Studies of resistance to aphids and viruses in *Arabidopsis thaliana* and *Capsicum annuum*

Ebenezer Antwi Gyamera

Hughes Hall

University of Cambridge

This dissertation is submitted for the degree of Doctor of Philosophy

April 2019

Dedicated to

God and Mama Agnes Mensah

Acknowledgements

I am very grateful for the support and guidance from my supervisor Prof. John P. Carr and all the members of the Virology group.

I couldn't have taken up my offer of admission here at Cambridge University without the financial support from the Cambridge Africa programme (Cambridge Trust). I am very thankful for their generosity. I'm also very grateful to the Philosophical Society of Cambridge University for awarding me a studentship toward the end of my research.

Finally, I am most grateful for the encouragement and motivation from my Ghanaian family, friends here in Cambridge and the wonderful international community I have come to know. You're awesome. Thank you.

Declaration

This is the result of my own work and includes nothing which is the outcome of work done in collaboration except as specified in the text. It does not exceed the word limit set by the Degree Committee for Biology. The work within it has not been submitted for a degree, diploma, or other qualification at any other university.

Ebenezer Antwi Gyamera

Hughes Hall

Summary

Cucumber mosaic virus (CMV) causes significant losses in crop production. In this study, methods to enhance plant resistance to CMV infection and infestation by *Myzus persicae*, a vector that transmits CMV, were assessed. There were two parts to the project. In the first, the feasibility of using exogenous salicylic acid (SA) application to induce resistance to CMV infection in pepper (*Capsicum annuum*) plants were investigated. In the second, I investigated the basis of the heightened resistance to *Myzus persicae* infestation observed in *Arabidopsis thaliana* plants carrying mutant alleles for Argonaute (AGO) 1 to determine if it was due to accumulation of a cyanogenic compound, 4-hydroxyindole carbonyl nitrile (4-OH-ICN). Exogenous application of 1 mM SA to pepper plants prior to CMV inoculation induced resistance to CMV, which was exhibited as a delay in the onset of symptoms. The level of CMV disease incidence among the treated pepper plants was also lower than the control-treated CMV-inoculated plants by 22 days post inoculation. Thus, exogenous SA treatment may have potential to protect pepper plants. Mutations in the 4-OH-ICN biosynthetic genes *cyp82C2* or *fox1* did not diminish the resistance to aphids observed in Arabidopsis *ago1.25* mutant plants. Aphid growth rates and fecundity on *ago1/cyp82C2* or *fox1/ago1* mutant plants did not significantly differ from aphid performance on wild-type plants. This indicates that deregulation of 4-OH-ICN biosynthesis does not contribute to aphid resistance in *ago1* mutant Arabidopsis plants. Progeny of crosses involving *ago1.25* mutant plants with mutant plants compromised in biosynthesis of SA (*salicylic acid induction deficient 2*, also called *isochorismate synthase 1*: *ics1*) were generated. Mutation in *ICS1* affected the *ago1.25* phenotype so that plants were less deformed and stunted and were less resistant to aphid infestation than *ago1.25* plants. However, not all the *ago1/ics1* progeny had milder phenotypes. The *ago1.25/ics1* progeny that retained their *ago1-like* phenotypes also retained their heightened resistance to aphid infestation. However, the milder *ago1*-like phenotypes of the *ago1.25/ics1* progeny cannot be explained by their *ago1* and *ics1* mutations. Results obtained with *ago1.46/nahG* cross indicated that SA accumulation is required for the *ago1* developmental phenotype in Arabidopsis. Furthermore, a cross between *ago1.25* mutant plant and a transgenic plant expressing the 2b RNA silencing suppressor protein of CMV were also generated. This cross resulted in plants which were severely deformed and with an even greater resistance to aphid infestation than their *2b* transgenic or *ago1.25* mutant parents. Many of the *2b*/*ago1* cross were sterile but a few of the cross formed siliques containing either few or no seeds at all.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

4-OH-ICN	4-hydroxyindole carbonyl nitrile
ABA	abscisic acid
ACS6	1-amino cyclopropane-1-carboxylic acid synthase 6
AFB1	auxin signalling F-Box 1
AP	*Apetala*
ARF	auxin response factor
Argonaute	AGO
AS-PCR	allele specific polymerase chain reaction
AUX/IAA	repressors of auxin-induced gene expression
AvrPtoB	Avirulence protein AvrPtoB
BAK1	BR1-associated receptor-like kinase 1
BTH	S-methylbenzo-[1,2,3]-thiadiazole-7-carbothiate
CaActin	*Capsicum annuum* actin
CaPR1	*Capsicum annuum* PR-1
CAPS	cleaved amplified polymorphism
CC	coiled coil
CCA1	circadian clock associated 1
cDNA	complementary DNA
cl	chlorophyll retainer mutant
CMV	*Cucumber mosaic virus*
COI1	Coronatine-Insensitive 1
CP	coat protein
CRL	CULLIN RING LIGASE

CUL3	Cullin 3A
dCAPS	derived cleaved amplified polymorphism
DCL	Dicer-like
DDH	aspartic acid-aspartic acid-histidine motif
DEPC	diethyl pyrocarbonate
dNTP	deoxyribonucleotide triphosphate
DPI	days post-inoculation
EDS1	*Enhanced Disease Susceptibility 1*
EDTA	ethylenediaminetetraacetic acid
eIF4E	eukaryotic translation initiation factor 4E
ET	ethylene
F1	filial generation 1
FLS2	*Flagellin Sensing 2*
FNY	Fast New York strain of CMV
Fox1	flavin-dependent oxidoreductase
GA	gibberellic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gDNA	genomic DNA
GGP1	γ-glutamyl peptidase
GH3	Gretchen Hagen 3
GSH	glutathione
GTase	glycosyltransferase
HD ZIP III	class III homeodomain leucine zipper
HRT	hypersensitive response to *Turnip crinkle virus*
HSP	heat shock protein

IAOX	indole-3-acetaldoxime
ICS1	isochorismate synthase 1
INA	2,6-dichloroisonicotinic acid
JA	jasmonic acid
JAZ	jasmonate Zim Domain Protein
LB	lysogeny broth
LRR	leucine-rich repeat
LZ	leucine zipper
m7G	7-methylguanine
MAPK	mitogen-activated protein kinase
MID	middle domain
MIM	microRNA target site mimic gene
MOP	methoxypsoralen
MP	movement protein
MRGR	mean relative growth rate
MS-agar	Murashige and Skoog agar
nahG	gene encoding salicylate hydroxylase
NASC	Nottingham Arabidopsis Stock Centre
NB	nucleotide-binding
NDR1	non-specific disease resistance 1
NPR1	non-expressor of PR-1
NRIP1	N-interacting protein 1
OD_{600}	optical density at 600 nm
PACT	PKR-Associated protein X

PAD4	phytoalexin deficient 4
PAMP	pathogen-associated molecular pattern
PAZ	Piwi, Ago and Zwille
PCS1	phytochelatin synthase 1
PEG	polyethylene glycol
PHB	*Phabulosa*
PHV	*Phavoluta*
PIWI	P-element induced wimpy testis
PR	pathogenesis-related
PRR	pattern recognition receptors
PTGS	post-transcriptional gene silencing
PVX	*Potato virus X*
R Protein	Resistance Protein
RanGAP2	ranGTPase-activating protein 2
RAR1	required for MLA 12 resistance 1
RCY1	recyclin-1
RISC	RNA induced silencing complex
RLK	receptor-like kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
RPM	revolutions per minute
RPP4	disease resistance protein RPP4
RTM	*Restricted TEV Movement*
Rx1	*Resistance against Potato virus X 1*
SA	salicylic acid

SAG101	senescence-associated gene 101
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
SGT1	*Suppressor of the G2 Allele of SKP1*
SID	*Salicylic acid induction deficient*
siRNA	short-interfering RNA
SNP	single nucleotide polymorphism
SPL	*Squamosa promoter binding protein-like*
STK	serine/threonine kinase
TAE	Tris-acetate-EDTA
TasiRNA	*trans*-acting short interfering RNA
TAV	*Tomato aspermy virus*
TCV	*Turnip crinkle virus*
TEV	*Tobacco etch virus*
TIR	Toll/Interleukin-like Receptor
TMV	*Tobacco mosaic virus*
TPS	terpene synthase
Vat	*Virus aphid transmission*
Vte2	homogentisate phytyltransferase 1, chloroplastic
WT	wild type

Chapter 1

General Introduction

1.1 Overview

Approximately 47% of all emerging plant diseases are caused by viruses (Anderson et al., 2004). The rate at which viruses can mutate into new strains and their ability to manipulate both plant hosts and vectors make viruses a potent threat to crop production (Varma and Malathi, 2003; Mauck et al., 2010; Howard and Fletcher, 2012; Ingwell et al., 2012). For example, in *Capsicum annuum* (pepper) production, disease caused by *Cucumber mosaic virus* (CMV), one of the most destructive of all plant viruses, can result in up to 80% loss of marketable fruit yield (Avilla et al., 1997; Scholthof et al., 2011). In some cases, the disease is perpetuated through subsequent generations of pepper plants through seed-borne transmission of the virus (Ali and Kobayashi, 2010).

Many plant viruses are vectored by insects. Therefore, most of the control measures against virus epidemics focus on controlling insect vector populations. The use of predatory insects, reflective mulches, natural and synthetic pesticides, semiochemicals and engineered genetic resistance have all been employed in controlling insect vector population sizes with varying degrees of success (Taylor, 1955; Regnault-Roger et al., 1997; Groen et al., 2017). For instance, aphids which vector half of all insect-transmitted viruses, including CMV, have effectively been controlled through the use of synthetic pesticides such as Carbofuran, Acephate, Pirimicarb, Oxydemeton-methyl and Parathion in the field. However, in many cases effective control of the aphid vectors by pesticides do not result in reduced virus disease incidence since CMV can be acquired and transmitted within seconds before the effect of the insecticide is manifested (Adams et al., 1976; Avilla et al., 1997; Ng and Perry, 2004).

Aside from the ineffectiveness of synthetic pesticides against transmission of viruses such as CMV, the insect vectors may also develop resistance to the pesticides. Excessive use of synthetic pesticides is also associated with mammalian toxicity and environmental pollution. Therefore, the use of pesticides is not ideal in controlling virus epidemics (Regnault-Roger et al., 1997; Perring et al., 1999).

Another method that has received significant attention in mitigating disease incidence is the use of biological or non-biological agents to induce systemic resistance in plants prior to infection (Palukaitis et al., 2017). Induction of systemic acquired resistance (SAR) by salicylic acid (SA) or its synthetic analogues has been studied intensively (Gaffney et al., 1993; Oosterdorp et al., 2001).

The SA analogue S-methylbenzo-[1,2,3]-thiadiazole-7-carbothiate (BTH) which is also commercially available as BION, ACTIGARD or BOOST is a synthetic activator of SAR and has been used to induce resistance against various pathogens including *Cucumber chlorotic yellow virus* in melon, *Tobacco mosaic virus* (TMV) in tobacco and CMV in tomato (Friedrich et al., 1993; Oostendorp et al., 2001; Palukaitis et al., 2017). In this study the feasibility of inducing systemic acquired resistance in pepper plants through exogenous SA application as a prophylactic measure against CMV incidence was assessed. This was to contribute to a collaborative research between our lab and researchers at the South Korean Rural Development Authority on the role of SA in mitigating CMV disease incidence in pepper plants. I was also interested in how vectoring by aphids could be inhibited therefore I investigated the phenomenon that makes *ago1* mutant plants more resistant to aphid infestation (Westwood et al., 2013). SA and the cyanogenic metabolite 4-hydroxyindole-carbonyl nitrile (4-OH-ICN) were hypothesized to contribute to the heightened resistance to aphids in *ago1* mutant plants.

1.2 The Genus *Capsicum*

There are 39 species in the genus *Capsicum*. Nearly all these species are diploid with a basic chromosome number of 12. *Capsicum* species are mostly self-compatible and are largely facultative inbreeders. Only 11 *Capsicum* species are considered true peppers/chillies, namely; *Capsicum annuum*, *Capsicum frutescens*, *Capsicum chinense*, *Capsicum baccatum*, *Capsicum cardenasii*, *Capsicum praetermissum*, *Capsicum chacoense*, *Capsicum galapagoense*, *Capsicum eximium*, *Capsicum tovarii* and *Capsicum pubescens* (Pickersgill, 1991; Pickersgill, 1997; Wang and Bosland, 2006; Qin et al., 2014). All *Capsicum* species, with the exception of *Capsicum anomalum*, originated in the New World and are currently cultivated throughout the tropics and subtropics and in intensive agricultural systems within temperate regions (Smith, 1951; Pickersgill, 1991).

Due to much similarities among specific individual species within the *Capsicum* genus, *Capsicum* species are further grouped into three distinct complexes based on morphological characteristics, chromosome banding and hybridization studies, namely; the *Capsicum annuum* species complex, the *Capsicum baccatum* species complex and the *Capsicum pubescens* species complex. Each of these species complexes contain species with varying degrees of cross compatibility (Ince et al., 2010; Ortiz et al., 2010). The five domesticated *Capsicum* species; *Capsicum annuum*, *Capsicum frutescens*, *Capsicum baccatum*, *Capsicum pubescens* and *Capsicum chinense* are distributed among these three *Capsicum* species complexes. The *Capsicum annuum* species complex alone (*Capsicum annuum*, *Capsicum frutescens*, *Capsicum chinense* and *Capsicum galapagoense*) contains three (*Capsicum annuum*, *Capsicum frutescens* and *Capsicum chinense*) of the five domesticated *Capsicum* species. *Capsicum annuum* is the most widely cultivated and economically important species among all the domesticated species of *Capsicum* peppers (Pickersgill, 1991; Pickersgill 1997).

1.2.1 *Capsicum annuum*

Domestication of *Capsicum annuum* occurred in the Highlands of Mexico. However, the species still contains wild accessions. Cultivated varieties of *Capsicum annuum* are mostly classified as *Capsicum annuum* var. *annuum* to distinguish them from their wild relatives. *Capsicum annuum* plants are early to late maturing, 1.0 to 2.5-foot-tall herbaceous plants with either glabrous or pubescent leaves. There are both pungent and non-pungent varieties of *Capsicum annuum*. The majority of Mexican peppers, the pungent peppers of Asia and Africa and all the non-pungent pepper varieties cultivated in the temperate regions are *Capsicum annuum* species (Smith, 1951; Pickersgill, 1991; Pickersgill, 1997; Wang and Bosland 2006). Most of the varieties within the *Capsicum annuum* species have clear or dingy white corollas with largely, solitary pedicels. Fruit size, shape and colour differ widely among the various cultivated varieties of *Capsicum annuum*. The different *Capsicum annuum* fruit colours are as a result of gene mutations in the carotenoid biosynthesis pathway. Red fruited *Capsicum annuum* peppers accumulate capsanthin and capsorubin pigments in their fruits upon maturity. Biosynthesis of both capsanthin and capsorubin are catalysed by the *Capsanthin Capsorubin Synthase* gene. Colour in yellow-fruited *Capsicum annuum* cultivars is controlled by the *Y* gene which is recessive to the *Capsanthin Capsorubin Synthase* gene. Upon fruit maturity, chlorophyll pigments break down. However, in the *Chlorophyll retainer* (*cl*) pepper

mutant, the lack of chlorophyll breakdown upon fruit maturity coupled with the accumulation of carotenoids make the fruits of *cl* mutant pepper brown upon fruit maturity (Smith, 1951; Paran and Van Der Knaap 2007). The pepper cultivar used in this study is the heirloom, non-pungent *Capsicum annuum* cultivar California Wonder (Figure 1.1), which in 2002 was reported to exist only in name due to the large genetic variability that were found among California Wonder plants sampled from different sources. However, this genetic variability was more intense among California Wonder seed sources of different origins (Votava and Bosland 2002).

Figure 1.1: California Wonder pepper seedlings (left) and fruits (right).

1.2.2 Viral Diseases and *Capsicum* Pepper Production

Breeding crops with improved agricultural value and the move toward genetic uniformity in cultivated crops are usually associated with compromised natural disease resistance which predisposes crops to increased pathogen invasion (Terry and Joyce 2004; Gurr and Rushton 2005). *Capsicum* peppers are susceptible to over 35 plant viruses, and over half of these viruses are transmitted by aphids (Green and Kim 1991). CMV is one of the most destructive causes of disease of pepper worldwide. CMV-infected plants are severely stunted with distorted leaves that show mosaic and chlorotic symptoms (Figure 1.2) (Ali and Kobayashi, 2010; Murphy et al., 2016). CMV-infected plants produce small and wrinkled fruits with little or no market value (Agrios et al., 1985).

Figure 1.2: Fny-CMV infected California Wonder *Capsicum annuum* plant (left) in comparison to a healthy pepper plant (right).

1.3 *Cucumber mosaic virus*

CMV is the type species in the *Cucumovirus* genus within the *Bromoviridae* family of viruses. More than 1,200 species of plants are susceptible to CMV infection (Ali and Kobayashi 2010; Smith et al., 2000). The various strains of CMV are grouped into two major subgroups, I and II based on sequence similarities and serological properties. Subgroup I strains are further categorized into IA and IB strains. Both the IA strains to which the Fast New York (Fny) strain of CMV belongs, and the II strains of CMV are distributed worldwide whilst the IB strains are largely found in Asia (Nouri et al., 2014). Aphids are the primary vectors of CMV transmission. CMV is thought to manipulate its aphid vector to enhance the acquisition and transmission of the virus (Ali and Kobayashi, 2010; Mauck et al., 2010; Ziebell et al., 2011; Westwood et al., 2013; Groen et al., 2016).

The CMV genome is composed of three positive sense RNA molecules (Figure 1.3). Each is encapsidated separately in icosahedral particles each with a diameter of about 29 nm (Peden and Symons, 1973). RNA1 encodes the 1a protein that has helicase and methyltransferase domains. RNA2 encodes the viral RNA-dependent RNA polymerase subunit of the viral replicase, the 2a protein. However, RNA2 also contains the open reading frame for the antiviral silencing suppressor protein, 2b, which is not translated from RNA2 but rather from subgenomic RNA4A. Proteins encoded directly from both RNA1 and RNA2 are involved in virus replication (Peden and Symons, 1973; Rizzo and Palukaitis, 1988; Rizzo and Palukaitis, 1990; Shi et al., 2002; Jacquemond, 2012). RNA3 also contains the open reading frames for

both the capsid protein and the movement protein. The capsid protein is translated from the subgenomic RNA4 (Figure 1.3). (Peden and Symons, 1973; Suzuki et al., 1991).

Some strains of CMV are also associated with RNA molecules called satellite RNAs which rely on CMV for replication, encapsidation and transmission. For instance, the beet isolate of CMV supports the satellites xjs2 and xj2 (Shang et al., 2009). Since xjs2 and xj2 satellites parasitize CMV, their presence is associated with reduced CMV accumulation and CMV-induced symptoms in *Nicotiana glutinosa* (Shang et al., 2009).

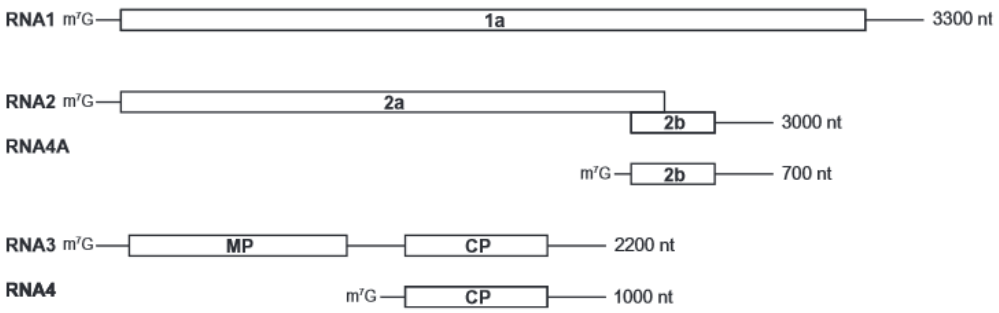


Figure 1.3: CMV Genome showing open reading frames for; the 1a protein with a methyl-transferase domain and a helicase motif; the 2a protein which functions as a viral RNA-dependent RNA polymerase; the movement protein (MP); the coat protein (CP) which is essential for genome encapsidation and aids insect transmission of virions; and the 2b, a multi-functional protein that functions as a symptom determinant and also involved in suppression of host induced RNA silencing (Jacquemond, 2012).

