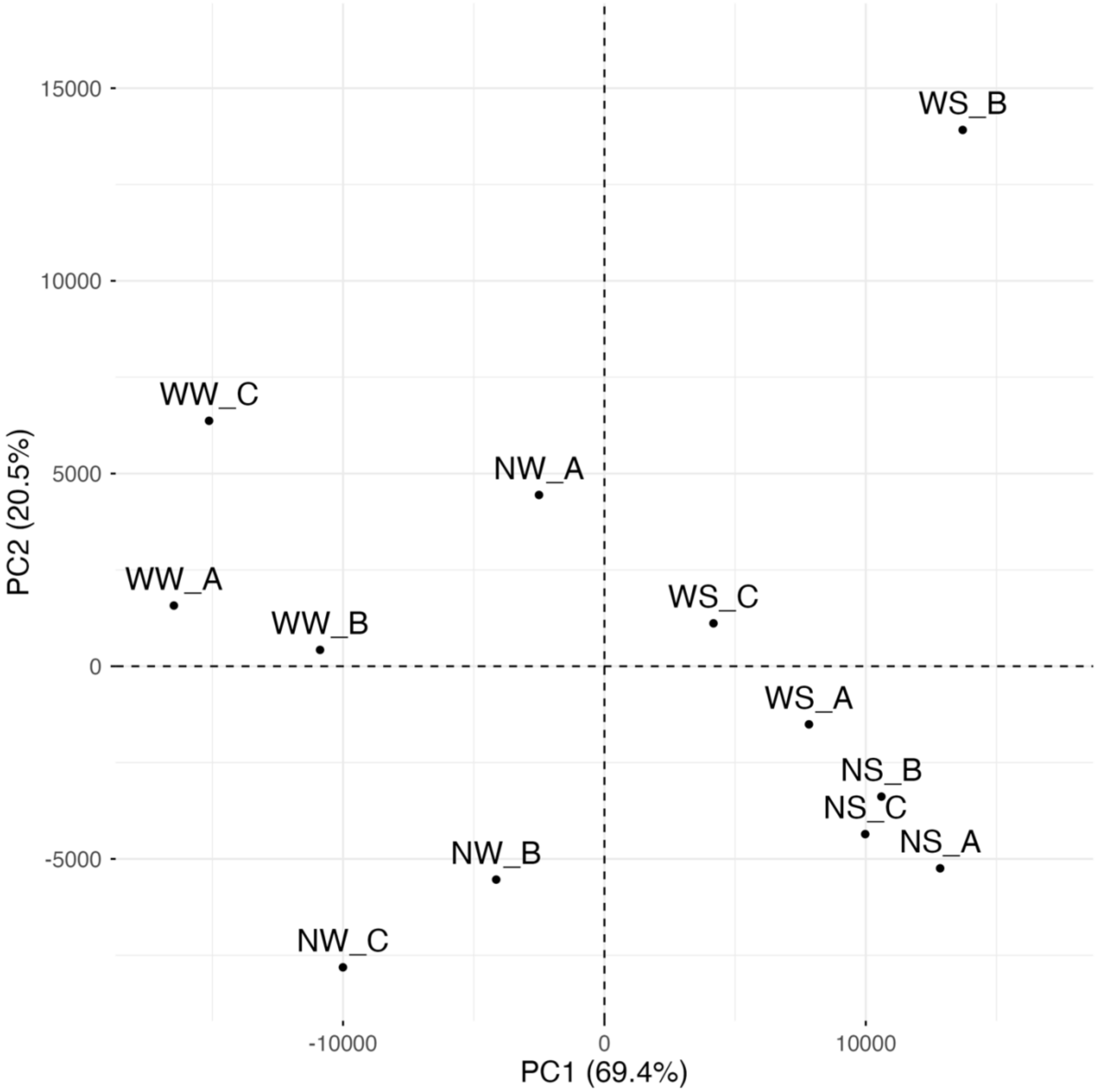


Figure 4.4 – Plants treated individually or sequentially with NHP and SA display distinct transcriptomic profiles when separated by PCA. *The treatments are denoted as follows: water + water = 'WW', NHP + water = 'NW', water + SA = 'WS' and NHP + SA = 'NS'. Each biological repeat is labelled A, B or C.*

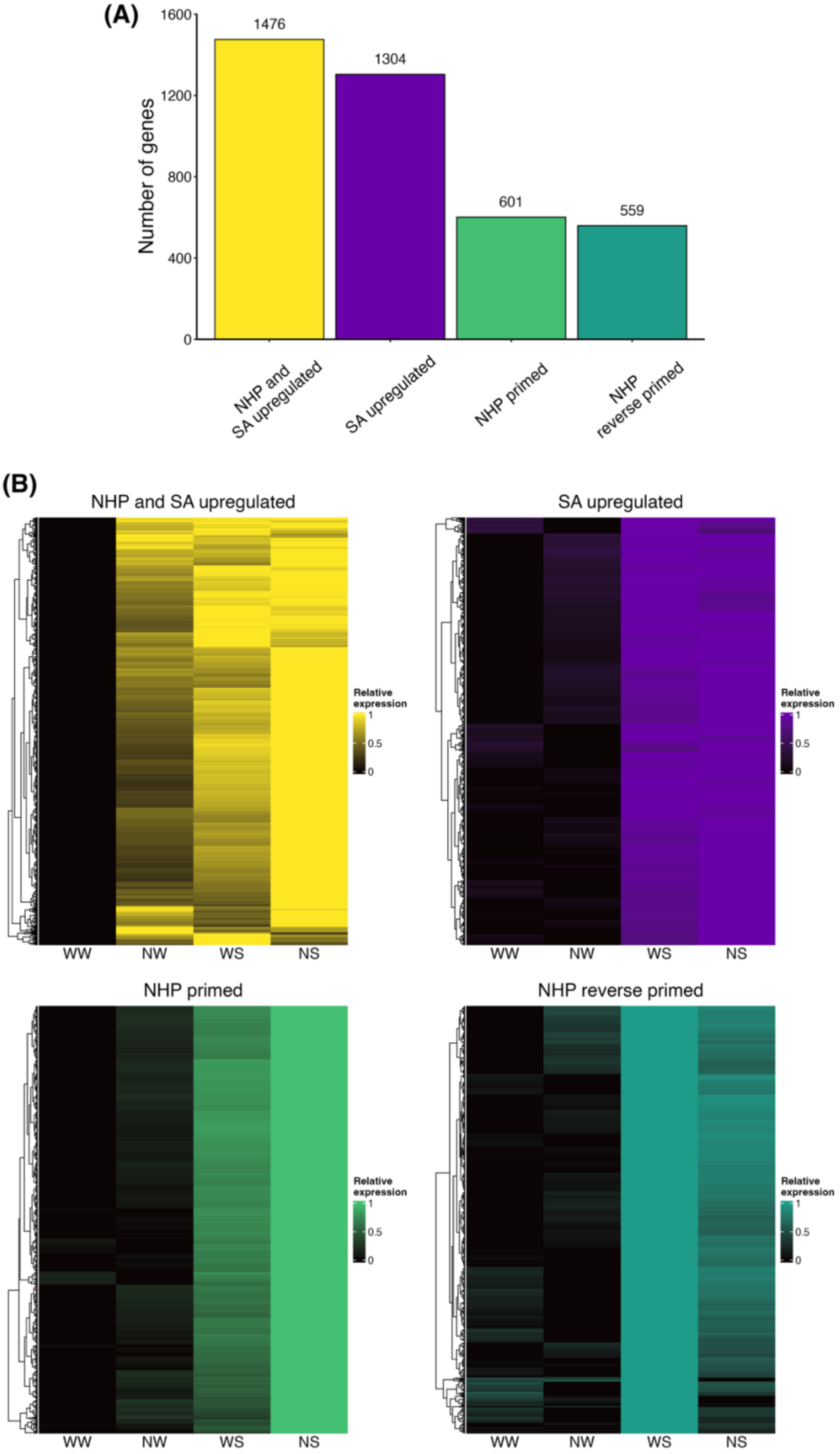


***Figure 4.5 - NHP diversely modulates SA-induced transcriptional reprogramming**. Wild type seedlings were treated as described in Figure 4.1. Total RNA was extracted and SA-responsive gene expression measured by RNA-*

*seq. **(A)** RNA-seq clusters of NHP-primed, SA-induced genes. Clusters were identified and assigned by hierarchical clustering using the hclust R package. **(B)** Heatmaps of clustered genes showing expression patterns across the four treatment conditions. 'Relative expression' is presented as transcripts per million values scaled from 0 to 1 across the four treatments. The treatments are denoted as follows: water + water = 'WW', NHP + water = 'NW', water + SA = 'WS' and NHP + SA = 'NS'.*

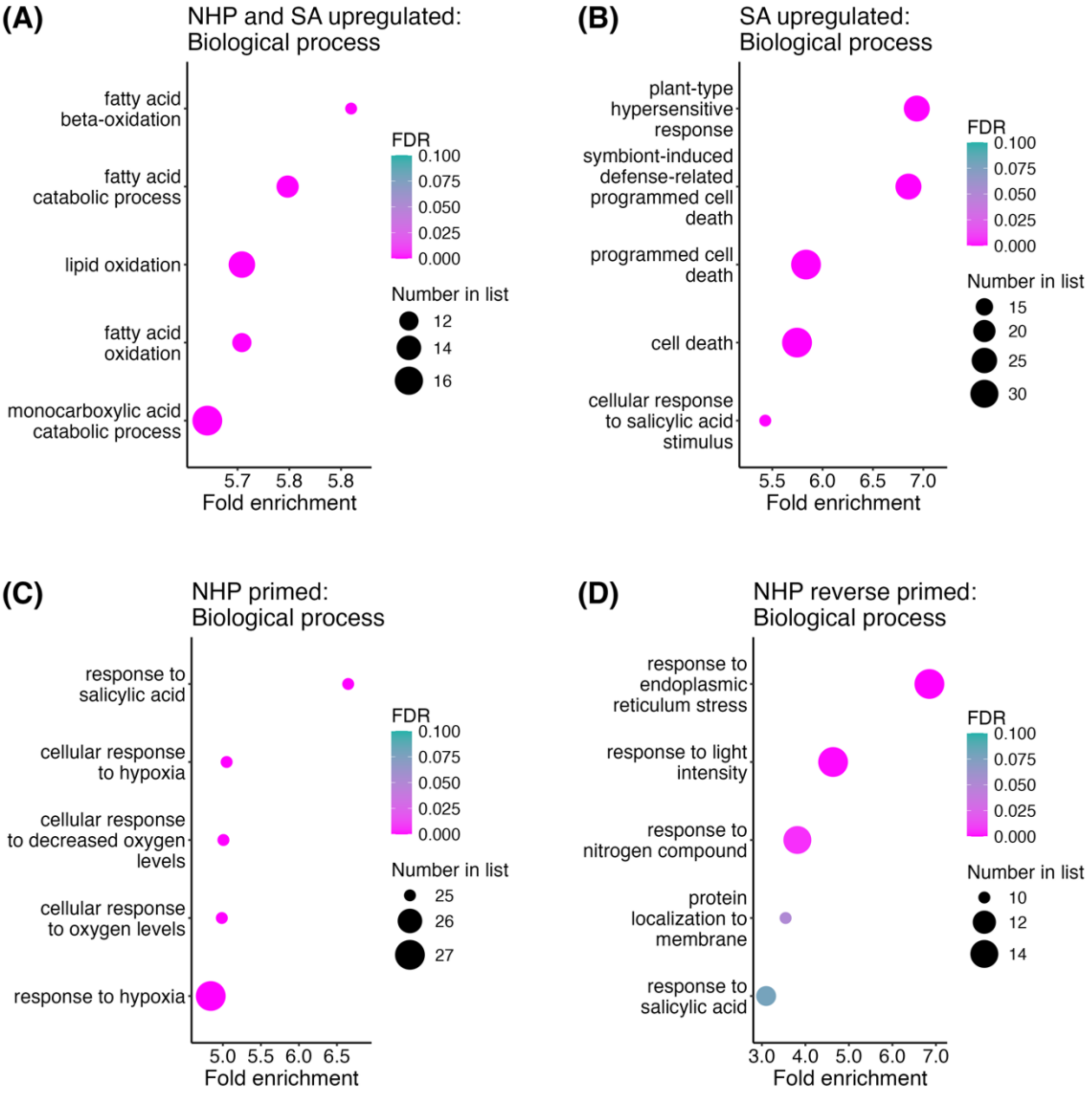


Figure 4.6 – NHP primed gene clusters show distinct enrichment with different GO terms. *Top 5 most enriched GO terms in each cluster of SA-upregulated genes ($p \leq 0.05$, $FDR \leq 0.1$).*

SA-downregulated genes separated into the same categories as SA upregulated, with an additional "NHP upregulated, SA downregulated" group, wherein NHP is directly antagonistic to SA signalling. Among SA-downregulated genes, NHP directly regulated more genes than it primed, with 48.0% (1,290 genes) classed as NHP and SA downregulated and 25.2% (678 genes) classed as NHP upregulated, SA downregulated. Priming was much less prevalent than in SA-upregulated genes, with only 9.4% (253 genes) reverse primed and 4.7% (128 genes) primed. The remaining 12.5% (337 genes) showed no NHP-modulated effect. Again, I performed GO term analysis on the SA-downregulated clusters. Across all clusters, most displayed enrichment for photosynthesis, translation or both. For example, the NHP and SA downregulated cluster contains two photosynthesis light harvesting terms and two ribosome assembly terms (Figure 4.8A). Both SA downregulated and NHP up/SA down categories contain photosynthesis terms (Figure 4.8B and C), while NHP reverse primed genes contain ribosome biogenesis and translation terms (Figure 4.8D). Overall, these data suggest that NHP directly promotes and represses different aspects of photosynthesis, whilst both directly and indirectly fine-tuning translational responses to SA. In this analysis, the NHP primed cluster did not display any significant enrichment with GO terms, likely due to its small size.

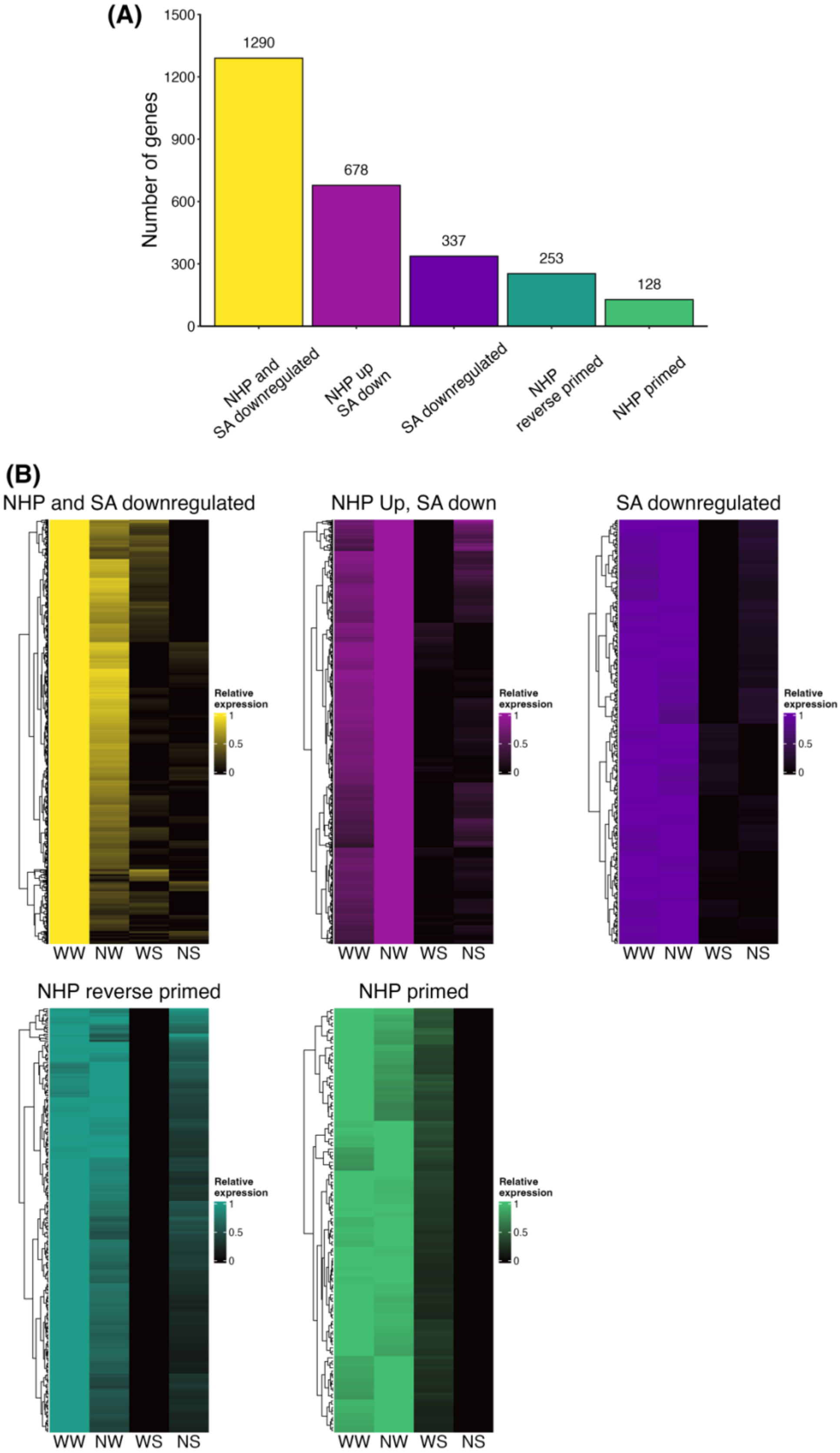


Figure 4.7 – NHP directly antagonises SA-mediated repression. *Wild type seedlings were treated as described in Figure 4.1. Total RNA was extracted and SA-responsive gene expression measured by RNA-seq.* ***(A)*** *RNA-seq clusters*

of NHP-primed, SA-repressed genes. Clusters were identified and assigned by hierarchical clustering using the hclust R package. ***(B)*** *Heatmaps of clustered genes showing expression patterns across the four treatment conditions. 'Relative expression' is presented as transcripts per million values scaled from 0 to 1 across the four treatments. The treatments are denoted as follows: water + water = 'WW', NHP + water = 'NW', water + SA = 'WS' and NHP + SA = 'NS'.*

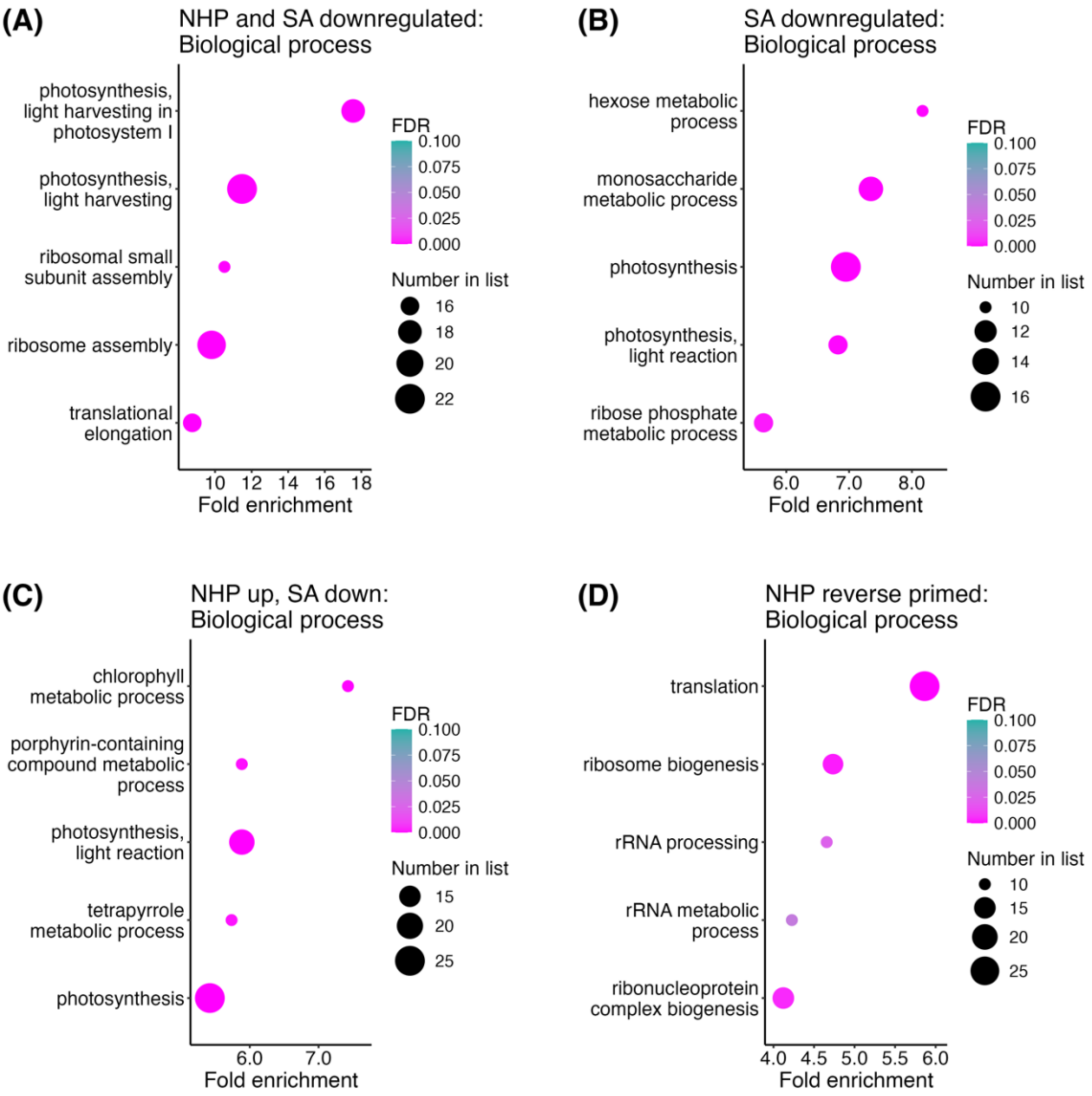
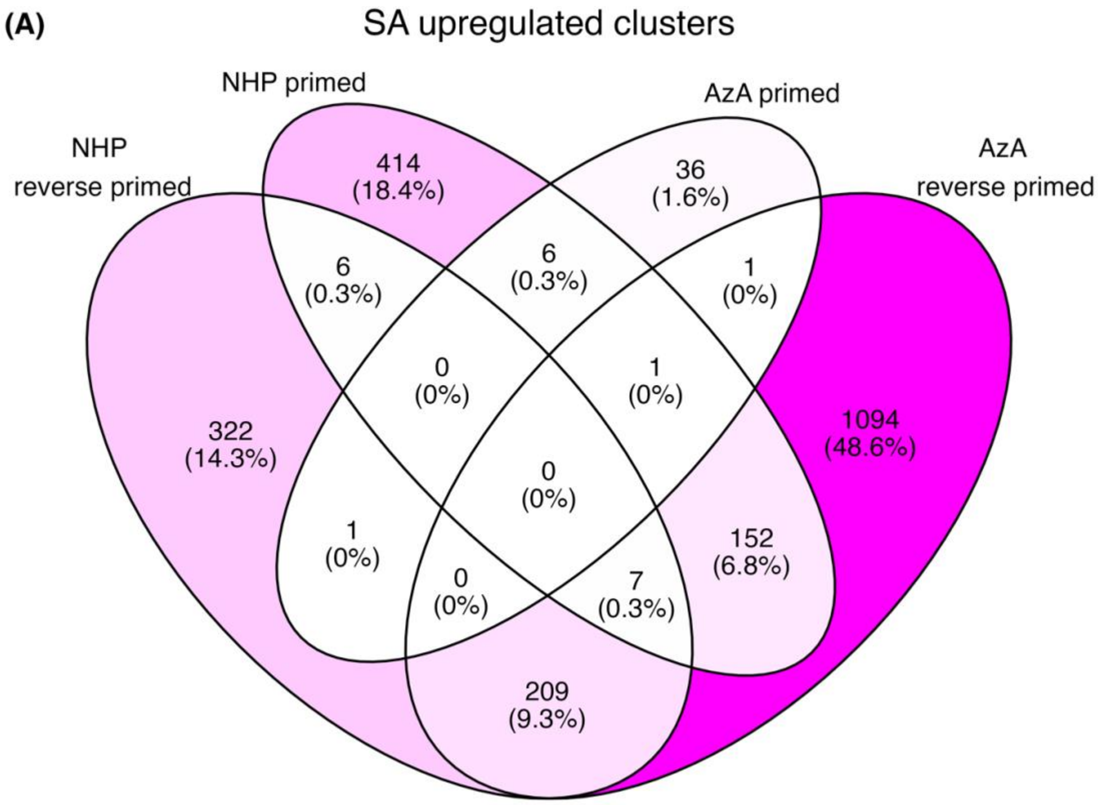


Figure 4.8 – NHP antagonises SA-mediated suppression of photosynthesis and translation *Top 5 most enriched GO terms in each cluster of SA-downregulated genes (p ≤ 0.05, FDR ≤ 0.1).*

Taken together, these data suggest that NHP fine tunes SA signalling during systemic immunity. The majority of NHP-mediated modulation is synergistic rather than antagonistic, suggesting NHP plays a more complementary role to SA signalling than the inhibitory effects of AzA reported in the previous chapter. GO term analysis reveals that NHP enhances SA signalling and metabolic modulation, while leaving the hypersensitive response (HR) untouched. NHP also fine tunes SA-regulated modulation of photosynthesis and translation, suggesting the role of NHP in systemic signalling is to increase the intensity of SA signalling, while adjusting the regulation of metabolic processes to facilitate the strengthened immune response.

4.2.3 – AzA and NHP prime different sets of genes

I next aimed to determine whether there is any overlap between the gene targets of NHP and AzA. While AzA and NHP appear to act in distinct ways on different components of SA signalling, the GO term analyses did not rule out whether they act independently or with some level of crosstalk. To investigate whether AzA and NHP target similar groups of genes, I compared the assigned clusters of SA-responsive transcripts primed or reverse primed by AzA or NHP (Figure 4.9). Of the 1497 NHP primed or reverse primed genes, only 26.7% (399) were also modulated by AzA, suggesting there is a small amount overlap between AzA and NHP primed genes. Moreover, for SA-downregulated genes there was nearly no overlap between AzA and NHP, with only 11 NHP primed genes and 12 reverse primed genes displaying modulation by AzA (Figure 4.9B). Overall, these data support that AzA and NHP have distinct and largely non-overlapping roles in modulating the SA-responsive transcriptome.



