

UNIVERSITY OF BIRMINGHAM

The epigenetic control of the establishment and transmission of long-term priming in tomato

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A thesis submitted to the University of Birmingham for the degree of DOCTOR OF PHILOSOPHY

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December 2023

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Abstract

Tomato (*Solanum lycopersicum)* is a major crop world-wide however its production is heavily limited by fungal pathogens such as *Botrytis cinerea*. Due to the toxicity of postharvest pesticide application, alternative control methods such as priming are being investigated. Here, it is demonstrated that priming with the broad acting elicitor β -amino butyric acid (BABA) results in long-lasting induced resistance (IR) in fruit and progeny of tomato plants only when initiated at an early developmental stage. Whole genome bisulfite sequencing analysis revealed seedling-specific changes, which occurred in the context of lower global CHH methylation levels. Large differences were observed in the transcriptome and methylome following early and late BABA-treatments suggesting a greater epigenetic imprinting capacity of young plants. Importantly, grafting experiments demonstrated that BABA-IR is transmissible from primed rootstocks to unprimed scions. In these resistant scions, a subset of mobile 24 nt small RNAs associated with genes were identified which display an enhanced expression in response to *B. cinerea* infection in fruit. These results suggest a functional association of a systemic signal with long-lasting IR in tomato. Additionally, investigations were conducted on the ability of strawberry seedlings (*Fragaria* x *ananassa*) to be chemically primed against *B. cinerea*, with large phenotypic differences seen in strawberry compared to tomato. This PhD therefore addresses current knowledge gaps on the long-lasting nature of priming including its maintenance and transmission. Elicitor screenings have investigated the influence of developmental stage on the plant's imprinting capacity and stress memory formation. Through use of integrated omics approaches, (epi)genetic markers of long-lasting priming in tomato have been identified which may also serve as targets for durable resistance in other crops.

Statement regarding published work

Large amounts of the introduction, results and discussion of chapters 3 and 4 have been published as a paper currently under review which is available as a pre-print (https://doi.org/10.1101/2023.10.09.561512). Appendix III contains a published paper in Quantitative Plant Biology titled “Data science approaches provide a roadmap to understanding the role of abscisic acid in defence”. The authors of this work declare the content of this thesis belongs solely to Katie Stevens who is the first author of these publications. First author Katie Stevens contributed to these publications by designing experimental analysis and pipelines, conducting all experiments, collecting data, performing sequencing and statistical analysis, and writing and editing the manuscripts. The senior author Dr Estrella Luna Diez contributed to this work providing feedback during the writing process and making corrections on the manuscripts.

Katie Stevens



Dr Estrella Luna Diez



Acknowledgments

Thank you firstly to my supervisor Dr Estrella Luna Diez for selecting me as your first PhD student in Birmingham. I am extremely grateful to you for setting the foundations for such a fantastic, challenging research project and providing me with so many opportunities to develop as a well-rounded researcher. Thank you to my second supervisor Dr Marco Catoni for your time and guidance with analysis, and importantly your invaluable research suggestions which helped shape this project.

The work and publication on abscisic acid and machine learning would not have been possible without Professor Iain Johnson, I am very grateful for your help and guidance on this project. Thank you to Dr Mike Roberts for your helpful inputs on both this PhD project and publications.

I would like to thank my colleagues in the plant science department and the members of S202/S200 for their shared knowledge, advice, and friendship. Thank you to Mark and Rosa for your support in and out of the lab over the past four years. Finally, a special thankyou to Hannah who I cannot imagine doing this PhD without.

Thank you to my friends and family. Thanks Beth, Yolanda, Elliot, Ashley, and my sister Emma for your constant support and love. Thank you to the bucas for the shared miles and pub trips who made the last few years so much better. Finally, to my mum and dad for providing me with the opportunity to wholeheartedly pursue my interests. I would not have managed without your love and encouragement.

Thank you to the BBSRC for funding this PhD through the MIBTP and to Saturn Bioponics for supporting this project. I am grateful for the funding and resources to complete this project as well as the extension of this studentship due to the Covid-19 pandemic.

Declaration

I declare that this thesis is entirely my own work with the exception of the following:

- The amino acid extractions and phytohormone analysis experiments in Fig 25 and Fig 26 were conducted at University Jaume by Dr Victoria Pastor an expert collaborator in metabolomic analysis and Dr Estrella Luna Diez.

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List of abbreviations

5-AzaC = 5-azacitidine

AABA = α-aminobutryic acid

ABA = Abscisic acid

ACRE = Avr9/Cf-9 rapidly elicited gene

AGO = ARGONAUTE

AMF = Arbuscular mycorrhiza fungi

AOC = ALLENE OXIDE CYCLASE

AP = Aspartyl protease

AsPRS = Aspartyl-tRNA synthetase

ATAC-seq = Transposon accessibility-based assays

AzA = Azealic acid

BABA = β-aminobutyric acid

bp = Base pairs

BTH = Benzothiadiazole

CAPE1 = Cap derived peptide 1

CDIP = cell-death inducing protein

CK = Cytokinin

CMT - CHROMOMETHYLASE

COI1 = CORONATINE-INSENSITIVE1

COPII = Coat protein complex II

CWDE = cell wall degrading enzyme

DCL = DICER-LIKE

DE = Differentially expressed

DEFRA = Government Department for Environment, Food and Rural Affairs

DEG = Differentially expressed gene

DML = DEMETER-LIKE

DMR = Differentially methylated region

DMSO = Dimethylsulfoxide

dpi = Days post inoculation

DRM2 = DOMAINS REARRANGED METHYLTRANSFERASE 2

DSI = Disease severity index

dsRNA = Double stranded RNA

epiRILs = Epigenetic recombinant inbred lines

ER = Endoplasmic reticulum

ET = Ethylene

ETI = Effector triggered immunity

EU = European Union

FAIRE - Formaldehyde-Assisted Isolation of Regulatory Elements

FDR - False discovery rate

FLC = FLOWERING LOCUS C

FPKM = Fragment per million mapped reads

FW = Fresh weight

GA = Gibberellin

GABA = y-aminobutyric acid

GM = Genetically modified

GO = Gene ontology

H3K27me3 = Trimethylation of lysine 27 residue of histone 3

H3k4me2 = Dimethylation of lysine 4 residue of histone 3

H3K4me3 = Trimethylation of lysine 4 residue of histone 3

H3K9ac = Acetylation of Lys 9 residue of histone 3

Hpa = *Hyaloperonospora arabidopsidis*

HPLC = High performance liquid chromatography

HR = Hypersensitive response

HSP = Heat shock protein

Hx = Hexanoic acid

I3CA = Indole-3-carboxylic acid

IAA = Indole-3-acetic acid

IAMPs = Insect-associated molecular patterns

IAR = Intergenerational-acquired resistance

IBI1 = Impaired in BABA-induced Immunity 1

IC = Isochorismate

INA = Dichloroisonicotinic acid

IR = Induced resistance

ISR = Induced systemic resistance

JA = Jasmonic acid

JA-Ile = JA-isoleucine

JAZ = Jasmonate ZIM-domain

kb = Kilobase

KYP = KRYPTONITE

LHT1 = LYSINE HISTIDINE TRANSPORTER 1

LOX = LIPOXYGENASE

LTR = Long-terminal repeat

MAPK = Mitogen activated protein kinase

MeJA = Methyl jasmonate

MeSA = Methyl-salicylate

MET = METHYLTRANSFERASE

miRNA = Micro-RNA

MS = Mass spectrometry

NB-LRR = Nucleotide binding – leucine rich repeat

NPR1 = NON-EXPRESSOR OF PROTEIN 1

nt = Nucleotide

OPDA - 12-oxo-10,15(Z)-phytodienoic acid

PAL = PHENYLALANINE AMMONIA-LYASE

PAMP = Pathogen associated molecular pattern

PFHA = Perfluoroheptanoic acid

PHL = Postharvest loss

ppb = Parts per billion

PR = PATHOGENESIS RELATED

PRR = Pathogen recognition receptor

PTGS = Post-transcriptional gene silencing

PTI = Pathogen triggered immunity

PTM = Post translational modification

R gene = Resistance gene

RBH = R-β-homoserine

RdDM = RNA-dependent DNA methylation

RGR = Relative growth rate

RNAi = RNA interference

ROS = Reactive oxygen species

ROS1 = Repressor of silencing1

rpm = Revolutions per minute

SA = Salicylic acid

SAR = Systemic acquired resistance

SIGS = Spray induced gene silencing

SIMR = Stress-induced morphogenic response

siRNA = Small interfering RNA

sRNA = Small RNA

ssRNA = Single stranded RNA

TAR = Trangenerational acquired resistance

TAL = Transcription activator like

TE = Transposable element

TF = Transcription factor

TMV = Tobacco mosaic virus

TQD = Triple quadrupole mass spectrometer

UPLC = Ultra-performance liquid chromatography

VOZ = Vascular Plant One Zinc Finger

WGBS = Whole-genome bisulfite sequencing

WHY1 = DNA-binding protein WHIRL

Chapter 1:

General Introduction

1.1 The fight against global postharvest loss

Climate change presents a major global issue; more frequent and more extreme weather events are predicted to occur, leading to increased disease pressure which will have detrimental impacts on crop yields and lead to billions of pounds of economic losses annually (Dhankher and Foyer, 2018). In addition, the global population is predicted to reach 9 billion by 2050, calorie demand is subsequently expected to increase by at least 60% (Fao, 2008, Kader, 2005). A second 'green revolution' is therefore required to ensure global food security in the face of our current climate crisis. Current food production however is heavily constrained by plant diseases caused by pathogens and pests. It is estimated that 44% of all global food loss is due to postharvest losses (PHL), this is likely to be exacerbated with the more extreme weather conditions predicted to occur globally (Lipinski, 2013). The FAO defines PHL as measurable qualitative and quantitative food loss along the supply chain, starting at the time of harvest until consumption or end use (De Lucia, 1994). Losses can therefore occur at any stage of the supply chain such as during harvesting, in postharvest storage, during packaging, in distribution or as consumer waste (Hodges et al., 2011). All of these losses can reduce both the quantity and quality of food and subsequently the income of farmers and food producers (Stathers et al., 2020). The reduction of PHL is being recognised as a valuable solution to the food security crisis and targets have been set by the United Nations to reduce food losses across the global supply chain (UnitedNations, 2015).

There are many factors which can contribute to PHL such as bruising, wilting and injury, however crop loss due to pathogenic fungi is the major driver (Zhang et al., 2021). The fungal pathogens that lead to the greatest PHL include *Botrytis cinerea, Alternatia alternata, Fusarium* spp, *Rhizopus stolonifer,* and *Penicillium* spp. Fruit and vegetables are particularly susceptible to PHL due to their high moisture content and low pH levels but are essential for adequate nutrition in human diets (Tripathi and Dubey, 2004). It is estimated that about 30% of fruit is lost annually worldwide due to PHL (OECD, 2014). Unfortunately, the prevalence of PHL is expected to increase in response to the predicted climatic changes of hotter, wetter

weather and increased carbon dioxide levels (Siddiqui et al., 2015). For example, higher temperatures lead to faster photosynthetic rates, reduced firmness in fruit and subsequently increased vulnerability to pathogens (Srivastava, 2019).

1.2 *Botrytis cinerea* and its current control

B. cinerea, commonly known as grey mould, is a necrotrophic fungal pathogen that can affect over 1000 plant species including economically important crops such as potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), and berries. *B. cinerea* therefore leads to huge economic losses of up to €100 billion per year, it is considered the second most important plant fungal pathogen globally (Dean et al., 2012). Plants possess pre-formed barriers against fungal penetration, thus *B. cinerea* typically enters through host openings such as wounds or plant stomata. Alternatively, *B. cinerea* can utilise cell wall degrading enzymes (CWDEs) such as pectin methylesterase 1 to facilitate the infection process (Cheung et al., 2020). Following penetration, *B. cinerea* must fight constitutively possessed phytoanticipins and mycotoxic metabolites such as α -tomatine and camalexin. Once inside the plant, *B. cinerea* releases phytotoxic secondary metabolites such as botrytial, botcinin and oxalic acid as well as cell-death inducing proteins (CDIPs). Furthermore, *B. cinerea* has been demonstrated to hijack host metabolism where it can regulate cell death through its transcription factors Lsd1 and Lol1 as well as induce effector-triggered hypersensitive response via Sgt1 (Bi et al., 2023). *B. cinerea* can therefore cause damage at both the pre- and postharvest stage. *B. cinerea* fails to develop in unripe fruit such as tomatoes, it can consequently initiate ethylene (ET) production to accelerate ripening and the infection process. Additionally, *B. cinerea* infections can be latent until optimal conditions of high humidity and temperature are met, thus increasing the difficulty of detection and effective control (Romanazzi et al., 2016).

The most common approach against postharvest fungal infections such as *B. cinerea* is the use of chemical treatments which are typically applied by dips or sprays. A 1994 study estimated that 23 million kg of fungicides are applied to fruit and vegetables every year (Ragsdale and Sisler, 1994). Whilst the use of these chemicals has played a vital role for increasing global food production, they are frequently associated with toxicity, environmental pollution, and potentially dangerous side-effects in humans (Zikankuba et al., 2019). Furthermore, resistance against chemical treatments can develop quickly, leading to the need for repeated application (Tripathi and Dubey, 2004). Over the past decade there has been an increasing consensus that the use of and reliance on chemical treatments must be reduced. Extensive chemical control methods are growing out of favour and are heavily legislated against in the European Union (EU) (Handford et al., 2015, Hillocks, 2012). There is consequently an increasing demand to discover and incorporate the use of alternative sustainable crop protection strategies. Such strategies must not incur major fitness costs to the crop as it is essential to maintain both high yield and quality. The UK Government's Department for Environment, Food and Rural Affairs' (DEFRA) is therefore aiming to transform UK farming practices to improve production and are injecting up to £90 million of funding through UKRI (UKRI, 2022). As a result of the UK's departure from the EU, there are potentially new opportunities to incorporate innovative technologies into horticultural production, previously not practiced within the EU.

1.3 Plant innate immunity

A potential strategy for improving food security is through the manipulation and direct targeting of the immune system of crops. Greater understanding of the fundamental mechanisms behind plant-pathogen responses in model plant systems can therefore contribute to a more sustainable food production. The higher plant immune system is complex and consists of two layers of immunity; basal, innate immunity and systemic acquired resistance (SAR). Basal immunity involves two well characterised branches; pathogen associated molecular pattern

(PAMP) triggered immunity (PTI) and effector triggered immunity (ETI) (Zhang and Zhou, 2010). Plants display PTI upon recognition of pathogen signatures by pathogen recognition receptors (PRRs) which leads to enhanced defence responses (Felix et al., 1999). Plant PTI induced responses include stomatal closure, callose deposition, rapid bursts of reactive oxygen species (ROS) and mitogen-activated protein kinase (MAPK) cascades (Bigeard et al., 2015). ROS bursts function in plant immunity through both direct toxicity towards pathogens and initiating signalling cascades with downstream defence components such as phytohormones (Mengiste, 2011).

If plants have successfully defended themselves against this initial attack, pathogens release effector molecules in an attempt to silence the hosts defences. Pathogen effector molecules are recognised by nucleotide binding leucine rich repeat (NB-LRR) proteins which are encoded by plant resistance genes (*R* genes*)* (McHale et al., 2006). NB-LRRs are highly specific and have co-evolved to detect pathogen effector molecules, this is classically described as the 'gene for gene model' (Flor, 1971). The first isolated race-specific *R* gene was the tomato *Pto* gene which provides resistance to *Pseudomonas syringae* pv *tomato* races carrying the *avrPto* gene (Ronald et al., 1992). NB-LRR activation therefore leads to ETI, which induces a stronger level of defence throughout the plant via metabolic and hormonal changes. ETI typically results in programmed cell death known as a hypersensitive response (HR) (Dangl and Jones, 2001). This well characterised system of basal immunity was first illustrated with the influential 'zig-zag' model (Fig 1) (Jones and Dangl, 2006). In the past decade the current understanding of both PTI and ETI have advanced, for example the influence of environmental conditions has been demonstrated to be highly influential to this model and plant-pathogen specificities have been identified (Pritchard and Birch, 2014). Importantly, the main branches of PTI and ETI have been revealed to display complex mutual interactions with multiple ETI responses such as the HR co-regulated by PTI in a NB-LRR - specific manner as well as the up-regulation of PRR signalling components during ETI (Yuan et al., 2021).

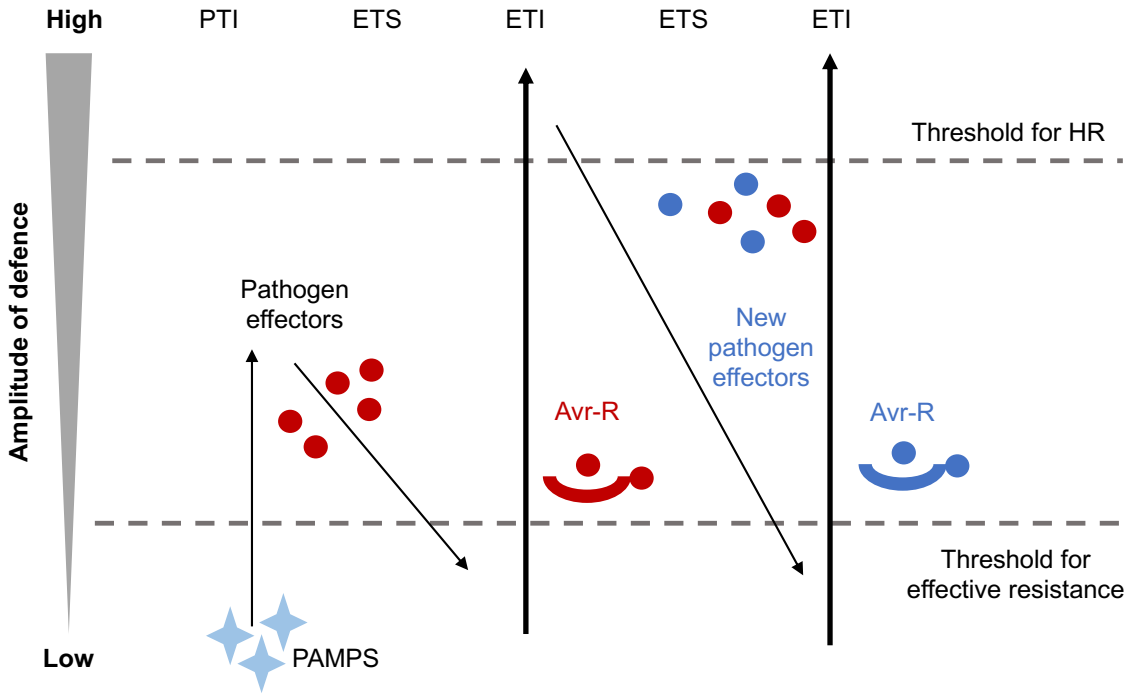


Figure 1: The 'zig zag' model of plant innate immunity. Recognition of pathogen associated molecular patterns (PAMPS) (blue stars) leads to pathogen triggered immunity (PTI). Pathogens deliver effectors (red circles) which induces effector triggered susceptibility (ETS). Recognition of effectors by an NB-LRR protein (Avr-R) activates effector triggered immunity (ETI), an increased amplitude of defence leads to a hypersensitive response (HR). New effector molecules (blue) are released repeating this process. Figure recreated from Jones and Dangl 2006.

1.4 Plant hormones

A variety of plant hormones have been identified as essential components of the innate defence response as well as SAR. The primary hormones involved in plant biotic defence signalling are salicylic acid (SA), jasmonic acid (JA) and ET. The use of both mutants and external application of these hormones has helped reveal their complex roles in plant defence signalling. Jasmonates are derived from oxylipins and consist of JA and its derivatives methyl jasmonate (MeJA) and JA-isoleucine (JA-Ile). They have a role in many key physiological plant processes such as stomatal opening and flower development but are most well characterised for their essential role in plant defence against necrotrophic pathogens such as *B. cinerea* and

Fusarium oxysporum (Acharya and Assmann, 2009, Grant and Lamb, 2006, Ishiguro et al., 2001). The production of JA and JA-Ile is typically triggered by accumulation of systemin, an 18 amino acid polypeptide derived from the precursor prosystemin (Ruan et al., 2019). Recognition of JA-Ile by its receptor CORONATINE-INSENSITIVE1 (COI1) leads to degradation of jasmonate ZIM-domain (JAZ) proteins, which act as repressors. This subsequently releases downstream signalling molecules such as WRKY, MYC and NAC transcription factors (TFs) (Ruan et al., 2019). In addition, JA is importantly associated with defence against herbivorous insects and mechanical wounding, for example *coi1* mutants display enhanced susceptibility against insect attack (Verhage et al., 2011).

SA is a beta-hydroxy phenolic acid with an essential role in plant defence responses. It is produced via two independent pathways: the phenylalanine ammonia-lyase (PAL) pathway and the isochorismate (IC) pathway. SA is known to play a critical role in the regulation of cell death and is primarily associated with defence against biotrophic and hemi-biotrophic pathogens (Bari and Jones, 2009). SA is both necessary and sufficient for the induced resistance SAR response, during which both localised and systemic levels of SA increase (Grant and Lamb, 2006). SA signalling is regulated through NON-EXPRESSOR OF *PATHOGENESIS RELATED* (*PR)* GENES 1 (NPR1). NPR1 interacts with TGA TFs to activate SA-responsive *PR* genes (Durrant and Dong, 2004). *npr1* mutant plants have been shown to display enhanced disease susceptibility with altered *PR* gene expression, whilst overexpression of *NPR1* has been shown to confer broad spectrum resistance to fungi in *Brassica juneca* (Ali et al., 2017, Glazebrook et al., 1996). JA and ET are both known to be antagonised by SA leading to a disruption of plant defence responses against necrotrophic pathogens and herbivore attacks (Anderson et al., 2004, Audenaert et al., 2002, Glazebrook et al., 1996, Mur et al., 2006). For example, in Arabidopsis the TF WRKY70 activates SA-responsive genes and represses JA-responsive genes (Caarls et al., 2015). The importance of this hormone balance in plants is further emphasised by the ability of selected pathogens to mimic and manipulate endogenous hormone levels. For instance, *P. syringae* produces

coronatine, a JA-signalling mimic, in order to disrupt SA-mediated defences. This infection strategy is also seen in *B. cinerea* which produces both abscisic acid (ABA) (De Torres Zabala et al., 2009) and cytokinin (CK) (Robert-Seilaniantz et al., 2011). The balance of hormone cross talk is therefore critical for prioritisation of defence signalling pathways and for plants to ensure the correct resistance response is activated.

Finally, ABA is an essential plant hormone involved in a wide range of physiological processes including both biotic and abiotic stress signalling (Lee and Luan, 2012). ABA is most generally known for its role in water stress responses, with endogenous levels increasing during drought (Finkelstein et al., 2002). ABA is considered to have a positive function in early defence responses due to its essential role in stomatal closure, however its role in later infection stages is controversial. ABA is known to antagonise the major defence pathways of SA, JA, and ET through hormone cross talk, typically leading to an increase in disease susceptibility (Anderson et al., 2004, Audenaert et al., 2002). The effect of ABA however is not clearly associated with a specific plant species or pathogen lifestyle (Asselbergh et al., 2008b), and contradictions are seen even within individual plant-pathosystems (Melotto et al., 2006, Mohr and Cahill, 2007).

1.5 Induced resistance (IR)

Whilst plants do not have an adaptive immune system as seen in mammals, induced resistance (IR) allows plants to respond to environmental stimuli and develop enhanced defence capacities through phenotypic plasticity. IR can be triggered by a variety of signals such as recognition of a pathogen or herbivorous insect, treatment of a chemical, or root colonisation by microbes (Boller and Felix, 2009, Hilker and Fatouros, 2015, Oostendorp et al., 2001). The two main examples of IR are SAR and induced systemic resistance (ISR). IR importantly provides the benefit of transmitting the initial defence signal beyond the local site of recognition, thus leading to enhanced resistance systemically across the entire plant.

SAR was first demonstrated experimentally in 1961 when inoculation of half of a tobacco leaf with tobacco mosaic virus (TMV) led to enhanced resistance in distal leaves when faced with a later challenge (Ross, 1961). Since this discovery, SAR has been recognised in a wide range of species and is known to prompt large transcriptional changes which are NPR1 dependent. A range of abiotic stimuli have been shown to trigger SAR including SA, β-aminobutyric acid (BABA) and benzothiadiazole (BTH), a functional analogue of SA. SAR is typically characterised by upregulation of *PR1* and increased levels of SA (Fu and Dong, 2013). Several mobile signalling molecules have been identified as transmitters of SAR including methyl-salicylate (MeSA) and azealic acid (AzA) (Durrant and Dong, 2004), however there remains a current lack of consensus on if they have a shared pathway and how these signals are perceived in systemic tissues (Fu and Dong, 2013).

IR can additionally be triggered by herbivore-associated stimuli such as insect oral secretions and insect-associated molecular patterns (IAMPs) at the site of tissue injury (De Vos et al., 2006). These triggers can induce a faster, more efficient defence response in attacked plants. Herbivore-IR signalling is associated with jasmonate responses with JA mutants displaying enhanced susceptibility to herbivores (Pieterse et al., 2014). Additionally, MeJA treatment has experimentally been shown to enhance tomato responses against herbivores through increased trichome production (Boughton et al., 2005). Finally, ISR was identified in 1991 by three independent research groups demonstrating that plant growth promoting rhizobacteria such as *Pseudomonas florescens* and *Trichoderma* spp lead to enhanced resistance (Alstr et al., 1991, Van Peer et al., 1991, Wei et al., 1991). This phenomenon has since been identified in hundreds of plant species (Pieterse et al., 2014). ISR is often regulated by SA-independent mechanisms, with JA and ET signalling mutants proving to be defective in *P. fluorescens* ISR (Knoester et al., 1999, Pieterse et al., 1998, Pozo et al., 2008).

1.6 Priming

Connected yet distinct to SAR is the phenomena of priming. Priming involves a faster and more efficient response to stress due to prior exposure to a stimulus (Fig 2). Priming can lead to increased basal resistance levels, so plants are more prepared to respond to future pathogen attacks (Conrath et al., 2006). The phenomenon of priming was first identified in parsley cell cultures 30 years ago through pre-treatment of SA, dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) leading to enhanced defence responses such as phytoalexin secretion, cell wall modifications and oxidative bursts (Kauss et al., 1992a, Kauss et al., 1992b). Priming stimuli can vary widely from pathogen or microbe derived compounds, insect derived stimuli, chemicals, or abiotic stimuli (Mauch-Mani et al., 2017). Whilst in animals memory formation is dependent on a nervous system comprised of neurons and synapses, in plants secondary regulators such as calcium, ROS and phytohormones play a critical role in the 'memory response' (Hilker and Fatouros, 2015). Priming induces physiological changes to plants which can occur at the transcriptomic, metabolic, proteomic, and epigenetic level. Such changes are often referred to as the 'priming fingerprint' and have been studied extensively in species such as Arabidopsis. Priming typically leads to broad-spectrum resistance with minimal fitness and growth costs to the plant (Conrath et al., 2002, van Hulten et al., 2006).

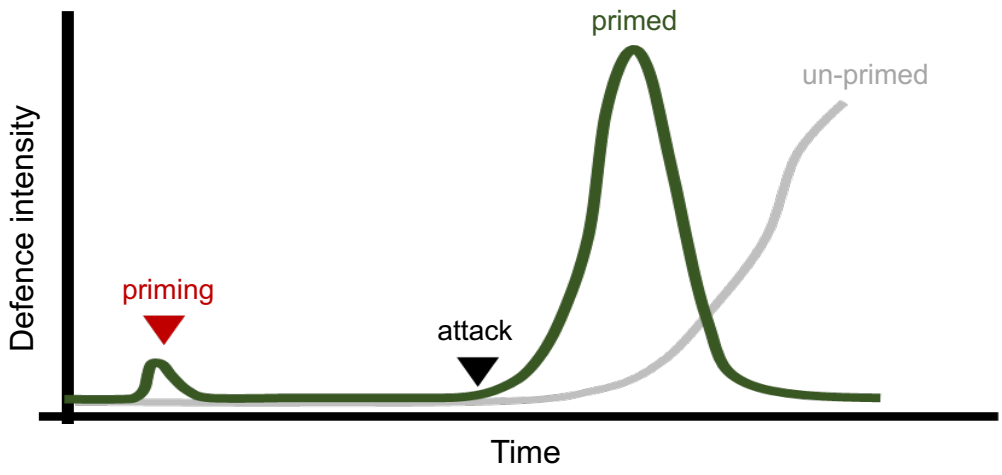


Figure 2: Priming response model. Primed plants display a faster, stronger immune response (green) against attack compared to un-primed plant (grey).

The term ‘priming’ typically relates to plants biotic memory however priming for abiotic stimuli and stresses such as hardening for cold is well characterised (Berry and Dean, 2015). The duration of memory, or priming, is typically in proportion to how long the exposure to the initial stimulus was. Memory can therefore be further characterised into short and long-term. Short term memory relies on facilitating active transcription, it is typically marked by alterations to expression of TFs regulating appropriate defence genes and short-term changes to metabolites (Hilker and Fatouros, 2015, Harris et al., 2023). Two different types of transcriptional memory responses have been described depicting different temporal responses (Fig 3). ‘Type I’ involves the sustained induction (activation or repression) of memory genes that exceeds the duration of the priming stimulus (Fig 3a). ‘Type II’ involves modified gene expression during exposure to a similar stress cue, for example an enhanced re-induction. Importantly, in-between the initial and subsequent stimulus the transcription of Type II memory genes returns to baseline levels (Fig 3b) (Oberkofler et al., 2021).

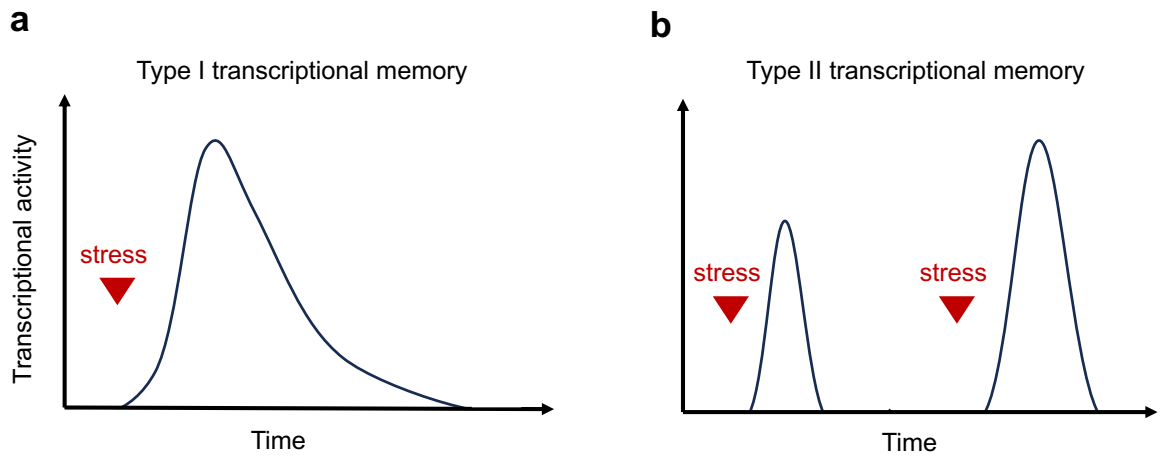


Figure 3: Transcriptional memory responses a) Type I transcriptional memory displaying sustained transcriptional activation. **b)** Type II transcriptional memory displaying modified transcriptional activity on a repeated stress exposure. Figure adapted from Oberkofler et al., 2021.

Long-term memory in contrast may involve more pervasive marks such as methylation, acetylation and ubiquitination and is typically associated with changes to chromatin status (Harris et al., 2023). These epigenetic changes can subsequently lead to transcriptional reprogramming by keeping the region in a permissive or repressive state (Bhar et al., 2021). A well characterised example of long-term abiotic memory is vernalisation, in which prolonged low temperatures in winter is 'remembered' and subsequently promotes flowering in the spring. Varying epigenetic marks to *FLOWERING LOCUS C* (*FLC*) therefore act as switches to mediate its expression between an active and repressed state (Berry and Dean, 2015). Generally, the formation of long-term memories in plants is likely to be the exception rather than the rule (Crisp et al., 2016). From an evolutionary, and resource allocation perspective it is vital that plant memories are 'forgotten' and not regularly transmitted to progeny. As each individual and generation will experience multiple, unique stresses, erasure allows the establishment of new memories. Far fewer studies have been conducted however on the erasure of plant memories and biotic stress recovery in particular.

1.7 Chemically induced priming

A variety of chemical compounds have been shown to induce pathogen resistance in plants by targeting essential defence pathways. For example, hexanoic acid (Hx) has been demonstrated to be an effective elicitor in Arabidopsis against *B. cinerea.* This resistance has been shown to be largely dependent on JA-signalling (Kravchuk et al., 2011). The importance of callose deposition in defence priming is demonstrated by priming of indole-3-carboxylic acid (I3CA), a compound identified during metabolomic analysis of BABA-primed plants. I3CA itself has therefore been shown to be an effective inducer of resistance against *Plectosphaerella cucumerina* in Arabidopsis and increases endogenous levels of ABA (Gamir et al., 2018). Spray application of Aza has been demonstrated to induce SA-dependent defences in Arabidopsis against *P. syringae* leading to enhanced SA levels and increased *PR1* expression in plants (Jung et al., 2009, Shah and Zeier, 2013). Chemical priming has been demonstrated

to be highly effective in both dicot and monocot crops, with foliar application of diproline leading to systemic IR in tobacco and rice (De Kesel et al., 2020, Wu et al., 2017). In rice, diproline has been demonstrated to provides transgenerational protection against *Meloidogyne graminicola*. This resistance is linked to Fe, ET, and nitric oxide-mediated signalling (De Kesel et al., 2023).

Tomato plants have been shown to be effectively primed by a range of elicitors through approaches including soil drench, foliar spraying, and seed treatment (Mauch-Mani et al., 2017). Hx has been shown to be effective against both *B. cinerea* and *P. syringae,* interestingly different tomato cultivars displayed cultivar specific levels of Hx-induced callose deposition and resistance (Vicedo et al., 2009). The foliar application of ChitoPlant, a water-soluble chitosan-based formulation led to IR against *B. cinerea* in leaf tissue, this was shown to be due to accumulation of JA and JA-Ile as well as priming of callose deposition (De Vega et al., 2021). Seed priming of 0.1 mM MeJA led to enhanced resistance in 4-week-old tomato seedlings against *F. oxysporum* with plants displaying elevated levels of SA through the PAL pathway (Król et al., 2015). Additionally, the use of beneficial microbes such as arbuscular mycorrhiza fungi (AMF) have been demonstrated to be highly effective in tomato. For example, inoculation with *R. irregularis* led to induced resistance against *Xanthomonas campestris* pv. *Vesicatoria.* This IR was associated with systemic changes to the expression level of genes associated with ET regulation in leaf tissue (Cervantes-Gámez et al., 2016). Inoculation with the AMF *Funneliformis mosseae* led to a substantial increase in expression of *PR1, PR2 and PR3* as well as upregulation of defence genes *LOX* (*LIPOXYGENASE*), *AOC* (*ALLENE OXIDE CYCLASE*), and *PAL* during infection with *Alternari solani.* Furthermore, use of JA mutants demonstrated this is central to AMF-IR against *A. solani* in tomato (Song et al., 2015). Thus, a broad range of effective chemical priming agents have been identified in the past decades which can target specific defence pathways to induce resistance.

1.8 BABA induced priming

A common priming stimulus studied is the 4-carbon non-protein amino acid BABA. There are other isomers of β-aminobutyric acid, including y-aminobutyric acid (GABA) and α-aminobutryic acid (AABA) however only BABA, the rarest of all three, can induce full resistance (Thevenet et al., 2017). BABA plays a role in inducing priming of defence and was first identified to induce resistance in 1963 against the oomycete pathogen *Aphanomyces euteiches* (Papavizas and Davey, 1963). Whilst BABA-induced priming and SAR are both forms of IR, BABA induced priming is distinct from SAR as it has an additional mode of action via ABA-dependent mechanisms. Furthermore, BABA-IR is highly effective against both biotrophic and necrotrophic pathogens in addition to abiotic stresses (Cohen et al., 2016). BABA is now known to involve two independent pathways: SA-dependent and SA-independent defence mechanisms (Ton and Mauch-Mani, 2004, Zimmerli et al., 2001). The SA-independent branch of BABA-IR is associated with ABA signalling and callose deposition; ABA deficient mutants *aba1-5* and *pmr4* callose mutants do not display BABA-IR against necrotrophic pathogens (Ton and Mauch-Mani, 2004). SA-dependent BABA-IR functions through the NPR1-dependent pathway (Van der Ent et al., 2009). BABA treatment has been shown to lead to an increase in defence regulatory TFs, including 21 members of the WRKY family which have a role in regulating SA-defences (Van der Ent et al., 2009). Furthermore, *npr1* mutants displayed a limited increase of these TFs further illustrating its important role in BABA-IR (Van der Ent et al., 2009).

The stereospecific nature of BABA helped the discovery of its receptor in Arabidopsis as *IMPAIRED IN BABA-INDUCED IMMUNITY 1* (*IBI1*), an aspartyl t-RNA synthetase (AspRS) (Luna et al., 2014b). BABA exists in two enantiomers, R and S, with most research utilising the racemic mixture of RS BABA to induce broad spectrum resistance. Stereomer specific differences have been reported in phytotoxicity with foliar spraying of R-BABA being more phytotoxic than S-BABA (Singh et al., 2023). BABA binds to the receptor blocking the AspRS's canonical function, priming the protein and triggering accumulation of uncharged tRNA. The

R-BABA enantiomer has been shown to display greater affinity than S-BABA to the AspRS binding domain. This leads to GCN2-dependent eIF2α phosphorylation and stress-related growth repression (Luna et al., 2014b) (Fig 4). In an unstressed state IBI1 is associated with the endoplasmic reticulum (ER). Pathogen infection however leads to IBI1 translocation from the ER to the cytoplasm, this translocation is augmented in BABA primed plants (Luna et al., 2014b). Schwarzenbacher unravelled further the IBI1-BABA interaction and the role of Vascular plant One Zinc finger (VOZ) TFs. This study identified that VOZ contribute to BABA-IR by enhancing callose associated cell wall defence. This study additionally identified a further link between BABA-IR and ABA in which VOZ TFs are inducible by ABA but repress pathogen induced ABA signalling during BABA-IR (Schwarzenbacher et al., 2020). This reveals that ABA has varying roles in BABA-IR depending on the specific stress faced by the plant, further adding to the controversy of this hormone in plant defence.

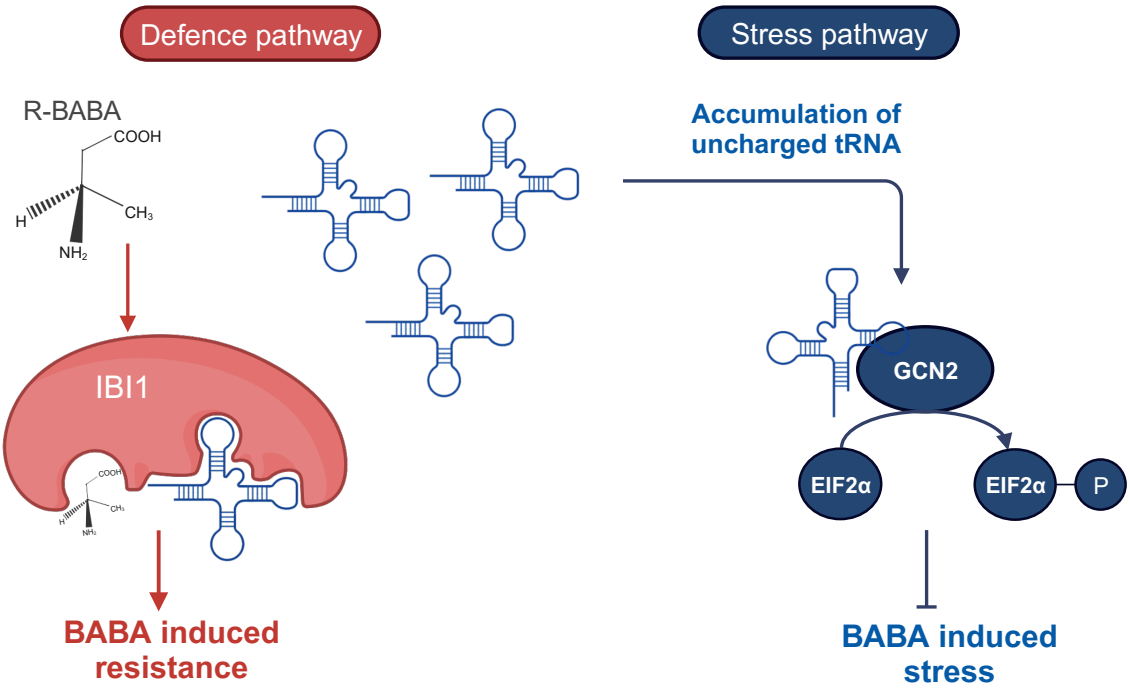


Figure 4: Model of BABA perception Left: binding BABA to *IMPAIRED IN BABA-INDUCED IMMUNITY 1 (IBI1),* blocks canonical aspartyl-tRNA synthetase (AspRS) activity, priming the protein for pathogen-inducible defence activity in the cytoplasm. Right: Accumulation of uncharged tRNAs triggers GCN2-dependent phosphorylation of eIF2α and suppression of plant growth. Figure adapted from Luna et al 2014b.

Following the discovery of the BABA receptor, the presence of endogenous BABA within plants was identified (Thevenet et al., 2017). BABA is found at low levels in plant tissue, 5-15 ng/g^{-1} fresh weight (FW) in Arabidopsis leaves and 10-37 ng/g^{-1} FW in roots. BABA levels were shown to increase when exposed to biotic stress, with levels in Arabidopsis plants increasing 15-fold following *P. syringae* infection. Furthermore, BABA is found in the model moss plant *Physcomitrella patens,* indicating that it is widely present in plants as part of their basic metabolism (Thevenet et al., 2017). Although initially studied in Arabidopsis, BABA has been proven to be an effective elicitor in a variety of important crops. Application of BABA as a seed treatment in tomato has been shown to induce resistance against *O. neolycopersici* without detrimental effects to plant growth (Worrall et al., 2012).

BABA has more recently been shown to induce long-lasting protection in plants at the postharvest level. A study by Wilkinson et al identified that BABA-IR can protect fruit during postharvest against *B. cinerea* following application at an early time point. Furthermore this work found that BABA treatment is ineffective when plants are treated after the onset of fruit production (Wilkinson et al., 2018). Whilst BABA treatment leads to short term changes in growth and delayed fruit ripening, at maturity no differences in size or water content are seen in fruit of BABA treated plants. Metabolomic analysis in fruit of BABA treated plants revealed long-lasting changes in their metabolome profiles, particularly in compounds associated with stress responses and plant-pathogen interactions. Lipids and secondary metabolites were significantly up- and down-regulated in BABA-treated plants. Whilst levels of SA, JA and their derivatives did not differ between treatments, levels of ABA were two times greater in BABA treated plants. Interestingly, exogenous application of ABA to water treated plants led to enhanced susceptibility. In BABA pre-treated plants however, ABA treatment led to enhanced resistance thus highlighting the complex role of ABA in BABA-IR (Wilkinson et al., 2018). BABA treatment has additionally been shown to provide broad spectrum, postharvest resistance in fruit tissue against pathogens with varying infection strategies (Luna et al., 2020). Metabolic markers were identified and revealed that no common markers were shared

amongst *B. cinerea*, *Phytophthora infestans and P. syringae* infected fruit tissue, highlighting the highly specific primed metabolic response to pathogen infection following BABA treatment.

1.9 BABA induced stress

Despite the benefits BABA provides in broad spectrum resistance, it currently presents drawbacks in its commercial application by inducing a stress phenotype due to overstimulation. A high concentration of BABA in Arabidopsis has been shown to induce a phenotype similar to a stress-induced morphogenic response and is associated with perturbations in amino acid homeostasis (Wu et al., 2010). BABA treated seedlings have been shown to display inhibited cell division in meristematic root tissue, increased lateral root density, reduced vegetative growth, and anthocyanin accumulation (Wu et al., 2010, Zimmerli et al., 2001). In addition, it has been shown that BABA accumulates in fruit tissue following application at the seedling stage, suggesting that it is metabolised slowly within plants (Wilkinson et al., 2018). Furthermore, there are potential limitations in its direct use due to toxicity risks as BABA has been shown to antagonise the mammalian inhibitory neurotransmitter glycine (Schmieden and Betz, 1995). BABA treatments have been shown to have large effects on essential nutrients within plants. Tiwari et al demonstrated that BABA treated citrus plants displayed significantly reduced levels of iron, magnesium, phosphorus, sodium, sulphur, and zinc compared to control plants. Authors hypothesise that this is associated with deleterious effects on development (Tiwari et al., 2013). Interestingly, BABA has been shown to display in vitro Fe-chelating properties via its carboxylic and amino groups. It has been shown to modify in planta Fe availability and ferritin accumulation at both the transcript and protein level, demonstrating BABA's influence on homeostasis (Koen et al., 2014). Additional evidence from phenotypic, metabolite accumulation and gene expression analysis displayed similarities between BABA treated and Fe-deficient plants suggesting that BABA treatment and Fe deficiency likely share common signalling pathways. Finally, Fe deficiency was shown to lead to enhanced resistance against *B. cinerea.* The authors

hypothesise that by chelating Fe in planta BABA leads to activation of defence reactions, as demonstrated by common defence-related pathways in BABA and Fe deficiency treatments (Koen et al., 2014).

1.10 (*R*)-β-homoserine (RBH)

In 2018 Buswell et al identified (*R*)-β-homoserine (RBH), a structural analogue of BABA which displays minimal side effects on plant growth which functions independently of IBI1. RBH leads to enhanced resistance in Arabidopsis against *P. cucumerina* and *Hyaloperonospora arabidopsidis* (*Hpa),* additionally RBH induces resistance in tomato against *B.* cinerea without triggering a reduction in relative growth rate (RGR) (Buswell et al., 2018). RBH was found to function independently of SA signalling. Metabolic analysis found that RBH-IR primes camalexin production for resistance against the biotrophic oomycete *Hpa*, and JA/ET-dependent mechanisms for protection against the necrotrophic fungus *P. cucumerina*, thus demonstrating its ability to prime distinct pathways depending on pathogen lifestyle. It is currently unknown if RBH is produced naturally within the plant, or if it contributes to postharvest or transgenerational resistance in tomato. Recently the receptor of RBH was identified through screening T-DNA insertion lines as the high-affinity amino acid transporter LYSINE HISTIDINE TRANSPORTER 1 (LHT1) (Tao et al., 2022). LHT1 has previously been shown to be involved in SA-dependent resistance and controversially can play both positive and negative roles in plant disease resistance (Liu et al., 2010). Tao et al identified that LHT1 was essential for mediating the uptake of both RBH and BABA from the soil in Arabidopsis. This work identified that both BABA- and RBH-IR is enhanced by *LHT*1 over expression, additionally LHT was suggested to be the dominant transporter of BABA from soil. LHT1 is unlikely to be the exclusive BABA transporter however as *lht1* mutants still displayed residual levels of BABA-IR and stress following high BABA doses (Tao et al., 2022).

1.11 The epigenetic basis of long-lasting priming

Priming against both abiotic and biotic stress has been demonstrated to be long-lasting and in certain cases, transgenerational (discussed later). The mechanisms behind this have been extensively explored, with epigenetic changes being identified as a critical driver and mark of long-lasting memory. The main epigenetic marks implicated in priming are DNA methylation marks, which are importantly heritable, chromatin modifications and changes to small RNA (sRNA) abundance. The machinery behind these important processes is discussed below.

1.12 DNA methylation

One of the most widely studied epigenetic marks is DNA methylation. DNA methylation involves the addition of a methyl group by covalent bonding at the fifth carbon position of DNA cytosine bases to form 5-methylcytosine. Whilst symmetric CG methylation is found across both plants and mammals, plants have evolved unique methylation machinery components and methylation in three cytosine contexts; CG, CHG and CHH (H represents any non-guanine base). DNA methylation can act as a mode of regulatory control on gene expression, it is found widely across the genome in promoter regions, gene bodies and transposable elements (TEs) to repress activity of harmful elements. TEs are classified into RNA transposons (Type I) and DNA transposons (Type II). Type I TEs display a copy-and-paste mechanism involving an RNA intermediate (Boeke et al., 1985). The most abundant RNA transposons are classified by the presence of long-terminal repeats (LTRs). The two major superfamilies of LTR TEs are *Copia* and *Gypsy*. In contrast Type II TEs move around the genome using a cut-and-paste mechanism (Brown et al., 1987). In plants, TEs are typically located within or near genes or promoters thus influencing gene expression. Most retrotransposons are inactive under normal conditions but are activated under stress indicating their crucial role in stress adaptation. Methylation of TEs can prevent their movement and importantly maintain genome stability (Erdmann and Picard, 2020). It has been proposed that hypomethylation of TEs provides a blueprint for priming of defence related

genes, either via *cis*- or *trans*- acting mechanisms on defence associated genes (Wilkinson et al., 2019).

DNA methylation is directed by the RNA-dependent DNA methylation (RdDM) pathway using sRNAs as guides (Fig 5) (Wassenegger et al., 1994). RdDM is dependent on the plant specific RNA polymerases Pol IV and Pol V, descendent from Pol II (Onodera et al., 2005, Wierzbicki et al., 2008). The majority of RdDM that occurs in plants is canonical. This is the most well characterised form of RdDM which maintains DNA methylation at heterochromatic regions characterised by inactive repetitive sequences (Cuerda-Gil and Slotkin, 2016). Initially, Pol IV transcribes heterochromatic regions to produce single stranded RNA (ssRNA) 30-45 nt in length, these short ssRNAs are next converted into double stranded RNAs (dsRNAs) via RNA-DEPENDENT RNA POLYMERASE 2 (RDR2). These dsRNAs are then cleaved by the endoribonuclease DICER-LIKE 3 (DCL3) into 24 nt sRNAs. 24 nt strands are then loaded into various ARGONAUTE (AGO) proteins (AGO4, AGO6, AGO8 or AGO3) (Duan et al., 2015). This creates a protein-sRNA duplex which can locate and bind to complementary sequences. This duplex binds to complementary transcripts along a 'RNA scaffold' produced by Pol V. Finally, DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) is recruited to methylate nearby target DNA (Erdmann and Picard, 2020).