1.4 Plant-Pathogen Interactions

1.4.1 Movement of CMV in Host Plants

Plant viruses can only replicate within a living host cell (Maule and Wang 1996). To sustain infection, a virus must be able to replicate and move out of the initially infected cell into neighbouring cells. Movement of viruses between cells is a highly regulated process that occurs through the symplastic connections (plasmodesmata) joining neighbouring cells (Maule and Wang 1996). Both the capsid protein and the movement protein are required for cell-to-cell movement of CMV but are not necessary for replication (Canto et al., 1997; Li et al., 2001). CMV is inoculated into epidermal cells by probing aphids. Once in the epidermal cell layer, the viral proteins are expressed by the host translational machinery.

The movement protein is known for its targeting of the plasmodesmal pores of the host including the specialized branched plasmodesmata that link the companion cells to the sieve elements. The movement protein also has the capacity to alter the gating properties of the plasmodesmata to permit transportation of molecules whose size exceeds the size exclusion limit of the plasmodesmata across cells (Blackman et al., 1998). After the expression of the viral proteins and subsequent virus replication, the viral genomic RNAs bind to the movement protein, and possibly the subunits of the viral capsid to form a ribonucleoprotein complex ready for transportation across cells through the plasmodesmata. First, the virus moves radially along the epidermal layer and then into the mesophyll layer to gain access to the host's vascular system (Blackman et al., 1998; Kang et al., 2010) . Once the virus enters the mesophyll cell layer, access to the vascular parenchyma and companion cells are gained through the bundle sheath cells. The virus then enters the sieve element through the specialized plasmodesmata connecting the companion cells to the sieve elements (Figure 1.4). Further virus accumulation and virion assembly is believed to occur within the sieve element. Virions are mostly found within membrane-like structures on the parietal layer of the sieve element (Blackman et al., 1998; Li et al., 2001).

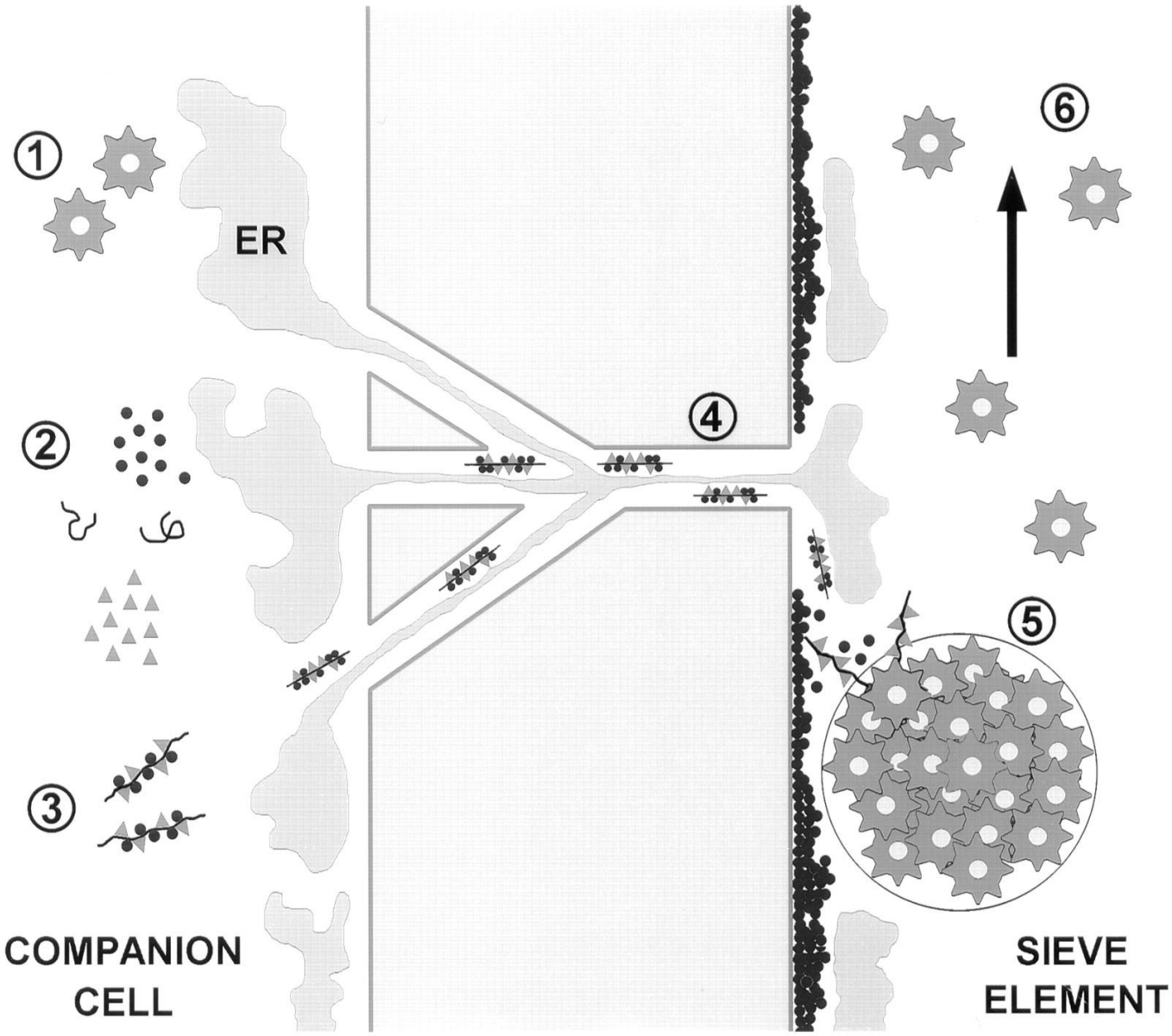


Figure 1.4: CMV movement into the vascular tissues. (1) CMV virion arrival in the companion cell, (2) disassembly of the CMV virion into proteins and nucleic acids (3) formation of the plasmodesmata-mobile ribonucleoprotein complex (4) movement of the ribonucleoprotein complex across the plasmodesmata (5) re-assembly of CMV ribonucleoprotein complex into virions in the sieve element (6) systemic movement of CMV virions along with photoassimilates (Blackman et al., 1998).

1.4.2 Plant Perception and Response to Pathogen Invasion

In comparison to the vast number of pathogens that could potentially infect plants, disease is the exception. Natural selection pressure imposed by pathogens on plants has resulted in the evolution of complex pathogen recognition and host defence mechanisms in plants (Staskawicz et al., 1995; Rausher, 2001). Every single plant cell possesses the capability to defend itself upon pathogen attack and transduce signals that prime neighbouring cells against the invading pathogen. Depending on the signal perceived from the invading pathogen, the host plant can activate and fine-tune the most appropriate defence response necessary to inhibit or compromise the fitness of the invading pathogen (Feys and Parker, 2000; McDowell and Woffenden, 2003). These defence mechanisms may be pre-existing or may be induced upon perception of the invading pathogen. For a successful infection to occur the pathogen must avoid or suppress the elaborate host defence mechanisms presented by the host plant (Staskawicz et al., 1995; Gurr and Rushton, 2005).

Plants possess broad perceptual mechanisms incorporating both pathogen specific resistance gene products and non-pathogen specific perceptual mechanisms that work through receptors capable of recognizing pathogen associated molecular patterns (Gurr and Rushton, 2005). Many of the proteins translated from resistance (*R*) gene mRNAs possess protein-protein interaction or recognition domains such as leucine-rich repeat domains or leucine zipper domains, in addition to signalling domains such as kinase domains, nucleotide-binding sites and toll/interleukin receptor-like domains. Hence, R proteins do not only detect pathogens but also have signalling roles that culminate in the activation of defence-related genes (Staskawicz et al., 1995; Feys and Parker, 2000).

R proteins constantly monitor cellular structures for pathogen invasion. The precision with which a host's R protein detects pathogen invasion determines the host's degree of resistance to that pathogen. In few instances, the R protein may directly detect the corresponding avirulence gene product of the invading pathogen. However, in many instances the R protein may indirectly detect pathogen invasion through detection of pathogen-modified host proteins. Plants possessing race-specific resistance to a pathogen may contain only one or few major *R* genes. Race-specific R proteins are more precise in detecting the specified pathogen, although race-specific R protein-mediated resistance are usually not durable and may be non-functional if there is a mutation in the pathogen's avirulence gene product. Plants possessing race non-specific forms of R protein-mediated resistance to pathogens usually possess a cluster of *R* genes which collectively contribute to the observed resistance. Because of the

presence of many major *R* genes contributing to the resistance, race non-specific resistance are more durable and are not easily loss if there is a mutation in the avirulence gene product of the pathogen (McDowell and Woffenden, 2003; Gurr and Rushton, 2005).

R-protein mediated recognition of a pathogen through either a direct detection of an avirulence gene product or pathogen-modified host proteins activates a torrent of host defence responses that may either slow down or prevent successful establishment of the pathogen in the host plant. The precision of R proteins in detecting pathogen invasion and the tight association of R protein-mediated pathogen detection to host defence signalling response represent a counter-evolution of plants against pathogen attack (Staskawicz et al., 1995; Rausher, 2001). Aside *R* gene-mediated pathogen detection, plants are also capable of detecting pathogen associated molecular patterns. For instance, the Leucine-rich repeat (LRR) receptor-like protein kinase, Flagellin sensing 2 (FLS2), is capable of recognizing the conserved 22 amino acid sequences in the flagellin of a bacteria and signal for the activation of the appropriate defence responses (Gurr and Rushton, 2005).

1.4.3 Host Signalling Upon Pathogen Perception

Successful recognition of a pathogen by the host plant leads to immediate responses at the site of infection such as a burst in reactive oxygen species (ROS), accumulation of nitrogen oxides, reinforcement of cell walls and the accumulation of plant hormones including salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) coupled with accumulation of pathogenesis-related (PR) proteins and phytoalexins (Staskawicz et al., 1995; Gurr and Rushton, 2005).

Collectively, plants synthesize more than 100,000 secondary metabolites, largely from the phenylpropanoid, isopropanoid, alkaloid or polyketide pathways. Many of these secondary metabolites play important roles in host defence responses. In most cases, all plant species within a taxon incorporate similar secondary metabolites in their host defence responses. For instance, plants in the *Leguminosae* and *Solanaceae* families largely utilize isoflavonoids and sesquiterpenes in their host defence responses, respectively. Many of these induced or constitutively expressed secondary metabolites possess antimicrobial properties. Therefore, the capability of a pathogen to infect plants synthesizing these metabolites depends on their counter-ability to encode enzymes that detoxify these host secondary metabolites. For instance, the tomato leaf spot fungus *Septoria lycopersici* encodes the saponin degrading

enzyme tomatinase that enables the fungus to infect tomato plants irrespective of the host's accumulation of saponin upon infection. Avenacin is an oat saponin which is constitutively expressed and stored in epidermal cells. It has an antimicrobial property to the oat fungus *Gaeumannomyces graminis*. *Gaeumannomyces graminis* that loses its ability to express the avenacin degrading enzyme avenacinase also loses its pathogenicity on oats (Dixon 2001; Gurr and Rushton 2005).

1.4.4 PAMP-Triggered Immunity

Plants perceive the presence of microbes including potential pathogens using plasma membrane-localized receptor-like kinases (RLK) termed pattern recognition receptors (PRRs) (Figure 1.5). Most PRRs contain LRR ecto-domains that are capable of recognizing and binding to conserved sequences characteristic of both pathogenic and non-pathogenic microbes. These conserved microbial components detectable by PRRs are called pathogen associated molecular patterns (PAMPs) or alternatively microbe associated molecular patterns since not all microbes are pathogens. A PAMP mostly constitutes an integral component of the pathogen and therefore are not easily discarded by the pathogen. For instance, components of bacterial flagellin and fungal and oomycete cell walls are recognized by plants as PAMPs. The *Arabidopsis thaliana* (hereafter referred to as Arabidopsis) FLS2 RLK has the capacity to detect a sequence of conserved 22 amino acids in the N-terminus of bacterial flagellin (flg22) as a PAMP. Although, PAMPs are mostly described for non-viral pathogens, viral components such as capsid proteins could also be recognized by plants as PAMPs (Figure 1.5). There are over 56 Arabidopsis genes that encode plasma-membrane localized RLKs capable of intercepting PAMPs from diverse sources (Trujillo and Shirasu, 2010; Tsuda and Katagiri, 2010). The strategic localization of RLKs across the plasma membrane ensures that that they offer an early protection against pathogen invasion. By interacting with other associated host factors, PRR recognition of PAMPs triggers a cascade of downstream signalling events that culminates in a form of basal defence called PAMP-triggered immunity. For example, through interacting with the host RLK protein BRI1-ASSOCATED RECEPTOR-LIKE KINASE 1 (BAK1), the recognition of the flg22 PAMP by FLS2 initiates a series of protein kinases activation, accumulation of signalling molecules and transcriptional reprogramming. One of the protein kinases that are activated downstream of PAMP recognition is the mitogen activated protein kinase (MAPK) MPK6. Within minutes of PAMP recognition, MPK6 is activated to phosphorylate the ET biosynthetic

enzyme 1-amino cyclopropane-1-carboxylic acid synthase 6 (ACS6) resulting in ET biosynthesis and ET-mediated defence signalling (Jones and Dangl, 2006; Tsuda and Katagiri, 2010). PAMP recognition also triggers SA biosynthesis. Accumulation of SA enhances the recruitment of a phosphorylated form of the host protein called Non-Expressor of PR-1 (NPR1) into a CUL3-based ligase for ubiquitinylation and proteolysis. The presence of the ankyrin repeat domain of the NPR1 enables it to involve in protein-protein interactions such as interacting with the TGA2 transcription factor. TGA transcription factors act as activators of JA-mediated defence responses and repressors of *PR* genes expressions. Hence, the physical interactions of an ubiquitinylated NPR1 with the *PR* genes repressor, TGA2, result in the proteolysis of both the NPR1 and TGA2 promoting the expression of *PR* and other defence related genes (Trujillo and Shirasu, 2010). Over 1,000 Arabidopsis genes are significantly upregulated within 30 minutes of PAMP recognition. Among these upregulated genes are *PR* genes. Many of the PR proteins encoded by *PR* genes have antimicrobial properties. For instance, PR-2 possesses a glucanase activity, PR-3, PR-4 and PR-7 act as chitinases. PR-10 possesses a ribonuclease activity and could potentially play an antiviral role in basal immune response but this has not been investigated. Specific sets of *PR* gene expressions are only induced by SA. *PR-1, PR-2, PR-5* and *PR-10* are SA dependent whilst *PR-3, PR-4* and *PR-13* are JA dependent. Due to the broad spectrum of pathogenic and non-pathogenic PAMPs detectable by the PRRs and the cost associated with defence signalling and defence responses, PTI are usually of a slow start. However, PTI could be boosted with continuous and sustained perception of PAMPs (Jones and Dangl, 2006; Sels et al., 2008; Katagiri and Tsuda, 2010).

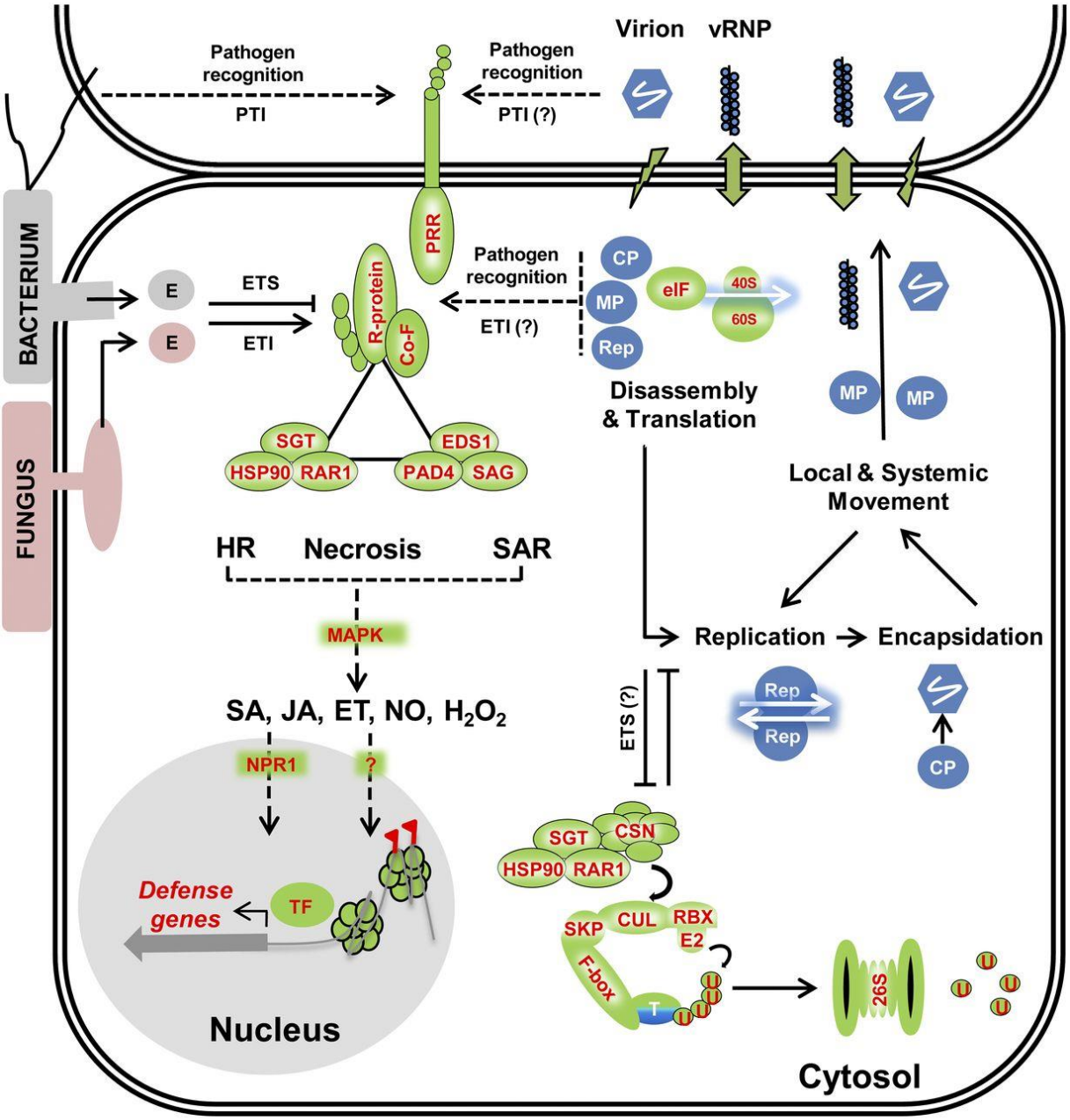


Figure 1.5: Comparison of immune responses to viral and non-viral pathogens. Virus components such as capsid proteins could potentially be perceived as a PAMP by the host plant to trigger PTI (Mandadi and Scholthof, 2013).

1.4.5 Strategies Adopted by Pathogens to Overcome PTI

To overcome PTI signalling and PTI-mediated host defence responses, adapted pathogens employ the use of pathogen-encoded molecules called effectors (van't Slot and Knogge, 2002). There are diversities of pathogen effectors utilizing varying mechanisms to counteract host basal defence responses. The capsid proteins of both *Potato virus X* (PVX) and *Turnip crinkle virus* (TCV) and the p50 helicase domain of the 126/183 kDa protein of (TMV) are some of the well-known viral components that also function as effectors. Some effectors act as biological mimics of signalling molecules such as SA to counteract a host's JA-mediated defence response against the pathogen. For instance, the toxin coronatine produced by some pathogenic bacteria can bind to the CULLIN RING LIGASE (CRL) subunit COI1 to activate the degradation of JAZ transcriptional repressors, as a result, activating JA-mediated defence responses to counteract the SA-mediated signalling and defence responses required to trigger stomatal closure. Other effectors also work by inhibiting MAPK cascades downstream of PAMP recognition. For instance, the C-terminus domain of the bacterial effector AvrPtoB acts as an active ubiquitin ligase that targets kinases involved in PRR and FLS2 mediated signalling and ubiquitinylate them for proteolysis, thereby enhancing host susceptibility through the impairment of PTI signalling components downstream of PAMP recognition (Martin et al., 2003; Jones and Dangl, 2006; Trujilo and Shirasu, 2010).