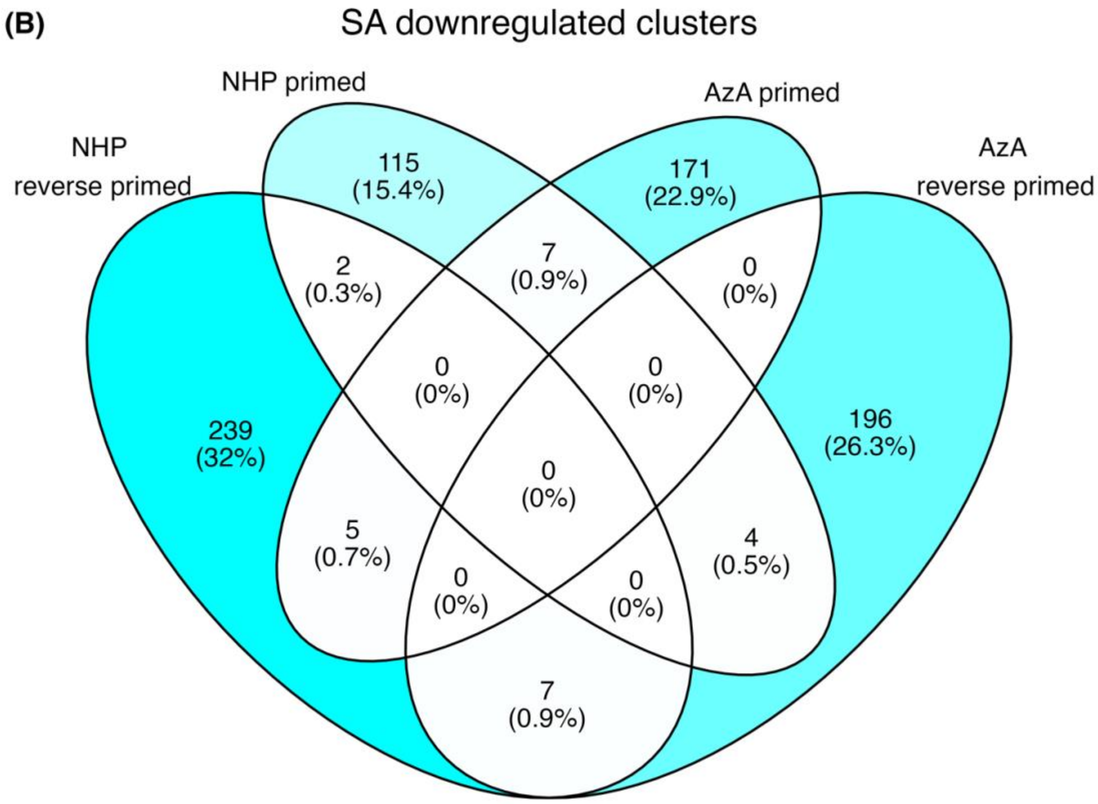
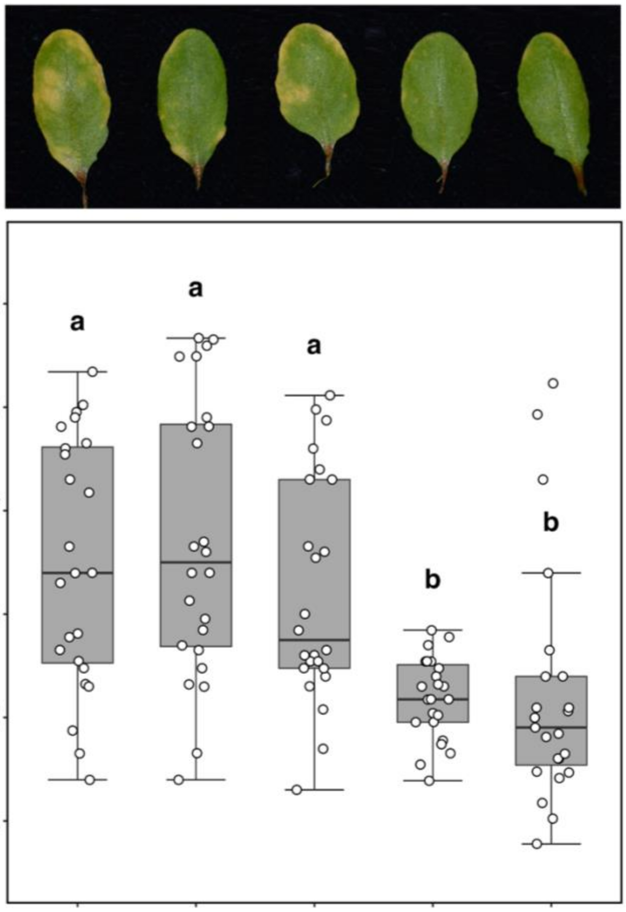


Figure 4.9 – AzA- and NHP-primed genes show little overlap. *Venn diagrams showing the number of SA upregulated **(A),** and SA downregulated **(B)** genes assigned to AzA and NHP primed clusters.*

4.2.4 – NHP signalling primes SA-responsive immunity

As NHP targeted a different gene set to AzA and unlike AzA, boosted SA signalling, I then assessed whether NHP can potentiate SA-induced immunity in adult plants. To test this, the disease assay from Chapter 3 was adapted, following the same priming and elicitation treatments 48 and 24 hours respectively before infection. As NHP comes in small volumes, for spraying I used the NHP precursor, pipecolic acid (Pip). The effects of Pip and NHP are comparable as both induced an observable priming effect (Appendix B). Adult *Arabidopsis* plants were sprayed with water or 1 mM Pip 48 hours before infection, followed by treatment with water, 0.05 mM SA, or 0.5 mM SA 24 hours before infection. Plants were then infected with *Pseudomonas syringae pv. maculicola* (*Psm*) ES4326 and its growth assessed 3 days after infection. I found that treatment with 1 mM Pip or 0.05 mM SA alone induced no significant response compared to water treatment, whereas 0.5 mM SA induced a strong reduction in bacterial growth (Figure 4.10). However, sequential treatment with 1 mM Pip followed by the low concentration of 0.05 mM SA induced a significant reduction in growth to the same level as a high concentration of 0.5 mM SA alone (Figure 4.10). This result supports the conclusions from my transcriptomic data, as it demonstrates that NHP signalling enhances SA-induced immunity by at least 10-fold.



1mM Pip	-	-	+	+	-
0.05mM SA	-	+	-	+	-
0.5mM SA	-	-	-	-	+

Figure 4.10 – Pre-treatment with NHP precursor pipecolic acid potentiates SA-inducible immunity against Psm ES4326. *Twenty-eight day-old wild-type plants were treated 48 hours before infection by spraying with water or 1 mM Pip, followed by treatment with water, 0.05 mM SA or 0.5 mM SA, 24 hours before infection. Leaves were then infiltrated with 5 x* 10^6 *colony forming units (cfu)/ml Psm ES4326. Leaf discs were analysed for bacterial growth 3 days post infection. Error bars represent interquartile range x1.5, while letters denote statistically significant differences between samples (Tukey ANOVA; α = 0.05, n = 24). Representative photos of leaves are displayed above the respective boxplots.*

4.2.5 – NHP stabilises NPR1, which contributes to, but does not wholly explain priming

With the importance of NHP in a plant immune response confirmed, my next goal was to investigate regulators of NHP-mediated priming. Therefore, I decided to investigate NPR1, which is indispensable for both SA-mediated local and systemic immune responses, is required for NHP-induced gene expression, and accumulates in response to NHP treatment (Cao *et al.*, 1994; Yildiz *et al.*, 2021; Nair *et al.*, 2021). Because NPR1 is tightly regulated by proteasome-mediated degradation (Spoel *et al.*, 2009), I first assessed whether NHP influences NPR1 ubiquitination. To this end, *Arabidopsis* seedlings expressing GFP-tagged NPR1 (*35S::NPR1-GFP* in *npr1-1* background) were treated sequentially with NHP and SA as described above, then ubiquitinated proteins pulled down using tandem ubiquitin binding entities (TUBEs). Ubiquitinated proteins were separated by SDS-PAGE and transferred to nitrocellulose for western blotting. Ubiquitinated NPR1 was detected by western blotting with an anti-GFP antibody. I found that NHP treatment alone induced ubiquitination of NPR1 to similar levels as SA, but NHP did not prime SA-mediated ubiquitination (Figure 4.11). This suggests that NHP is capable of directly altering the ubiquitination state of NPR1, which may feed into regulating the stability.

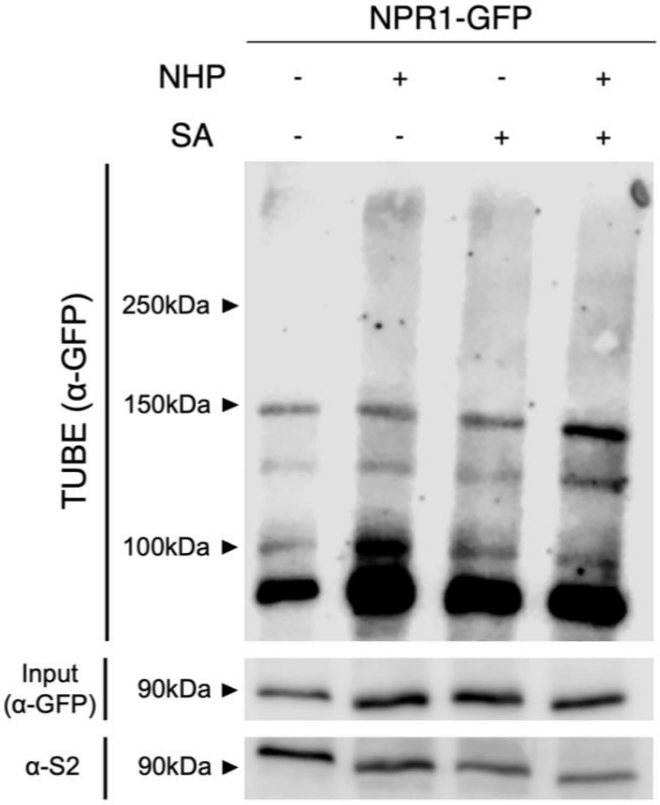


Figure 4.11 – NHP induces ubiquitination of NPR1. *Fourteen-day-old 35S::NPR1-GFP (npr1-1 background) seedlings were treated by immersion in water or 1 mM NHP for 18 hours, followed by treatment with water or 0.5 mM SA, supplemented with proteasome inhibitor MG132 for 6 hours. Ubiquitinated proteins were pulled down with TUBEs, and ubiquitinated NPR1 visualised by western blotting with an anti-GFP antibody. Equal loading of protein was assessed using an anti-proteasome (S2) antibody. Blot representative of a pattern observed in three independent experiments.*

As NHP was capable of modulating NPR1 ubiquitination, I then tested whether NHP could modulate NPR1 stability. *NPR1-GFP* seedlings were treated sequentially with NHP and SA as described above. Total protein was extracted, and NPR1 detected by western blotting with an anti-GFP antibody. As expected, both SA treatment alone and NHP treatment alone induced accumulation of NPR1 (Figure 4.12A). However, NHP pre-treatment did not enhance SA-inducible NPR1 accumulation. To assess whether the accumulated NPR1 is active or inactive, I then measured accumulation of NPR1 in its active monomeric/dimeric state or the inactive oligomer. To that end I extracted protein in presence or absence of reducing agent to respectively disrupt or maintain the disulfide-linked NPR1 oligomer. While NPR1 is now known to act as a dimer (Kumar *et al.*, 2022), the monomeric form significantly accumulates after SA treatment in non-reducing conditions (Mou *et al.*, 2003), so monomer accumulation can be considered a marker of accumulation of active NPR1. Reminiscent of SA, NHP alone induced accumulation of both NPR1 oligomer, monomer, and possibly dimer, but pretreatment with NHP did not further promote SA-mediated monomerisation (Figure 4.12B).

Non-oligomeric NPR1 is tightly regulated by post-translational modifications and proteasome-mediated degradation (Spoel *et al.*, 2009; Skelly *et al.*, 2019; Wang *et al.*, 2022), so I then aimed to determine if NHP induces monomeric NPR1 accumulation by modulating its mRNA expression or protein stability. Using qPCR I measured NHP-mediated expression and priming of endogenous *NPR1* gene expression, finding that NHP did not induce or prime its expression (Appendix C). This suggests that NHP, like SA, may promote stabilisation of NPR1 protein. To test this, I treated *NPR1-GFP* seedlings with water or NHP overnight and then added cycloheximide (CHX) to inhibit protein translation. I found that NHP treatment increased NPR1-GFP stability such that after 6 hours of CHX treatment, NPR1-GFP was still detected by western blot, whereas the protein was undetectable in the presence of only water after just 4 hours (Figure 4.12C). SA stabilises NPR1 protein, but also promotes NPR1 degradation to maximise expression of NPR1 target genes (Spoel *et al.*, 2009). Therefore, I then repeated this assay to determine whether NHP modulates SA-mediated NPR1 turnover. Seedlings were treated overnight with water or NHP, then with SA for 4 hours before CHX treatment and time course sampling as described above. NHP did not substantially alter the rate of SA-mediated degradation of NPR1 protein, suggesting NHP does not prime or influence NPR1's stability to modulate SA- and NPR1-dependent gene expression.

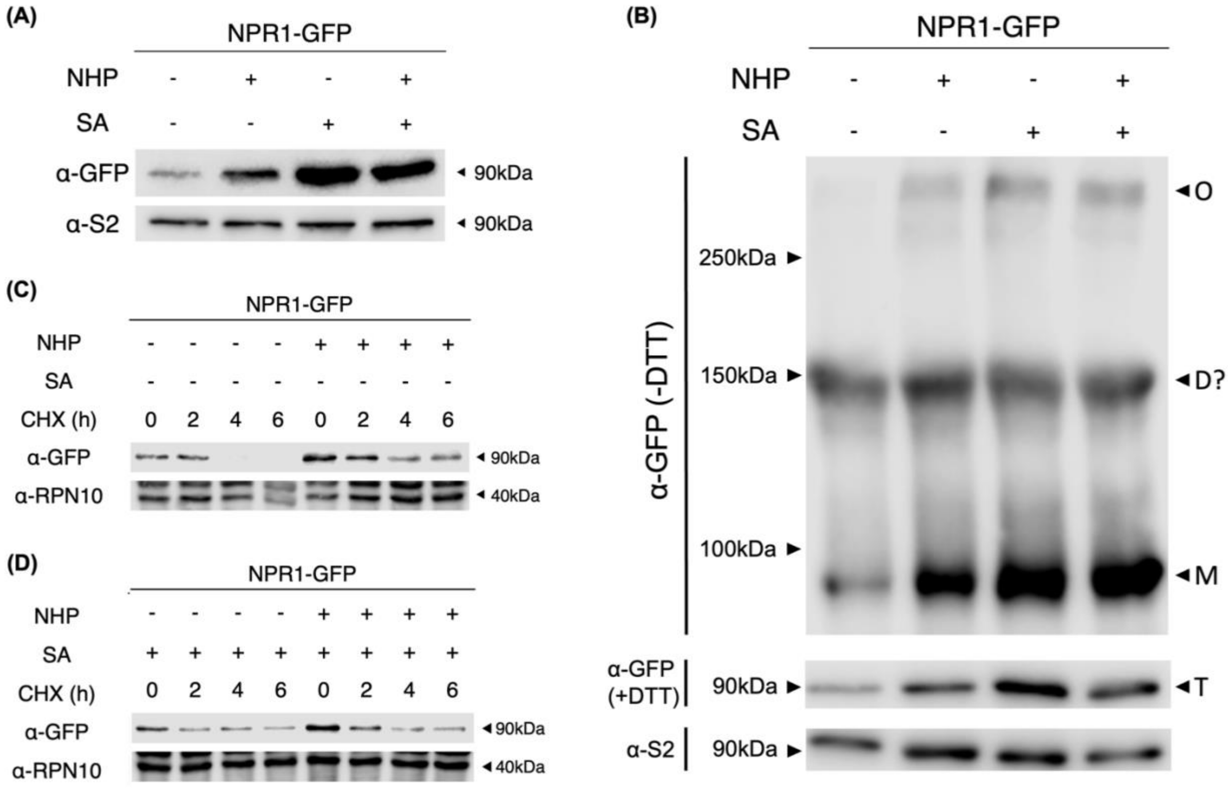


Figure 4.12 – NHP promotes monomeric NPR1 accumulation by stabilisation. *Fourteen-day-old 35S::NPR1-GFP (npr1-1 background) seedlings were treated by immersion in water or 1 mM NHP for 18 hours, followed by treatment with water or 0.5 mM SA for 6 hours.* ***(A)*** *Total protein was extracted and, NPR1 was visualised by western blotting with an anti-GFP antibody. Equal loading of protein was assessed using an anti-proteasome (S2) antibody. Blot representative of a pattern observed in three independent experiments.* ***(B)*** *Total protein (T) was extracted in a non-reducing sample buffer, monomeric (M) and oligomeric (O) NPR1 was visualised by western blotting with an anti-GFP antibody. An unknown band (D?) was observed at 150kDa that has been previously described when performing this experiment on this line (Tada et al., 2008), it may be an NPR1-GFP dimer. Equal loading of protein was assessed using an anti-proteasome (S2) antibody. Blot representative of a pattern observed in three independent experiments.* ***(C)*** *35S::NPR1-GFP seedlings were treated as described in (A), but 2 hours after removal of NHP, 100µM CHX was added. Samples were taken immediately after adding CHX and thereafter at 2, 4 and 6 hour timepoints. Total protein was extracted and, NPR1 was visualised by western blotting with an anti-GFP antibody. Equal loading of protein was assessed using an anti-proteasome (RPN10) antibody. Blot representative of a pattern observed in three independent*

experiments. ***(D)*** *35S::NPR1-GFP seedlings were treated as described in (A), but 2 hours after addition of SA, 100µM CHX was added. Samples were taken immediately after adding CHX and thereafter at 2, 4 and 6 hour timepoints. Total protein was extracted and, NPR1 was visualised by western blotting with an anti-GFP antibody. Equal loading of protein was assessed using an anti-proteasome (RPN10) antibody. Blot representative of a pattern observed in three independent experiments.*

To investigate whether enhanced accumulation of NPR1 is sufficient for priming, I then assessed NHP-mediated *PR1* priming in mutants that hyperaccumulate NPR1. This included *nonexpressor of pr genes 3 and 4 (npr3/4)*, *cullin 3 (cul3a/b)* and *ube4* (also known as *muse3*) mutants, all of which are involved in ubiquitination and degradation of NPR1 (Fu *et al.*, 2012; Spoel *et al.*, 2009; Skelly *et al.* 2019). In all three mutants, NHP primed SA-induced *PR1* gene expression (Figure 4.13), suggesting that NHP-mediated priming is not solely due to enhanced NPR1 accumulation. Overall, these results demonstrate that NPR1 accumulation alone is insufficient to induce priming. Instead, NPR1 accumulation could be a mechanism of enabling priming, i.e. increased NPR1 levels enable the increased strength of a primed SA response.

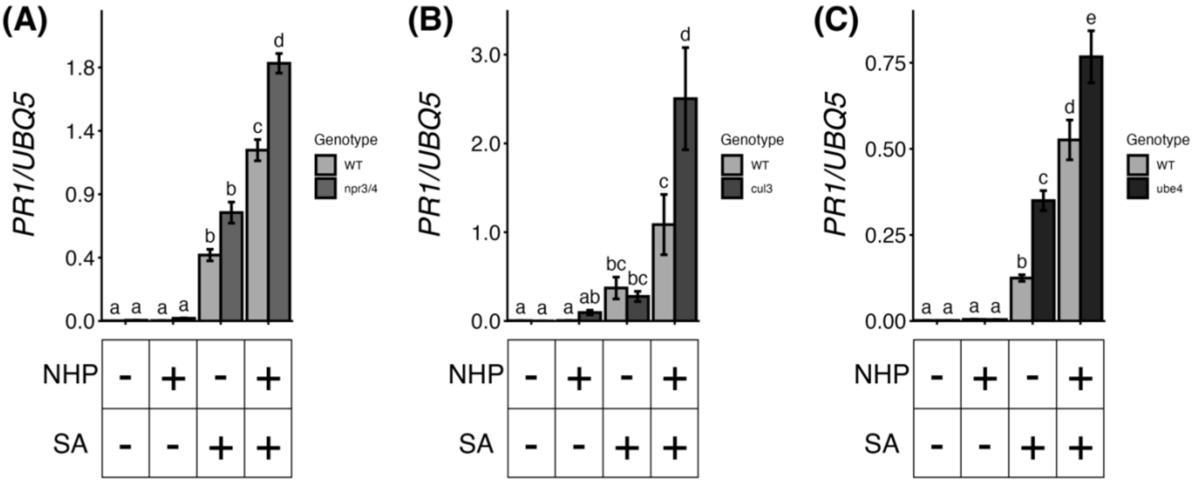


Figure 4.13 – Elevated NPR1 levels alone are not sufficient to induce priming. *Fourteen-day-old wild-type and npr3/4* ***(A)****, cul3a/b* ***(B)*** *or ube4* ***(C)*** *seedlings were treated by immersion in water or 1 mM NHP for 18 hours, followed by treatment with water or 0.5 mM SA for 6 hours. Total RNA was extracted and the expression PR1 measured by*

qPCR. Reporter genes were normalised against constitutively expressed UBQ5. Letters denote statistically significant differences between samples (Tukey ANOVA; α = 0.05, n = 3).

4.2.6 – NHP promotes global ubiquitination, but proteasomal degradation is not required for priming

Interestingly, in the controls for the NPR1 ubiquitination assays I also saw that NHP induced accumulation of total cellular ubiquitin (Appendix D). To investigate this further, I treated wild-type and *npr1-1* mutant seedlings overnight with NHP, then extracted total protein and measured cellular ubiquitin by western blotting with an anti-ubiquitin antibody (P4D1). Here I found that in both wild-type plants and *npr1* mutants, NHP induced moderate increases in cellular ubiquitin conjugates (Figure 4.14A), suggesting NHP induces global cellular ubiquitination independent of NPR1.

Ubiquitin is best known for promoting protein degradation via the proteasome, but also acts as a non-proteolytic signal, regulating protein interactions and function. To decipher if NHP-induced cellular ubiquitination could trigger priming through protein degradation or signalling, I repeated *PR1* priming assays in presence of the proteasome inhibitor MG132. First, I treated plants overnight with water or NHP, supplementing with MG132 2 hours after the start of treatment to inhibit protein degradation during the priming stage. Adding MG132 in the NHP-priming stage increased *PR1* expression regardless of treatment, but did not perturb NHP-mediated priming (Figure 4.14B). I then repeated this assay but supplemented with MG132 only at 2 hours after immersion in SA to inhibit protein degradation during activation of a primed response. As previously reported (Spoel *et al.*, 2009), this treatment scheme reduced overall *PR1* expression, but again did not disrupt the NHP-mediated launching of a primed response (Figure 4.14C). Overall, these findings may suggest that NHP-induced global ubiquitination primarily has non-proteolytic signalling functions rather than promoting protein degradation.

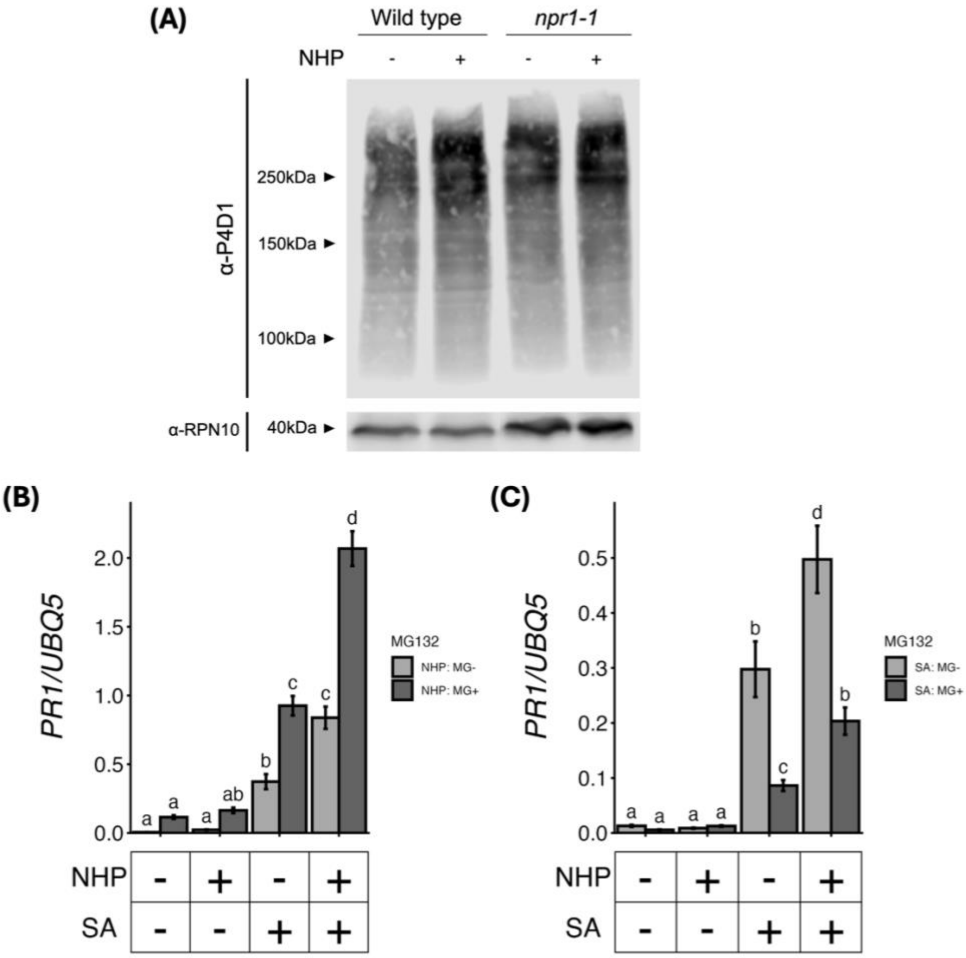


Figure 4.14 – NHP induces global ubiquitination but proteasomal degradation is not required in the priming or activation of NHP-primed SA signalling. (A) *Fourteen-day-old wild type and npr1-1seedlings were treated by immersion in water or 1 mM NHP, supplemented with MG132 for 18 hours. Total protein was extracted and, ubiquitinated proteins were visualised by western blotting with an anti-ubiquitin antibody. Equal loading of protein was assessed using an anti-proteasome (RPN10) antibody. Blot representative of a pattern observed in three independent experiments.* ***(B)*** *Fourteen-day-old wild-type seedlings were treated by immersion in water or 1 mM NHP supplemented with MG132 for 18 hours, followed by treatment with water or 0.5 mM SA for 6 hours. Total RNA was*

extracted and the SA-responsive expression of PR1measured by qPCR. ***(C)*** *Seedlings were treated as in (A) but supplemented with MG132 during SA treatment rather than NHP. Total RNA was extracted and the SA-responsive expression of PR1measured by qPCR. Reporter genes were normalised against constitutively expressed UBQ5. Letters denote statistically significant differences between samples (Tukey ANOVA; α = 0.05, n = 3).*

4.3 – Discussion

4.3.1 – Interactions between NHP and SA

NHP is a well-established systemic immune signal, but its relationship with SA signalling is not as well understood. In this chapter I show that NHP modulates SA-responsive gene expression, primarily boosting but also selectively inhibiting certain SA-responsive gene sectors. In accordance, I demonstrate that NHP signalling strongly potentiates SA-induced immunity against pathogen infection. In search of the mechanisms by which NHP promotes SA signalling, I found that NHP ubiquitinates and yet stabilises the key immune regulator NPR1. NHP also increases global cellular ubiquitination, but its priming effects do not rely on proteasome-mediated protein degradation, suggesting that NHP may instead utilise non-proteolytic ubiquitin signalling.