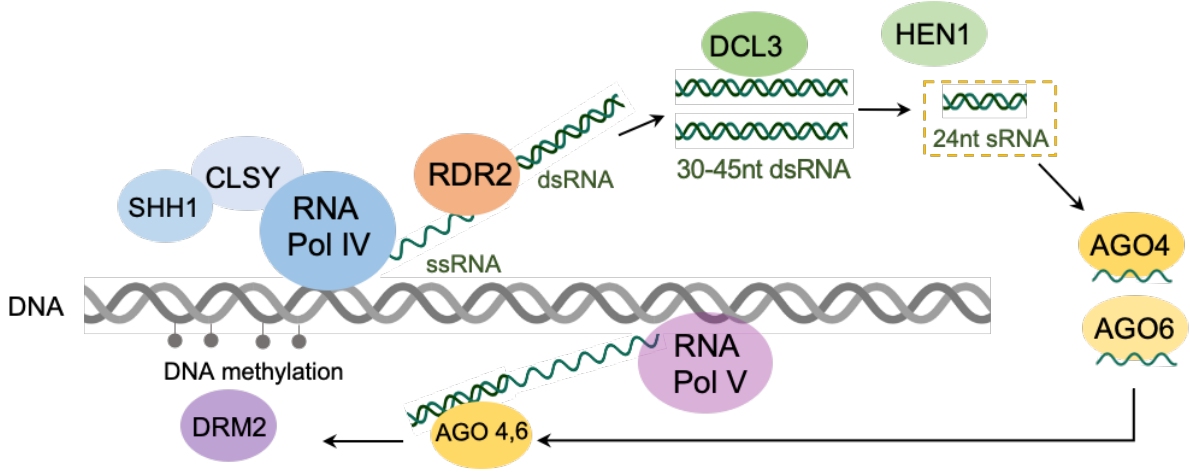


Figure 5: Schematic depicting canonical RNA directed DNA methylation (RdDM). Pol IV is recruited to a genomic target region by SAWADEE HOMEODOMAIN HOMOLOGUE 1 (SHH1), here Pol IV transcribes a single stranded RNA (ssRNA) aided by CLASSY 1 (CLSY1) protein. This is copied into double stranded RNA (dsRNA) by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2). DICER-LIKE 3 (DCL3) processes the dsRNA molecule into 24 nt siRNAs which are then methylated at their 3' end by HUA ENHANCER 1 (HEN1). siRNAs are incorporated into ARGONAUTE (Orta et al.) effector complex proteins AGO4 or AGO6 which is recruited by Pol V to a scaffold RNA. DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) is recruited and catalyses *de novo* methylation in heterochromatic regions. Figure adapted from Erdmann and Picard 2020, Matzke and Mosher 2014.

In contrast, the non-canonical RdDM pathway functions in establishing DNA methylation at new target loci such as TE insertions. Non-canonical RdDM involves the production of 21-22 nt sRNAs from a variety of sources, this process is less firmly characterised than canonical RdDM with new mechanisms being identified still. Generally, TE-derived mRNAs transcribed by Pol II are processed into dsRNAs by RNA-DEPENDENT POLYMERASE 6 (RDR6) and further cleaved into 21-22 nt sRNAs by DCL2 and 4 (Cuerda-Gil and Slotkin, 2016). Most siRNAs are loaded onto ARGONAUTE 1 (AGO1), however some associate with AGO6.

Methylation is maintained by METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASES 2 and 3 (CMT2 and CMT3). MET1 regulates CG methylation, CMT2 and CMT3 regulate CHH and CHG respectively. Whilst symmetrical DNA methylation is stable and maintained through cell division and transmitted to progeny, CHH methylation is not maintained after DNA replication and must occur *de novo*. Loss of methylation can either occur actively or passively. Passive demethylation is due to a lack of methylation maintenance whereas active demethylation occurs via DNA glycosylases/lyases in the DEMETER-LIKE (DML) family. This base excision repair pathway involves the removal of a 5-methylcytosine base by cleaving the phosphodiester backbone, this gap is then refilled with an unmethylated cytosine (Parrilla-Doblas et al., 2019). There are 4 described demethylases in Arabidopsis, REPRESSOR OF SILENCING1 (ROS1), DEMETER (DME), DEMETER-LIKE2 (DML2) and

DEMETER-LIKE3 (DML3). ROS1 was the first identified and is the most well characterised, it functions in maintaining activity of transposable elements and transgenes through hypomethylation (Gong et al., 2002, Lei et al., 2015). Active methylation and demethylation are highly linked to ensure homeostasis of DNA methylation levels; a negative feedback loop exists by which ROS1 is under transcriptional control by RdDM (Lei et al., 2015, Williams et al., 2015). Importantly, stressful environmental conditions may lead to changes in methylation patterns which can influence new resistance traits (Law and Jacobsen, 2009).

1.13 The role of DNA methylation in long-lasting IR

Many research groups have explored the epigenetic basis, and specifically the role of DNA methylation in IR. For example, the Arabidopsis hypermethylated *ros1-4* mutant impaired in active DNA de-methylation is more susceptible to biotrophic pathogens such as *Hpa* and is impaired in transgenerational acquired resistance (TAR) (López Sánchez et al., 2016). Additionally, four hypomethylated regions were identified in Arabidopsis that were associated with resistance against *Hpa* by genome wide priming of defence-related genes (Furci et al., 2019). Recent work by Wilkinson et al demonstrated the epigenetic basis of JA-signalling in long-lasting resistance in Arabidopsis. Defence against herbivory was revealed to be associated with RdDM and ROS1-dependent methylation. Hypomethylation of TEs and their associated siRNAs were involved in regulating long lasting IR. The authors proposed a model in which long-term JA-IR is mediated by ROS1-dependent demethylation of *ATREP2* TEs, this generates AGO1-binding 21 nt siRNAs which subsequently prime distant defence genes (Wilkinson et al., 2023).

The highly dynamic basis of priming induced methylation changes has been studied in potato (Kuźnicki et al., 2019). BABA treatment was shown to cause immediate, genome-wide DNA hypermethylation. The maintenance of this primed state however involved a subsequent demethylation of the initial hypermethylated state. Here *MET1,* the controller of CG

methylation, did not show differential expression and was therefore not considered important for BABA-induced DNA methylation in potato. In contrast, the DNA methyltransferases *CMT3* and *DRM2* showed enhanced up-regulation, displaying the importance of *de novo* methylation and the RdDM pathway. Kuznicki et al also identified *R3a* as a resistance gene displaying high variability in methylation coupled with enhanced transcription as the gene was de-methylated in priming maintenance. Furthermore, *R3a* was less methylated and more highly transcribed in subsequent progeny of BABA primed plants. This study illustrates the importance of multiple time points in the analysis of priming fingerprints to determine how such a state is initiated, maintained and transmitted (Kuźnicki et al., 2019).

Recent work by Catoni et al used whole genome bisulfite sequencing (WGBS) to assess the impact of BABA on DNA methylation in Moneymaker tomato plants. Methylation analysis was conducted in leaf tissue of 3-week-old plants, a week after BABA treatment. BABA treated plants displayed a global loss of DNA methylation, most dramatically in the CHH context where BABA led to a 38% reduction. Differentially hypomethylated regions were highly concentrated in chromosome arms, where genes are most frequently found. When overlapping these differentially methylated regions (DMRs) with differentially expressed genes (DEGs) in *B. cinerea* infection however, authors did not observe a clear association between methylation and transcriptional priming, suggesting BABA is functioning through indirect or *in trans* regulatory mechanisms (Catoni et al., 2022). This study provides valuable insight into how BABA remodels the methylation landscape and suggests a role of CHH methylation in priming in tomato. Further research is required however to determine if these DNA methylation changes are contributing to resistance against *B. cinerea* in fruit tissue where postharvest losses are most costly. Determining how permanent these DMRs are and if they can be manipulated would provide the opportunity to target ‘markers’ for long-lasting protection and potentially trans-generational resistance. Additionally determining if these changes are observed in other cultivars, or are common marks seen following alternative elicitor treatments such as RBH would provide insight into how conserved these resistance mechanisms are.

1.14 The role of chromatin modifications in long-lasting IR

In addition to DNA methylation, many studies are assessing the influence of chromatin structure in regulating the expression of defence genes to facilitate a fast immune response (Ramirez-Prado et al., 2018). Chromatin consists of 147 bp of DNA wrapped around each histone octamer with two copies of each four core histone proteins (H2A, H2B, H3, H4). Histones can be post translationally modified (PTM) through chromatin remodelling complexes in many ways such as through the loss or attachment of acetyl or methyl groups, phosphorylation, or glycosylation (Zhang et al., 2016). The PTM of histones is a highly dynamic process that has a significant role in gene regulation by impacting both chromatin accessibility and structure (Zhao et al., 2019). Different histone marks are associated with certain transcriptional states and are often additionally linked to levels of methylation. For example, DNA hypermethylation typically coincides with repressive histone marks such as trimethylation of histone H3 at lysine 27 (H3K27me3) which characterises transcriptionally inactive, closed heterochromatin (Ramirez-Prado et al., 2018). Methylation and repressive histone marks therefore frequently interact creating a positive feedback loop to maintain a closed, repressive chromatin state.

A study by Jaskieqicz et al identified that treatment with BTH, a synthetic SA analogue, induces di- and tri-methylation of histone H3 at lysine 4 (H3K4me3 and H3k4me2) as well as increased acetylation. Pre-stress chromatin modification of defence gene promoters was therefore shown to display a role in plant defence against *P. syringae* in Arabidopsis (Jaskiewicz et al., 2011). There have been multiple studies linking defence priming with modifications of histones on defence gene promoters. For example, Lopez et al demonstrated that mutants in Pol V, are primed to activate SA-inducible defence genes. This gene priming is associated with H3K4me3 and acetylation of histone H3 at Lys9 (H3K9ac) (López et al., 2011). Furthermore, long-lasting maintenance of BABA-IR by SA-dependent priming requires

functional KRYPTONITE/SUVH4 (KYP), a histone methyltransferase. KYP methylates lysine 9 residues on histone H3 (H3K9) resulting in gene silencing through interaction with CMT3 (Luna et al., 2014a). It is hypothesised that KYP silences SA-suppressive genes through histone modifications. These chromatin modifications may facilitate gene expression activation following subsequent stress due to increased DNA accessibility. However, it has not currently been demonstrated that histone modifications are transmissible in plants through meiosis. A likely hypothesis is that this long-lasting BABA-IR is attributed to changes in cytosine methylation status, for example methylation of histone H3 at Lys9 (H3K9me) has been shown to be tightly coupled with CHG methylation (Jackson et al., 2002). Whilst demonstrated in Arabidopsis and potato, it is currently unknown if BABA treatment leads to chromatin modifications in tomato plants associated with defence against pathogens.

1.15 Transgenerational resistance

In the past decade many studies have explored the mechanisms behind long-lasting and heritable resistance against pathogens. In 2012 three independent groups identified the phenomena of transgenerational priming (Luna et al., 2012, Rasmann et al., 2012, Slaughter et al., 2012). Transgenerational resistance via JA signalling was observed in tomato and Arabidopsis plants following exposure to caterpillar feeding (Rasmann et al., 2012). Priming through either mechanical damage, MeJA treatment or caterpillar feeding led to enhanced resistance and decreased herbivore growth. However, mutants blocked in jasmonate perception such as *coi1* or in the biosynthesis of siRNAs failed to display inherited resistance. Two siRNA mutants were used, a *nuclear RNA polymerase d2a nuclear RNA polymerase d2b (nrpd2a nrpd2b)* double mutant and *a dicer-like2 dicer-like3 dicer-like4 (dcl2 dcl3 dcl4)* triple mutant, thus demonstrating the essential role of siRNA biogenesis in transgenerational induced resistance (Rasmann et al., 2012).

Luna et al showed that progeny of Arabidopsis plants exposed to *Pst*DC3000 displayed enhanced resistance against *Hpa* and *Pst*DC3000, thus demonstrating transgenerational defence priming (Luna et al., 2012). This primed resistance was associated with activation of *PR1* and the NPR1 associated WRKY TFs *WRKY6, WRKY53, WRKY70.* This enhanced gene expression was also shown to be associated with changes to chromatin structure at the promoter region of these genes, in particular H3K9ac. Despite the identification of increased gene expression and chromatin modifications there is currently no robust evidence that chromatin modifications are transmissible and thus the driving force behind transgenerational resistance. The role of methylation was therefore explored in this study. Mutants in non-CpG DNA methylation, *ddc*, were used and showed higher levels of resistance than control lines against *Pst*DC3000, suggesting that the transgenerational SAR is transmitted through hypomethylation at non-CG sites (Luna and Ton, 2012).

Finally, Slaughter et al demonstrated that the progeny of BABA primed Arabidopsis plants display enhanced disease resistance against *P. syringae* and *Hpa.* Additionally, the F2 progeny displayed enhanced sensitisation and were 'primed to be primed' (Slaughter et al., 2012). However, this 'primed to be primed' phenotype was reduced in the Arabidopsis BABA priming mutant *ibs1.* In contrast to experiments by Luna et al, F2 plants of BABA treated plants did not display primed *PR1* expression in the absence of BABA treatment, with memory having to be rescued. This was observed in progeny of plants primed by both BABA and *P. syringae* infection. The methylation status of the *PR1* promoter was analysed through WGBS and no direct changes in methylation were found in cis-elements known to be important for *PR1* expression. It is therefore likely that *PR1* expression may be under activity of hypomethylated *trans*-acting elements or TFs. Greater analysis is therefore required to understand the epigenetic mechanisms driving this *PR1* dependent long-lasting resistance through WGBS. The differences in the long-lasting nature of priming observed between Slaughter, Luna and Rasmann is potentially due to differences in both timing and method of stress application. Luna applied recurrent pathogen stress of *Pst*Dc300 compared to a single application by

Slaughter (Luna et al., 2012, Slaughter et al., 2012). It is therefore important to understand both the optimal time point of priming application to generate a strong long-lasting response and how much priming stimulus needs to be applied to elicit a robust response.

1.16 The role of mobile siRNA signals

siRNAs are known to be a critical modulator of RdDM and plant defence responses as discussed above. Very recent work has illustrated the role of DCL3a, the protein responsible for the biosynthesis of 24 nt siRNAs, in intergenerational-acquired resistance (IAR) against root knot nematodes *Meloidogyne graminicola*. This work demonstrated that DCL3a-mediated DNA methylation is specifically targeted to stress-responsive genes, particularly those involved in phytohormones and signalling. This subsequently leads to a unique transcriptional response of these genes termed “spring loading” and enhanced resistance (Meijer et al., 2023). Further identification of specific siRNAs which are directing transcriptional responses of stress-associated genes may therefore provide a potential target for manipulation and crop improvement strategies.

Within plants two main classes of siRNAs exist, critically these molecules are known to be mobile within plants with 21-22 nt playing a role in short-distance signalling and 24 nt in long-distance signalling. The movement of siRNAs across grafting junctions has been demonstrated frequently. Grafting is a naturally occurring process that has been used as a tool for decades in horticulture and was reported to be used over 2,000 years ago in China (De Vos et al., 2006). Grafting has been used extensively in Solanaceous crops to improve disease resistance, cold temperature tolerance, salt stress and oxidative stress tolerance (Manickam et al., 2021 Wang et al., 2023, Martínez-Andújar et al., 2021, Wang et al., 2023, Zhang et al., 2021). It is not fully understood how grafting can induce large biological effects in recipient tissue, it is thought that movement of small noncoding RNAs (sRNAs and miRNAs) which can travel bidirectionally within the vasculature and facilitate physiological,

transcriptional, and epigenetic changes such as DNA methylation may play a role (Jeynes-Cupper and Catoni, 2023). For example, Molnar et al demonstrated through use of Arabidopsis siRNA biogenesis mutants that transgene derived siRNAs were transmissible across graft unions. These siRNAs were shown to initiate post-transcriptional gene silencing (PTGS) of a transgene in root tissue. This important study additionally demonstrated that mobile 24 nt siRNAs could direct DNA methylation in the genome of recipient grafted cells (Molnar et al., 2010). Furthermore, Cao et al demonstrated that DNA methylation changes in grafted plants may play a role in phenotypic changes which are heritable (Cao et al., 2016). Significant progress on our understanding of both siRNAs and epigenetics has therefore been obtained through the tool of grafting. Whilst grafting has been used extensively to improve vigour and desirable crop traits, the use of a primed root stock to induce postharvest resistance has not been investigated. Considering that long-lasting priming is increasingly being recognised to be associated with siRNA signalling, further investigations incorporating priming elicitors and grafting can uncover valuable information on the mechanisms behind this phenomenon.

1.17 Timing and cell type specificities of epigenetically-regulated priming

The timing of priming treatment is known to be critical for achieving postharvest resistance (Wilkinson et al., 2018). Differences have previously been identified in priming responses between cell types. For example, the shoot apical meristem displays an enhanced transcriptomic response and resetting compared to seedlings as a whole, demonstrating tissue dependent regulation of heat stress memory (Olas et al., 2021). The developmental stage at which a plant experiences a priming stimulus may additionally determine how much it invests in priming of stress responses and how long-lasting the priming fingerprint remains. Young plants are likely to benefit from a long-term stress memory if it helps to minimise costs of future inducible stress responses and tailor resources for growth and defence (Hilker and Fatouros, 2015). What facilitates this enhanced ability to ‘remember’ however is relatively

unexplored. Plant age has shown to have a significant influence on global methylation profiles. CG and CHG methylation levels are lower in fruit tissue than 4-week-old leaf tissue, with the reverse pattern seen in CHH context (Zhong et al., 2013). However, it is unknown how changes in developmental stage-dependent DNA methylation mediate the establishment of long-lasting postharvest priming. Determining the optimal timepoint for priming treatment is critical for generating effective long-term immunological memory, thus understanding the optimal application period for priming compounds is essential for growers.

1.18 Using epigenetics as a solution for disease resistance

Advances in understanding of crop stress responses has facilitated the use of genetically edited organisms in agriculture (Hua et al., 2019). Epigenetic diversity and induced epigenetic variation however presents a new, underutilised line of research for crop improvement. This could involve natural variation in epigenetic diversity, inducing epigenetic variants through chemical treatment, mutations in epigenetic machinery or epigenome editing (Zhi and Chang, 2021). Recently epigenetic editing has been utilised in cassava against bacterial blight demonstrating stable methylation interference for use in crop improvement. Previous work has demonstrated that gene editing can be utilised to prevent exploitation of susceptibility (S) genes. S genes are targeted by transcription activator like (TAL) effectors that activate their transcription. TAL binding can be inhibited by DNA methylation. Use of an artificial zinc-finger containing the RdDM factor DEFECTIVE IN MERISTEM SILENCING 3 (DMS3) was used to induce CG methylation at the target site preventing transcriptional activation and subsequently reducing cassava bacterial blight symptoms (Veley et al., 2023) Alternative approaches for inducing epigenetic changes include utilising sRNA technology, for example high pressure spray application of dsRNA in tobacco targeted to a specific target gene promoter region has been shown to facilitate targeted methylation by RdDM (Dalakouras and Ganopoulos, 2021). The use of epibreeding, sRNA technology and chemical inhibitors require further development however present promising techniques for inducing TGS and potentially trans-generationally

maintained epigenetic changes. Thus, greater understanding and identification of genetic regions involved in immune priming which are epigenetically-regulated can identify potential targets for epigenetic modification to enhance disease resistance.

1.19 Utilising BABA-IR as a solution for postharvest losses in horticulture

Most of the existing knowledge in the field of IR and priming is from research conducted in Arabidopsis. However, priming presents an attractive solution to be incorporated into IPM of crops due to its low environmental impact and long-lasting effects. Greater understanding of the mechanisms behind this phenomenon can allow us to make targeted changes to the genome or epigenome. Additionally, the long-lasting nature of BABA-IR means it presents a good system for further understanding of the role of epigenetics in immunological memory as a whole. Tomato is a member of the Solanaceae family, which includes many economically important crops. It is a highly studied species in plant science, with its genome first sequenced using shotgun technology in 2012 (Tomato Genome Consortium, 2012). It is therefore a rich genetic resource and the model organism for fruit ripening and infection. Previous studies have already demonstrated that tomato is an effective system for studying long-lasting priming and epigenetic and metabolic mechanisms behind this (Wilkinson et al., 2018, Catoni et al., 2022). Furthermore, due to the extensive postharvest loss this crop faces this is a system which requires new effective control strategies.

An interesting species to study long-lasting priming is the nutrient rich crop strawberry (*Fragaria* x *ananassa*). In 2018, 8.4 million tonnes were grown globally (FAOSTAT, 2019) however they are particularly vulnerable to infection by *B. cinerea.* Strawberries can produce stolons called runners which enable vegetative reproduction, the production of these runners is controlled and activated by gibberellin (GA) (Hytönen and Kurokura, 2020). Due to the clonal nature of runners, strawberries are an attractive system to study next-generational priming. If an elicitor treated mother plant displays enhanced resistance, the priming phenotype may be

transmitted vegetatively to runners. This would be an effective solution for enhanced disease resistance which warrants further investigation.

Whilst tomato and strawberry both experience huge PHL, these crops are highly different to Arabidopsis which poses challenges for incorporating fundamental research into these systems. Firstly, the tomato genome is 950 Mb (Sato et al., 2012) compared to 135 Mb of the five-chromosome Arabidopsis (The Arabidopsis Genome, 2000). In addition, the components of these genomes varies largely; TEs make up approximately 60% of the total tomato reference genome compared to only 20% in Arabidopsis (Deneweth et al., 2022). Given the major role of TEs in epigenetic and transcriptional regulation, it is plausible that the epigenetic machinery and methylation responses vary largely between these two model species.

1.20 Thesis aims and objectives

This PhD thesis therefore aims to understand further the nature of long-lasting chemical priming for use in postharvest crop protection. Through application of the priming agent BABA, this project aims to understand the potential epigenetic basis behind the perception, maintenance, and systemic signalling of long-lasting priming in tomato. Recent work in tomato has demonstrated that timing of BABA treatment is important, and that ABA has a complex role in this priming response (Wilkinson et al., 2018). It is unknown however how plant developmental stage influences priming responses and the epigenetic changes underpinning this. It is therefore hypothesised that young seedlings display a greater capacity to be primed than older plants. Young plants may therefore display greater changes in DNA methylation and sRNA abundance which facilitate long-term and heritable memory. Although recent work has demonstrated BABA induced methylation changes are dynamic, how these are directly linked to resistance over the long-term is relatively unexplored. Furthermore, with the known stress responses induced by BABA treatment, it is important to isolate epigenetic changes associated with resistance specifically. The importance of plant age and the maintenance of

BABA associated epigenetic marks are therefore discussed in **Chapter 3.** BABA has been shown to lead to a robust systemic signal of resistance within plants at a postharvest level, as well as epigenetic changes within tomato plants (Catoni et al., 2022, Wilkinson et al., 2018). How this resistance signal is maintained and spread to other plant tissues however is yet to be explored. Here it is proposed that the BABA induced influence on RdDM, and subsequently sRNAs may be modulating this long-lasting immune memory. **Chapter 4** therefore uses grafting and sRNA sequencing to unpick the systemic nature of BABA-IR in tomato. Finally, whilst IR has been studied extensively in Arabidopsis, the full capabilities of chemical priming in crops has not been explored. Thus, in **Chapter 5,** chemical priming is utilised in strawberry through use of elicitors BABA and RBH. Due to the clonal nature of strawberries, and the inter-generational IR reported in Arabidopsis and rice (Luna et al., 2012, Meijer et al., 2023), this represents an attractive crop to optimise priming. This work is conducted in collaboration with iCASE industrial partner Saturn Bioponics who grow strawberries in hydroponic settings. Finally, work in this thesis aims to explore the controversial role of ABA in plant defence. With highly contrasting defence phenotypes reported within individual plants, this work aimed to use machine-learning tools to untangle the importance of age and pathogen lifestyle in influencing disease susceptibility. This therefore further explores the role of plant developmental stage on resistance and establishment of priming.

Overall, work in this PhD thesis aims to further characterise long-lasting chemical priming to facilitate its effective use in commercial settings to reduce PHL. By advancing our understanding on the importance of plant age, plants can be effectively treated to ensure maximum protection. Through identification of epigenetic changes underpinning long-lasting IR, markers of long-lasting resistance can be isolated for direct targeting in crops. Furthermore, by studying alternative crop systems such as strawberries and use of horticultural techniques such as grafting, this project aims to advance our knowledge of priming from early studies in Arabidopsis to generate practical tools for use in integrated pest management in a commercial setting.

Chapter 2:

Materials and Methods

2.1 Plant growth conditions

2.1.1 Tomato growth conditions

Tomato seeds (cultivars Micro Tom or Moneymaker) were placed in petri-dishes containing damp filter paper and maintained at 28 °C in the dark for 3-6 days to stimulate homogeneous germination. Germinated seeds were planted in individual pot propagators containing Levington M3 soil and cultivated under standard tomato growth conditions (16h- 8h/ day- night cycle; 25 °C/ 20 °C).

2.1.2 Strawberry growth conditions

Seeds of strawberry (cultivar Durban) were placed in petri-dishes containing damp filter paper and maintained in light for 10-14 days to stimulate homogenous germination. Germinated seeds were planted in individual pot propagators containing Levington M3 soil, or hydroponic rockwool plugs. For hydroponically grown plants, rockwool plugs were kept constantly damp with water. Following the establishment of roots, seedlings in rockwool plugs were placed into Saturn Growers provided by Agritech company Saturn Bioponics. Saturn Growers consist of a tower of vertical pots containing a pebble substrate to allow drainage, the top pot contains a sprayer which irrigates water automatically. Towers were attached to a 25-litre nutrient tank which irrigated towers for 3 minutes every hour. Once a week the tank was filled with water and 10 g of nutrient stock containing calcium nitrate, phosphate and sulphate provided by Saturn Bioponics was added alongside 1% hydrogen peroxide to prevent contamination and algal growth. All plants were cultivated in standard glasshouse conditions (16h- 8h/ day- night cycle; 25 °C/ 20 °C).

2.2 Plant treatments

2.2.1 Soil drench treatments

Tomato and strawberry plants were treated with various chemical treatments including β-aminobutyric acid (BABA) (catalogue number A4420-7 Sigma Aldrich), R-β-homoserine (RBH) (catalogue number 03694 Sigma Aldrich) and zebularine (catalogue number 2293 Biotechne). BABA treatments unless stated otherwise were conducted at a final concentration of 0.5 mM in the soil. BABA and RBH treatments were prepared freshly in distilled water and diluted to appropriate concentrations. A 50 mM stock solution was made of zebularine by dissolving 10 mg in 0.8765 mL of dimethylsulfoxide (DMSO) which was stored at -20 °C. Before use the stock solution was thawed and diluted in fresh distilled water to a final concentration of 80 µM. Zebularine control plants were treated with water plus the same volume of DMSO as zebularine added. BABA and RBH control plants were treated with the same volume of water. Soil drench treatments were conducted at 10x concentration with 10x less volume. Following all soil drench treatments plants were kept in trays to avoid run off contamination. One week after chemical treatments seedlings were removed from soil, roots were gently washed, and plants were replanted in new compost.

2.2.2 Strawberry seed elicitor treatments

Strawberry (cultivar Durban) seeds were germinated on filter paper soaked in 0.5 mM of BABA or 80 µM of zebularine. Seeds were kept on filter paper for 10-14 days until germination and then transferred into Levington M3 soil and cultivated under standard strawberry growth conditions.

2.3 Relative growth rate (RGR) analysis

The height of tomato plants was measured before BABA treatment at (t1) and one week after BABA treatment (t2), as previously described (Luna et al., 2016). Relative growth rate (RGR) was calculated using the formula = LN(height-t2)-LN(height-t1)/t2-t1 (Pérez-Harguindeguy et

al., 2013). To measure RGR in strawberry plants, photographs were taken of plants before treatment and one week after treatment. The total green area of leaves was quantified using photoshop and calculated using the formula = LN(area-t2)-LN(area-t1)/t2-t1(Pérez-Harguindeguy et al., 2013). During experiments fruit number and ripening was quantified by counting the number of green and red fruit at different times during growth.

2.4 Pathogen assays

2.4.1 *B. cinerea* cultivation and preparation

Botrytis cinerea was prepared as described in Luna et al, *B. cinerea* strain R16 (Faretra and Pollastro, 1991) was cultivated on potato dextrose agar (PDA, Difco). Spores were maintained by placing a small section of agar from a fully colonised plate into the centre of a new PDA plate. Plates were grown in the dark at room temperature. For infections, spores were scraped from agar plates in 10 ml of sterile water containing 0.01% Tween 20. Spores were filtered through Miracloth (EDM Millipore) and centrifuged for 10 min at 3,000 rpm. Spores were resuspended in sterile water and adjusted to a concentration of 106 spores/ml. To prepare inoculum the spore concentration was adjusted to 105 spores/ml in 10 mM glucose and KH_2PO_4 (pH 5) and incubated at room temperature for 2 hours.

2.4.2 *B. cinerea* tomato infections

Infections of leaves with *B. cinerea* were performed as previously described (Luna et al., 2016), with minor modifications. Each tomato leaf was infected with droplets of 5 µl inoculum containing $5x10^5$ spores/ml. Leaves were incubated in the dark at 100% humidity and 20 ^oC. Disease was scored by measuring lesion diameters with an electronic calliper at 2 days post infection (dpi). To infect fruit, ripe fruit were harvested and placed with pointed tips facing upwards on plastic frames sat on trays containing wet paper. A 2 mm deep wound was made close to the tip of each fruit using a needle. The wound was dried fully and 5 µl of inoculum

containing $5x10^5$ spores/ml was added to the wound. Trays were covered and plants were incubated in the dark at 100% humidity at room temperature (Wilkinson et al., 2018).

2.4.3 *B. cinerea* strawberry infections

B. cinerea was prepared as in Luna et al with some modifications. Briefly, spores were prepared to a final concentration of 5×10^5 in half strength potato dextrose broth (PDB) (Luna et al., 2016).

2.4.4 Disease scoring

Disease was scored by measuring the diameter of visible necrosis and classifying fruit in different categories of infection (Wilkinson et al., 2018). The following disease categories were used class I - no disease, class II - necrosis diameter < 8 mm, class III - necrosis diameter > 8mm, class IV - diameter larger than 8mm and external mycelium, class V - tissue collapse.

2.5 Tomato grafting

Tomato plants were grafted using the cleft method (Lee and Oda, 2002). The scion tissue end was cut into a V- shape to narrow the stem, the scion was inserted into the rootstock which has a cut in the centre of the stem. Plants were either wrapped in parafilm to secure the graft and/or clipped. Plants were supported with small cane sticks. Following grafting plants were kept in 100% humidity for five days. Over the course of a week the humidity was gradually reduced by cutting a hole in the bags covering each individual plant until eventual removal of the bag.

2.6 Nucleic acid extractions

2.6.1 DNA extractions

DNA extractions were conducted for whole genome bisulfite sequencing (WGBS) analysis. DNA was extracted from 100 mg of ground frozen leaf and fruit tissue using Qiagen DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions and resuspended in 30 µl RNA-se free H_2O. DNA quantity and quality was determined using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific) at 230, 260 and 280 nm.

2.6.2 RNA extractions

RNA was extracted from 100 mg of frozen leaf and fruit tissue using TRIzol (catalogue number 15596026 Thermo Fisher). 1 ml of TRIzol was added to each sample and centrifuged for 10 minutes at 4 °C at 12000 g, the supernatant was transferred to a new tube with 200 µl of chloroform added. Samples were vortexed and centrifuged for 15 minutes at 4 °C at 12000 g. The upper aqueous phase was transferred to a new tube containing 250 µl isopropanol and 250 µl of high salt precipitation solution (0.8 M sodium citrate e 1,2 M NaCl). RNA was left to precipitate for 1 hour in -20 °C. Samples were centrifuged for 10 minutes at 4 °C at 12000 g. The supernatant was removed, and pellet was washed with 1 ml of 70% ethanol and span at 7500 g for 5 minutes at 4 °C. The ethanol wash was repeated twice. Supernatant was removed, pellet was left to dry briefly before resuspension in RNA-se free H_2O. RNA quantity and quality was determined using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific) at 230, 260 and 280 nm.

2.7 Sequencing analysis

2.7.1 Library preparation and mRNA sequencing

mRNA sequencing analysis was conducted on RNA extracted from both leaf and fruit tissue infected and uninfected with *B. cinerea.* Leaf and fruit tissue were collected at 48 and 72 hrs post infection respectively. Library preparation and sequencing was conducted by Novogene using NovaSeq 6000 PE150 at a sequencing depth of 20 million paired reads per sample. For leaf tissue a total of 550514406 clean reads were generated across 12 samples, with an average of 45876201 clean reads per sample. An average of 90% of nucleotides per sample had a Phred quality score of > 30. For fruit tissue a total of 779771438 clean reads were generated across 17 samples, with an average of 45868908 clean reads per sample. An average of 93% of nucleotides per sample had a Phred quality score of > 30. To confirm that the sequencing quality was sufficient, sample quality was assessed using FASTQC (version 0.11.9) (Andrews, 2010). Adapters were removed from samples using Trimmomatic (version 0.39) (Bolger et al., 2014). Reads were aligned to the tomato genome (ITAG4.0) using HISAT2 (version 2.2.1) (Kim et al., 2019). Samples had an average mapping efficiency of 95.1% and 90.5% for leaf and fruit samples respectively. Samtools (version 1.12) (Li et al., 2009) was used to sort and index SAM files into BAM format. Read counts were generated using HTSeq (version 0.13.5) (Anders et al., 2015).

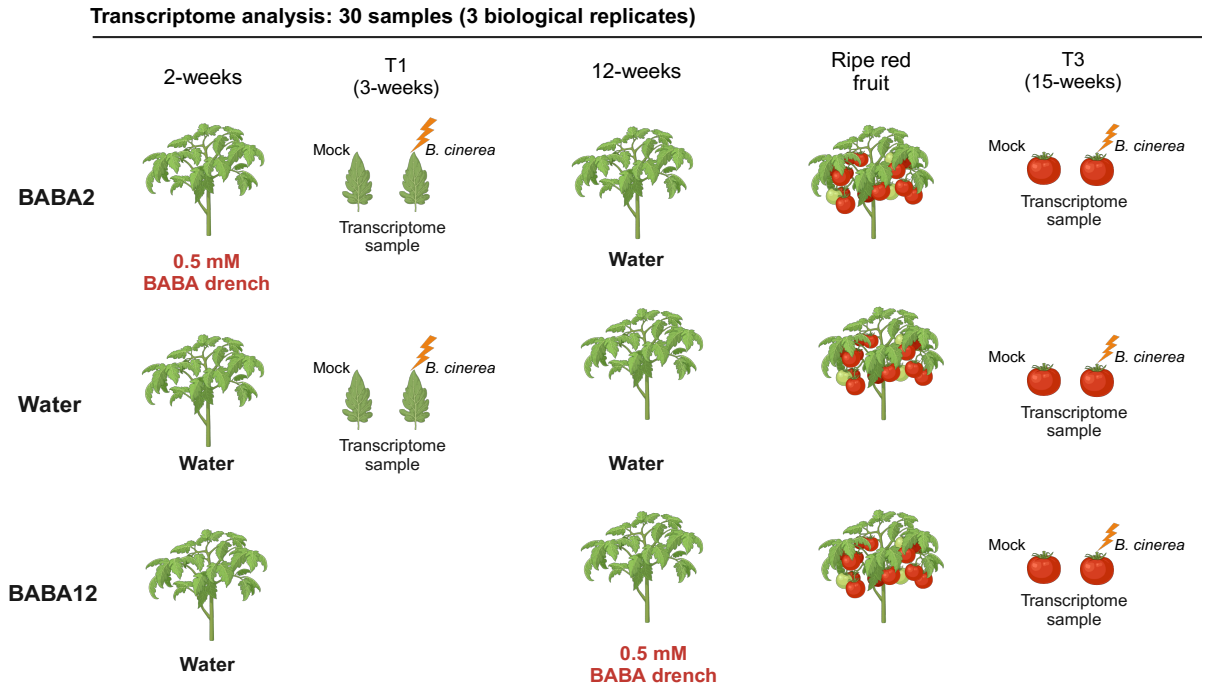


Figure 6: Experimental design of mRNA sequencing experiment. Plants treated with BABA at 2-weeks (BABA2), 12-weeks (BABA12) or water control. BABA2 and water leaf tissue was infected with *B.* cinerea one week following BABA treatment (T1), three biological replicate samples were collected from infected and mock tissue for all treatment groups (BABA2, Water). All plants were grown to maturity, ripe red fruit were collected and infected with *B. cinerea*, 3dpi infected and mock tissue was collected from three biological replicates from each treatment groups (BABA2, BABA12, Water). All samples were flash frozen for RNA extractions and mRNA sequencing. Figure created using BioRender.

2.7.2 Statistical analysis of sequence count files

Count files were analysed in R using DESeq2 (version 1.34.0) (Love et al., 2014). Genes with total read counts of under 10 were removed. A variance stabilising transformation (VST) was applied for normalisation, principal component analysis (PCA) plots were generated using R packages DESeq2 and ggplot2 (version 3.3.4) (Wickham 2016). Hierarchical clustering was conducted for samples based on Euclidean distances and produced using the R package pheatmap (version 1.012) (Kolde, 2019). Due to poor clustering, the sample of 'Water infection 2' was removed from subsequent statistical analysis in T3 RNA sequencing analysis. Differentially expressed genes (DEGs) involved in infection were identified for infected plants

of all three conditions (BABA2, Water, BABA12) using DESeq2 and a threshold adjusted p-value of 0.05 (Benjamini and Hochberg correction) (Benjamini and Hochberg, 1995). To identify the effect of early and late BABA treatments on the transcriptome without infection pairwise comparisons between BABA2 Mock vs Water Mock and BABA12 Mock vs Water Mock were conducted. For specific genes of interest Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values were calculated by normalising for the total number of fragments sequenced and the length of the gene (kb). The FPKM values for all samples were then plotted as a heatmap using the R package pheatmap (version 1.012) (Kolde, 2019).

2.7.3 Library preparation and whole genome bisulfite sequencing (WGBS)

All library preparation and sequencing was conducted by Novogene using NovaSeq 6000 PE150. Briefly, after quality control, positive control DNAs were added into the DNAs and were fragmented into 200-400 bp using Covaris S220 and Bisulfite treated (Accel-NGS Methyl-Seq DNA Library Kit for illumina, Swift). Methylation sequencing adapters ligation, size selection and PCR amplification was then performed before Illumina sequencing for 20 million paired reads per sample with 30 x sequencing depth. An average of 90.2% of nucleotides per sample had a Phred quality score of > 30. A total of 1.09938788×10^9 reads were generated across all 24 samples, with an average of 45.8 million clean reads generated per sample.

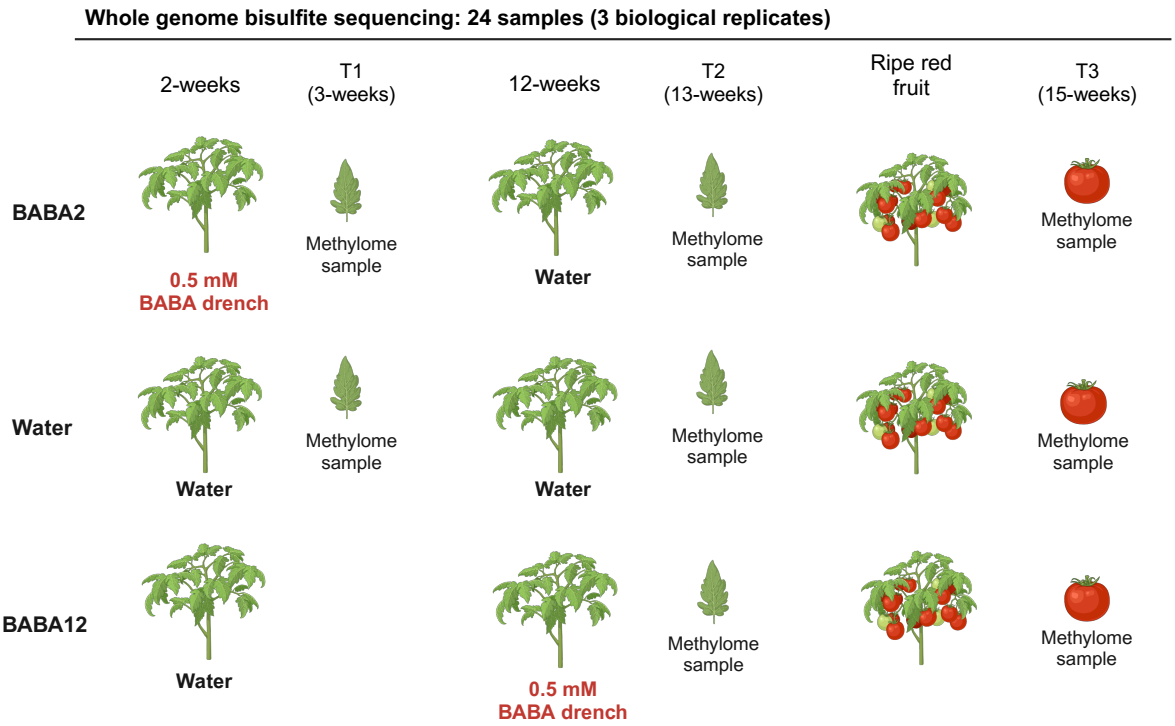


Figure 7: Experimental design of WGBS experiment. Plants treated with 0.5mM BABA via soil drench at 2-weeks (BABA2), or water control, leaf tissue was collected one week post treatment at T1 (3-weeks). Plants were treated at 12-weeks with 0.5 mM BABA via soil drench, water and BABA2 plants were treated with equal volume of water. One week post BABA12 treatment, at T2 (13-weeks) leaf tissue was collected. All plants were grown to maturity, the first ripe red fruit from all plants were collected at T3 (approx. 15 weeks). All samples were flash frozen for DNA extractions and WGBS. Figure created using BioRender.

2.7.4 Whole genome bisulfite sequencing (WGBS) data processing

The tomato genome version SL.4.0 was downloaded from Sol Genomics (Fernandez-Pozo et al., 2015). The genome was merged with the chloroplast sequence (NC_007898.3) to calculate the bisulfite conversion rate. The reference genome was bisulfite converted using Bismark (v0.16.3_bowtie2) (Krueger and Andrews, 2011). To confirm that the sequencing quality was sufficient, sample quality was assessed using FASTQC (version 0.11.9) (Andrews, 2010). Adapters were removed from samples using Trimmomatic (version 0.39) (Bolger et al., 2014) using the following parameters 'LEADING:30 TRAILING:30 SLIDINGWINDOW:4:15

MINLEN:16'. Reads were aligned to the SL4.0 tomato genome with Bismark using: 'Bismark -N 1 -L 20 -p 4 -X 1000 -score_min L,0,-0.6' which uses Bowtie for mapping (Krueger and Andrews, 2011). The average mapping efficiency was 80% with a minimum value of 77% and 82.2% respectively. Samples were de-duplicated using Samtools (version 1.12) (Li et al., 2009). CX files were created with Bismark using: 'bismark_methylation_extractor –comprehensive --multicore 8 --bedGraph --CX --buffer_size 10G'. The bisulfite conversion rate was calculated for each sample using the chloroplast sequence, conversion rates varied from 98.41- 99.66%. An estimated conversion rate was applied following (Catoni and Zabet, 2021) in order to account for non-converted DNA.

2.7.5 Analysis of DNA methylation

Differentially methylated regions (DMRs) were identified at the following timepoints between BABA2 and water-treated replicates, and BABA12 and water-treated replicates at different time points: T1 (3 weeks) Water and BABA2 leaf tissue one week post BABA soil drench; T2 (13 weeks) Water, BABA2 and BABA12 leaf tissue one week post BABA soil drench; and T3 (15 weeks) Ripe red fruit in water, BABA2 and BABA12. The package DMRcaller (version 1.26.0) (Catoni et al., 2018) was used to identify DMRs in each cytosine context (CG, CHG and CHH) in R (version 4.1). The following parameters were used: function computeDMRsReplicates, the 'bins' method was used with a bin size of 200 bp, methylationDiff of 0.2, minCytosinesCount of 2, minReadsPerCytosine of 4, and a minimum *p*-value of 0.05. To identify DMRs unique to the 'BABA2' treatment group, overlap was conducted between BABA2 and BABA12 DMRs, with only BABA2 DMRs used for further downstream analysis. To identify genomic features, the ITAG4.0 tomato reference genome was used. Repeat Modeler was used to identify repeats (Prashant et al., 2019). The R package GenomicRanges (version 1.46.1) (Lawrence et al., 2013) was used to assess overlap between DMRs and genomic features.

2.7.6 Small RNA (sRNA) sequencing analysis

sRNA sequencing analysis was conducted on RNA extracted from scion leaf tissue from BG, HG and MG plants 6 weeks post grafting. Library preparation and sequencing was conducted by Novogene using NovaSeq SE50 at a sequencing depth of 20 million paired reads per sample. A total of 310240010 clean reads were generated across 12 samples, with an average of 25853334 clean reads per sample. An average of 97.5% of nucleotides per sample had a Phred quality score of > 30. To ensure that the sequencing quality was sufficient, sample quality was assessed using FASTQC (version 0.11.9) (Andrews, 2010). Samples were trimmed using Trimmomatic (version 0.39) to remove adapter sequences and obtain reads between 16-30 bp in length (Bolger et al., 2014). Trimmed reads were uniquely mapped to the tomato reference genome using Shortstack (version 3.8.5) (Axtell, 2013).

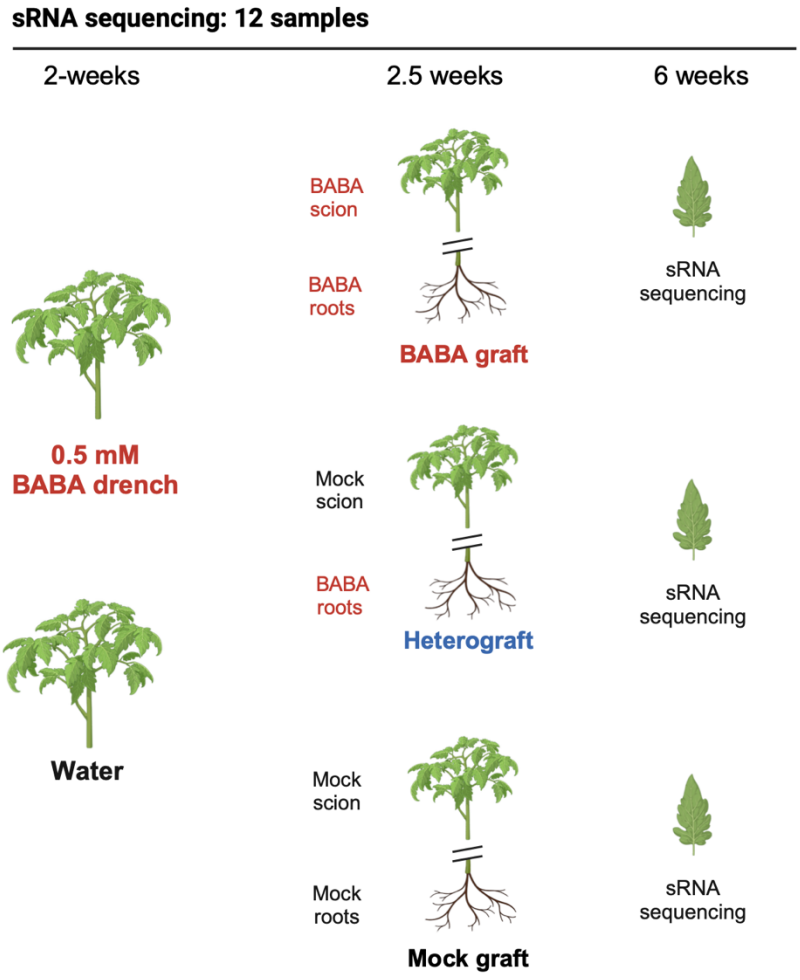


Figure 8: Experimental design of sRNA sequencing experiment. Plants treated with 0.5mM BABA via soil drench at 2-weeks, or water control. 3 days post BABA treatment grafting was conducted in the following combinations: BABA graft = BABA scion and BABA rootstock, Heterograft = BABA roots and mock scion, Mock graft = mock roots and mock scion. Six weeks after BABA treatment went grafted plants had recovered, leaf tissue was collected from scions from all treatments. Leaves were flash frozen for RNA extraction and sRNA sequencing. Figure created using BioRender.

2.7.7 Small RNA (sRNA) cluster analysis

Clusters were identified as 20-24 nt in size using the R package mobileRNA (https://github.com/KJeynesCupper/mobileRNA). For each replicate the consensus cluster size was identified and non-dicer-derived clusters were excluded from the analysis. Differentially expressed clusters were identified between HG and MG, BG and MG and HG and BG using the DESeq2 method. Location of DE clusters was found using GenomicRanges (version 1.46.1) (Lawrence et al., 2013) by overlapping clusters (+/- 1 kb) with known genomic features using a log threshold change of 1 or -1 for up- and down-regulated, respectively; a false discovery rate (FDR) threshold of 0.05 was applied to control significance (Benjamini and Hochberg correction (Benjamini and Hochberg, 1995).

2.7.8 Gene Ontology (GO) enrichment analysis

GO enrichment analysis was performed on differentially expressed genes and genes overlapping differentially methylated regions or sRNA clusters (+/- 2 kb). Analysis was performed by using the online tool ShinyGO (Ge et al., 2020) with Fisher's exact test, false discovery rate correction and a *p* value cut-off of 0.05.

2.8 Metabolite analysis

2.8.1 Targeted phytohormone analysis

Phytohormone analysis was conducted by sampling one leaf from each plant, 3-4 plants per condition were used. 30 mg of ground dried plant material was weighed, 1 ml of extraction solution (900 µl MiliQ water, 100 µl MeOH) and a pool of internal standards containing abscisic acid-d6 (ABA-d6), salicylic acid-d5 (SA-d5), indole acetic acid-d5 (IAA-d5), dehydrojasmonic acid (hJA; Sigma-Aldrich) were added to each sample and kept on ice for 30-45 minutes. Samples were ground using a tissue lyser (3 rounds x 20 seconds) and kept cold. Samples were centrifuged for 20 minutes at 14,000 rpm in 4 °C. The upper aqueous phase was recovered and brought to a pH of 2.5 with acetic acid (30-50%) on ice. 2.5 mL of diethyleter was added to the supernatant, vortexed and centrifuged at 4000 rpm for 3 minutes, this was repeated. The two organic phases were combined and evaporated in a centrifugal evaporator (Speedvac) at room temperature to produce a pellet. Samples were redissolved in 100 µl MeOH and 900 µl H_2O MQ leading to a final internal standard concentration of 100 ng $ml^{-1.}$ The precise quantification for SA, ABA and IAA was performed by using external calibration curves with each pure chemical, dhJA was used for 12-oxo-phytodienoic acid (OPDA) quantification (Sánchez-Bel et al., 2018).