1.4.6 Host R Proteins

There are more than five major classes of R-proteins in the plant kingdom. Class 1 category of R proteins contains a serine/threonine kinase (STK) domains with a myristylation motif at their N-terminal. Class 2-R proteins contain LRR and nucleotide binding (NB) domains with a putative leucine zipper (LZ) or coiled coil (CC) domain at their N-terminal. Class 3-R proteins also contain LRR-NB domains but with a toll interleukin-like receptor (TIR) domain at their N-terminal. Class 1, 2 and 3 categories of R-proteins do not possess any extracellular or transmembrane domains. There are over 150 genes in the Arabidopsis genome that encode proteins with NB-LRR domains. The LRR domains are located at the carboxyl terminal of the NB-LRR R-proteins with the NB domain in the middle of the R-protein (Martin et al., 2003; Spoel and Dong, 2012). The variable N-terminal domains could either be a CC, TIR, putative BED zinc finger domain or a Solanaceae domain. The N-terminal domains are known to be involved in indirect pathogen detection whilst the LRR domains engage in a direct pathogen recognition. Both class 4 and class 5 categories of R-proteins possess

extracellular LRR domains. There are other groups of R-proteins which are not categorized under these classes such as the RTM1 and RTM2 proteins that confer passive resistance to *Tobacco etch virus* (TEV) in Arabidopsis (Martin et al., 2003; Caplan et al., 2008). The RCY1 R-protein against CMV-Y together with the Rx1 and Hypersensitive response to TCV (HRT) R-proteins against PVX and TCV are class 2 R-proteins with CC, NB and LRR domains. The N protein, which confers resistance against TMV, is a class 3 R-protein with TIR, NB and LRR domains (Takashashi et al., 2012; Mandadi and Scholthof, 2013; Nicaise, 2014). The intramolecular interactions among the domains of the R-proteins and their interactions with other associated proteins are crucial for their role in mediating defence response against pathogens. R-proteins have surveillance roles and largely monitor host cellular proteins for effector perturbations (Spoel and Dong, 2012; Mandadi and Scholthof, 2013).

1.4.7 Activation of Host R-Proteins

Recognition of pathogen effectors by R-proteins are mostly done in concert with a group of host proteins termed as R-protein co-factors. For instance, the Rx R-protein requires interaction with the R-cofactor ranGTPase-ACTIVATING PROTEIN 2 (ranGAP2). The CC domain of Rx1 forms a heterodimer with ranGAP2 which in turn is believed to interact directly with the capsid of PVX resulting in the activation of the Rx protein. ranGAP2 is also believed to induce the dislocation of the Rx protein from the nucleus into the cytosol where it is activated (Nicaise, 2014). The N R-protein also requires interaction with the R-cofactor N-INTERACTING PROTEIN 1 (NRIP1), a chloroplast-localized sulfurtransferase (Figure 1.6). The p50 helicase of TMV recruits NRIP1 from the chloroplast into the cytosol. The dislocation of the NRIP1 protein into the cytosol and its interaction with the TMV p50 helicase act as a pre-recognition protein complex that interacts with the TIR domain of the N R-protein resulting in its activation. The N R-protein only physically interacts with NRIP1 in the presence of p50. NRIP1 may be targeted by TMV helicase due to its potential role in host defence responses. For instance, the expression of *AtSEN1*, the Arabidopsis homolog of the *Nicotiana benthamiana* NRIP1 is SA regulated. *AtSEN1* is also involved in basal defence responses (Caplan et al., 2008; Nicaise, 2014).

To limit the occurrence of autoimmunity and unrequired immune responses, the activation and accumulation of R-proteins are kept under strict control through interactions with other

host proteins including the eukaryotic chaperone complex comprising of the Heat Shock Protein 90 (HSP90), the Required for MLA 12 Resistance 1 (RAR1) and Suppressor of the G2 Allele of SKP1 (SGT1) proteins. In the absence of effectors, the HSP90/RAR1/SGT1 chaperone complex folds the R-proteins into an inactive but an effector-recognition competent conformation. In this conformation, the LRR domain blocks the exchanges of nucleotides at the NB site domain through intramolecular interactions among the NB, N-terminal domain and the LRR domain. In certain cases, accumulations of R proteins are known to follow a dynamic pattern synchronous with the lifecycle of its cognate pathogen. For instance, the *RPP4 R*-gene which confers resistance to the Arabidopsis downy mildew pathogen *Hyaloperonospora. arabidopsidis* is under the control of the circadian gene *circadian clock associated 1* (*CCA1*). Peak accumulation of RPP4 occurs at dawn which also coincides with the sporulation of *H. arabidopsidis*. The recognition of effector molecules either through a direct interaction between the effector and the R-protein or through an interaction of the R-protein with an effector-modified host protein results in the release of the NB site causing an activation of the R-protein. After the activation of the R-protein the HSP90/RAR1/SGT1 complex again facilitates the necessary changes in the conformation of the R-protein required for the induction of downstream signalling events (Spoel and Dong, 2012; Jones and Dangl 2006).

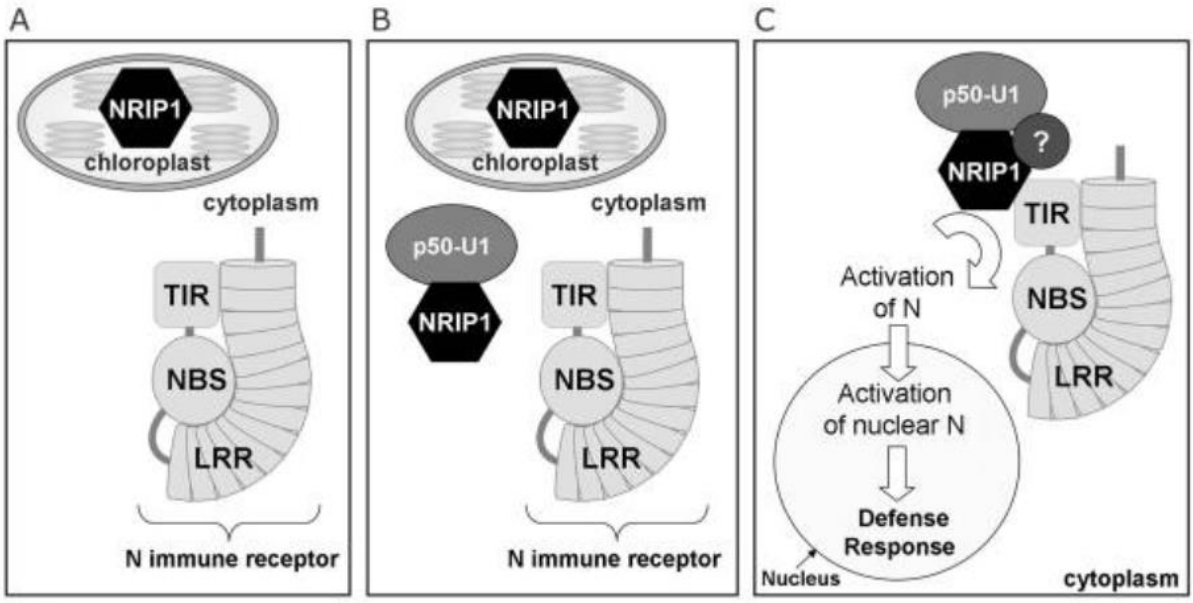


Figure 1.6: The tobacco N R-protein requires interaction with the R-cofactor, N Receptor-Interacting Protein 1 (NRIP1) to mediate resistance to TMV. NRIP1 interacts with N only in the presence of the TMV helicase p50 (B). However, interaction with other host proteins or a functional NRIP1 may be required for the activation of the N R-protein (C) (Caplan et al., 2008).

1.4.8 Activated R-Protein mediated Signalling and Effector-Triggered Immunity

Typically, activated R-proteins with CC or TIR domains require interactions with Non-Specific Disease Resistance 1 (NDR1) or Enhanced Disease Susceptibility 1 (EDS1) proteins respectively, to mediate resistance against pathogens. Although, there are a few exceptions. The HRT R-protein possesses a CC domain at its N-terminus but requires interaction with EDS1 for the extreme resistance to TCV. Specific interactions between the viral effectors, the R-proteins, R-cofactors, the chaperone complex and NDR1 or EDS1, phytoalexin deficient 4 (PAD4) and senescence associated gene 101 (SAG101) mediate distinct downstream changes that culminate in an effector triggered immunity through a MAPK signalling cascade. These R-protein-mediated downstream events include changes in SA, JA and ET accumulation levels, as well as accumulation of ROS such as hydrogen peroxide and superoxide radicals just as in PTI (Figure 1.7) (Mandadi and Scholthof, 2013; Nicaise, 2014).

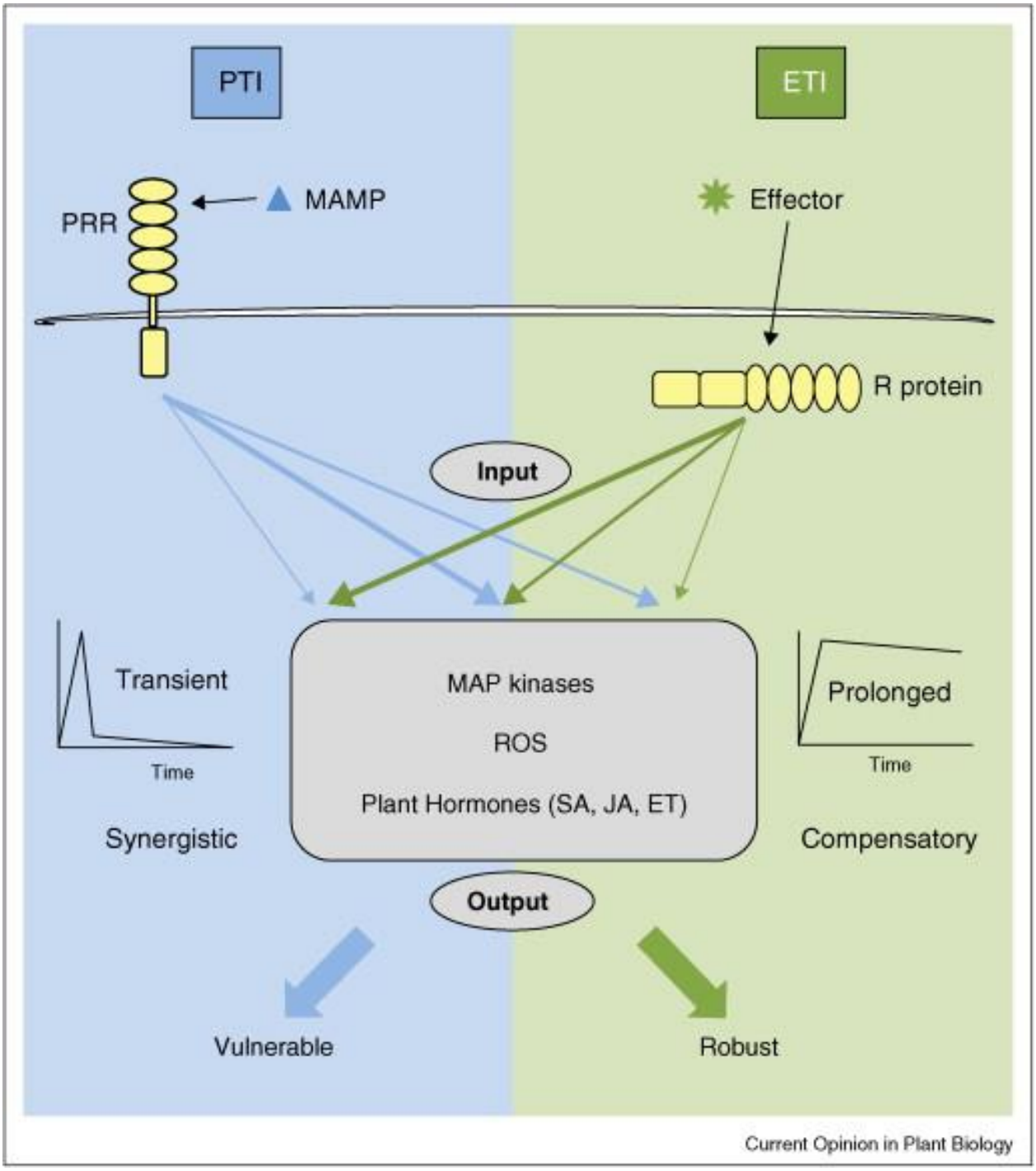


Figure 1.7: Common signalling pathways are used by both PAMP-Triggered Immunity and Effector Triggered Immunity in diverse ways (Tsuda and Katagiri, 2010).

Although both PTI and ETI seem to employ the use of similar signalling networks, immune responses in ETI are more robust and prolonged than in PTI. For example, in both PTI and ETI there are accumulations of ROS following a PAMP or an effector recognition. However, in PTI, the ROS accumulation is solitary and transient compared to the biphasic ROS accumulation that occurs in ETI responses consisting of early transient ROS accumulation followed by a second more robust and sustained ROS burst (Tsuda and Katagiri, 2010). EDS1, which is crucial for the expression of hypersensitive response (HR)-associated cell death closely interacts with PAD4. PAD4 is essential for SA accumulation, expression of *PR-1* and biosynthesis of phytoalexins such as camalexin. Different host plants employ different mechanisms to bring about cell death. R-protein mediated cell death is in part mediated by metacaspases which are analogous to the animal caspases that mediate apoptosis in animal cells. The Arabidopsis *Metacaspase1* gene plays crucial role in *R*-gene mediated cell death. SA signalling is also thought to promote autophagy which results in cell death (Martin et al., 2003; Spoel and Dong, 2012).

However, cell death has been shown to be unnecessary for restricting CMV to infected cells. In the HR-cell death deficient *dnd1* Arabidopsis mutant carrying the CMV-Y resistance gene *RCY1*, resistance to CMV-Y is kept intact despite the significantly compromised HR-cell death in *dnd1* mutants. SA signalling and induction of *PR* genes expressions around the site of infection were also kept intact. There are other *R*-gene mediated resistance to viruses where cell death is not required for containing the virus. For instance, in both *Rx* and *Rsv-1* mediated resistance to PVX and *Soybean mosaic virus*, HR-cell death is not present. Although, the Rsv1-*Soybean mosaic virus* interaction has the potential of resulting in an HR cell death-like phenotype (Takahashi et al., 2012). Aside from HR-mediated cell death, the host ubiquitinylation system also plays a role in containing virus spread. For instance, the movement proteins of TMV and *Turnip yellow mosaic virus* are specifically heavily ubiquitinylated and targeted for proteolysis by the host ubiquitinylation system resulting in decrease virulence of the viruses due to restricted movement (Mandadi and Scholthof, 2013).

1.5 CMV-Resistant *Capsicum* Accessions and Resistance Breeding

There are many pepper accessions and cultivars that possess different forms of tolerance or resistance to either one or several strains of CMV. The majority of these CMV resistance or tolerance genes/loci were discovered in wild relatives of cultivated *Capsicum annuum*. In

most cases, resistance to CMV infection is conditioned by a number of partially dominant or recessive genes that contribute quantitatively to the resistance. For instance, the very strong CMV-HB isolate-resistance possessed by the pepper inbred line BJ07047 is conditioned by two major partially dominant genes and a number of additive genes which all contribute to the resistance in BJ07047 (Yao et al., 2013). In a few cases, pepper accessions were found to possess resistance to multiple strains of CMV, as is the case of the *Capsicum frutescens* breeding line BG2814-6 which has good resistance to CMV strains CMV-V26, CMV-V27, CMV-V28, CMV-Ny, CMV-Fny and CMV-Ca, although the resistance is incompletely penetrant (Grube et al., 2000). The commercial pepper cultivar Bukang possesses one of the rarest forms of CMV resistance found among CMV-resistant pepper cultivars and accessions. Resistance to CMV in Bukang is conditioned by a single dominant gene, *Cmr1*, which is located on chromosome 2 of the pepper genome. The Cmr1 protein inhibits the systemic movement of the CMV strains CMV-Fny and CMV-P_0. However, the major source of CMV resistance genes in pepper breeding programmes is obtained from the small fruited pungent *Capsicum annuum* cultivar called Perennial. Other commonly used sources of CMV resistance genes are the *Capsicum frutescens* breeding lines BG2814-6 and LS1839-2-4 (Kang et al., 2010; Kang et al., 2012). Besides the challenge of cross incompatibilities, inheritance of CMV resistance genes can be very complex and is sometimes associated with undesirable agronomic traits. For instance, CMV resistance in the pepper breeding line BC5FC is associated with reduced fruit size. *CMV11.1*, the most important quantitative trait locus conferring the CMV resistance phenotype in BC5FC is located in the same region of BC5FC genome as two quantitative trait loci, *fw3.2* and *fw4.1*, that influence fruit size. The TMV-resistance locus, *L*, also occurs on the same linkage group as *CMV11.1* on chromosome 11. Hence, increased tolerance to CMV infection can result in increased susceptibility of pepper to TMV (Chaim et al., 2001).

1.6 Salicylic Acid

SA is a natural plant secondary metabolite common to plants including peppers and Arabidopsis. It plays crucial roles in a diversity of physiological and metabolic processes in plants. SA is known to regulate plant growth and development and also influences seed germination and fruit yield (Malamy and Klessing, 1992; Hayat et al., 2010).

Typically, basal endogenous SA levels range between 0.05 µg – 5.0 µg/g leaf fresh weight. However, in some species basal endogenous SA levels can be as high as 30 µg or 100 µg/g leaf fresh weight, as is the case for *Oryza sativa* (rice) and *Dioon edule* (chestnut dioon), respectively (Delaney, 2010). SA contributes to both local and systemic forms of plant defence responses (Feys and Parker 2000). It acts as a pathogen-induced signalling molecule that is crucial for the swift activation of an array of defence related genes. In many incompatible host-pathogen interactions, such as the interaction between TMV and TMV-resistant (*NN* genotype) tobacco, endogenous SA levels increase by 20-fold upon perception of the virus (Malamy et al., 1990). SA can contribute also to basal resistance. The ability of a particular poplar (*Populus* spp.) variety to resist infection by the pathogenic fungus *Dothichiza populea* correlates directly to the amount of SA that accumulates in the bark (Malamy and Klessing, 1992; Kunkel and Brooks, 2002; Hayat et al., 2010). Arabidopsis mutants that are defective in SA synthesis such as *eds1*, *eds4*, *eds5*, salicylic acid induction deficient-2 (*sid2,* also known as *ics1*), and *pad4* or are constitutively expressing a bacterial gene encoding the SA degrading enzyme salicylate hydroxylase (nahG), which does not permit the mutant to accumulate SA in response to stress, are more susceptible to infection compared to wild-type (WT) plants (Kunkel and Brooks 2002). SA accumulation precedes the accumulation of PR proteins. The extent of *PR-1* and *PR-2* expression in both inoculated and non-inoculated leaves of resistant cultivars in response to infection directly correlates with increased endogenous SA levels (Chen et al., 1995). Whilst SA above certain concentrations is known to be directly toxic to pathogens such as *Collectotrichum falcatum*, *Agrobacterium tumefasciens* and *Fusarium oxysporum*, they are not known to be directly detrimental to plant virus. TMV virions pre-treated with SA before inoculation still retain their infectivity. The PR proteins induced by SA are also not known to directly affect virus infectivity (Malamy and Klessing, 1992; Chen et al., 1995; Durner et al., 1997).

1.6.1 Biosynthesis of Salicylic Acid in Plants

SA is synthesized from either the phenylpropanoid or the isochorismate pathways in plants (Figure 1.8). Radiolabelled ^{14}C-phenylalanine, ^{14}C-cinnamic acid and ^{14}C-benzoic acid fed to tobacco plants were quickly synthesized into radiolabelled SA (Yalpani et al., 1993). In the phenylpropanoid pathway, the enzyme, phenylalanine ammonia lyase catalyses the conversion of phenylalanine into trans-cinnamic acid which is then converted into benzoic acid or ortho-coumaric acid. The conversion of benzoic acid into SA is catalysed by benzoic

acid-2-hydroxylase, an enzyme which is also induced by virus infection in plants (Yalpani et al., 1993; Hayat et al., 2010). The isochorismate pathway is proposed to be the primary SA biosynthetic pathway in Arabidopsis (Figure, 1.8). Isochorismate synthase 1 (ICS1) catalyses the conversion of chorismate into isochorismate in this biosynthetic pathway (Wildermuth et al., 2001). Mutants defective in the *ICS1* gene, such as *sid1* and *sid2* only accumulate 5-10% of the SA that accumulates in WT plants after infection (Wildermuth, et al., 2001; Delaney, 2010; Hayat et al. 2010).