As discussed in Chapter 3.3.1, the approach to studying mobile signals in this chapter differs from previously established methodology. Most research has focused on the role of mobile NHP signalling through methods such as treating roots with NHP and exploring transcriptional and immune responses in leaves (Bernsdorff *et al.* 2016; Yildiz *et al.*, 2021). Here, I assessed how previous exposure to NHP influences future SA signalling to investigate how NHP induces a primed immune state. In the case of NHP, the direct responses to signal treatment are well characterised (Yildiz *et al.*, 2021), so it is not a significant concern that the approaches in this chapter provide less information on direct NHP signalling. The NHP-mediated priming response described in this chapter complement previous research in NHP signalling, providing some explanations for how NHP pretreatment enhances immunity.

Unlike AzA, the sequential NHP-SA treatment has been performed and shown an effect prior to this study. Previously, NHP was shown to prime immune responses and SA-responsive gene expression (Yildiz *et al.*, 2021), but only of the SA marker gene *PR1*. Here, I confirmed this effect, showing that NHP pretreatment enhanced SA-mediated expression of *PR1* and further marker genes *WRKY38* and *WRKY62* (Figure 4.1). To investigate whether these effects extend to the whole transcriptome, I then performed RNA-seq analysis and found that NHP modulated the expression of 75.2% of SA-responsive genes (Figures 4.3 and 4.6). Like AzA (Figures 3.2 and 3.5), NHP signalling was both synergistic and antagonistic with SA signalling. However, unlike AzA, the NHP priming effects were more supportive than repressive, as NHP enhanced the expression of 52.7% of SA-responsive transcripts and impaired the expression of only 22.5%. GO term analysis of the synergistic NHP clusters showed that NHP enhanced various aspects of SA signalling, including lipid metabolism, hypoxia responses and suppression of photosynthesis (Figures 4.5 and 4.7). Conversely, NHP inhibited SA-mediated endoplasmic reticulum signalling and suppression of translation and photosynthesis through a mixture of direct regulation and priming. These results show that NHP is more complex than just an enhancer or suppressor of SA, and rather works to fine tune SA signalling in systemic immunity. This is exemplified by the presence of photosynthetic genes in both NHP upregulated and downregulated clusters, suggesting

NHP tailors metabolic processes to best support a primed immune response. Of particular interest was also that the cluster unaffected by NHP but upregulated by SA was primarily enriched with HR genes (Figure 4.5), suggesting that the NHP priming enhances immunity without increasing rates of cell death. These results fit the expectations of a systemic signal. During SAR, cell death is suppressed as primed immune responses are sufficient to combat infection without HR (Rate and Greenberg, 2001). Accordingly, NHP-mediated priming will not prime HR genes and only enhance the SA signalling pathways that are required for SAR.

The high overlap between NHP and SA signalling raises an important question: does NHP require SA biosynthesis to prime immunity? Previous research into the SA biosynthesis mutant *ics1* (*sid2*) suggests that SA biosynthesis is vital for NHP signalling. In *ics1* mutants, NHP pretreatment is markedly less effective in enhancing disease resistance against both bacterial and oomycete pathogens (Hartmann *et al.* 2018; Yildiz *et al.*, 2021). However, in these experiments inducible SA biosynthesis is lost in both the priming phase and the pathogen response phase, which makes it unclear whether SA biosynthesis is required for establishment of priming or for the launching of primed responses. To dissect this, I repeated priming assays on SA biosynthesis mutants *ics1* and *pal1*. I found that NHP priming is not lost in these mutants (Figure 4.2), indicating that SA is not required for NHP to establish immune priming. This conclusion is supported by published evidence that NHP-inducible gene expression in systemic tissues is reduced, but not abolished, in *ics1* mutants (Yildiz *et al.*, 2021). Indeed, NHP does induce the accumulation of SA in systemic tissues, but upon more precise investigation this was revealed to primarily be inactive SA glucoside, SAG (Yildiz *et al.*, 2021). While accumulation of inactive signalling molecules will undoubtably contribute to a primed immune response, these data suggest that SA biosynthesis is not required for NHP-mediated priming of SA signalling.

Previous research into primed immune responses shows that SAR is mostly an enhancement of existing immune response pathways rather than a unique signalling pathway. For example, pathogen- or NHP-primed plants display increased accumulation of SA, NHP, branched chain amino acids, camalexins and reactive oxygen species (Hartmann *et al.*, 2018; Yildiz *et al.*, 2021; Lowe *et al.* 2023; Foret *et al.*, 2025). Here, I suggest that these observations are a result of NHP priming SA-responsive gene expression and therefore SA-mediated production of molecules and metabolites in response to infection. I further investigated this hypothesis by assessing the effect of NHP on SA signalling at the immune response level. Spraying leaves with the NHP precursor Pip or a low concentration of SA (0.05 mM) had no effect on resistance to the pathogen *Psm* (Figure 4.10). However, spraying sequentially with Pip followed by 0.05 mM SA showed that Pip primed the plants such that this low amount of SA was now sufficient to induce an immune response comparable to a ten times higher concentration of 0.5 mM SA. Overall, my results and previous research show that NHP primes the SA-responsive transcriptome to trigger an enhanced and tailored immune response.

4.3.2 – Interactions between NHP and AzA

Previously I found that AzA displayed no ability to enhance SA-mediated immunity, and primarily dampened components of SA-induced transcriptional reprogramming (Figures 3.2 and 3.8). From this I proposed that AzA may largely prevent unfavourable side effects of enhanced SA signalling, while a different mobile signal may be responsible for priming the immune response (Figure 3.8). Here, I show that NHP primarily acts synergistically with SA signalling and potentiates SA-mediated immunity without influenced HR gene expression (Figures 4.5 and 4.10), suggesting that NHP is the partnering signal.

So, if AzA and NHP have different effects on SA signalling, do they also target different SA-responsive gene sectors? To assess this, I compared the assignment of genes to each priming cluster and found a striking lack of overlap between each NHP- and AzA primed cluster (Figure 4.9), suggesting that NHP and AzA target distinct components of SA signalling. This is further supported by the lack of overlap in GO terms enriched in each cluster. For example, AzA reverse primed genes enriched for the hypersensitive response, but these genes were not regulated by NHP (Figures 4.5B and 3.4A), suggesting that NHP does not manipulate hypersensitive cell death, whereas AzA does. While there is some overlap in GO terms antagonised by each signal, such as reversal of photosynthesis suppression (Figures 3.7 and 4.8), each signal displays a distinct priming and immune response phenotype.

Crosstalk between synergistic and antagonistic immune signals is not unique to NHP, AzA and SA. For example, in response to necrotrophic pathogen infection both ethylene (ET) and jasmonic acid (JA) are required to fully launch an immune response (Penninckx *et al.*, 1998). Similarly, JA and abscisic acid (ABA) synergise to promote responses to abiotic stress, but ET plays a role in dampening ABA signalling (Kim *et al.*, 2021; Beaudoin *et al.*, 2000). Antagonistic interactions between SA and JA signalling are also well studied, with NPR1 acting as a key point of crosstalk (Nomoto *et al.*, 2021). Altogether these examples show that signal interplay tailors plant responses specifically to the threat encountered. While AzA and NHP largely do not cross paths, further study into how each signal feeds into and manipulates SA signalling will provide further insight into how they and other signals act to modulate plant immunity.

4.3.3 – NHP stabilises NPR1 to potentiate priming

While NHP-induced priming is largely independent of SA biosynthesis, it is entirely dependent on the master immune regulator NPR1 (Bernsdorff *et al.*, 2016; Yildiz *et al.*, 2021). As with SA biosynthesis, it is hard to show whether a lack of NHP-mediated priming in an *npr1* mutant is due to a lack of NHP signalling or the lack of pathogen-induced immunity. However, it has been shown that NHP-inducible systemic gene expression is lost in *npr1* mutants (Yildiz *et al.*, 2021), suggesting that NPR1 is indeed required for NHP signal transduction.

To assess how NHP may signal through NPR1, my first step was to investigate posttranslational modification of NPR1, as this tightly controls the stability, activity and targets of NPR1. Following monomerisation and nuclear transport, NPR1 is phosphorylated and SUMOylated, which alters its protein interaction partners and recruits ubiquitin E3 ligase CUL3 (Skelly *et al.* 2016; Spoel *et al.* 2009). Short chain ubiquitination by CUL3 promotes NPR1-mediated gene expression, but also recruits UBE4 to lengthen ubiquitin chains and mark NPR1 for degradation (Spoel *et al.*, 2009; Skelly *et al.*, 2019). Further ubiquitination by proteasome-interacting E3 ligases UBIQUITIN PROTEIN LIGASE 3 and 4 (UPL3/4), ensures appropriate degradation and turnover of NPR1 (Wang *et al.*, 2022). Through this mechanism, NPR1 activity is fine tuned to enable a strong burst of immune-responsive gene expression before rapid suppression of NPR1 to limit energy waste or autoimmunity. As NHP acts to fine tune

SA-responsive gene expression (Figures 4.3 and 4.6), it is plausible that it does so by specifically altering NPR1 regulation. To test this hypothesis, I performed tandem ubiquitin binding entity (TUBE) pulldowns to detect ubiquitinated NPR1. Here I found that NHP induces ubiquitination of NPR1 but does not enhance SA-mediated ubiquitination of NPR1 (Figure 4.11).

As NHP manipulates posttranslational modifications that regulate NPR1 stability, I next investigated whether NHP alters NPR1 accumulation and stability. Importantly, NPR1 accumulates in systemic tissues after infection and mutants with increased NHP levels also display increased NPR1 levels (Nair *et al.*, 2021; Kim *et al.*, 2020), suggesting a link between NHP and NPR1 regulation. It has since been shown that exogenous NHP application directly increases NPR1 levels, with a suggestion that this is through stabilisation as the increase in protein level is not mirrored by a corresponding increase in gene expression (Nair *et al.*, 2021). Here, I corroborate the finding that NHP induces NPR1 accumulation and confirm the hypothesis that this is through increased stabilisation. First, I found that both NHP and SA treatment increase NPR1 levels, but this effect is not cumulative (Figure 4.12A). I then treated plants with NHP and the translation inhibitor cycloheximide, which prevents new proteins from being produced so degradation can be measured. Here, I found that NHP enhances NPR1 stability (Figure 4.12C). This stabilising effect is also seen after SA treatment (Fu *et al.*, 2012; Figure 4.12D), but NHP did not show capability to enhance this further, suggesting that NHP and SA stabilise NPR1 through similar or redundant mechanisms.

With NHP-mediated stabilisation of NPR1 confirmed, the role of stabilised NPR1 in NHP signalling remains unknown. To address this, using non-reducing sample buffer to maintain NPR1 oligomers, I have also shown that NHP induces accumulation of monomeric/dimeric NPR1 (Figure 4.12B). NPR1 activity is in part controlled by oligomerisation, as oligomeric NPR1 is unable to translocate into the nucleus to activate immune signalling (Kinkema *et al.*, 2000; Tada *et al.*, 2008). The accumulation of monomeric/dimeric NPR1 in response to NHP treatment suggests that the accumulated NPR1 is in an active rather than inactive state. To further assess this theory, I then measured the strength of NHP-mediated priming in various mutants that display increased NPR1 levels. NPR3 and 4, CUL3 and UBE4 are proteins all involved in the posttranslational regulation and degradation of NPR1, and their respective mutants all display enhanced NPR1 protein levels (Fu *et al.*, 2012; Spoel *et al.*, 2009; Skelly *et al.* 2019). Here, I found that the NHP-mediated priming effect is cumulative with the enhanced signalling effect seen in each mutant (Figure 4.13). This shows that while NHP increases NPR1 protein levels, this alone is not sufficient for NHP-mediated priming. As NHP induces transcription in systemic tissues dependent on NPR1 (Yildiz *et al.*, 2021), NPR1 accumulation could be a precursor for NHP- or SA-responsive transcription. This suggests that NHP-mediated priming could be regulated by specific transcriptional responses in systemic tissues, facilitated by NPR1 accumulation.

4.3.4 – NHP signalling and ubiquitination

It is well established that ubiquitin has many roles other than just proteasomal degradation, with each linkage type and mixture of linkages promoting unique protein interactions and functions (Komander and Rape, 2012). As targeting by different ubiquitin ligases induces different effects on NPR1 (Spoel *et al.*, 2009; Skelly *et al.*, 2019), it is possible that NPR1 is ubiquitinated with different linkage chains to promote different roles. The finding that NHP induces NPR1 ubiquitination (Figure 4.11) raises the possibility that NHP signalling redirects NPR1 towards priming by modulating NPR1 modifications. However, without further experimentation to determine which linkage type(s) NHP adds to NPR1 we cannot be certain what the biological significance of this result is. However, when measuring total ubiquitinated protein as a control in the TUBE pulldowns, I observed that NHP was promoting an increase in total cellular ubiquitination. I corroborated this result, confirming that in both wild type and *npr1* mutants, NHP application increases the amount of ubiquitinated protein across the cell (Figure 4.14A). Again, without context of chain types and targets this result does not divulge much information. However, what it does reveal is that posttranslational modification is a previously undescribed mechanism of NHP signal transduction.

To further investigate the role of ubiquitination in NHP signalling, I repeated priming assays in the presence of the proteasome inhibitor MG132 during either the priming phase or the activating phase (Figure 4.14 B and C). In both cases the priming effect was unperturbed, suggesting that NHP-mediated priming does not require proteasome-mediated protein degradation. Interestingly, adding MG132 prior to SA boosted gene expression in all conditions (Figure 4.14B) and adding MG132 alongside SA reduced gene expression in all conditions (Figure 4.14C). These results may be explained by the accumulation of transcriptional regulators prior to SA treatment and the ineffective turnover of regulators after SA treatment, respectively. Regardless, both effects appear not to influence NHP signalling. Overall, these data suggest that non-proteolytic ubiquitination is a component of NHP signalling, but the targets remain elusive. Further research into which proteins are ubiquitinated by NHP, and what the consequence of these modifications are may give exciting insights into the direct mechanism of NHP perception and signal transduction.

4.3.6 – Conclusion

This chapter makes great progress towards understanding the role of NHP in systemic resistance (Figure 4.15). Here, I have shown that NHP-mediated priming modulates the SA-responsive transcriptome and enhances SA-inducible immune responses. NHP appears to target a different subset of genes to AzA, suggesting its role is distinct from AzA-mediated priming. The mechanism of priming is not well understood, but I find that NHP induces non-proteolytic accumulation of ubiquitinated proteins, revealing posttranslational modifications as a possible mechanism of NHP-mediated priming. I also find that NHP stabilises key SA regulator NPR1 to promote its accumulation. However, NPR1 accumulation alone is not sufficient to induce priming, suggesting that other pathways are deployed by NHP. NHP induces strong transcriptional responses in systemic tissues, raising the possibility that NHP may mediate priming through transcriptional pathways that could be facilitated by NPR1.

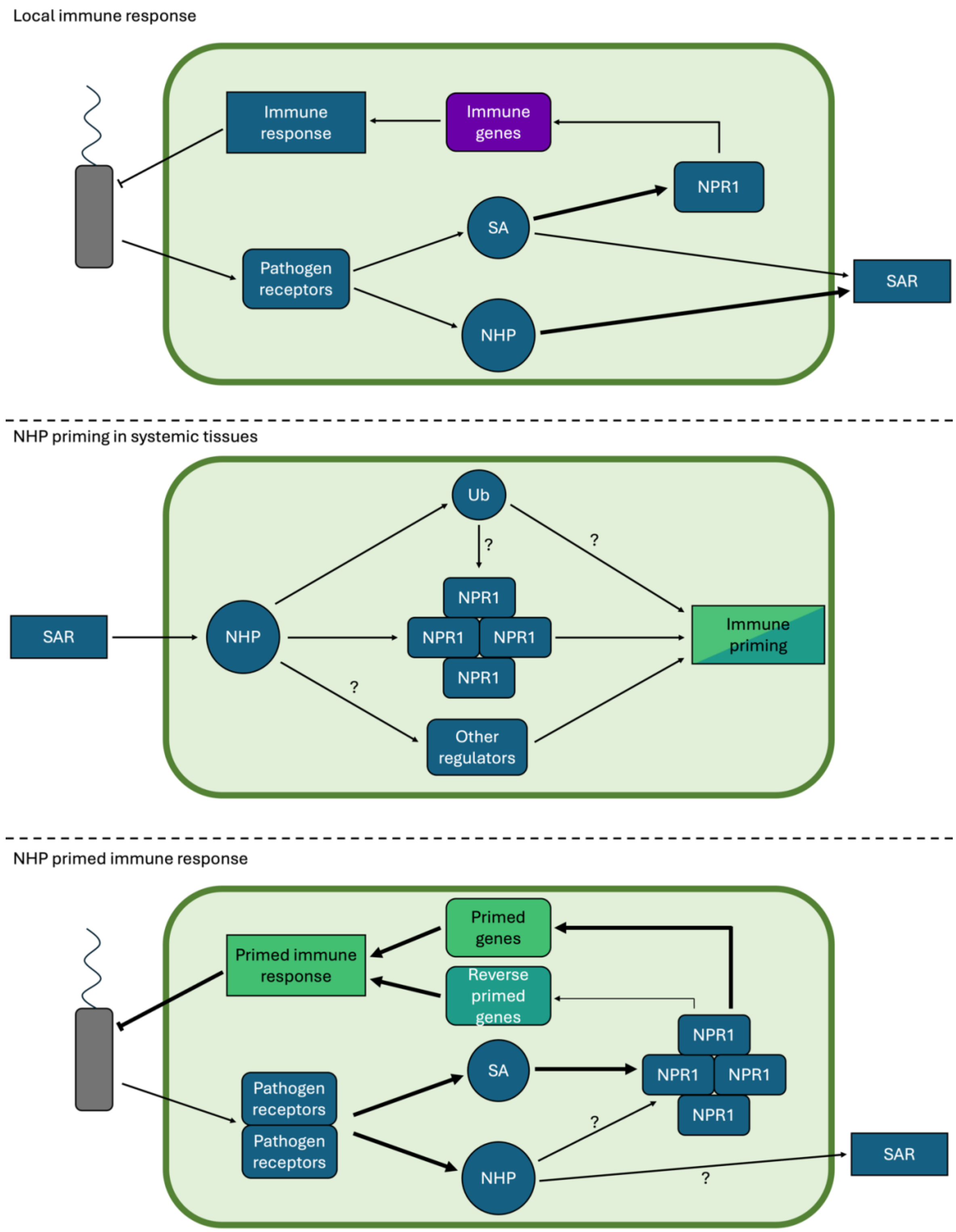


***Figure 4.15 – Schematic diagram of NHP signalling and priming in local and systemic tissues.** During local immune responses, pathogen perception triggers SA and NHP signalling. NHP is transported systemically to induce SAR, while SA induces an immune response through NPR1 and may contribute to SAR. In systemic tissues, NHP promotes NPR1 accumulation and ubiquitination, both of which may contribute to priming. NHP likely also activates other regulators, possibly transcriptional, to induce priming. In a primed cell, the accumulation of pathogen*

receptors enables a faster and stronger SA response. Accumulation of NPR1 also increases the strength of SA signalling and the expression of SA-responsive genes which have been primed or reverse primed. Expression of SA-responsive genes is now tailored to maximise immunity, successfully combating the infection. NHP may contribute to the primed response by further supporting NPR1 accumulation and may promote propagation of the systemic immune signal further.

Chapter 5 – WRKY38 and WRKY62 regulate NHP-mediated priming through chromatin remodelling

5.1 – Introduction

SA-mediated plant immunity is tightly controlled at the transcriptional level by transcription factors (TFs) and cofactors. The NPR1 coactivator is considered a 'master regulator' of salicylic acid (SA) signalling and systemic acquired resistance (SAR). NPR1 has several important interaction partners both within and beyond the nucleus (Powers *et al.*, 2024), but vitally it regulates the targeting and activity of TGA family TFs (Despres *et al.*, 2000). SA accumulation promotes interaction between NPR1 and TGA1/4, triggering recruitment to SA-inducible genes such as *PR1* to activate their expression (Despres *et al.*, 2003; Kesarwani *et al.*, 2007). Conversely, SA-mediated gene expression is suppressed by NIM-INTERACTING 1 (NIMIN1), which interacts with NPR1 and TGAs to regulate the extent of immune activation (Weigel *et al.*, 2005). Also vital in SA signalling are WRKY family TFs, many of which are expressed by TGAs, facilitated by NPR1 (Powers *et al.*, 2024). For example, WRKY18 is responsible for amplifying SA-responsive gene expression of ~25% of NPR1-regulated genes (Wang *et al.*, 2006). The data from previous research and this thesis have drawn a strong link between NPR1 and N-hydroxy pipecolic acid (NHP) (Yildiz *et al.*, 2021; Nair *et al.*, 2021; Figure 4.11), suggesting that NHP may signal through previously identified, immune-related transcription factors.

Of particular interest, WRKY70 has recently been identified as a regulator of NHP-responsive gene expression and immune induction (Foret *et al.*, 2025). Furthermore, *WRKY38* and *WRKY62* are both induced by NHP, and are required as a pair for systemic, but not local resistance (Foret *et al.*, 2025; Spoel *et al.*, 2009), demonstrating the importance of WRKYs in systemic signalling. The WRKY family of TFs are key regulators of development, senescence and multiple stress responses, including NHP signalling. There are 74 WRKY genes in *Arabidopsis*, separated into three groups based on their characteristics (Rushton *et al.*, 2010). All WRKY TFs contain a zinc finger domain and a ~60 amino acid residue WRKY domain, containing tryptophan (W), arginine (R), lysine (K) and tyrosine (Y). The WRKY domain is a DNA-binding domain that targets the conserved W-box sequence, TTGACY (where Y corresponds to C or T), but target specificity is also influenced by adjacent sequences. Group 1 WRKYs contain two WRKY domains and a C2H2-type zinc finger, Group 2 WRKYs contain a single WRKY domain and a C2H2 zinc finger, and Group 3 WRKYs also contain a single WRKY domain but instead have a C2HC-type zinc finger. Each group contains subgroup classifications with slight variations in other domains and intron positioning.

WRKYs from the same subgroup show high levels of homology, and often work in tandem through homo- or heteromerisation, such as WRKYs 18, 40 and 60 which interact synergistically to promote immune responses (Xu *et al.*, 2006). Many are transcriptional activators, such as WRKY50 that interacts with TGA2 and TGA5 to promote *PR1* expression (Hussain *et al.*, 2018). Several WRKYs also act as repressors by binding and blocking target promoters, including WRKY70 which binds the *SARD1* promoter to inhibit expression in the absence of pathogens (Zhou *et al.*, 2018). Finally, some WRKYs are linked to chromatin modifications, such as a selection of group 2B WRKYs that interact with histone-binding OBERON proteins to regulate drought stress responses (Du *et al.*, 2023). Moreover, SAR-associated WRKY38 and 62 both interact with a histone deacetylase (Kim *et al.*, 2008), suggesting their role in regulating systemic immunity could be linked to epigenetic genome regulation.

Epigenetics considers regulation of gene expression without alteration of the DNA sequence. There are several mechanisms of epigenetic control, one of which is through histone modifications. Nucleosomes are comprised of DNA wrapped around 8 histone subunits, each of which has protruding unfolded 'tails' (Cutter and Hayes, 2015). The histone tails can be post-translationally modified at several positions to precisely regulate protein interactions, subunit stability, and chromatin accessibility (Jenuwein and Allis, 2001). Histone modifications often regulate gene expression, but they are also important components in DNA repair and chromosome condensation (Peterson and Laniel, 2004). In the context of plant immunity, histone modifications are vital for proper immune function. For example, transcription of the immune-related genes *WRKY70* and *PR1* is associated with trimethylation of lysine 4 on subunit H3 (H3K4me3) deposited by ARABIDOPSIS HOMOLOG OF TRITHORAX 1 (ATX1), and mutation of *atx1* reduces immune gene expression and disease resistance (Alvarez-Venegas *et al.*, 2007). Histone subunits come in a number of different variants, which can be interchanged to regulate gene expression. In *Arabidopsis*, heat stress responses involve substitution of both H2 and H3 subunits to different variants (Zhao *et al.*, 2023). Histone marks are a key component of immune priming in humans. For example, macrophages harvested from immune challenged tissues displayed a global depletion of the repressive H3K27me3 mark compared to 'untrained' macrophages (Benjaskulluecha *et al.*, 2022). The 'trained' macrophages also displayed decreased lipopolysaccharide (LPS)-responsive accumulation of tumour necrosis factor alpha (TNFα), which corresponds to a primed immune response. While the evidence of histone-mediated immune memory is still in its infancy in plants, histones are highly conserved between plants and animals (Chaboute *et al.*, 1993). Therefore, the importance of histone modifications in human immune priming opens the possibility that NHP-mediated priming could manipulate histone modifications to prime SA-responsiveness.