Chromatographic separation was conducted as described in Sánchez-Bel et al. Briefly, 20 µl of sample was injected into a high-performance liquid chromatography (HPLC) Kinetex C18 analytical column with a 5 µm particle size, 2.1 100 mm (Phenomenex). Phytohormone quantification was conducted in an Acquity ultraperformance liquid chromatography system (UPLC) (Waters, Mildford, MA, USA), which was connected to a triple quadrupole mass spectrometer (TQD) (Waters, Manchester, UK). The chromatographic and mass spectrometry conditions were those published by Gamir et al (Gamir et al., 2012, Sánchez-Bel et al., 2018).

2.8.2 Amino acid profiling

Amino acid analysis was conducted by sampling one leaf from each plant, 3-4 plants per condition were used. 100 mg of ground freeze-dried plant material was added to 600 µl of extraction solution (400 µl of MiliQ water + 200 µl MeOH) and a mix of internal standards of Phe-9$C_{13}N_{15}$ and Thr-4$C_{13}N_{15}$ to a final concentration of 200 ppb. Samples were homogenised and 200 µl of chloroform was added before vortexing. Samples were kept on ice for 30 minutes and centrifuged at 14,000 rpm for 2 minutes. 400 µl of the upper aqueous phase was transferred to a fresh eppendorf. 600 µl of water was added to the organic phase before centrifuging again at 14,000 rpm for 2 minutes. The aqueous phase was recovered and added to the previous to form a total volume of 1 ml. 10 µl of 100 mM perfluoroheptanoic acid (PFHA) was added to form the ionic pair.

20 µl of sample was injected into a Kinetex 100 Å HILIC column with a 1.7 µm particle size, 2.1 100 mm (Phenomenex). Phytohormone quantification was conducted in an Acquity UPLC (Waters, Mildford, MA, USA) connected to a TQD (Waters, Manchester, UK). The solvent A (organic eluent acetonitrile and 20 mM of ammonium formate) and solvent B (ultrapure water + 20 mM of ammonium formate) chromatography gradient was as follows: 0-5 minutes: 10-20% B, 5-6 minutes: 20-60% B, 6-9 minutes: 50% B, 9-10 minutes: 50-20% B, 10-15 minutes: 0% B. The samples were ionized in positive (ESI+) mode, the fragments and energies used for the MS detection are listed in Table 1.

Table 1: Fragment and energy values used for MS detection of amino acids.

Parental/ fragment	Cone Volt (v)	Collision energy (eV)	Compound
76.10 > 76.10	15	5	Gly
90.00 > 43.90	20	10	Ala
106.00 > 60.00	18	12	Ser
116.00 > 70.30	28	15	Pro
118.00 > 72.30	25	12	Val
120.00 > 73.80	25	15	Thr
133.00 > 60.30	10	10	Asp
133.00 > 87.00	10	10	Asn
147.00 > 84.00	18	15	Lys
147.00 > 130.00	20	15	Gln
148.00 > 83.80	18	15	Glu
150.00 > 104.00	25	10	Met
166.00 > 119.80	20	15	Phe
175.00 > 70.40	30	17	Arg
205.00 > 188.00	18	12	Trp

Chapter 3:

Characterising the age-dependent changes associated with long-lasting BABA induced resistance

Declaration regarding published work in this chapter

Large amounts of the introduction, results and discussion of this chapter have been published as a paper currently under review which is available as a pre-print (https://doi.org/10.1101/2023.10.09.561512). The authors of this work declare the content of this chapter belongs solely to Katie Stevens who is the first author of this publication. The senior author Dr Estrella Luna Diez contributed to this work providing feedback during the writing process and making corrections on the manuscript.

Katie Stevens



Dr Estrella Luna Diez



3.1. Introduction

3.1.1 Threats to food security

The current food supply chain experiences major losses at the postharvest level due to both injury and infection by pathogenic fungi (Lipinski, 2013, Zhang et al., 2021). Tomato is a major global commodity, with 182.3 million tons of fruit produced in 2019, however, its yield is heavily restricted due to pathogens, and 50% of yield loss occurs at the postharvest stage (FAOSTAT, 2019). Pesticide use in tomato fruit postharvest is not allowed in commercial settings (Pétriacq et al., 2018). The current control measures in tomato against postharvest pathogens therefore include cold temperature storage and strict hygiene measures (Abbey et al., 2019). However, postharvest pathogens such as *Botrytis cinerea*, the causal agent of grey mould, cannot be successfully controlled with these strategies. *B. cinerea* affects plants and fruit of 230 crop species and its infection postharvest causes economic losses of up to $100 billion (Boddy, 2016). Therefore, new strategies are required. Greater understanding of tomato plant and fruit defence mechanisms would allow researchers to design strategies to control pre and postharvest fungal infections and reduce yield waste.

3.1.2 Plant priming

The adaptive component of the plant immune system can be referred to as priming (Mauch-Mani et al., 2017). Unlike direct activation of defence mechanisms, priming is a less costly defence strategy due to the targeted allocation of energy resources upon attack, thus resulting in a faster and stronger activation of defence mechanisms when they are required (van Hulten et al., 2006). Priming is considered to be broad spectrum and has been described in many different plant species, from Arabidopsis to *Malus pumila* (apple trees) (Cohen, 2002, Gur et al., 2021, Reuveni et al., 2003). Importantly, priming has been shown to be long-lasting (Catoni et al., 2022, Luna et al., 2020, Mageroy et al., 2020, Worrall et al., 2012) and to be transmitted to following generations (Luna et al., 2012, Rasmann et al., 2012, Slaughter et al., 2012).

A very well characterised priming chemical is the non-protein amino acid β-amino butyric acid (BABA), first identified in the 1960s (Davey, 1963). In subsequent decades BABA has been documented to be effective against both abiotic and biotic stresses in a range of species (Cohen et al., 2016). BABA-IR is associated with a range of changes to the plant such as enhanced physical protection through callose deposition, accumulation of Pathogenesis-related (PR) proteins and increased defence hormones such as salicylic acid (SA) and jasmonic acid (JA) (Hamiduzzaman et al., 2005, Schwarzenbacher et al., 2020, Ton et al., 2005, Ton and Mauch-Mani, 2004). BABA-IR works through both SA-dependent and -independent mechanisms (Ton and Mauch-Mani, 2004, Zimmerli et al., 2001). SA-independent IR is associated with abscisic acid (ABA) signalling (Ton and Mauch-Mani, 2004) and with priming of cell-wall defence through the effective deposition of callose. BABA treatment has been shown to lead to long-lasting protection when applied at the seedling stage in fruit tissue thus providing protection at the postharvest stage (Luna et al., 2020, Wilkinson et al., 2018). Metabolomic studies in tomato have demonstrated BABA's ability to prime multiple defence pathways (Luna et al., 2020) under the control of different defence-dependent hormones.

3.1.3 Epigenetically-regulated long-lasting priming

Long-lasting priming has been linked to epigenetic changes such as DNA methylation and the production of small interfering RNAs (siRNAs), as they can contribute to changes in gene expression (Catoni et al., 2022, Dowen et al., 2012, Hannan Parker et al., 2022, Rasmann et al., 2012, Slaughter et al., 2012). For instance, analysis of Arabidopsis epigenetic recombinant inbred lines (epiRILs) demonstrated that hypomethylated loci enhanced priming of SA-dependent and SA-independent defences against virulent *Hyaloperonospora arabidopsidis* (Furci et al., 2019). Moreover, siRNAs produced by the plant specific RNA-directed DNA methylation (RdDM) pathway have been associated with long-lasting and transgenerational induced resistance in Arabidopsis (Rasmann et al., 2012). Importantly, BABA has been shown

to be associated with changes in DNA methylation. For example, in potato, researchers recently demonstrated that spray treatments of 5 mM BABA led to hypomethylation of the promoter of the resistance gene *R3a* in parental plants and their progeny (Kuźnicki et al., 2019). In tomato, global changes to DNA methylation in the CHH context have been associated with long-lasting BABA-IR in the Moneymaker cultivar. Whilst many DMRs were found in promoters of differentially expressed genes in *B. cinerea* infection, the majority of primed genes were not differentially methylated (Catoni et al., 2022). Therefore, the mechanisms behind the long-lasting epigenetic nature of priming are currently still unclear.

3.1.4 Gaps in current understanding

The long-lasting nature of BABA-IR has yet to be fully explored and utilised for its potential role in postharvest research. Interestingly, tomato plants have been shown to have different methylation profiles depending on both fruit developmental stage and tissue type: CG and CHG methylation levels are lower in fruit tissue than 4-week-old leaf tissue, with the reverse pattern seen in CHH context (Zhong et al., 2013). However, how changes in developmental stage-dependent DNA methylation mediate the imprinting and the maintenance for long-lasting postharvest priming is yet to be explored. Furthermore, whilst a range of epigenetic marks have been associated with enhanced resistance, how long-lasting and dynamic these are over a plants lifetime is unknown. Therefore, in this chapter the influence of developmental stage on a plant's ability to maintain long-lasting priming against *B. cinerea* in tomato was tested. The impact of BABA treatment on a transcriptomic and epigenomic level at different developmental stages was assessed, and methylome analysis was conducted to test the hypothesis that young plants display greater epigenetic plasticity. Through whole genome bisulfite sequencing analysis, the durability of methylation marks was determined over the lifetime of plants, characterising the dynamic nature of defence priming. Finally, through the integration of various -omics approaches, potential markers associated with long-lasting BABA-IR in tomato were identified for the control of *B. cinerea* in fruit postharvest. Results

presented in this chapter investigate the influence of developmental stage on the plant's imprinting capacity and stress memory formation in plants. This work therefore aimed to aid in the identification of potential postharvest disease control strategies that exploit the plant immune system when it is at its maximum capacity of stimuli-dependent information acquisition.

3.2. Results

3.2.1 Plant developmental stage influences long-lasting BABA-IR

A time course experiment was used to investigate the dynamics of BABA-IR establishment in tomato (Fig 9a). A soil drench approach was used to prime with BABA the roots of plants at two different developmental stages, either at 2 or 12 weeks after germination (hereafter referred to as BABA2 and BABA12, respectively). Plant material was analysed at three developmental times, including leaves collected at 3- and 13-weeks post germination (referred to as T1 and T2, respectively) and in detached ripe, red fruit (referred to as T3). Mock-treated plants were used as controls for each of the timepoints considered. Disease progression of *B. cinerea* was assessed based on the size of lesions appearing after infection following inoculation of detached fruit harvested from treated plants, and in leaf tissue of the following plant generation. Long-lasting resistance was observed only in fruit collected from BABA2 plants and next-generational resistance in their progenies, while the infection of fruits from BABA12 plants and their progeny did not differ from that observed in mock-treated plants (Fig 9b and c). Although BABA can be phytotoxic in young seedlings, BABA2 treatment caused no significant reduction of relative growth rate (RGR) (Fig 9d). BABA12 plants displayed resistance in the short term against *B. cinerea* in leaf tissue only (Fig 9e).

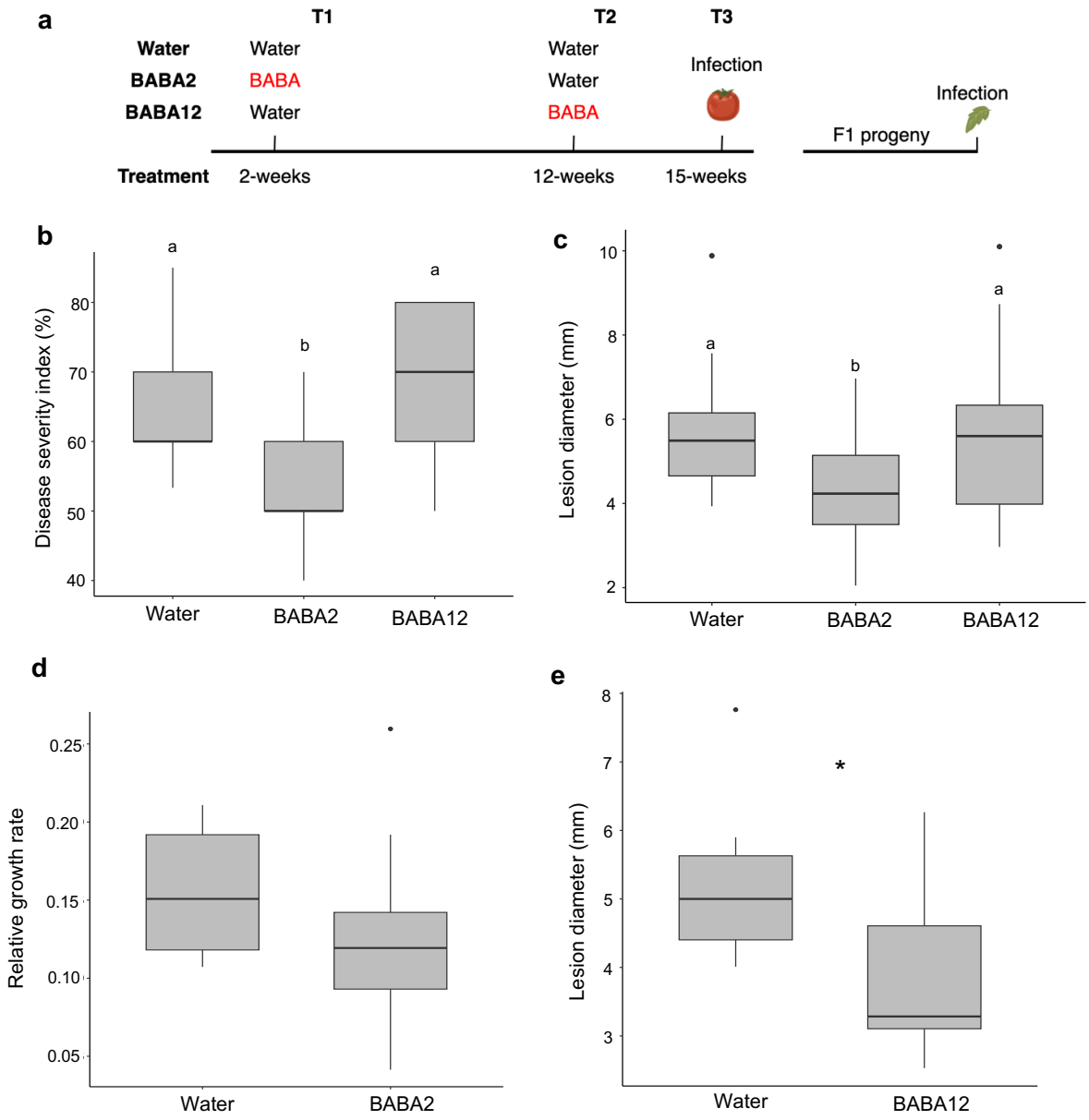


Figure 9: Experimental set up and phenotype data. a) Experimental setup, plants were treated with 0.5 mM BABA via soil drench at either 2 weeks (BABA2) or 12 weeks (BABA12). **b)** Disease severity index in ripe tomato fruit converted from the percentage of lesions in 6 disease categories at 2 days post infection (dpi) with *B. cinerea*. **c)** Lesion diameter in 3-week-old tomato progeny leaves caused by *B. cinerea* at 2 dpi. **d)** Average relative growth rates (in cm per cm per week; ± standard error of the mean [SEM] of plants between 2 and 3 weeks post 0.5 mM BABA treatment. **e)** Lesion diameter in 13-week-old tomato leaves caused by *B. cinerea* at 2dpi. Boxes denote 25th and 75th percentile, bars display min and max values. Different letters denote significant differences among treatment groups determined by One-Way ANOVA (tukey post hoc test; $p < 0,05$; n = 8-10). Asterisk denotes significant differences between treatments determined by a student's t-test ($p < 0,05$; n = 8-10).

3.2.2 BABA soil drench treatments before 6-weeks induce postharvest resistance

Having identified that soil drench treatments at 2-weeks generated postharvest resistance but 12-weeks failed to induce this resistance, BABA treatments were conducted at weekly timepoints to investigate the critical stage for forming long-lasting immune memory. Plants were treated with 0.5 mM BABA via soil drench from 2-12 weeks. Due to disease pressure, an insufficient number of replicates from each timepoint was generated. Individual timepoints were therefore grouped together into three treatment groups: 'BABA Y' (2-6 weeks), 'BABA O' (7-12 weeks) and water controls. A statistically significant difference in lesion diameter was observed between treatment groups, BABA Y plants displayed enhanced resistance compared to both water controls and BABA O (Fig 10). BABA treatment after 6-weeks therefore failed to induce postharvest resistance in fruit tissue, with plants displaying the same disease severity as water control plants.

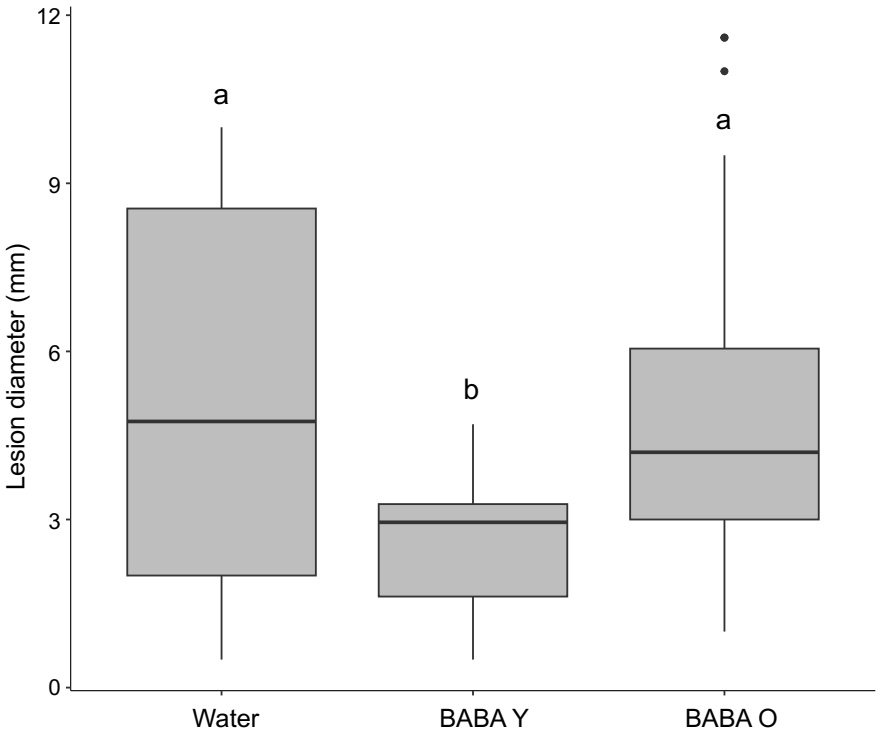


Figure 10: Impact of developmental stage on long-lasting-IR. Plants were treated with 0.5 mM BABA via soil drench at different timepoints between 2 and 12 weeks. 'BABA Y' refers to plants treated at 2-6 weeks, 'BABA O' represents plants treated between 6-12 weeks. Lesion diameter in ripe tomato fruit collected at 15 weeks was measured at 3 days post infection (dpi) with *B. cinerea.* Boxes denote 25th and 75th percentile, bars display min and max values. Different letters denote significant differences among treatment groups determined by One-Way ANOVA (tukey post hoc test; $p < 0.05$, n = 12-45).

3.2.3 Short term and long-term BABA priming are characterised by alternative transcriptomic profiles.

To test the priming mechanisms activated by BABA over both the short- and long-term, transcriptomic analysis was conducted on *B. cinerea* infected leaf (T1) and fruit tissue (T3) (Figure 6). Samples were analysed when disease symptoms were first observed at 24 and 48 hours after infection in leaves and fruit, respectively. Principal component analysis (PCA) revealed a clear separation between mock and infected samples in the short-term response. Furthermore, PC1 also differentiated BABA2 from control infected leaves (Fig 11a). In fruit tissue the impact of the infection was more pronounced (Fig 11b), but all the infected samples,

regardless of BABA treatment, clustered together. This result indicates that the BABA treatment has a stronger effect on gene expression in leaves shortly after its application, than in fruit at T3.

To identify genes that were common to responses at T1 and T3, and those that were unique to a single timepoint, comparisons were made between treatment-specific differentially expressed genes (DEGs) (Fig 11c-g) Lists of all T1 and T3 DEGs are available in Supplemental Table S1 and Supplemental Table S2 respectively. Considering that priming-based induced resistance is observed in fruit of BABA2 plants but not BABA12 and mock treated samples, it was hypothesised that by comparing the expression profiles among these conditions during infection, molecular determinants of BABA-IR could be distinguished from other unrelated effects of the BABA treatment. Primed genes were identified by filtering out those regulated by BABA treatment alone and those regulated by infection in the absence of BABA (Fig 11c-e). These genes were labelled as ‘BABA2 T1 primed’ and ‘BABA2 T3 primed’, depending on the timepoint when they were identified. A total of 598 and 971 DEGs were isolated in ‘BABA2 T1 primed’ and ‘BABA2 T3 primed', respectively. Therefore, it appears that early BABA treatment impacts a greater number of primed genes over the long-term compared to the short-term. 660 DEGs unique to BABA12 infection at T3 were also found (labelled ‘BABA12 T3 primed’). To assess how similar the DEGs after infection are between T1 and T3, 'BABA2 T1 primed’ and ‘BABA2 T3 primed’ genes were compared (Fig 11f). Only 25 genes out of a total 1569 genes were shared, indicating that plant tissue is a major driver of the transcriptomic priming profiles. Finally, comparing ‘BABA2 T3 primed’ genes with ‘BABA12 T3 primed’ indicated that 659 genes of a total 1,631 were shared (Fig 11g). However, since BABA12 displayed a susceptible phenotype in the long-term (Fig 9b and 6c), it is likely that those shared genes are not directly related to the enhanced resistance phenotype. Only 1 gene was unique to ‘BABA12 T3 primed’ genes. Only the 312 genes found differentially

expressed exclusively in the early BABA2 treatment, which could be linked to long-term resistance, were therefore considered in subsequent analyses.

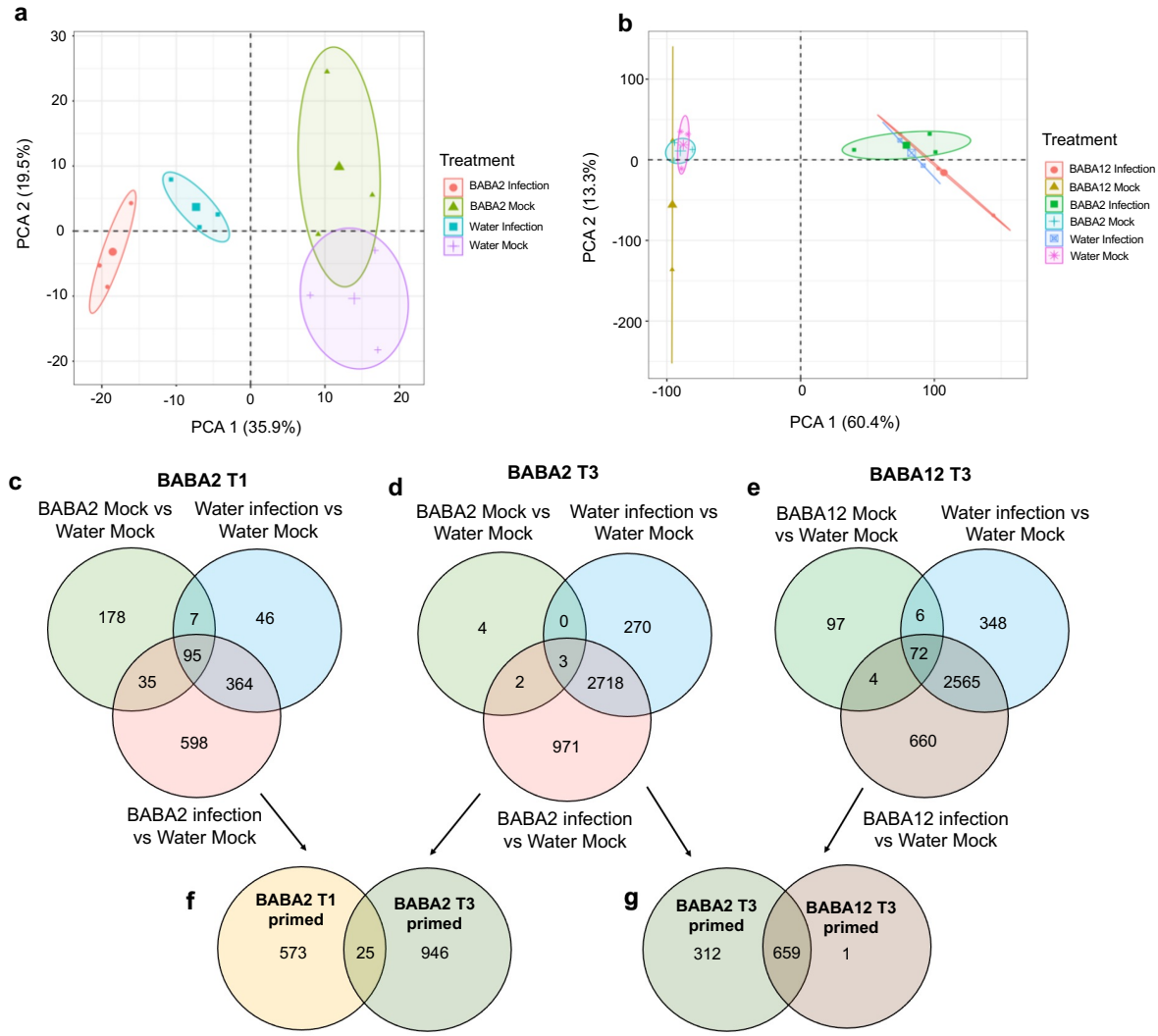


Figure 11: Transcriptome responses to *Botrytis cinerea* infection. Principal component analysis (PCA) of transcriptomic responses to *Botrytis cinerea* infection or mock inoculation in **a)** early BABA treated (BABA2) and control (water) 3-week-old tomato leaf tissue and **b)** BABA2, water and late BABA treated (BABA12) fruit tissue. Venn diagram comparisons between treatment-specific differentially expressed genes: **c)** BABA2 mock vs water mock, water infection vs water mock and BABA2 infection vs water mock at T1. **d)** BABA2 mock vs water mock, water infection vs water mock and BABA2 infection vs water mock at T3. **e)** BABA12 mock vs water mock, water infection vs water mock and BABA12 infection vs water mock. **f)** BABA2 T1 primed genes vs BABA2 T3 primed genes, **g)** BABA2 T3 primed genes vs BABA12 T3 primed genes.

3.2.4 BABA induced DNA methylation changes are dynamic and increase over time.

To explore whether epigenetic mechanisms are involved in long-term priming, changes in DNA methylation were investigated following treatments with BABA in each of the samples collected at the different timepoints of our analysis (Fig 7). Whole genome bisulfite sequencing (WGBS) was conducted to obtain DNA methylation profiles of each sample at a single cytosine resolution. No significant response to BABA treatment was seen at the global methylation level when each treated sample was compared to the corresponding mock control at the same timepoint (Fig 12a-c). However, substantial variation in global DNA methylation was observed depending on tissue type and developmental stage. For CG, lower levels of global methylation at T3 (fruit; 77.9%) than at T1 and T2 (leaves; 82.9% and 83.6%) (Fig 12a) were observed. Changes associated with the developmental stage were also seen in the CHH context, where global levels increased from 7.3% to 13.5% between T1 and T2 and to 15.4% in T3 in all treatment groups (Fig 12c). However, no variation in CHG methylation levels were found for either tissue type or developmental stage (Fig 12b).

To investigate the presence of specific DNA methylation patterns associated with BABA priming, differentially methylated regions (DMRs) were identified from samples treated with BABA and the corresponding mock control, for each timepoint. The total numbers of DMRs found in each context are listed in Table 2.

Table 2: Total number of 200 bp DMRs· in each cytosine context (CG, CHG, CHH). DMRs· compared to corresponding water control for each treatment group at each timepoint.

Timepoint	Treatment vs water	CG	CHG	CHH
T1	BABA2	714	1,720	1,751
T2	BABA2	1,125	2,137	2,777
	BABA12	692	1,783	2,297
T3	BABA2	1,518	2,858	5,425
	BABA12	2,104	3,552	2,836

Generally, the number of DMRs increases over time in all three cytosine contexts. This effect is most dramatic in the CHH context where the total number of DMRs increases over 3 times from T1 to T3 and 2 times from T2 to T3 in BABA2 plants (Table 2). In contrast, there was only a small increase in CHH DMRs between T2 and T3 for BABA12. There was a more noticeable increase in the numbers of CG and CHG DMRs in BABA12, which was not as pronounced in BABA2. Collectively, these results suggest that DNA methylation in tomato is primarily affected by the developmental stage, displaying increased variation in the CHH context and, less markedly, in the CG context. These results also indicate that the most prominent effect of BABA treatment occurs several weeks after the treatments, with the maximum difference measured in fruits, and many new DMRs arising weeks after BABA2 treatment.

The annotated features on the tomato genome were used to assess the overlap of BABA2 and BABA12 associated DMRs with features of interest. For all samples analysed, DMRs were primarily concentrated on Class I TEs in all cytosine contexts (Fig 13d), but also overlap a portion of genes and/or their promoters. Therefore, to identify differentially methylated genes (DMGs) that potentially contribute to priming, DMGs unique to the BABA2 phenotype were isolated. Lists of all DMGs are available in Supplemental Table S3. A total of 1,253 DMGs unique to BABA2 at T1, 1,427 at T2, and 2,957 at T3 were identified. Overlap of these DMGs revealed that only 54 were present in both T1 and T2, 115 in T1 and T3, 151 in T2 and T3. Only 7 DMGs were present at all three timepoints (Fig 13e). This indicates that early DMRs generated by BABA soil drench treatment at 2-weeks are generally not maintained into older leaves or fruit tissue.

As well as large changes between numbers, there are major differences in where DMRs are located between leaf and fruit tissue. For example, in the CG context 37% of BABA2 DMRs are found on Class I TEs, in T3 fruit tissue this increases to 50% (Appendix I, Fig 1a). This

difference between tissue types appears to be found in both BABA2 and BABA12 treated plants, for example within CHG DMRs, BABA12 T2 has 5% of DMRs found on genes, this increases to over 12% in T3 fruit tissue on genes. Differences between BABA2 and BABA12 treatments are seen at corresponding time points however, illustrating the importance of timing of BABA treatment. For example, in fruit tissue 5% of BABA2 CHG DMRS are located on genes however, in BABA12 plants 13% of CHG DMRs are found on genes (Appendix I, Fig 1b). Finally, differences are observed between individual cytosine contexts, for instance there are fewer Class I TEs in CHH context than CHG (approximately 32% in BABA2 T1 CHH vs 66% in BABA2 T1 CHG).

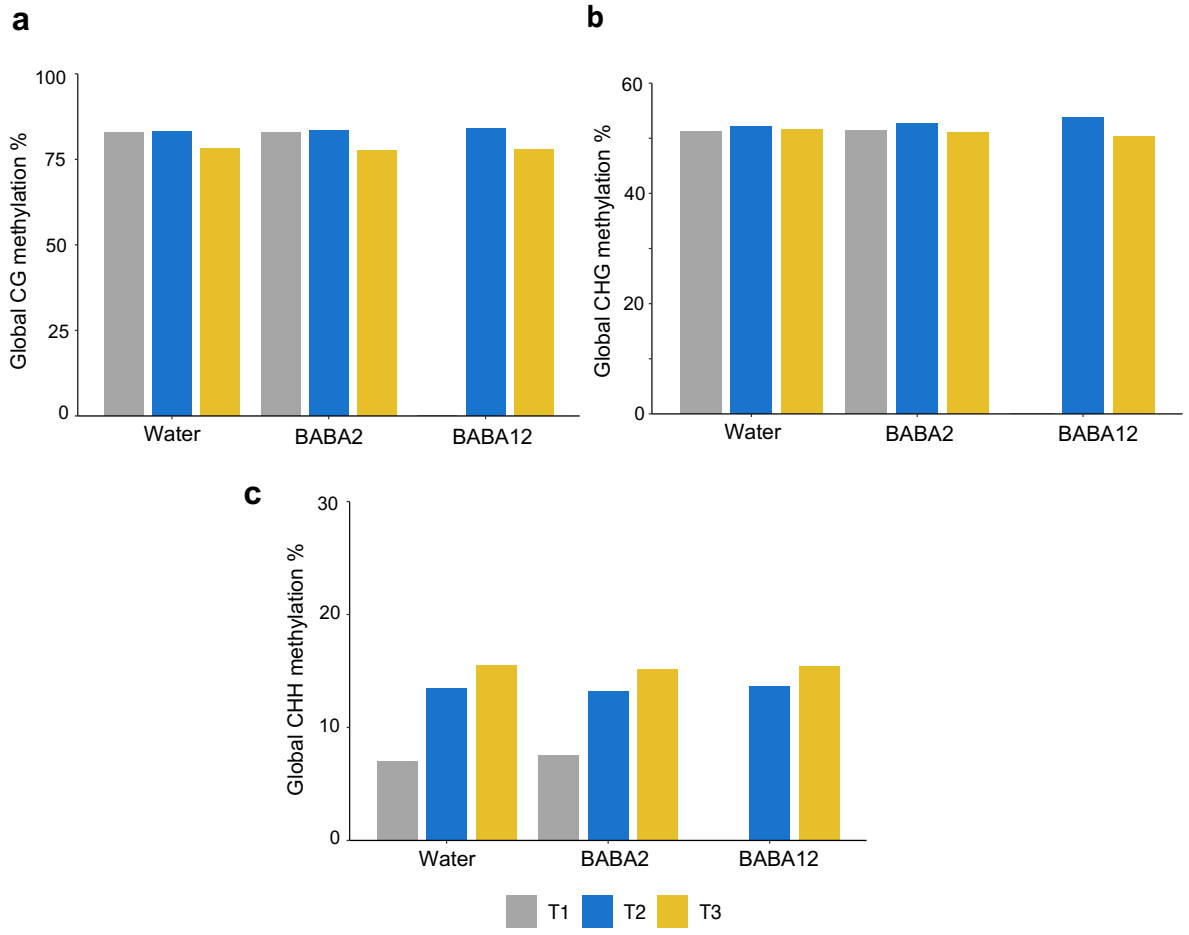


Figure 12: Global DNA methylation levels in tomato. Global methylation level across treatment groups in 3-week-old leaf tissue (T1, grey), 13-week-old leaf tissue (T2, blue), and ripe fruit tissue (T3, yellow) in each cytosine context **a)** CG, **b)** CHG, and **c)** CHH.

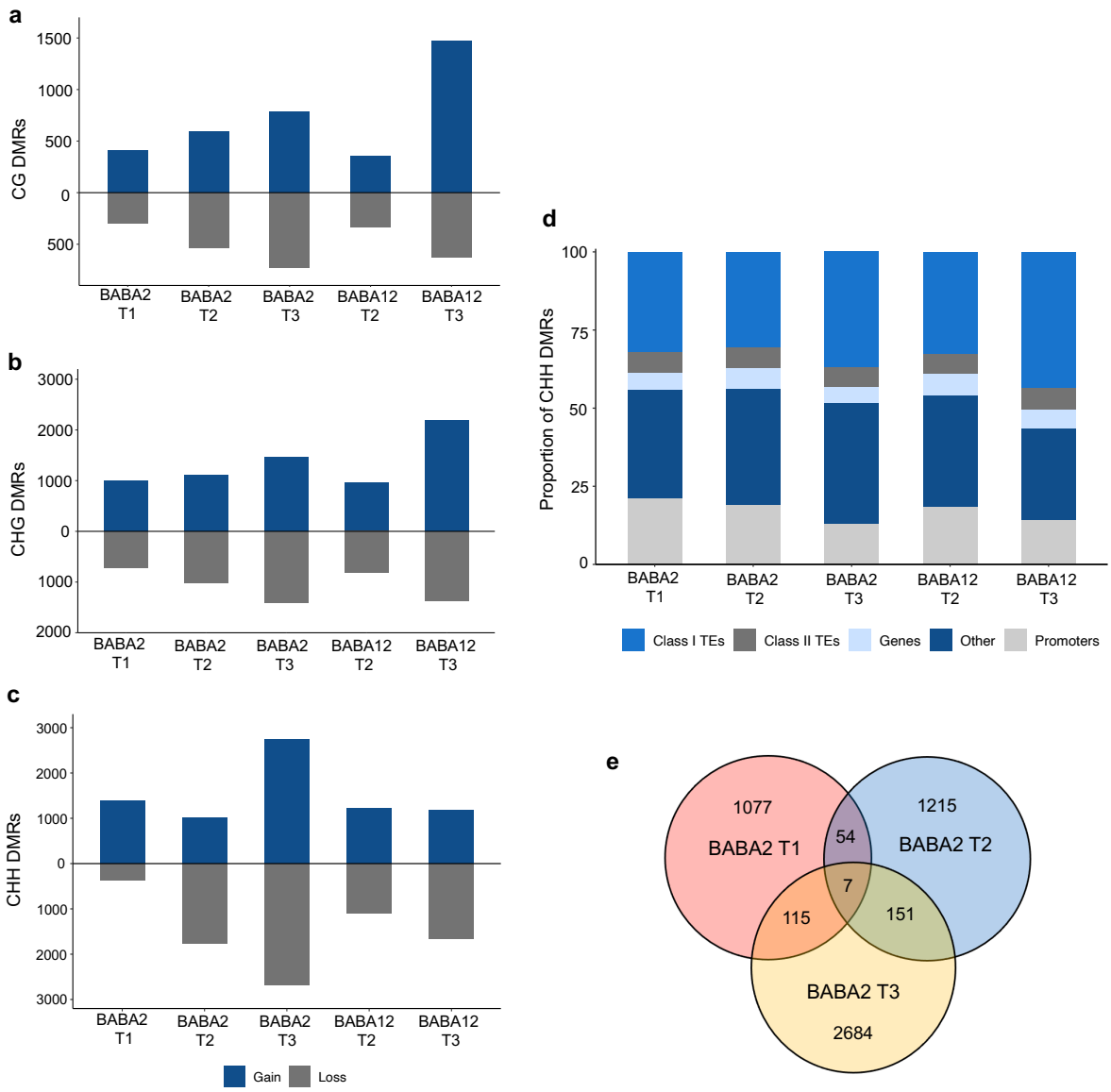


Figure 13: Differentially methylated regions in BABA treated plants. Total number of hyper-DMRs (blue) and hypo-DMRs (grey) across samples in each cytosine context **a)** CG, **b)** CHG, **c)** CHH. **d)** Location of DMRs across genomic features in BABA2 leaf tissue at 3-weeks. **e)** Total number and overlap of genes overlapping DMRs unique to BABA2 treated plants at T1 (red) T2 (blue) and T3 (yellow).

3.2.5 Genes associated with priming are not frequently methylated

To test whether transcriptional responses associated with BABA priming are under direct epigenetic regulation, DEGs resulting from the BABA treatment alone (i.e. BABA mock -

without infection; Fig 11c-e) and ‘BABA2 T1 primed’, ‘BABA2 T3 primed’ and ‘BABA12 T3 primed’ genes (Fig 11f and g) were compared with DMGs. Venn diagram analysis revealed that very few genes associated with a BABA transcriptional response were also differentially methylated (Fig 14a-c). At T1, only 25 out of 573 ‘BABA2 T1 primed’ genes were also differentially methylated in any cytosine context, the majority of which were hypermethylated and in the CHH context (21/25 and 13/25, respectively) (Table 3; Fig 14a). At T3, no DEGs responding to early BABA treatment without infection were differentially methylated and only 16 genes were both ‘BABA2 T3 primed’ and differentially methylated (Fig 14b). Similarly, to timepoint T1, a greater number of DMGs were in the CHH context but there was a more even distribution between methylation status and gene expression (9 hypermethylated DMRs, 9 down-regulated genes) (Table 3). Interestingly there was no clear classically associated pattern in terms of direction of DMR status and gene expression level. For example, the most up-regulated gene, an O-acyltransferase WSD1-like, was up-regulated 6.72-fold and hypermethylated in the CG context.

The data presented in Fig 11 and Fig 13h show that the effects of BABA on gene expression and DNA methylation vary substantially across the different treatment and sampling points. This raised the question if there might be greater similarity in biological impacts when viewed at higher levels of organisation. Gene ontology (GO) term enrichment analysis was therefore performed with the full non-redundant lists of DEGs and DMGs contained in the Venn diagrams in Fig 14a, b and c. BABA2 T1 genes were enriched in biological processes associated with “regulation of systemic acquired resistance”, “photosynthesis, light harvesting”, “systemic acquired resistance” and “regulation of immune system processes” (Fig 14d). BABA2 T3 genes displayed enrichment in the biological processes “endosperm development”, “rRNA (metabolic) processing”, “regulation of developmental processes”, “response to abiotic stimuli” (Fig 14e). Finally, BABA12 T3 genes were enriched in “tetraterpenoid biosynthetic proc.”, “carotenoid biosynthetic proc.” and “carotenoid metabolic proc.” (Fig 14f). There was therefore very little similarity between the biological processes

impacted by BABA2 at T1 (Fig 14d), BABA2 at T3 (Fig 14e) and BABA12 at T3 (Fig 14f). This reinforces the conclusion that different pathways are associated with short-term and long-term priming mechanisms generated by BABA and that the timing of application is crucial for the priming of specific pathways that result in enhanced resistance long-term.

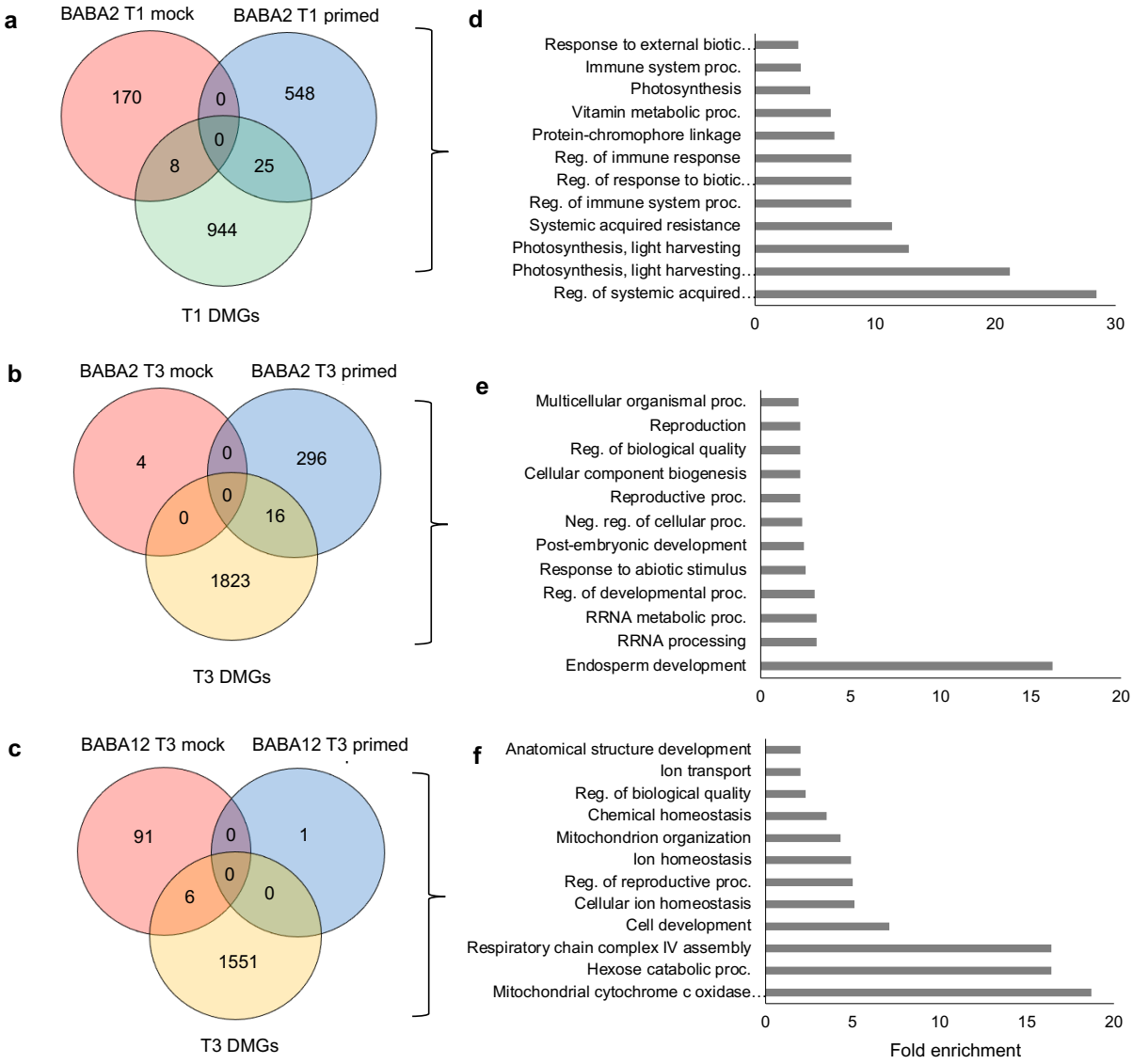


Figure 14: Overlap and enrichment of primed and differentially methylated genes. **a)** Genes associated with BABA2 T1 (red representing BABA2 treated only DEGs, blue representing BABA2 + infection DEGs ('primed'), green representing T1 BABA2 DMRs), **b)** Enrichment of BABA2 T1 genes of interest. **c)** Genes associated with BABA2 T3 (red representing BABA2 treated only DEGs, blue representing BABA2 + infection DEGs ('primed'), yellow representing T3 BABA2 DMRs). **d)** Enrichment of BABA2 T3 genes of interest. **e)** Genes associated with BABA12 T3 (red representing BABA12 treated only DEGs, blue representing BABA12 + infection DEGs ('primed'), yellow representing T3 BABA12 DMRs). **f)** Enrichment of BABA12 T3 genes of interest.

Table 3: Methylation status, gene expression and gene description of BABA2 primed genes which are simultaneously differentially methylated genes (DMGs).

Gene	DMR direction	Methylation context	Log2-Fold Change	Timepoint	ITAG 4.0 description
Solyc01g095960.4	Gain	CG	6.722	T1	O-acyltransferase WSD1-like
Solyc01g097390.4	Gain	CG	1.203	T1	NAD(P)-linked oxidoreductase, aldo/keto reductase family protein
Solyc01g099660.4	Gain	CHH	2.328	T1	Heat shock protein 70 family
Solyc01g163210.1	Gain	CHH	1.317	T1	Unknown protein
Solyc02g069260.3	Loss	CHG	1.049	T1	Argonaute2a
Solyc03g070380.3	Gain	CHH	1.268	T1	Methyl esterase 1
Solyc03g098010.3	Gain	CHH	1.701	T1	Phosphate starvation inducible gene TPSI1
Solyc04g006980.1	Gain	CHG	1.592	T1	TCP transcription factor 22
Solyc04g077810.1	Gain	CHH	1.161	T1	ZCF37
Solyc06g060560.3	Gain	CG	-1.478	T1	Hypothetical protein
Solyc06g066680.3	Gain	CHH	-1.673	T1	Mitochondrial carrier protein
Solyc06g070990.3	Loss	CHH	1.343	T1	WRKY transcription factor 74
Solyc07g009380.5	Gain	CHH	-1.185	T1	Xyloglucan endotransglucosylase-hydrolase 2
Solyc07g055950.3	Gain	CHH	1.778	T1	Protodermal factor 1
Solyc08g077170.3	Gain	CHH	-2.081	T1	Protein NRT1/ PTR FAMILY 7.3
Solyc08g077900.3	Gain	CG	1.413	T1	Expansin-like B1
Solyc08g079235.2	Gain	CG	1.071	T1	Unknown protein
Solyc08g080090.3	Gain	CG	-1.034	T1	Green flesh
Solyc08g160370.1	Gain	CHG	1.541	T1	Unknown protein
Solyc09g014820.3	Gain	CHG	2.296	T1	Protein LURP-one-related 15
Solyc09g072610.3	Loss	CHG	1.121	T1	Long-chain-alcohol oxidase
Solyc09g090500.3	Gain	CHH	1.761	T1	Cyclopropane-fatty-acyl-phospholipid synthase
Solyc10g008200.4	Gain	CHH	1.833	T1	Tyrosine aminotransferase
Solyc10g074440.2	Gain	CHG	1.475	T1	Chitinase
Solyc10g086710.3	Loss	CHH	1.147	T1	PAR1

Solyc01g008260.5	Gain	CHH	-1.372	T3	E2F-associated phosphoprotein
Solyc01g067090.4	Loss	CG	1.859	T3	Arginine-glutamic acid dipeptide repeat protein
Solyc01g080490.2	Loss	CHH	-3.596	T3	DUF679 domain-containing protein
Solyc02g070780.5	Loss	CHH	2.139	T3	DNA helicase
Solyc02g084400.4	Gain	CG	-4.645	T3	Protein kinase superfamily protein
Solyc02g084730.3	Gain	CHH	1.559	T3	rRNA adenine N(6)-methyltransferase
Solyc02g089780.3	Gain	CHG	-2.622	T3	Dihydroflavonol 4-reductase/flavanone protein
Solyc02g092250.3	Loss	CG	3.197	T3	Cytochrome
Solyc03g043850.3	Gain	CG	-1.445	T3	Cytochrome c oxidase subunit 6a, mitochondrial-like
Solyc04g056713.1	Loss	CHG	-2.187	T3	Aldehyde dehydrogenase family 2 member mitochondrial-like
Solyc05g047580.5	Gain	CHH	5.853	T3	Terpene cyclase/mutase family member
Solyc05g052240.3	Gain	CHH	-2.285	T3	Chalcone-flavonone isomerase family protein
Solyc06g066520.1	Gain	CG	6.218	T3	Avr9/Cf-9 rapidly elicited protein 146
Solyc07g063930.4	Gain	CHH	-1.374	T3	Organic cation/carnitine transporter 4
Solyc09g015170.4	Loss	CHH	4.269	T3	Receptor-like kinase
Solyc09g065180.3	Loss	CHH	-2.572	T3	Binding protein precursor AF106660

3.2.6 Methylation inhibitors induce effective short-term resistance against *B. cinerea*

WGBS analysis revealed that early BABA treatment induced an increase in hypermethylated CHH DMRs which was not seen in susceptible (BABA12) plants following late soil drench application. It was therefore hypothesised that application of zebularine, a cytosine analogue known to reduce levels of DNA methylation (Baubec et al., 2009), may have an opposite effect to our BABA priming agent and would induce susceptibility against *B. cinerea*. Two-week-old seedlings were therefore soil drenched with final concentrations of 40 µM and 80 µM zebularine, mock treated plants were treated with water containing equal quantities of dimethylsulfoxide (DMSO). Seedlings treated with 80 µM zebularine displayed a reduction in

RGR however this was not statistically significant, no difference was observed in plants treated with 40 µM zebularine (Fig 15a). One week after treatment, zebularine treated plants displayed enhanced resistance in leaf tissue against *B. cinerea* at 3 dpi compared to control treated plants (Fig 15b). This enhanced resistance however was not long-lasting; no postharvest resistance was observed in fruit of zebularine treated plants against *B. cinerea* (Fig 15c).