The bulk of the synthesized SA is conjugated into SA glucoside by the cytoplasm-localised SA glycosyltransferase, GTase which is activated by elevated levels of free SA in the cytoplasm (Chen et al., 1995). The SA glucoside is then stored in cell vacuoles. However, when plants are infiltrated with SA glucoside, they are first hydrolysed by the cell wall-localized β-glucosidases into free SA. Once in the cytoplasm, part of these free SA is converted back into the inactive SA glucoside by GTase and stored in cell vacuoles. Tobacco suspension cell cultures which have been rid of their cell walls are capable of absorbing free SA but not SA-glucoside added to the cell suspension culture. The swift conversion of free SA into the inactive SA glucoside may serve as a regulatory mechanism to moderate the activation of SA-induced defence responses in plants (Chen et al., 1995; Delaney, 2010; Hayat et al., 2010).

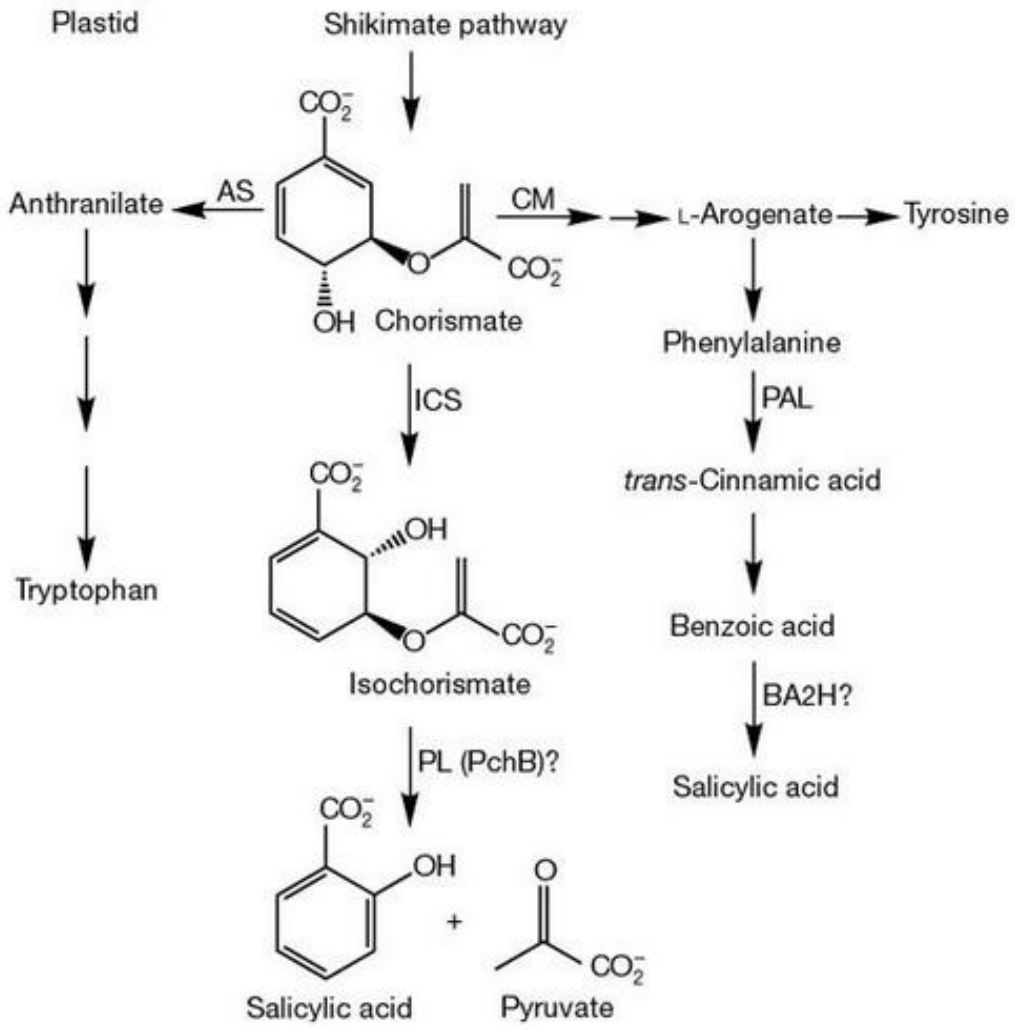


Figure 1.8: Proposed biosynthetic pathways for salicylic acid in plants (Wildermuth et al., 2001).

1.6.2 Exogenous Application of SA and the Induction of Systemic Acquired Resistance in Plants

SAR is a type of induced resistance that offers protection against a broad spectrum of plant pathogens over an appreciable length of time (Durant and Dong, 2004; Carr et al., 2010). SA is required for the induction SAR. Mutant or transgenic plants that are defective in SA synthesis, accumulation or perception are not capable of expressing SAR (Durner et al., 1997; Carr et al., 2010). However, not all pathosystems are responsive to SA induced SAR. For instance, the tobacco-*Potato virus Y* pathosystem is not affected by SA application (Malamy and Klessing, 1992; Hammerschmidt, 1999; Murphy et al., 1999). Exogenous application of SA or any of its biologically active synthetic functional analogues such as 2,6-dichloroisonicotinic acid (INA) or (BTH) can induce the expression of PR proteins and SAR in a number of plants including tobacco, cucumber and Arabidopsis, irrespective of the presence of a pathogen specific resistance gene in the host plant. For instance, treatment of both susceptible and resistant tobacco plants with high non-phytotoxic concentrations of SA induces the same set of nine genes that are induced in the incompatible interactions between TMV-resistant NN genotype tobacco and TMV (Malamy and Klessing, 1992; Durner et al., 1997; Delaney, 2010).

1.6.3 Effects of SA on Virus Infection Cycle

In many instances, SA accumulation during virus infection enhances virus disease resistance (Malamy et al., 1990; Chivasa 1997; Mayers et al., 2005; Falcioni et al., 2014). For example, infection of the TMV-resistant tobacco plant by TMV results in a 20-fold increase in SA accumulation and a parallel elevated *PR* expression. However, in a susceptible cultivar of tobacco, TMV infection increases SA accumulation by only 5-fold (Malamy et al., 1990). Also, SA over-accumulating mutant plants, such as the Arabidopsis mutant *constitutive expression of PR genes 5* (*cpr5*), are more resistant to virus infection compared to WT plants (Love et al., 2007). However, there are instances where SA accumulation is not accompanied by virus resistance (Whitham et al., 2003; Huang et al., 2005; Love et al., 2005). SA may promote resistance to virus infection through restricting virus accumulation or cell-to-cell movement in the inoculated leaf, delay systemic movement (Sections 1.6.3.1 and 1.6.3.2) or contribute to post-transcriptional gene silencing (PTGS) of viral transcripts through its effects on RNA-dependent RNA polymerase 1 (RDR1) (Section 1.6.3.3) (Xie et al., 2001; Murphy and Carr, 2002; Baebler et al., 2014; Lee et al., 2016).

1.6.3.1 SA Restricts the cell-to-cell Movement and Accumulation of Viruses in the Inoculated leaf

SA treatment induces cell-specific restriction on virus movement and accumulation (Murphy and Carr, 2002; Baebler et al., 2014). In tobacco, application of exogenous SA strongly inhibits both the movement of TMV out of the epidermal cell layer and its accumulation in the mesophyll palisade cells for at least 6 days after virus inoculation (Murphy and Carr, 2002). In potato, the *Ny-1 R* gene mediates an HR against PVY infection culminating in the restriction of the virus to the inoculated cells. *NahG-Ny-1* transgenic potato plants infected with PVY however develop systemic disease symptoms due to the unrestricted movement of the virus (Baebler et al., 2014). SA treatment also reduces the accumulation of CMV and PVX in the inoculated leaves of squash and tobacco plants respectively (Mayers et al., 2005; Naylor, et al., 1998).

1.6.3.2 SA Restricts the Systemic Movement of Viruses

Another mechanism through which SA induces resistance to virus infection is the inhibition of the systemic movement of the virus. In both tobacco and Arabidopsis, SA treatment induces resistance to the systemic movement of CMV resulting in a delay in the onset of systemic symptoms (Mayers et al., 2005). SA also contributes to resistance to PVX infection in tomato plants. SA treatment of tomato plants prior to virus inoculation leads to a delay in the detection of the virus in systemic leaves (Falcioni et al., 2014). Transgenic expression of *nahG* in the less PVY susceptible potato cultivar desiree results in enhanced PVY accumulation in systemic leaves and severe disease symptoms. However, pre-treatment of *nahG*-desiree potato plants with 1 mM INA prior to PVY inoculation significantly reduces systemic virus accumulation and symptom development (Baebler et al., 2011).

1.6.3.3 SA contributes to PTGS-Mediated Virus Resistance

SA treatment upregulates RDR1 gene expression and activity. RDR1 is a component of the PTGS pathway required for the amplification of silencing (Xie et al., 2001). The important role of RDR in antiviral silencing is ubiquitous. In tobacco, silencing *RDR1* increases susceptibility to TMV infection and facilitates the systemic spread of PVX in the host (Xie et al., 2001). In Arabidopsis, *rdr1* knock-out mutant plants accumulate high levels of *Tobraviruses* and *Tobamoviruses* in both inoculated and systemic leaves in comparison to

WT plants (Yu et al., 2003). Laboratory accessions of *Nicotiana benthamiana* are natural *rdr1* mutants. TMV infection of *Nicotiana benthamiana* results in plant death. However, a prior SA treatment before TMV inoculation delays the onset of necrosis in *N. benthamiana,* although the infection ultimately leads to the death of the plant. SA treatment of transgenic *N. benthamiana* expressing *RDR1* from *Medicago truncatula* prior to TMV inoculations results in recovery of disease symptoms (Lee et al., 2016).

1.7 4-Hydroxyindole-3-Carbonyl Nitrile, A Novel Cyanogenic Metabolite in Arabidopsis

4-OH-ICN was the first cyanogenic metabolite to be found in the Brassica family and the only cyanogenic metabolite in the entire plant kingdom that possesses the highly reactive α-ketonitrile moiety which readily undergoes nucleophilic abstraction in aqueous alkaline solution to release the toxic cyanide ions (Rajniak et al., 2015).

1.7.1 Biosynthesis of 4-OH-ICN in Arabidopsis

Production of cyanogenic metabolites occurs in more than 2,600 plant species. Synthesis of cyanogenic glucosides is from an amino acid-derived acetaldoxime (Ganjewala et al., 2010). Despite the abundance of cyanogenic metabolites in the plant kingdom, prior to the discovery of 4-OH-ICN, all the >50 known cyanogenic glucosides were synthesized from acetaldoxime intermediates derived from valine, isoleucine, leucine, phenylalanine, tyrosine or cyclopentenyl-glycine. 4-OH-ICN is the only cyanogenic metabolite which is synthesized from a tryptophan-derived acetaldoxime intermediate and also the only cyanogenic metabolite not known to be conjugated to a sugar moiety. There are four enzymes in the 4-OH-ICN pathway, which catalyse the conversion of indole-3-acetaldoxime (IAOx) into 4-OH-ICN and one of its hydroxylated breakdown derivatives (Figure 1.9). These are; the cytochrome P450 CYP71A12, the flavin-dependent oxidoreductase, FOX1, the cytochrome P450 CYP82C2 and the γ-glutamyl peptidase, GGP1 (Figure 1.9) (Ganjewala et al., 2010; Møller, 2010; Rajniak et al., 2015).

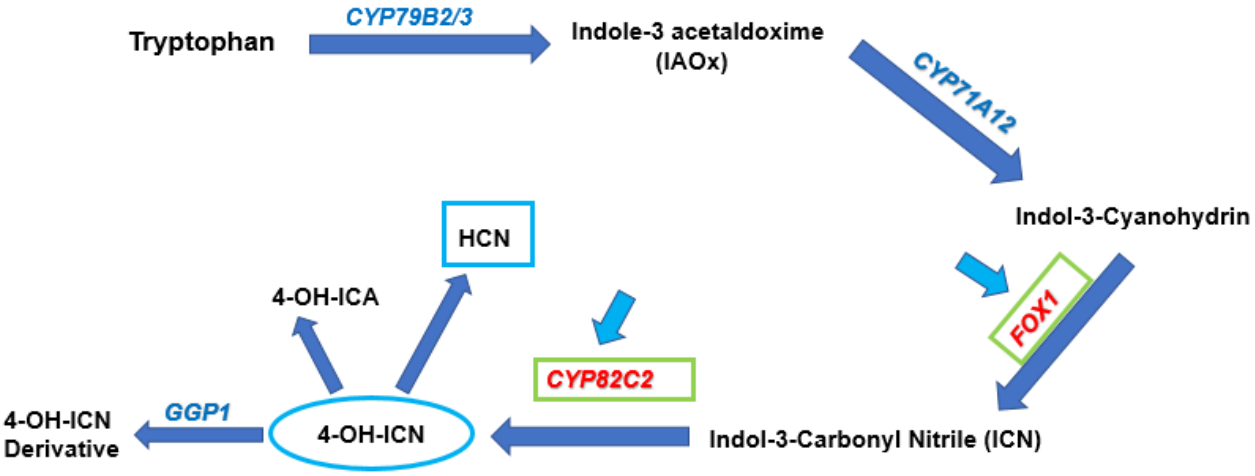


Figure 1.9: The biosynthetic pathway of 4-OH-ICN in Arabidopsis with emphasis on *FOX1* and *CYP82C2* (based on Rajniak et al., 2015).

1.7.1.1 Enzymes Involved in the Biosynthesis of 4-OH-ICN

1.7.1.1.1 CYP71A12

The cytochrome P450 enzyme CYP71A12 functions in both the camalexin and 4-OH-ICN biosynthetic pathways. It diverts IAOx into either indole-3-acetonitrile or indol-3-cyanohydrin in the camalexin, or 4-OH-ICN metabolic pathways, respectively. CYP71A12 is highly co-expressed with FOX1, an enzyme downstream of CYP71A12 in the 4-OH-ICN metabolic pathway. Arabidopsis plants with a mutated *CYP71A12* gene are susceptible to *Pseudomonas syringae* infection and are impaired in root exudation of camalexin (Rajniak et al., 2015; Moldrup et al., 2013). *CYP71A12* is upregulated by more than 35-fold in the *vte2* Arabidopsis mutant which constitutively express camalexin. In the absence of any biotic or abiotic stressors, *vte2* mutants accumulate a 100-fold more camalexin than WT plants (Sattler et al., 2006).

1.7.1.1.2 FOX1

The *FOX1* gene encodes a flavin-dependent oxidoreductase that contributes to the conversion of indole-3-cyanohydrin into indole-3-carbonyl nitrile (ICN). The *FOX1* gene is highly co-expressed with the *CYP71A12* gene in Arabidopsis. Compared to *fox1* mutant plants, WT Arabidopsis plants accumulate 3-5-fold more ICN. In the Arabidopsis mutant, *vte2*, *FOX1* expression is upregulated by 44-fold change even in the absence of elicitors (Rajniak et al., 2015; Sattler et al., 2006). Hydrogen peroxide accumulation also increases the expression of the *FOX1* gene by approximately 26-fold. On the other hand, arsenic toxicity is known to repress the expression of *FOX1* by 1.5-fold (Abercrombie et al., 2008; Inzé et al., 2012).

1.7.1.1.3 CYP82C2

Orthologous copies of Arabidopsis *CYP82C2* gene are also found in the genome of *Glycine max* (soybean) and *Populus trichocarpa* (black cottonwood). In the 4-OH-ICN biosynthetic pathway in Arabidopsis, CYP82C2 functions as an indolic hydroxylase that catalyses the hydroxylation of ICN at the number 4 carbon position to form the 4-OH-ICN (Kruse et al., 2008; Rajniak et al., 2015). Prior to the discovery of 4-OH-ICN, the endogenous substrate of CYP82C2 in Arabidopsis was not known. However, it was known that CYP82C2 was capable of hydrolysing an exogenously supplied furanocoumarin, 8-methoxypsoralen (8-

MOP) into 5-hydroxy-8-methoxypsoralen (5-OH-8-MOP) which was then glycosylated into 5-O-β-D-glucopyranosyl-8-MOP. Furanocoumarins are phytoalexins employed largely by plants in the *Apiaceae* and *Rutaceae* families as defence metabolites against fungal, bacterial and insect attack. Furanocoumarins are not known to occur naturally in Arabidopsis (Kruse et al., 2008). *CYP82C2* is induced by pathogens. *cy82C2* mutant plants are more susceptible to *Pseudomonas syringae* and *Botrytis cinerea* infection and also exhibit increased symptoms to the avirulent strains of the necrotrophic fungal pathogen *Alternaria brassicicola*. Mutant c*yp82C2* plants exhibit decreased induction of JA-responsive genes coupled with a reduced accumulation of JA-inducible indole glucosinolates. However, the accumulation levels of indole acetic acid and camalexin are not altered in *cyp82C2* mutant plants (Liu et al., 2010; Rajniak et al., 2015).

1.7.1.1.4 GGP1

The *GGP1* gene encodes a γ-glutamyl peptidase that functions downstream of 4-OH-ICN biosynthesis, converting 4-OH-ICN into one of the hydroxylated breakdown products. GGP1 also functions in the metabolic pathways of both camalexin and glucosinolates. In the camalexin and benzylglucosinolate metabolic pathways, a tripeptide glutathione (GSH) conjugated to indol-3-acetonitrile (IAN) acts as the GGP1 substrate. The IAN is conjugated to the tripeptide glutathione, γ-Glu-Cys-Gly, through a γ glutamyl peptide bond. Therefore, the role of GGP1 in the metabolic pathways of camelexin and benzyl glucosinolates is the hydrolysis of the γ-glutamyl peptide bond in the GSH-IAN conjugate (Geu-Flores et al., 2009; Lemarié et al., 2015; Rajniak et al., 2015). However, in the camalexin pathway, aside GGP1, the γ-glutamyl peptide bond could also be cleaved by other peptidases including, putatively, phytochelatin synthase 1 (PCS1) and the apoplastic localised γ-glutamyl transpeptidases 1 and 2 (GGT1 and GGT2) (Moldrup et al., 2013).

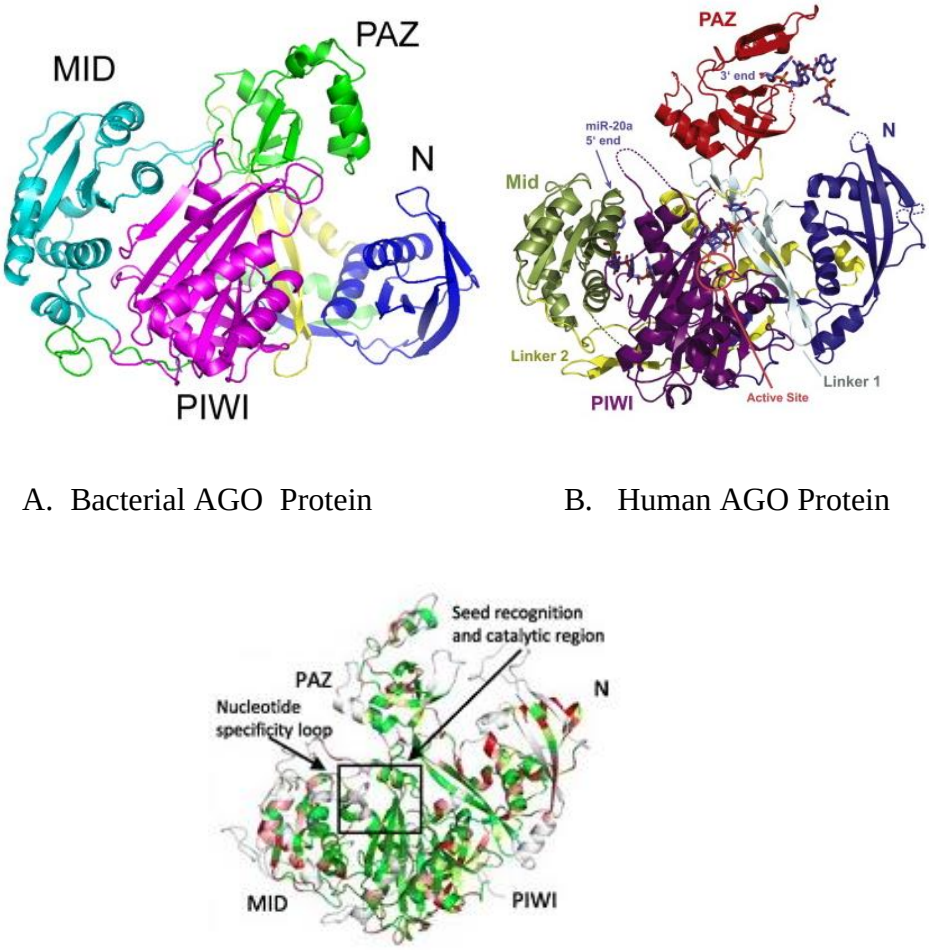
1.8 AGO Proteins

Argonaute (AGO) proteins belong to the P-element Induced Wimpy Testis (PIWI) superfamily of proteins. The “founding member” of the AGO protein family is the Arabidopsis AGO 1 which was discovered in 1998 based on the characteristic phenotype of the Arabidopsis *ago1* mutant plant which has deformed and narrow rosette leaves that were said to resemble the tentacles of the *Argonauta argo* octopus (Bohmert et al., 1998; Swarts et

al., 2014). AGO proteins are common to all six kingdoms of life with the exception of very few species that completely lack any AGO proteins. In the cell, AGO proteins are found in both the cytoplasm and in the nucleus. In the cytoplasm, AGO proteins localize to the cytoplasmic processing bodies and stress granules. In mammalian germ cells AGO proteins have also been found in specialized cytoplasmic structures called chromatoid bodies. In the nucleus AGO proteins localize to the Cajal, bodies which are associated with the nucleolus and are sites for ribonucleoprotein assembly (Detzer et al., 2011; Swarts et al., 2014).