A second major epigenetic signalling mechanism is through DNA methylation, where cytosines are methylated by specific methyltransferases (Zhang *et al.*, 2018). Methylation sites are usually palindromic, i.e. CG or CHG (where H corresponds to A, T or C), which enables cytosines on each strand to promote methylation of their partnering strand during DNA replication (Finnegan *et al.*, 1998). Uniquely, plants also display CHH methylation sites, although by comparison these are rarer. Increased methylation of cytosines in a DNA region promotes heterochromatin formation, significantly limiting chromatin accessibility (Luo *et al.*, 2018). Differential DNA methylation is vital for processes such as centromere maintenance, transposon suppression and gene regulation (Fedoroff, 2012). In *Arabidopsis*, pathogen-responsive DNA demethylation triggers expression of several immune genes, and loss of this response leads to susceptibility to *Pseudomonas syringae* (Yu, A. *et al.*, 2013). DNA methylation has also been linked to immune priming in other species. For example, monocytes collected from human patients with acute pneumonia displayed significantly lower LPS-responsive interleukin production, linked to DNA hypomethylation at specific regulatory genes (Brands *et al.*, 2021). Vitally, DNA methylation regulates transgenerational immune priming in plants. The progeny of infected *Arabidopsis* plants display inheritance of differential demethylated regions, which correlate to enhanced immunity in a dose dependent manner (Luna *et al.*, 2012; Stassen *et al.*, 2018). While there is some variation between kingdoms, the genomic locations and roles of DNA methylation are broadly similar between plants and animals

(Feng *et al.*, 2010), so DNA methylation could also play an important role in NHP-mediated immune priming.

In this chapter, analysis of RNA sequencing (RNA-seq) data revealed that WRKY family TFs are strongly associated with NHP-primed genes. In particular, the closely related WRKY38 and 62 pair are required for NHP mediated transcriptome priming and induced resistance. Previous research shows that WRKY38/62 interact with a histone deacetylase (Kim *et al.*, 2008), so I investigated whether NHP regulates chromatin accessibility using ATAC sequencing (ATAC-seq). NHP induced a marked increase in chromatin accessibility, which was almost entirely dependent on WRKY38/62. Analysis of chromatin peaks revealed that the majority of NHP-responsive peaks contain gene promoters, with many NHP-primed genes displaying WRKY38/62-dependent, NHP-responsive peaks. Overall, these data suggest that NHP utilises WRKY38/62 to prime immune responses by extensively remodelling the chromatin accessibility of immune genes and other genomic regions.

5.2 – Results

5.2.1 – Identification of WRKY transcription factors involved in immune priming

To identify transcriptional regulators of NHP-mediated immune priming, I assessed whether any TFs associate with groups of primed gene promoters. Using previously published DNA affinity purification sequencing (DAP-seq) data (O'Malley *et al.*, 2016), I ran Chi squared and Fisher's tests to identify TFs that significantly target NHP-primed, SA-responsive genes when compared to all genes in the dataset. Strikingly, 9 of the top 10 candidates (sorted by p-value) were WRKY family TFs (Table 5.1), suggesting that WRKY TFs could be involved in NHP-mediated priming of SA-responsive genes.

Table 5.1 – WRKY family transcription factors significantly associate with primed genes. *Using available DAP-seq data, the total number of TF targets was compared to the number of primed targets, and TFs ranked by p-values. 'All targets' shows the number of genes targeted by each TF across all genes tested in the DAP-seq (Total targets). 'Primed targets' shows the number of primed genes targeted by each TF across all NHP-primed genes (Total primed). The 10 lowest p-values (pval) when considering both tests are shown.*

TF ID	TF name	All targets	Total targets	Percent total	Primed Targets	Total Primed	Percent primed	Fishers pval	Chi squared pval
AT2G40750	WRKY54	781	8311	9.40%	112	584	19.18%	5.80E-12	5.02E-14
AT3G56400	WRKY70	2290	8311	27.55%	239	584	40.92%	2.27E-11	6.13E-12
AT2G40740	WRKY55	2456	8311	29.55%	250	584	42.81%	5.80E-11	2.32E-11
AT4G18170	WRKY28	3025	8311	36.40%	293	584	50.17%	6.13E-11	3.88E-11
AT5G63790	NAC102	2427	8311	29.20%	244	584	41.78%	4.36E-10	1.97E-10
AT2G38470	WRKY33	1806	8311	21.73%	191	584	32.71%	4.01E-09	1.11E-09
AT1G30650	WRKY14	2283	8311	27.47%	229	584	39.21%	3.18E-09	1.49E-09
AT1G29860	WRKY71	2336	8311	28.11%	232	584	39.73%	6.96E-09	2.82E-09
AT3G01970	WRKY45	2544	8311	30.61%	248	584	42.47%	5.42E-09	3.19E-09
AT1G62300	WRKY6	1450	8311	17.45%	159	584	27.23%	1.60E-08	4.13E-09

To test this hypothesis, I selected candidate WRKY mutants and assessed if they were still capable of priming *PR1* expression. WRKY54 and WRKY70 were the top 2 ranked TFs, and work in tandem to regulate SA signalling (Wang *et al.*, 2006; Zhou *et al.*, 2018), so were the first selected. WRKY18 is required for potentiation of NPR1-dependent gene expression (Wang *et al.*, 2006), so could also play a role in NHP-mediated signal potentiation. Finally, *wrky38*/*wrky62* double mutants were shown to lack systemic immune responses but maintain local immunity (Spoel *et al.*, 2009), so they could regulate SAR signalling in systemic tissues.

Fourteen-day-old wild type *Arabidopsis thaliana* seedlings were sequentially treated with NHP and SA as described in Chapter 4, after which *PR1* gene expression was measured by qPCR. First, *wrky54/70* mutants displayed a strong reduction in SA-responsiveness, but maintained *PR1* priming in response to NHP (Figure 5.1A). Second, *wrky18* mutants showed only a minor reduction in NHP-primed *PR1* gene expression (Figure 5.1B). These data suggest that WRKYs 18, 54 and 70 are not major components of NHP-mediated priming of SA-responsive gene expression. In contrast, *wrky38/62* mutants displayed slightly enhanced basal SA-responsiveness, but NHP was completely ineffective in priming SA-induced *PR1* expression (Figure 5.1C), suggesting WRKY38/62 are required for NHP signalling.

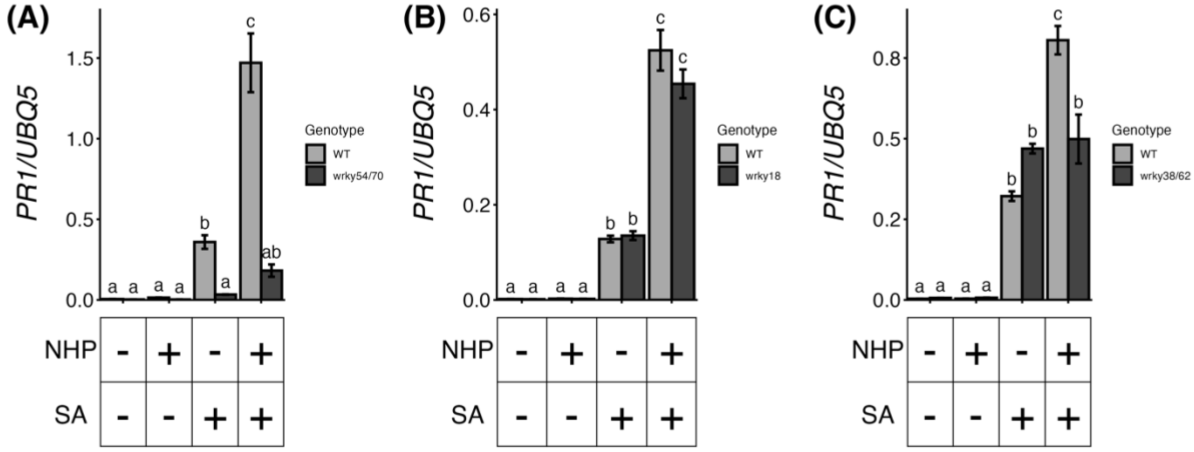


Figure 5.1 – WRKY38 and WRKY62 are required for NHP-mediated priming of SA-responsive PR1 expression. *Fourteen-day-old wild-type and either wrky54/70* ***(A)****, wrky18* ***(B)*** *or wrky38/62* ***(C)*** *seedlings were treated by immersion in water or 1 mM NHP for 18 hours, followed by treatment with water or 0.5 mM SA for 6 hours. Total RNA was extracted and the expression of SA marker gene PR1 was measured by qPCR. Reporter genes were normalised against constitutively expressed UBQ5. Letters denote statistically significant differences between samples (Tukey ANOVA; α = 0.05, n = 3).*

5.2.2 – WRKY38/62 are required for NHP-mediated priming of SA-dependent immunity

With the loss of NHP-mediated *PR1* priming identified, I then repeated the RNA-seq experiment described in Chapter 4.2.2, including both wild-type and *wrky38/62* plants. I again identified SA-responsive genes through linear modelling, then assigned NHP-mediated primed behaviours by clustering. In wild-type plants I identified 4,492 SA-responsive genes, with 62% (2,789 genes) displaying direct NHP-responsive expression or NHP-mediated priming. While this is a lower number of differentially expressed genes than the previous experiment (Figures 4.5 and 4.7), roughly 70% overlap is expected between different biological repeats of RNA-seq experiments (Lamarre *et al.*, 2018). 54% of differentially expressed genes in this chapter's RNA-seq are also observed in Chapter 4's RNA-seq, so this slight variation is within acceptable boundaries.

Furthermore, the absolute number of primed genes is similar in both experiments (Figures 4.3 and 5.2). In the *wrky38/62* mutants, a larger number of 5,289 SA-responsive genes were identified, which is in line with the observed increase in SA-responsiveness of *PR1* (Figure 5.1C). Interestingly, 86% of SA-responsive genes (4,562) displayed direct NHP-responsive expression or NHP-mediated priming in the mutant, suggesting that NHP signalling is considerably dysregulated in *wrky38/62* mutants.

To fully characterise the effects of the *wrky38/62* mutation, I compared the priming behaviour of SA-responsive genes in wild-type plants to their behaviour in the mutant. This analysis revealed that NHP-mediated priming is massively dysregulated in *wrky38/62* mutants (Figures 5.2 and 5.3). For example, of the 552 SA upregulated, NHP primed genes in WT, only 59 remain primed in *wrky38/62*. At the same time, of the 1,192 SA-upregulated genes, 562 are reverse primed in the mutant, showing that in the absence of *WRKY38/62*, NHP-mediated priming becomes dysregulated.

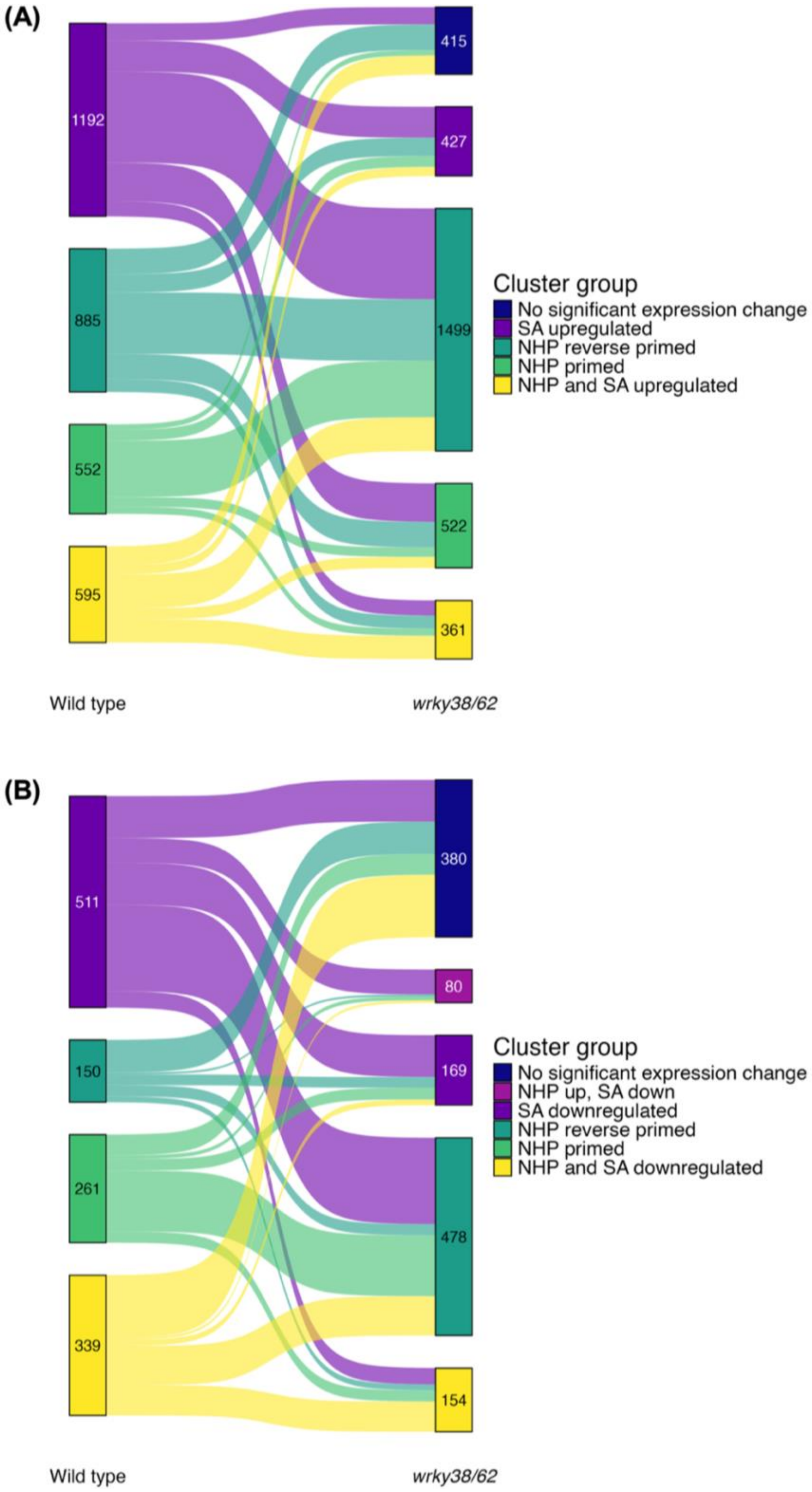


Figure 5.2 – NHP-mediated transcriptional reprogramming is dysregulated in wrky38/62 mutants. Wild type and wrky38/62 seedlings were treated as described in Figure 5.1, *total RNA was extracted, and SA-responsive gene expression measured by RNA-seq. **(A)** Assignment of genes to each priming category in SA upregulated genes. **(B)** Assignment of genes to each priming category in SA downregulated genes.*

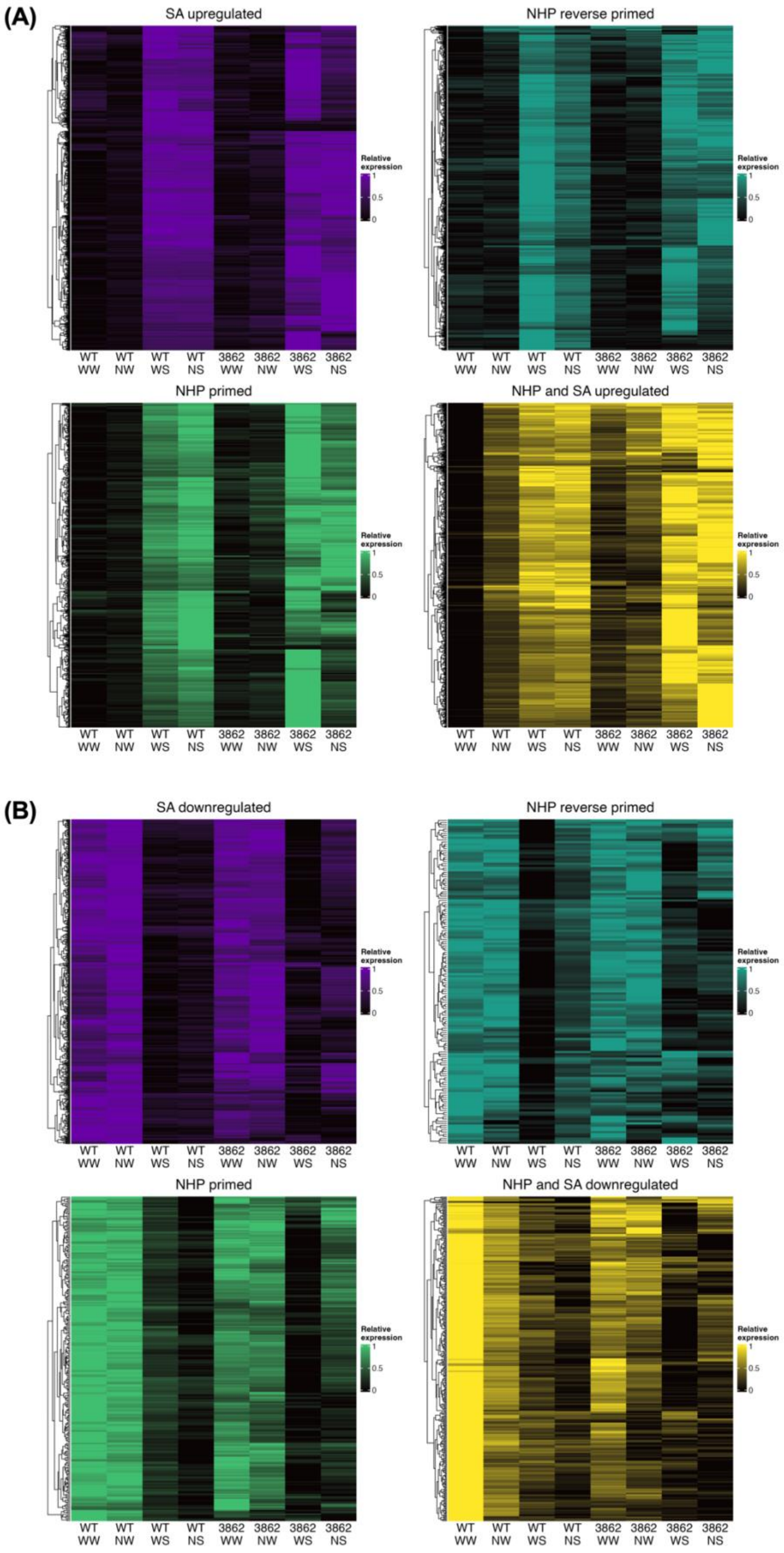

(A)
SA upregulated
NHP reverse primed
NHP primed
NHP and SA upregulated
(B)
SA downregulated
NHP reverse primed
NHP primed
NHP and SA downregulated
Relative expression
1
0.5
0
WT WW
WT NW
WT WS
WT NS
3862 WW
3862 NW
3862 WS
3862 NS

Figure 5.3 – Gene expression patterns in wild type plants and wrky38/62 mutants. *Heatmaps of clustered genes showing expression patterns across the four treatment conditions in* ***(A)*** *SA upregulated genes and* ***(B)*** *SA downregulated genes. The wild type and wrky38/62 genotypes are denoted as 'WT' and '3862'. 'Relative expression' is presented as transcripts per million values scaled from 0 to 1 across the four treatments. The treatments are denoted as follows: water + water = 'WW', NHP + water = 'NW', water + SA = 'WS' and NHP + SA = 'NS'.*

I then assessed whether the transcriptional dysregulation observed in *wrky38/62* mutants correlates with a perturbed immune response. To this end, I repeated the *Pseudomonas. syringae pv maculicola* (*Psm*) disease assays as described in Chapter 4.2.4. Twenty-eight-day-old WT and *wrky38/62* plants were sequentially treated with pipecolic acid (Pip) and then SA before infection with *Psm* ES4326. In line with previous reports, the *wrky38/62* mutant demonstrated enhanced basal resistance and was still responsive to SA, with 0.5 mM SA inducing a significant reduction in bacterial growth (Figure 5.4). In both genotypes, treatment with 0.05 mM SA or 1 mM Pip was not sufficient to enhance immunity. In the wild type plants, sequential treatment with 1 mM Pip, then 0.05 mM SA was sufficient to enhance immunity. However, this effect was lost in the *wrky38/62* mutant, showing that WRKY38 and WRKY62 are integral to appropriate regulation of NHP-mediated priming of SA-dependent transcriptional reprogramming and immunity.

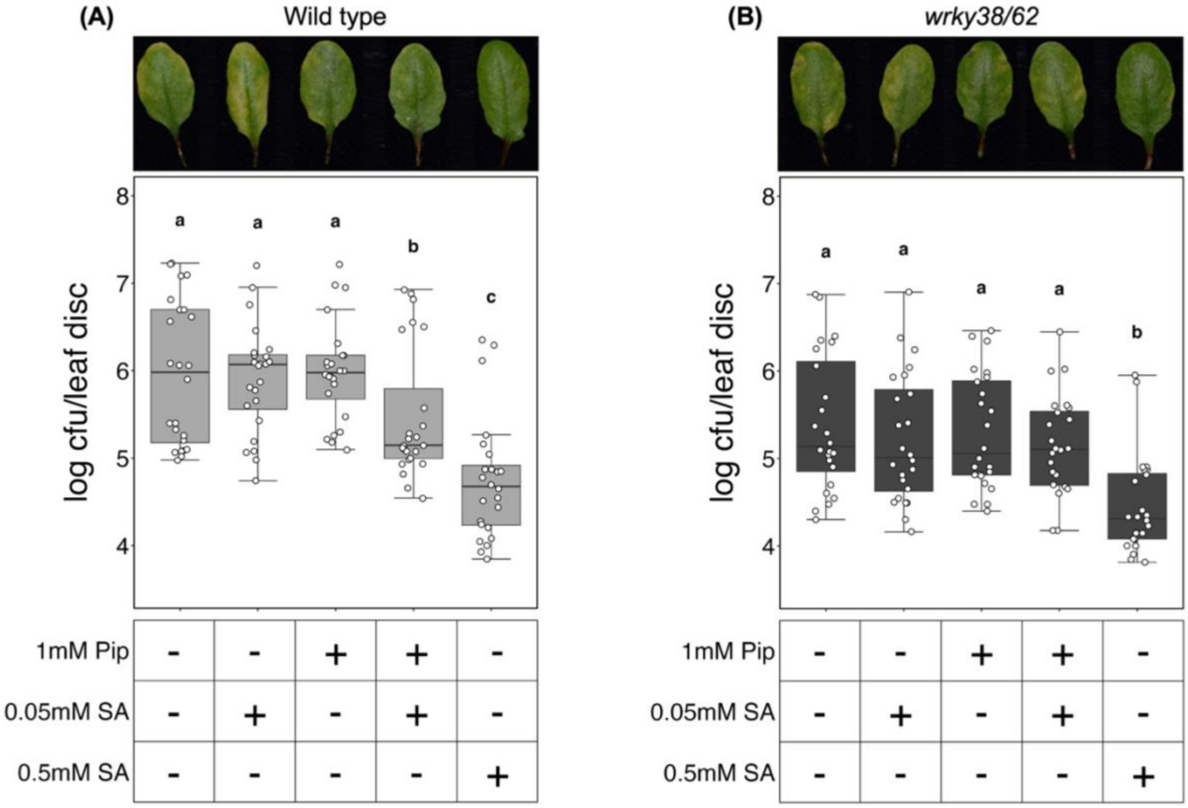


Figure 5.4 – Pipecolic acid is unable to potentiate SA-inducible immunity against Psm ES4326. *Twenty-eight-day-old wild-type* ***(A)*** *and wrky38/62 plants* ***(B)*** *were treated 48 hours before infection by spraying with water or 1mM Pip, followed by treatment with water, 0.05 mM SA or 0.5 mM SA, 24 hours before infection. Leaves were then infiltrated with 5 x 10^6 colony forming units (cfu)/ml Psm ES4326. Leaf discs were analysed for bacterial growth 3 days post infection. Error bars represent interquartile range x1.5, while letters denote statistically significant differences between samples (Tukey ANOVA; α = 0.05, n = 24). Representative photos of leaves are displayed above the respective boxplots.*

5.2.3 – NHP modulates chromatin accessibility via WRKY38/62

Next, I explored how WRKY38/62 might regulate NHP-mediated priming of the SA-responsive transcriptome. Little is known about the mechanism by which WRKY38/62 signal, but they have been shown to interact with histone deacetylase HDA19 (Kim *et* *al.*, 2008). Because histone deacetylases play a key role in chromatin remodelling, a core mechanism activated during SAR (Conrath *et al.* 2015), I hypothesised that WRKY38/62 may be involved in remodelling chromatin containing NHP and SA-responsive genes.

To investigate whether NHP signalling influences the chromatin state, I performed Assay for Transposase-Accessible Chromatin (ATAC-seq) sequencing on wild-type and *wrky38/*62 plants sequentially treated with NHP and SA. As before, fourteen-day-old *Arabidopsis* seedlings were treated with 1 mM NHP or water for 18 hours, followed by 0.5 mM SA or water for 6 hours. Fresh nuclei were extracted from the plants on which I performed Illumina tagmentation reactions. The DNA in fresh nuclei consists of heterochromatin and euchromatin structures, so tagmentation transposons insert adapters preferentially into the more accessible DNA. Thus, “open” more accessible euchromatin regions are sequenced to a significantly greater depth than the heterochromatic DNA. Using MACS2 peak analysis software (Zhang *et al.*, 2008), I identified significant peaks in DNA accessibility and selected enriched peaks with a minimum of log2-fold change in each sample for further analysis.