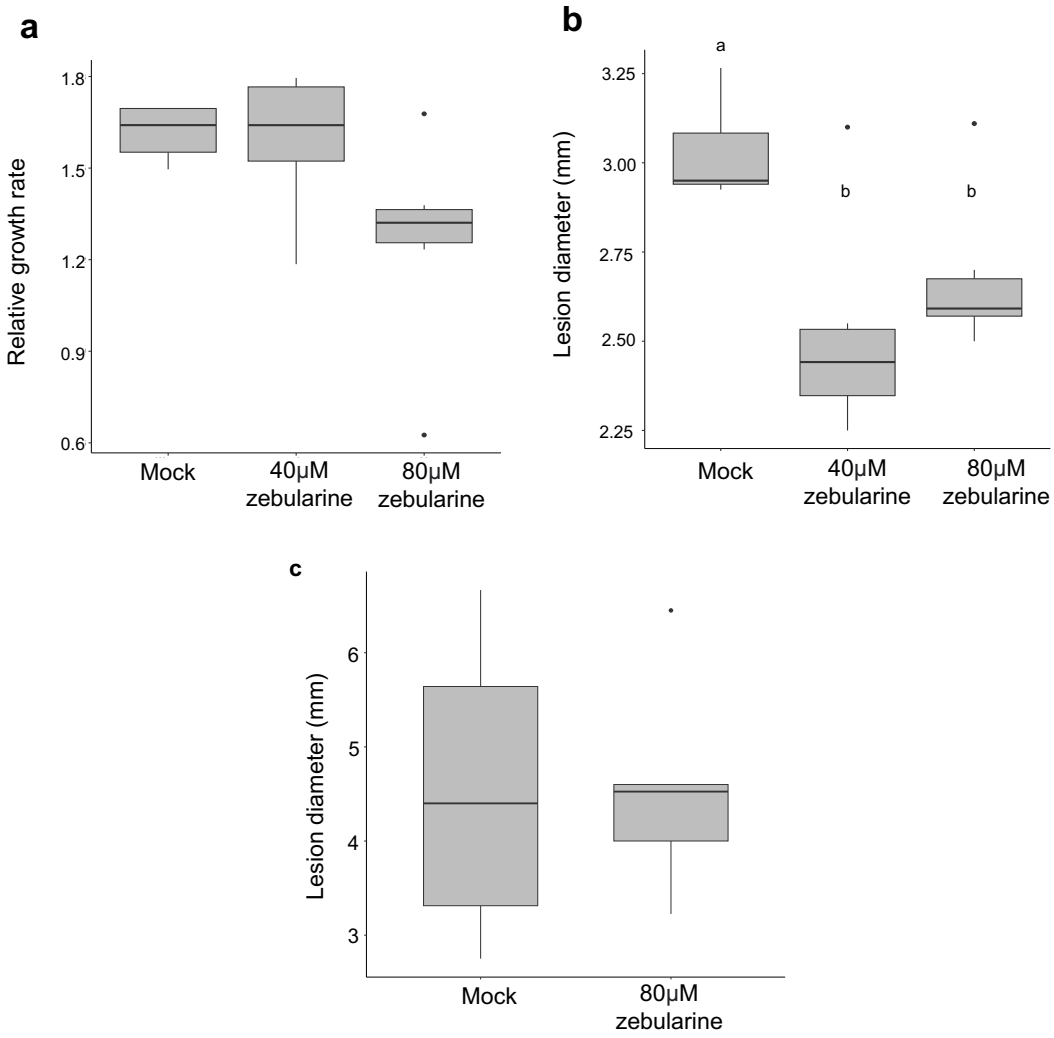


Figure 15: Phenotype of zebularine treated plants. **a)** Average relative growth rates (in cm per cm per week; ± standard error of the mean [SEM] of plants between 2 and 3 weeks post zebularine soil drench treatment. **b)** Lesion diameter in 3-week-old tomato leaves infected with *B. cinerea* at 2 days post infection (dpi). **c)** Disease severity index in ripe tomato fruit converted from the percentage of lesions in 6 disease categories at 2 dpi with *B. cinerea*. Boxes denote 25th and 75th percentile, bars display min and max values. Different letters in b denote significant differences among treatment groups determined by One-Way ANOVA (LSD post hoc test; $p < 0.05$, n = 5-6).

Following the unexpected finding of strong short-term zebularine IR in leaf tissue, further experiments were conducted. Combinations of BABA and zebularine were used to assess if these chemicals displayed a synergistic or complementary effect. Seeds were therefore pre-germinated on 80 µM of zebularine. Seedlings germinated on 80 µM zebularine displayed a statistically significant reduction in their height compared to water controls (Fig 16a). At 2.5 weeks soil was drenched to a final BABA concentration of 0.5 mM. The following combinations were tested: Mock Water, Mock BABA, Zebularine Water and Zebularine BABA. Resistance assays were conducted at three timepoints: 1 week post BABA treatment in leaf tissue, 6 weeks post BABA treatment in leaf tissue and in ripe red fruit tissue. One week post treatment, water germinated plants displayed enhanced resistance against *B. cinerea* following BABA treatment compared to mocks. However, seeds germinated on zebularine displayed the highest level of resistance irrespective of BABA treatment (Fig 16b). Six weeks post BABA treatment, mock BABA plants displayed the same resistance level as plants germinated on 80 µM zebularine (Fig 16c). Finally, all plants germinated on zebularine failed to display enhanced resistance following *B. cinerea* infection in fruit tissue. The highest levels of resistance in fruit tissue were seen in Mock BABA plants (Fig 16d). This therefore demonstrates that whilst zebularine provides highly effective short-term resistance against *B. cinerea,* this protection is not as long-lasting as BABA treatments. Furthermore, this phenotype suggests that these two chemicals may be targeting different pathways to induce resistance.

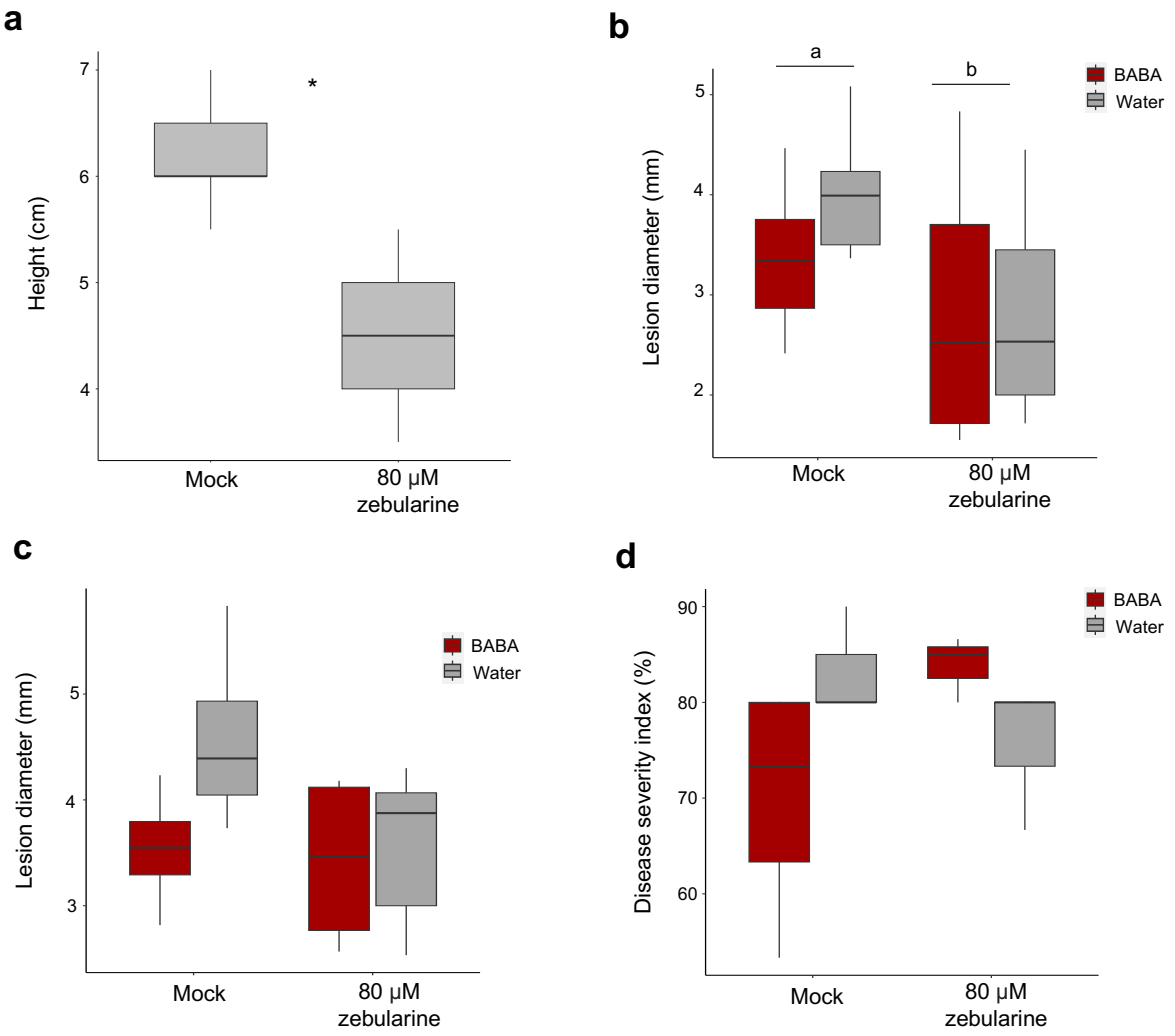


Figure 16: Phenotype data of zebularine and BABA treated plants. a) Average height of 2-week-old tomato plants germinated on 80 µM zebularine-soaked filter paper or mock treated. **b)** Lesion diameter in *B. cinerea* infected leaf tissue 1-week post 0.5 mM BABA (red) or water (grey) soil drench treatment in zebularine of mock germinated seedlings at 2 days post infection (dpi). **c)** Lesion diameter in *B. cinerea* infected leaf tissue 6-weeks post 0.5 mM BABA or water soil drench treatment in zebularine of mock germinated seedlings at 2 dpi. **d)** Disease severity index (DSI) in *B. cinerea* infected fruit tissue 6-weeks post BABA or water soil drench treatment in zebularine of mock germinated seedlings at 3 dpi. Boxes denote 25th and 75th percentile, bars display min and max values. Asterisk denotes significant differences between groups determined by a student's t-test ($p < 0,05$; n = 3-8). Different letters denote significant difference between groups determined by a Two-way ANOVA ($p< 0.05$, n = 3-8).

3.3. Discussion

3.3.1 The importance of age in the establishment of durable defence priming

In this chapter the hypothesis that plant age is a critical factor for the generation and maintenance of long-lasting defence priming has been explored. Moreover, this work has investigated how epigenetic mechanisms such as DNA methylation play a role in this phenomenon. It was shown that treatment of 2-week-old tomato seedlings (BABA2) with the chemical priming elicitor BABA leads to long-lasting postharvest resistance in fruit, and that this resistance phenotype was not observed if BABA treatment is conducted in older 12-week-old plants (Fig 9b). These results corroborate previous research where only priming of seedlings, unlike treatments of 10- and 12-week-old plants, led to long-lasting postharvest resistance (Wilkinson et al., 2018).

Work in this chapter delved further into the specificity of the timing of BABA application, with treatments conducted on a weekly basis to determine at which point between 2 and 12 weeks the ability of plants to generate a long-lasting priming response is lost. Due to problems with disease pressure it was not possible to generate a high number of replicates, however pooling timepoints into categories revealed a clear phenotype. Plants that received BABA treatments before 6-weeks produced fruit with enhanced resistance against *B. cinerea,* with soil drench after this failing to induce significant resistance (Fig 10). This critical timepoint coincides with the production of flowers and fruit in the Micro Tom cultivar used. This raises the hypothesis that early BABA treatment may lead to pivotal changes which impact the regulation of key genes involved in fruit defence, thus facilitating long-term priming and resistance. Application of BABA after fruit production has been established may therefore fail to lead to pivotal changes which facilitate long-term immunological memory, thus explaining the short term, but not long-term resistance observed in BABA12 treated plants (Fig 9e). Separate mechanisms regulating age dependent long-term resistance and short-term resistance would additionally explain why postharvest application of BABA has been shown to be effective in inducing

enhanced resistance in a variety of crop species (Wang et al., 2019). Experiments were additionally performed to test transgenerational resistance after priming at different developmental stages. Similar to the long-lasting resistance observed in fruit, only priming treatments of young seedlings led to heritable resistance against *B. cinerea* in following generations (Fig 9c). Given existing knowledge on the heritability of epigenetic marks, one may speculate this resistance is attributed to changes in DNA methylation facilitated by sRNAs (Bond and Baulcombe, 2014). Profiling the DNA methylation landscape in progenies would therefore provide insight into important regulators of this. Furthermore, continuing to test the resistance in successive generations would reveal how robust this resistant phenotype is, as well as insight into what point plants 'forget' this immunological memory. These results have therefore demonstrated that the timing of BABA treatment is critical for maintenance of long-lasting postharvest defence, and next-generational resistance against *B. cinerea*.

3.3.2 Tissue specific transcriptomic responses in BABA primed plants infected with *B. cinerea*

The durable postharvest defence phenotype has been linked to priming of different defence mechanisms. In this study, the transcriptomic priming profiles from two different timepoints (i.e. T1 in leaves and T3 in fruit) were analysed with and without infection with *B. cinerea*. This transcriptome analysis therefore complements previous investigations which focused on the study of long-lasting priming through metabolomic analysis (Luna et al., 2020, Wilkinson et al., 2018). Both approaches have elucidated mechanisms by which long-lasting resistance is generated and maintained, however, a crucial difference has been unravelled in mock fruit samples where no infection was applied. BABA treatment led to a clear signature at the transcriptional level in T1 (Fig 11a), but this was lost at T3 with only 7 genes differentially expressed (Fig 11b, d). This indicates that whilst the memory of BABA treatment is maintained sufficiently to contribute to postharvest resistance, is not linked with major global changes in basal transcription. In contrast, previous metabolomics studies of BABA2-treated tomatoes

demonstrated major changes in uninfected fruit, which were suggested to play a role in long-lasting resistance (Luna et al., 2020, Wilkinson et al., 2018). BABA treatment therefore has a longer-lasting priming fingerprint in fruit tissue in the metabolome compared to the transcriptome. These differences in the persistence of metabolite accumulation or gene transcription have been identified previously in priming (Schwachtje et al., 2019) and have been attributed to faster rearrangement of the transcriptional profile and long-term storage of metabolites in the vacuole, or rearrangement of metabolites to their inactive conjugates (Schwachtje et al., 2019).

Interestingly, transcriptome analyses performed after infection with *B. cinerea* demonstrated major differences in the transcriptomic priming profiles of both young leaf (T1) and red fruit tissue (T3) (Fig 11a, b). These differences suggest that BABA treatment is likely to be having a transcriptional pivotal effect and that tissue type is a major driver of response to *B. cinerea* infection. Whilst the latter offers a straightforward explanation to these differences, this alone is not sufficient as a considerable number of DEGs unique to the BABA2 treatment were observed in fruit tissue, whilst BABA12 infected plants displayed only one unique DEG in fruit tissue when compared to BABA2 (Fig 11g). Thus, whilst tissue type is the major determinant of BABA priming, only the early application of BABA treatment is leading to greater specificity in the priming transcriptional response, which then can contribute to the resistance phenotype observed in BABA2.

3.3.3 Age dependent methylation profiles

Interestingly, WGBS data demonstrates that CHH methylation levels in leaf tissue doubles substantially over 10 weeks from 7% to 14% (Fig 12c). This is in agreement with various studies that have demonstrated that global methylation levels increase with chronological age in many plant species (Dubrovina and Kiselev, 2016). For example, Valledor et al demonstrated that needle maturation of *P. radiata* is associated with an increase in global

DNA methylation of ~3% over 12 months (Valledor et al., 2010). Taking this into consideration and the fact that BABA treatment of seedlings results in a unique gain in CHH methylation (Fig 13f), this raises the hypothesis that young seedlings display greater epigenetic plasticity, which allows them to facilitate a strong priming imprinting following BABA treatment. Following the identification of the optimal timepoint for BABA application (Fig 10), it would be interesting to determine if low basal CHH methylation does influence epigenetic plasticity by assessing the global CHH methylation profile over a time course. Additionally, the use of technology such as formaldehyde-assisted isolation of regulatory element sequencing (FAIRE-seq) or transposon accessibility-based assays (ATAC-seq) may provide further insight into the genomic landscape at the time of successful imprinting of priming. Whilst notable differences in the chromatin accessibility and subsequent transcription factor binding of cell types has been demonstrated, little has been explored in the effect of developmental stage in crops (Sijacic et al., 2018). Work in Arabidopsis has demonstrated that repression of *WRKY53* expression by the DNA binding protein WHIRLY1 (WHY1) is dependent on developmental stage, with chromatin immunoprecipitation assays displaying major differences in protein accumulation between 6- and 8-week-old plants (Van Peer et al., 1991). Furthermore, histone modifications of the *WRKY53* promoter have been shown to be developmental stage dependent (Liu et al., 2019). Thus, given the epigenetic and transcriptional changes known to occur following priming treatment, as well as age-dependent changes to accessibility and transcriptional control, BABA treatment may be influencing key pathways in an age-dependent manner to facilitate long-lasting resistance.

3.3.4 Characterising the epigenetic changes induced by BABA priming

Epigenetic modifications have been shown to be involved in the acquisition and maintenance of long-lasting induced resistance and priming (Alonso et al., 2019, Crisp et al., 2016). Specifically, plant stress memories have been shown to be associated to changes in DNA methylation which are maintained throughout the life of a plant and even transgenerationally

(Quadrana and Colot, 2016, Stassen et al., 2018). DNA methylation has been previously associated with long-lasting resistance in a variety of species (Catoni et al., 2022, Hannan Parker et al., 2022, Wang et al., 2021). In tomato using the cultivar Moneymaker, Catoni et al recently demonstrated that BABA led to global hypomethylation following 0.5 mM treatment, which was most dramatic in the CHH context where levels were reduced by 38% (Catoni et al., 2022). To address the hypothesis that young plants display greater epigenetic imprinting capacity, WGBS was performed at different timepoints and from different tissue types after BABA treatment at 2-week-old and 12-week-old plants. Interestingly, contrary to what was reported by Catoni et al on BABA-induced global methylation changes, no global changes were observed following BABA treatment at 2 or 12 weeks (Fig 12). This demonstrates that the priming treatment here does not lead to major epigenetic changes in Micro Tom. This contrasting observation within the same plant species and priming elicitor may be due to the known differences on BABA perception between the tomato cultivars used in the studies. BABA is known to trigger a stress phenotype associated with its perception by the plant (Luna et al., 2014b) and the relocation of resources from growth to defence (van Hulten et al., 2006). This manifests mainly as a growth reduction. Whereas in these experiments in Micro Tom no statistically significant BABA-induced reduction in RGR was observed (Fig 9d), in Moneymaker, seedling treatment with 0.5 mM BABA results in a statistically significant ~20% reduction in the RGR (Luna et al., 2016). Therefore, it is easy to speculate that the global changes in methylation levels in Moneymaker are associated with BABA-induced stress upon perception of the elicitor. In addition, experiments in the relative Solanaceae species potato have reported that BABA leads to genome-wide DNA hypermethylation. However, this phenotype was only observed at very early time points (i.e., 1h to 6h) after spraying of BABA and it was lost after 24h. Again, one may speculate that the rapid global changes observed here may respond to the stress-inducing nature of BABA, which upon spraying has been described to trigger cell death (Bengtsson et al., 2014). Experiments in Micro Tom here have shown that BABA does not lead to major changes in epigenetic reprogramming. Furthermore, the experimental design used in this study of both BABA2 which induces long-term resistance

and BABA12 which fails to do so, allows us to specifically isolate the effects of BABA-IR from BABA-triggered stress.

Despite the absence of global methylation changes found between treatments (Fig 12a-c), DMR analysis unravelled crucial differences between short-term and long-term priming responses. For instance, DMRs produced initially after BABA2 treatment are not maintained throughout the life of the plant, thus further supporting the observation that BABA has a pivotal effect in DNA methylation changes. In addition, young plants had an unbalanced response in DMRs to BABA treatment, with more hypermethylated DMRs present than hypomethylated (Fig 13a-c). This observation was particularly pronounced in the CHH context (Fig 13c). In contrast, this pattern was not observed one week post late BABA treatment (BABA12 T2) (Fig 13c). Moreover, differences were also seen at T3 where BABA2 fruit have twice the number of DMRs than BABA12 fruit. These are highly interesting observations as CHH methylation has been described to play a relevant role in response to stimuli and stress (López et al., 2011), and in the last decade, it has become an epigenetic modification marking the maintenance and expression of long-lasting priming (Catoni et al., 2022, Wang et al., 2021). Finally, the observation that DMRs associated to BABA2 are not maintained from seedlings to fruiting (Fig 13e) extends the hypothesis towards the importance of foundational changes at early stages of development, which could result in pivotal effects thus setting the basis of future development of methylation profiles underpinning priming and resistance. Importantly, in addition to the described changes in CHH methylation, our ‘primed genes’ (Fig 11 and 14) most frequently overlap with DMRs in the CHH context at both T1 and T3. These findings therefore highlight the need for further investigation into the mechanisms impacting CHH methylation, which is controlled by the RdDM machinery through the production of mobile sRNAs. The RdDM pathway has been frequently associated with defence against pathogens and with the expression and maintenance of long-lasting priming. Therefore, one could speculate that the RdDM pathway plays a role in the establishment and maintenance of long-lasting priming in tomato.

3.3.5 The overlap of BABA primed DEGs and DMRs

Whilst analysis of transcriptomic and methylation datasets demonstrates that generally primed genes are not under direct epigenetic control. A small number of genes display strong correlation in their expression and methylation status (Table 3). For example, the receptor-like kinase, Solyc09g015170.4 was up-regulated 4.269-fold and displayed loss of CHH methylation at T3. The protein kinase superfamily protein, Solyc02g084400.4, in contrast displayed CG hypermethylation and was subsequently down-regulated 4.645-fold. Protein kinases are known to be critical for innate immune responses, these proteins are dynamically modulated to ensure precise regulation of the intensity and duration of immune responses to ensure a balanced trade-off between growth and defence (Tang et al., 2017). Interestingly a NRT1/PTR family protein, Solyc08g077170.3, displayed CHH hypermethylation and 2.081-fold downregulation in infected leaf tissue. These proteins are nitrate and peptide transporters and have more recently been demonstrated to have broad substrate specificity, transporting plant hormones IAA, ABA, gibberellin (GA) and glucosinolates (Chiba et al., 2015, Corratgé-Faillie and Lacombe, 2017). Whilst the role of transporters have so far been implicated for their role in perception of BABA, given the major perturbations in hormone levels known to be induced by BABA treatment, how transporters are primed and perhaps epigenetically-regulated may warrant further investigation. Here a correlation between methylation status and expression in infected tissue has been identified following early BABA treatment. Transcriptional control of defence genes has been demonstrated to be a critical regulatory aspect of plant immunity. Recently, *ROS1*-directed demethylation of defence gene promoters was shown to be critical to ensure binding of PAMP-responsive WRKY-TFs (Halter et al., 2021). Further analysis is required to understand the downstream impact of BABA-induced methylation changes to these defence associated genes.

Transcriptome analysis identified a range of DEGs with recognised functions in immunity which were differentially methylated in the opposite direction to what is classically associated with transcriptional regulation (Table 3, Fig 11). For example, the *heat shock protein 70*

(*HSP70)* is hypermethylated in the CHH context and simultaneously up-regulated 2.328-fold in *B. cinerea* infected primed leaf tissue. HSP70 is known to participate in both PTI and ETI and is linked to endoplasmic reticulum (ER) supported immunity functions as well as regulation by defence associated phytohormones such as ABA, JA, ET and cytokinin (CK) (Berka et al., 2022). Interestingly in grapes (*Vitis vinifera*), priming with BABA has been suggested to lead to activation of *NPR1*. Mutants of *HSP24* were impaired in NPR1 and SA-associated signalling and displayed enhanced susceptibility. The authors suggest that BABA priming post-translationally modifieds VvHSP24 which leads to activation of VvNPR1 and resistance (Li et al., 2021). In fruit tissue, Solyc06g066520.1, Avr9/Cf-9 rapidly elicited gene 146 (*ACRE146*) is highly up-regulated, 6.22-fold and hypermethylated in the CG context (Table 3). ACRE genes are known to be involved in both early plant defence responses and R gene mediated resistance. Recently, two ACRE genes; *ACRE180* and *ACRE75* were shown to display primed expression in chitosan treated tomato plants during infection with *B. cinerea* in fruit tissue. Furthermore, over-expression of these genes in *N. benthamiana* led to enhanced resistance (De Vega et al., 2021). Here *ACRE146* displays a similar priming profile and differential methylation. Further analysis on the dynamic nature of both methylation status and expression level may provide insight into if these may be potential important marker genes regulating postharvest resistance which additionally may be under epigenetic control.

3.3.6 The impact of methylation inhibitors on resistance phenotypes

Work in this chapter demonstrated how both the timing of elicitor treatment and the chemical applied can influence the longevity of the immune response generated. For example, BABA12 treatment led to enhanced resistance in leaf tissue one week post treatment but no resistance in fruit tissue (Fig 9c-e). Interestingly, zebularine soil drench treatment led to a stronger short-term resistance than BABA treatment however this phenotype was not as robust as BABA-IR, and crucially no zebularine treatments led to durable resistance at the postharvest stage against *B. cinerea*. The use of chemical methylation inhibitors such the well characterised 5-

azacytidine (5-AzaC) and zebularine have provided an effective tool for investigating effects of induced hypomethylation on plant stress responses. Application of 5-AzaC has recently been demonstrated to lead to large perturbations in secondary metabolites (Yang et al., 2022). The effect of methylation inhibitors has also been investigated against pathogens. For example, foliar spraying of 5-AzaC on rice plants led to enhanced resistance against root knot nematodes, DNA hypomethylation was therefore suggested to be part of early defence mechanisms (Atighi et al., 2020). Considering the original motivation for use of zebularine treatment was due to the increase of gain in CHH DMRs one week post BABA treatment, the enhanced resistance response following zebularine treatment is seemingly contradictory to this. It is likely however that the methylation landscape following both BABA and zebularine treatments is highly dynamic. For example, a time course study following 5-AzaC treatment demonstrated a large decrease in global methylation 7 days post treatment, which at 14 days was reversed (Yang et al., 2022). Nevertheless, the role of hypomethylation in generating a basal immune response is well documented in monocots and dicots (Atighi et al., 2020). Considering this, it would be interesting to assess the global methylation profile of leaf tissue at earlier timepoints following BABA treatment e.g., 6-24 hours, to assess if hypomethylation is observed in any cytosine context. Additionally, assessing how conserved the effective resistance in zebularine and BABA treatments are at the transcriptomic and metabolomic level would provide further insight in downstream signalling.

3.3.7 Conclusions

The results presented here have aimed to develop protection strategies against *B. cinerea* in tomato. Results demonstrate that long-lasting BABA-induced resistance is marked by changes in CHH methylation and that this phenotype is not maintained throughout the life of the plant, thus likely having a pivotal effect as plants and fruit develop. Large variations in global CHH methylation levels were observed, with young seedlings displaying significantly lower levels compared to adult plants. Furthermore, young plants displayed a large number of

CHH hyper-DMRs following BABA treatment, suggesting that young seedlings may display higher epigenetic plasticity at early stages of plant development when basal CHH levels are lower. The impact of the methylation inhibitor zebularine was tested on short and long-term resistance demonstrating that hypomethylation is associated with highly effective short-term resistance against *B. cinerea* however this resistance is not durable into postharvest. These findings could therefore be exploited into commercial settings, for protection against diseases pre and postharvest when plants display maximum capacity for stimuli-dependent information acquisition.

Chapter 4:

Investigating the early perception and systemic signal of BABA-induced resistance in tomato

Declaration regarding published work in this chapter

Large amounts of text in the results and discussion of this chapter have been published as a paper currently under review which is available as a pre-print (https://doi.org/10.1101/2023.10.09.561512). The authors of this work declare the content of this chapter belongs solely to Katie Stevens who is the first author of this publication. The senior author Dr Estrella Luna Diez contributed to this work providing feedback during the writing process and making corrections on the manuscript.

Katie Stevens



Dr Estrella Luna Diez



4.1. Introduction

4.1.1 BABA perception and signalling

The priming elicitor β-amino butyric acid (BABA) was first identified in the 1960s (Davey, 1963) and has subsequently been shown to be an effective priming agent in over 40 plant species (Cohen et al., 2016). The ability of BABA to prime tomato was first demonstrated in 1994 and has since been shown to occur naturally in tomato leaves (Cohen and Gisi, 1994, Cohen et al., 2016). More recently, BABA has been demonstrated to lead to long-lasting and postharvest resistance in tomato (Luna et al., 2016, Wilkinson et al., 2018, Catoni et al., 2022). Through the use of mutant screens in Arabidopsis, the receptor of BABA, *IMPAIRED IN BABA-INDUCED IMMUNITY 1 (IBI1)* was identified, which encodes an aspartyl-tRNA synthetase (AspRS) (Luna et al., 2014b). It is currently unknown if the same BABA receptor exists in other species such as tomato. The binding of BABA to IBI1 activates this enzyme for defence activity in the cytoplasm and blocks AspRS enzyme activity. This leads to the accumulation of uncharged tRNA and plant stress (Luna et al., 2014b). The accumulation of tRNA subsequently leads to changes in amino acid pools within the plant, such as increased levels of aspartic acid, proline, asparagine and serine (Singh et al., 2010). Given the known changes to metabolism following BABA treatment in Arabidopsis, and the recent global methylation changes reported in tomato leaves following BABA treatment, it warrants investigation to determine if perception of BABA treatment leads to similar perturbations in primary metabolites in tomato which may be subsequently associated with epigenetic changes controlling immunity (Catoni et al., 2022, Luna et al., 2020).

4.1.2 Systemic signalling in plants

Results in **Chapter 3** demonstrated that BABA soil drench treatment of young seedlings (two weeks old, BABA2) leads to long-lasting effects in the methylome over the plant's lifetime and critically, enhanced resistance in tissue that was not present at the time of application. Given that the presence of BABA is not sufficient to induce resistance (Wilkinson et al., 2018), this

indicates that long-lasting resistance after BABA treatment is likely maintained due to a systemic signal. Systemic signalling in plants, often referred to as systemic acquired resistance (SAR), was first identified in tobacco (*Nicotiana tabacum*) following inoculation with tobacco mosaic virus (TMV) (Ross, 1961). SAR is classically associated with enhanced resistance to biotrophic pathogens and enhanced salicylic acid (SA) defence signalling, the mobile signal for SAR has been shown to be methyl-salicylate (MeSA) (Park et al., 2007). The vascular tissue, comprising of the xylem and phloem, is the primary site of long-distance signalling in plants. Important phytohormones including abscisic acid (ABA), jasmonic acid (JA), SA, gibberellin (GA) and/or their derivatives have been shown to be mobile and present in phloem sap and are thought to have a role in systemic defence signalling (Hoad, 1995, Notaguchi and Okamoto, 2015). Grafting experiments have provided insight into the transport of many mobile signals involved in biotic and abiotic stress tolerance. For example, in tomato, the wild cultivar *Solanum habrochaites* is used as a rootstock to enhance the cold resistance of commercial varieties by enhancing JA accumulation in scion tissue (Wang et al., 2023). In addition to phytohormones, proteins and peptides, the phloem transports mobile RNAs such as mRNAs, long non-coding RNA and small RNA (sRNA) transcripts (Jeynes-Cupper and Catoni, 2023).

4.1.3 Mobile epigenetic signals

Over the past three decades, RNA as a mobile signal within plants has been explored through use of microinjections, analysis of phloem sap and grafting experiments (Liu and Chen, 2018). Grafting has demonstrated that mobile RNAs can travel bidirectionally between source and sink (root to shoot and shoot to root). High throughput sequencing analysis has identified a large number of mobile RNAs and their ability to direct changes in gene expression via alterations to the epigenome (i.e., DNA methylation). The most well characterised mobile RNAs are sRNAs, 20-24 nt in size, which are typically divided into two classes – micro RNAs (miRNAs) and small interfering RNAs (siRNAs). siRNAs can be further grouped based on their

length. siRNAs 21-22 nt in length are classically associated with short distance signalling and transcriptional gene silencing, whilst 24 nt are involved in long-distance signalling and can direct changes in the DNA methylation status of specific loci through the RNA-directed DNA methylation (RdDM) pathway. The RdDM pathway has been shown to be directly involved in the regulation of immune responses (described in **Chapter 1, 1.12)** (López et al., 2011, López Sánchez et al., 2016). Recently, a key RdDM component DICER-LIKE-3 (DCL3), responsible for the biogenesis of 24 nt siRNAs, has been shown to be necessary for inducing long-lasting and intergenerational acquired resistance against parasitic nematodes *Meloidogyne graminicola* (Meijer et al., 2023). Given the known function of 24 nt siRNAs in next generational resistance and their long-distant range of movement, they may play an important role in the long-lasting, systemic resistance observed postharvest in tomato fruit.

4.1.4 Gaps in understanding

Despite the benefits of BABA in inducing long-lasting resistance, its use in commercial settings has not been successfully incorporated due to reported growth reductions and potential toxicity with high concentrations (Slaughter et al., 2012, van Hulten et al., 2006, Wu et al., 2010). Greater understanding of both its perception, and the potential epigenetic mechanisms driving long-lasting resistance may allow the uncoupling of this resistance response from stress induced phenotypes. Here the hypothesis that postharvest resistance is regulated by a systemic signal is tested. Furthermore, considering the large number of changes associated with CHH methylation identified in **Chapter 3,** the hypothesis that this resistance is driven by changes in sRNA accumulation is explored. Results in this chapter therefore aimed to further elucidate the mechanisms behind both the perception and transmission of BABA-IR. By incorporating analysis on the transcriptome, methylome and sRNA profiles, new findings on the early effects of BABA and critically, the systemic nature of BABA-IR are discussed. Through the use of grafting experiments and analysis of sRNAs, potential gene candidates

under epigenetic regulation have been identified which may be important regulators of BABA induced postharvest resistance in tomato.

4.2. Results

4.2.1 Early BABA treatment leads to up-regulation of defence associated genes

To understand further the mechanistic basis of long-lasting BABA-IR in tomato, a 0.5 mM BABA soil drench treatment was conducted on 2-week-old plants. One week after treatment, leaf tissue was collected from plants for transcriptomic analysis (Fig 6). Principle component analysis (PCA) revealed a clear separation between BABA and control (water) treated plants, with greater variation observed in water treated plants (Fig 17a). BABA treatment led to significant transcriptional changes with the upregulation of 289 genes (> 1 log fold change) and downregulation of 26 genes (< 1 log fold change). Lists of all T1 DEGs are available in Supplemental Table S1. Gene Ontology (GO) enrichment analysis revealed that these up-regulated genes were significantly enriched in GO terms associated with “regulation of systemic acquired resistance”, “immune response”, and “response to biotic stimuli” (Fig 17b). Down-regulated genes were enriched in GO terms associated with ‘DNA replication’ and ‘cytokinesis’ (Fig 17b).

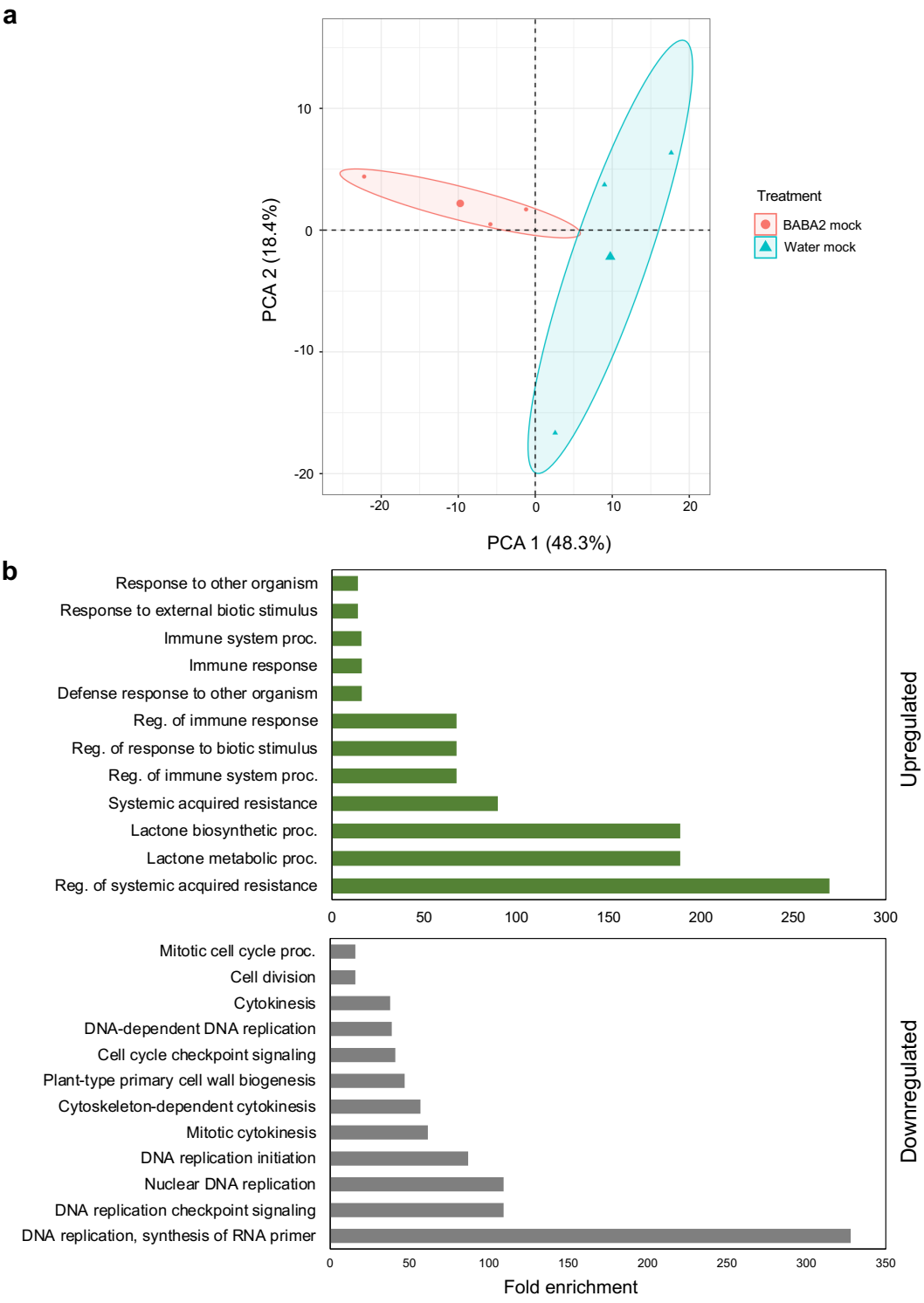


Figure 17: T1 transcriptomic data a) Principal component analysis (PCA) plot of 0.5 mM BABA soil drench treated and mock (water) treated plants. **b)** Statistically significant enriched GO term biological processes in BABA2 treated up-regulated and down-regulated DEGs

4.2.2 BABA induced DEGs are generally not differentially methylated

Leaf tissue was collected one-week post BABA soil drench treatment for whole genome bisulfite sequencing (WGBS) analysis to obtain DNA methylation profiles at a single cytosine resolution (Fig 7). In order to determine if genes DE due to BABA treatment were under direct regulation of DNA methylation, the methylation proportion of these genes (up- and down-regulated) (including 2000 bp upstream promoter regions) was assessed from CX report files (Fig 18a-c). Overall, no major difference in methylation status was seen in any cytosine contexts between BABA and mock treated plants, as indicated by the lack of clustering between treatment groups (Fig 18). This suggests that generally, differential gene expression due to BABA treatment is not associated with direct changes to the DNA methylation status of the promoter or gene body.

In **Chapter 3** differentially methylated regions (DMRs) associated to BABA treatment were identified in each cytosine context (CG, CHG and CHH) and overlapped with annotated genes on the tomato genome to isolate differentially methylated genes (DMGs). Despite the lack of a general correlative effect in methylation status and expression, directly overlapping DMGs with DEGs in Fig 17 isolated 11 genes with differential expression and methylation due to BABA treatment (Table 4). With the exception of Solyc09g007010, *Pathogenesis-related protein 1* (*PR1*) gene, all genes were hypermethylated and the majority in the CHH or CHG context. A number of genes were associated with pathogen defence signalling and specifically defence-hormone signalling, for example *PR1* Solyc09g007010.1 and *PR5*, Solyc08g080620.

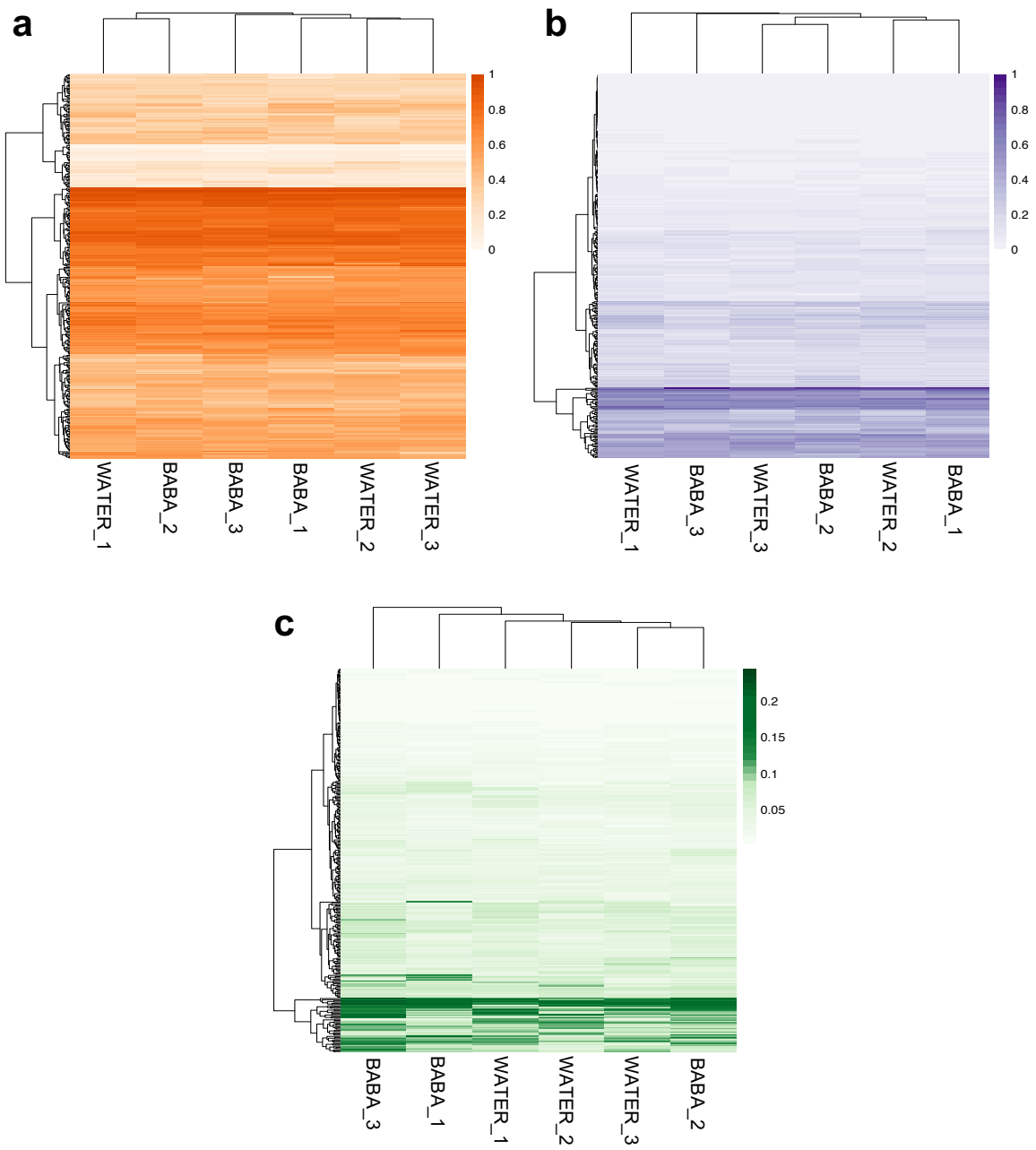


Figure 18: Methylation proportion of BABA T1 differentially expressed genes. Heatmap displaying methylation proportion in leaf tissue of water and 0.5 mM BABA treated plants. **a)** CG methylation context **b)** CHG methylation context and **c)** CHH methylation context.

Table 4: Up- and down-regulated DEGs due to BABA treatment simultaneously differentially methylated at T1. Gene name, ITAG description, methylation status and log-2-fold change.

Gene name	Description	T1 DMR	Log2-fold change
Solyc07g056210.5	Heavy metal-associated isoprenylated plant protein 47	CHG Gain	2.75
Solyc08g080620.1	PR-5x	CHG Gain	1.03
Solyc09g007010.1	Pathogenesis-related protein 1	CHG Loss	1.90
Solyc01g099990.3	F-box domain, Phloem protein 2-like protein	CHG Gain	2.47
Solyc07g006480.4	Receptor-like kinase	CG Gain	1.57
Solyc03g033795.1	AAA-ATPase At3g50940-like	CHH Gain	1.04
Solyc08g008150.1	Late embryogenesis abundant hydroxyproline-rich glycoprotein family	CHH Gain	1.25
Solyc09g075210.3	Late embryogenesis abundant protein Lea5	CHH Gain	1.00
Solyc10g085010.3	PAR1-like protein	CHH Gain	1.34
Solyc09g011260.2	Cysteine/histidine rich C1 domain protein	CHH Gain	1.78
Solyc05g009490.3	Unknown protein	CHH Gain	3.27

4.2.3 BABA signalling can be transmitted across graft junctions to induce long-lasting postharvest resistance.

Results in **Chapter 3** demonstrated that early BABA treatment triggers long-lasting IR in tissue not present at the time of BABA treatment, and that new DMRs specifically associated with BABA2 emerge weeks after the treatment (Fig 13). These observations suggest that long-lasting resistance after BABA treatment is maintained due to a systemic signal. To test this hypothesis directly, grafting experiments were conducted to determine whether BABA-treated tissues could transmit resistance to untreated tissues. Naive scions obtained from plants mock-treated with water were grafted onto BABA-treated rootstocks (hetero graft; HG). As controls, homografts were generated using both rootstocks and scions obtained from plants treated with BABA (BABA graft; BG) or mock-treated (mock graft; MG) (Fig 19a). Resistance against *B. cinerea* was tested in scion leaf tissue 6 weeks after grafting (corresponding to 6 weeks and 3 days post BABA treatment) and in ripe fruit tissue. Remarkably, leaf and fruit tissue from HG plants displayed the same level of resistance as BG, with significantly smaller lesions compared to the MG condition (Fig 19b). No statistically significant differences between graft treatments were found between the average weight of ripe fruit collected or the average number produced at 15-weeks (Appendix II, Fig 1). These results therefore indicate that BABA-IR is transmissible to untreated scions by grafting with no deleterious effects in fruit production.

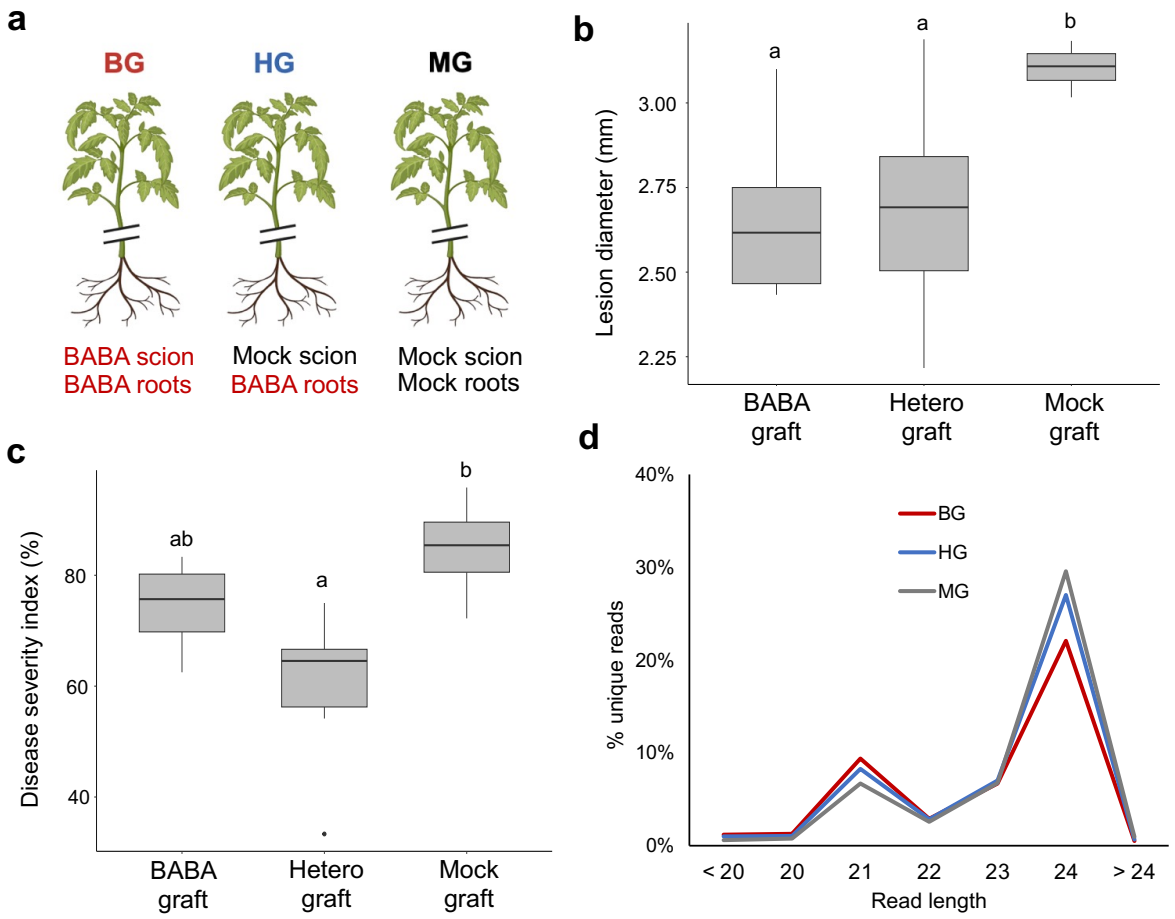


Figure 19: Grafting setup and phenotype. a) Experimental set up for grafting: from left to right BABA graft (BG) consisting of BABA scion and BABA treated roots scion, heterograft (HG) consisting of water scion and BABA roots, Mock graft (MG) consisting of Mock scion and Mock roots. **b)** Lesion diameter in tomato leaves, 6-weeks post grafting, infected with *B. cinerea* at 3 days post infection (dpi). **c)** Disease severity index in ripe tomato fruit converted from the percentage of lesions in 6 disease categories at 6 dpi with *B. cinerea*. **d)** Proportion of unique reads 20-24 nt in length across treatment groups (BG: red, HG: blue, MG: grey). Boxes denote 25th and 75th percentile, bars display min and max values. Different letters denote significant differences among treatment groups (LSD post hoc test; $p < 0,05$, $n = 3$-8).