1.8.1 Molecular Structure of AGO Proteins

The first crystal structures of AGO proteins to be solved were of bacterial and archaeal origins such as the AGO protein of the rod-shaped bacterium *Aquifex aeolicus* which is only 700 amino acid long. *Aquifex aeolicus* AGO folds into an elliptical-like molecule measuring approximately 55 x 70 x 90 Å (Figure 1.10A). Structures of larger eukaryotic AGO proteins including the human AGO-2 protein (Figure 1.10B) and *Nicotiana attenuata* AGO proteins (Figure 1.10C) were deciphered later. Despite the considerable differences between prokaryotic AGOs and their eukaryotic counterparts, they all share remarkable similarities in terms of function and structure. The ability of AGO proteins to control protein synthesis, affect mRNA stability and contribute to production of secondary small RNAs is attributed to their structural domains and architectural conformation (Parker and Barford, 2006; Rashid et al., 2007; Swarts et al., 2014).



A. Bacterial AGO Protein

B. Human AGO Protein

C. Plant AGO Protein

Figure 1.10; Crystal structures of AGO proteins from A) *Aquifex aeolicus* refined at 3.2 Å resolution; B; human AGO-2 protein bound to miR-20a guide strand; C) *Nicotiana attenuata* AGO-1a. All three structures are very similar despite belonging to AGO proteins found in different kingdoms (Rashid et al., 2007; Singh et al., 2015; Elkayam et al., 2012).

Eukaryotic AGO proteins possess the four core domains; namely the PIWI domain, the Piwi, Ago and Zwille (PAZ) domain, the MID domain and the N-terminal domain along with interdomain and intradomain linkers. The domains of eukaryotic AGO protein fold into a bi-lobal structure with the N-terminal, PAZ and the interdomain linker-1 constituting one lobe whilst the PIWI together with the MID serve as the second lobe forming a crescent-shaped base. Both lobes are held together by interdomain linker-2. The positioning of the lobes is such that a positively charged groove is created in between the lobes. In the majority of cases prokaryotic AGO proteins lack a complete catalytic motif within their PIWI domain. Approximately, only 40% of prokaryotic AGO proteins possess a PAZ domain (Song et al., 2004; Swarts et al., 2014).

1.8.2 The Formation of RNA Induced Silencing Complex

The spatial orientations of the domains of AGO proteins result in the formation of a positively charged groove that is ideal for interactions with the negatively charged phosphate backbone and 2'-OH moieties of small RNAs (Song et al., 2004).

Small RNA duplexes such as short interfering (si)-RNA duplexes are recognized and bound to the PAZ domain based on their 3' dinucleotide overhangs. One strand of the loaded small RNA duplex is retained as the guide strand whilst the complementary strand is disposed as a passenger strand. The resulting AGO-small RNA effector complex is referred to as RNA induced silencing complex (RISC). The relative thermodynamic stabilities of the small RNA strands influence the selection of the guide strand and the passenger strand. In *Drosophila*, the double stranded RNA binding domain protein R2D2 forms a heterodimer with dicer and binds the end of the more stable strand of the siRNA duplex ensuring the strand with the less stable 5' thermodynamics is retained in AGO protein as the guide strand. Human TRBP and PACT proteins are analogous to *Drosophila* R2D2 and may also aid in selection of guide and passenger strands. The catalytic motif of the PIWI domain may cleave the passenger strand to facilitate its disposal ensuring only a single stranded guide remain loaded into the AGO protein (Song et al., 2004; Lasse and Meister, 2007).

The incorporation of the 5' phosphorylated end of the guide strand into the MID binding pocket strongly bends the guide strand and exclude the base of the first nucleotide from the 5' end from participating in base-pairing with the target strand. Bases 2 to 6 of the guide strand from the 5' end which is also termed as the seed sequence is the primary sequence

responsible for interaction of the guide strand with the target messenger RNA (mRNA) (Figure 1.11). The seed sequence assumes an A-like helical formation with its bases pointing outwardly and accessible for base-pairing with the target sequence. The target strand enters the binding groove with its 5’ end toward the PAZ domain with the N-terminal domain serving as a grip to position the target strand (Song et al., 2004; Hutvagner and Simard, 2008; Swarts et al., 2014).

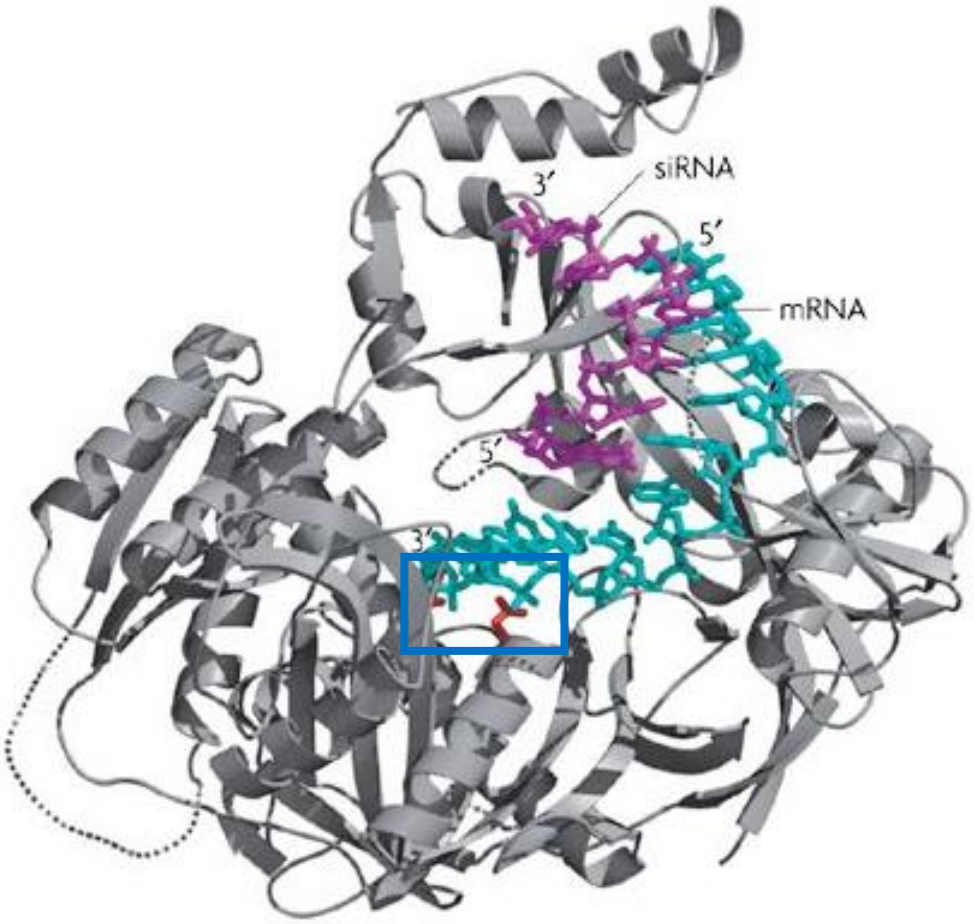


Figure 1.11: Crystal structure of a programmed *Pyrococcus furiosus* AGO protein binding its mRNA target (shown in turquoise). The catalytic motif DDH required for mRNA cleavage is shown in red (highlighted by the blue rectangular inset) (Hutvagner and Simard, 2008).

Interactions of the AGO protein with the small RNAs largely involve the sugar-phosphate backbone of the small RNAs which ensures the availability of the bases of the guide strand for base-pairing with the target sequence. In *Thermus thermophilus* AGO, two arginine

residues lock the 10th and 11th bases of the guide strand from the 5' end into an orthogonal formation slightly disrupting the preformed helical formation of the seed sequence. The seed sequence of the guide strand and the complementary sequences on the target strand then engage in a Watson-Crick base-pairing in the region of the seed sequence with the N-terminal possibly blocking nucleation beyond the seed region of the guide strand (Ender and Meister 2010; Swarts et al., 2014).

Both the 5' and 3' ends of the guide strands still remain anchored in the MID and PAZ domains during nucleation. However, for extensive nucleation between the target and the guide strands, the 3' end of the guide strand in certain cases are known to be released from the 3' binding pocket of the PAZ domain which may be essential to overcome torsional constraints associated with the extensive base-pairing. The release of the 3' end of the guide strand from the PAZ domain during nucleation may expose the 3' end of the guide strand to 3' end modifying exonucleases, possibly explaining why guide strands that form extensive base-paring with their target sequence are 2'-O-methylated. Once the seed sequence and the target sequence are hybridized, the duplex slides down toward the 3' end of the guide strand in order to position the nucleotide 10 and 11 from the 5' end of the guide in the proximity to the catalytic motif (Figure 1.11). Complementarity between the seed sequence and the target sequence is critical for AGO slicing. If there are significant mismatches within the hybrid duplex AGO action may rather result in translation inhibition instead of cleaving. In cleaving competent AGOs, a glutamic acid residue called the glutamate finger is inserted into the cleaving pocket of the PIWI domain completing the catalytic tetrad (Parker and Barford, 2006; Song and Tor, 2006; Ender and Meister 2010).

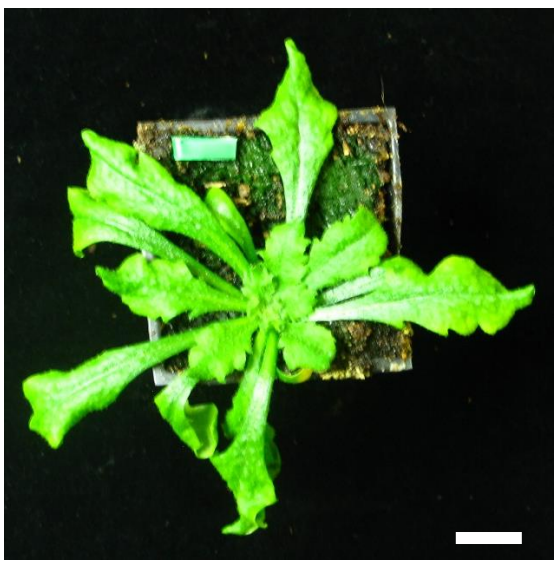
Slicing of the target RNA is facilitated by the presence of two metal cations within the vicinity of the catalytic motif of the PIWI domain. One cation binds to the scissile phosphate of the target RNA and the carboxylate side chain aspartate residue whilst the other cation contributes to the stabilization of the transition states and the reaction products. The catalytic tetrad cleaves the phosphodiester bond of the target RNA at the site opposite nucleotide 10 and 11 of the guide strand resulting in a cleaved RNA product with a 3'-OH and 5'-phosphate group (Parker and Barford, 2006; Ender and Meister 2010).

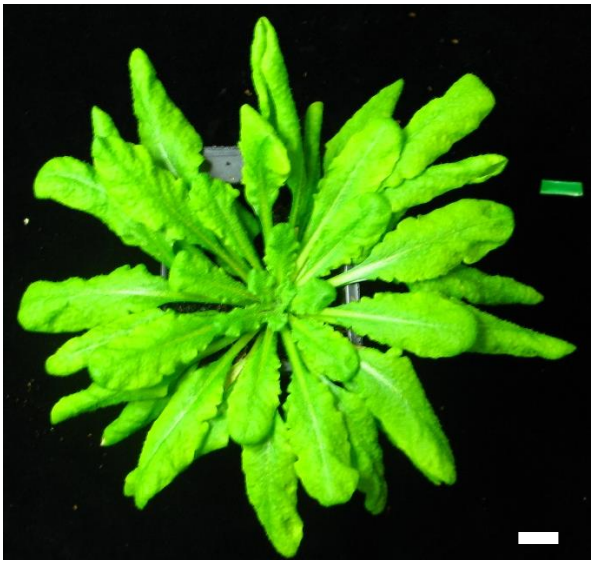
However, not all AGO proteins are cleaving competent possibly due to their lack of the catalytic tetrad or other factors that may be essential to attain cleaving competency. These cleaving incompetent AGO proteins exert their function through inhibition of translation of

the target mRNAs. Although lack of polyA tail in a target mRNA may not completely preclude the transcript from AGO-mediated inhibition of translation however, a functional 7-methylguanine (m7G) cap is essential for targeting mRNAs for translational inhibition. The interactions of the m7G cap of the target mRNA with the m7G binding pocket of the MID domain may result in the unavailability of the m7G cap to eukaryotic initiation of translation factor 4 (eIF4E) resulting in repression of translation. It is also postulated that AGO proteins may prevent the circularization of targeted mRNAs which is essential for initiation of translation hence repressing the translation of targeted mRNAs (Song et al., 2004; Parker and Barford, 2006; Hock and Meister, 2008; Ender and Meister 2010).

1.8.3 Role of AGO Proteins in Plant Growth and Development

AGO proteins in association with siRNAs, miRNAs and tasiRNAs and also Dicer-like enzymes that produce these small RNAs play central role in modulating the processes that directly affect plant growth and development (Vaucheret et al., 2008). This is evident in the pleiotropic developmental deformities seen in plants with mutations in one or more of their *AGO* genes. For instance, an Arabidopsis plant with a mutated *AGO1* gene is associated with unexpanded and pointed cotyledons and also possesses distinctively narrow dark green and thick rosette leaves that do not show any clear distinction between the leaf petiole and the leaf blade (Figure 1.12) (Bohmert et al., 1988; Allen et al., 2005).





Arabidopsis *ago1.25* mutant plant **Arabidopsis WT plant**

Figure 1.12; Phenotype of an Arabidopsis *ago1.25* mutant plant in comparison with a WT Col-0 Arabidopsis plant (Scale bars = 1cm).

The regulatory role of AGO proteins is evident in almost every stage of plant growth and development. Subsequent to fertilization, miRNA-guided AGO proteins are required for the transition from maternally expressed messages into zygotic expression programme where genetic information from both parental sources contribute to the unique identity and differentiation patterning of the newly formed zygote (Nodine and Bartel, 2010). During the early stages of embryogenesis AGO proteins guided by miRNA repress the expression of differentiation promoting transcription factors to maintain the potential of the pre-globular cells to generate varied cell types during the globular stage of embryogenesis. For instance, many of the transcription factors that are precociously expressed in *dicer-like1* (*dcl1*) -5/+ are targets of either mir156, mir159, mir166, mir319 or mir824. mir156-guided AGO-1 and mir165/6 guided AGO-1 are required to repress the differentiation and seed maturation promoting Squamosa promoter binding protein-like (SPL) transcription factors and Class III Homeodomain Leucine Zipper (HD ZIP III) transcription factors Phavoluta (PHV) and Phabulosa (PHB), respectively during early embryogenesis. The juvenile phase of Arabidopsis development is characterised by high accumulation of mir156. Accumulation of mir156 is associated with the principal characteristic of a juvenile plant such as round rosette leaves, lack of trichomes on the abaxial domains of leaves and limited leaf serration.

Overexpressing mir156 prolongs the juvenile phase of Arabidopsis development (Nodine and Bartel, 2010; Yu et al., 2015a).

Transitioning from the juvenile phase to vegetative adult phase of development is in part controlled by AGO 7-generated *trans*-acting siRNA 3 (TAS3) tasiRNAs. Downregulation of *auxin response factor 3* (ARF3) transcripts by AGO-7 generated tasiRNAs prevents precocious transitioning from the juvenile phase to vegetative adult phase. Arabidopsis plants with mutation in their AGO-7 gene transition to adult phase earlier than WT plants. For instance, abaxial trichomes, an adult phenotype, appear about 2 leaf position ahead of the appearance of abaxial trichomes on WT plants. Also, elongation of rosette leaves, another phenotype of adult plants, occur earlier in *ago7* mutant plants than in WT plants (Fahlgren et al., 2006).

Formation of flowers with functional male and female structures is a mark of the reproductive phase of angiosperm development and is crucial for the perpetual existence of angiosperms. miRNA-guided AGOs are essential for the reproductive success of a plant (Jung et al., 2014). During flower formation mir172 accumulates to high levels in stamen primordia compared to its low level of accumulation in petal primordia. Altering this varied localized accumulation of mir172 in the stamen and petal primordia results in the partial conversion of stamen to petals (Figure 1.13A). During the reproductive phase, mir172-guided AGO proteins target Apetala (AP) and Apetala-like (AP-like) proteins either through mRNA slicing or through inhibition of translation. Repressing AP and AP-like proteins which act as repressors of flowering at the vegetative phase of development creates a conducive environment to promote flowering. Transgenic expression of recombinant AP2 that cannot be regulated by mir172 results in the formation of petaloid stamen and in severe cases complete conversion of stamen to petals (Figure 1.13 B and C). Therefore, the high accumulation of mir172 in the stamen primordia during floral development is essential to promote stamen cell fate through the localized downregulation of the petal cell fate promoting AP2 protein. As expected, overexpression of mir172 results in precocious flowering (Chen 2004; Wollmann et al., 2010; Yu et al., 2015a).

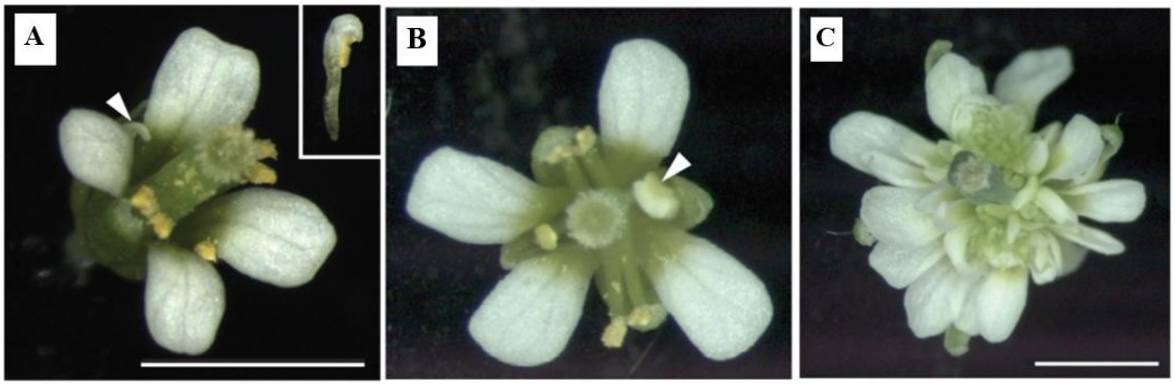


Figure 1.13: Arabidopsis flowers from A) pAP3:MIM172 transgenic plant showing stamen that is partially converted into petals; B) pAP3:amiR-AP2 transgenic plant with petaloid stamen; C) pAP3:rAP2 transgenic plant showing proliferation of petals (Wollmann et al., 2010).