In wild-type plants, 3,287 peaks were identified in water treated samples, whereas NHP treatment increased the number to 3,884. SA treatment reduced the number of peaks to only 2,716, while NHP-primed SA treated plants displayed 2,957 peaks (Figure 5.5). With the peaks identified, I then assessed peak intensity and location. Using Deeptools (Ramirez *et al.*, 2016), I compared peak intensity around transcription start sites in each sample by visualising read accumulation with heatmaps (Figure 5.6). Interestingly, despite SA reducing the total number of peaks, the total peak intensity increased (Figure 5.6A), suggesting SA promotes the accumulation of fewer open regions but with more accessibility. The strongest peak signal and the most peaks were present after NHP treatment, suggesting that NHP manipulates the chromatin state of wild-type *Arabidopsis* plants in a different manner to SA. In *wrky38/62* mutants the accessibility signal was dramatically lower. Water-treated samples displayed only 2,088, and while NHP did increase the number of peaks it was only to 2,234 (Figure 5.5). SA signalling also appeared dysregulated, with SA treatment increasing the peaks to 2,475, while NHP-primed SA treatment reduced the number of peaks to 1,643. This suggests that overall chromatin accessibility is reduced in *wrky38/62* mutants, with the effect of NHP significantly reduced and the effect of SA reversed compared to the wild type (Figure 5.6B).

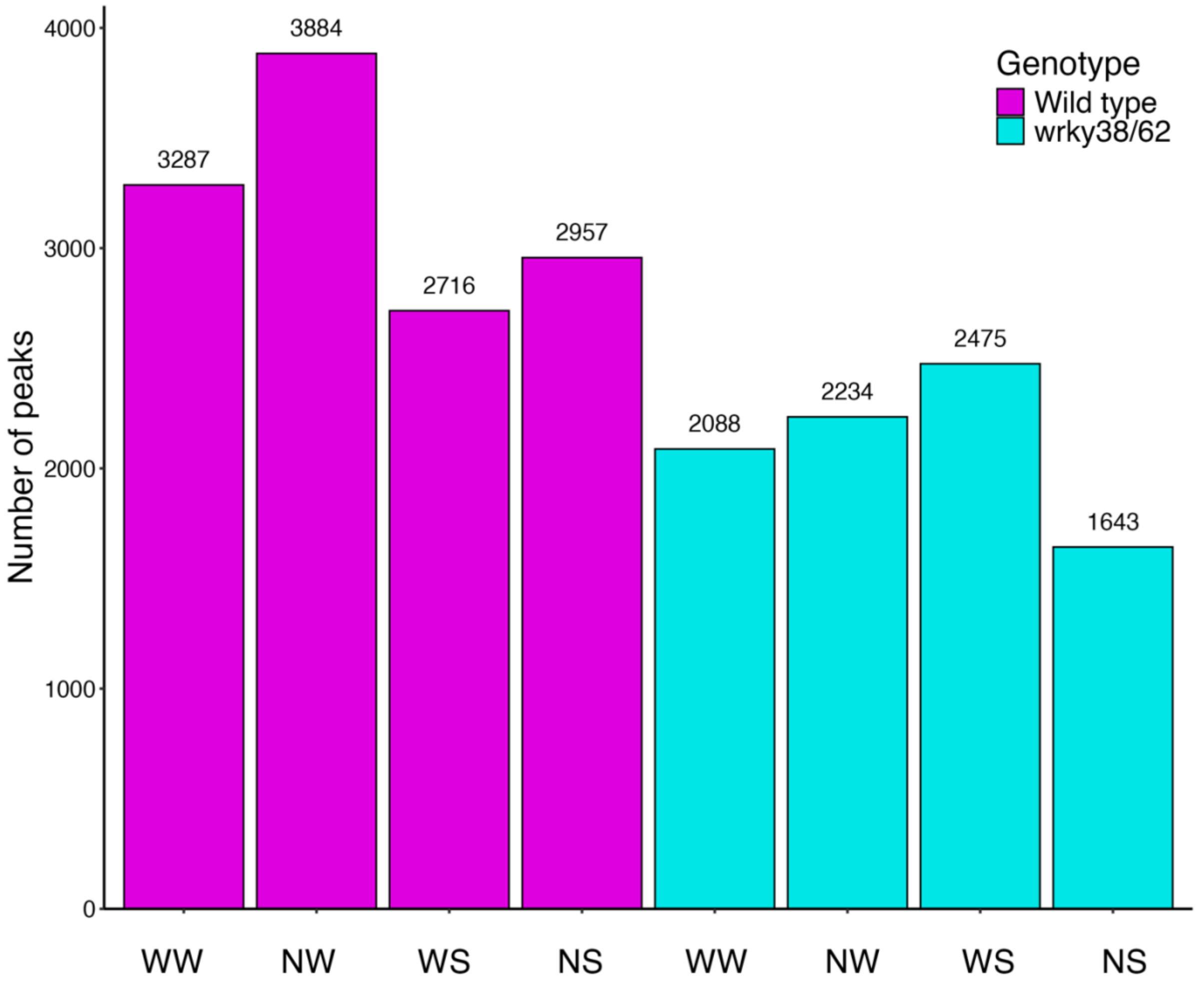


Figure 5.5 – Chromatin accessibility is regulated by NHP, SA and WRKY38/62. *Fourteen-day-old wild-type and wrky38/62 seedlings were treated by immersion in water or 1 mM NHP for 18 hours, followed by water or 0.5 mM SA for 6 hours. Whole nuclei were extracted and used in tagmentation reactions before DNA sequencing. Log2-fold enriched peaks, representing 'open' chromatin were detected using MACS2 software and counted in each sample. Numbers above bars indicate the number of peaks in each treatment. The treatments are denoted as follows: water + water = 'WW', NHP + water = 'NW', water + SA = 'WS' and NHP + SA = 'NS'.*

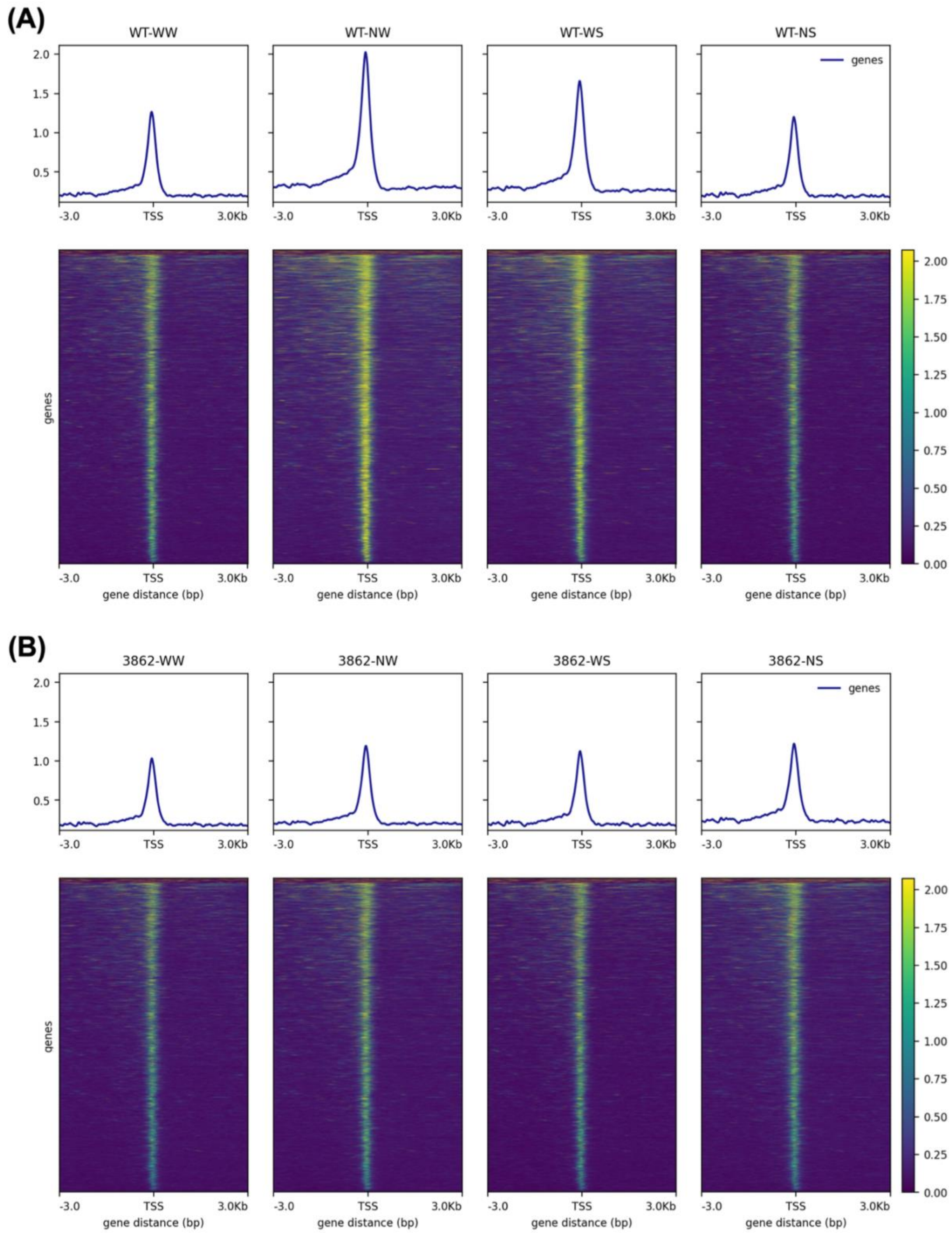


Figure 5.6 – NHP modulates chromatin accessibility, dependent on WRKY38 and WRKY62. *Visualisation of chromatin accessibility 3 kb up- and downstream of transcription start sites (TSS). Upper profile plots represent total accumulation of reads across all genes with a log2 fold enrichment higher than 2. Lower heatmaps represent accumulation of reads per gene with a log2 fold enrichment higher than 2. Heatmap colour represents relative*

accessibility, where a higher number of reads and therefore higher accessibility is represented by increasingly yellow colouration. The wild type and wrky38/62 genotypes are denoted as ‘WT’ and ‘3862’. Axis and heatmap labels represent relative transcript accumulation scaled from 0 to 2 across all genes. The treatments are denoted as follows: water + water = ‘WW’, NHP + water = ‘NW’, water + SA = ‘WS’ and NHP + SA = ‘NS’.

While the total number of peaks in each sample is a useful metric, I also compared the presence of each peak across every sample. The called midpoint of each peak was not identical between samples as slight variations in read length shifted the apex of coverage between samples. Some peaks were clearly the same response in different samples, but the exact midpoint varied by 20-30bp. Therefore, a peak in any given sample was considered the same peak if it was within 100bp of a peak in a different sample. Each unique peak was assigned an identification (id) number, and the accuracy of this metric confirmed by counting the incidence of each peak id across samples. At this cutoff, there were no incidences of two distinct peaks allocated the same id due to their proximity, suggesting this approach accurately assigned ids to each peak.

Strikingly, in wild-type samples, NHP treatment induced 862 new peaks (16.6% of all detected peaks) that were not seen after any other treatment (Figure 5.7A), demonstrating that NHP likely increases chromatin accessibility. I therefore investigated whether peaks were opened or closed in response to NHP and SA treatments. A peak was considered “opened” if the log2 fold change in peak enrichment between water and NHP treatment was greater than 0.5, and vice versa for closed peaks. Furthermore, combined NHP and SA treatment opened a unique set of 344 peaks, showing that NHP also modulates SA-mediated chromatin unwinding. As expected, the number of unique NHP-responsive peaks was much lower in *wrky38/62*, comprising only 7.3% (235) of all detected peaks (Figure 5.7B). The number of peaks in the combined NHP-SA treatment also decreased considerably to 110 unique peaks, supporting that NHP's effect on SA is reduced in *wrky38/62*. While the total number of peaks was lower in *wrky38/62*, the number of SA-responsive peaks almost doubled, which may explain why SA responses are stronger in this mutant.

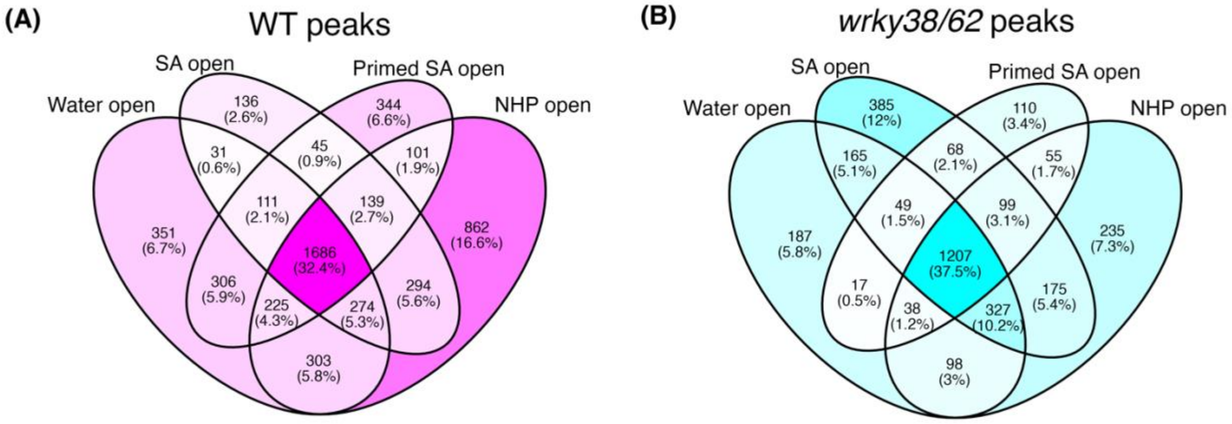


Figure 5.7 – Many NHP responsive peaks are lost in wrky38/62. *Comparison of overlap of peaks found in each treatment condition in wild type* ***(A)*** *and wrky38/62* ***(B)*** *samples.*

To further assess the importance of WRKY38/62 in NHP-mediated chromatin regulation, I compared the presence of each NHP opened and closed peak across the two genotypes. This approach identified 1,396 peaks opened by NHP and 799 peaks that were closed by NHP in wild type, with 564 opened and 418 closed in *wrky38/62*. Comparing the peak ids in each opened and closed group revealed that only 7% of peaks opened and closed in WT samples were NHP responsive in *wrky38/62* (Figure 5.8 A and B). Instead, several new peaks were identified in this mutant. Curiously, enrichment analysis with MEME-suite (Bailey *et al.*, 2009) did not find significant enrichment of any specific DNA motifs in *wrky38/62*-dependent peaks, suggesting that

WRKY38/62-responsive peaks are not regulated by one specific sequence. To determine if the NHP-responsive peaks unique to each genotype harboured any specific roles, I then determined the relative genomic location of each peak. This showed that the majority of opened and closed peaks were in or around the transcription start site (TSS), while fewer peaks were associated with transcription end sites (TES), introns/exons, and intergenic sites (Figure 5.7C). This distribution was largely unaffected by genotype. Comparison of the genomic location of peaks showed that the peaks present in both genotypes were not associated with a specific region, and the peaks were lost evenly across each genomic feature (Figure 5.7D). Although the peaks were lost evenly, this demonstrates that the majority of NHP-responsive peaks lost in *wrky38/62* mutants are within the TSS, which may explain the significant disruption in gene expression in the mutant.

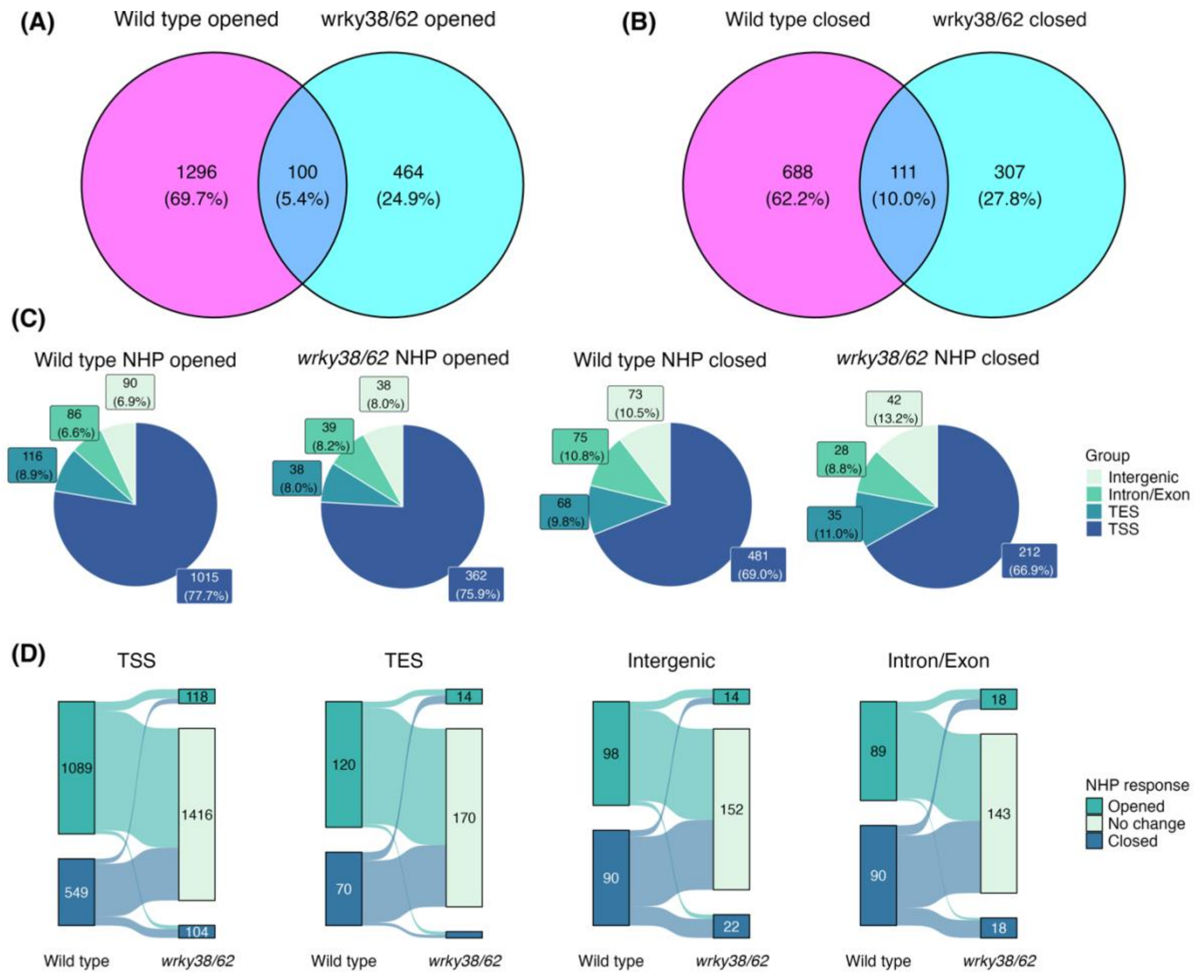


Figure 5.8 – NHP-responsive peaks are mostly lost in wrky38/62, but not in a specific genomic region. (A-B) *Comparison of the overlap of NHP-responsive peaks in each genotype.* ***(C)*** *The genomic location of each NHP-responsive peak.* ***(D)*** *Comparison of NHP responsive peaks in genotype, separated by genomic region of the peak.*

5.2.4 – Different NHP-responsive clusters display distinct chromatin profiles

With a clear link between NHP and chromatin remodelling established, I next examined if NHP-mediated chromatin remodelling was associated with NHP-mediated priming of SA-responsive gene expression. First, I compared the peak signal profiles 3 kb up- and downstream of the TSS of genes belonging to each NHP-primed category with that of NHP unprimed genes. This showed that while the peak signal directly at the TSS is increased by NHP regardless of expression, there were also many small regions of NHP-responsive peak signal within primed and reverse primed genes upstream of the TSS that were not present in SA upregulated genes (Figure 5.9A). To verify this effect, I selected highly expressed, *WRKY38/62*-dependent candidate genes from each cluster and compared read signal in their promoters. *ERF105* is an NHP-primed gene, showing clear NHP-induced opening of the chromatin within 1 kb of the TSS (Figure 5.9B). *CAMTA5* is a reverse primed gene, showing an NHP-responsive peak situated further upstream (approx. 2 kb) from the TSS than the primed genes (Figure 5.9C). *WRKY62* itself is an SA upregulated gene but shows no NHP-mediated response and no NHP-mediated peak in its promoter (Figure 5.9D). Thus, NHP-primed or reverse primed reporter genes appear to harbour NHP-responsive chromatin regions, whereas genes unresponsive to NHP may not.

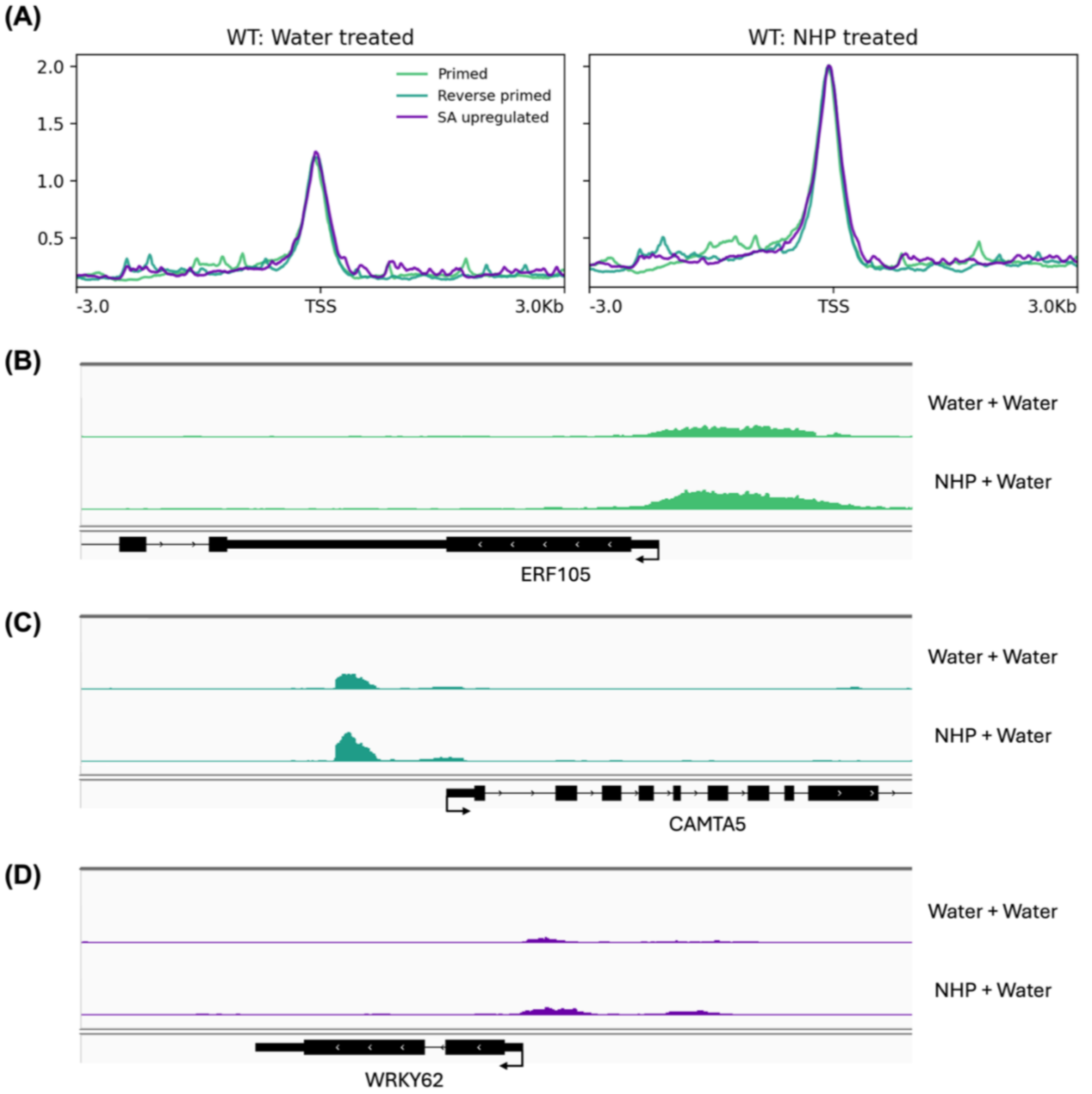


Figure 5.9 – NHP-responsive peak signal varies between NHP-primed clusters. (A) *Comparison of NHP-responsive chromatin signal in NHP primed, NHP reverse primed, and SA upregulated genes. Axis labels represent relative transcript accumulation scaled from 0 to 2 across all genes.* ***(B)*** *NHP-responsive chromatin signal in the ERF105 promoter.* ***(C)*** *NHP-responsive chromatin signal in the CAMTA5 promoter.* ***(D)*** *NHP-responsive chromatin signal in the WRKY62 promoter.*

With a relationship between priming behaviours and peak positions identified, I then assessed whether the NHP-responsive peaks in NHP-primed genes were perturbed in *wrky38/62* mutants. First, I compared the NHP-responsive peak signal in primed genes, reverse primed genes, and SA upregulated genes across the wild type and *wrky38/62* (Figure 5.10A). Interestingly, the NHP-responsive signal was lower in *wrky38/62* regardless of transcriptomic behaviour, likely reflecting the overall reduction in peaks in the mutants. I then compared the NHP-responsive peak signal in wild type versus *wrky38/62* to assess whether genes that lose NHP responsiveness in the mutant also lose peak signal. In *ERF105*, the water treated signal was only slightly reduced, but the NHP-responsive signal was considerably lower in the mutant compared to wild-type (Figure 5.10B). The same response was seen for *CAMTA5*, where again only the NHP-treated peak signal differed substantially between the wild type and mutant, whereas the water treated peak signals were similar (Figure 5.9C). By contrast, in the *WRKY62* promoter there was little difference between wild type and mutant in either water or NHP treated samples (Figure 5.10D). Taken together, these data suggest that when the NHP-primed genes are *WRKY38/62* dependent, their NHP-mediated chromatin accessibility is also *WRKY38/62* dependent.