4.2.4 BABA induces graft transmissible and non-transmissible effects in sRNA accumulation

Considering that the greatest changes in DNA methylation in resistant plants were found in the CHH context and that this type of methylation is known to be mediated by RdDM, the hypothesis that the resistant phenotype in HG is associated to a change in accumulation of mobile small RNAs (sRNAs) was tested. Scion leaf tissue was harvested 6 weeks post grafting and sRNA sequencing was conducted (Fig 19). The highest proportion of uniquely mapped sRNA reads were 24 nt in length across all grafting types, ranging from 26% to 34% in BG and MG respectively. The second most abundant proportion of reads were 21 nt in length varying from 9% to 13% in MG and BG, respectively (Fig 19d). Differentially expressed (DE) clusters were identified. Lists of all DE sRNA 24 nt and 21/22 nt clusters are available in Supplemental Table S4 and Supplemental Table S5 respectively. The majority of DE clusters were sRNAs 24 nt in size across all treatments- (HG v MG: 23,117/48,907, HG v BG: 113,349/123,306, BG v MG: 99,417/117,621). When compared to MG plants a much larger number of DE clusters in the BG plants were observed (17,323 up-regulated and 100,298 down-regulated) than the HG plants (10,150 up-regulated and 38,757 down-regulated) as demonstrated by greater separation in clustering on the PCA (Appendix II, Fig 2). On the other hand, HG vs BG contained 14,790 up-regulated DE clusters and 108,516 down-regulated DE clusters. Across all treatments therefore, the majority of DE clusters were down-regulated. These results additionally indicate that the HG scions maintained an intermediate sRNA profile between MG and BG plants, however, are more similar to the former condition. This suggests that BABA treatment induced both graft transmissible and non-transmissible effects of sRNA accumulation.

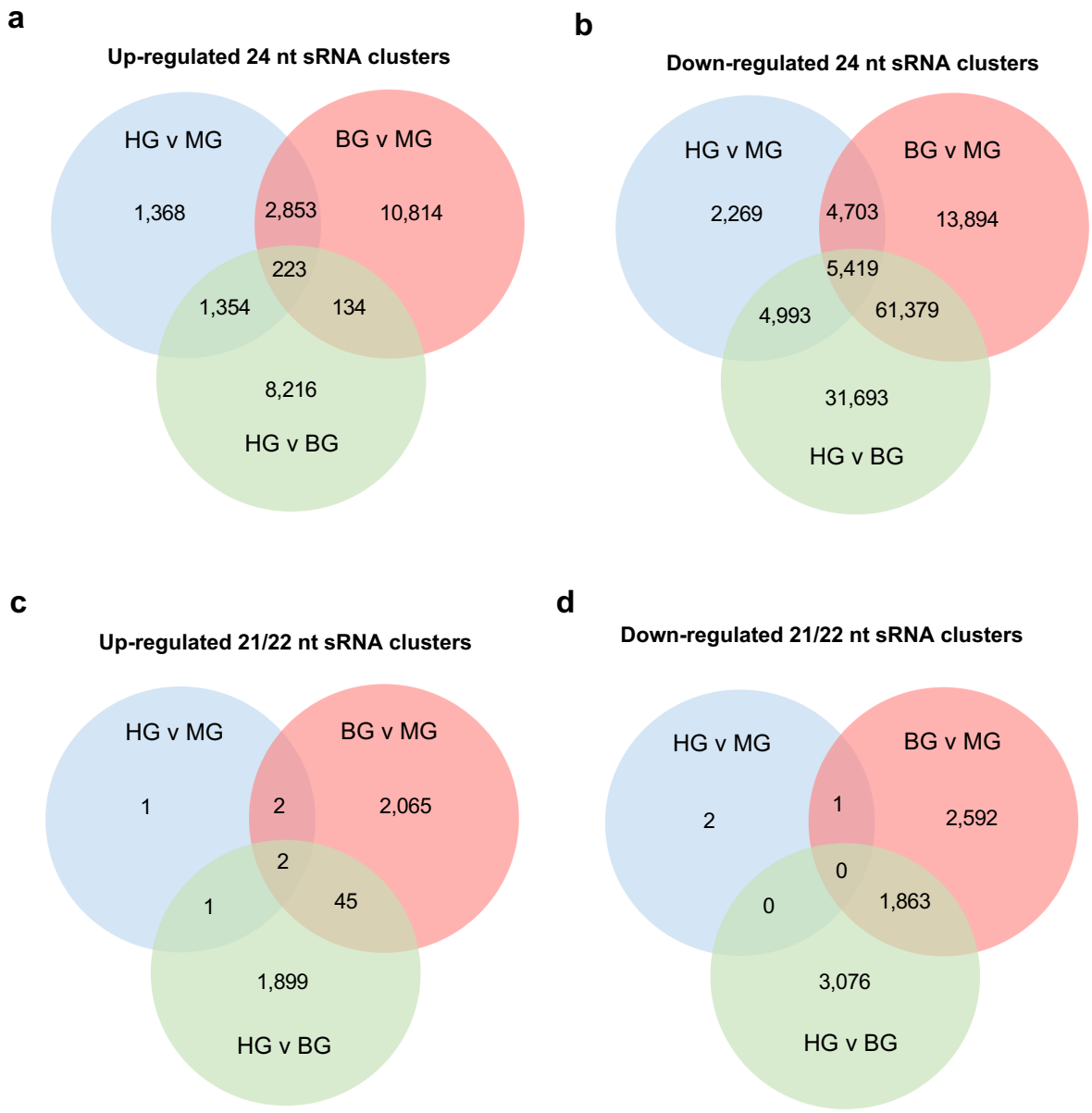


Figure 20: Correspondence between DE sRNA clusters. Venn diagram circles denote HG v MG – blue, BG v MG - red or HG v BG - green. **a)** Overlap of up-regulated 24 nt clusters. **b)** Overlap of down-regulated 24 nt clusters. **c)** Overlap of up-regulated 21/22 nt clusters. **d)** Overlap of down-regulated 21/22 nt clusters.

To assess how shared sRNA profiles were in resistant plants and identify potentially mobile clusters, DE clusters (HG v MG and BG v MG) were overlapped. sRNA clusters conserved to both HG and BG are referred to as ‘mobile clusters’ henceforth. Comparing DE clusters revealed that BABA treatments led to substantial changes in sRNAs not associated with a resistance phenotype which may be attributed to stress responses. Overlapping DE 24 nt clusters identified 2,853 up-regulated and 4,703 down-regulated mobile clusters (Figure 20a-b). Due to the very low number of DE 21/22 nt clusters in HG compared to MG, only 2 and 3 up and down-regulated mobile clusters were conserved across resistant treatments (Fig 20c-d). The location of DE clusters was assessed by counting the number of overlaps between DE clusters and genomic features including gene bodies, promoters, Class I TEs and Class II TEs. Overall, both up and down-regulated 24 nt clusters were equally distributed across genomic features, however differences were seen between specific grafting treatments. For example, 24 nt clusters DE in BG were more frequently found in promoter regions and Class II TEs than in MG or HG. Additionally, mobile 24 nt clusters were more frequently located on Class I TEs compared to in the BG condition (Appendix II, Figure 3a).

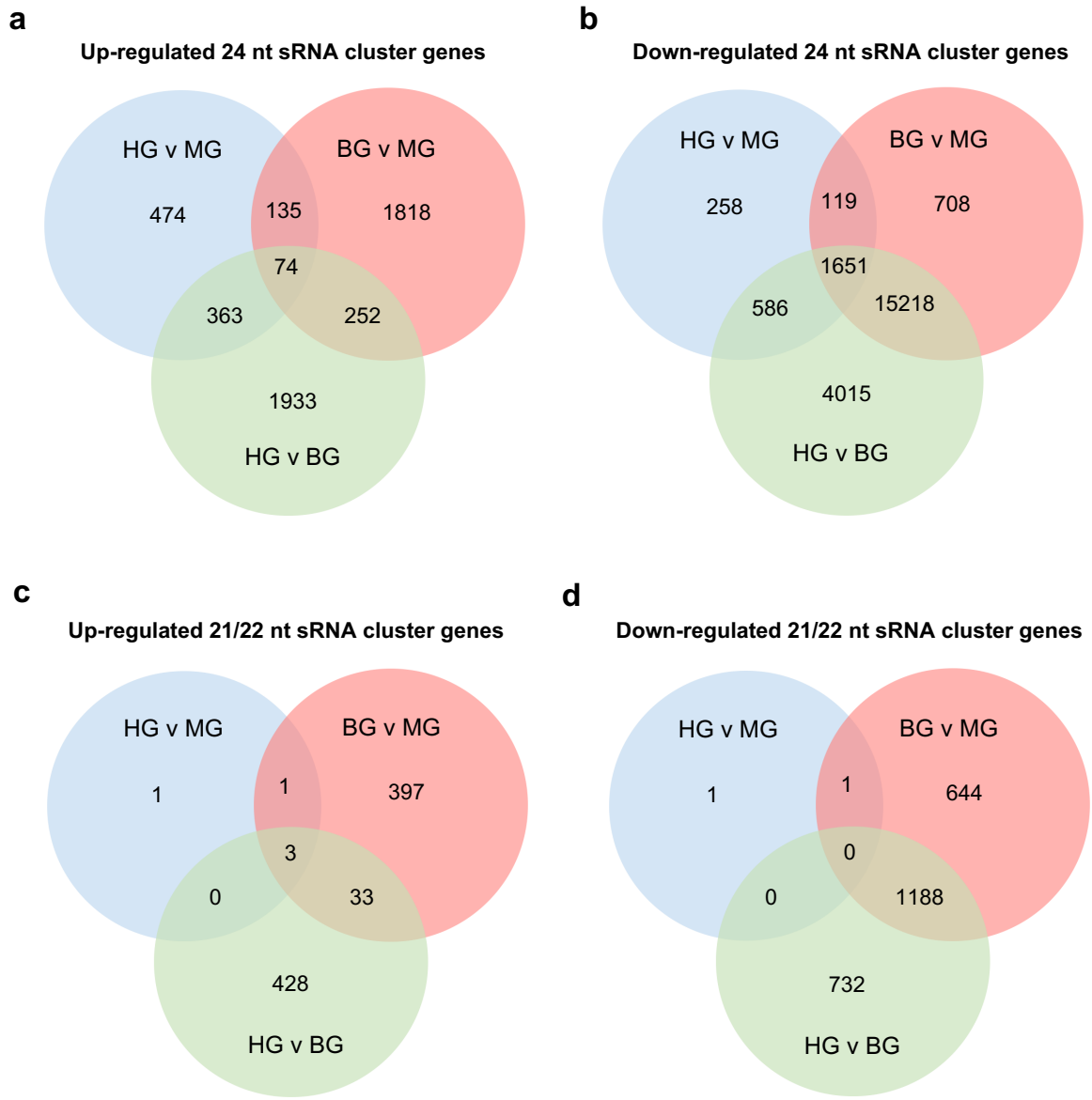


Figure 21: Correspondence between genes overlapping DE sRNA clusters. Venn diagram circles denote HG v MG - blue, BG v MG – red, or HG v BG - green. **a)** Overlap of up-regulated 24 nt cluster genes. **b)** Overlap of down-regulated 24 nt cluster genes. **c)** Overlap of up-regulated 21/22 nt cluster genes. **d)** Overlap of down-regulated 21/22 nt cluster genes.

4.2.5 Genes associated to up-regulated 'mobile' 24 nt sRNAs display enhanced expression in fruit infected with *B. cinerea*

To identify BABA-induced effects more directly linked to long-term resistance, genes overlapping (+/- 1 kb) DE mobile clusters were identified. 135 and 119 genes overlapping up-regulated and down-regulated 24 nt size mobile clusters were isolated respectively (Figure 21). Only 1 gene overlapping up-regulated (Solyc07g150127.2: zinc finger, GRF-type) and 1 gene overlapping down-regulated (Solyc01g010020.3: calmodulin) 21/22 nt mobile clusters were found (Fig 21) Further analysis of sRNAs was therefore focussed on clusters 24 nt in size. To assess if these mobile 24 nt sRNAs were associated with priming responses, the expression levels of mobile cluster overlapping genes was assessed by comparing their fragments-per-kilobase-million (FPKM) values across all treatment groups for infections and BABA treatments at T1 and T3 (Fig 22).

In the 135 genes overlapping with up-regulated mobile clusters, a clear trend of enhanced overexpression in response to infection in BABA2 fruit tissue (T3), compared to all other conditions was observed (Fig 22a). For these genes, statistically significant enrichment was observed in the following biological process: "COPII-coated vesicle budding" (103.4-fold enrichment, FDR p-value 0.025), "aspartate family amino acid biosynthetic processes" (74.4-fold enrichment, FDR p-value 0.025), "vesicle budding from membrane" (71.6-fold-enrichment, FDR p-value 0.025), and "aspartate family amino acid metabolic processes" (51.7-fold-enrichment 0.029 p-value FDR). Notably, such enhanced response to infection seen in BABA2 T3 is largely specific for these 135 genes, and it is not observed when all genes overlapping up-regulated 24 nt clusters from the only BG condition are considered (2279 genes) (Fig 22b). Similarly, this effect was not observed for the BABA2 T3 119 genes associated with down-regulated mobile clusters (Fig 22c), or in the BABA2 T1 condition for both genes associated with up-regulated and down-regulated clusters (Fig 22e-f). Therefore, these results suggest that mobile sRNAs accumulating in resistant naive tissue grafted on

BABA-primed rootstock are specifically associated with a set of genes with an enhanced response to infection in tomato fruit expressing long-lasting induced resistance.

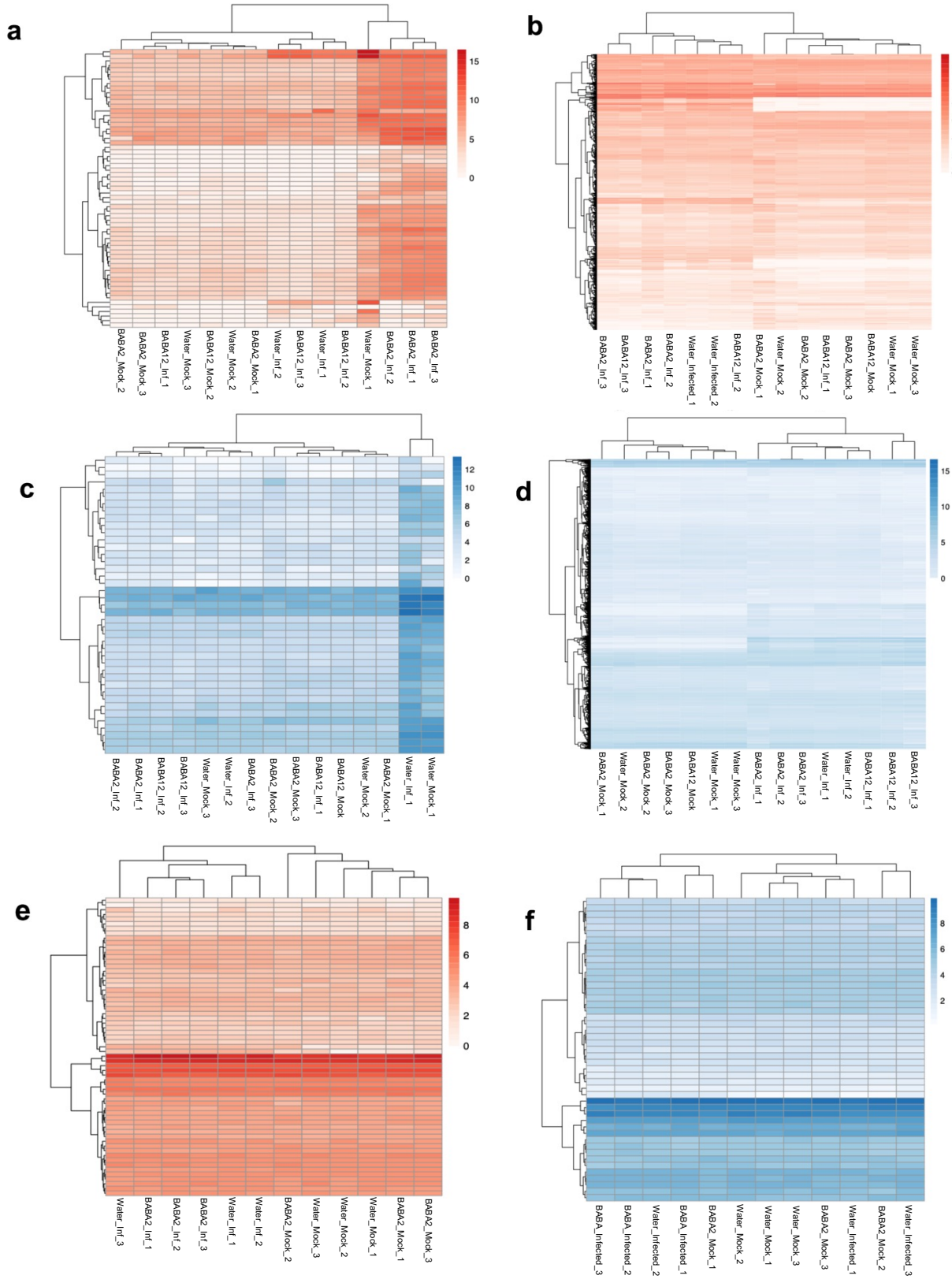

a
BABA2_Mock_2
BABA2_Mock_3
BABA12_Inf_1
Water_Mock_3
BABA12_Mock_2
Water_Mock_2
BABA2_Mock_1
Water_Inf_2
BABA12_Inf_3
Water_Inf_1
BABA12_Inf_2
Water_Mock_1
BABA2_Inf_2
BABA2_Inf_1
BABA2_Inf_3
15
10
5
0
b
BABA2_Inf_3
BABA12_Inf_3
BABA2_Inf_1
BABA2_Inf_2
Water_Infected_1
Water_Infected_2
BABA12_Inf_2
BABA2_Mock_1
Water_Mock_2
BABA2_Mock_2
BABA12_Inf_1
BABA2_Mock_3
BABA12_Mock
Water_Mock_1
Water_Mock_3
12
10
8
6
4
2
0
c
BABA2_Inf_2
BABA2_Inf_1
BABA12_Inf_2
BABA12_Inf_3
Water_Mock_3
Water_Inf_2
BABA2_Inf_3
BABA2_Mock_2
BABA2_Mock_3
BABA12_Inf_1
BABA12_Mock
Water_Mock_2
BABA2_Mock_1
Water_Inf_1
Water_Mock_1
12
10
8
6
4
2
0
d
BABA2_Mock_1
Water_Mock_2
BABA2_Mock_2
BABA2_Mock_3
BABA12_Mock
Water_Mock_1
Water_Mock_3
BABA2_Inf_1
BABA2_Inf_2
BABA2_Inf_3
Water_Inf_1
Water_Inf_2
BABA12_Inf_1
BABA12_Inf_2
BABA12_Inf_3
15
10
5
0
e
Water_Inf_3
BABA2_Inf_1
BABA2_Inf_2
BABA2_Inf_3
Water_Inf_1
Water_Inf_2
BABA2_Mock_2
Water_Mock_3
Water_Mock_2
Water_Mock_1
BABA2_Mock_1
BABA2_Mock_3
8
6
4
2
0
f
BABA_Infected_3
BABA_Infected_2
Water_Infected_2
BABA_Infected_1
BABA2_Mock_1
Water_Mock_2
Water_Mock_1
Water_Mock_3
BABA2_Mock_3
Water_Infected_1
BABA2_Mock_2
Water_Infected_3
8
6
4
2

Figure 22: Expression of mobile cluster associated genes. Heatmap displaying normalised fragments per kilobase of transcript per million mapped reads (FPKM) value of genes containing **a)** Up-regulated differentially expressed 24 nt sRNA clusters conserved to HG and BG phenotype. FPKM values display expression level in fruit tissue of mock and *B.* cinerea BABA2, Water and BABA12 plants. **b)** Up-regulated differentially expressed 24 nt sRNA clusters in BG phenotype. FPKM values display expression level in fruit tissue of mock and *B. cinerea* infected BABA2, Water and BABA12 plants. **c)** Down-regulated differentially expressed 24 nt sRNA clusters conserved to HG and BG phenotype. FPKM values display expression level in fruit tissue of mock and *B.* cinerea BABA2, Water and BABA12 plants. **d)** Down-regulated differentially expressed 24 nt sRNA clusters in BG phenotype. FPKM values display expression level in fruit tissue of mock and *B. cinerea* infected BABA2, Water and BABA12 plants **e)** Up-regulated differentially expressed 24 nt sRNA clusters conserved to HG and BG phenotype. FPKM values display expression level in leaf tissue of mock and *B.* cinerea BABA2 and Water plants. **f)** Down-regulated differentially expressed 24 nt sRNA clusters in BG phenotype. FPKM values display expression level in leaf tissue of mock and *B. cinerea* infected BABA2 and Water plants.

4.2.6 Primed genes associated with mobile sRNAs have no change in their methylation status

To gain further insight into the primed expression of genes associated with mobile sRNAs, the methylation status of the gene and promoter region (2000 bp upstream) was assessed (Fig 23a-f). The methylation proportion of the 135 genes of interest was calculated for all BABA2 and water samples at T1 (leaf tissue 3-weeks) and T3 (ripe fruit tissue) (Fig 23a-f). Surprisingly no major differences were observed in the methylation status of our primed genes between BABA and water treated plants, in any condition or cytosine context. Therefore, whilst this selection of 135 genes associated with up-regulated mobile 24 nt sRNA clusters display enhanced gene expression during infection, this is not associated with changes to the methylation status of these genes or their promoters.

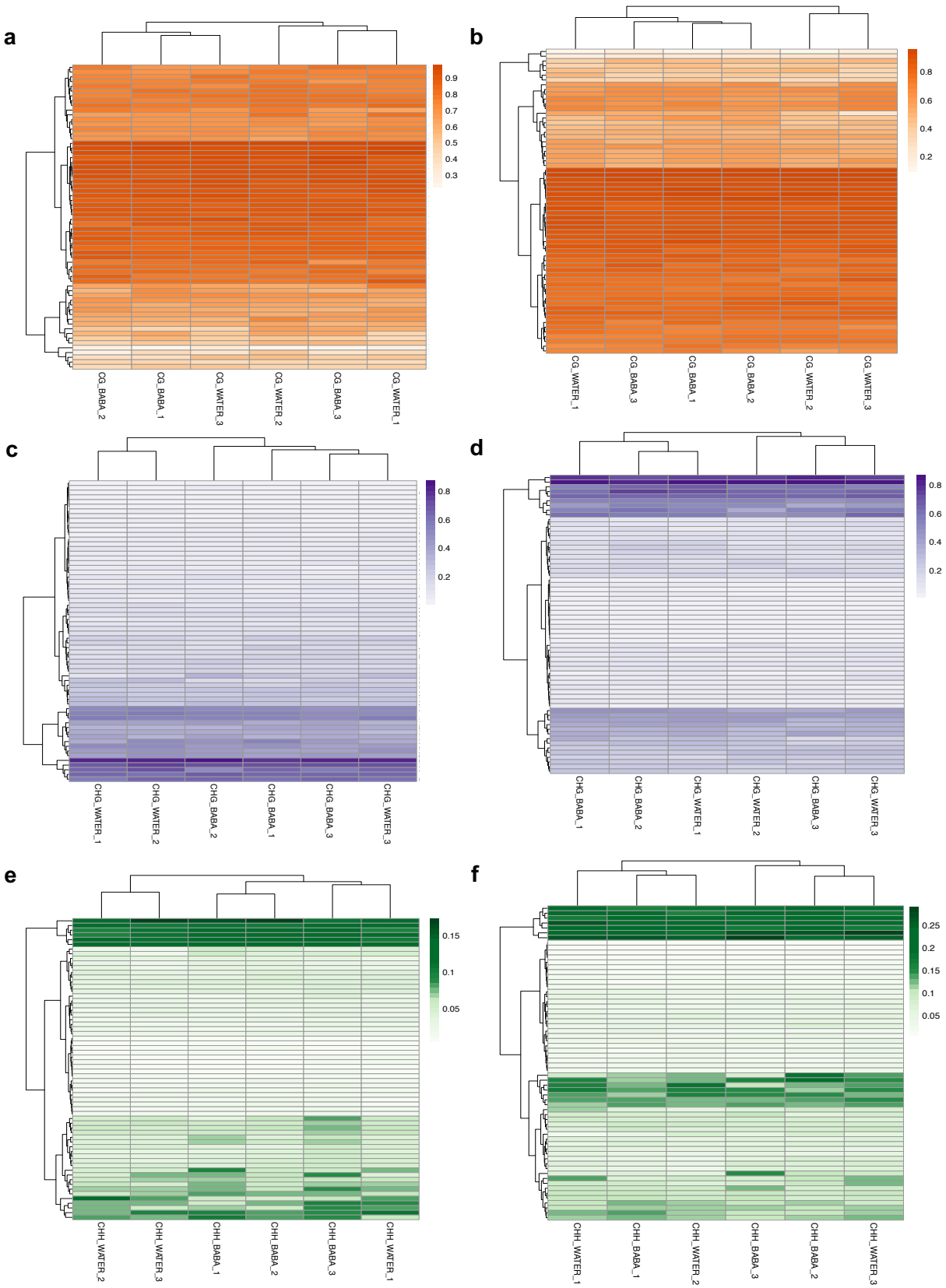


Figure 23: Methylation proportion of mobile primed genes and promoters. Heatmaps display methylation in BABA2 and water treated plants **a)** T1 CG context. **b)** T3 CG context. **c)** T1 CHG context. **d)** T3 CHG context. **e)** T1 CHH context and **f)** T3 CHH context.

4.2.7 Transposons associated with mobile sRNAs have no differences in their methylation status or expression

To further investigate what may be regulating the primed expression of genes associated with mobile clusters, transposons overlapping (+/-1 kb) up- and down-regulated mobile 24 nt clusters were isolated (Appendix II Fig 4). The following were selected: 335 class I TEs overlapping up-regulated 24 nt mobile clusters, 502 class I TEs overlapping down-regulated 24 nt mobile clusters, 134 class II TEs overlapping up-regulated 24 nt mobile clusters, 153 class II TEs overlapping down-regulated 24 nt mobile clusters. The methylation status of these transposons was determined for all methylation contexts (CG, CHG and CHH) across all timepoints (T1, T2 and T3) (Appendix II Fig 5-8). No differences were observed in the methylation status of Class I or Class II transposons overlapping up or down-regulated mobile 24 nt clusters between water and BABA treated tissue at any timepoint or methylation context. The expression status of these Class I and Class II transposons was assessed in fruit tissue by comparing their FPKM values across infections and BABA treatments at T3. Whilst a small subset of transposons displayed a unique expression profile (Fig 24), no clear overall primed response was observed as seen in genes in Fig 22.

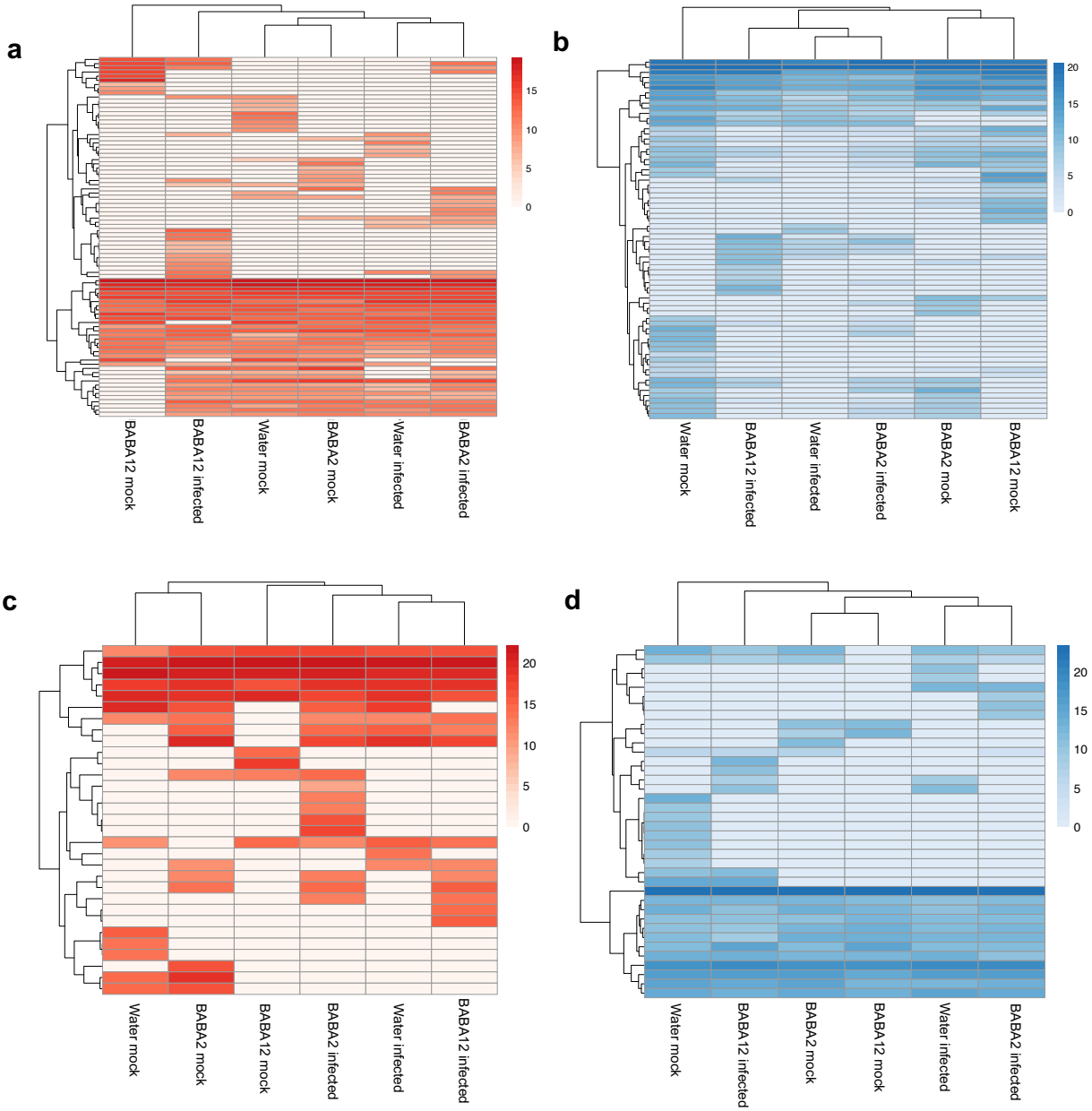


Figure 24: Expression of TEs overlapping mobile sRNA clusters. Mobile clusters are those conserved in HG and BG phenotypes only. **a)** Class I transposons containing up-regulated differentially expressed 24 nt sRNA mobile clusters. FPKM values display expression level in fruit tissue of mock and *B.* cinerea BABA2, Water and BABA12 plants. **b)** Class I transposons containing down-regulated differentially expressed 24 nt sRNA mobile clusters. FPKM values display expression level in fruit tissue of mock and *B. cinerea* infected BABA2, Water and BABA12 plants. **c)** Class II transposons containing up-regulated differentially expressed 24 nt sRNA mobile clusters. FPKM values display expression level in fruit tissue of mock and *B.* cinerea BABA2, Water and BABA12 plants. **d)** Class II transposons containing down-regulated differentially expressed 24 nt sRNA mobile clusters. FPKM values display expression level in fruit tissue of mock and *B. cinerea* infected BABA2, Water and BABA12 plants.

4.2.8 BABA treatment leads to major changes in the amino acid content of leaf tissue

Following the finding of enhanced expression in genes enriched in GO terms associated with aspartic acid signalling, further analysis was conducted on amino acid signalling in response to perception of BABA treatment. In Arabidopsis, binding of BABA to the receptor IBI1 has been shown to lead to an increase in uncharged tRNA and accumulation of aspartic acid (Luna et al., 2014b). It is currently unknown however if the IBI1 BABA receptor in Arabidopsis is present in tomato. To assess the similarity of this metabolic response in tomato, the relative quantity of selected amino acids was measured in leaf tissue one week post 0.5 mM BABA soil drench treatment of 2-week old seedlings. Major differences were observed between BABA and water treated seedlings. BABA treated plants displayed a statistically significant reduction in levels of glycine, alanine, threonine, and glutamate (Fig 25). BABA treated seedlings contained larger quantities of asparagine as well as a significant increase in aspartate. The increase of these amino acids however was not statistically significant due to large variability between individuals. This preliminary data however suggests that the perception of BABA in tomato does lead to a similar increase in aspartic acid levels, as well as major disruptions to primary metabolism, as observed in Arabidopsis (Luna et al., 2014b).

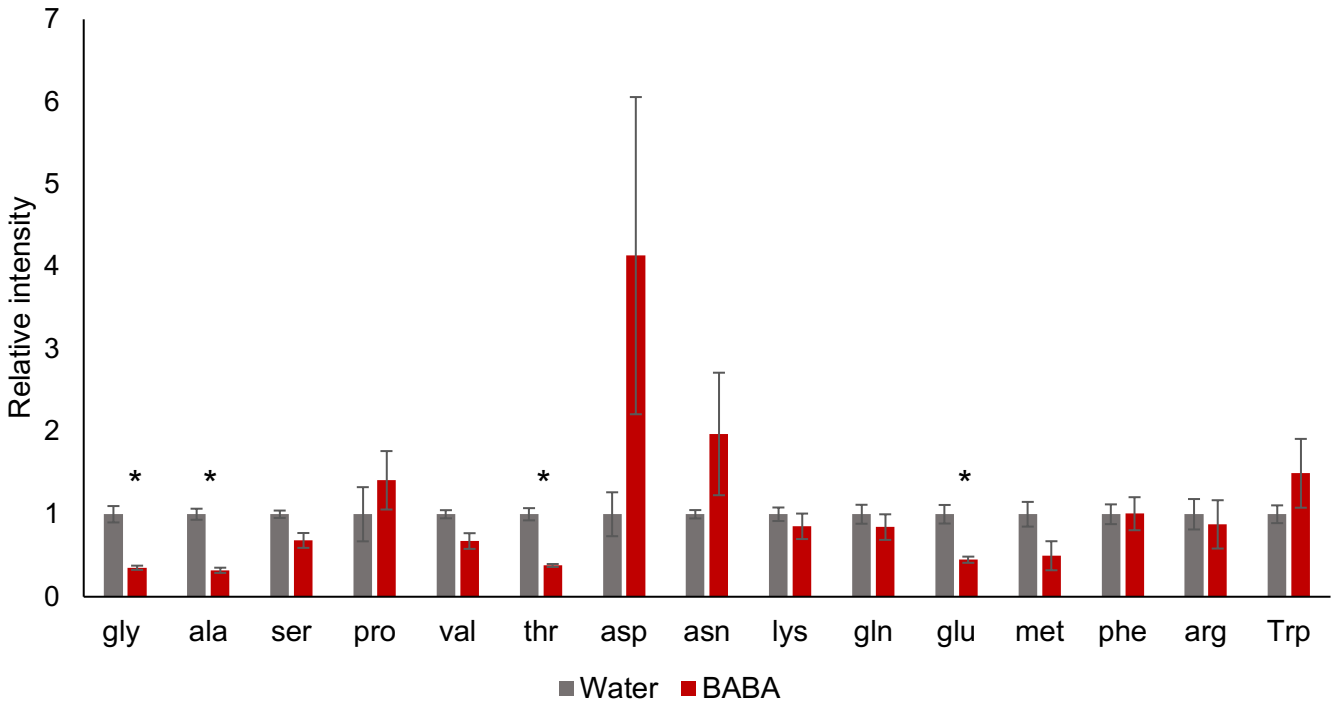


Figure 25: Relative amount of amino acid in 0.5 mM BABA treated seedlings: Glycine, alanine, serine, proline, valine, threonine, aspartate, asparagine, lysine, glutamine, glutamate, methionine, phenylalanine, arginine, tryptophan in BABA2 treated leaf tissue compared to water treated seedlings. Asterisks denote statistically significant difference between BABA and water treated seedlings (Student's t-test; $p < 0.05$, $n = 3$-4).

4.2.9 Fruit of resistant grafted plants display reduced levels of salicylic acid

To gain further insight on potential mechanisms regulating this systemic resistance, targeted phytohormone analysis was conducted on fruit tissue from HG, BG, and MG plants. Significant differences were seen in levels of salicylic acid (SA) in HG and BG compared to MG plants. Additionally, BG plants displayed lower levels of indole-3-acetic acid (IAA) compared to MG and HG, and higher levels of 12-oxo-10,15(Z)-phytodienoic acid (OPDA) than MG and HG (Fig 26). Whilst not significant, resistant plants displayed higher quantities of ABA compared to MG plants.

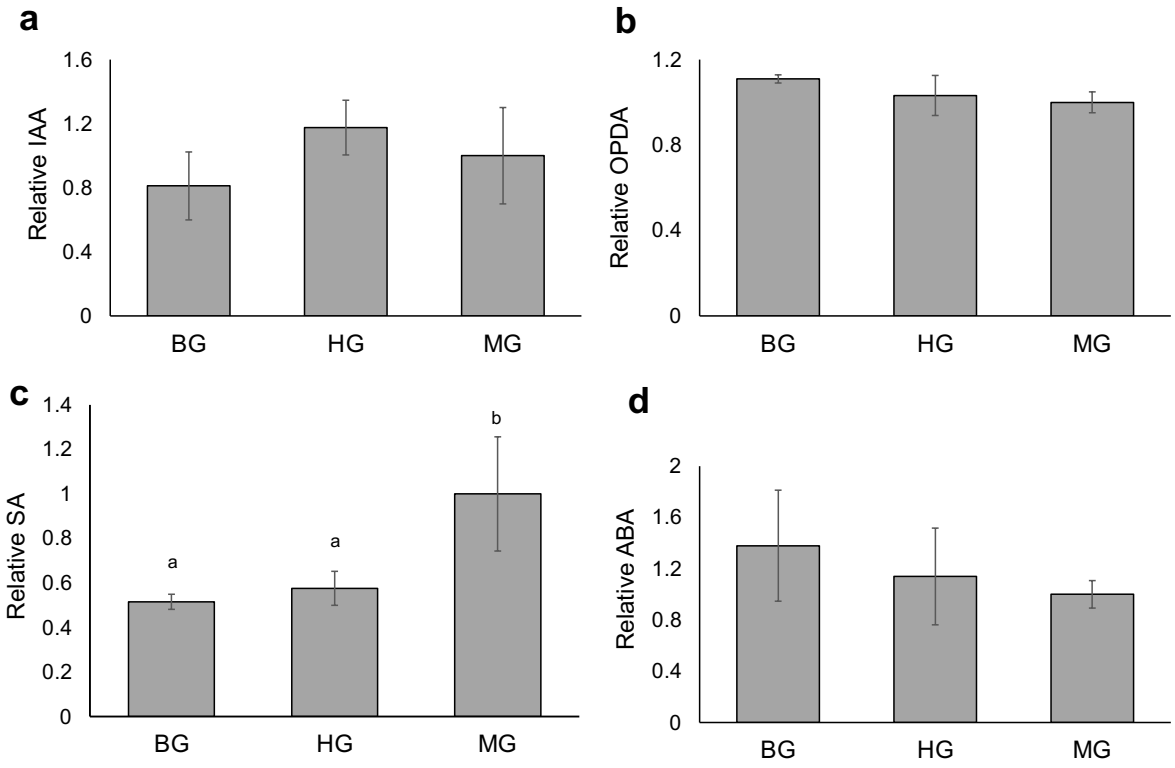


Figure 26: Influence of grafting treatment on phytohormone content in fruit tissue from HG, BG, and MG plants. Bars represent relative mean quantity of **a)** IAA, **b)** OPDA, **c)** SA, **d)** ABA compared to MG. Boxes denote 25th and 75th percentile, bars display min and max values. Different letters denote significant differences between treatment groups determined by One-Way ANOVA (LSD post hoc test; $p < 0.05$, n = 3-4),

4.2.10 Progeny of heterograft plants display mild enhanced resistance compared to mock graft plants

Finally, the durability of BABA-IR was investigated by conducting pathogen assays in progeny of grafted plants. From three biological replicate plants from each grafting treatment, fruit were harvested, and seeds were collected. Progeny were grown and resistance against *B. cinerea* was tested in leaf tissue of 4-week-old plants. No statistically significant difference was seen between treatment groups, however enhanced resistance was seen in HG leaf tissue compared to MG (Fig 27).

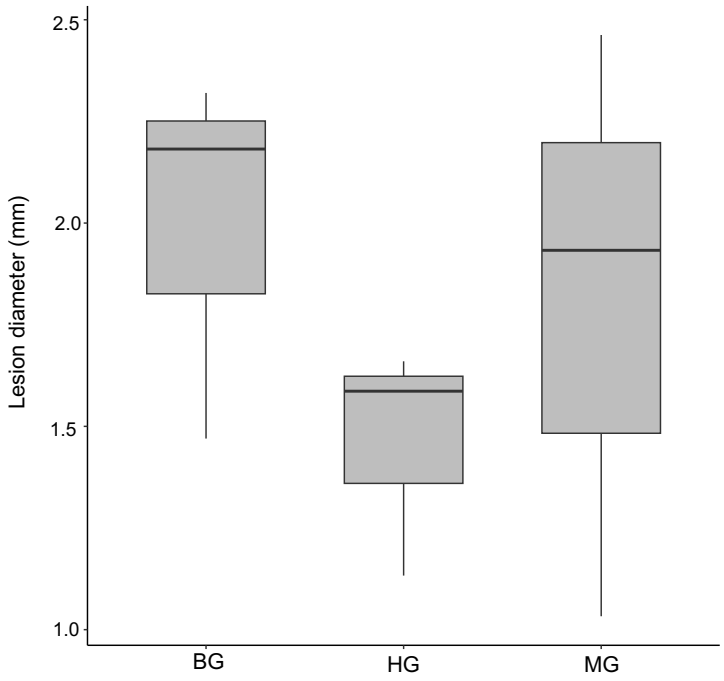


Figure 27: Phenotype of grafted progenies. Lesion diameter in tomato leaves from progeny of grafted plants infected with *B. cinerea* at 3 dpi. Boxes denote 25th and 75th percentile, bars display min and max values.

4.3. Discussion

4.3.1 Identification of BABA primed defence associated genes

Transcriptomic analysis of tomato leaf tissue revealed that BABA soil drench treatment leads to upregulation in GO terms classically associated with defence signalling such as “systemic acquired resistance”. In this chapter, genes with known roles in defence displaying differentially expression and methylation due to BABA treatment were identified. For example, Solyc09g007010, *PR1* is hypomethylated in the CHG context and up-regulated 1.9-fold in expression, *PR5*, Solyc08g080620 is hypermethylated in the CHG context and displayed slight up-regulation in expression. Previous studies in Arabidopsis have shown that BABA primed plants sprayed with SA displayed enhanced expression of NPR1 associated genes such as *PR1* and *PR5* (Luna et al., 2014b). PR5, the defence osmotin has additionally shown to be up-regulated in BABA primed potato during infection (Bengtsson et al., 2014). Interestingly, Peptidase A1 domain-containing protein, an aspartyl protease (AP), was DE at T1 following BABA treatment and was differentially methylated in the CHH context at T1, T2 and T3. This gene is additionally DE in *B. cinerea* infected fruit tissue; therefore, this may be a novel potentially epigenetically-regulated gene involved in long-lasting BABA induced defence priming. BABA has been demonstrated to lead to an increase in aspartyl proteases following application in potato (Andreu et al., 2006). The increase of AP genes is thought to increase the amino acid pool for synthesis of defence proteins for pathogen defences. Importantly, tomato aspartyl protease has been shown to lead to the cleavage of PR1-b, triggering the release of a cap derived peptide 1 (CAPE1) amino acid peptide which induces defence and SAR responses. CAPE1 is derived from the 11 C-terminal residues of PR1 and has been demonstrated to act as a novel DAMP like elicitor (Chen et al., 2014, Han et al., 2023). Spraying tomato plants with CAPE1 has been shown to reduce spread of *P. syringae* pv tomato DC3000 as well as a mild anti-herbivore response. This enhanced resistance is linked to increased JA and SA, and H_2O_2 production (Chen et al., 2014). Whilst PR1 has been shown to be differentially expressed and methylated due to BABA treatment (Table 4), it is

unexplored if BABA treatment leads to PR1 cleavage and production of CAPE1. Proteomic analysis may reveal if this process is involved in BABA-IR and potentially an important epigenetically-regulated defence pathway.

Across the transcriptome dataset there was no clear correlation between level of gene expression and methylation status. For example, the unknown protein Solyc05g009490.3 was up-regulated 3.27-fold yet displayed a gain of CHH methylation. This contradicts the generally assumed knowledge that enhanced methylation reduces transcription (Law and Jacobsen, 2009). These observations may be due to known dynamic changes in gene expression following priming treatment and the limitations of collecting a single timepoint. In some cases, however, DNA methylation in promoters can induce gene transcription. This has been demonstrated in demethylase mutants closely related to ROS1, (*SlDML2*) in Micro Tom in which a broad role for DNA demethylation in gene regulation has been shown. Hundreds of genes involved in fruit ripening display a correlation with DNA hypomethylation in their promoter region and repression of transcription (Lang et al., 2017). Therefore, DNA methylation may be enhancing the binding of transcriptional activators or preventing the binding of transcriptional repressors (Zhang et al., 2018).

4.3.2 Characterising the systemic signal of BABA-IR

The observation in **Chapter 3** that DMRs associated to early BABA treatment are not maintained from seedlings (T1) to fruiting (T3) supports the hypothesis on the importance of foundational changes at early stages of development and tissues that experience early BABA exposure. Considering that tissues present early during development are influencing responses in fruit that were not present at the time of BABA2 treatment, this raised the question of whether the long-lasting resistance is due to a systemic signal. Therefore, the use of grafting and BABA soil drench treatments were combined revealing that long-lasting resistance is transmissible across graft junctions to untreated naive scions (Fig 20b-c). The

absence of long term-resistance observed in BABA12 plants excludes that this primed state is due to residual BABA transported to and accumulated in fruit tissue (Wilkinson et al., 2018).

Efforts therefore focused on the characterisation of this mobile signal by investigating the accumulation of sRNAs, which are known to be mobile and able to transmit traits across a graft junction (Jeynes-Cupper and Catoni, 2023). Previous grafting experiments performed with transgenic plants have demonstrated that 24 nt sRNAs can lead to epigenetic changes in recipient scion tissue (Molnar et al., 2010). Importantly, the RdDM pathway has been frequently associated with defence against biotic stresses and with the expression and maintenance of long-lasting priming. For example, the double mutant *drm1drm2* displays enhanced susceptibility against the necrotrophic pathogen *P. cucumerina* (López et al., 2011). Furthermore, the *nrpe1* mutant was shown to display enhanced resistance against *Pseudomonas syringae* DC3000. Enhanced SA- and reduced JA-mediated defences were observed demonstrating the importance of Pol V in regulating effective immune responses (López et al., 2011). Therefore, one could speculate that the RdDM pathway plays a role in the establishment and maintenance of long-lasting priming in tomato by impacting mobile sRNAs that alter methylation in naive tissue.

To identify siRNAs potentially driving the resistance observed in our experiments, sRNA sequencing was conducted on leaf tissue from grafted plants. Interestingly, while BABA treated plants (Li et al., 2009) display a change in the accumulation of many sRNA clusters, only a small portion are maintained in grafted scions (HG) and are therefore associated with long-lasting resistance. This suggests that the majority of the BABA induced effects on sRNA accumulation are not involved in graft-transmissible IR. The large number of DE sRNA clusters in BG plants may be attributed to a combined stress response of BABA treatment and grafting. The finding in **Chapter 3** of late accumulation of DMRs may suggest a potential role of non-canonical, *de novo* RdDM. Whilst BG and HG plants displayed a higher percentage of total unique reads 21 nt in length than MG plants (Fig 19d), only 3 total mobile 21/22 nt sRNAs

were found in resistant plants (Fig 20c-d). This indicates that 21/22 nt sRNAs are not a long-distance graft transmissible signal contributing to the resistant phenotype in plants with BABA rootstocks. In contrast, the most abundant class of sRNA clusters in all treatments were 24 nt in size, which are more classically associated with long-distance systemic signalling and canonical-RdDM. (Molnar et al., 2010). Conserved DE 24 nt sRNA clusters and their overlapping genes were therefore identified from our resistant plants (BG and HG). The 135 genes associated with up-regulated clusters displayed a clear pattern of enhanced expression during fruit infections in resistant BABA2 plants (Fig 22a). Crucially, enhanced expression of these mobile cluster associated genes was not seen in BABA12 or water plants. Moreover, the same expression pattern was not observed in leaf tissue or across our down-regulated cluster associated genes, (Fig 22). Whereas this could be due to the dynamic nature of transcriptomic responses during pathogen infection and the limitations of collecting single timepoints (Guan et al., 2020), these results suggest that early BABA treatment generates a systemic signal that leads to dynamic changes in the priming response in fruit.