Decline in mir156 accumulation during the reproductive phase also promote flowering. Since certain class of mir-156 regulated SPLs such as SPL3 are able to directly bind and activate flowering promoting genes such as AP1, low accumulation of mir156 during the reproductive phase is translated into high accumulation of flowering promoting SPLs. Constitutive expression of *SPL3* results in early flowering (Cardon et al., 1997; Wang et al., 2009).

AGO-9 is also required for specifying cell fate during megagametogenesis in the ovule. In a WT plant, about 94.2% of the ovules contain a single megaspore mother cell in the pre-meiotic ovule however, in *ago9* mutant plants several abnormally enlarged cells differentiate in the pre-meiotic ovule. However, only one of these abnormally enlarged differentiated cells undergoes meiosis and gametogenesis. AGO-9 is therefore crucial for restricting cell differentiation to a single sub-epidermal cell in the pre-meiotic ovule (Olmed-Monfil et al., 2015).

Another important role played by AGO proteins during the reproductive phase of Arabidopsis growth is the contribution to the production of volatile organic compounds. *Terpene Synthase 21* (*TPS21*) is an SPL9 regulated gene that encodes the enzyme sesquiterpene synthase required for the biosynthesis of (E)-β-caryophyllene sesquiterpene during flowering. Since SPL9 is downregulated by mir156, downregulation of mir156 during flowering phase ensure

the accumulation of SPL9 which in turn promotes the synthesis of sesquiterpenes during flowering (Yu et al., 2015b).

1.8.4 Interaction of CMV 2b Protein with RISC

The 2b protein encoded by CMV recognizes and interacts with AGO proteins (Gonzalez et al., 2010). The 33 amino acid residue C-terminal to the double stranded RNA binding domain of the 2b protein is thought to be critical for its interaction with AGO proteins (Gonzalez et al., 2010; Duan et al., 2012). AGO-2b interactions could result in inhibition of the catalytic activity of the PIWI domain preventing silencing of viral transcripts. The 2b protein can also sequester virus derived siRNAs to avert their incorporation into the RISC complex (Figure 1.14) (Gonzalez et al., 2010; Duan et al., 2012; Gonzalez et al., 2012).

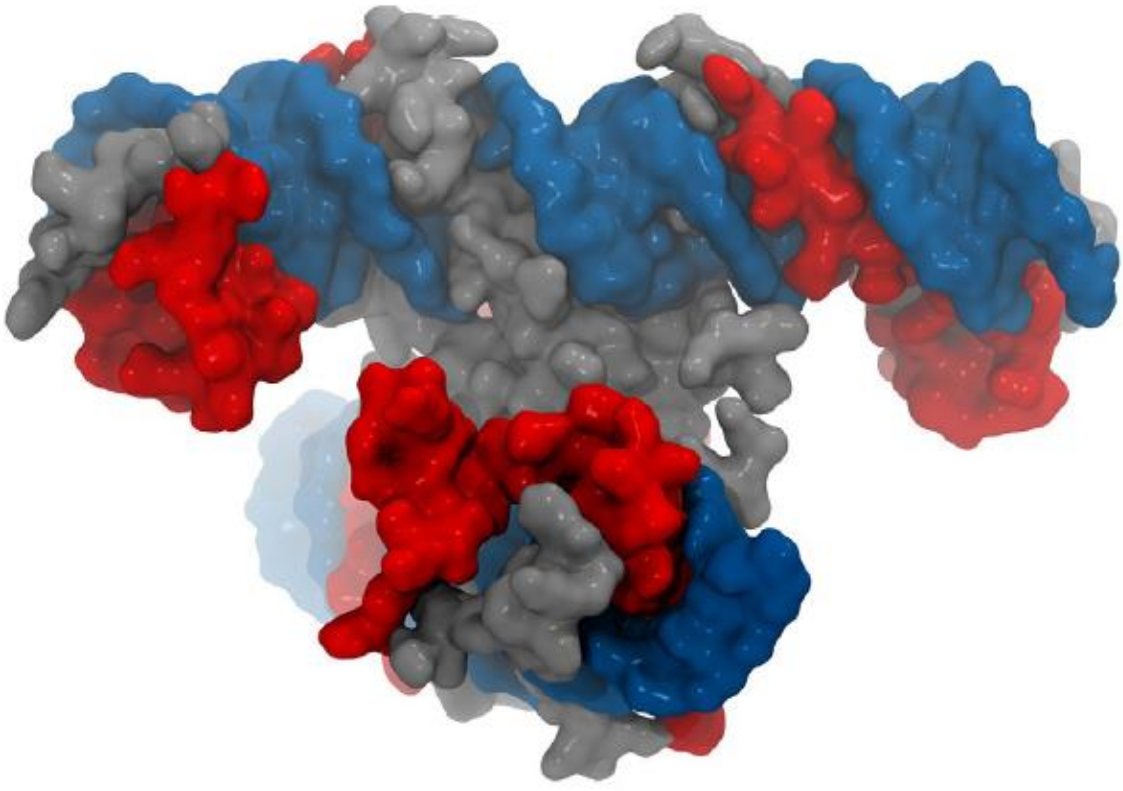


Figure 1.14: The Fny-CMV 2b protein (grey) in a complex with two siRNA strands (blue). The red coloured subunits of the 2b proteins are believed to be the units of the 2b protein that directly interacts with the AGO proteins (Salanki et al., 2018).

Mutation in the 2b protein therefore can significantly alter its interaction with AGO proteins or small RNAs (Gonzalez et al., 2010; Hamera et al., 2012). For example, a truncated CMV Fny-2b protein containing only the N-terminal 50 amino acids only partially inhibits the catalytic activity of AGO1 protein (Zhang et al., 2006). Also, a spontaneous single amino acid substitution in the 2b protein of the mild cross protection CM95 mutant strain of CMV results in the severe CM95R CMV mutant strain. The severity of CM95R infection is as a result of the ability of its 2b protein to stably bind siRNAs compared to the 2b protein of the mild CM95 strain that weakly bind siRNAs (Goto et al., 2007).

1.9 Aphids

The *Aphididae* family consists of over 4,300 species with specialized mouthparts that enable direct feeding from the phloem of host plants. Aphids can reproduce both sexually and asexually depending on the day length, temperature, population size and available nutrition. The ability of aphids to produce prolific wingless clones that can reach maturity within 5 days and establish a colony without mating and winged clones that can disperse to start a colony in different location enable aphids to accumulate to very high densities over a large area within a short period of time (Powell et al., 2006; Goggin, 2007).

Extraction of phloem resources by aphids can impact plant growth and development resulting in wilting, reduced stem elongation, gall formation or reduced crop yield (Larson and Whitham, 1991; Girousse et al., 2005). However, the most significant role of aphids as agricultural pests is their ability to transmit pathogenic plant viruses. Aphids are efficient vectors of important plant viruses. More than 200 species of aphids can vector over 100 plant viruses to over 30 plant families including plant families with economically important agricultural crops. Prominent among these aphid vectors of plant viruses is *Myzus persicae* (van Emden et al., 1969; Goggin 2007).

1.9.1 *Myzus persicae*

The *Myzus persicae* species complex consists of biotypes, races and strains adapted to various geographical and climatic conditions (van Emden, 1969). *Myzus nicotianae* and *Myzus antirrhinii* which were previously classified as independent species are currently grouped under the *Myzus persicae* complex. Mitochondrial *cytochrome oxidase II* and *Elongation Factor-1α* gene sequences from *Myzus nicotianae* are identical to those of *Myzus*

persicae and are both distinct from other *Myzus* species such as *Myzus hemerocallis* and *Myzus varians* (Clements et al., 2000; Radcliffe and Ragsdale, 2002). At optimum temperatures of between 20 - 27.5 ºC, a *Myzus persicae* aphid can produce up to an average of 85.33 nymphs in its lifetime (Auclair, 1963). In warmer climates *Myzus persicae* aphids reproduce largely through parthenogenesis. However, in temperate regions, the decrease in day length that occurs during late summer and autumn induces the production of male and female sexual morphs that mate and produce overwintering eggs (Blackman, 1974).

1.9.2 Host Selection by Aphids

Aphids employ a combination of sensory cues to locate and infest host plants. Generally, aphids tend to land on both host and non-host plants at the same frequency. Accumulation of aphids on host plants rather than on non-host plants is therefore as a result of the differential rate of dispersal after initial contact with a plant. After a contact with a plant is initiated, an aphid assesses the topology of the leaf, presence of trichome exudates and cuticular waxes and subsequently begin to sample the epidermal/mesophyll content of the host by short probes with its piercing and sucking mouthpart. Once probing is initiated, the secondary metabolite profile and the nutritional quality of the host determines aphid arrestment or departure (Figure 1.15) (Powell et al., 2006).

Myzus persicae prefer settling on young leaves despite the high accumulation of aphid deterring secondary metabolites in these leaves. The preference of *Myzus persicae* for young brassica leaves may be due to the high amino acid and sugar content obtained from the phloem of young brassica leaves. Phloem sap from young cabbage leaves is rich in arginine, serine, asparagine and glutamine in comparison to older leaves. Hence *Myzus sp.* perform better on young cabbage leaves irrespective of the high glucosinolate content. Therefore, in terms of host selection, the nutritional quality of the host may take precedence over the host defence responses against the aphid (Zust and Agrawal, 2016; Cao et al., 2018).

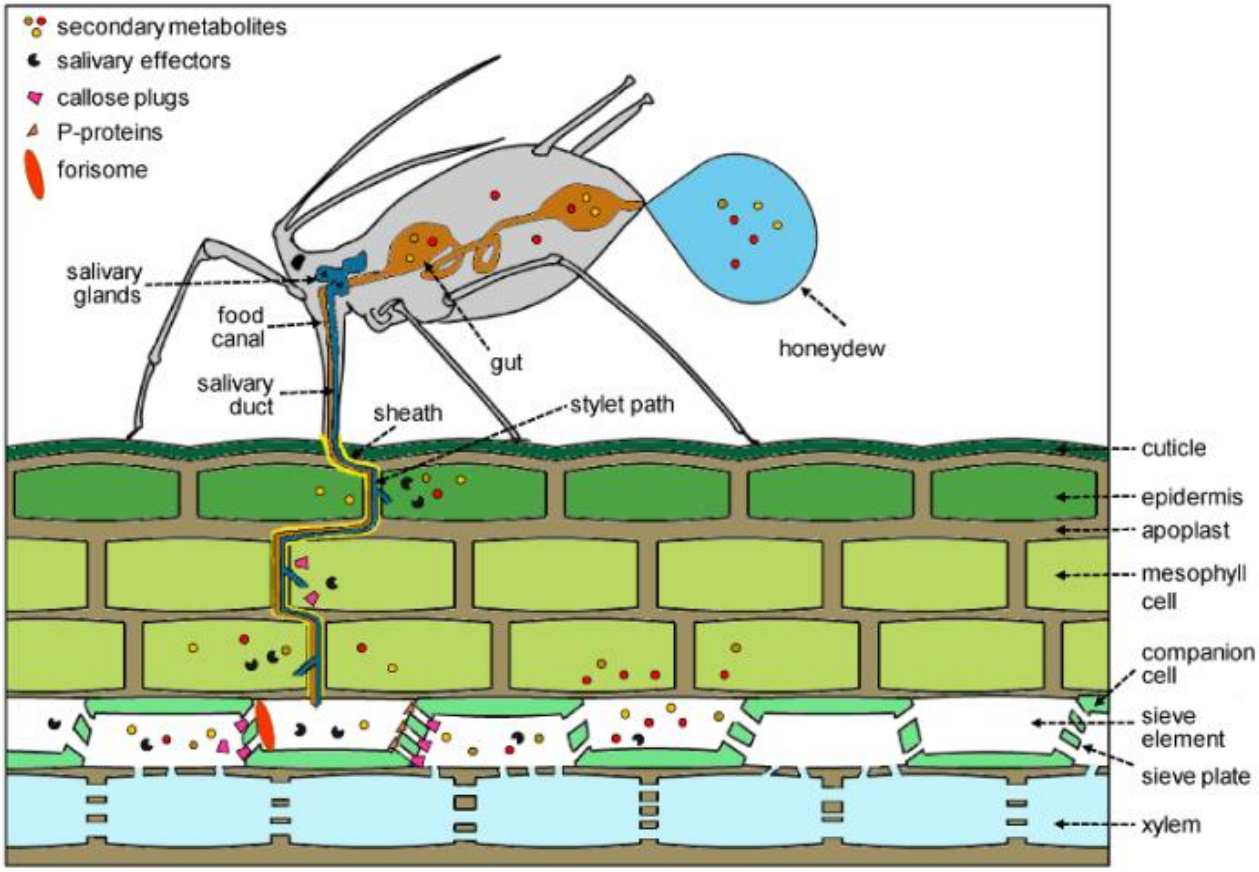


Figure 1.15: A diagram of an aphid feeding from the sieve element of a plant. The quality of the phloem sap and the extent of exposure to the host defence metabolites may influence aphid arrestment or deterrence (Diagram from Nalam et al., 2019).

1.9.2.1 Role of Visual Cues in Host Selection by Aphids

The ability to distinguish between a nutritious host from an inferior host prior to physical contact is essential for the survival of insect herbivores (Mattson, 1980; Stutz et al., 2017). Many aphid species including *Macrosiphoniella artemisiae* (*Artemisia* aphid) and *Rhopalosiphum padi* (bird cherry-oat aphid) rely profoundly on visual cues in locating their host (Gish and Inbar, 2006; Schroder et al., 2014). For instance, in the dark only 17% of all *Macrosiphoniella artemisiae* aphids that dropped from their *Artemisia arborescens* host were able to relocate their host compared to a 92% return rate when the experiment was performed under sunlight (Gish and Inbar, 2006).

The age of a plant, its chlorophyll content, nutritional value or physical attributes of the foliage influence how a plant reflects light and its preference as a host by insect herbivores (Prokopy and Owens, 1983). For instance, plants with very bright autumn colours tend to be an honest visual cue for plants with both abundant free amino acid content in their sap and an increased defence signalling (Hamilton and Brown, 2001; Holopainen and Peltonen, 2002). In line with this, trees with very bright autumn colours are usually heavily infested in the autumn by migratory specialist aphids seeking quality host plants as a source of food and oviposition site (Holopainen and Peltonen, 2002). Also, the migration of bird cherry-oat aphids to their *Prunus padus* (bird cherry) winter host coincides with the period when the sap of the host is high in free amino acid content. During late spring and summer when the free amino acid content drops, bird cherry-oat aphids return to their *Hordeum vulgare* (barley) summer hosts (Sandstrom, 2000). Aphids may therefore have evolved to use the bright autumn colours of trees as a visual cue for hosts with high nitrogen sap content (Holopainen and Peltonen, 2002).

In many instances, infection of a plant by a virus results in drastic changes that affect plant-vector interactions (Carr et al., 2018). For example, leaf yellowing, which also occurs in senescent autumn foliage is a common symptom associated with many virus diseases (Farkas and Solymosy, 1965; Zaitlin and Hull, 1987). Yellow is preferentially selected by many aphid species (Fereres et al., 1999; Hodge and Powell, 2008; Schroder et al., 2014). For instance, pea aphids preferentially settle on the chlorotic leaf tissues from *Pea enation mosaic virus* (PEMV) infected, *Bean yellow mosaic virus* (BYMV) infected or *Broad bean mottle virus* (BBMV) infected plants in comparison to healthy plant tissues largely due to their attraction to the yellow coloration of the virus infected tissues and not based on the ability of the insect to act as a vector for the virus (Hodge and Powell, 2008). In tobacco, infection by the aphid

vectored viruses PVY or CMV affects how light is transmitted from these virus-infected tobacco plants. PVY or CMV infection significantly reduces the extent of polarization of light reflected from the abaxial side of the virus infected leaves. Interestingly, non-insect vectored viruses (TMV and Pepino mosaic virus) are not able to cause this effect (Maxwell et al., 2016).

1.9.3 Host Manipulation by Aphids to Enhance Nutrition

Aphids can actively manipulate their host to enhance the quality and the nutritional content of the phloem sap. Galling aphids such as *Pemphigus betae* create *de novo* physiological sinks in the form of galls to import host resources for their use. Aphid experiments involving the use of carbon-14 indicate that a basal *Pemphigus betae* gall can import as much as 26% of its carbon-14 from neighbouring leaves. As a result, *Pemphigus betae* found in basal galls have higher fecundity rate (Larson and Whitham, 1991).

Other evidence for the active manipulation of host plants by aphids to enhance their nutrition is the high amino acid and sugar content of previously *Myzus persicae* infested Chinese cabbage leaves. *Myzus persicae* feeding on previously infested Chinese cabbage leaves gain 33% more weight and a 110% increase in colony size (Cao et al., 2016).

1.9.4 Aphids as Vectors of Plant Viruses

Insect species within the *Homoptera* family can vector a total of over 380 plant viruses. Prominent among these vectors are aphids. Over 200 species of aphids can acquire and transmit various plant viruses (Nault, 1997; Goggin, 2007).

Based on the mode of acquisition and transmission by insect vectors, plant viruses are categorized as non-persistently transmitted, semi-persistently transmitted, persistently transmitted-circulative, or persistently transmitted-propagative viruses (Figure 1.16) (Nault, 1997).

Non-persistently transmitted viruses are acquired through brief intercellular perforations of the epidermal/mesophyll layer of an infected plant. These brief probes usually last no longer than 10 seconds. Two hypotheses are proposed to explain the acquisition of non-persistently transmitted virus. These are the stylet-borne/stylet contamination hypothesis and the ingestion-egestion hypothesis (Pirone and Harris, 1977).

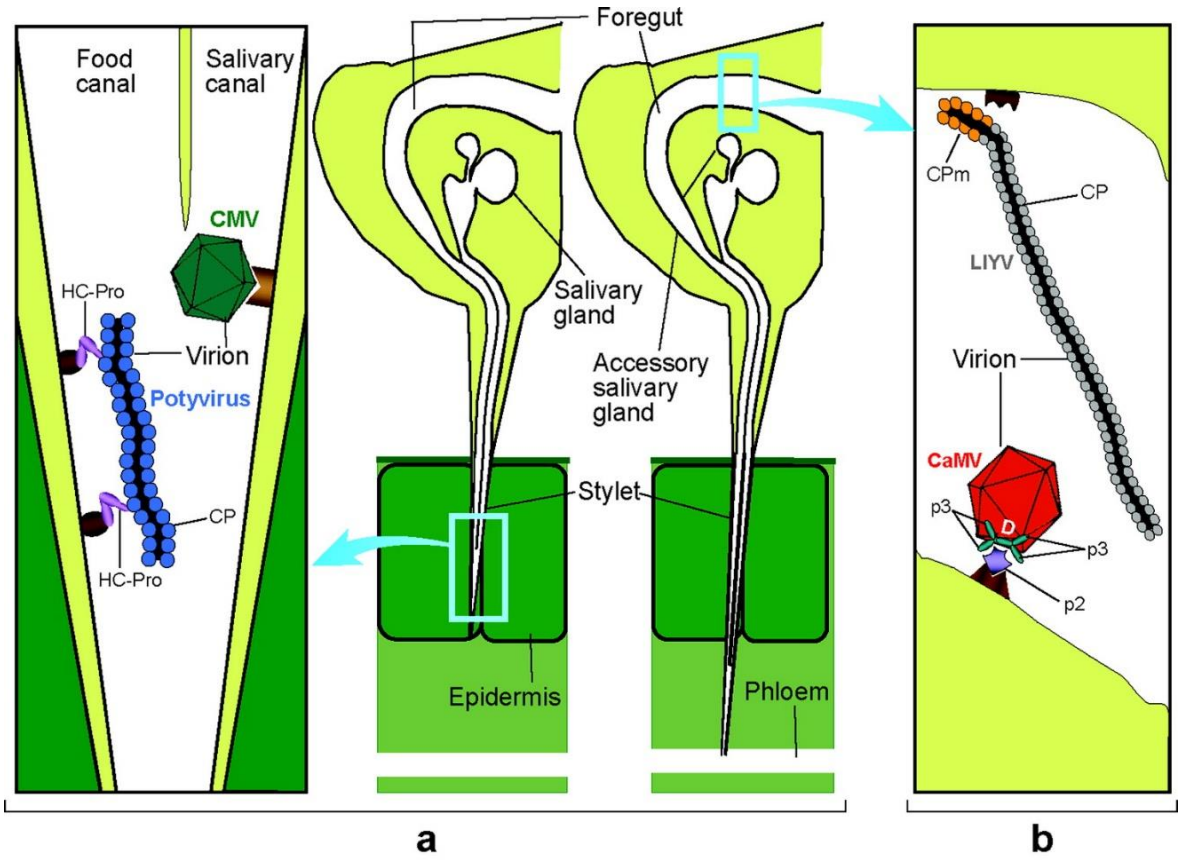


Figure 1.16: Non-persistent (a) and semipersistent (b) transmission of plant viruses by hemipteran vectors. CMV acquisition by aphids does not require virus encoded helper components (From Ng and Falk, 2006).