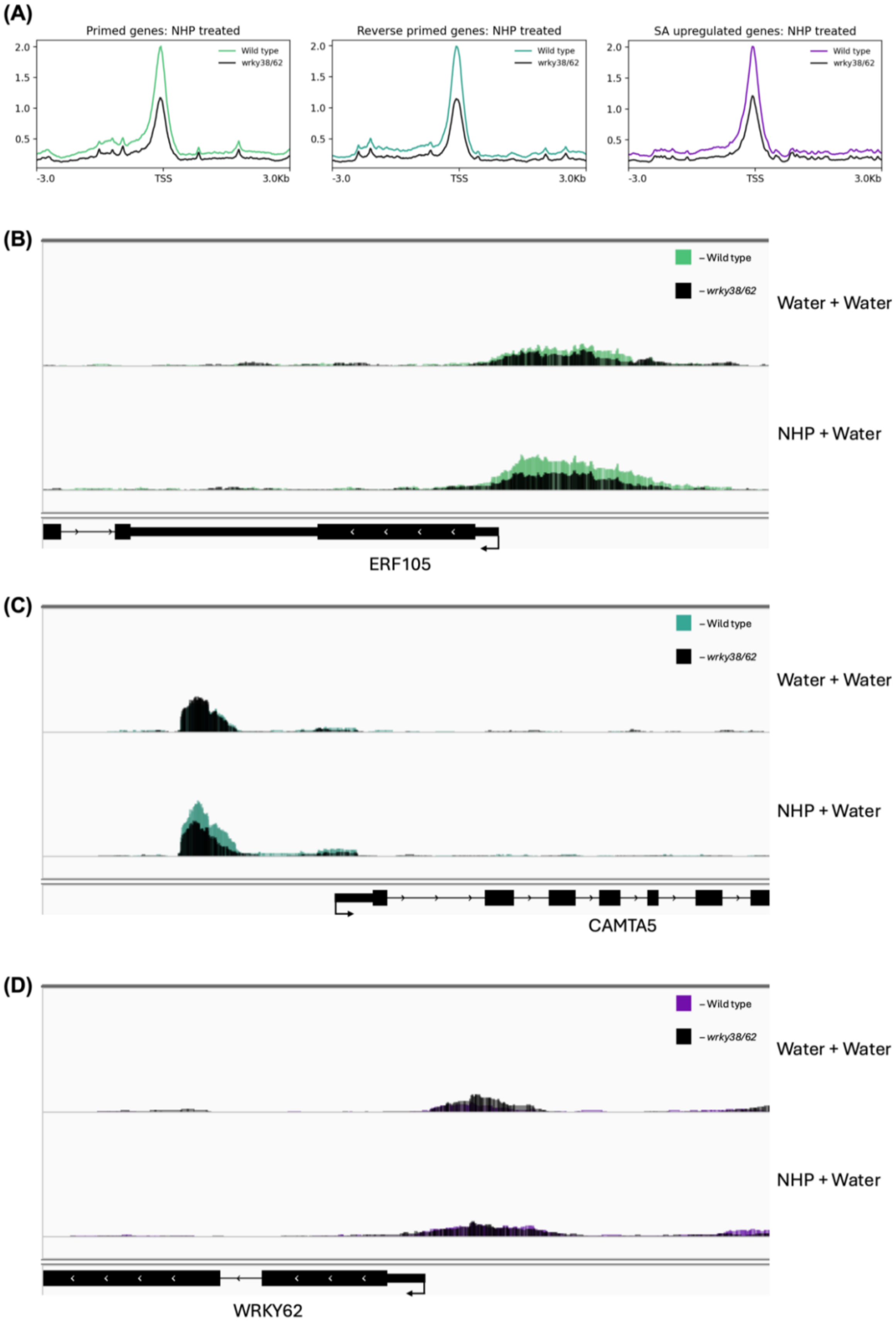


***Figure 5.10 -WRKY38/62 dependent gene's promoters contain WRKY38/62 dependent peaks. (A)** Comparison of NHP-responsive chromatin signals in wild type and wrky38/62 samples. Axis labels represent relative transcript*

accumulation scaled from 0 to 2 across all genes. ***(B)*** *WRKY38/62-dependent, NHP-responsive chromatin signal in the ERF105 promoter.* ***(C)*** *WRKY38/62-dependent, NHP-responsive chromatin signal in the CAMTA5 promoter.* ***(D)*** *WRKY38/62-dependent, NHP-responsive chromatin signal in the WRKY62 promoter.*

While this hypothesis is credible, it is so far only supported by single gene candidates. Therefore, I compared the overlap between NHP-responsive peak signals and NHP-responsive genes identified in the previous RNA-seq. For this analysis I separated genes into categories based on their transcriptomic response to NHP and SA: (i) NHP primed (including both primed and reverse primed genes), (ii) NHP regulated (not including NHP regulated genes that also respond to SA), (iii) NHP and SA regulated, and (iv) SA regulated, while also considering both up- and downregulation within these categories. I then separated peaks into those opened or closed by NHP, and whether they were WRKY dependent or independent. This analysis found that 40% of genes primed by NHP underwent NHP-responsive, *wrky38/62*-dependent chromatin opening or closing events (Figure 5.11). Similarly, 43% of genes cumulatively regulated by SA and NHP showed changes in chromatin accessibility dependent on *WRKY38/62*. This relationship was harder to assess for genes regulated only by NHP and not by SA, with only 38 genes (37%) in this group displaying NHP-responsive peaks. Overall, these data suggest there may be a relationship between NHP-mediated potentiation of SA-responsive gene expression and changes in chromatin accessibility. Notably, there were also many NHP-responsive peaks in NHP-unresponsive genes, and many NHP-responsive genes without NHP-responsive peaks, suggesting the relationship between NHP-mediated chromatin regulation and NHP-mediated gene regulation is more complex than a ‘peak = priming’ hypothesis.

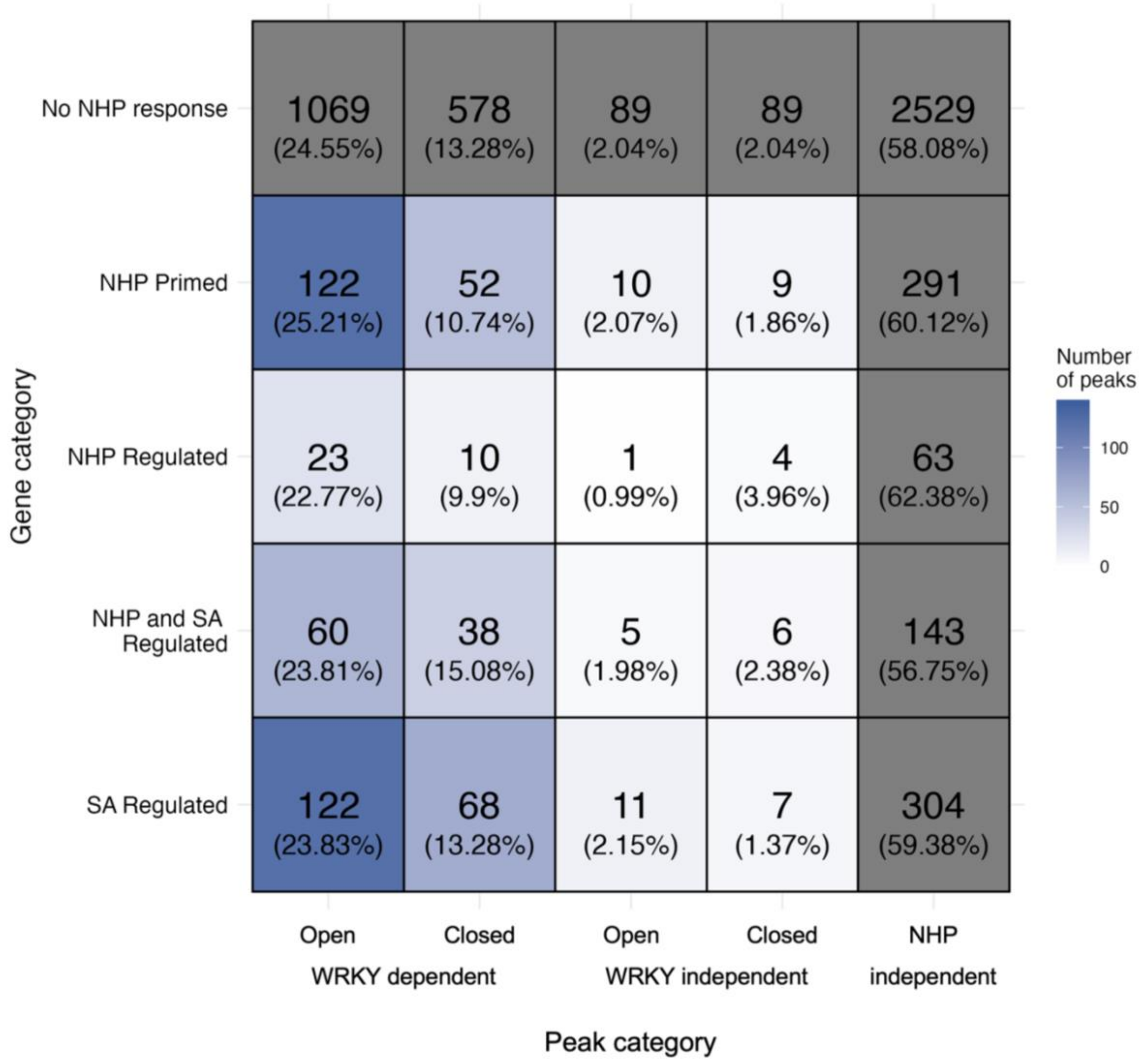


Figure 5.11 – Many, but not all NHP-primed genes contain NHP-responsive peaks. *Comparison of NHP regulation and wrky38/62 dependence of peaks present in NHP primed genes, NHP regulated and SA regulated genes. Numbers in each tile indicate the number of genes of each transcriptional and chromatin category.*

5.2.5 – NHP-responsive peaks associate with some AzA-responsive genes

As a large number of NHP-responsive peaks did not correlate with NHP-primed genes, I then compared the presence of NHP-responsive peaks in AzA-primed genes to assess whether NHP could influence AzA-responsive genes. Using the same gene categories as before, a similar proportion of AzA-responsive and AzA-primed genes contained NHP-responsive peaks (Figure 5.12). This suggests that while there is little overlap between the genetic targets of NHP and AzA (Figure 4.9) there is some level of crosstalk between NHP and AzA at the chromatin level. Whether this is synergistic, antagonistic, or a mixture of both is unknown. It would be informative to repeat the ATAC-seq experiment with AzA treatment rather than NHP to investigate whether AzA induces unique regions of differential accessibility and whether it reciprocally alters chromatin at NHP-primed genes.

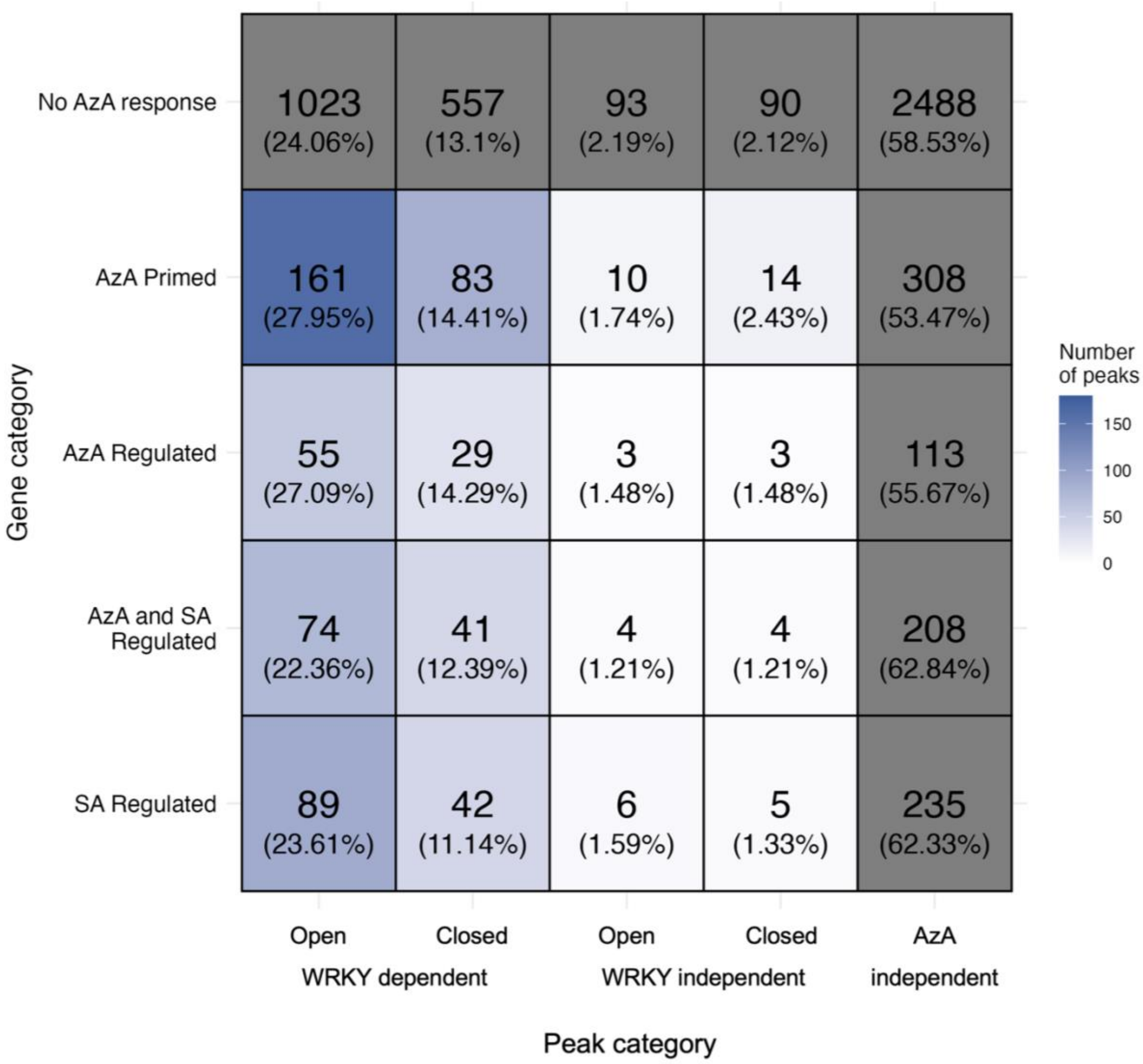


Figure 5.12 – Many, but not all AzA-primed genes contain NHP-responsive peaks. *Comparison of NHP regulation and wrky38/62 dependence of peaks present in AzA primed genes, AzA regulated and SA regulated genes. . Numbers in each tile indicate the number of genes of each transcriptional and chromatin category.*

5.2.6 – WRKY38/62-linked chromatin remodellers contribute to priming

The data in this chapter strongly suggest that NHP promotes priming by inducing chromatin modifications in a *WRKY38/62* dependent manner. Therefore, I searched for and studied potential regulators of NHP-mediated chromatin remodelling and assessed whether they are also required for priming. The first candidate was HDA19, as this protein was previously shown to interact with both WRKY38 and WRKY62 (Kim *et al.*, 2008). The second candidate was JUMONJI14 (JMJ14), as this is a H3K4 histone demethylase shown to be required for immune gene expression and NHP biosynthesis. I treated fourteen-day-old wild-type, *hda19* and *jmj14* seedlings sequentially with NHP and SA, and measured *PR1* gene expression as previously described (Figure 4.1). The *hda19* mutant displayed unperturbed SA-responsive gene expression and marginally reduced NHP-mediated priming (Figure 5.13A), which was consistently observed across independent assays. As HDA19 is part of a large protein family, it is plausible that multiple HDAs act redundantly, thereby limiting the reduction in priming observed here. Conversely, the *jmj14* mutants displayed a strong reduction in SA-responsiveness, but still maintained an NHP-dependent priming effect (Figure 5.13B). Therefore, while JMJ14 is important for SA signalling, it is not required for establishing the primed state.

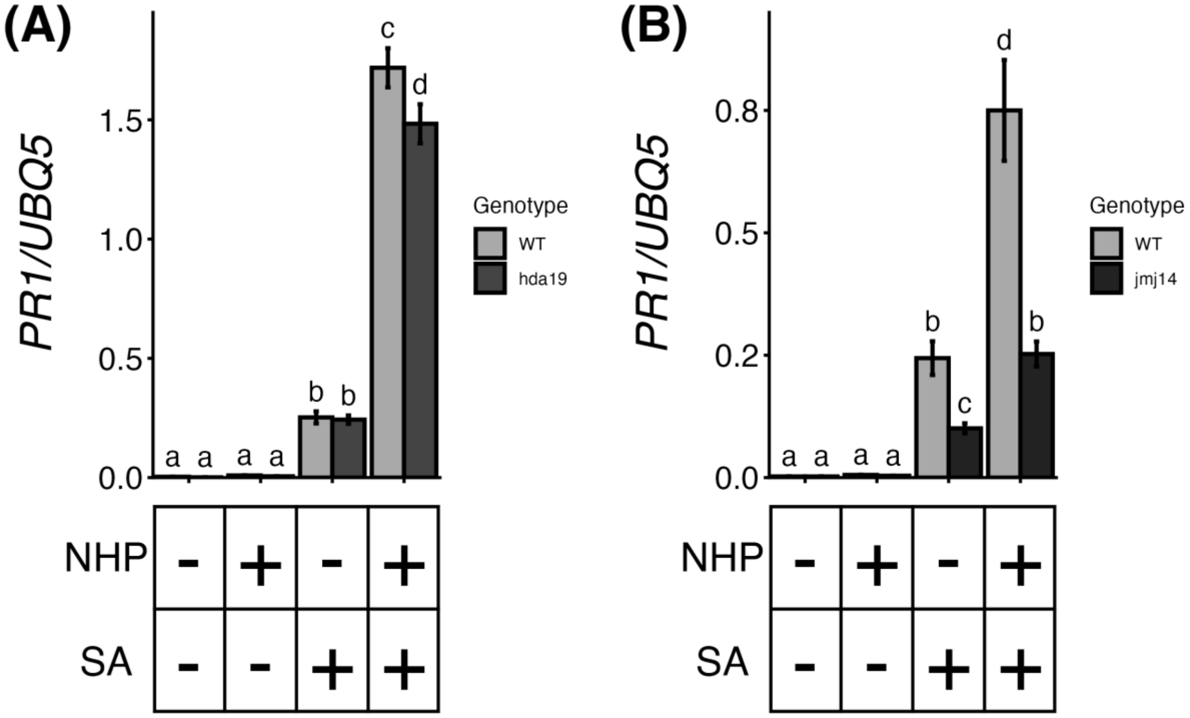


Figure 5.13 – Chromatin remodeller mutants display reduced NHP-mediated priming. *Fourteen-day-old wild-type and either hda19* ***(A)*** *or jmj14* ***(B)*** *seedlings were treated by immersion in water or 1mM NHP for 18 hours,*

followed by treatment with water or 0.5mM SA for 6 hours. Total RNA was extracted and the expression of SA marker gene PR1 was measured by qPCR. Reporter genes were normalised against constitutively expressed UBQ5. Letters denote statistically significant differences between samples (Tukey ANOVA; α = 0.05, n = 3).

5.3 – Discussion

5.3.1 – The role of WRKYs in systemic immunity

While NHP is a vital component of systemic immune signalling, how NHP facilitates priming and what the downstream signalling components are, is not well studied. In this chapter I reveal that NHP-mediated priming requires the transcription factor pair WRKY38 and WRKY62. Analysis of the *wrky38/62* double mutant shows that NHP-mediated transcriptional reprogramming and immune potentiation are substantially dysregulated. In search of a priming mechanism, I also found that NHP induces large scale chromatin remodelling across the genome, increasing and decreasing chromatin accessibility at 862 and 111 unique locations respectively, many of which are in close proximity to genes primed by NHP. This effect is significantly disrupted in *wrky38/62* mutants, suggesting that these transcription factors prime immune responses through chromatin remodelling. Finally, I show that priming is partially disrupted in the absence of WRKY38/62-associated histone deacetylase *HDA19*, suggesting that NHP primes immunity by histone modification directed by WRKY38/62.

WRKYs are a large family of plant transcription factors, several of which are vital for proper regulation of immunity at several stages. For example, WRKY33 is a core component of early immune signalling, as it is activated by immune-induced MITOGEN

ACTIVATED PROTEIN KINASE 3 and 6 (MPK3/6) and promotes pipecolic acid and camalexin biosynthesis (Wang *et al.*, 2018). Recently, it was shown that cells adjacent to the site of infection activate a distinct transcriptome from infected cells, called the "bystander" cell state (Nobori *et al.*, 2025). The transcripts of bystander cells are enriched in WRKY target motifs, suggesting that WRKY TFs are also a component of systemic immune signalling. Here, I find that WRKY motifs are highly enriched in NHP-primed genes, suggesting that WRKYs play an important role in NHP signalling as well as NHP biosynthesis. Consistent with this, a role for WRKY70 in NHP signalling has recently been revealed, as *wrky70* mutants displayed reduced NHP-mediated gene expression at some loci, and reduced NHP-mediated ROS accumulation (Foret *et al.*, 2025). In local immunity, WRKY70 acts in tandem with WRKY54 to negatively regulate SA biosynthesis by suppressing expression of *SARD1* (Zhou *et al.*, 2018). Interestingly, while *wrky70* mutants display enhanced disease resistance, *wrky54/70* double mutants display impaired resistance (Foret *et al.*, 2025; Chen *et al.*, 2021), suggesting that to understand the full effects of this duo they should be studied in tandem. Here, I corroborate that WRKY54/70 are important together, finding that *wrky54/70* display strongly impaired SA-responsiveness (Figure 5.2A). However, I still observed NHP-mediated priming of *PR1*, indicating that NHP-mediated priming is not reliant on WRKY54/70.

WRKY18 is another core component of immune signalling. WRKY18 interacts with WRKY40 and WRKY60 to regulate responses to both pathogen attack and abiotic stresses (Xu *et al.*, 2006; Chen *et al.* 2010). WRKY18 also displays a close link to NPR1 signalling, as *wrky18* mutants exhibit reduced SA-responsive expression of ~25% of NPR1 target genes (Wang *et al.*, 2006). With an association between NHP and NPR1 previously established (Figure 4.11; Nair *et al.*, 2021), WRKY18 arose as a candidate regulator of NHP-mediated, NPR1-linked signalling. However, while *wrky18* mutants displayed a very slight reduction in priming, NHP still induced a significant amount of priming in these mutants (Figure 5.2B). This suggests that WRKY18 may somewhat amplify NHP-mediated priming but is not a major component required for establishing the primed state.

Finally, I tested the pair of WRKY38 and WRKY62, because interestingly, *wrky38/62* double mutants display impaired systemic immunity, but unperturbed (and slightly enhanced) local immunity (Spoel *et al.*, 2009), making them excellent candidates as regulators of systemic NHP signalling. A further link between WRKY38/62 and NHP has been drawn, as analysis of genes expressed within 15 minutes of NHP application revealed that *WRKY38* and *WRKY62* are rapidly and strongly expressed after NHP treatment (Foret *et al.*, 2025). Thus, I tested NHP-mediated priming in *wrky38/62* mutants and found that not only was *PR1* priming abolished, but the whole NHP-modulated transcriptome was dysregulated in these mutants. NHP-mediated immune potentiation was also lost, suggesting that the loss of systemic immunity is due to a loss of NHP-responsiveness in *wrky38/62* mutants. Taken together, these findings indicate that while several WRKYs contribute to NHP-mediated priming, it is the early activation and redundant signalling functions of WRKY38 and WRKY62 after NHP signal perception that sets priming in motion.

5.3.2 – How do WRKY38/62 induce NHP-mediated priming of immunity?

As WRKY38/62 are indispensable for NHP-mediated priming this poses the question: What is the function of WRKY38/62? Research into WRKY38 and 62 is limited, but there are some suggestions of their roles in immune signalling. WRKY62 is expressed in response to both SA and jasmonic acid (JA) treatment, dependent on NPR1, with WRKY62 acting to suppress JA signalling (Mao *et al.*, 2007). Meanwhile WRKY38 was directly identified in bystander cells of infected tissues (Nobori *et al.*, 2025), suggesting a link to systemic signalling. Of particular importance, it was shown that WRKY38/62 both directly interact with histone deacetylase HDA19 (also known as HDA1), which is also induced by SA treatment, JA treatment and pathogen infection (Kim *et al.*, 2008). Immune responses and *PR1* expression are both impaired in *hda19* mutants, suggesting that HDA19 plays an important role in plant immunity. While the exact purpose of the interaction between WRKY38/62 and HDA19 has not been ascertained, these data imply that WRKY38/62-associated chromatin remodellers could be important components of fine-tuning immune responses for the desired output.

My data strongly support this hypothesis, as I show not only that NHP remodels chromatin, but it does so dependent on WRKY38/62. Chromatin accessibility was reduced across the whole genome of *wrky38/62* mutants, even in uninduced cells (Figure 5.6), suggesting WRKY38/62 have broad roles in regulating chromatin accessibility. Analysis of the NHP-primed transcriptome revealed that the majority of chromatin that was remodelled in response to NHP treatment, contained NHP-responsive genes whose expression was dependent on WRKY38/62 (Figure 5.11). However, I did not identify any significant sequence or expression patterns in *wrky38/62*-dependent chromatin regions, so without further experimentation it is difficult to determine if these regions are targeted directly by WRKY38/62. Moreover, it remains unclear if WRKY38/62 trigger chromatin remodelling, which then leads to enhanced gene expression, or if chromatin remodelling is a consequence of

WRKY38/62-activated gene promoters. Future study should focus on cloning epitope-tagged WRKY38 and WRKY62 for use in chromatin immunoprecipitation sequencing (ChIP-seq), as this could be analysed in tandem with RNA-seq and ATAC-seq data to create a database of where WRKY38/62 bind, whether the chromatin is remodelled in the same region, and whether that results in priming or expression of immune genes contained within.