Gene expression analysis in **Chapter 3** revealed that tissue type is a major driver of the transcriptomic response to *B. cinerea* infection. It is therefore striking that sRNA sequencing analysis conducted from harvested leaf tissue correlates with major changes in fruit transcriptional changes. There are few examples of primed transcriptional defence responses in fruit tissue as a consequence of root treatments. Colonisation of tomato roots with arbuscular mycorrhiza (AM) fungi has been shown to lead to substantial transcriptional changes in genes associated with fruit ripening, transporter and defence associated genes (Zouari et al., 2014) however the exact mechanisms behind this are unknown. Changes relating additionally to sRNA signalling however are sparse. Soil drench application of BABA treatment may be leading to direct production of sRNAs in root tissue which are later transported via the phloem to newly produced leaf and fruit tissue. Here, abundant 24 nt sRNAs may prime and direct changes in gene expression, with the most predominant priming response in infected fruit tissue. Alternatively, root application of BABA treatment may be

leading to the production of an unidentified signal (metabolite or protein). Movement of this signal may therefore trigger local, tissue specific changes in abundance of sRNA molecules by impacting RdDM machinery as opposed to the direct movement of siRNAs from root to newly produced organs. Future screening of sRNAs in root and fruit tissue following BABA soil drench may elucidate if these are produced directly in roots and reveal if sRNAs are predominantly transported to fruit to contribute to dynamic changes in expression. Alternatively, the use of compatible mixed species grafts with unique genomes, for example tomato and eggplant, would allow direct identification of which source genome sRNAs are originating from. Finally, to reveal the mechanistic basis of such signals, the use of mutants in key RdDM machinery such as Pol IV or DCL3 to block the production of 24 nt sRNAs may confirm their importance. Combining the use of RdDM mutants and grafting for example will reveal their role in both production and spread of this signal of long-lasting BABA-IR.

4.3.3 A potential epigenetic role of aspartic acid signalling in BABA-IR

Among the genes that were found to be functionally associated with sRNA clusters and priming in this experiment, significant enrichment was observed in the biological process “COPII-coated vesicle budding” with two SEC23 transport proteins displaying enhanced expression in fruit tissue infected with *B. cinerea*. Whilst the role of these genes has not been fully explored in plant pathogen defence, growing evidence has shown their role in abiotic stresses and ABA signalling (Li et al., 2022, Li et al., 2021). Furthermore, studies of *NPR1* the key regulator of SAR show that to prepare cells for secretion of PR proteins more secretory machinery components must be created (Wang et al., 2006). The role of secretory machinery in priming through use of elicitors such as BABA has not been investigated, however may be a component in the enhanced transcriptional defence responses found in resistant primed plants.

The enrichment of “aspartate family amino acid biosynthetic processes” offers a highly interesting result, as priming by BABA in Arabidopsis has been directly associated with

aspartic acid-related processes, following identification of its receptor IBI1 (Luna et al., 2014b). BABA has been shown to block the aspartic domain of AspRS, leading to a constant production of aspartic acid. IBI1 can switch from canonical AspRS activity to non-canonical defence activity upon pathogen infection. BABA induced priming therefore stimulates this change in activity and leads to the translocation of IBI1 from the endoplasmic reticulum to the cytoplasm (Luna et al., 2014b). An asparagine synthetase, SOLYC04G055200.3, was found in the mobile T3 primed gene list thus providing further evidence of a role of amino acid processes related to aspartic acid. Interestingly, this asparagine synthetase has been shown to be important in defence against microbial pathogens and is mainly found in root tissue where BABA was applied and up taken in this study (Hwang et al., 2011, Olea et al., 2004).

Amino acid profiling conducted in this study further demonstrates the importance of aspartic acid metabolism and provides evidence that tomato may display a similar perception response as reported in Arabidopsis. In plants treated with BABA via soil drench, leaf tissue displayed enhanced quantities of aspartic acid and asparagine (Fig 25). This is in contrast to a recent study by Janotík et al which specifically investigated the impact of aspartic acid on tomato (Micro Tom) plants. Here, tomato leaflets were submerged in 5 mM BABA for 24 hours prior to amino acid profiling and pathogen assays. No increase in aspartic acid was observed following submersion treatment in BABA (Janotík et al., 2022). Further differences between these studies are shown in the differences in in levels of Phe, Try, and Trp, precursor amino acids of the phenylpropanoid pathway, as well as Gly, Thr and Ala which displayed significant reductions in our study (Fig 25). The differences in results between the same cultivar may be attributed to application method of BABA, with root treatment leading to an increase in aspartic acid in both Arabidopsis and tomato (Luna et al., 2014b). Considering these differences and findings on the systemic nature of this signal here, it may be necessary for the perception and triggering of this systemic signal to occur specifically in root tissue. More complex grafting experiments using selected small sections of BABA treated scion rather than an entire BABA

treated root stock may provide further insight into if the root stock is in fact critical for the generation and transmission of this signal.

4.3.4 Phytohormone profiling in grafted fruit

To further characterise the resistance phenotype observed in this study, profiling of major phytohormones in grafted fruit tissue was conducted. A statistically significant reduction in levels of SA were observed in BG and HG plants compared to MG, with resistant plants on average having half the quantity of SA (Fig 26). This was the only shared pattern between resistant plants, suggesting it may be contributing to the enhanced postharvest resistance attributed to BABA treatment on root stock. Given the known antagonism between SA and JA signalling, low SA levels may be driving the enhanced resistance against the necrotrophic pathogen *B. cinerea* in fruit tissue. Previous analysis in BABA treated fruit tissue demonstrated a statistically significant increase in ABA levels in fruit of BABA2 treated seedlings (Wilkinson et al., 2018). No statistically significant difference was observed here however BG plants displayed higher level of ABA in fruit tissue with HG displaying an intermediate quantity. Combining the use of mutants in hormones such as SA or ABA and grafting may provide further insight. Similarly epigenetic changes may be directing these differences in phytohormones. Work by Meijer et al demonstrated that *dcl3a* mutants, impaired in 24 nt sRNA production, displayed hypomethylation in gene promoters controlling defence hormone pathways, specifically in JA/ET responses (Meijer et al., 2023). Further analysis on both mobile sRNAs and phytohormones may therefore indicate which is the driving factor in systemic resistance observed in this study.

4.3.5 Conclusions

The work in this chapter was part of an approach to understand further the role of epigenetics in the perception and signalling of BABA-IR in tomato for use in postharvest protection. This has led to the discovery that long-lasting BABA-IR is transmissible to naïve tissue through

graft junctions leading to postharvest resistance. sRNA sequencing of grafted plants identified a group of mobile 24 nt sRNAs which displayed enhanced gene expression during infection in fruit tissue which was importantly not observed in BABA treated plants as a whole. These genes displayed GO term enrichment in biological processes associated with vesicle trafficking and aspartic acid metabolism. Amino acid profiling demonstrated that BABA treatment leads to major changes in metabolism, including but not limited to an increase in aspartic acid and asparagine. This therefore provides further information on the role of aspartic acid signalling in BABA perception, and importantly, sRNA directed systemic signalling in tomato. Considering the relevance of grafting to the horticulture industry due to enhanced plant vigour (Cerruti et al., 2021, Jeynes-Cupper and Catoni, 2023), these results could be exploited into commercial settings, for both enhanced growth and disease protection.

Chapter 5:

Investigating chemically induced priming in strawberry

5.1. Introduction

5.1.1 Strawberry production and threats

Fragaria vesca, the woodland wild strawberry is a herbaceous plant in the *Rosaceae* family found widely across the globe. Grown commercially however is the modern strawberry, *Fragaria x ananassa* which originated in the 18th century in Europe (Liston et al., 2014). Highly valued for its flavour, the modern cultivar is rich in nutrients, phenolic compounds, and vitamin C (Liston et al., 2014). *F. ananassa* is the dominantly produced variety that accounts for approximately 60% of the world's production, with 8.4 million tonnes grown globally in 2018 (FAOSTAT, 2019, Amil-Ruiz et al., 2011). Due to the soft, fleshy nature of strawberry fruit they are particularly perishable, and are vulnerable to infection by a wide range of pathogens including the necrotrophic fungus *Botrytis cinerea*. Strawberries are characterised as a non-climacteric fruit; they do not continue to ripen postharvest so must be harvested near full ripeness. This involves a trade-off for growers as softer, riper fruit increases susceptibility to pathogens, *B. cinerea* infection can therefore lead to losses of up to 25% for unprotected strawberries (Essghaier et al., 2009).

F. ananassa are a particularly unique system due to their octoploid genome ($2n = 8x = 56$). The full sequencing of the strawberry genome was conducted in 2018 revealing the evolutionary history of the octoploid genome and insight on the dominant sub-genome *F. vesca* (Edger et al., 2019). Due to the high amount of inbreeding within strawberries, reduced genetic diversity has increased their vulnerability to abiotic and biotic stresses. For example, cultivars introduced by breeding programmes in the USA from the 1960's to 90's are from clones with only 17 cytoplasm sources (Dale and Sjulin, 1990). Fungal pathogens on strawberry are typically controlled by chemical treatments however due to environmental and consumer risks alternative approaches are needed. Furthermore, there are fears of potential cross resistance to unrelated fungicides due to their reduced genetic diversity (Leroux et al.,

2010). A potential solution to be incorporated into integrative pest-management in strawberry for postharvest protection is the phenomena of priming.

5.1.2 Chemical immune priming

Plants can be considered to contain both 'innate' and 'adaptive' immune systems, with the learned component being referred to as priming. Prior exposure to a stimulus can therefore lead a plant to produce a stronger, more efficient immune response which demands less energy than direct activation of defence responses (Conrath et al., 2006). A well characterised priming elicitor is β-amino-butyric acid (BABA) which has been studied for its use in many crops against a range of abiotic and biotic stresses (Cohen et al., 2016). BABA has been shown to induce long-lasting and transgenerational resistance in Arabidopsis and tomato, however research by Raghuram et al demonstrated that BABA fails to induce resistance in strawberry against *B. cinerea* (Luna et al., 2012, Raghuram et al., 2019, Wilkinson et al., 2018). The structural homologue of BABA, R-β-Homoserine (RBH), has been shown to induce effective long-lasting resistance with reduced non-target effects in both changes to metabolism and vegetative growth. RBH has been shown to function through both shared and distinct mechanisms to BABA and is effective against both necrotrophic and biotrophic pathogens (Buswell et al., 2018). During infection with *H. arabidopsidis,* RBH primes effective callose and camalexin production however this resistance was shown to be independent of salicylic acid (SA) signalling. In strawberry, RBH has been shown to lead to resistance in *F. vesca* leaves following soil drench of 8–12-week-old plants. RBH-IR was characterised by the priming of *PATHOGENESIS-RELATED 4* (*PR4*) following *B. cinerea* infection (Raghuram et al., 2019). Due to the polar nature of RBH it is a potential candidate for use in hydroponically cultivated strawberry via the nutrient supply stream (Buswell et al., 2018).

5.1.3 Priming in strawberry

The use of priming agents has been explored in strawberry within the past decade for use in postharvest storage. For example, Saavedra et al demonstrated the effectiveness of methyl jasmonate (MeJA) treatment to induce postharvest resistance against *B. cinerea* during storage of Chilean strawberry fruit (*Fragaria chiloensis)*. This enhanced resistance was associated with primed expression of *PR* genes and β-1,3-glucanases (Saavedra et al., 2017). In contrast to treating whole plants by soil drench, direct treatment of fruit with 10 mM BABA has additionally been shown to improve postharvest resistance in strawberry against *B. cinerea* (Wang et al., 2016). This resistance was associated with higher H_2O_2 levels, the promotion of chitinase, β-1,3 glucanase and PAL activity as well as enhanced *PR* gene expression levels during *B. cinerea* infection. The impact of BABA treatment on postharvest physiology such as sweetness scores was measured, with 10 $mmol^{-1}$ of BABA leading to improved sensory attributes after storage (Wang et al., 2016). The use of elicitors as an early seedling soil drench treatment to induce postharvest in fruit has yet to be fully explored in strawberry. Considering the potential toxicity drawbacks associated with BABA and postharvest chemical treatments as a whole, seedling treatments are a more attractive approach for pathogen control (Zikankuba et al., 2019). Furthermore, previous studies and results in **Chapter 3** of this thesis have demonstrated the importance of an early application time point to generate postharvest resistance in tomato (Wilkinson et al., 2018). Due to the clonal reproductive nature of strawberries through the production of stolons known as runners, strawberries are an attractive species to study next-generational priming. If elicitor-treated mother plants display enhanced resistance, the priming phenotype may be transmitted vegetatively to runners.

5.1.4 Role of hypomethylation in priming defence

The long-lasting nature of defence priming, including BABA-IR has been shown to be associated with epigenetic changes such as DNA methylation which are a heritable mark

(Johannes et al., 2009). DNA methylation involves the addition of a methyl group to a cytosine. Importantly, loss of DNA methylation (hypomethylation) has been shown to be associated with long-lasting defence priming. For example, in Arabidopsis *Pst*DC3000 induced transgenerational resistance and global DNA hypomethylation, the DNA hypomethylation triple mutant *drm1drm2cmt3* was shown to share this resistant phenotype (Luna and Ton, 2012). Additionally in tomato, application of BABA has been associated with global hypomethylation in the CHH context and long-lasting resistance against *B. cinerea* (Catoni et al., 2022). Due to the known influence of DNA methylation on resistance, different research groups have explored the use of methylation inhibitors such as 5- azacytidine (5-AzaC) which can be used to generate hypomethylated epialleles. For instance, 5-AzaC treatments produced a hypomethylated *Brassica rapa* population with phenotypic variation across traits including seed protein and oil content (Amoah et al., 2012). In rice, spraying of 5-AzaC led to enhanced resistance against root knot nematodes, further demonstrating the importance of DNA hypomethylation in early defence mechanisms (Atighi et al., 2020). Despite its effectiveness 5-AzaC is a highly toxic compound, therefore other methylation inhibitors have been optimised for safer use including zebularine (Baubec et al., 2009). Zebularine is a cytidine analogue that incorporates into the DNA double helix, this depletes DNA methyltransferases and leads to a reduction in DNA methylation (Santi et al., 1984). Due to the known association of hypomethylation and long-lasting resistance, using methylation inhibitors to induce epigenetic modifications may induce resistance in crops such as strawberry.

5.1.5 Gaps in current understanding

Work in this chapter explores the use of chemical priming agents to induce postharvest resistance in strawberry. The well characterised elicitors BABA and RBH as well as the chemical zebularine were tested to assess their ability to prime resistance at short and long-term stages for use in postharvest. Having identified in **Chapter 3** that young plants displayed

an enhanced capacity to be primed, seedlings were used in this study for elicitor treatments. It is unknown currently if BABA or any elicitors can induce resistance in clonal systems, this therefore represents an attractive solution for enhanced disease resistance. The impact of chemically induced hypomethylation has not been explored for use in strawberry, therefore the methylation inhibitor zebularine was selected in this study. In this chapter disease resistance against *B. cinerea* was therefore tested across a time course following a screen of different elicitor treatments and application methods.

5.2. Results

5.2.1 BABA fails to induce resistance in strawberry seedlings

The effect of BABA was assessed in hydroponically grown plants using a range of concentrations from 0.05 mM to 0.5 mM. Relative growth rate (RGR) was determined following BABA application and calculated from the total green area of individual plants. No differences in RGR were seen in any treatment condition (Fig 28a). One week after elicitor treatment disease resistance assays were conducted on detached leaf tissue against *B. cinerea*. No enhanced resistance was observed in any BABA treated plants (Fig 28b).

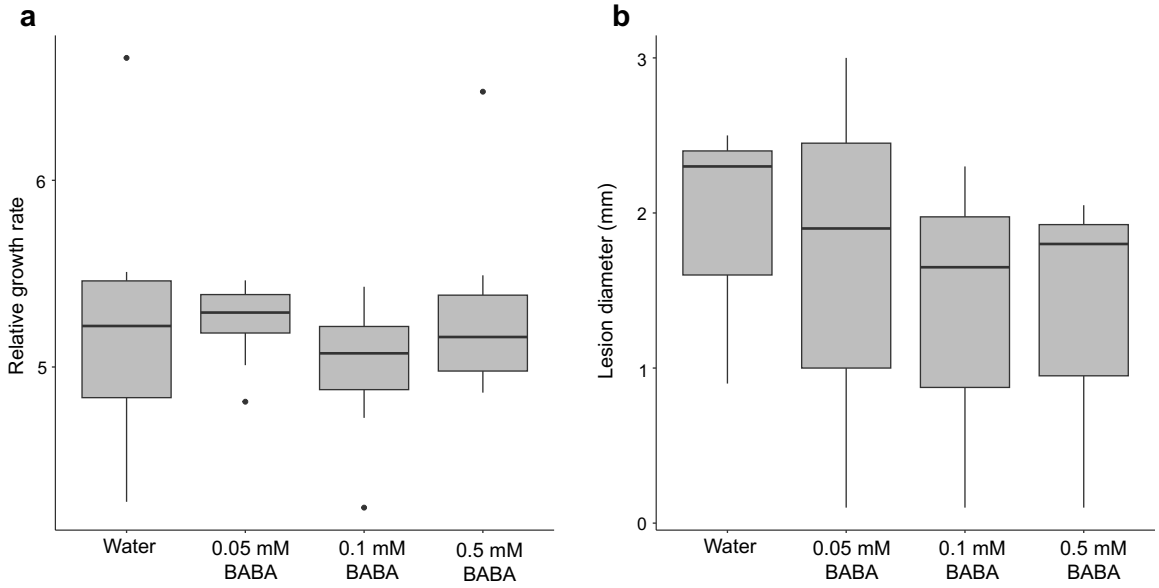


Figure 28: Phenotype of BABA treated seedlings. a) Average relative growth rates (in cm per cm per week; ± standard error of the mean [SEM] of plants between 2 and 3 weeks post 0.05 mM, 0.1 mM or 0.5 mM BABA treatment. **b)** Lesion diameter in 3-week-old strawberry leaves infected with *B. cinerea* at 2 days post infection (dpi). Boxes denote 25th and 75th percentile, bars display min and max values.

Due to both the lack of stress phenotype and absence of IR, even in high concentrations, it was questioned if BABA was being effectively taken up by root tissue during soil drench treatments. Therefore, alternative application methods were used including germinating seeds on BABA-soaked filter paper and foliar spraying with BABA. Interestingly, no effect was seen

in these plants (Fig 29a-b), indicating that irrespective of application method BABA has no effect on resistance in strawberry.

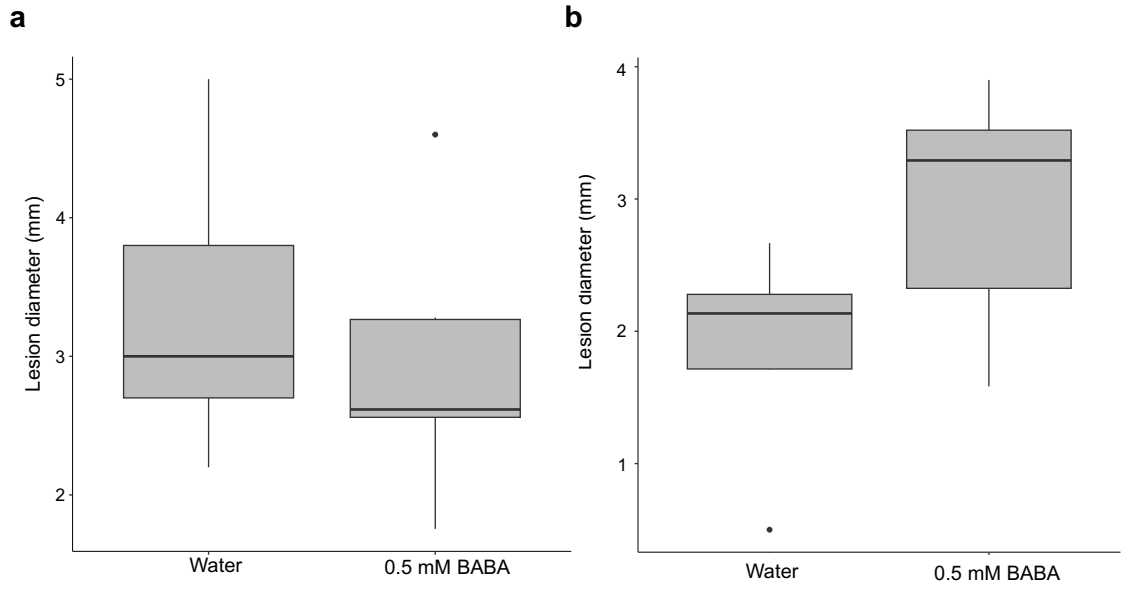


Figure 29: Resistance of BABA seed and spray treated plants against *B. cinerea*. a) Strawberry seeds were germinated on 0.5 mM BABA-soaked filter paper or water, resistance against *B. cinerea* was tested in leaf tissue, lesion diameter was measured in seedlings 2 days post infection (dpi). **b**) Strawberry seedlings were sprayed with 0.5 mM or water control, lesion diameter in 3-week-old seedlings infected with *B. cinerea* at 2dpi. Boxes denote 25th and 75th percentile, bars display min and max values.

5.2.2 RBH induces effective resistance in soil grown strawberry

The effect of an alternative elicitor, R-β-homoserine (RBH) was therefore trialled in strawberry. Seedlings grown in soil were treated to a final concentration of 0.5 mM RBH via root soil drench. Three days after elicitor treatment, resistance was tested via leaf detach assay against *B. cinerea*. Statistically significant resistance was seen in RBH treated plants (Fig 30a). No significant difference was seen in the relative growth rate (RGR) of plants treated with 0.5 mM RBH (Fig 30b). Plants were grown to maturity and resistance was later tested in fruit tissue. A reduction in disease severity was seen in RBH treated plants compared to water control however due to a lack of biological replicates statistical analysis was not conducted (Figure 30c). Data was collected on the development of plants including number of ripe fruit (Fig 30d).

Plants treated with 0.5 mM RBH displayed a delay in fruit production and ripening, however an increase in final total ripe fruit was observed (Fig 30d).

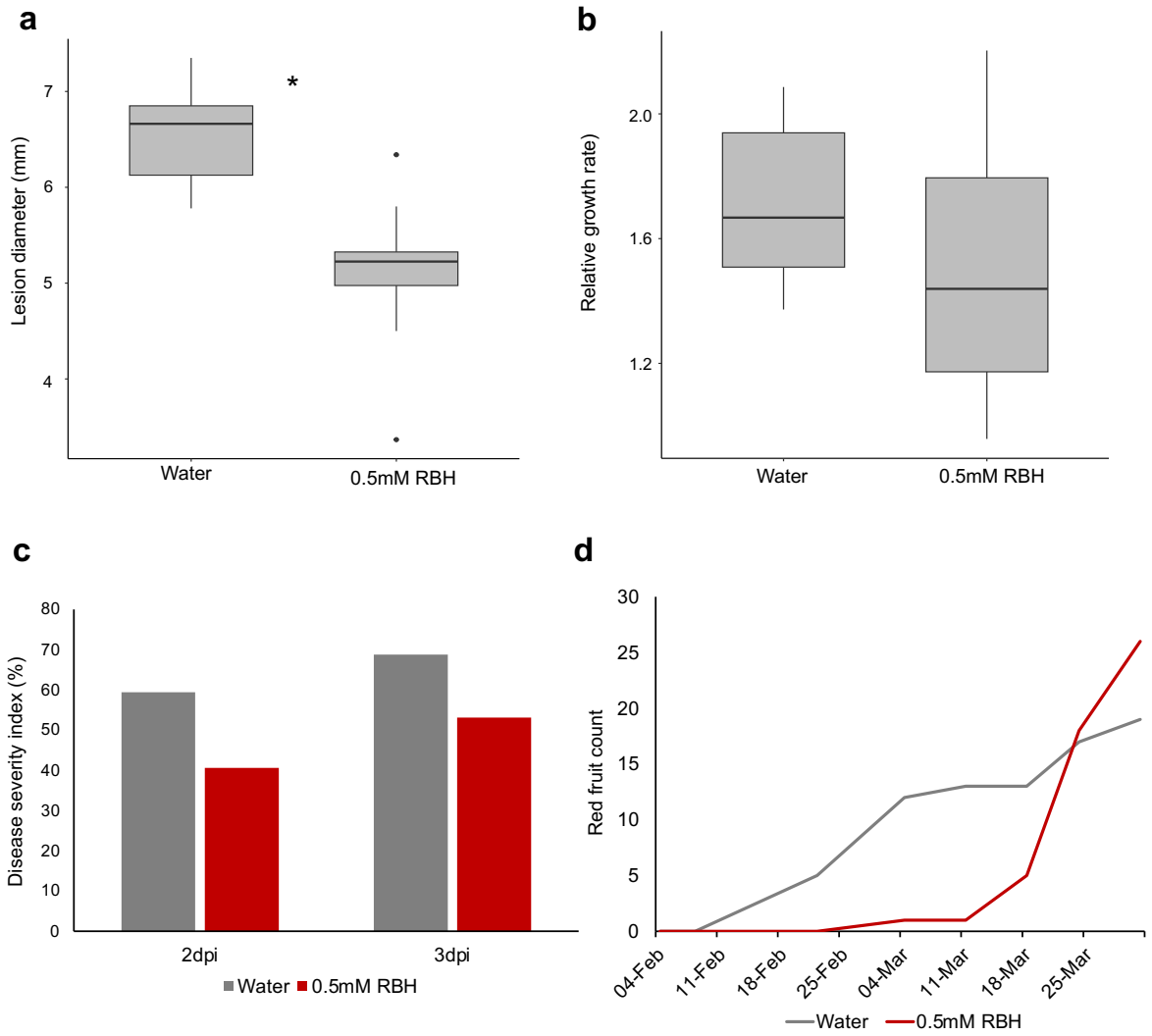


Figure 30: Phenotype of soil grown strawberries treated with RBH. **a)** Resistance against *B. cinerea* in leaf tissue treated with 0.5mM RBH or water. **b)** Average relative growth rates (in cm per cm per week; ± standard error of the mean [SEM] of plants between 0- and 3-days post 0.5 mM RBH treatment. **c)** Disease severity index in ripe strawberry fruit converted from the percentage of lesions in 4 disease categories at 3 days post infection (dpi) with *B. cinerea*. **d)** Fruit developmental data in water (grey) and 0.5 mM RBH (red) treated plants, lines represent total number of red ripe fruit across all replicates between February and April 2022. Boxes denote 25th and 75th percentile, bars display min and max values. Asterisk denotes significant difference between treatment groups determined with Student's t-test ($p < 0.05$, $n = 6$).

5.2.3 RBH fails to induce next-generational resistance in runners

Having identified short term resistance and mild postharvest resistance in soil grown plants treated with RBH, runners from 0.5 mM RBH and water parent plants were removed and grown in fresh soil. Runners were grown and the resistance of leaf tissue against *B. cinerea* was tested. No enhanced resistance was observed in runners from primed plants (Fig 31a). It was therefore assessed if runners from RBH parent plants displayed an enhanced capacity to be primed. Runners from 0.5 mM RBH plants were therefore soil drenched with either 0.05 mM RBH, 0.5 mM RBH or water, resistance assays were conducted in leaf tissue against *B. cinerea*. No statistically significant differences were observed across treatment groups in progeny demonstrating no inherited 'primed to be primed' effect (Fig 31b).

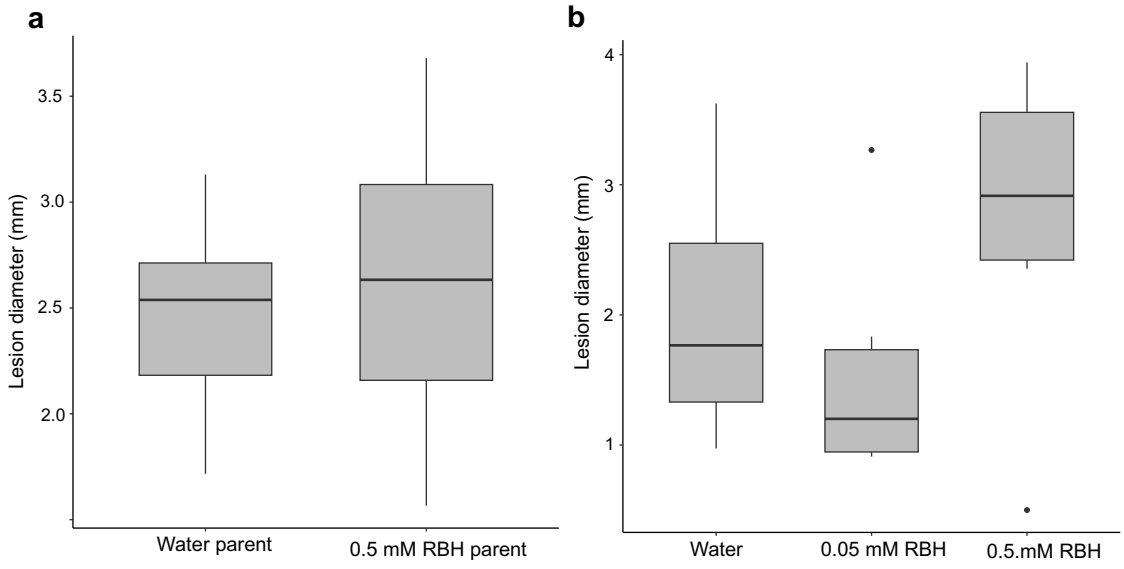


Figure 31: Resistance of runner seedlings against *B. cinerea*. a) Resistance phenotype of leaves of untreated runners taken from either 0.5 mM or water treated parent plants against *B. cinerea* at 2 days post infection (dpi). **b)** Resistance phenotype of runners taken from 0.5 mM parent plants, runners were treated with either 0.05 mM, 0.5 mM or water soil drench, lesion diameter in runners infected with *B. cinerea* at 2 dpi. Boxes denote 25th and 75th percentile, bars display min and max values.

5.2.4 RBH induces concentration dependent resistance in hydroponic seedlings

Having observed enhanced resistance in soil grown plants treated with 0.5 mM RBH, the use of this elicitor was tested in a hydroponic system in towers provided by the Agritech company Saturn Bioponics. A concentration range between 0.05 mM and 5 mM of RBH was used and applied to seedlings by saturating their rockwool plugs. Three days after elicitor treatment, resistance was tested by leaf detach assay against *B. cinerea*. Statistically significant resistance was observed in 0.5 mM and 5 mM treatments compared to water controls. This demonstrated a concentration specific resistance response (Fig 32a). Resistance was tested in leaf tissue again 4 weeks after initial elicitor treatment however no enhanced resistance was observed (Fig 32b). Plants were grown until fruiting; to determine the impact of RBH on plant development, weekly measurements were collected on flower and fruit production as well as fruit ripening. Both water and 5 mM RBH treated plants failed to produce a sufficient number of fruits to conduct comprehensive resistance assays. Data was collected for 0.05 mM and 0.5 mM plants, 0.05 mM RBH plants produced twice the average number of fruits compared to 0.5 mM treated (Fig 32c).

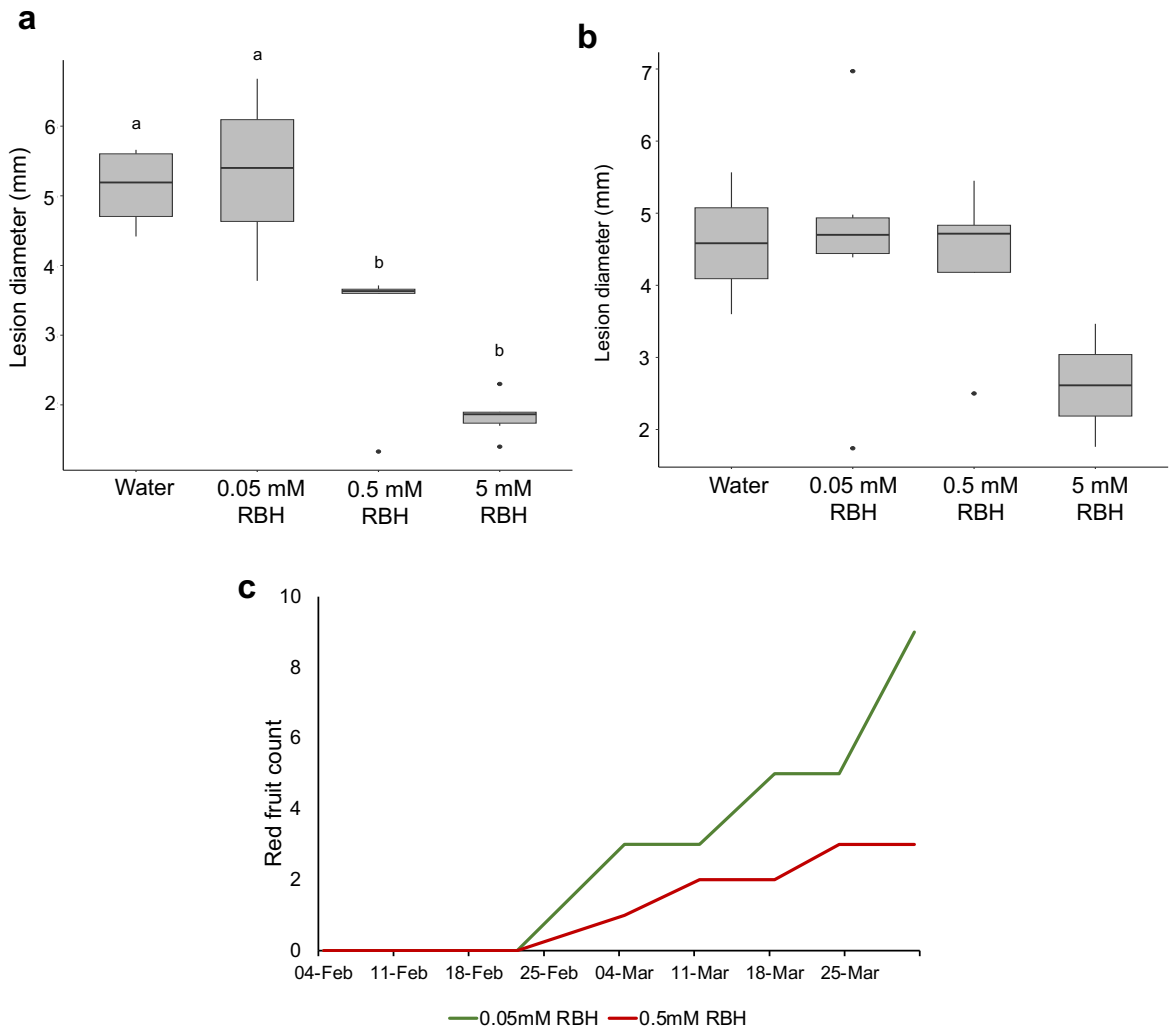


Figure 32: Phenotype of hydroponically grown RBH treated strawberries. Seedlings treated with water, 0.05 mM RBH, 0.5 mM RBH or 5 mM RBH. **a)** Resistance in leaf tissue against *B. cinerea* one-week post elicitor treatment at 3 days post infection (dpi). **b)** Resistance in leaf tissue against *B. cinerea* 4 weeks post elicitor treatment and 3 dpi. **c)** Fruit developmental data in 0.05 mM and 0.5 mM RBH treated plants, lines represent total number of red ripe fruit across all replicates between February and April 2022. Boxes denote 25th and 75th percentile, bars display min and max values. Different letters denote significant difference between groups determined by One-Way ANOVA (tukey posthoc test; $p < 0.05$, n = 5-7).

5.2.5 Zebularine induces effective short-term resistance in strawberry

Finally, following the finding in **Chapter 3** that zebularine induces effective short-term resistance in tomato seedlings, the effect of this methylation inhibitor was tested in strawberry. Zebularine was applied through both seed germination and a soil drench treatment. No difference in lesion diameter of *B. cinerea* infected plants was seen following seed germination on 80 µM zebularine (Fig 33a). Soil drench treatment of 80 µM zebularine one week before inoculation with *B. cinerea* led to statistically significant resistance in leaf tissue (Fig 33b).

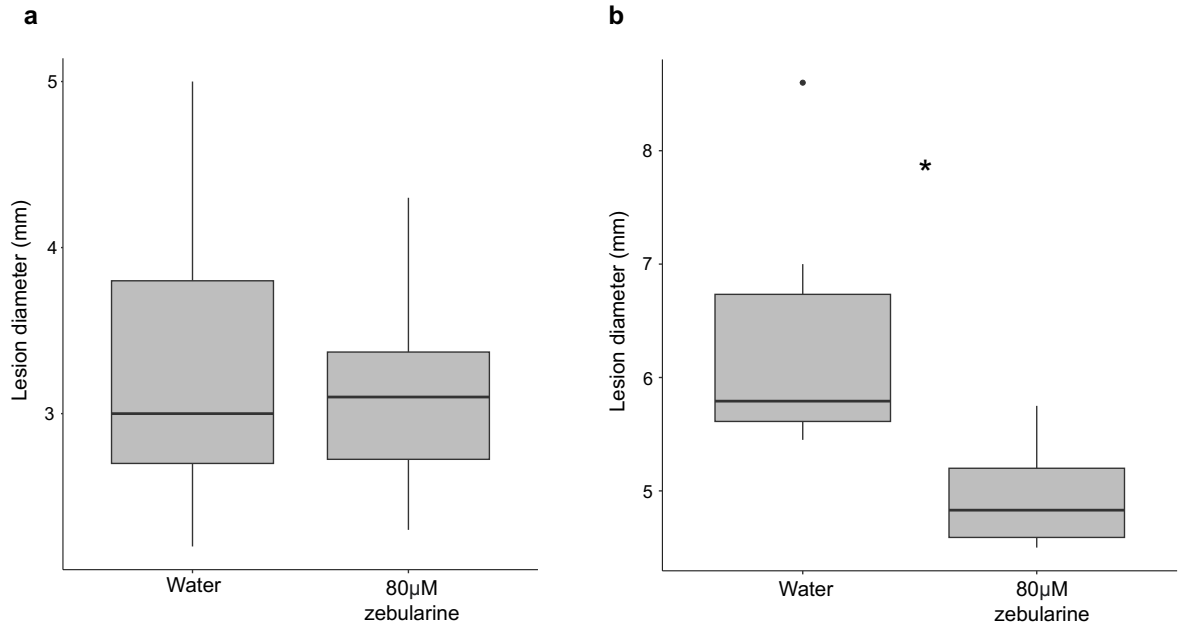


Figure 33: Phenotype of zebularine treated seedlings. a) Strawberry seeds were germinated on filter paper soaked with 80 µM zebularine or water (+ DMSO), resistance against *B. cinerea* was tested in leaf tissue, lesion diameter in seedlings 2 days post infection (dpi). **b)** Strawberry seedlings were soil drenched with 80 µM or control, lesion diameter in 4-week-old seedlings cause by *B. cinerea* at 2dpi. Boxes denote 25th and 75th percentile, bars display min and max values. Asterisk denotes statistically significant difference between treatments determined by Students t-test, ($p < 0.05$, n = 8-10).

5.3. Discussion

5.3.1 RBH IR in strawberry plants

Work in this chapter investigated the effect of various priming elicitors in strawberry (*Fragaria ananassa,* cultivar Durban) against *B. cinerea*. Whilst BABA has been shown to be effective at inducing robust resistance in a range of crops (Cohen et al., 2016), it proved to be ineffective in strawberry seedlings. Interestingly however, RBH, a structural analogue of BABA was found to induce resistance in leaf tissue in a concentration dependent manner, without inducing a major stress response following application (Fig 30a-b). Resistance in RBH treated seedlings was seen mildly at the postharvest level. Whilst plants treated with higher concentrations of RBH appeared to have delayed fruit production and ripening, due to inadequate biological replicates this was not confirmed statistically (Fig 30c-d). Two growth conditions were used for experiments with RBH, both soil grown and hydroponic growth towers with plants in rockwool plugs. No overall differences were seen between growth mediums in the efficacy of RBH demonstrating its effective root uptake (Fig 30, Fig 32).

5.3.2 BABA-IR vs RBH-IR

The discrepancies seen in resistance between BABA and RBH in strawberry is intriguing. Whilst RBH has been shown to function via separate pathways to BABA (Buswell et al., 2018), these elicitors have been shown to be taken up through the same receptor in Arabidopsis (Tao et al., 2022). Recent work revealed through production of T-DNA insertion lines that the amino acid transporter LYSINE HISTIDINE TRANSPORTER 1 (LHT1) is critical for the uptake and systemic distribution of both RBH and BABA in Arabidopsis. Competition experiments additionally revealed that LHT1 has higher affinity for BABA than RBH (Tao et al., 2022). Our results here in strawberry seemingly contrast these findings, with only RBH leading to enhanced resistance and no observed effect of BABA in resistance or stress phenotypes (Fig 31a-b). Additionally, no effect was seen on disease resistance following spray treatment of BABA (Fig 29b). This suggests that plants were not recognising or taking up BABA as

treatments of even high concentrations (over 0.5 mM in preliminary trials not presented here) did not lead to stress or resistance phenotypes typically reported (Luna et al., 2016). Metabolic analysis detecting the presence of BABA would provide clarity on these results considering that BABA is shown to metabolise slowly in planta (Wilkinson et al., 2018). Secondly, investigating the presence and functionality of known amino acid transporter homologs in strawberry may provide insight on if these elicitors are being transported in the same way as described in Arabidopsis. Finally, it is worth noting that this work was conducted in a single strawberry variety. A screening analysis of different cultivars may reveal if this is a general response in strawberry. Unpublished recent work from the lab has demonstrated that there are large differences within cultivars. Preliminary screens have shown that whilst strawberry can be successfully primed, cultivars display large variation in both their innate susceptibility to *B. cinerea*, and their ability to be primed by varying elicitors (Morena et al, unpublished data).

5.3.3 IR-responses in diploid vs octoploid strawberry

The effectiveness of RBH and BABA in strawberry has been investigated by other research groups, however treatments were conducted in wild *F. vesca* plants which were 8-12 weeks old (Raghuram et al., 2019). This study similarly identified that RBH soil drench induces effective resistance against *B. cinerea*. However, BABA soil drench led to susceptibility against *B. cinerea* in a concentration dependent manner. This therefore suggests that there may be differences between the elicitor perception and/or induced resistance responses of diploid wild *F. vesca* and octoploid *F. ananassa* hybrids. Whilst it has now been identified that BABA is produced as an endogenous stress hormone in Arabidopsis and a variety of species, it has not been tested specifically in strawberry (Thevenet et al., 2017). It may therefore warrant further investigation if BABA is produced naturally in strawberry. The polyploid genome of *F. ananassa* originated over 300 years ago, recent genome sequencing revealed that the *F. vesca* sub-genome is dominant and may dominate the disease resistance response

(Edger et al., 2019). Polyploidy events in plants have facilitated the emergence of a range of important agronomic traits, hence polyploid species have been shown to display enhanced tolerance and are often found in more extreme conditions. Interestingly, experiments examining salt resistant varieties of rice identified that salt tolerance was enhanced in tetraploid rice compared to diploid. This enhanced resistance was associated with an increase in CHH methylation levels and the suppression of TEs adjacent to stress-responsive genes, which was not observed in diploid plants (Wang et al., 2021). With the relatively limited genetic resources in strawberry, studies investigating how the octoploid genome may influence the ability of strawberry to maintain epigenetic memories and transmit these to progeny compared to its diploid originator is yet to be unexplored. This poses an interesting research question from both an evolutionary and epigenetic perspective.

5.3.4 Investigating next generational IR in strawberry

Work in **Chapter 3** demonstrated that BABA-IR in tomato against *B. cinerea* was transmissible to progeny. Indeed, many examples of transgenerational resistance following priming treatment have been identified (Luna et al., 2012, Slaughter et al., 2012). It is known that phenotypic variation in clonal plants can occur due to heritable changes in DNA methylation (Wibowo et al., 2022). Considering the asexual reproductive strategy of strawberry, it was investigated if runners produced by resistant parent plants also displayed enhanced resistance which may be attributed to inherited epigenetic changes. Our experiments failed to observe enhanced resistance in progeny from RBH treated plants. It may be hypothesised that the resistance phenotype in our parent plants was not sufficient to lead to long-lasting epigenetic changes and thus transmission of this signal. Therefore, higher concentrations, or repeated application of elicitor treatments may lead to effective transmission to progenies. Future characterisation of differentially expressed genes (DEGs) and differentially methylated regions (DMRs) in resistant strawberry plants will provide potential markers for long-lasting resistance. The heritability of these marks will be able to be analysed in runner populations.

Potential candidates could be identified from mining existing data and quantified via quantitative-PCR or use of methylation-sensitive restriction enzymes. For example, Raghuram et al identified *PR1* as a DEG in RBH-primed infected plants (Raghuram et al., 2019). This gene was also both differentially expressed and differentially methylated in tomato seedlings treated with BABA in **Chapter 4** of this thesis. Further characterisation of candidates like this will therefore provide insight into how conserved priming mechanisms are against *B. cinerea* in strawberry and tomato.

5.3.5 A role of epigenetics in the strawberry immune responses

Previous research has demonstrated an important role of hypomethylation as an early plant immune response (Yu et al 2013). For example, a study in rice used a pre-treatment with 50 μM of 5-azacitidine (5-AzaC) and found that this reduced susceptibility to root knot nematodes, this response was shown to be conserved in both monocot and dicot species (Atighi et al., 2020). Work in **Chapter 3** of this thesis supported these findings demonstrating that soil drench treatment of the methylation inhibitor zebularine provides effective short-term resistance however not long-term resistance. Soil drench with the 80 μM in strawberry induced effective resistance against *B. cinerea* (Fig 33). Plants displayed an extreme stress phenotype from this treatment however and fruit was not produced preventing the full characterisation of long-lasting resistance effects. Considering the lack of postharvest zebularine-IR in tomato, and the absence of a strong postharvest resistance response in other strawberry experiments however, it is unlikely that this individual zebularine treatment would induce postharvest resistance in strawberry fruit.

5.3.6 Conclusions

Results in this chapter reveal that RBH is a promising elicitor to provide resistance against *B. cinerea* in strawberry seedlings. RBH was effective in multiple growth mediums and therefore could be added to water-based hydroponic nutrient solutions for use in commercial settings.

The impact on full growth parameters such as fruit quality and resistance against other biotrophic and insect pests however must be assessed prior to full-scale use. BABA failed to induce resistance or stress through any application method or concentration trialled in strawberry. This raises questions on whether strawberry possess a functional receptor to uptake this compound or if this was a cultivar specific response, further work is therefore required. Whilst strawberry contains many unique traits compared to tomato such as a non-climacteric ripening and an octoploid genome, its clonal reproductive strategy in particular makes this an interesting species for studying long-lasting resistance. Further work optimising repeated application of RBH in a range of cultivars may reveal its potential for use in next-generational resistance.

Chapter 6:

General discussion

6.1 Summary

Advancing our current understanding of the plant immune system presents a promising way to manipulate crops in order to enhance yields and prevent losses at the postharvest level. The fungal necrotrophic pathogen *Botrytis cinerea* is a major driver of postharvest loss and new crop-protection strategies with minimal environmental impact are required. Priming represents a part of the adaptive immune system in plants, in which exposing a plant to an initial stimulus leads to a stronger, faster immune response when a second stress is encountered. Work in **Chapter 3** demonstrated the influence of plant developmental stage on the formation and maintenance of long-lasting β-amino butyric acid (BABA) induced priming in tomato. In **Chapter 4**, details of the perception and transmission of this long-lasting resistance signal, including its movement across graft junctions were outlined. Finally, in **Chapter 5** the effectiveness of a selection of elicitors were trialled in strawberry to elucidate their potential in inducing postharvest resistance in this important crop vulnerable to *B. cinerea*.

Work presented in this thesis used a variety of multi-omics sequencing techniques, metabolic profiling, pathogen assays and grafting experiments. Given the known role of BABA in inducing a wide range of changes within the plant, including phytotoxicity and stress, experiments were designed in an attempt to isolate changes purely associated with systemic resistance. For example, in **Chapter 3** BABA12 plants which displayed a large range of transcriptomic and methylation changes without postharvest resistance against *B. cinerea* were used as a negative control compared to resistant BABA2 plants to isolate -omics changes more likely attributed to resistance. In **Chapter 4,** BABA-homograft plants were selected as a control compared to hetero-graft plants to isolate potentially mobile effects rather than the impact of a BABA treatment alone**.** In this PhD a large number of -omics datasets were generated with unique sampling timepoints, this therefore presented a challenge to capture dynamic, precisely regulated immune responses. Nevertheless, new insights have been revealed on the priming response in tomato. Here, results and hypotheses generated

from this thesis are discussed alongside suggestions for future work for use in inducing postharvest resistance.

6.2 The importance of plant age in generating an effective priming response

Extensive research has characterised the defence processes associated with BABA-IR in a range of plant species (Cohen et al., 2016). Although the timing of elicitor treatment has previously been demonstrated to be important for establishing long-lasting priming in tomato, the reasons driving this age-dependent phenotype were unknown (Wilkinson et al., 2018). Here, it is shown that an early priming timepoint is critical for establishing long-lasting resistance in fruit tissue and resistance in progenies. Whilst treatments at 2-weeks led to effective resistance in this study, weekly treatments suggested that this early priming window may be extended, with soil drench treatments until 6-weeks leading to postharvest resistance (Fig 9, Fig 10). This 6-week timepoint coincides with flower and initial fruit production in our Micro Tom cultivar (Carvalho et al., 2011). Given the epigenetic reprogramming known to occur in fruit tissue (Zhong et al., 2013), the significance of this may warrant further investigation. Whole genome bisulfite sequencing (WGBS) analysis in **Chapter 3** revealed that young and old plants display unique responses to BABA treatment in their methylation profiles (Fig 13). Furthermore, major differences were seen in the basal global methylation status of young and old tomato plants, with young seedlings displaying a significantly lower global CHH methylation level (Fig 12). It is hypothesised that this may be contributing to the enhanced phenotypic plasticity observed in this study. It is currently unknown if this lower CHH methylation level is associated with any other epigenetic changes which may be influencing the ability of these plants to form long-lasting memories of stress. Further characterisation through techniques such as formaldehyde-assisted isolation of regulatory DNA elements (FAIRE) or transposase-accessible chromatin sequencing (ATAC-seq) may reveal if these plants display differences in their chromatin architecture and accessibility correlating with this low global level of CHH methylation. The observation that priming of young seedlings induces

formation of long-lasting ‘memories’, but that older plants fail to do so raises interesting questions on both life-history strategies and growth-defence trade-offs. The establishment of stress memories is generally regarded as an exception rather than a rule, as formation of strong memories may need to be reset due to potential dynamic future environmental conditions (Crisp et al., 2016). Nevertheless, work presented here may suggest that the ability to form immunological memories is more likely, and has a better adaptive advantage, in young plants compared to older plants.