The primary determinant for the transmission of CMV by *Myzus persicae* is the viral coat protein (Perry et al., 1998). Specific mutations in the CMV coat protein significantly reduce its transmissibility by *Myzus persicae*. For instance, a single amino acid substitution in the coat protein of Fny-CMV can significantly reduce transmission efficiency by *Myzus persicae*. Substituting the coat protein of the non-transmissible mutant M-CMV with the coat protein of aphid-transmissible *Tomato aspermy virus* (TAV) restored transmission by *Myzus persicae* (Chen and Francki, 1990).

Volatile organic compounds and other secondary metabolites produced by plants play crucial role in either attracting or repelling insect herbivores (de Vos and Jander, 2010; Groen et al., 2016). Plant viruses are thought to exploit the plant-vector interactions to facilitate their transmission. An important example is the manipulation of the squash (*Cucurbita pepo*) plant- aphid interactions by CMV. CMV infection alters the blend of volatiles emitted and the accumulation of distasteful secondary metabolites in plant tissues. The volatiles enhance attraction of *Myzus persicae* and *Aphis gossypii* to the infected plants. However, these aphids are repelled after brief sampling of the mesophyll cell content of the infected plant due to its poor quality as food or increased accumulation of defence metabolites (Mauck et al., 2010).

1.10 Role of AGO and small RNAs in Insect Resistance in Plant

Insect infestations result in the upregulation of miRNA-encoding genes in plants (Xia et al., 2015). miRNA-guided AGO complexes are required for tuning various aspect of plant defence against insect herbivory such as regulation of *R* genes, plant hormones and secondary metabolites (Robert-Seilaniantz et al., 2011; Xia et al., 2015). Hence, mutant plants impaired in miRNA processing or RNA silencing such as Arabidopsis *dicer-like 1* (*dcl1*) and *ago1* mutant plants display altered resistance to aphid infestations (Kettles et al., 2013; Westwood et al., 2013).

1.10.1 AGO-Mediated Regulation of *R* genes against Insect Herbivory

Expression of host defence-related genes such as *R* genes are tightly regulated so as to limit the chances of autoimmunity or channelling of resources into host defence responses in the absence of a tangible threat (Li et al., 2012; Tang et al., 2012). AGO-mediated RNA silencing is one of the mechanisms by which plants prevent the constitutive or precocious expression of *R* genes (Li et al., 2012). Generally, a specific set of miRNAs is enough to regulate an entire

class of related *R* genes. For instance, in *Medicago truncatula*, over 60% of all the NB-LRR encoding genes are targeted by a set of miRNA-guided RISC which further generates secondary siRNA that are capable of amplifying the silencing (Zhai et al., 2011). Similarly, *miR482* in *Lycopersicum esculentum* and *miR1885* in the Brassicas also target NB-LRR-encoding genes for silencing (He et al., 2008; Shivaprasad et al., 2012).

In *Cucumis melo* (muskmelon) the *Virus aphid transmission* (*Vat*) *R* gene confers resistance against *Acyrthosiphon pisum* (pea aphid) colonization and virus transmission (Villada et al., 2009). During the incompatible pea aphid-*Vat* muskmelon interactions over 70 miRNAs are upregulated. Six of these upregulated miRNAs are believed to be essential for the *Vat*-mediated defence against pea aphids by virtue of their potential role in suppressing auxin signalling (Sattar et al., 2016).

1.10.2 AGO-Mediated Regulation of Plant Hormones and Secondary Metabolites against Insect Herbivory

Impaired miRNA processing in Arabidopsis *dcl1* mutant plants results in the upregulation of camalexin and JA. Consequently, *Myzus persicae* perform poorly on Arabidopsis *dcl1* mutant plants (Kettles et al., 2013). Similarly, perturbations in auxin signalling also alter plant-insect interactions (Erb et al., 2012). *miRNA 393* targets Transport Inhibitor Response/Auxin Signalling F-Box Protein (*TIR/AFB*) auxin response receptors for degradation, in the process acting as a negative regulator of auxin signalling (Robert-Seilaniantz et al 2011; Windels et al., 2014). In *miR393* over-expressing Arabidopsis plants, the reduced auxin accumulation is associated with the increased accumulation of indole glucosinolates, potent antixenotic metabolites involved in insect resistance in plants (Robert-Seilaniantz et al., 2011).

1.10.3 RNA interference-based Insect Vector Population Management

The protein-based insect pest control strategy that protects plants against insect attack through the transgenic expression of insecticidal protein from *Bacillus thuringiensis* in crops is not very effective against controlling hemipteran herbivores such as aphids (Kola et al., 2015). RNA-based insect control methods that introduce either siRNA or dsRNA into insect cells to induce silencing of vital insect genes could rather prove more hopeful against the management of hemipteran herbivores (Zhang et al., 2013; Kola et al., 2015). The sequence-specificity of this method and the ability of insects exposed to an RNAi trigger to pass the

RNAi signal to subsequent insect generations coupled with the environmentally friendly nature of the procedure make RNAi-based insect control method a viable alternative to explore (Coleman et al., 2015; Kola et al., 2015;).

A number of vital genes involved in various physiological processes in insects have all been used as the basis for designing RNAi triggers with varied outcomes including mortality, reduced fecundity, inability to feed or enhanced susceptibility of the targeted insects to pesticides (Price and Gatehouse, 2008; Yang et al., 2011; Yu et al., 2013; Coleman et al., 2015). Micro-injection of pea aphids with *siApCOO2* resulted in a 100 % silencing of the insect's *COO2* mRNA transcripts and subsequent mortality 8 days earlier than aphids that received mock injection (Mutti et al., 2006) Up to 60% of *Myzus persicae COO2* transcripts were also silenced after the aphids fed from *dsMpCOO2*-expressing transgenic plants. Subsequently, the reproduction rate of the RNAi-expressing aphids dropped by more than half (Mutti et al., 2006; Price and Gatehouse, 2008; Coleman et al., 2015). Similarly, the reproduction rate of *Myzus persicae* exposed to *dsMpRack1* transgenic plants were also reduced by 40% (Coleman et al., 2015). Other genes that have been targeted to effectively control *Myzus persicae* infestations are *MpPInto1*, *MpPInto2*, *Macrophage migration inhibiting factor 1* (*MMIF-1*) and *hunchback* (Price and Gatehouse, 2008; Pitino et al., 2011; Coleman et al., 2015).

1.11 Aims and Objectives

Plant viruses cause some of the most destructive and difficult to control diseases in crop production. The goal of this project was to explore ways to mitigate incidence of virus diseases and its associated economic cost in crop husbandry. Specifically, the basis of the heightened resistance to the CMV vector *Myzus persicae* in Arabidopsis *ago1.25* mutant plant (Westwood et al., 2013) and the feasibility of exogenous application of SA in inducing resistance to CMV disease in pepper plants were investigated.

Myzus persicae perform poorly on *ago1.25* mutant plants in terms of growth and reproduction rate. In addition, aphid infestation of an *ago1.25* mutant plant causes severe stunting and in certain cases premature hyper-senescence of older *ago1.25* mutant plant leaves. Previous experiments in our lab show that the heightened defence to aphid infestation in *ago1.25* mutant plants is not due to feeding deterrence since aphid tend to spend more time actively feeding on an *ago1.25* mutant plant in comparison to a WT plant (Westwood et al.,

2013). It is plausible that aphids feeding on *ago1.25* mutant plants are exposed to a combination of aphid growth-impairing AGO1-regulated metabolites resulting in the poor performance of aphids on this hypomorphic mutant plant. Therefore, part of the aim of my research was to identify the nature of the AGO1-regulated metabolite(s) responsible for the heightened defence to *Myzus persicae* in Arabidopsis *ago1.25* mutant plant. The other part of my research was to find the most effective mode of SA application and the optimum SA concentration required to induce resistance to CMV disease in sweet pepper plants.

Hypotheses

I examined two hypotheses in this thesis. First, I examined if exogenous application of SA could protect pepper against CMV. Subsequently, I explored the hypothesis that 4-OH-ICN and/or SA play roles in the increased resistance of Arabidopsis *ago1* mutant plants to aphids.

To test these hypotheses, the experiments were designed to answer the following questions;

- Part 1: Does 4-OH-ICN biosynthetic genes (*FOX1*, *CYP82C2*) play a role in the heightened aphid defence observed in *ago1.25* mutant plants?
- Part 2: Does the SA biosynthetic gene, *Isochorismate synthase 1 (ICS1)* play a role in the heightened aphid defence observed in *ago1.25* mutant plants?
- Part 3: Does the expression of the Fny-CMV 2b protein in *ago1.25* background affect the heightened aphid defence observed in *ago1.25* mutant plants?
- Part 4: Does exogeneous application of SA induce resistance to CMV disease in pepper plants?

Chapter 2

General Materials and Methods

2.1 Reagents and Sterilization of Equipment

2.1.1 Chemical and Molecular Biology Reagents

The reagents used in this study were largely obtained from Sigma-Aldrich (Gillingham, Dorset, UK), Fisher Scientific (Loughborough, Leicestershire, UK) and Melford Biolaboratories Limited (Chelsworth, Ipswich, UK). In instances where reagents were obtained from other sources, the manufacturers of these reagents were indicated in the text.

2.1.2 Sterilization of Solutions and Equipment

Growth media, thermostable solutions, pipette tips, re-usable plastic pestles and microfuge tubes were sterilized by autoclaving for 15 minutes at 121 ºC. Ceramic mortars and pestles were washed with 1% (v/v) sodium hypochlorite, rinsed, dried and baked for 2 hours at 180 ºC. Plastic tubes, syringes and other single use plastics were supplied in sterile state by the manufacturers.

2.2 Plant Materials

2.2.1 Sources of Arabidopsis Seeds

All mutant lines were in the background of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia-0 (generally referred to as Arabidopsis in this thesis). Mutant *isochorismate synthase 1* (*ics1*) seeds, WT Arabidopsis seeds and Fny-CMV *2b* (line 3.13F) transgenic seeds were all readily available from colleagues and from the laboratory seed stock. Mutant seeds, their origins and appropriate references are listed in Table 2.1. Mutants affecting biosynthesis of 4-OH-ICN were resistant to the herbicide 4-Amino-N-(2-pyrimidinyl)benzenesulfonamide sodium salt (sulfadiazine sodium salt). Since the obtained T_3 seeds were collected from segregating T_2 plants, the seeds were screened to identify homozygous mutant plants. Seeds of the RNA silencing *AGO-1* mutant *ago1.46* were also obtained from the Nottingham Arabidopsis Stock Centre (NASC). The single nucleotide

polymorphism (SNP) *ago1.46* mutant plant was generated by the University of Pennsylvania using the mutagen ethyl methanesulfonate (Alonso et al., 2003; Smith et al., 2009).

2.2.2 Source of Sweet Pepper, Chinese cabbage and *Nicotiana benthamiana* Seeds

Seeds for California Wonder cultivar of *Capsicum annuum* L. (sometimes referred to in this thesis as pepper or California Wonder) and *Brassica rapa* L. *subsp. pekinensis* (Chinese cabbage) were obtained from King's Seeds Ltd, Colchester, UK. The pepper is recognized by the Committee for *Capsicum* Gene Nomenclature as a WT cultivar for the *Capsicum annuum* species (Daskalov and Poulos, 1994). It produces large non-pungent fruits with thick flesh. It is often used in pepper research as a standard cultivar. However, recent studies identified significant genetic variabilities among various accessions of California Wonder pepper plants (Votava and Bosland, 2002). California Wonder pepper was selected for this study due to its importance as a vital crop and its susceptibility to CMV infection (Montasser, 1998). *N. benthamiana* seeds were obtained from the lab stock.

Table 2.1: Sources of WT and mutant seeds used in this study

Name	Gene Mutated	Type of Mutation	Activity	NASC ID/Parental Line	Reference
cyp71A12	*CYP71A12*	T-DNA insertion	Converts IAOx to indole-3 - cyanohydrin	GK-127H03	Rajniak et al., 2015
fox1	*FOX1*	T-DNA insertion	Converts indol-3-cyanohydrin to ICN	GK-813E08	Rajniak et al., 2015
cyp82C2	*CYP82C2*	T-DNA insertion	Converts ICN to 4-OH-ICN	GK-261D12	Rajniak et al., 2015
ggp1	*GGP1*	T-DNA insertion	Converts 4-OH-ICN to a 4-OH-ICN Derivative	GK-960B11	Rajniak et al. 2015.
ago1.25	*AGO1*	SNP (Missense)	Involved in gene silencing	Lab stock	Morel et al., 2002
ago1.46	*AGO1*	SNP (Missense)	Involved in gene silencing	N67862	Smith et al., 2009
ics1(sid2.1)	*ICS1*	SNP (Nonsense)	Involved in salicylic acid biosynthesis	Lab stock	Wildermuth et al., 2001
cyp82C3	*CYP82C3*	T-DNA insertion	Cytochrome P450	N570325	Alonso et al., 2003
2b (3.13F)	Fny-CMV 2b ORF + 3' NTR	Transgenic mutant	Suppressor of Silencing	Lab stock	Lewsey et al., 2007
Col-0 WT	WT Arabidopsis	WT	WT	Lab stock	
California Wonder	WT Pepper plant	WT	WT	Kings Seeds, Colchester,	

				UK	
Nicotiana benthamiana	WT *Nicotiana benthamiana*	WT	WT	Lab stock	

SNP = Single nucleotide polymorphism; T-DNA = Transfer DNA; ORF = Open reading frame; NTR = Non-translated region.

2.3 Seed Sterilization and Growth Conditions

2.3.1 Sterilization of Arabidopsis and Pepper Seeds

Arabidopsis seeds were first soaked in 1 ml 70% (v/v) ethanol for 2 minutes and then surface sterilized with disinfectant consisting of 5% sodium hypochlorite and 0.5% (w/v) SDS for 10 minutes and later rinsed at least three times with autoclaved deionized distilled water.

Pepper seeds were soaked in 1% (v/v) sodium hypochlorite solution for 10 minutes with intermittent agitations. Afterwards, the seeds were then rinsed with tap water for at least three times and dried on tissue paper. The surface-sterilized pepper seeds were then plated in tissue culture plates lined with wetted filter paper. Seeds were kept in an incubator set at 25 ºC until full emergence of radicle.

2.3.2 Plating and Germination of Arabidopsis Seeds

Surface-sterilized Arabidopsis seeds were plated on 1% (w/v) Murashige and Skoog (MS) agar (comprising of MS medium salt with a pre-added micro and macro elements, sucrose and phytoagar). Both MS salt and Phytoagar were obtained from Duchefa Ltd, Haarlem, The Netherlands. Plated seeds were stratified at 4 ºC for a minimum of two days and then moved to a long day (16 hours light, 8 hours darkness) growth incubator (Conviron, Winnipeg, Manitoba, Canada) with a temperature of range of 20-21 ºC. The plates were kept in the incubator for a period of 18-19 days until they were taken out and transplanted.

Arabidopsis seeds that were used in generating experimental plants were sown directly onto saucers containing F2 Levington compost, (Fisons Plc., Ipswich, UK) sprayed with tap water and sealed tight with Saran wrap to maintain high humidity. The seeds were then cold

stratified for a minimum of 2 days at 4 ºC and then moved to short day growth room (8 hours light, 16 hours darkness) for approximately 2 weeks before being pricked out.

2.3.3 Transplanting of Arabidopsis and Pepper Seedlings

Arabidopsis seedlings were pricked-out into plastic growth trays (Desch Plantpak, Mundon, Essex, UK) containing F2 Levington Compost. The compost was mixed with 0.002% (w/v) Intercept systemic insecticide (active ingredient; imidacloprid: Scotts, Bramford, Ipswich, UK) and sand to control insect pests and improve soil aeration, respectively. Plants that were taken off the automated watering system were manually watered. All the main experimental plants were grown under short day conditions with 8 hours of light and 16 hours of darkness.

Pre-germinated pepper seeds were pricked out into trays containing M3 Levington compost mixed with sand and Intercept insecticide. The transplanted pepper plants were placed on the automated watering system until the day of SA application where they were taken out of the automated watering system and watered manually. All pepper plants were grown under long day conditions with 16 hours of light and 8 hours of darkness.

The relative humidity, temperature and light flux in the growth rooms were 60%, 22 ºC and 200 µmol.

2.4 Generation of Double Mutant Plants

2.4.1 Crossing Arabidopsis Plants

Seeds of the individual genotypes needed for the crosses were sown and maintained under long day conditions as previously described. At the inflorescence stage, each of the participating parental genotypes were either assigned as a male parent which was to provide pollen for the cross or as a female parent which was to provide an unfertilized ovum for the cross. In cases where reciprocal crosses were required the male parents were later used as female parents whilst the female parents were used as male parents. For the female parents, a healthy-looking inflorescence was selected. With the aid of tweezers all the fully opened flowers were nipped off leaving only the unopened but mature-looking floral buds to be used for the cross. Each bud was carefully opened by removing all the sepals and petals to expose the stamen and the pistil. Afterwards, all the stamens were removed with the aid of a tweezer

leaving only the pistil in place. For a male parent, fully opened mature flowers were selected as sources of pollen for the cross. Flowers from the male parent were nipped-off by the peduncle with the aid of tweezers and dusted onto the stigma of the freshly emasculated female flower for transfer of pollen from the male parent onto the stigma of the female parent. Crosses were then labelled indicating the male parent and the female parent and then returned to the growth room. Within approximately 3 days from crossing, crosses that resulted in successful fertilisation still maintain a green style showing signs of elongation whilst styles from unsuccessful crosses become necrotic and wither off. After approximately 3 weeks from crossing, the F_1 siliques will be fully elongated, mature and drying up. Prior to the onset of natural dehiscence, the F_1 siliques are harvested into microfuge tubes and labelled.

Table 2.2: List of crosses and double mutant plants generated for this study

Cross	**Phenotype**
ago1.25* x *cyp82C2	*ago1.25* mutant plant defective in 4-OH-ICN biosynthesis
fox1 ago1.25	*ago1.25* mutant plant defective in 4-OH-ICN biosynthesis
ago1.25* x *ics1	*ago1.25* mutant plant defective in SA biosynthesis
ago1.46* x *nahG	*ago1.46* mutant plant defective in SA accumulation
2b* x *ago1.25	Transgenic expression of Fny-CMV 2b gene in *ago1.25* background

2.4.2 Maintaining F_1 Progeny from Crosses

The F_1 seeds are collected from their siliques, surface-sterilized, plated on MS agar, cold stratified for approximately 3 days and maintained under long day conditions for 2 weeks prior to picking out into F2 Levington compost for growth under long day conditions. Except crosses involving transgenes, all F_1 plants resemble a WT plant irrespective of their parental phenotypes due to their heterozygosity. Leaf and/or floral tissues are then collected from the bolting/mature F_1 plants for gDNA extraction to confirm their heterozygosity using the appropriate primers listed in Appendix I. The F_1 plants are then grown until silique maturation. The plants are then taken off the watering system at this stage to facilitate the silique drying process. After thorough drying, the F_2 seeds are harvested from each F_1 plant and labelled appropriately.