Interestingly, in *Arabidopsis* DAP-seq experiments, WRKY38 and WRKY62 were tested, but no specific binding motif was identified (O'Malley *et al.*, 2016). WRKY38/62 are group 3A WRKYS, and no member of the group 3A WRKYs displayed significant binding motifs in the DAP-seq experiment. WRKY group 3 is defined by the presence of a C2HC-type zinc finger rather than a C2H2 type, and subgroup A all contain a 50 amino acid C terminal domain that is highly charged and acidic (Kalde *et al.*, 2002). The more 'classical' C2H2 zinc finger primarily binds DNA, but C2HC zinc fingers are not as specific, with evidence showing that they can also bind proteins and nucleosomes (Fedotova *et al.*, 2017; Akhtar and Becker, 2001). This may explain why WRKY38/62 and the other group 3A WRKYs do not show specific DNA sequence recognition. One possible explanation is that group 3A WRKYs act as 'pioneer' transcription factors, which bind heterochromatin and trigger its remodelling to enable other transcription factors to bind the now accessible DNA (Mayran and Drouin, 2018). This would explain why no motifs were detected in the DAP-seq data, as the group 3A WRKYs can only bind their targets when they are packaged in nucleosomes and not in a chromatin-free context. This also can explain why no enriched motifs were discovered in *WRKY38/62* dependent peaks in this study, as *WRKY38/62* dependency is not defined by a motif in the target genes DNA.

In support of this hypothesis is the breadth of chromatin regulated by WRKY38/62 in my ATAC-seq. Even in uninduced cells, loss of *WRKY38/62* drops the number of accessible peaks from 3,287 to 2,088, suggesting WRKY38/62 are vital for maintaining chromatin accessibility across the genome. WRKY38/62 are also some of the first WRKYs induced by NHP, with transcripts detectable just 15 minutes after treatment (Foret *et al.*, 2025). WRKY motifs are not detected in the promoters of expressed genes until 3 hours after NHP treatment, suggesting WRKY38/62 could unwind a wide group of target genes minutes after signal perception, enabling their expression by a later wave of WRKYs, possibly including WRKY18, WRKY54 and WRKY70. Furthermore, subgroup 3A WRKYs all contain a C terminal acidic domain. While acidic domains have several possible functions, they are frequently found in chromatin interacting proteins (Wu *et al.*, 2017), providing further evidence that this subgroup could possibly interact with chromatin components rather than DNA directly. The above proposed ChIP-seq experiment would help assess if WRKY38/62 are pioneer TFs, as this will enable direct comparison of transcription factor binding with chromatin state. If the data support this hypothesis, then the result can be verified by supplying nucleosomes to purified TFs and detecting electrophoretic mobility shifts on an agarose or polyacrylamide gel (Yu and Buck, 2021).

5.3.3 – NHP peaks do not perfectly correlate with priming

An unexpected observation from the ATAC-seq was that the majority of NHP-regulated chromatin accessible peaks do not contain primed SA-responsive genes (Figure 5.10). One explanation for this result could be that genes other than SA-responsive genes are primed by NHP. Indeed, these accessible peaks could be associated with primed genes expressed during pattern-triggered immunity by ROS, Ca_{2+}, or MAPK signalling. Furthermore, immunity to biotrophic pathogens crosstalks with several necrotroph-responsive phytohormones, such as jasmonic acid (JA) and ethylene (ET) (Hou and Tsuda, 2022). As transgenerational priming against biotrophs has been associated with reduced resistance to necrotrophs (Luna *et al.*, 2012), these chromatin accessible peaks may be present in JA- or ET-responsive genes, regulating their future responsiveness to prioritise SA-responsive immunity. Finally, in Chapter 3, I show that other mobile signals such as AzA are important contributors to systemic resistance, and there are several other potential mobile signals, including glycerol-3-phosphate (G3P) and dehydroabietinal (DA) (Chanda *et al.*, 2011; Chaturvedi *et al.*, 2012). While I have shown that NHP-primed genes and AzA-primed genes display very little overlap (Figure 4.9), here I show that many AzA-primed genes contain NHP-responsive peaks (Figure 5.12). Therefore, NHP could aid the signalling and priming processes of other transported signals through wide scale chromatin loosening.

Another surprise was that many SA-responsive, NHP-unprimed genes also displayed *WRYK38/62* dependent peaks. However, this could also be linked to the complexity of an immune response to a pathogen compared to a single signal. By remodelling chromatin, WRKY38/62 may prepare the SA-responsive transcriptome for increased or decreased responsiveness to other immune signals to optimise SA signalling for a primed immune response. It would be interesting to assess the interactions between NHP and other plant hormones using the same framework created in this study. Assessing the impact of NHP pretreatment on the JA-responsive transcriptome would give insight into whether NHP modulates immune responses against necrotrophs. It would also be useful to perform RNA-seq in pathogen infected plants, to determine whether NHP peaks are in genes activated by pathogen infection, but not SA alone. Due to the level of crosstalk between immune response pathways and the persistent exposure of plants in the wild to biotic and abiotic stresses, it is highly likely that NHP will feed into other plant stress signalling pathways.

5.3.4 – Conclusion

So, how does NHP signalling induce priming? The data in this chapter make two big strides towards answering this question. First, the WRKY family transcription factor pair WRKY38/62 are indispensable for NHP-mediated priming. Second, NHP-mediated priming is closely associated with chromatin remodelling, as NHP induces WRKY38/62-dependent chromatin unwinding at hundreds of unique sites across the genome, especially in primed genes. From these results I conclude that WRKY38/62 are likely pioneer transcription factors, responding to NHP signalling to loosen chromatin across the genome to induce the primed state. Taken together, these data suggest that while NHP directly regulates multiple genes in systemic tissues to induce priming, it also induces epigenetic signalling pathways to promote long lasting primed states (Figure 5.14).

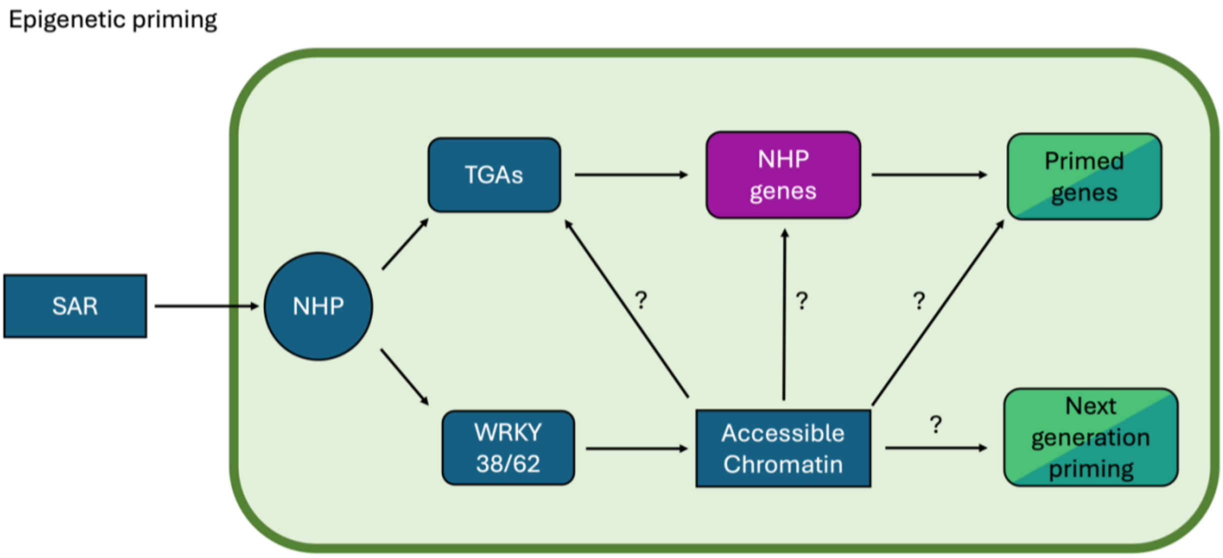


Figure 5.14 – Schematic diagram of NHP signalling in systemic tissues. *When NHP accumulates in systemic tissues, it activates transcriptional cascades driven by TGA family TFs to activate NHP-responsive genes. NHP also promotes expression of WRKY38/62, which regulate chromatin accessibility across the genome. The increased accessibility may contribute to one of several roles, including promoting NHP-mediated gene expression, priming of SA-responsive genes, or transgenerational priming pathways.*

Chapter 6 – Discussion

In plants, pathogen perception triggers immune activation in both local and systemic tissues. Local immune responses trigger the translocation of phloem mobile signals from the site of infection to systemic tissues to establish systemic acquired resistance (SAR). A key feature of SAR is immune priming, in which plant immunity is sensitised such that it responds faster and stronger to subsequent challenge stimuli. While several mechanisms of priming have been identified (Conrath *et al.*, 2015), if all mobile signals contribute to priming and how they do so is not understood.

In this study, I assessed whether pretreatment with the phloem mobile signals azelaic acid (AzA) and N-hydroxy pipecolic acid (NHP) was sufficient to prime immune responses regulated by the immune hormone salicylic acid (SA). In Chapter 3, I show that AzA acts to suppress specific SA-responsive transcriptional responses and failed to prime SA-dependent immune responses against the bacterial pathogen *Pseudomonas syringae pv. maculicola* (*Psm*) ES4326. Conversely, in Chapter 4, I find that NHP primarily potentiates SA signalling, and sensitised SA-induced immunity against infection. In search of a regulator of NHP signalling, I found that NHP increases stability of the SA receptor NPR1, but that accumulation of NPR1 alone was not sufficient to explain priming. In Chapter 5, I discover a pair of transcription factors (TFs), WRKY38 and WRKY62, that are indispensable for NHP-mediated priming. I demonstrate that NHP induces widespread changes to chromatin accessibility in a WRKY38/62-dependent manner. Taken together, this thesis reveals fascinating new molecular insights into the unique roles of these phloem mobile signal and their interactions with

SA signalling and uncovers new links between NHP and epigenetic priming established during SAR.

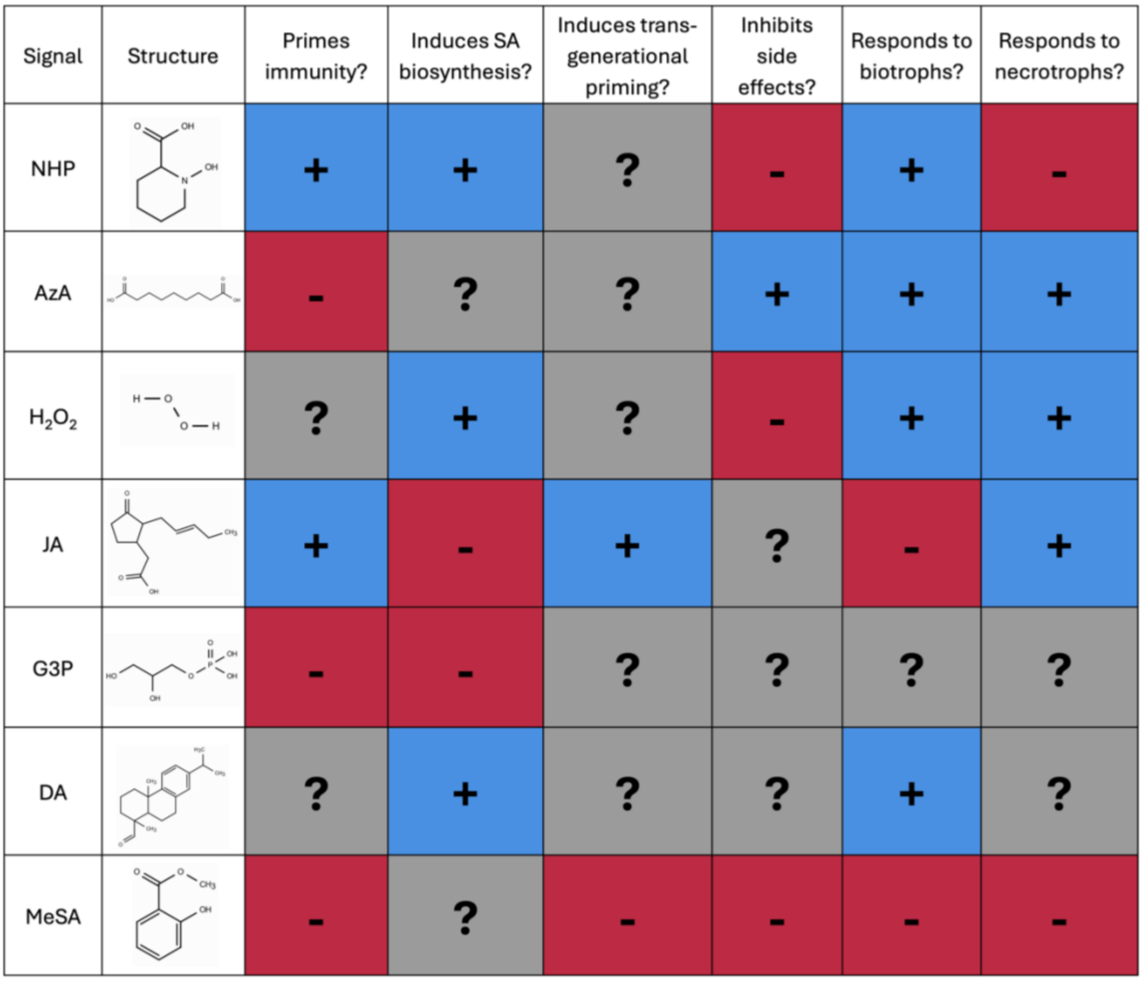
6.1 – Phloem mobile signals play non-redundant roles in SAR

While NHP and AzA are both linked to priming, less is known about the pathways they prime and the extent of priming controlled by each signal. In Chapter 3, I show that AzA primes SA-responsive expression of some, but not all SA marker genes (Figure 3.1). Surprisingly, RNA sequencing (RNA-seq) revealed that AzA primarily ‘reverse primes’ SA-responsive genes, reducing their SA-responsive expression (Figures 3.4 and 3.6). Although AzA substantially dampened parts of the SA-responsive transcriptome, it did not affect SA-inducible immunity in *Arabidopsis*. Overall, these data suggest that while AzA contributes to fine-tuning SAR, it cannot establish a complete SAR state by itself. In Chapter 4, I show that NHP primes multiple SA marker genes (Figure 4.1). Looking at the entire transcriptome, NHP enhanced SA signalling through synergistic expression and priming of SA responsive genes (Figures 4.5 and 4.7). Accordingly, the NHP precursor, pipecolic acid, sensitised plants such that 10-fold lower levels of SA were sufficient to induce a strong immune response (Figure 4.10).

From these data, I hypothesised that NHP and AzA play different roles in SAR. Comparison of the AzA- and NHP-primed transcriptomes revealed that the majority of genes primed by each signal do not overlap (Figure 4.9). Moreover, gene ontology (GO) term analyses showed that both signals altered the expression of SA-responsive genes with diverging roles. These findings indicate that AzA and NHP have distinct roles in establishing SAR. AzA predominantly antagonised specific sectors of SA signalling, suppressing genes involved in programmed cell death and catabolism, while promoting the expression of photosynthetic genes usually suppressed by SA (Figures 3.5 and 3.7). NHP had more varying effects in that it enhanced SA-mediated suppression of photosynthesis and immune signalling, while antagonising SA-mediated suppression of translation (Figure 4.6 and 4.8). Altogether these results suggest that NHP acts to boost the strength of SA responses, while AzA suppresses responses associated with local immune responses and possibly preventing side effects that may otherwise arise from enhanced immune activation. The role for NHP as an enhancer of SA signalling is further substantiated by multiple studies that show treatment with NHP alone can enhance immunity in plants (Yildiz *et al.*, 2021; Nair *et al.*, 2021; Foret *et al.*, 2025). The role of AzA as a selective inhibitor of SA signalling is harder to prove, but evidence suggests SAR signalling includes fine tuning of immunity and not only sensitisation (Durrant and Dong, 2004). During SAR the strength of immune responses is enhanced, but cell death associated with the hypersensitive response (HR) is suppressed, possibly because primed cells can combat infection without triggering HR (Fu and Dong, 2013). Sustained immune activation causes growth inhibitory side effects as energy is focused away from growth and development (He *et al.*, 2022), so it is entirely plausible that the role of AzA is to reduce unwanted responses associated with local immunity that may occur when SA signalling is boosted by NHP in systemic tissues. This would also explain why previous research has rarely found AzA capable of enhancing immune responses (Zoeller *et al.*, 2012; Nagy *et al.*, 2016), as it acts to dampen ‘side effects’ of priming rather than directly enhancing immunity.

These distinct roles of NHP and AzA in SAR suggests that other mobile signals may each play unique roles. Jasmonates and hydrogen peroxide (H_2O_2) are both confirmed mobile signals, while glycerol-3-phosphate (G3P), dehydroabietinal (DA) and methyl-salicylate (MeSA) are all potential mobile signals (Figure 1.3). Jasmonates are required for signal perception in systemic tissues and H_2O_2 propagates cell-to-cell SA biosynthesis (Gaikwad *et al.*, 2023; Cao *et al.*, 2023). Both of these processes are not regulated by NHP nor AzA, but occur when SAR is induced by a pathogen (Zhang *et al.*, 2010), suggesting jasmonates and H_2O_2 also modulate unique processes to establish SAR. While the importance of G3P and DA has not been confirmed, they are strongly associated with AzA signalling (Yu *et al.*, 2013; Chaturvedi *et al.*, 2012). As with AzA and NHP signalling, the answer to the roles of these signals may not come from study in isolation, but instead study alongside other signals to enable a response or identify a specific pathway targeted by G3P or DA.

It is plausible that a unique formulation of signals is produced depending on the lifestyle and infection strategy of the encountered pathogen to tailor the priming response. Indeed, it was previously shown that pathogens trigger specific signal signatures (De Vos *et al.*, 2007). Overall, my findings that NHP and AzA target very different immune pathways suggest that together phloem mobile signals make up a 'cocktail' that fine tunes the exact nature of a systemic response depending on the infectious or eliciting agent (Figure 6.1). Despite strong links between several mobile signals, previous research rarely considered the effects of multi-signal treatment as in this study. Thus, the mobile signal 'cocktail hypothesis' could be tested further by investigating if sequential or simultaneous signal treatment enables or enhances immunity compared to a single treatment alone.



Signal	Structure	Primes immunity?	Induces SA biosynthesis?	Induces trans-generational priming?	Inhibits side effects?	Responds to biotrophs?	Responds to necrotrophs?
NHP		+	+	?	-	+	-
AzA		-	?	?	+	+	+
H_2O_2		?	+	?	-	+	+
JA		+	-	+	?	-	+
G3P		-	-	?	?	?	?
DA		?	+	?	?	+	?
MeSA		-	?	-	-	-	-

Figure 6.1 – Overview of the confirmed and speculative roles of each described mobile signal in this study.

6.2 – Systemic signalling by phloem mobile signals

Next, I investigated the molecular mechanisms by which NHP induces priming. In Chapter 4, I show that NHP promotes accumulation of the SA receptor protein NPR1 by increasing its stability (Figure 4.11). I also show that NHP induces ubiquitination of NPR1 (Figure 4.13), which could affect its stability or activity (Spoel *et al.*, 2009; Skelly *et al.*, 2019; Wang *et al.*, 2022). However, NHP still primed SA signalling in SA-sensitised mutants that display elevated levels of NPR1 (Figure 4.12), suggesting that NPR1 accumulation alone does not explain NHP-mediated priming.

Despite its structural similarity to SA, NHP was found to not interact with NPR1 *in vitro* (Nair *et al.*, 2021), suggesting at least one unidentified NHP receptor is required to transduce the NHP signal to NPR1. This also raises the possibility that NHP signalling occurs through nodes other than just NPR1. NHP-inducible genes often include pathogen receptors and early signalling components, such as receptor-like kinases, MAP kinases and NLR resistance proteins (Yildiz *et al.*, 2021). Buildup of inactive receptors and signalling components potentiates pathogen-inducible responses (Conrath *et al.*, 2006), so the expression of these genes is likely a mechanism of NHP-mediated priming. NHP also induces expression of SA biosynthesis genes and glucosyltransferase UGT76B1 (Mohnike *et al.*, 2021), which promotes the accumulation of inactive SA as SA-O-ß-glucoside (SAG). If the SAG is quickly converted to free SA upon infection this may also contribute to the potentiation of SA signalling in an immune response.

A core feature of NHP-mediated priming is metabolic priming, as pretreatment with NHP increases the pathogen inducible production of SA, NHP, camalexin and several amino acids (Lowe *et al.*, 2023). Priming of camalexin biosynthesis in particular is linked to specific histone modifications that enable a rapid response when required (Zhao *et al.*, 2021). Epigenetic priming is another well described component of SAR, where both DNA methylation and histone modification pathways have been linked to immune priming (Luna *et al.*, 2012; Jaskiewicz *et al.*, 2011). In the absence of immune activation, histone remodelling and DNA methylation can poise gene expression such that when a pathogen is encountered, the gene is activated at faster or slower speeds. NPR1 is also associated with chromatin remodelling (Koornneef *et al.*, 2008; Jin *et al.*, 2018), so it is possible that NHP manipulates the epigenetic state at primed genes through NPR1. Furthermore, 3 of the 6 genes in the camalexin biosynthesis pathway are upregulated in response to NHP treatment (Yildiz *et al.*, 2021), suggesting camalexin biosynthesis is primed by both epigenetics and inactive transcript/protein accumulation.

Interestingly, I found that NHP induced accumulation of total ubiquitinated proteins, but priming was not perturbed by inhibition of proteasomal degradation (Figures 4.13 and 4.14). Beyond gene expression, NPR1 also acts as a substrate adapter for the ubiquitin E3 ligase CUL3 to promote ubiquitination of signalling proteins (Fu *et al.*, 2012). With the evidence that NHP induces gene expression through NPR1, it is plausible that NHP could also induce ubiquitination of signalling proteins through NPR1. TUBE pulldowns such as those in Figure 4.11 sometimes may not differentiate between a ubiquitinated protein and those interacting with it, so it is also possible that some of the protein detected in Figure 4.11 are being ubiquitinated by NPR1 rather than being ubiquitinated NPR1 itself. However, NHP-responsive ubiquitination was still detected in *npr1* mutants (Figure 4.14A), so at a global cell scale it is likely not dependent on NPR1. This suggests that NHP may not only alter cellular gene expression, but also signal through manipulation of post-translational modifications.

6.3 – Epigenetic signalling by mobile signals

6.3.1 – The role of epigenetics in NHP signalling

In search of other NHP priming regulators, in Chapter 5, I discovered a pair of TFs indispensable for NHP signalling. WRKY family TFs, WRKY38 and WRKY62, have previously been reported as indispensable for pathogen-induced SAR (Spoel *et al.*, 2009). Here, I show that *wrky38/62* mutants are defective in NHP-mediated priming of SA-responsive gene expression and immunity (Figures 5.2 and 5.4). Previous research shows that WRKY38/62 interact with histone deacetylase HDA19 (Kim *et al.* 2008), suggesting they could influence chromatin dynamics. Thus, I performed ATAC sequencing (ATAC-seq) to investigate if NHP mediates priming by modulating chromatin accessibility. NHP application alone induced broad scale changes in chromatin accessibility, with this effect lost in *wrky38/62* mutants (Figures 5.6 and 5.8). To determine whether chromatin accessibility correlates with priming, I compared the NHP-responsive peak profiles of NHP-primed genes, showing that certain primed genes and reverse primed genes contain clear regions of NHP-opened or closed chromatin in their promoters (Figure 5.9). These changes in chromatin accessibility were lost in *wrky38/62* mutants, correlating with a loss of priming (Figure 5.10), strongly suggesting that NHP-responsive, *WRKY38/62*-dependent chromatin changes promote NHP-mediated priming. I then assessed NHP-mediated priming in *hda19* mutants, finding that *PR1* priming is partly perturbed but not abolished (Figure 5.13). This suggests that HDA19 is not the sole disseminator of NHP-mediated chromatin remodelling, and other chromatin regulators may be involved in WRKY38/62-dependent priming.

The data in Chapter 5 show that NHP induces broad changes in chromatin accessibility, and when this effect is perturbed, priming is dysregulated. Together these data strongly indicate that NHP-mediated priming is driven by NHP-responsive epigenetic signalling. A limitation of the data in this study is that while ATAC-seq provides great insights into the dynamics of chromatin accessibility, it does not detect which epigenetic modifications are controlling chromatin remodelling. NHP may signal through multiple epigenetic pathways, as both histone modification and DNA methylation are important in plant immune regulation (Alvarez-Venegas *et al.*, 2007; Yu *et al.*, 2013).

Comparison of NHP-regulated chromatin accessibility with the NHP-regulated transcriptome suggests that several processes occur at NHP-regulated chromatin regions (Figure 5.11). One is active transcription. While NHP does not uniquely regulate many genes, a fair number of genes are regulated by both NHP and SA (Figures 4.5 and 4.7). Epigenetic signalling is vital for regulation of transcriptional responses, such as is in the concept of ‘transcriptional bursting’. Here, genes do not simply exist in ‘on’ or ‘off’ states, but instead active expression occurs by repetitive bursts of transcription to steadily accumulate mRNAs (Tunnacliffe and Chubb, 2020). This process is tightly regulated by epigenetic modifications, as rhythmic acetylation and deacetylation of histones controls burst frequency (Nicolas *et al.*, 2018). The association between NHP and NPR1 (Figure 4.11; Nair *et al.*, 2021) suggests NHP may directly regulate chromatin, as NPR1 promotes chromatin remodelling to regulate gene expression (Powers *et al.*, 2024). Early NHP signalling relies on NPR1-associated TGA family TFs such as TGA2/5/6, but NHP does not induce the expression of any TGA genes (Yildiz *et al.*, 2023; Foret *et al.*, 2025). Therefore, NHP-mediated NPR1 accumulation may promote chromatin loosening at TGA target sites to promote gene expression by TGAs already present in the cell. Furthermore, if WRKY38/62 can be verified as pioneer TFs as proposed in Chapter 5, this would strongly indicate that NHP does directly induce chromatin remodelling by inducing expression of pioneer TFs to enable gene expression.