In general, differentially methylated regions (DMRs) produced at an early timepoint of BABA application were not maintained over the lifetime of plants in any cytosine context (Fig 13). However, continued changes in the landscape of DMRs were observed, particularly in the CHH context, which were unique depending on the timepoint of elicitor application. These observations led to the generation of a ‘foundational hypothesis’ describing how changes at an early developmental timepoint may have accumulative, knock on effects in the subsequent methylation and transcriptomic responses. Akin to building a house, large changes to the foundations in the early building phase are more likely to have knock on effects on the entire architecture and structure of the building. An early BABA treatment in seedlings is therefore having a pivotal effect on the accumulation of epigenetic changes and subsequently the primed transcriptomic response over the entire lifetime of the plant. This is reflected in both the continuous accumulation of CHH DMRs after the time of priming treatment and the greater transcriptional response observed during infection at T3 in BABA2 plants (Fig 13, Fig 22). Whilst the late BABA12 treatment is leading to changes within the plant, as demonstrated by T3 transcriptomic data, in the house analogy these late treatment changes are less impactful on the structure as a whole and may be on a higher level and represent a crack in a window. This late treatment may therefore cause a more localised response which is not influencing a long-lasting defence response in the plant.

6.3 Exploring the role of abscisic acid in defence responses

Whilst plant age has been shown to be critical for the establishment of long-lasting priming, there are other determining factors influencing BABA-IR such as the phytohormone abscisic acid (ABA). ABA has been shown to be essential for BABA-IR, with resistance against *Hyaloperonospora arabidopsidis* impaired in ABA mutants due to absence of callose deposition (Ton and Mauch-Mani, 2004). Metabolomic analysis revealed that tomato seedlings primed with BABA displayed higher levels of ABA in fruit tissue at the postharvest stage. Interestingly however, external application of ABA to fruit led to an increase in susceptibility in un-primed plants against *B. cinerea*, which was not observed in BABA primed plants (Wilkinson et al., 2018). The complex role of ABA is also seen in early defence responses, where it can have a positive or negative influence. For example, ABA deficient *sitiens* tomato mutants have enhanced resistance against *B. cinerea* and *Odium neolycopersici* (Achuo et al., 2006). Due to the Covid-19 pandemic preventing experimental work at the start of this PhD in 2020, a computational project was designed to investigate the role of ABA in defence responses (Appendix III). Data was collected from primary research articles for use in machine learning analysis in an attempt to unravel the complex role of ABA in resistance. Predictive models (decision tree and random forest) demonstrated that plant age is a major factor influencing the susceptibility of tomato plants to necrotrophic pathogens such as *B. cinerea* following exogenous application of ABA (Appendix III). In this work, plant age as well as ABA concentration were shown to be major predictors of resistance responses. The influence of these computational predictions was later tested experimentally in tomato. Treating four-week-old plants with ABA led to a mild increase in susceptibility in higher concentrations, however eight-week-old plants displayed significant enhanced resistance against *B. cinerea* (Appendix III Fig 3). Interestingly in grafting experiments in **Chapter 4**, an increase of endogenous ABA was seen in HG and BG plants (Fig 26). Whilst not statistically significant, these plants displayed enhanced resistance corroborating results of previous studies (Wilkinson et al., 2018). The influence of ABA in fruit tissue is particularly complex given its influence and cross talk with ethylene (ET) levels as well as fruit ripening. ABA can

influence ripening in different ways depending on both timing and mode of application (Diretto et al., 2020). These observations as a whole further demonstrate the contradictory yet pivotal role of ABA in defence, particularly in tomato. Furthermore, given the essential role of ABA in BABA-IR (Ton and Mauch-Mani, 2004), this suggests that ABA is essential for short and long-term priming responses however it has a clear contrasting role depending on plant age and organ type. Greater characterisation of its importance in fruit resistance and priming in seedlings will provide further clarity. Considering its role in priming and hormone cross talk as a whole, it may play a critical role acting to buffer and prevent costly singular defence responses. ABA is therefore a highly influential factor influencing both short and long-term defence. To gain further insight into mechanisms regulating long-term and next-generational resistance in this project however, epigenetic changes were investigated.

6.4 The importance of RNA directed DNA methylation in priming

Methylation differences were seen frequently in the CHH methylation context in **Chapter 3**, with young plants displaying a large increase of hyper-DMRs following BABA treatment, and a continuous increase in CHH DMRs throughout the lifetime of the plant (Fig 13). In contrast, plants treated at twelve weeks (BABA12) demonstrated larger differences in the non-CHH context. This may suggest that early BABA treatment is leading to changes in RNA directed DNA methylation (RdDM) activity, the plant-specific pathway responsible for CHH DNA methylation regulation. The involvement of RdDM in both early plant defence response and priming has been described in both monocot and dicot species (Catoni et al., 2022, López et al., 2011, López Sánchez et al., 2016, Meijer et al., 2023). Whilst RdDM has been implicated for its role in long-lasting priming, a direct correlation associated to gene expression, methylation status and siRNA expression is not always observed in the literature or indeed in this work (Catoni et al., 2022). Notably, promoters and transposons associated with up-regulated small RNAs (sRNAs) identified in **Chapter 4** did not as a whole display an increase in their proportion of methylation in any cytosine context (Fig 23). This may be attributed to a

failure to capture dynamic processes in our data-analysis. For example, sRNA sequencing data was conducted on leaf tissue 6-weeks post grafting and 6.5-weeks post BABA treatment compared to our WGBS conducted on 3-week-old, 13-week, and ripe fruit tissue. Alternatively, the lack of correlation between methylation status and gene expression in **Chapter 3** may be attributed to uncaptured *in trans* mechanisms at play regulating methylated genes and potentially defence pathways.

6.5 Advances in our understanding of the systemic signal of BABA-IR in tomato

Due to the sessile nature of plants, they have evolved many sophisticated processes in order to facilitate their survival such as systemic acquired resistance and the production of volatile signals (Kachroo and Kachroo, 2020, Shah and Zeier, 2013) Precisely how these systemic signals are generated and perceived is a popular research topic. Results in **Chapter 3** demonstrated that elicitor soil drench of roots generated a long-lasting resistance response in tissue that was not present at time of application. As methylation analysis failed to identify long-lasting DMRs corresponding to a continued control of transcriptional regulation, it was therefore questioned if this phenotype may be due to a systemic mobile signal. The influence, or direct movement of sRNAs was subsequently investigated through grafting experiments. Combining the use of elicitor treatments and grafting of BABA primed rootstock to naïve scion tissue revealed that this was a phenotype regulated by a mobile signal (Fig 19). These findings therefore provide novel evidence on the systemic regulation of this long-lasting BABA-IR. The use of primed root stocks may therefore be explored for use in applied settings, combining cultivars displaying varying degrees of both resistance and the ability to be primed may optimise disease resistance and crop quality. For example, experiments conducted in Moneymaker display reduced BABA-IR at the postharvest stage and are known to exhibit a more extreme stress response to BABA treatments (Appendix II Fig 9) (Luna et al., 2016). This therefore represents a potential system to induce resistance in fruit through use of a Micro Tom primed rootstock and Moneymaker scion.

6.6 Identification of mobile sRNA candidates in resistant plants

sRNA sequencing analysis revealed a subset of genes overlapping up-regulated 24 nt sRNAs in resistant grafted plants only (HG and BG) which displayed primed transcription during *B. cinerea* infection in fruit tissue (Fig 22). This pattern was not seen in 24 nt sRNAs across all BG plants, importantly demonstrating this is a unique primed response associated with a mobile signal. These primed genes of interest displayed significant enrichment in biological processes including “Aspartic acid metabolism” and “COPII mediated vesicle trafficking”. The first of these processes presents a highly interesting result considering existing knowledge on the importance of aspartic acid signalling in BABA perception and stress responses (Luna et al 2014b). The BABA receptor in Arabidopsis is known to be an aspartyl-tRNA synthetase and levels are shown to increase in planta following BABA root drench treatment (Luna et al 2014b). Beyond the role of vascular Plant One Zinc Finger protein (VOZ) transcription factors and ABA, little is known on the downstream signalling of this initial perception process (Schwarzenbacher et al., 2020). Data presented in **Chapter 4** may therefore provide evidence on a potential epigenetic role of aspartic acid signalling in systemic long-lasting resistance. Further characterisation on the role of aspartic acid signalling, particularly the hypothesised receptor of BABA in tomato and alternative candidates such as asparagine synthetases, will provide insight into the relaying of this signal from perception in root tissue to inducing long-lasting resistance postharvest. Considering the stress associated phenotype of BABA this may additionally identify pathways to bypass the growth-defence trade-off seen in other cultivars and species.

The analysis of sRNAs in **Chapter 4** and genes in **Chapter 3** has identified potential candidates for further analysis and direct targeting to assess their role in inducing postharvest resistance. Current legislation issues and expensive costs of development and commercialisation of genetically modified (GM) technology mean that new crop protection

strategies are desired. Therefore, the manipulation of RNA interference (RNAi) and RdDM via exogenous sRNA application presents a novel avenue for commercial application to target specific genes of interest or susceptibility factors. For example, recent work in Arabidopsis has provided proof-of-concept data demonstrating that exogenous uptake of synthetic miRNA through roots leads to induction of post-transcriptional gene silencing (PTGS) and targeted phenotypic differences in root architecture (Betti et al., 2021). Thus, direct application of identified sRNAs in this work may be an attractive tool to induce targeted gene silencing through root treatment, spray induced gene silencing (SIGS) or use of epibreeding.

6.7 Outstanding questions regarding the systemic signal of BABA-IR

The discovery that BABA induces a mobile systemic signal raises many questions on the mechanisms behind this which warrant further investigation. Two potential hypotheses for this phenotype are currently suggested:

1. BABA soil drench treatment is leading to an accumulation of sRNAs in root tissue which are being directly transported to aerial tissues and subsequently directing changes in gene expression during infection.
2. BABA treatment is leading to the production and transport of an alternative signal which impacts RdDM machinery triggering local changes in sRNAs in leaf and fruit tissue.

The use of RdDM mutants in components such as Pol IV, DCL3 or RDR2 and further profiling of BABA induced sRNAs in different plant organs, including the root and leaf tissue will provide insight into the mechanisms behind this. Additionally, characterisation of phloem exudates in BABA treated plants will provide insight into alternative signals such as mRNA, amino acids or hormones perhaps involved in this resistance signal.

Whilst it has been demonstrated that this signal can be transmitted to naïve tissue, which is not present at time of treatment, the timing of BABA treatment has additionally been shown to be critical for establishing long-lasting resistance. These two observations raise the question on the importance of plant age and tissue type for both the perception, signal generation, and response generated due to BABA treatment. Using grafting experiments to combine both old and young plants would provide insight onto the driving forces behind this. For example, if resistance was seen through grafting an old, BABA-primed root stock onto a young naïve scion, this would demonstrate that the 'mobile signal' is generated irrespective of plant age, however a young 'primeable' scion with a 'plastic' epigenome is necessary to generate the long-lasting memory. Alternatively, if resistance was not generated from the grafting of young scion tissue onto an old root stock primed with BABA this would demonstrate the importance of the age of root tissue in generating this response – this may be dependent either on local gene expression or the low global methylation status.

6.8 Major differences in priming responses between tomato and strawberry

Results in **Chapter 5** demonstrate that strawberry and tomato plants display major differences in their ability to be primed by BABA. Whilst this elicitor has been demonstrated to be broad acting, no resistance was reported against *B. cinerea* following application through soil drench, seed germination or spray treatment (Fig 28, Fig 29). The absence of a stress phenotype even with application of high concentrations has led to the hypothesis that BABA may not be effectively taken up or perceived by strawberry plants. This is an interesting finding considering that the elicitor RBH in contrast did induce short-term resistance through soil drench application (Fig 30). Considering the previous reports of shared perception of these two similar elicitors in Arabidopsis, this raises additional questions on the amino-acid transporters in strawberry (Buswell et al., 2018, Tao et al., 2022). Further work is required in other cultivars to confirm if this is a shared phenotype, in addition to repeated elicitor treatments to improve the postharvest resistance generated from RBH treatment. Effective

levels of postharvest resistance in parent plants may motivate future research in runner populations. If epigenetic marks associated with resistance could be identified, the use of clonal systems would be an attractive tool to study their maintenance and transmission in the future.

6.9 Final conclusions

Work in this thesis aimed to further characterise the long-lasting nature of BABA induced defence priming in tomato for use against *B. cinerea*. Whilst epigenetic mechanisms have been implicated for their role in long-lasting resistance, they have until now been uncharacterised for use in postharvest in tomato. Time course analysis provided valuable insight into changes in DNA methylation associated with resistance and plant development, characterising what is suggested to be enhanced epigenetic plasticity. Importantly work in this PhD identified that BABA-IR is graft transmissible and thus attributed to a systemic signal. Whilst the exact mechanisms behind this are still to be elucidated, a group of mobile 24 nt sRNAs have been identified which are associated with enhanced transcription during resistant *B. cinerea* infected fruit tissue only. Interestingly, these sRNAs are enriched in biological processes associated with aspartic acid signalling, an important process already implicated for its role in BABA perception in Arabidopsis (Luna et al 2014b). This work has therefore identified a range of genetic and sRNA candidates whose role in IR warrant further investigation through targeted approaches. Furthermore, the discovery of graft-transmissible BABA-IR presents a potential tool for both improving our fundamental knowledge on epigenetic IR signalling and use in horticultural industries to reduce postharvest losses to fungal pathogens.

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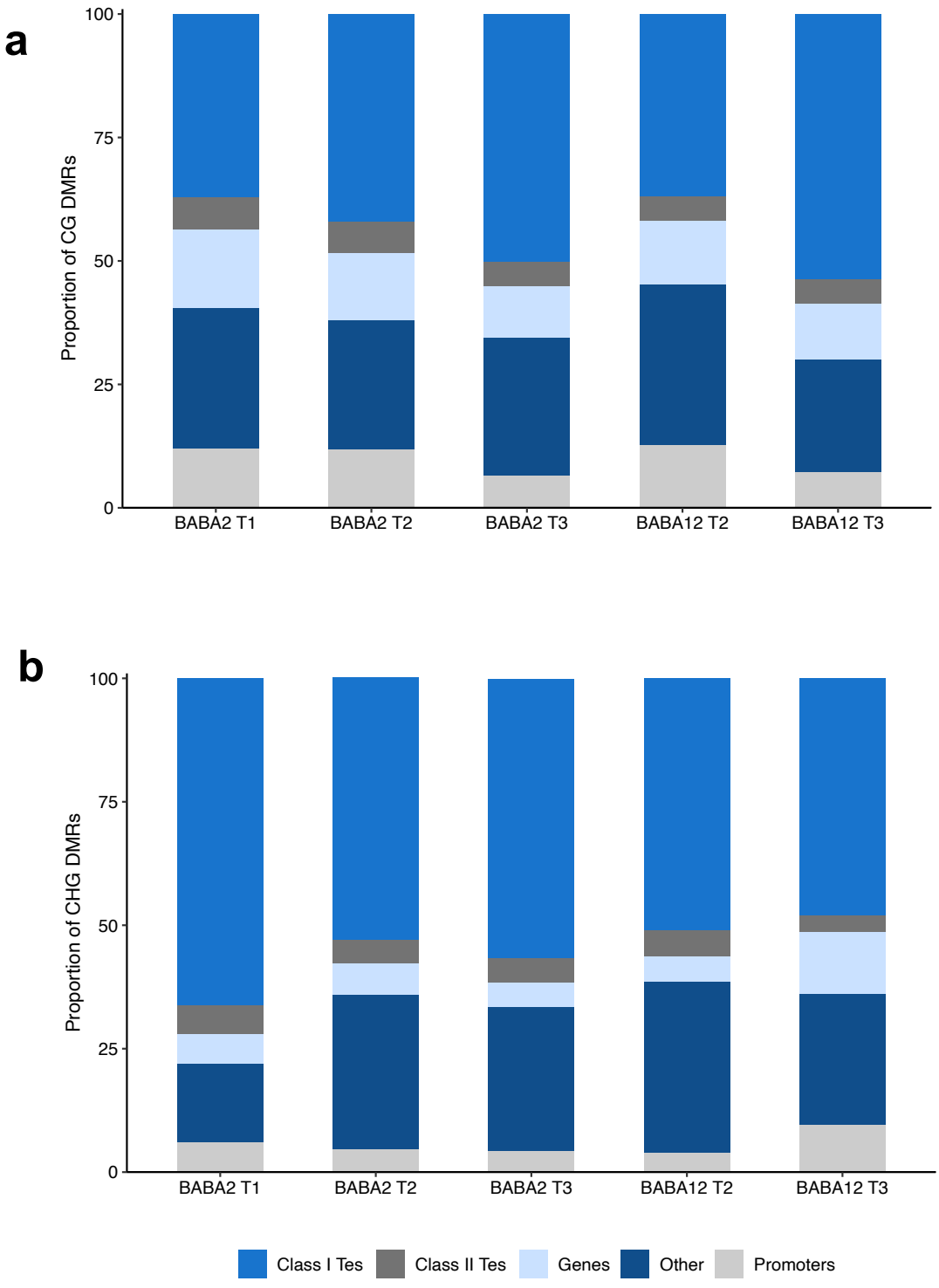
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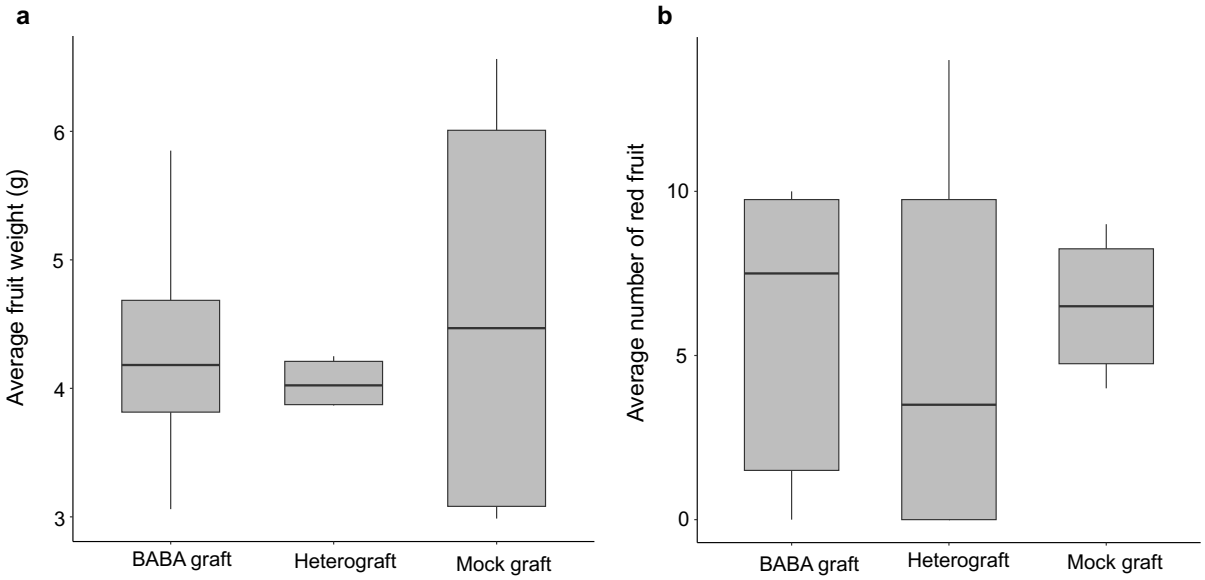
Appendices

Appendix I: Chapter 3 supplementary figures

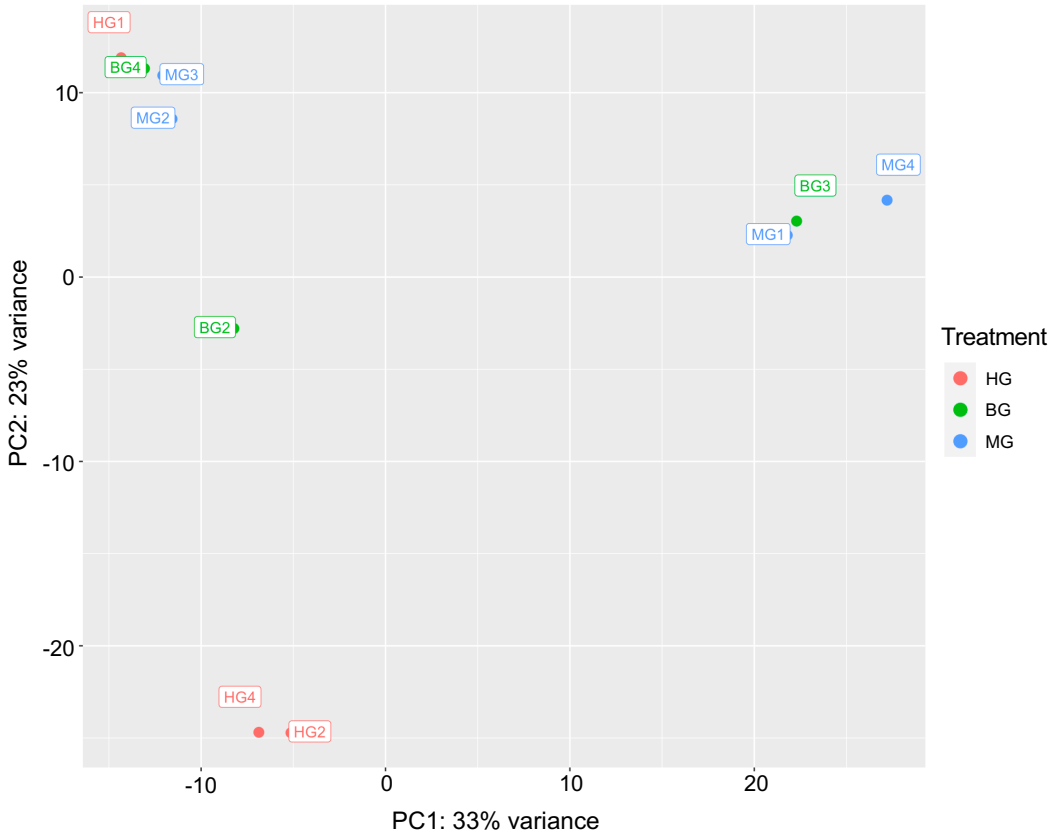


Appendix I Fig 1: Location of non-CHH DMRs across genomic features a) Location of CHG DMRs across genomic features, **b)** Location of CG DMRs across genomic features.

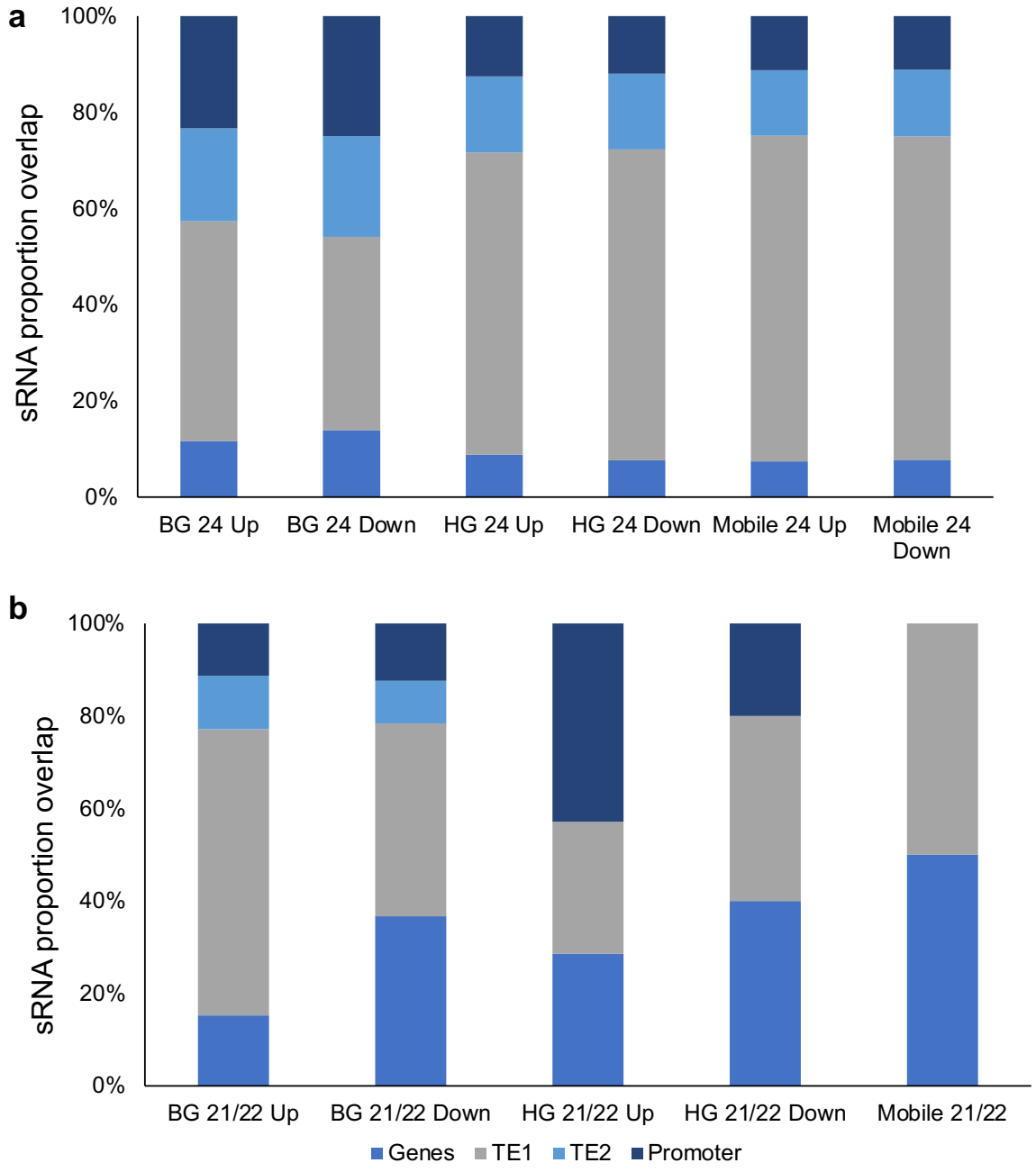
Appendix II: Chapter 4 supplementary figures



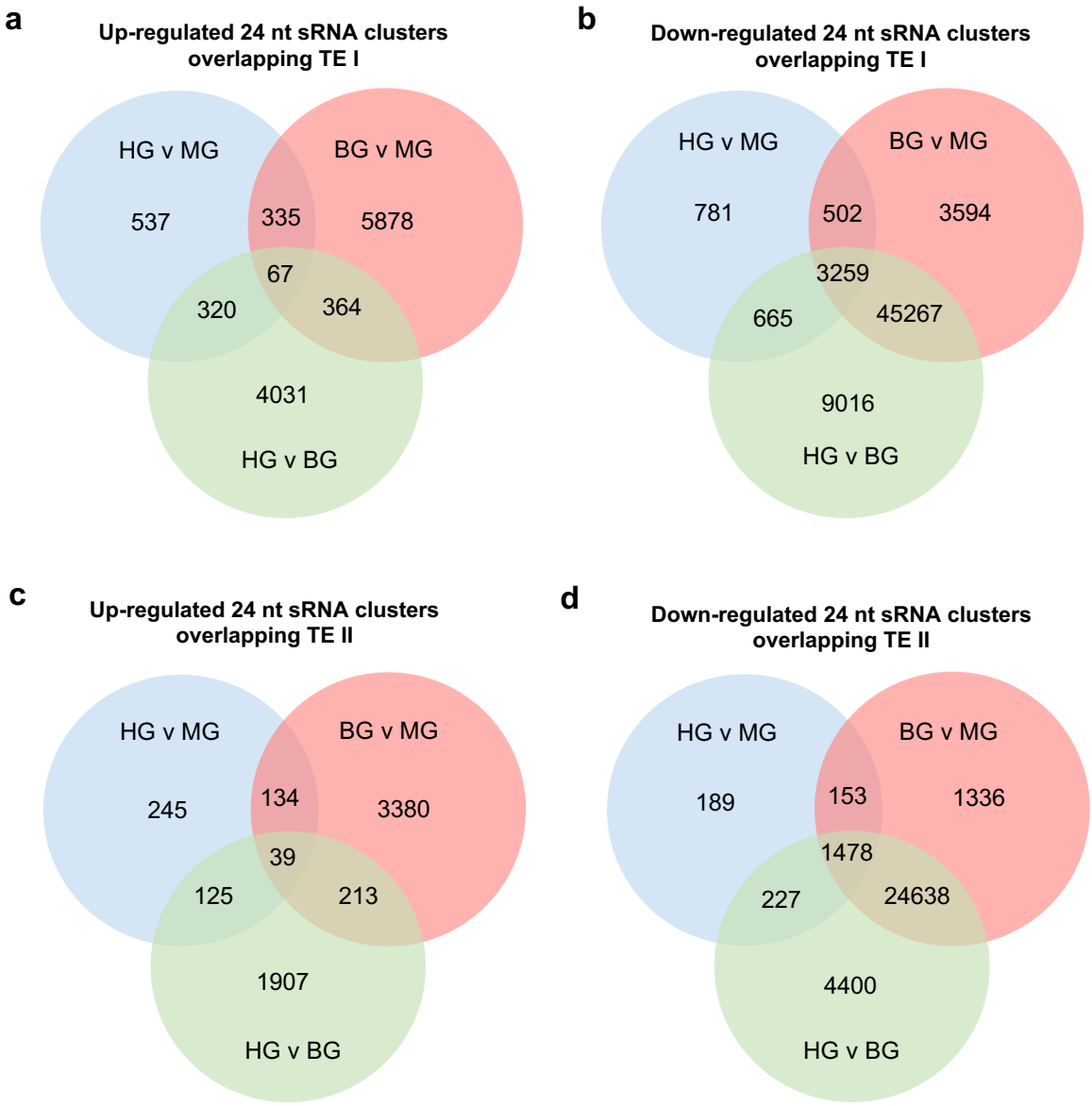
Appendix II Fig 1: Phenotype of fruit in grafting treatments. a) Average fruit weight (g) calculated from the first three fruit produced on each individual plant. **b)** Average number of ripe red fruit on 15-week-old plants.



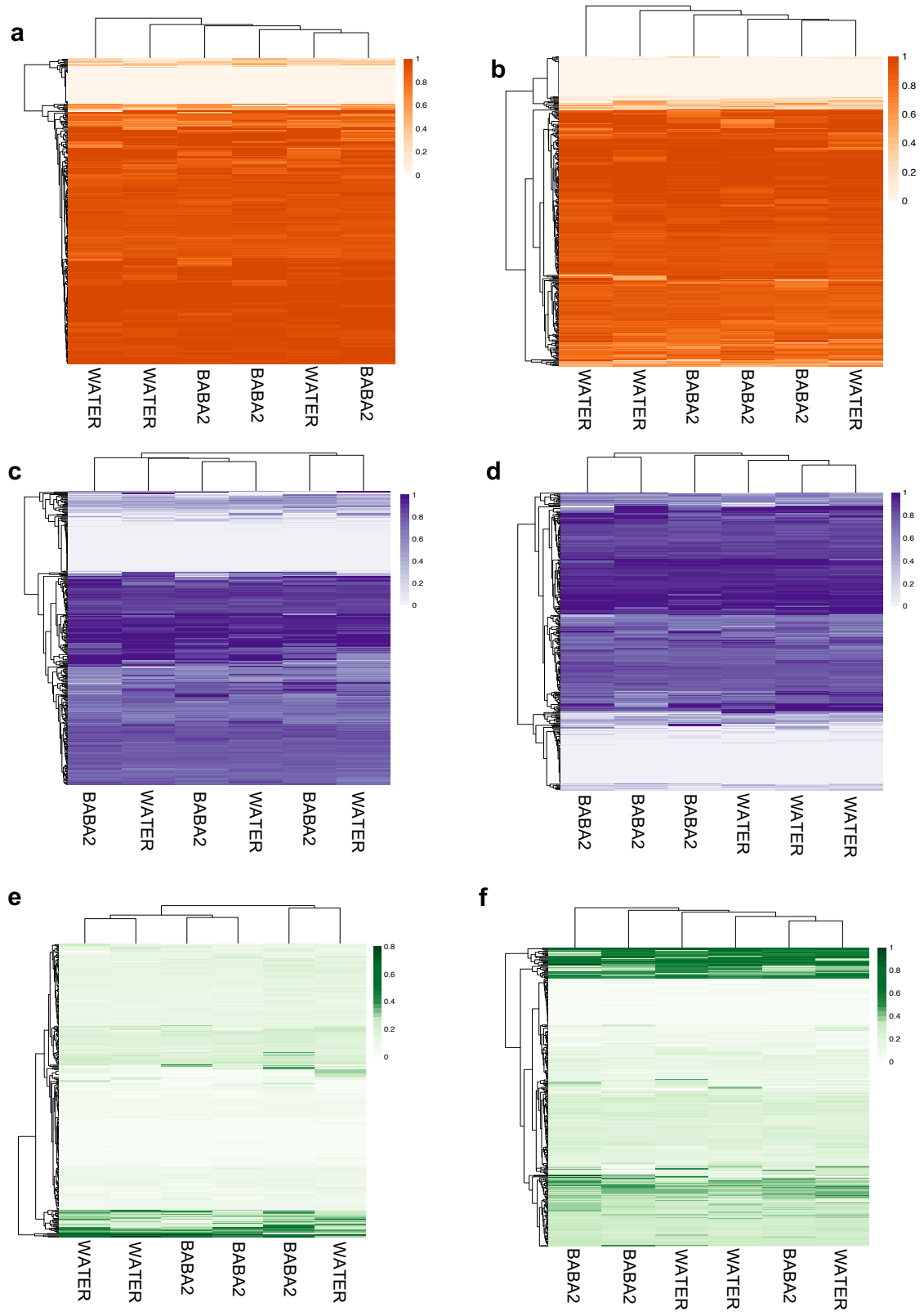
Appendix II Fig 2: **sRNA sequencing principal component analysis (PCA) plot** of sRNA sequencing from grafted plants. HG = heterograft, BG = BABA graft, MG = mock graft.



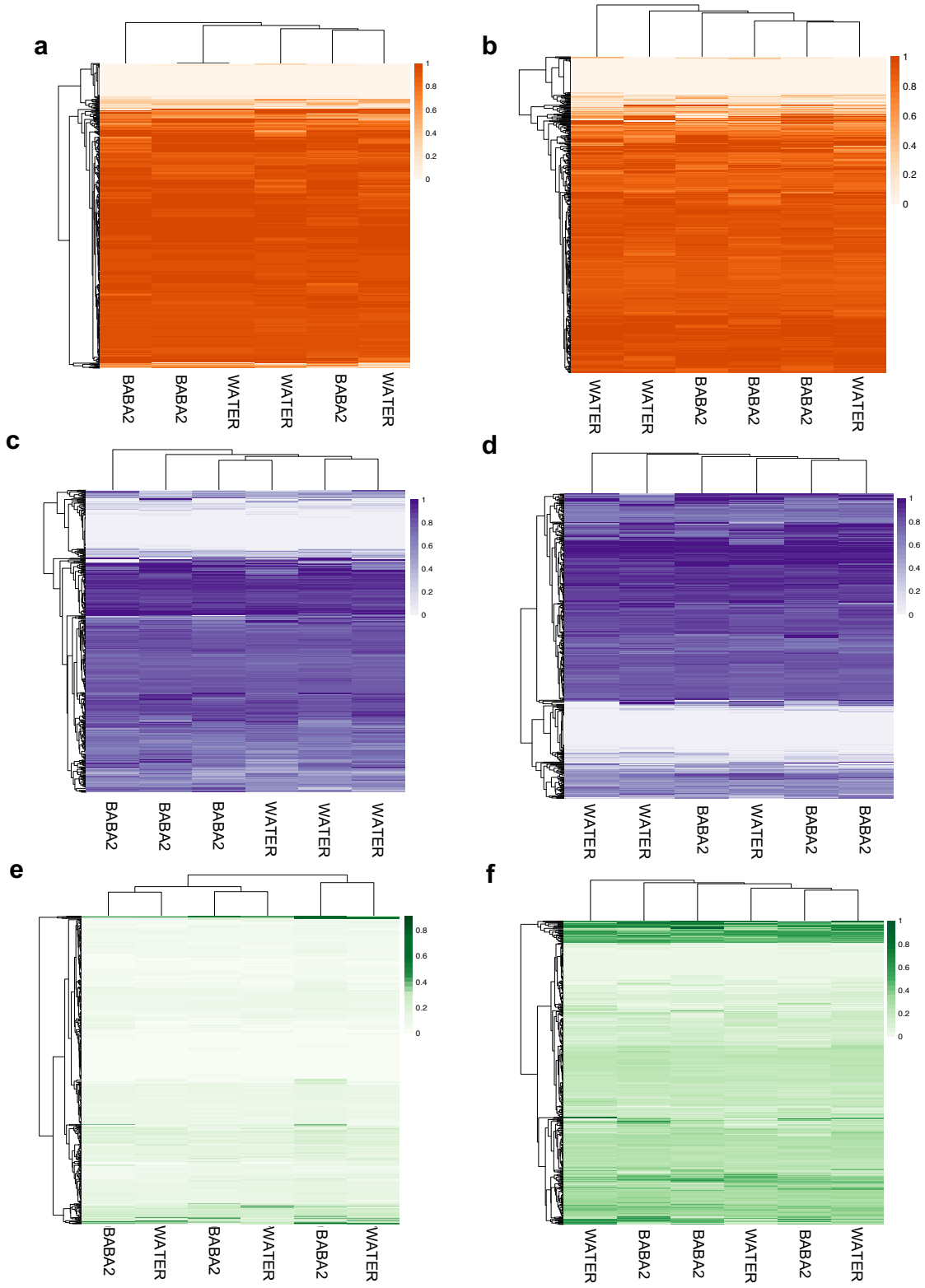
Appendix II Fig 3: Proportion of total number of overlaps of sRNAs with annotated genomic features (Genes, Class I Transposons, Class II transposons, promoters). **a)** 24 nt sRNAs, BG up and down DE compared to MG, 24 nt HG up and down DE compared to MG, 24 nt Mobile (uniquely conserved to BG and HG only) up and down DE**. b**) 21/22 nt sRNAs, BG up and down DE compared to MG, 21/22 nt HG up and down DE compared to MG, 21/22 nt Mobile (uniquely conserved to BG and HG only) up and down DE.



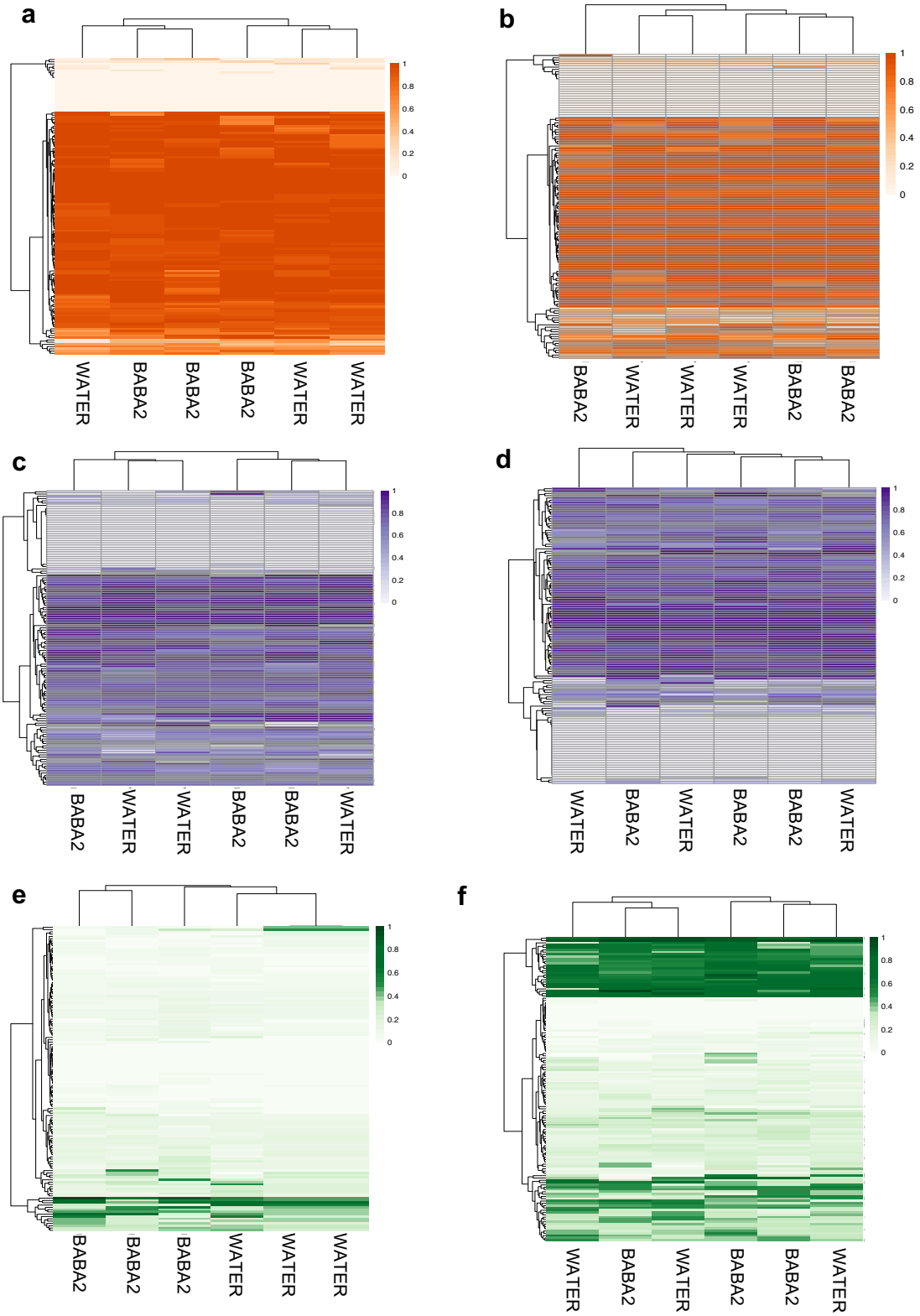
Appendix II Fig 4: Correspondence between TEs overlapping DE sRNA clusters. Venn diagram circles denote HG v MG - blue, BG v MG – red, or HG v BG - green. **a)** Overlap of up-regulated 24 nt clusters on class I TEs. **b)** Overlap of down-regulated 24 nt clusters on class I TEs. **c)** Overlap of up-regulated 24 nt clusters on class II TEs. **d)** Overlap of down-regulated 24 nt clusters on class II TEs



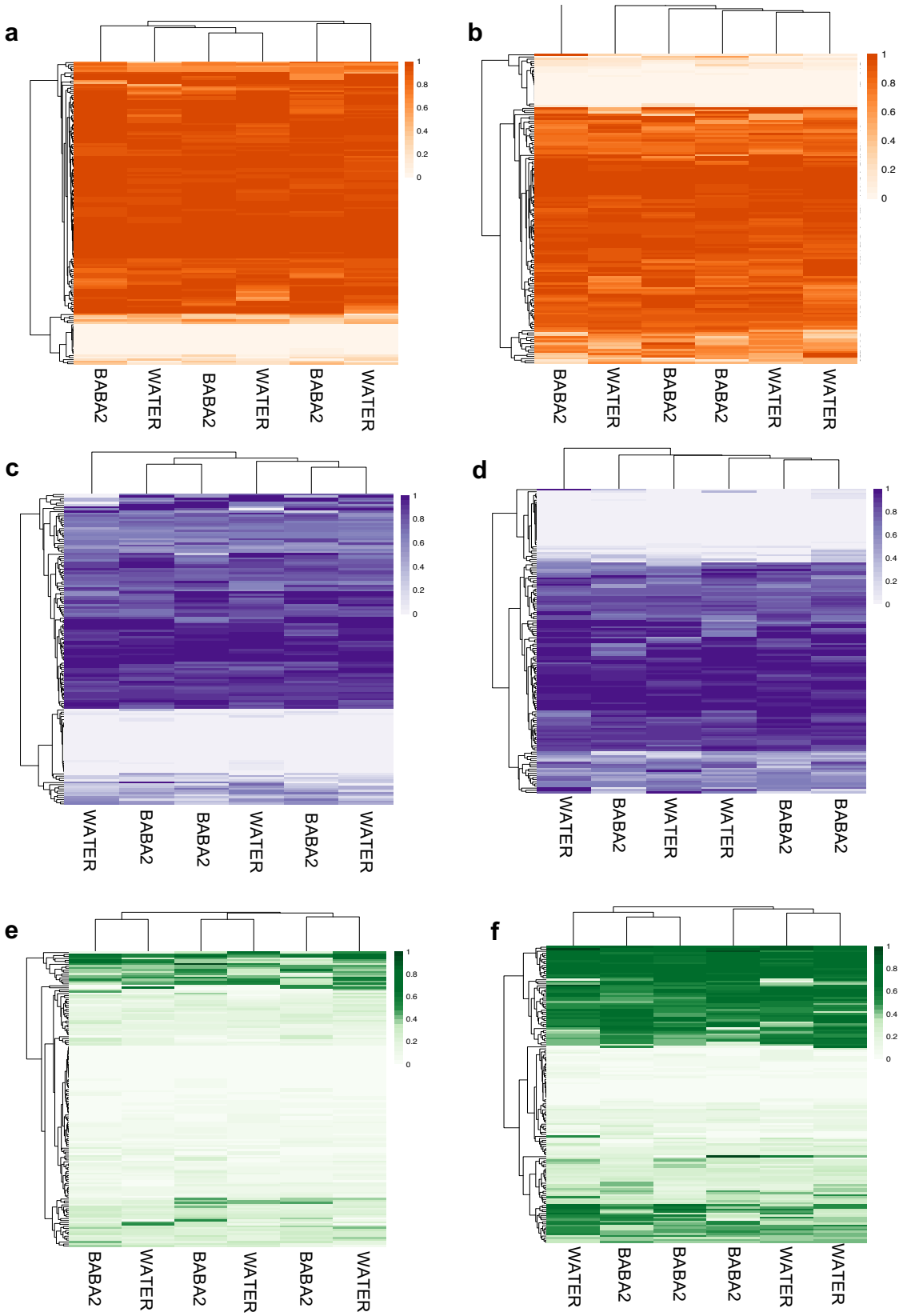
Appendix II Fig 5: Heatmaps displaying methylation proportion of Class I transposons overlapping up-regulated mobile 24 nt clusters. **a)** CG methylation status T1, **b)** CG methylation status T3, **c)** CHG methylation status T1, **d)** CHG methylation status T3, **e)** CHH methylation status T1, **f)** CHH methylation status T3.



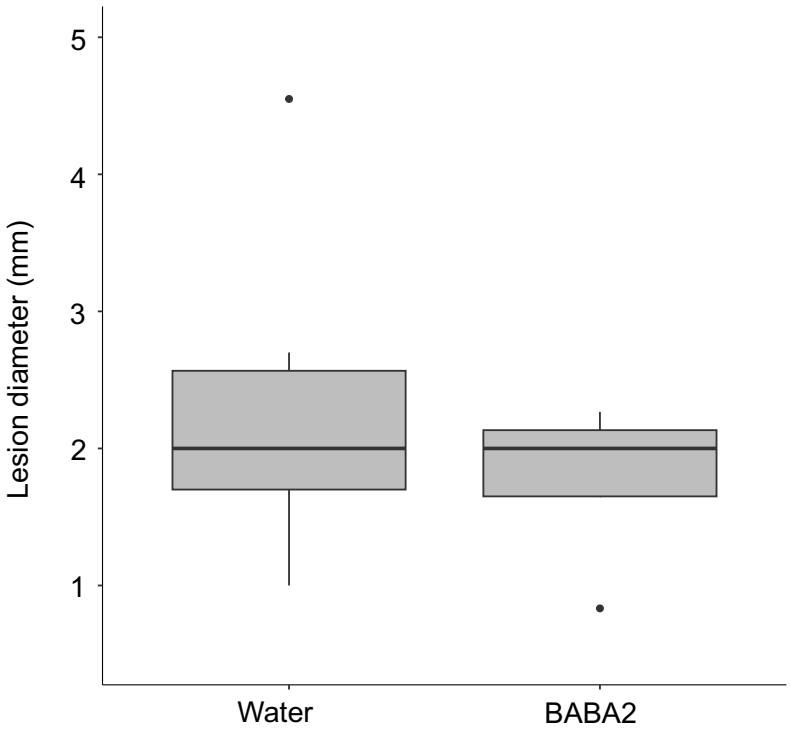
Appendix II Fig 6: Heatmaps displaying methylation proportion of Class I transposons overlapping down-regulated mobile 24 nt clusters. **a)** CG methylation status T1, **b)** CG methylation status T3, **c)** CHG methylation status T1, **d)** CHG methylation status T3, **e)** CHH methylation status T1, **f)** CHH methylation status T3.



Appendix II Fig 7: Heatmaps displaying methylation proportion of Class II transposons overlapping up-regulated mobile 24 nt clusters. **a)** CG methylation status T1, **b)** CG methylation status T3, **c)** CHG methylation status T1, **d)** CHG methylation status T3, **e)** CHH methylation status T1, **f)** CHH methylation status T3.



Appendix II Fig 8: Heatmaps displaying methylation proportion of Class II transposons overlapping down-regulated mobile 24 nt clusters. **a)** CG methylation status T1, **b)** CG methylation status T3, **c)** CHG methylation status T1, **d)** CHG methylation status T3, **e)** CHH methylation status T1, **f)** CHH methylation status T3.



Appendix II Fig 9: Postharvest resistance in Moneymaker tomato. Lesion diameter (mm) in ripe tomato fruit at 2 days post infection (dpi) with *B. cinerea.*

Appendix III:

Data science approaches provide a roadmap to understanding the role of abscisic acid in defence

Declaration regarding published work in this chapter

This appendix contains a published paper in Quantitative Plant Biology titled “Data science approaches provide a roadmap to understanding the role of abscisic acid in defence”. The authors of this work declare the content of this chapter belongs solely to Katie Stevens who is the first author of this publication. Co-author Iain G. Johnston contributed to this study by helping KS design the data analysis pipeline and guided statistical analysis. The senior author Dr Estrella Luna Diez contributed to this work providing feedback during the writing process and making corrections on the manuscript.

Katie Stevens



Dr Estrella Luna Diez



Introduction

Abiotic and biotic defence responses are important evolutionary mechanisms that allow plants to adapt to their dynamic environments (Peck and Mittler, 2020). A variety of sophisticated signalling networks modulate plant responses to challenges, helping to enhance survival. Hormone signalling and crosstalk play major roles in these responses, which can also be influenced by plant age, organ type and environment (Berens et al., 2017). However, as plants encounter many biotic challenges, including pathogens with varying lifestyles and methods of attack, there is no one-size-fits-all approach to defence. Therefore, hormones have unique roles in multiple, often antagonistic, complex defence pathways (Robert-Seilaniantz et al., 2011).

Abscisic acid is an essential hormone involved in both biotic and abiotic stress signalling (Lee and Luan, 2012). ABA is found universally across vascular plants and is involved in a wide range of physiological processes. For instance, through interaction with developmental hormones, ABA modulates plant growth, seed germination, stomata closure and development and embryo maturation (Vishwakarma et al., 2017). Moreover, ABA is essential for adaptation to drought and salt stress (Casson and Hetherington, 2010, Sah et al., 2016) and has a positive role in ensuring plant survival during abiotic stress. For example, water supply limitation is associated with an increase in ABA across the entire plant that leads to reduced stomatal conductance (Vishwakarma et al 2017). However, the function of ABA in defence against biotic challenges, including pathogens, is varied, which has led to mounting controversy in the field of plant biology (Asselbergh et al., 2008a, Cao et al., 2011, Ton et al., 2009).

ABA is widely considered to be a positive influence on early pathogen invasion due to its essential role in stomatal closure (Melotto et al., 2006). However, controversy arises in the later infection process. On one hand, ABA is known to be involved in multiple defence pathways and often interacts antagonistically with other hormones such as salicylic acid (SA),

jasmonic acid (JA) and ethylene (ET) through 'hormone crosstalk' (Anderson et al., 2004, De Torres Zabala et al., 2009, Oide et al., 2013). SA is involved in resistance against biotrophs and its production is enhanced following colonisation with the bacterial biotroph *Pseudomonas syringae* (Delaney et al., 1995). However, SA has a negative role in resistance to necrotrophs such as *Botrytis cinerea* (El Oirdi et al., 2011). In contrast, JA and ET display the reverse effect of SA and enhance resistance against necrotrophs and herbivory (McDowell and Dangl, 2000). These hormones therefore behave antagonistically, and ABA is typically shown to have a negative influence on resistance as it can disrupt SA-, JA- and ET-mediated defence signalling (Anderson et al., 2004, Audenaert et al., 2002). On the other hand, ABA has been linked to the expression of defence responses controlled by these plant hormones, including deposition of callose and reactive oxygen species production, which mediate resistance against biotrophic and necrotrophic pathogens (Asselbergh et al., 2007). In addition, crucially, a positive role of ABA in defence has been linked to the requirement of an intact ABA signalling pathway for direct and priming of callose deposition (Flors et al., 2005, Flors et al., 2008, Luna et al., 2011, Schwarzenbacher et al., 2020, Ton and Mauch-Mani, 2004, Vicedo et al., 2009). Therefore, the role of ABA on the expression of defence mechanisms appears highly varied, with positive and negative effects via different mechanisms during different circumstances.

The effect of ABA does not appear to be associated with plant species or pathogen lifestyle (Asselbergh et al., 2008b), and contradictions have been shown even within a single model plant pathosystem. For example, whereas ABA-treated Arabidopsis plants display enhanced susceptibility against *P. syringae* (Melotto et al., 2006, Mohr and Cahill, 2007), ABA-deficient *aba3-1* Arabidopsis mutant displays enhanced susceptibility against *P. syringae* (Melotto et al., 2006). In economically important crops such as tomatoes, ABA deficient *sitiens* tomato mutants have enhanced resistance against *B. cinerea, Odium neolycopersici* and *Erwinia chrysanthemi* (Achuo et al., 2006, Asselbergh et al., 2007, Asselbergh et al., 2008b, Audenaert et al., 2002). In contrast, long-lasting induced resistance in tomato fruit is marked

by an accumulation of ABA (Wilkinson et al., 2018). ABA is therefore a key component of defence at all phases of infection, yet its influence remains to be understood.

To make progress on our understanding of such complex questions, machine learning (RF) and supervised learning techniques are increasingly being used for studying plant pathogen interactions, specially motivated by the increased production of large -omics datasets and imaging capabilities (Sperschneider, 2020, Sun et al., 2020, van Dijk et al., 2021). For instance, ML has facilitated analysis of gene networks in immune response (Dong et al., 2015), pathogen effector protein localisation (Sperschneider et al., 2018) and plant disease diagnostics using image-based learning (Mishra et al., 2019). Here, we have used ML strategies to investigate the contrasting effects of ABA in resistance against pathogens. Through the collection of hundreds of data points from a total of 30 scientific peer-reviewed publications, we used tools from ML to attempt to unpick the complex interplay of effects shaping heterogeneous ABA influences, combined with new experiments to further explore these data-driven findings. Overall, our study highlights key factors that influence the effect of ABA in resistance and provides a tool for future studies towards facilitating the understanding of the role of ABA in plant responses against biotic stresses.

Methods

Data collection and preparation

Quantitative data regarding disease resistance was collected during March-June 2020 from literature involving ABA (Table S1). We searched Web of Science using broad search terms including “abscisic acid”, “plant pathogen” and “disease resistance”. Papers were selected as they included quantitative data regarding disease susceptibility from experiments directly involving pathogen infection and manipulation of ABA either through use of ABA signalling and biosynthesis mutants, exogenous application of ABA, and/or ABA inhibitors. From those papers, 13 shared variables were recorded from the three general classes of plant, ABA treatment, and pathogen. From the plant, we recorded the species, variety, genotype, mutant, mutant type, plant age and tissue. From ABA, we recorded the treatment, application method, and concentration applied. From the pathogen, we recorded the type, lifestyle, and species. All variable information was taken directly from papers used, however values of plant age for orange, grape and pepper were imputed based on known cultivar information. A large number of disease scoring methods were used across publications such as lesion diameter (mm), % spreading lesions, and categorical scoring. A disease severity index (DSI) was therefore used in an attempt to unify the heterogeneity of disease scoring in our dataset. All observations involving ABA **(ABAphenotype)** were compared to the level of resistance in control treatments **(Controlphenotype)** for each individual publication. DSI was represented as a ‘percentage resistance change’ of all data points, forming a continuous measure of percentage resistance change as a result of ABA, as described below (Table S1, Figure 1).

‘Percentage resistance change’ = 100 - (ABA**phenotype***100/Control**phenotype**) = x%

Computational meta-analysis

Two supervised learning approaches, described below, were used to identify the most important factors to predict disease resistance (Breiman et al., 1984). All analysis was

conducted in R (Version 1.4.1717). For each method of classification, data was randomly split into training (75%) and test (25%) data. Models were generated using the training data and model accuracy was assessed using test data. Final models were generated using all data available. Two models were created: a decision tree (DT) predicting a binary response of resistance 'susceptible' or 'resistant', depending on if the percentage resistance change was a positive or negative value, and a random forest (RF) model. The same variables and parameters were used for both DT and RF. To decide which variables to include in our models and guard against overfitting, the Akaike Information Criterion (AIC) score was used, providing a trade-off between the (maximum likelihood) fit of the model on the training data set and the complexity of the model, reflected by the number of parameters. The model with the lowest AIC score was selected.

Classification and regression tree (CART)

Decision trees (DT) were constructed using the package rpart (v 4.1-15) (Therneau et al., 2015) with the data described in Table S1 and Table S2. DT1 describes data collected from existing literature exclusively (Table S1), DT2 includes observations from new experiments described in this study in attempt to refine our original model (Table S2). Rpart uses the classification and regression tree (CART) algorithm to construct trees based on recursive binary partitioning, which splits the data based on the best predictive variable. The chosen variables and specific splits are determined by the Gini impurity index which measures the disorder in a set of data points. Gini impurity is calculated as the probability of incorrectly labelling a data point assuming random labelling according to the distribution of all classes in the set (Breiman et al., 1984). Splits in trees therefore aim to maximise the decrease in impurity; this process is repeated to build the tree. Seven variables were selected for DT analysis based on the optimal AIC score. Trees produced via the CART algorithm are typically large and overfitted, to reduce complexity trees are pruned. Pruning is determined by the rpart 'complexity parameter' value of the smallest tree with the smallest cross validation error (Therneau et al., 2015). The CART algorithm additionally provides a measure of variable

importance. Variable importance is determined by the accumulated contribution of each variable across all nodes and trees where it is used (Breiman, 2001, Breiman et al., 1984). Precision and recall statistics of all models were calculated from true positive (TP), false positive (FP), true negative (Kettner and Dörffling) and false negative (FN) values. The final model predictive performance was determined by creating Receiver Operating Characteristic (ROC) plots and obtaining Area Under Curve (AUC) values. ROC plots and AUC values were made using the package ROCR (v 1.0-11) (Sing et al.).

Random forest

Random forest analysis was performed using the package randomforest (v 4.6-14) (Liaw and Wiener, 2002) using the data described in Table S3 and Table S4. RF uses the CART algorithm, as described above, to construct a set of different individual trees, which are assembled into a 'forest'. Here, each tree provides a classification 'vote', and the average vote of all trees in the forest is selected. Through resampling with replacement, random forests guard against overfitting, and RFs can improve accuracy and predictive abilities in classification compared to singular CART trees (Breiman, 2001). In our approach, the number of trees and cross validation was optimised to provide the lowest out-of bag (OOB) estimate of error rate. The final RF model used in this study contained 400 trees and 10 folds cross-validation.

Simplified machine learning models

Further DT and RF analysis were performed to test the importance of specific variables using the following minimised datasets: DText/RFext - entire dataset minus extreme exogenous concentrations of ABA (i.e. above 750 µM) and DTage/RFage – entire dataset minus plant age over 6 weeks.

Plant materials and growth conditions

Tomato seeds (cultivar Micro Tom) were maintained in damp, humid, dark conditions to stimulate germination. Germinated seeds were transferred to individual 80mL pots containing Scott's Levington M3 soil. For analysis of older plants, three-week-old plants were transplanted into 3l pots until experiments were performed. Plants were grown in greenhouses under a daily light integral of 8.64mol m^{-2} d^{-1} and 25 °C /20 °C temperatures. Experiments were performed between July and November 2020.

Abscisic acid treatment

Leaf material was treated with freshly prepared stock of 20mM Abscisic Acid (De Torres Zabala et al.) (Sigma Catalogue number A1049) dissolved in ethanol. The ABA stock was diluted in water to the different concentrations used in this study: 20µM, 50µM, 100µM, 500µM. The control treatment was made by applying water. All treatment solutions were adjusted to the amount of ethanol present, determined by the highest concentration of ABA. Leaves from 4-week and 8-week-old plants were excised and placed in a plastic tray, laid flat, with stems wrapped in wet paper as previously described (Luna et al., 2016, Worrall et al., 2012). Two leaves per plant and a total of six plants (six biological replicates) were used to test the effect of ABA in leaves. ABA treatment was performed by spraying the treatment solutions onto leaves. Leaves were then allowed to dry before moving trays to 100% humidity in the dark for 24h before pathogen infection.

***Botrytis cinerea* cultivation and infection**

Botrytis cinerea (isolate BcI16) was grown on plates of potato dextrose agar (PDA;Difco), plates were kept in the dark at room temperature for 4 weeks. Infections of leaves with *B. cinerea* were performed as previously described (Luna et al., 2016) by applying droplets of 5µL inoculum containing $5x10^5$ spores/ml. Leaves were incubated in the dark at 100% humidity and 20°C. Disease severity was recorded at 3-days post infection (dpi) by measuring the diameter of the necrotic lesions using a Vernier calliper.

Phytophthora infestans cultivation and infection

Phytophthora infestans 88069td10 was grown on rye medium supplemented with geneticin for 2-3 weeks at 20 °C as previously described (Whisson et al., 2007). Plates were flooded with 10ml water and spores scraped from the plate surface and incubated at 4°C for 1-3 hours. Zoospores released from sporangia were decanted. A desired concentration of $5x10^4$ spores/ml was obtained. The underside of each leaf was infected with six droplets of 10µl inoculum. Plants were incubated at 100% humidity, 16hour/8hour light/dark cycles and 18 °C /15 °C temperature cycle. Disease severity was recorded at 7-days post infection. Scoring was done by classifying lesions into different categories of colonisation. Class I: healthy, Class II; lesions on leaf underside only, Class III; lesions in under and upper leaf surface, Class IV: prominent lesions on both leaf sides, Class V: spreading lesions causing tissue damage, Class VI: total leaf collapse. Category distributions were used to calculate disease severity indexes using the formula (Chiang et al., 2017):

$$Disease\ severity\ index = \sum \frac{(Class\ frequency\ x\ score\ of\ rating\ class)}{(Total\ number\ of\ observations\ x\ maximal\ disease\ index)}$$

Statistical analysis

Data from the experiments with *B. cinerea* and *P. infestans* were analysed using R (v 1.2.1335). In the analysis of lesion diameter and disease severity index, normality of distributions was assessed through qq plots and Shapiro-Wilk tests, and homogeneity of variance was assessed through Levene's tests. Data for which the null hypothesis of normality was not rejected was analysed through one-way ANOVA data; cases where normality was rejected were analysed with Kruskal-Wallis tests. Distributions with homogenous variances were analysed through Least Significant Differences post-hoc tests. Distributions with non-homogeneous variances were analysed with Dunnett's post-hoc tests. When data did not support rejection of normality, two-tailed t-tests were used to compare differences between water control and grouped treatments of different ABA concentrations. Experiments were repeated twice with similar results.

Results

Data exploration and sorting

A total of 224 data points were collected from a total of 30 peer reviewed papers directly reporting resistance phenotypes (Figure 1, Table S1). 194 data points were from studies using Arabidopsis or tomato as the experimental species, representing 58% and 29% of the data, respectively (Figure 1A). The majority of data represented experiments conducted in leaves, with only 14/225 data points in fruit tissue (Figure 1B). There were 55 total different genotypes in this dataset that were categorised by type of mutation into three classes: wildtype (De Vega et al.), ABA mutant (including signalling and synthesis variants), and 'Other' (primarily callose or other hormone-disrupted mutants). ABA mutants represented 46% of the data points (Figure 1C). Regarding plant age, 94% of datapoints were obtained in plants of the age of or younger than 6 weeks (Figure 1D). 23%, 49% and 28% of the data points correspond to biotrophic, necrotrophic and hemibiotrophic pathogens, respectively (Figure 1E). Fungi represented the majority of the data with 66% of the points, while bacteria made up 30% and oomycetes made up 4% (Figure 1F). Exogenous concentrations of ABA ranged from 0 to 100,000uM, although 95% of the data points were between 10 and 750uM (Figure 1G). 109 datapoints had an exogenous ABA concentration value of 0 due to experiments involving ABA mutants rather than external application. In this dataset, the level of 'percentage resistance change' varied from -800 to 91. 132 data points were labelled 'Susceptible' (negative change in resistance) and 93 datapoints 'Resistant' (positive change in resistance) (Figure 1H).

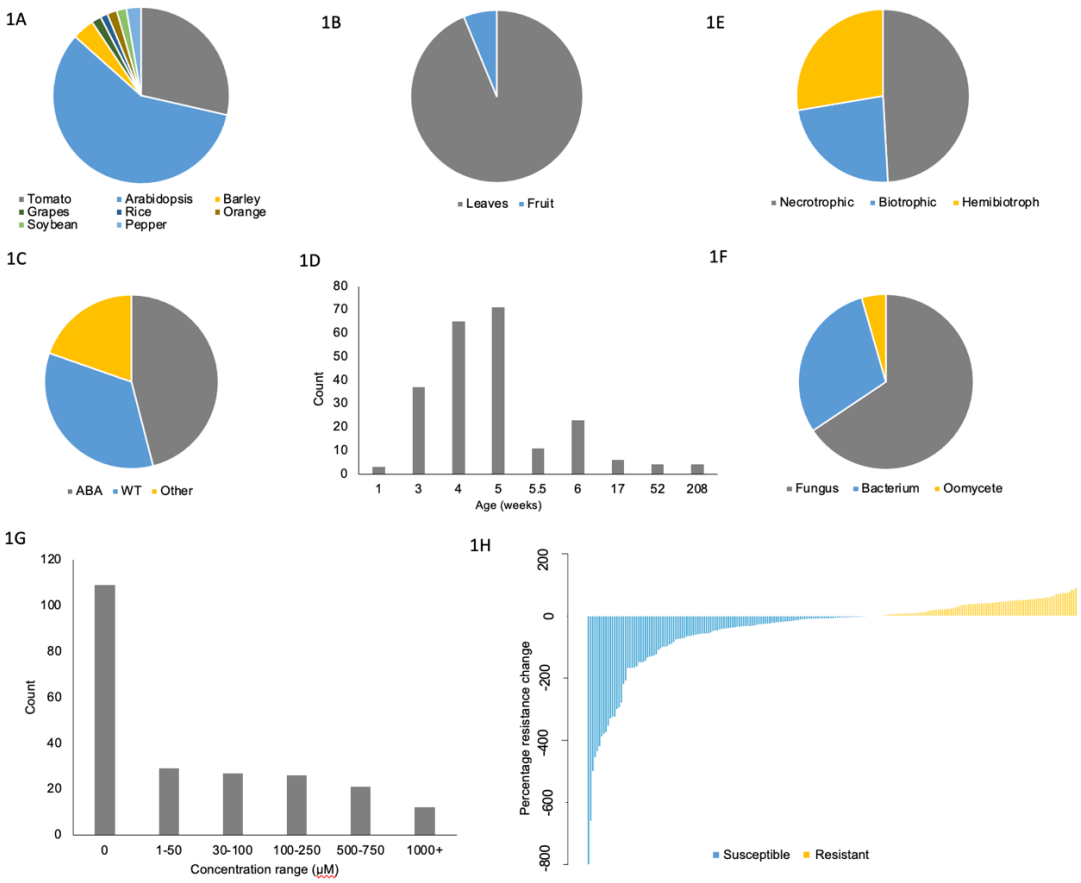


Figure 1: Characteristics of our amalgamated dataset. Proportion of plant species (A), plant tissue (B), mutant type (C), count distribution of plant ages (weeks) (D), pathogen lifestyle (E), pathogen type (F), and count distribution of Exogenous ABA concentration range (G). (H) Distribution of percentage resistance value. Data described in Table S1.

CART models

The CART algorithm was used to produce a decision tree (DT) to unpick which variables are involved in, and the most influential in, determining resistance phenotypes linked to ABA. A DT predicting a binary response of resistance had a predictive accuracy on unseen data of 76% and an AUC value of 77%, ROC curve is displayed in Figure S1a. DT had a precision score of 69% and a recall score of 78% (further details in Table S6). Rpart determined the most important variables in DT (Table 1). ABA concentration (in µM) was determined as the most important variable. Plant age (in weeks), plant species and mutant type were also determined as important predictive variables.

Random Forest models

A random forest model was created for predicting a binary resistance output. Increasing the number of trees used in the forest increased the predictive accuracy of the model, however beyond 400 trees the predictive capabilities of our model did not improve. The final RF model was therefore generated by using 400 trees. This model had an out-of-bag (OOB) estimate of error rate of 21%. The error rate in unseen data for predicting 'resistance' was 27%, the error rate for predicting 'susceptibility' was 15%, (further details in Table S6). As in the DT model, ABA concentration (μM) was considered the most important variable in the RF model (Table 1). Similar to DT models, RF predicts plant age (in weeks), plant species and pathogen lifestyle as important variables.

Simplified decision trees and random forest analysis

Decision trees (DTs) were reconstructed using minimised datasets to assess if accuracy could be improved from 76% and whether alternative predictions would be produced (Figure S2; Table S5). Removing extreme exogenous concentrations of ABA (i.e., above 750 uM - DText) reduced predictive accuracy to 61%, DText had an AUC value of 77% (further details in Table S6). DText has minimal structural changes with a single change in node positioning shifting the plant age threshold from 5.3 to 5.5 weeks (Figure S2A; Table S5). Removing extreme age datapoints of plants over 6 weeks (DTage) reduced predictive accuracy to 69% and produced a more simplified tree with the root node split of concentration changed to < 90uM (Figure S2B; Table S5). DTage had a relatively unchanged AUC value of 76% (further details in Table S6).

We then tested whether the RF out-of-bag (OOB) estimate of error could be improved. RFext marginally increased the OOB error rate to 22% demonstrating that inclusion of these datapoints in the RF analysis gives us the best possible prediction within the noise of the data.

Finally, the OOB error rate was unchanged in RFage, (further details for RFext and RFage in Table S6).

Table 1: Importance of variables in DT and RF determined by rpart and RF. Data described in Table S1 and Table S3.

Model	**Variable**	**Predictive importance**
DT	Exogenous ABA concentration (μM)	21.7
	Plant age (weeks)	13.54
	Plant mutant	12.98
	Plant species	11.87
	Pathogen lifestyle	9.89
RF	Exogenous ABA concentration (μM)	35.02
	Plant species	15.34
	Pathogen lifestyle	14.55
	Plant age (weeks)	12.7

Decision tree predictions

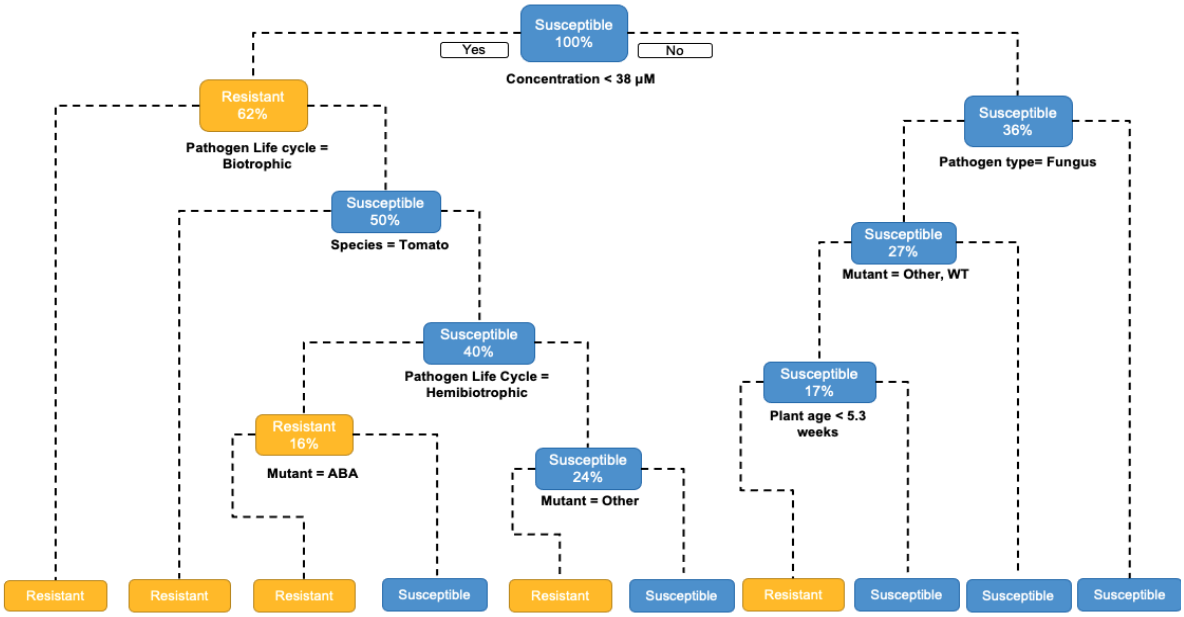


Figure 2: Decision tree (DT) predicting a binary resistance response of 'Susceptible' or 'Resistant'. Yellow boxes indicate resistance and blue boxes indicate susceptibility. Percentage number in box represents proportion of dataset remaining following node split. DT is read from top root node to bottom leaf nodes, if the question asked at a node is 'yes' the left side is followed. Data described in Table S1.

The DT (Figure 2) predicted that exogenous ABA concentrations above 38µM more frequently lead to susceptibility. This tree predicted that exogenous concentrations below 38µM infected with biotrophic pathogens display resistance and that with exogenous ABA concentrations below 38µM infected with non-biotrophic pathogens tomato plants display resistance. The DT predicts that exogenous ABA concentrations above 38µM infected with non-fungal pathogens display susceptibility. This DT shows the impact of age in resistance: it predicts that with exogenous ABA concentrations above 38µM, during fungal infections 'other' mutant plants younger than 5.3 weeks display greater resistance than plants over 5.3 weeks.

Effect of ABA on disease resistance against *Botrytis cinerea* and *Phytophthora infestans.*

Our RF and DT results highlighted that exogenous ABA concentration, pathogen lifestyle and plant age are important variables in resistance predictions. Seeking to refine our quantitative understanding of these influences, we therefore experimentally tested these specific variables. Tomato was used as the experimental plant species as it appears as a split in our decision tree (Figure 2) and is one of the most represented species in our data (Table S1).

The effect of exogenous ABA application at different concentrations on susceptibility against *B. cinerea* was assessed in tomato plants of different ages (i.e. four week and eight-week-old). ABA treatment had a statistically significant effect on the phenotype in leaves from 4-week-old plants against *B. cinerea* (Figure 3A; ANOVA, p= 0.04), with ABA generally causing an increase in susceptibility, although post-hoc test (least significant difference, LSD) does not show any specific statistically significant differences. In contrast, in older plants, ABA concentrations of 50μM and above enhanced resistance against *B. cinerea* (Figure 3B; Kruskal-Wallis, p = 0.001). The concentration of 100μM led to a significant, dramatic reduction in disease severity (Figure 3B). These results suggest that the effect of ABA on susceptibility against *B. cinerea* is influenced by ABA concentration and the plant age.

The effect of ABA application was also studied against the biotrophic pathogen *P. infestans.* In young leaves, ABA had a statistically significant effect on susceptibility against this pathogen (Figure 3C; ANOVA, p= 0.012), with the concentration 500μM causing increased susceptibility in four-week-old leaves (p= 0.029). In older leaves, ABA treatment increased susceptibility in all instances (Figure 3D; ANOVA, p= 0.04). However, lower range concentrations had a more severe effect on disease incidence, with 20μM leading to statistically significant increase in susceptibility (p = 0.003).

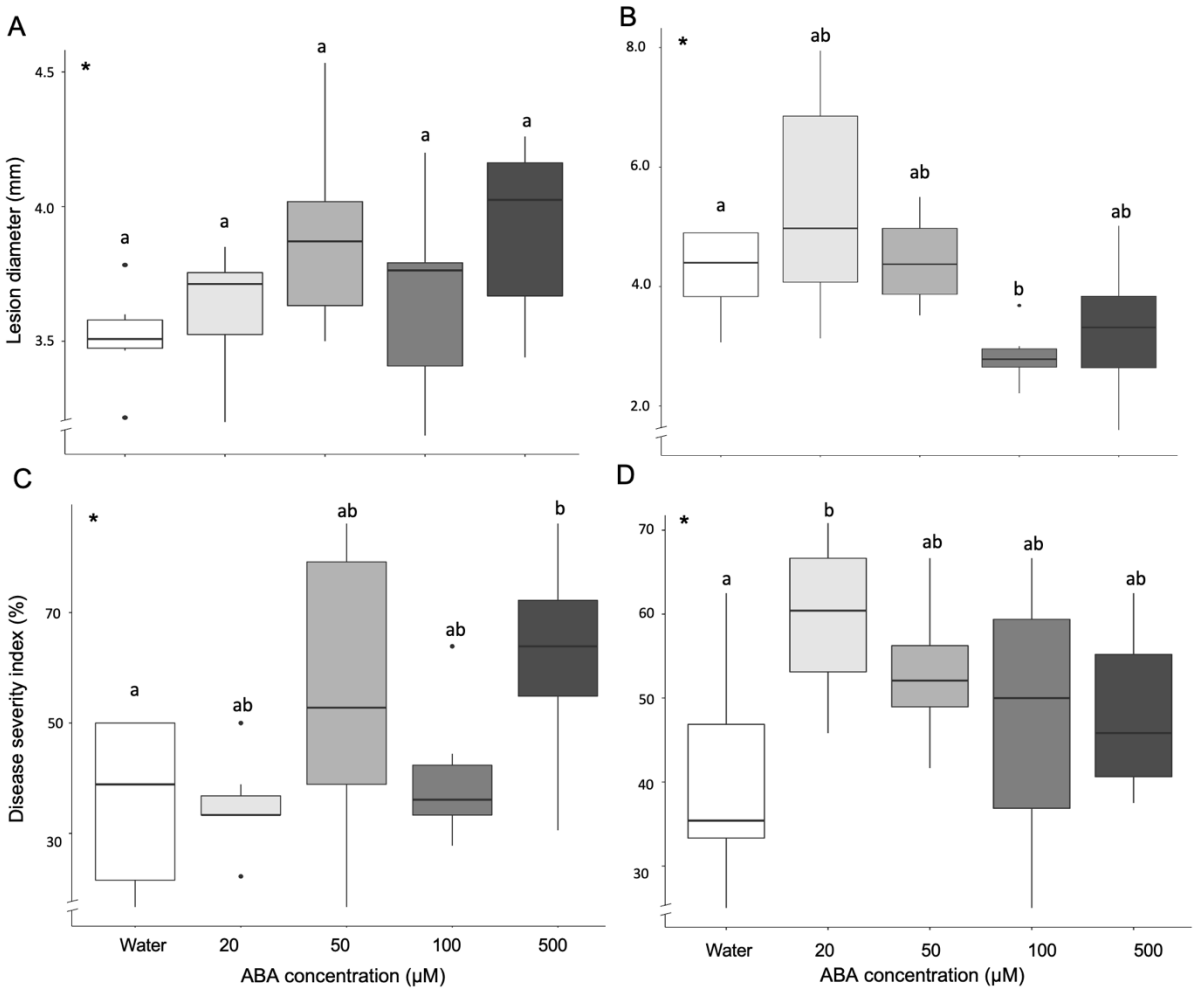


Figure 3: Disease resistance phenotypes in tomato. Four and eight-week-old tomato leaves were sprayed with 20, 50, 100, 500 µM of ABA 24 hours pre inoculation. (A) Lesion diameters in four-week-old tomato leaves caused by *Botrytis cinerea* at 3 dpi. (B) Lesion diameters in eight-week-old tomato leaves caused by *B. cinerea* at 3 dpi. (C) Disease severity index in four-week-old tomato leaves converted from the percentage of lesions in 6 disease categories at 7dpi with *Phytophthora infestans*. (D) Disease severity index in eight-week-old tomato leaves converted from the percentage of lesions in 6 disease categories at 7dpi with *P. infestans*. Boxes denote 25th and 75th percentile, bars display min and max values. Asterisks indicate statistically significant differences at each specific age and treatment (ANOVA for A, C and D, Kruskal-Wallis for B $p < 0,05$; $n = 6$). Different letters denote significant differences among treatment groups (LSD for A and D and Dunnets' for B and C post hoc tests; $p < 0,05$; $n = 6$).

Decision trees with extended experimental data

Comparing our DT predictions (Figure 2) to our experimental results (Figure 3) highlighted similarities and differences in our model. Our DT predicted only 1/8 of our *B. cinerea* infection results correctly. The prediction for *P. infestans* infected plants was more accurate with 6/8 experimental results being predicted by DT correctly. For a complex question like the role of ABA, such imperfect performance is not surprising. We do not claim to be solving the question of how ABA influences defence in all circumstances, but rather demonstrating a quantitative approach for knowledge curation and generation. To this end, we took the next step in what we propose as an iterative process: refining. In an attempt to refine an initially imperfect model, data was included from new and rationally chosen experiments (Table S2, Table S4). We thus added our experimental findings to our dataset, forming DT2 (Figure 4). The predictive abilities of DT2 remained relatively unchanged, with a predictive accuracy on unseen data of 70%, and an increased AUC value of 85%. DT2 had precision and recall values of 83% and 58% respectively demonstrating increased precision however lower power (Table S6). However, DT2 is now capable of better capturing the new experiments above. For *B. cinerea* infections 6/8 of our experimental results were predicted correctly from DT2, 6/8 were predicted correctly from *P. infestans* infection. The most important variable in DT2 was maintained as exogenous ABA concentration. Addition of our experimental data to our model altered some later splits in the DT2 compared to DT1 leading to differences in the overall topology. The split of age was lowered from 5.3 weeks to 4.5 weeks and an additional node was created. For concentrations higher than 38μM a node was created splitting for specific plant species (Arabidopsis, orange, rice, and tomato) and further predicted that exogenous ABA concentrations greater than 375μM lead to resistance (Figure 4). These changes subsequently increased the number of final leaf nodes from 10 in DT1 to 11 in DT2, potentially allowing a more fine-grained classification.

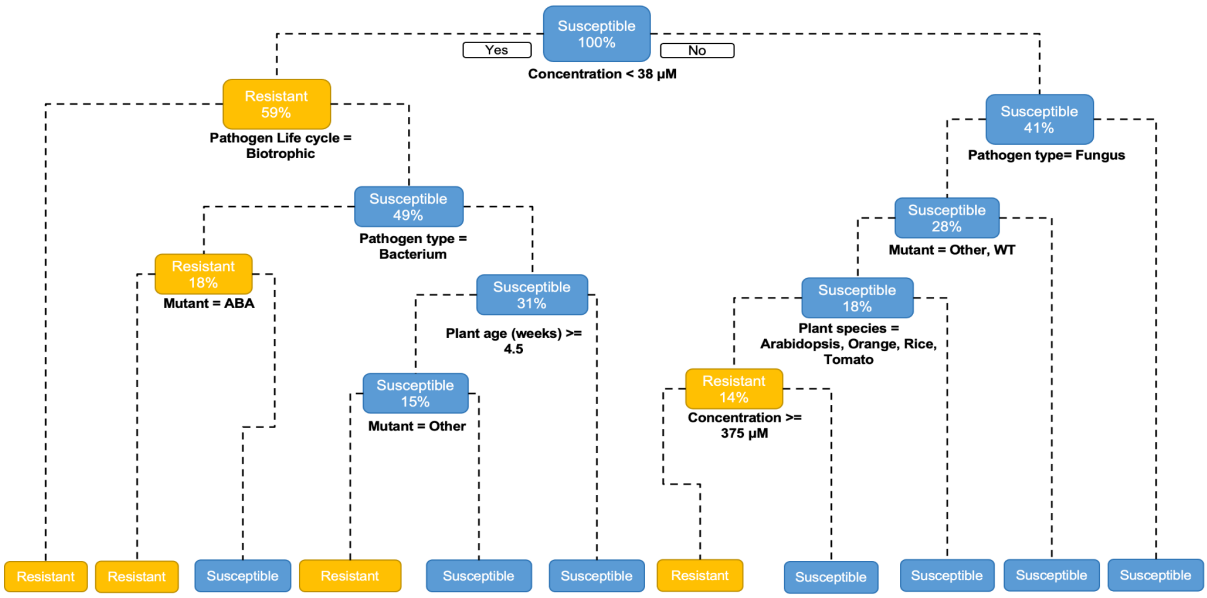


Figure 4: Decision tree (DT) including our experimental data predicting a binary resistance response of ‘Susceptible’ or ‘Resistant’. Yellow boxes indicate resistance and blue boxes indicate susceptibility. Percentage number in box represents proportion of dataset remaining following node split. DT is read from top root node to bottom leaf nodes, if the question asked at a node is ‘yes’, the left side is followed Data described in Table S2.

Discussion

The role of ABA in pathogen defence has remained highly ambiguous (Ton et al., 2009). In this study we used supervised ML techniques to attempt to resolve these relationships and determine the most influential factors in ABA resistance phenotypes. In our predictions and experiments, overall, we can observe pronounced roles for ABA on susceptibility to pathogens. Exogenous ABA concentration, pathogen lifestyle and plant age were shown to be important influencers of resistance, therefore illustrating ABA's regulatory role in the activation of major defence pathways and its specificity in individual pathosystems.

Importance of the concentration

DTs and RF analyses predict the exogenous concentration as the primary determinant factor on the role of ABA. Our DT predicts that exogenous concentrations higher than 38μM, result in susceptibility (Figure 2). Our experimental work showed in general terms that ABA application at high concentrations is associated with susceptibility (Figure 3). However, our experimental work did not show a consistent pattern of susceptibility as exogenous concentration of ABA was increased or as it passed a particular concentration threshold. For instance, we were unable to observe changes at the threshold concentration predicted and instead variable levels of susceptibility were observed (Figure 3). Moreover, whereas increasing exogenous concentrations of ABA led to enhanced susceptibility, the concentration of 100μM was extremely effective in triggering resistance against *B. cinerea* (Figure 3C). The differences observed between the predictions and the experimental work may be in part due to differences in the basal levels of ABA in plants. Achuo *et al*., demonstrated major discrepancies in the endogenous ABA levels found in Moneymaker tomato plants. Basal quantities varied within identical experimental set ups from 4.08×10^{-4} μM (408 picomol) g^{-1} FW to approximately 2.5×10^{-3} μM (2,500 picomol) g^{-1} FW (Achuo et al., 2006). Therefore, basal concentrations of ABA can be different depending on the experimental conditions and could explain discrepancies between predictions and observations.

Importance of the plant age and pathogen lifestyle

DT analysis allowed us to explore the effect of plant age and pathogen lifestyle. Generally, DT predicted that ABA triggers resistance against biotrophic pathogens with low exogenous ABA concentrations. When it comes to the effect against fungal pathogens, DT predicts that ABA affects the resistance phenotype in an age-dependent manner. Our DT predicts susceptibility in older plants, however experimental work has confirmed that ABA triggers resistance to the necrotrophic fungal pathogen *B. cinerea* only in older leaves.

The reasons behind the enhanced resistance observed after ABA treatments in older plants may be due to age related resistance (ARR). It is well reported that plant age influences pathogen resistance, with young juvenile leaves displaying enhanced susceptibility (Kus et al., 2002). This is often due to important defence functions, such as PR gene expression, being upregulated in older leaves (Kus et al., 2002, Li et al., 2020). Moreover, different developmental stages, such as the transition to floral stage, are associated with increased resistance in a range of species including Arabidopsis, tobacco and tomato (Develey-Rivière and Galiana, 2007, Hu and Yang, 2019). This resistance has been linked to a positive correlation between development and defence-related processes such as phytoalexin production and resistance gene expression (Bell, 1969, Li et al., 2020). The DT model produced in this study however did not predict this ARR. This is likely due to the small age interval of our data, as 3-6 weeks seems to be the most popular age range for infection studies (Figure 1, Table S1). Furthermore, a limitation of our data collection could also be responsible for the lack of ARR in the DT predictions, mostly based on a threshold of age. Whereas we have recorded and assumed that below ~four weeks old is considered “young” and ~eight weeks is considered “old”, this may not be true for all plant species studied. For instance, a four-week-old Arabidopsis plant may no longer be considered young, and an eight-week-old orange tree cannot be considered old. A more accurate assessment of ARR would therefore

come from the analysis of the impact of ABA in resistance at different developmental stages for each plant species.

Our results show that pathogen lifestyle determines the effect of ABA in resistance; this can be linked to the modulation of major defence networks associated with the infection strategies. It is well characterised that whereas biotrophic pathogens result in the activation of SA-dependent defences (Mohr and Cahill, 2007), the plant activates JA and ET-dependent defences in response to necrotrophic pathogens (McDowell and Dangl, 2000). Moreover, there is a comprehensively understood antagonistic interaction between SA and JA (Anderson et al., 2004, De Torres Zabala et al., 2009). It is known that *B. cinerea* produces a virulence factor, EPS, that directly modulates the JA/SA antagonism. EPS suppresses JA defences, reduces *proteinase inhibitor I* and *II* (*PI I* and *PI II*) expression and leads to accumulation of SA and NPR1 expression (El Oirdi et al., 2011). Tomato hosts resistance to *P. infestans* can occur through production of defence proteins that have been shown to be ET and SA dependent (Smart et al., 2003). Importantly, it is also known that there are multiple antagonistic responses between ABA and SA, JA, and ET. Therefore, it is easy to speculate that the effect of ABA in resistance against pathogens with different lifestyles is the result of ABA impacting the activation of those hormone-dependent signalling pathways. To our knowledge this is the first example of the impact of exogenous ABA application on tomato resistance against the biotroph *P. infestans.* Previous studies have been carried out on potato slices against this pathogen demonstrating that ABA treatments increase susceptibility (Henfling et al., 1980, Liu et al., 2020). Our experimental work also demonstrates that treatments with 500μM ABA result in susceptibility against *P. infestans* (Figure 3C). Therefore, it is likely that this phenotype is due to disruption of SA defence signalling by ABA. Future studies analysing both genotypes altered in SA and JA signalling will help confirm if ABA application is contributing to susceptibility due to the direct activation or repression of these essential defence pathways.

Application of machine learning tools to complex biological mechanisms

We suggest that tools from ML (here particularly decision tree and random forest classification approaches) provide an approach for further clarification of the role of ABA. This approach is, unsurprisingly, limited by the amount of data available - as demonstrated by our original decision tree, which had reasonable predictive performance on test data but failed to capture behaviour in several new experiments, and the reduced performance of our minimised DTs and RFs. For instance, DT predicts resistance in 4-week-old leaves against *B. cinerea* (Figure 2), however susceptibility is observed experimentally (Figure 3). Additional analyses were conducted to assess how robust our models were in respect to outliers. This demonstrated that extreme ABA concentrations do not dramatically change the structure or predictive capacity of our DT (Figure S2A). Removing the most extreme age values had a greater structural effect on our DTs: whilst the root node remained as 'exogenous ABA concentration' the value increased to 90µM. This indicates that the threshold for susceptibility is dependent on plant age and or tissue type. Later nodes were altered more substantially, though this is not unexpected as an age split appears in the DTs trained on the full dataset, already indicating that higher ages lead to different behaviour. As this outlier-removed analysis removes experiments from fruit material, it is easy to speculate that the observation on the change in ABA concentration may be a tissue specific effect related to the known tight link between ABA and fruit development and ripening.

These approaches are not proposed as an immediate solution, but rather as a framework for iteratively refining and improving our understanding. Hence, in an attempt to improve the prediction of the initial data we added our experimental data into the model (Figure 4). This improved our DT predictions in the tomato-*B. cinerea* pathosystem, increasing correct predictions from 1/8 to 6/8. Therefore, the addition of only 16 new data points (i.e., 7.1% change in the original data) led to changes in DT predictions and far greater accuracy against our own experimental results. This improvement points to the importance of adding under-represented data to the model. Here, the capacity for improvement can likely be attributed to

the limited current dataset, in particular the narrow age range represented in tomato with no data points included in leaves from plants older than 5.5 weeks. We believe that these ML approaches can both help unify and interpret observations from heterogeneous studies and to identify relevant characteristics in the role of ABA in defence. As more data is added, particularly on sparsely represented cases and surrounding key tree splitting points, further clarity and prediction refinement will be achieved. Thus, we invite researchers to input their results into the model, especially from data in under-represented groups, to improve its prediction capacity. This could allow production of refined species-specific predictive models to determine the threshold level of ABA concentration and test specific questions such as if our findings of the age-dependent effect of ABA against necrotrophs is specific to tomato or can be extrapolated to other plant species.

Moving forward with experimental work on the role of ABA in defence.

Due to its essential role in abiotic defences, ABA signalling is a common target for engineering stress tolerant plants. Therefore, greater understanding of its controversial role in biotic defences is essential to ensure phenotypes are resistant to both abiotic stress and pathogen challenge. Methodologies used in ABA research vary significantly, this is testament to the many functions of ABA and physiological processes in which it is involved. For example, data examined from literature in this study included repeated ABA spray application, root soaking, biosynthesis mutants, signalling mutants and salt stress treatment, all of which will lead to variable contributions to resistance (Achuo et al., 2006, Audenaert et al., 2002, Song et al., 2011). Despite these, we have been able to identify key characteristics that could determine phenotypes. Whereas it is clear that ABA triggers susceptibility against biotrophic pathogens in a concentration dependent manner, this is not as straightforward against necrotrophic pathogens, where plant age and specific concentrations are relevant., it is important to understand the function of hormone crosstalk in evolution and survival, as they are an essential characteristic to prevent singular resource allocation against a single stressor (Vos et al., 2015). ABA has been demonstrated to be fully involved in the expression of priming of

defence, which does not result in the direct activation of defence mechanisms but the fine-tuning of the response upon subsequent challenges. Due to the key role of ABA in defence crosstalk and priming, it could be hypothesised that ABA plays a central role in buffering any single effort, contributing to noisiness, and therefore playing a central role in the evolution of defence strategies. Therefore, this study can serve as a tool to test that hypothesis and to guide future studies involving the role of ABA in disease resistance.

Data and code availability

All data and code is publicly available at https://github.com/PlantPriming/ABAresistance

Supplementary figures

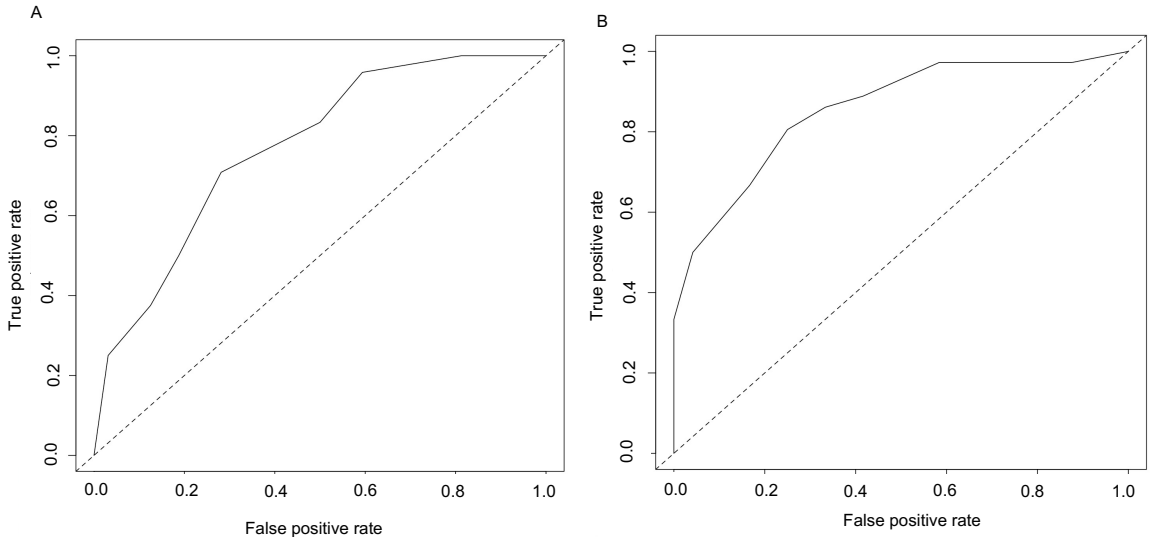


Figure S1:

Receiver operating characteristic curve (ROC) indicating false and true positive rates of decision tree (DT) models. (A) ROC curve of DT1, (B) ROC curve for DT2.

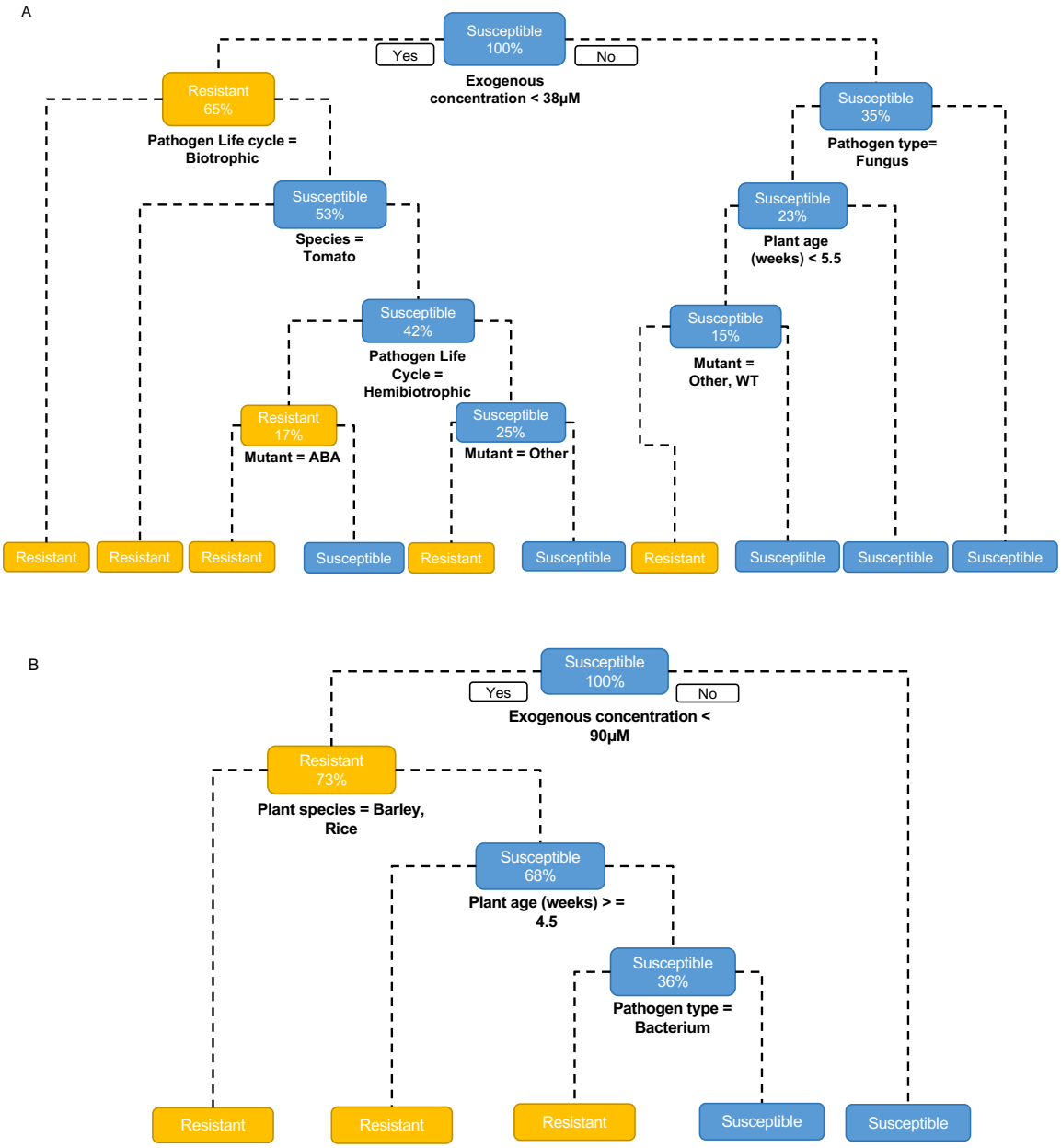


Figure S2: Decision trees with minimised datasets predicting a binary resistance response of 'Susceptible' or 'Resistant'. (A) DText with extreme concentrations of exogenous ABA removed from the dataset, (B) DTage with plant ages over 6 weeks removed from the dataset. Yellow boxes indicate resistance and blue boxes indicate susceptibility. Percentage number in box represents proportion of dataset remaining following node split. DT is read from top root node to bottom leaf nodes, if the question asked at a node is 'yes', the left side is followed Data described in Table S2.