NHP-responsive chromatin regions did not only associate with actively transcribed genes, as 174 NHP-primed genes displayed NHP-responsive chromatin reorganisation without NHP-responsive expression (Figure 5.11). Epigenetic modifications are strongly associated with priming due to their long-lasting nature, especially marks such as histone acetylation that tend to increase accessibility without activating gene expression (Nitsch *et al.*, 2021). For example, exposure to stress induces deposition of H3K4 trimethylation (H3K4me3) and H3K9 acetylation (H3K9ac) at the promoters of immune response regulators *WRKY6*, *WRKY29* and *WRKY53* (Jaskiewicz *et al.*, 2010). Interestingly, in this case H3K4me3 is decoupled from transcription, suggesting this mark can also prime genes by inducing a transcriptionally capable state in the absence of inducing stimuli. During drought stress it has been shown that RNA polymerase II accumulates and ‘stalls’ at several drought-responsive loci. After a second exposure to drought, the expression of these genes was enhanced, showing they were primed by stalled RNA polymerase (Ding *et al.*, 2012). This could also be a mechanism of NHP-mediated priming, and could be enabled by accumulation of H3K4me3 marks at inactive genes. As histone deacetylase *HDA19* contributes to priming (Figure 5.13), this strongly suggests that NHP-mediated priming at least in part involves modulation of histone acetylation status. Another epigenetic mechanism of priming could be modulation of histone subunits, as histone subunits can also be interchanged with a variety of consequences. Replacement of the H2A subunit with

H2A.Z is important for SA-responsive gene expression, and may stably contribute to enhanced immune sensitivity (Conrath *et al.*, 2011). At the DNA level, long lasting priming of jasmonic acid (JA) signalling is maintained by DNA demethylation (Wilkinson *et al.*, 2023). As differential DNA methylation modulates chromatin accessibility, the NHP-responses observed by ATAC-seq could be a result of priming by DNA methylation and demethylation.

There is also evidence that priming can be transmitted across generations, as the progeny of immune-challenged plants display enhanced disease resistance and enhanced immune-responsive gene expression, suggesting they have inherited the primed state from their parents (Luna *et al.*, 2012). Histone modifications are again associated with transgenerational resistance, as the progeny of stressed plants display higher levels of H3K9ac at key immune genes, such as *PR1*, *WRKY6* and *WRKY53* (Luna *et al.*, 2012). Moreover, it has been convincingly demonstrated that DNA methylation is vital for transgenerational priming. Hypomethylation mutants display immunity mimicking priming, and do not gain any resistance from parental stress, suggesting that hypomethylation regulates transgenerational immunity (Luna *et al.*, 2012). Indeed, over 1000 differentially methylated regions have been identified in the progeny of stressed plants, where the duration of stress exposure has a dose dependent effect on the levels of DNA methylation (Stassen *et al.*, 2018). Specifically, differential methylation is detected at the *PR1* promoter after infection (Slaughter *et al.*, 2012), revealing a direct mechanism of priming that could be deployed by NHP.

6.3.2 – Which epigenetic pathways could be employed by NHP?

DNA methylation and histone modification are closely linked, as DNA methylation state regulates the nature of histone modification, and certain histone modifications can recruit or block DNA methylases and demethylases (Rose and Klose, 2014). Therefore, it is difficult to ascertain whether NHP-mediated alterations in chromatin accessibility are associated with DNA or histone modification. There are several experiments that could be performed to verify if NHP induces DNA methylation, histone modification, or both. Bisulfite sequencing is an approach that measures DNA methylation, as the addition of bisulfite before sequencing converts unmethylated cytosines to uracil (Kruegar *et al.*, 2012). Bisulfite sequencing of NHP treated WT and *wrky38/62* plants could identify if alterations in DNA methylation are associated with NHP-induced accessible chromatin regions and NHP-primed genes therein. To survey chromatin modifications chromatin immunoprecipitation (ChIP-seq) with H3K9ac antibodies (Zhou *et al.*, 2010) would be informative due to the association between acetylation and gene priming mentioned above. Comparison of an NHP treated H3K9ac ChIP-seq with the ATAC-seq data could show if histone acetylation sites are induced by NHP and if they correlate with NHP-primed genes. As WRKY38/62 are required for priming and interact with HDA19 (Kim *et al.*, 2008), this experiment should also be performed in *wrky38/62* mutants. If NHP-responsive acetylation or deacetylation is lost in *wrky38/62* mutants, this would give further support to indicate that NHP-mediated priming involves manipulation of histone acetylation states mediated by WRKY38/62.

Interestingly, despite their inherent association, there appears to be a disconnect between the roles of histone modification and DNA methylation in systemic and transgenerational immunity. DNA methylation is often more stably inherited across generations than histone marks, so proves important for priming between generations as well as within (Lopez-Sanchez *et al.* 2016; Stassen *et al.*, 2018; Wilkinson *et al.*, 2023). Taken together, and especially if WRKY38/62 are pioneer TFs, it is possible that NHP induces epigenetic marks that signal for chromatin reorganisation to prime immune responses, and this effect is maintained across generations by differential DNA methylation at primed sites. The previously mentioned bisulfite and ChIP sequencing experiments would provide evidence that may validate this hypothesis.

6.3.3 – Interactions between NHP and other mobile signals

Surprisingly, the majority of NHP-responsive chromatin regions did not appear in NHP primed genes (Figure 5.11). However, in this study I only investigated NHP-mediated priming of SA-responsive genes. One possible explanation is that NHP also primes other signalling pathways such as JA or ethylene (ET). Jasmonate signalling is required for SAR (Gaikwad *et al.*, 2023), suggesting the JA signalling pathway also contributes to priming. There is a strong level of crosstalk between SA and JA signalling (Hou and Tsuda, 2022; Nomoto *et al.*, 2021), with each signal generally repressing the other. Therefore, NHP may also modulate JA signalling to further enhance SA signalling or to balance crosstalk to differentiate SAR from local immunity. Developmental responses are also deployed during immune responses, such as stomatal closure triggered by abscisic acid (ABA) (Hsu *et al.*, 2021). Again, NHP could prime, or reverse prime the sensitivity of various ABA-responsive genes through chromatin remodelling to regulate developmental responses during SAR.

This is the first report linking NHP signalling to chromatin remodelling, but SAR has been linked to epigenetics for several years. ‘Memory’ of resistance within generations is associated with increased deposition of chromatin loosening histone modifications, and inheritance of immune priming is driven by modulation of DNA methylation (Jaskiewicz *et al.* 2010; Luna *et al.*, 2012). Furthermore, AzA-mediated priming is also linked to epigenetics through MORPHEUS MOLECULE 1 (MOM1), an epigenetic regulator that suppresses the expression of a set of transposons through a DNA methylation-independent pathway (Vaillant *et al.*, 2006). In *mom1* mutants, disease resistance and pathogen-responsive *PR1* expression are enhanced but are not further enhanced by AzA (Torre *et al.*, 2023). AzA treatment was shown to reduce the expression of *MOM1* in systemic tissues, suggesting that transported AzA could modulate inducible gene expression through chromatin remodelling. Surprisingly, many AzA responsive genes displayed NHP-manipulated chromatin regions (Figure 5.12), suggesting that there may be a degree of crosstalk between NHP- and AzA-mediated chromatin remodelling. JA- and β-aminobutyric acid (BABA)- mediated priming are also associated with epigenetics (Wilkinson *et al.*, 2023; Stevens *et al.*, 2024), revealing that chromatin organisation is important across several types of systemic signalling. Mobile signal specificity could even be provided by signal specific chromatin remodellers. For example, HDA19 and MOM1 may target different groups of genes or induce different histone modifications. Crosstalk between signals could be synergistic, with NHP contributing to AzA-mediated priming to amplify the response. It is also possible that crosstalk could promote signal primacy, where NHP overrides chromatin modifications induced by other signals to replace epigenetic priming against necrotrophs with epigenetic priming against biotrophs.

Future study into the interactions between NHP and other signals such as AzA, JA, ET and ABA could provide vital insights into the breadth and function of NHP signalling. This study details a simple and replicable framework to study priming that could easily be adapted to study NHP-mediated priming in genes responding to other phytohormones. Given the differences in AzA- and NHP-mediated priming, it is likely that several pathways contribute to priming and mobile signals prime multiple signalling pathways (Figure 6.2). Moreover, knowledge of the type and nature of chromatin modifications induced after NHP or AzA treatment could be developed by ChIP-seq with modification-specific antibodies. These data would expand on the ATAC-seq, identifying not just whether chromatin is accessible, but if different modifications are associated with gene priming or reverse priming, and giving insight into other chromatin remodellers that may regulate AzA- and NHP-mediated priming.

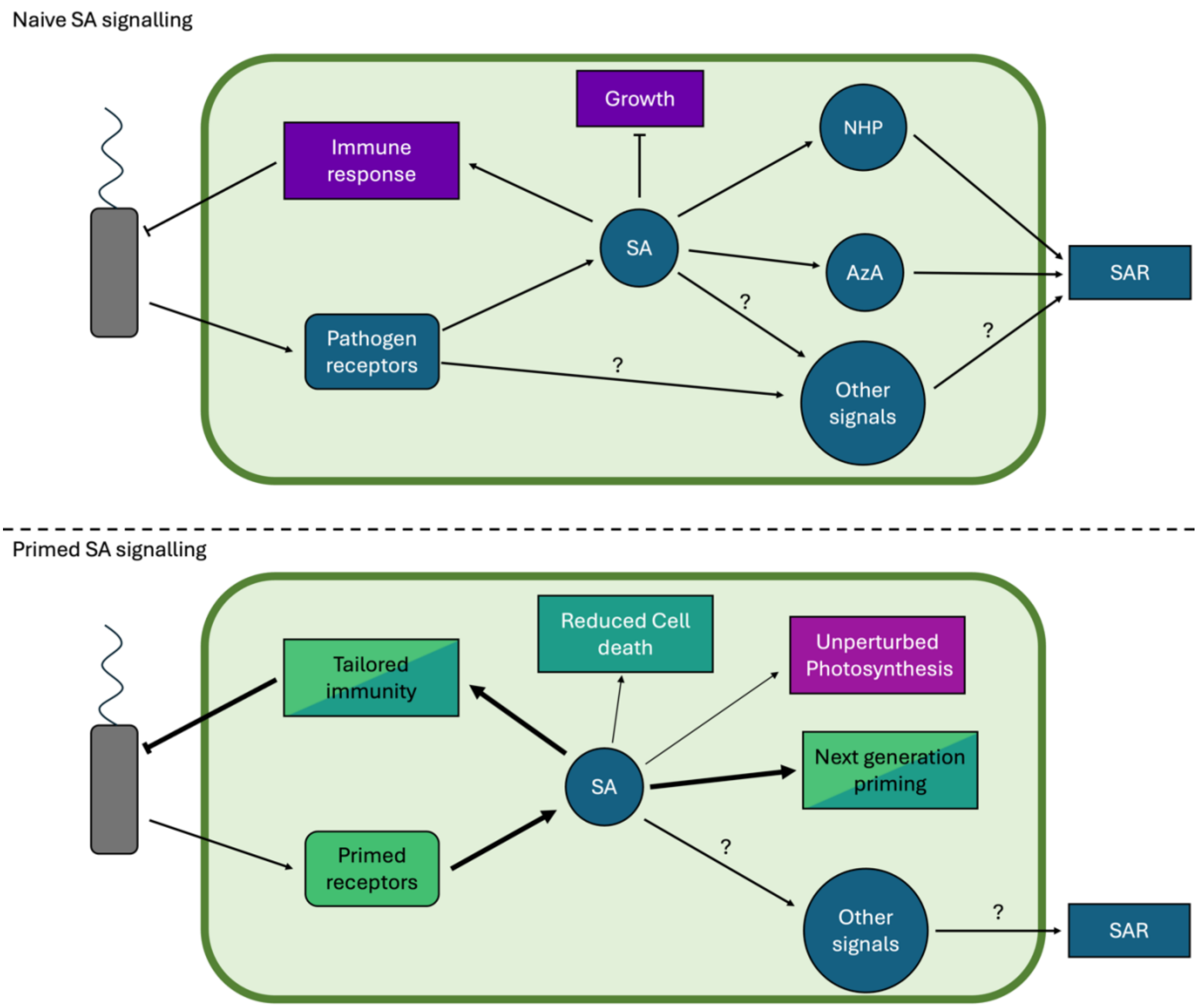


Figure 6.2 – Schematic diagram of priming by mobile signals. *In Naïve cells, pathogen perception triggers SA signalling, which launches and immune response at the expense of growth. NHP and AzA are produced by SA signalling, and other mobile signals are produced either directly by SA or through other immune signalling pathways. The cocktail of mobile immune signals primes SA signalling in systemic tissues. When a primed response is launched, increased receptor density increases the speed and strength of SA signalling, while epigenetic modifications tailor downstream SA signalling to maximise the response. Tailoring of SA signalling also reduces cell death responses and photosynthesis suppression to maintain growth. SA signalling contributes to the establishment of transgenerational priming, and may produce more mobile signals to pass on the priming response to other tissues.*

6.4 – From gene priming to agriculture

The value of the research in this thesis is in its potential for application in agriculture. Plant pathogens cause devastating yield losses annually and disproportionately in the developing world. Current development of control measures, such as fungicides and pesticides, are not capable of keeping up with pathogen evolution (Savary *et al.*, 2019; Hawkins *et al.*, 2018). However, a promising approach to pathogen control is to harness and enhance the plant's own immune system, as this evolves with pathogenic threats and is not directly cytotoxic. Several immune elicitors have been developed and tested for use in agriculture, but due to yield tradeoffs and inconsistencies, they lack commercial popularity compared to chemical control measures (Yassin *et al.*, 2021). Phloem mobile signals contribute to systemic priming, a response that enhances immunity without growth trade off, and that remains relatively consistent in field conditions (Gozzo and Faoro, 2013). Mobile signals such as NHP could be developed into synthetic analogues that are efficient to make and long lasting in storage, allowing them to be routinely applied to crops to enhance immunity without drawbacks.

However, before these signals can be applied to agriculture, we need to understand the exact impact of their use to avoid ecological or agricultural side effects. A vital side effect to avoid is reduction in yield. While previous elicitors act by pre-emptively activating immune responses, NHP primes inducible immunity (Heil *et al.*, 2000; Yildiz *et al.*, 2021), so will not expend energy unless a pathogen is encountered. Here, I present analysis of the extent of SA-responsive genes primed by NHP, from which potentially problematic genes could be identified, and demonstrate that the mechanism is likely epigenetic, which is less likely to impact long term yield. Another important consideration is the role of AzA. AzA alone does not appear to prime immunity (Figure 3.8; Zoeller *et al.*, 2012), so it seems not to be a suitable elicitor on its own. However, this study finds that AzA primarily acts to suppress the side effects of SA signalling, possibly in tandem with other signals that enhance SA signalling. Therefore, when this research is used to develop commercial elicitors, possible side effects could be limited by adding AzA to the formulation to reduce the undesirable effects from boosted SA signalling primed by NHP analogues.

The other potential use of this research is in informing targets for gene editing. Developing and applying immune priming sprays may be effective, but could prove crude due to the breadth of NHP signalling pathways and targets (Yildiz *et al.*, 2021; Foret *et al.*, 2025; Figures 4.13 and 5.6). However, by identifying regulators of a portion of NHP-mediated priming, precision gene editing technologies could be used to develop crop cultivars in a pre-primed state. This comes with the added benefit of reducing agrochemical production and treatment, which may be a cause of environmental pollution (Zhang, 2018). With the discovery that WRKY38/62 regulate NHP-mediated priming but not local immunity, this study may have identified ideal candidates for gene editing. While it has not yet been confirmed, in Chapter 5, I propose that WRKY38/62 could be pioneer TFs, loosening chromatin without inducing gene expression. Therefore, overexpression of WRKY38/62 could be used to induce a primed state in crops by increasing chromatin accessibility without the need for prior pathogen or stress exposure. Furthermore, precise editing in promoter binding sites could alter the targets of WRKY38/62, either increasing or reducing their agrochemical- or stress-responsiveness. This would enable fine tuning of a priming response to activate specific pathways and not undesirable ones. Altogether, there are several approaches that could be taken to translate this research into field applications, all of them promising and flexible enough to meet the evolving demands of modern agriculture.

6.5 – Conclusion

This thesis takes great strides towards understanding the role of phloem mobile signals in systemic immune signalling and the mechanisms of systemic priming. First, I demonstrate that two mobile signals, AzA and NHP, manipulate systemic SA signalling, with each contributing a unique role to fine-tune primed immune responses and differentiate them from local. In particular I show that NHP modulates the SA-responsive transcriptome, and potentiates SA-responsive immune responses. I then discover a dedicated set of NHP signalling TFs, WRKY38 and WRKY62, which are required for NHP-mediated priming. Finally, I show for the first time that NHP and WRKY38/62 induce large scale epigenetic remodelling to manipulate chromatin accessibility at thousands of sites. However, while many NHP-primed genes display NHP-responsive chromatin remodelling, there are more regions in the genome with NHP-modulated chromatin that do not respond to NHP and/or SA. Therefore, I conclude that NHP may also prime other immune pathways to further fine tune immune responses during SAR. Overall, this thesis details previously unknown pathways of priming by phloem mobile signals and suggests unique roles for each signal. These data have strong potential for application in agricultural settings to either chemically or genetically enhance innate immune responsiveness in crops, strengthening food security across the globe.

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Appendix A

Table A.1 – Primers used for RT-qPCR in this study

Name	±	Sequence (5’-3’)
UBQ5	F	CCAAGCCGAAGAAGATCAAG
	R	ACTCCTTCCTCAAACGCTGA
PR1	F	CTAAGGGTTCACAACCAGGC
	R	AAGGCCCACCAGAGTGTATG
WRKY38	F	CCGGTTTACCGAACCACTTA
	R	GGCTTTCCTTCTCCTGATCC
WRKY62	F	GCCTACACCAAGGACCAGAA
	R	AGAGGTGGAGGAGGAGAAGC
NPR1/NPR1-GFP	F	TCCACATCGAAATCAACCG
	R	GTCCAGCTCGACCAGGATG

Table A.2 – Transposon specific primers used in genotyping

Name	Sequence (5’-3’)
LBb1.3	ATTTTGCCGATTTCGGAAC
LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
Spm32	TACGAATAAGAGCGTCCATTTTAGAGTGA

Appendix B

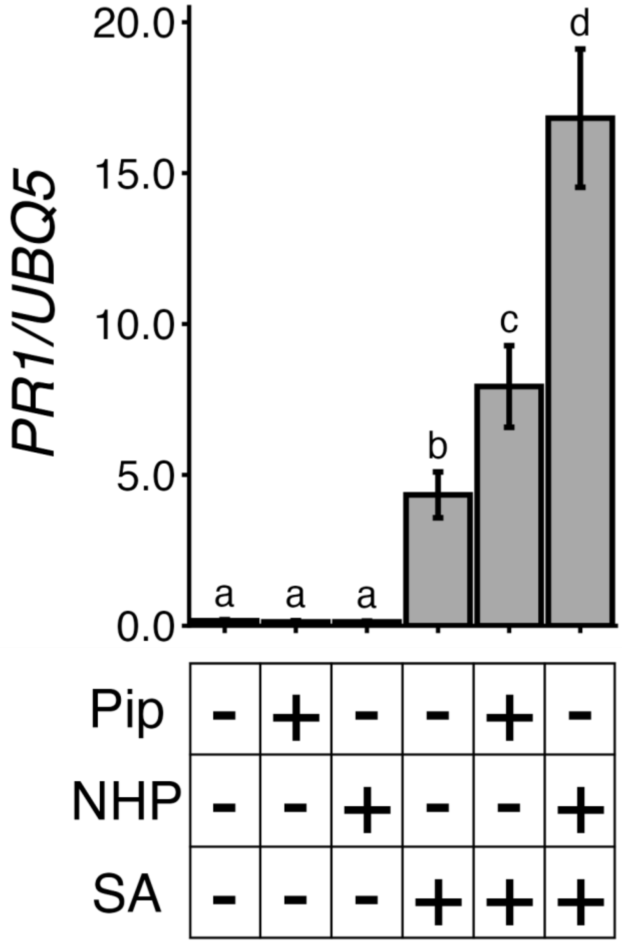

Figure B – Pip and NHP prime SA-responsive gene expression. *Fourteen-day-old wild-type seedlings were treated by immersion in water, 1 mM Pip or 1 mM NHP for 18 hours, followed by treatment with water or 0.5 mM SA for 6 hours. Total RNA was extracted and the expression PR1 measured by qPCR. Reporter genes were normalised against constitutively expressed UBQ5. Letters denote statistically significant differences between samples (Tukey ANOVA; α = 0.05, n = 3).*

Appendix C

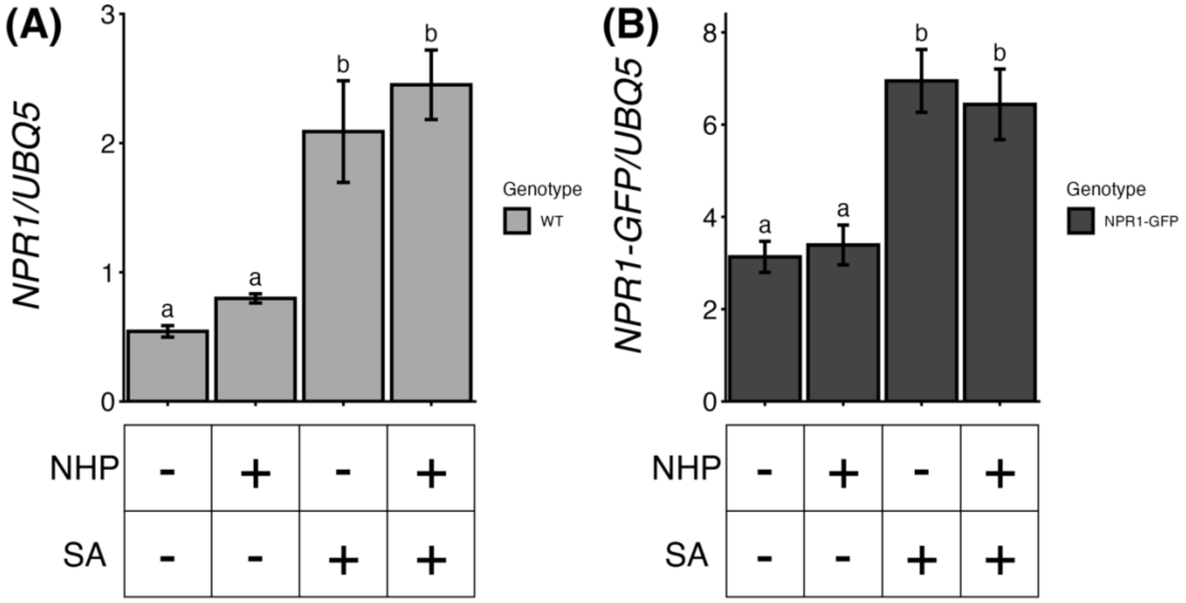

Figure C – NHP does not induce or prime SA-responsive expression of NPR1 or 35S::NPR1-GFP. *Fourteen-day-old wild-type **(A)** or 35S::NPR1-GFP **(B)** seedlings were treated by immersion in water, or 1 mM NHP for 18 hours, followed by treatment with water or 0.5 mM SA for 6 hours. Total RNA was extracted and the expression NPR1 **(A)** or NPR1-GFP **(B)** measured by qPCR. Reporter genes were normalised against constitutively expressed UBQ5. Letters denote statistically significant differences between samples (Tukey ANOVA; α = 0.05, n = 3).*

Appendix D

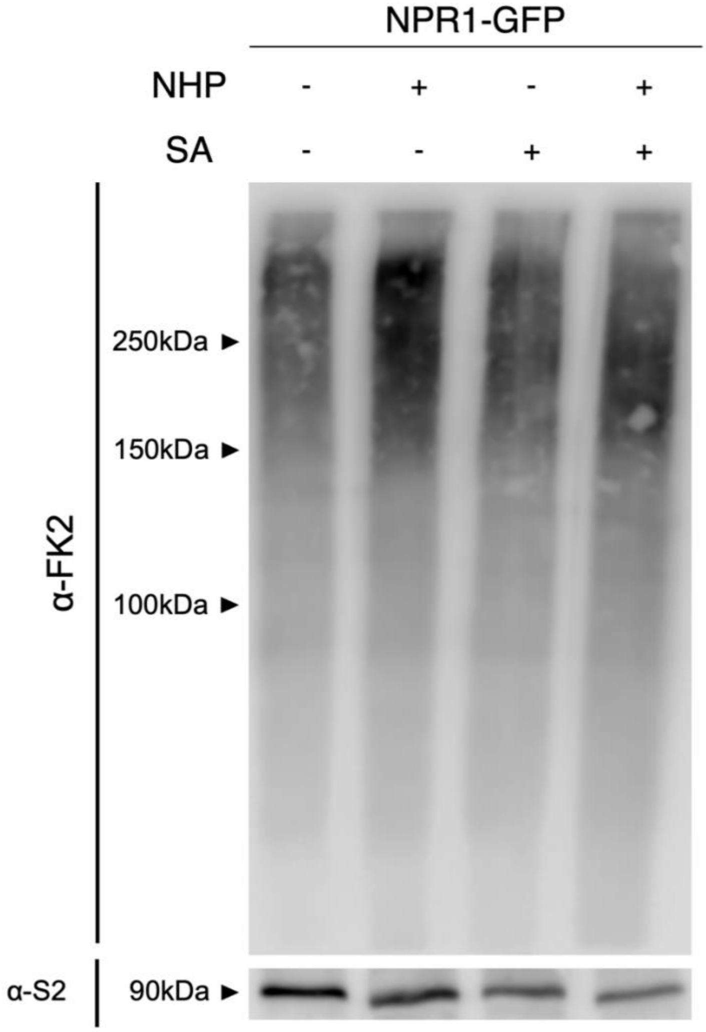

Figure D – NHP induces global changes in ubiquitin levels. *Fourteen-day-old 35S::NPR1-GFP (npr1-1 background) seedlings were treated by immersion in water or 1 mM NHP for 18 hours, followed by treatment with water or 0.5 mM SA, supplemented with proteasome inhibitor MG132 for 6 hours. Ubiquitinated proteins were pulled down with TUBEs, and ubiquitinated total ubiquitinated proteins visualised by western blotting with an α-FK2 antibody. Equal loading of protein was assessed using an anti-proteasome (S2) antibody.*