2.4.3 Identifying Double Mutants Among Segregating F_2 Populations

In cases where one of the parental mutant plant has an antibiotic selection marker such as in the case of the 4-OH-ICN biosynthetic mutant plants which were resistant to the herbicide sulfadiazine (sul), the segregating F_2 seeds were plated on sulfadiazine sodium salt - containing MS agar, cold stratified for approximately 3 days and maintained in a long day room for 2 weeks prior to pricking out. At this stage, the segregants without the antibiotic resistance either begin to show signs of chlorosis or are necrotic making it easier to narrow down on the double mutants. In crosses involving both *ago1* mutant and any of the 4-OH-ICN biosynthetic mutant plants, since mutation in any of the 4-OH-ICN genes does not affect the parental phenotype of *ago1* in the F_2 double mutant, all the F_2 progeny that have the *ago1.25* phenotype and are able to survive the sul-MS agar medium are pricked out into compost and grown under long day conditions. At the rosette stage, gDNA are collected for genotyping to identify the 4-OH-ICN biosynthetic/*ago1* double mutants from the 4-OH-ICN biosynthetic heterozygote/*ago1* mutants which have the same phenotype as the double mutants and are also able to survive the sul-MS agar medium. In crosses where none of the parental mutants has an antibiotic resistance marker such as in the case of crosses involving the two single nucleotide polymorphism mutants *ics1* and *ago1.25*, identification of the F_2 double mutant is solely through sanger sequencing and Allele-Specific PCRs. Identified F_2 double mutants are then grown till bolting and allowed to self-fertilize. Mature F_3 seeds are then harvested and used for generating experimental plants.

2.5 Salicylic Acid Treatment

2.5.1 Preparation of Salicylic Acid Solutions

SA solutions were prepared from pure grade SA (molecular mass of 138.12 g/ml) with a 99% purity (Lancaster Synthesis, Eastgate, England). Stock solutions of between 0.5 mM and 1.0 mM concentrations were first made and later diluted to the required concentrations using tap water without any additional ethanol. In the majority of the experiments, SA was applied as foliar sprays and/or watered to plants at the cotyledonous to first true leaf stage. Other modes of SA application included imbibition of pepper seeds in SA solutions and submersion of whole pepper seedlings into SA solutions for a specified duration.

All pepper plants that received SA treatments through foliar sprays or through both foliar sprays and watering were inoculated a day after the last SA treatment regime. Thus, the plants were treated with SA for three consecutive days and on the fourth day, they were inoculated with CMV. These inoculated plants were largely at their first true leaf stage. Pepper plants that were treated with SA through drenching were pricked-out into trays and watered regularly with tap water until emergence of their first true leaf after which they were inoculated. SA imbibed seeds were pre-germinated in plates lined with filter paper moistened with SA solution. The pre-germinated seeds were then pricked-out into trays and watered with tap water until full development of cotyledonous leaves before inoculation.

2.6 Preparation of CMV inoculum

The Fny-CMV strain was used for all virus inoculations in this research. CMV inocula were generated from infectious cDNA clones.

2.6.1 Preparation of Luria Broth (LB) Media

Liquid LB medium was prepared by dissolving 25 g of Miller's LB broth base (Invitrogen, UK) per litre of distilled water. Solid LB media contained 0.75% (w/v) phytoagar. Both liquid and solid LB media were autoclaved prior to using. A 120 µl volume of 50 mg/ml stock kanamycin was added to 60 ml of a melted LB solid medium. An aliquot of 20 ml each of the medium was transferred into three separate agar plates and allowed to solidify.

2.6.2 Preparation of *Agro*-inoculation Suspension

LB plates were streaked with *Agrobacterium tumefasciens* strain *GV3101* carrying infectious clone for a specific CMV RNA (CMV RNA1, CMV RNA 2 or CMV RNA3). The bacteria were incubated at 28 ºC for two days to isolate colonies. A 9 ml aliquot of liquid LB medium was mixed with 100 µg/ml kanamycin and 3 ml each was then dispensed into three 50ml conical tubes. Each tube was inoculated with a single colony of the *Agrobacterium tumefasciens* carrying a specific CMV RNA. Liquid cultures were incubated overnight at 30 ºC on a G24 Environmental mechanical shaker (New Brunswick Scientific, Edison, USA) set at 250 rpm. Dilutions of 1:10 of the overnight cultures were made to measure optical density at 600nm (OD_{600}). Appropriate volumes of each bacteria culture were then added to liquid LB medium containing kanamycin and incubated overnight at 30 ºC with shaking at 250 rpm. The optical densities of RNA1, RNA2 and RNA3 cultures were 1.83, 2.13 and 2.73 respectively. A volume of 3.28 ml RNA1 culture, 2.82 RNA2 culture and 2.20ml RNA3 culture were then pipetted into microfuge tubes and centrifuged at 12,000 g. The supernatants from all three cultures were discarded and the remaining bacteria pellets were pooled together in a 50 ml infiltration buffer made from 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.5 and 10ml $MgCl_2$.

2.6.3 Agro-inoculation of *Nicotiana benthamiana* Seedlings

Small nicks were made on the epidermal layer of the abaxial surface of *N. benthamiana* leaves. The *A. tumefasciens* suspension was, using a needleless syringe pressed against the nicks on the leaves, injected into the intracellular spaces, with the thumb providing counter-pressure from the adaxial surface of the leaves (Schob et al., 1997). Agro-inoculated plants were then placed in a growth room to allow CMV infection to develop. Systemic CMV symptoms were visible 10-days post inoculation. CMV infection was confirmed 14-days post infiltration using a lateral flow serological test (Agristrip kit, Bioreba AG, Switzerland).

2.6.4 Purification of CMV Virions

Whole leaves and upper portions of CMV-infected *N. benthamiana* plants were blended with 1.5 ml thioglycolic acid and 300 ml ice-cold buffer A (0.5 M sodium citrate, 5 mM disodium EDTA) and 300 ml of chloroform. The mixture was then centrifuged at 10,000 rpm for 15 minutes at 4 ºC in an Avanti J-25 centrifuge (Beckman Coulter, Fullerton, CA, USA) fitted with JA-20 fixed angle rotor. The aqueous phase was collected by filtering through a layer of

chilled pre-wetted muslin cloth. The collected filtrate was added with 2.7g of PEG and centrifuged at 10,000 rpm (Beckman JA-20 rotor) for 10 minutes at 4 ºC. The supernatant was discarded and the pellets were re-suspended in buffer A at 4 ºC. The suspension was then centrifuged at 4 ºC for 10 minutes at 6,000 rpm (JA-20 rotor). The supernatant was collected and ultracentrifuged at 40,000 rpm for 1 hour 15 minutes at 4 ºC in a Beckman Ti70 rotor. The pellets were then re-suspended in 5 ml buffer B (5 mM sodium tetraborate pH 9.0, 0.5 mM disodium EDTA, 2% (v/v) Triton-X 100) at 4 ºC on a mechanical shaker at high speed settings. The re-suspended solution of virions was then centrifuged at 6,000 rpm (JA-20 rotor) for 10 minutes at 4 ºC in a JA 20 rotor. The supernatant was collected and ultra-centrifuged at 40,000 rpm (Beckman Ti70 rotor) for 1 hr 15 mins at 4 ºC over a 5 ml cushion of buffer C (5 mM sodium tetraborate pH 9.0, 0.5 mM disodium EDTA) containing 10% (w/v) sucrose. The supernatant was discarded and the pellets re-suspended overnight at 4 ºC in buffer C containing 0.02% (w/v) sodium azide as a preservative. The concentration of the purified virions in mg/ml was then calculated by measuring the absorption at 260 nm and dividing the absorbance by the 1 mg/ml extinction coefficient (Lot and Kaper, 1976; Roossinck and White, 1998).

2.7 Extraction of Total RNA from CMV-infected *N. benthamiana* leaves for Mechanical Inoculations

A stock of *N. benthamiana* seedlings were mechanically inoculated with sap prepared from CMV-infected *N. benthamiana* plants. The sap inoculum was made by pulverizing systemically infected *N. benthamiana* plants in a mortar with a pestle and diluting to the required consistency with distilled water. Prior to the CMV inoculation, the *N. benthamiana* seedlings to be inoculated were first dusted with silicon carbide powder (Carborundum) 320 grit (Alfa Aesar, Lancashire, UK) after which the freshly prepared sap inoculum was rubbed onto the Carborundum-dusted leaves. Excess sap and Carborundum on the inoculated leaves were washed off using water dispensed from a spray bottle. The inoculated plants were then covered with lids and returned to the growth room. Two to three weeks after inoculation systemically infected leaves were collected and stored at -80 ºC until total RNA extraction. Extraction of total RNA for mechanical inoculations followed similar protocol as described in section 2.9.1 but without DNAse treatment of the extracted total RNA.

2.8 Mechanical Inoculation of Plants with CMV Virions or RNA

In all the pepper experiments, the inoculated organs were the cotyledons. Purified virions of CMV were diluted to the desired inoculum concentration with sterile water. Both cotyledons of each pepper plant to be inoculated were dusted with Carborundum. With the aid of an inoculum-dipped Q-tip, several strokes were made across the adaxial surface of the cotyledons with a gentle counter pressure supplied by the index finger from the abaxial surface. After the inoculations, excess Carborundum was washed off with water and the plants were covered with transparent lids to enhance survival of the inoculated leaves. Inoculated plants were then returned to the growth room for periodic monitoring of symptom development.

Arabidopsis plants were inoculated 2-weeks post pricking-out with total RNA extracted from Fny-CMV infected *N. benthamiana*. Arabidopsis plants to be inoculated were first dusted with Carborundum as previously described. A total of approximately 5 µl of the 500 ng/µl RNA inoculum was then pipetted onto the Arabidopsis leaves and with the aid of the index finger, the inoculum was rubbed onto the leaf. Excess Carborundum was washed off with water. The inoculated plants were covered with lids and returned to the growth room.

2.9 General Nucleic Acid Manipulation

2.9.1 Extraction of Total RNA

Leaf tissues were pulverized in liquid nitrogen into a fine powder in pre-chilled mortars. Approximately 2 ml of TRIzol-like extraction buffer (38% v/v Tris-buffered phenol, 0.8M guanidine thiocyanate, 0.4M ammonium thiocyanate, 0.1M sodium acetate, pH 5.0, 5% (v/v) glycerol) was added to the ground sample, mixed thoroughly and then transferred into 1.5 ml microfuge tubes. The samples were spun at 13,200 rpm in a Z 400 K centrifuge (Hermle Labortechnique GmbH, Germany) for 10 minutes at a temperature of 4 ºC. A 1 ml volume of the supernatant was transferred into fresh 1.5 ml microfuge tubes with 200 µl chloroform-isoamyl (24:1). The samples were then vortexed for approximately 5 seconds and placed on ice for a minimum of 10 minutes after which they were spun at 13,200 rpm (Z 400 K centrifuge) for 15 minutes. The upper aqueous phase was transferred into fresh tubes and added with equal volume of isopropanol. After approximately 10 seconds of vortexing, the samples were kept at -20 ºC for at least 1 hour to allow the precipitation of nucleic acid. The

suspensions were centrifuged at 13,200 rpm (Z 400 K centrifuge) for 10 minutes to collect the nucleic acid pellets. The pellets were washed with 1 ml 70% (v/v) ethanol by vortexing and centrifugation at 13,000 rpm (Z 400 K centrifuge) for 5 minutes. The pellets were re-suspended in 750 µl sterile water and mixed with an equal volume of 4M LiCl and incubated at -20 ºC overnight. They were then centrifuged at 13,200 rpm (Z 400 K centrifuge) for 15 minutes and the resulting pellets were re-suspended in 400 µl sterile water. Each of the re-suspended pellets were mixed with 200 µl buffer phenol solution (pH 4.8) and 200 µl chloroform:isoamyl alcohol (24:1) and vortexed for approximately 15 seconds before being centrifuged at 13,200 rpm (Z 400 K centrifuge) for 10 minutes. Approximately, 360 µl volume of the supernatant of each sample was transferred into a clean 1.5 ml microfuge tube and mixed with 720 µl ethanol and 36 µl 3M sodium acetate (pH 5.3) and kept at -20 ºC for at least 1 hour for RNA precipitation. The samples were then centrifuged at 13,200 rpm (Z 400 K centrifuge) for 15 minutes to collect the RNA pellets. The RNA pellets were washed with 1 ml 70% ethanol and allowed to dry in a laminar flow hood. The RNA pellets were re-suspended in 45 µl sterile water.

2.9.2 DNAse Treatment of Plant Total RNA

DNAse treatment was carried out using the TURBO DNA-free Kit (AMBION, Austin, TX, USA). The 45 µl re-suspended total RNA was mixed with 5 µl 10x TURBO DNA-free buffer and 1 µl TURBO DNAse to remove any remnant genomic DNA fragments in the extracted total RNA. The DNAse reaction was performed at 37 ºC for 30 minutes. In order to stop the activity of the DNAse, 5 µl TURBO DNAse inactivation reagent was added to the mixture and briefly vortexed. The mixture was allowed to stand for 5 minutes at room temperature and afterward centrifuged at 13,000 rpm (Heraeus Biofuge pico centrifuge) for approximately 2 minutes. A 45 µl volume of the supernatant of each sample was then transferred into a fresh 1.5 ml microfuge tube. Samples of 1 µl each from the total RNA preparations were analysed spectrophotometrically at 260 nm using a Nanodrop 1000 (Thermo Fisher Scientific, Walton, MA, USA) against diethylpyrocarbonate (DEPC)-treated water.

2.9.3 Complementary DNA synthesis

Approximately 500 ng of DNAse-treated total RNA was mixed with 0.5 µl random sequence oligonucleotide primers (Thermo Fisher Scientific, Walton, MA, USA) and diluted with

sterile water to a volume of 5 µl in a 1.5 ml microfuge tube and incubated at 70 ºC for 5 minutes and placed on ice for at least 10 minutes. The 5 µl mixture was mixed with 4 µl GoScript 5x reaction buffer, 3 µl $MgCl_2$ and 1 µl full complement (deoxyribonucleotide triphosphate) dNTP mix, 1 µl RNAse-out enzyme and 1 µl GoScript reverse transcriptase (Promega, Southampton, UK). The mixture was made up to a volume of 20 µl with sterile water. The reverse transcription reaction was carried out in a thermocycler (Applied Biosystems SimpliAmp Thermal Cycler) for a single cycle of 5 minutes at 25 ºC; 60 minutes at 42 ºC and 15 minutes at 70 ºC.

2.9.4 Non-quantitative Polymerase Chain Reactions

PCR routinely consisted of 1 µl cDNA template, 5 µl Biomix Red buffer (Bioline), 0.2 µl each of the appropriate primers. The reaction mixture was made up to 10 µl with sterile water. PCR was performed in a thermocycler (Applied Biosystems SimpliAmp Thermal Cycler). Each of the 30-40 cycles was as follows: denaturing of cDNA, 45 seconds at 94 ºC; primer annealing, 45 seconds at 55 ºC; primer extension, 30 seconds at 72 ºC. There was an initial 3 minutes denaturing step at 94 ºC prior to the cycles and a final 72 ºC step for 1 minute after the completion of all the required cycles.

2.9.5 Quantitative PCRs

Each cDNA was diluted 10-fold with sterile distilled water. Each 15 µl qRT-PCR reaction consisted of 7.5 µl SensiMix SYBR reaction buffer (Bioline), 2 µl template cDNA, 4.9 µl deionized water and 0.3 µl each of both forward and reverse primers. The reactions were assembled in a 96 well PCR plate (Bio-Rad, Hemel Hempstead, Herts, UK). The plates were sealed with sealing film (Bio-Rad) and pulse-centrifuged at low speed to collect reactions in the bottom of each well. Reactions were run in duplicates on a Bio-Rad C1000 thermal cycler connected to a CFX96 Real-Time PCR Detection System and a PC running CFX manager software (Bio-Rad). Initial denaturation was conducted at 95 ºC for 2 minutes followed by 44 cycles of 20 seconds at 95 ºC, 30 seconds at 57 ºC and 20 seconds at 72 ºC. Fluorescence was recorded at the end of each cycle.

The raw data in a .csv file format was exported from CFX manager to LinRegPCR software where the baselines, threshold cycle numbers and amplicon amplification efficiency were computed. Mean fold change expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and

Schmittgen, 2001; Ramakers et al., 2003; Yuan et al., 2008). Pepper actin (LOC107840006) and Arabidopsis glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as reference genes in their respective experiments. Both reference genes are thought to be stably expressed under most standard experimental conditions (Kozera and Rapacz, 2013).

2.9.6 Agarose Gel Electrophoresis and Gel Photography

Depending on the experiment, an aliquot of 4 – 8 µl of each PCR reaction was run on a 1.0% (w/v) agarose gel prepared with 1x TAE buffer [0.04 M Tris, 0.001 M EDTA, 0.1142 (v/v) glacial acetic acid)]. The gel was mixed with 1 µl/ml ethidium bromide, allowed to solidify and later submerged in TAE buffer in an MHU-1010 gel rig (Flowgen) and electrophoresed at 60V to 120V using Power Pac 3000 (Bio-Rad). Adjacent lanes were loaded with appropriate size DNA markers from Bioline. The ethidium bromide labelled bands were visualized with a UV transilluminator and the images were captured with a thermal printer.

2.9.7 Extraction of Amplified DNA Fragments from Agarose Gels

Extraction of DNA fragments from agarose gel was performed using the QIAquick Gel Extraction Kit (Qiagen, Crawley, West Sussex, UK). With the aid of a UV transilluminator, the fragment of amplified DNA to be purified was excised from the TAE agarose gel using a scalpel blade and placed into a microfuge tube. Each gel was added with 3 parts buffer QC in relation to the weight of the excised gel in the microfuge. The excised gel was incubated at 50 ^{o}C in a water bath until dissolved. The weight of the gel in isopropanol was added to the dissolved gel, vortexed thoroughly and pipetted into a spin column already inserted into a collection tube. The column was centrifuged for 1 minute at 13,000 rpm (Heraeus Biofuge pico centrifuge) and the collected flow-through was discarded. The column was inserted back into the collection tube and 500 µl of buffer QG was added onto the membrane of the column. The column was centrifuged at 13,000 rpm for 1 minute to discard the flow-through. Approximately 750 µl volume of buffer PE was pipetted onto the column and allowed to stand for approximately 2 - 5 minutes after which the column was centrifuged at 13,000 rpm for a minute to wash the membrane-bound DNA in the column. The flow-through was discarded and the column was spun again to remove any residual wash buffer from the column. The column was then removed from the collection tube and transferred into a clean 1.5 ml microfuge tube. Approximately, 50 µl buffer EB (10mM Tris.Cl, pH 8.5) was added

onto the membrane in the column and allowed to stand at room temperature for approximately 2-4 minutes. Afterwards, the column as centrifuged at 13,000 rpm (Heraeus Biofuge pico centrifuge) for 1 minute to elute the purified DNA into the microfuge tube.

2.9.8 Purification of PCR Products

Purification of PCR product was carried out using Monarch PCR and DNA Cleanup Kit (New England Biolabs, UK). A 5 µl PCR reaction to be cleaned up was pipetted into a clean 1.5 ml microfuge tube and diluted with 3 parts TE buffer. The 20 µl diluted PCR reaction was then mixed with 40 µl DNA binding buffer and flipped gently. The sample was then loaded onto the matrix of a clean column inserted into a collection tube and centrifuged at 13,000 rpm for 1 minute. After the centrifugation, the column was removed and the flow-through was discarded. The column was re-inserted into the collection tube and a 200 µl of DNA wash buffer was added onto the matrix of the column. The column was centrifuged again at 13,000 rpm (Heraeus Biofuge pico centrifuge) for 1 minute and the flow-through was discarded. The DNA washing up step was repeated twice before the column was removed and inserted into a clean 1.5 ml microfuge tube. Approximately 6 - 8 µl of the DNA elution buffer was carefully pipetted onto the matrix of the column and allowed to stand at room temperature for a minimum of 1 minute. Afterwards, the column was centrifuged at 13,000 rpm (Heraeus Biofuge pico centrifuge) at for 1 minute to collect the purified DNA products. The concentration of the purified DNA was checked with the aid of a Nanodrop spectrophotometer as previously described.

2.9.9 DNA Sequencing

Purified amplified DNA at a concentration of 1 ng/µl per 100 bp were sent to Source BioScience UK Ltd (Cambridge, UK) together with aliquot of the appropriate primers listed in Appendix I at a concentration of 3.2 pmol/µl for automated Sanger sequencing (Smith et al., 1986). The returned sequencing data were checked using the Basic Local Alignment Search Tool (BLAST) to confirm that the amplified DNA fragment was the gene of interest (Altschul et al., 1990).

2.10 Allele-Specific PCR Primer Design

Allele-specific PCR primers which limit the amplification of one SNP DNA fragment over the alternative allele were designed using the WebSNAPER Allele-Specific PCR Primer design software tool (https://pga.mgh.harvard.edu/cgi-bin/snap3/websnaper3.cgi). After entering the DNA sequences of both the WT DNA fragment and the SNP mutant DNA fragment to be amplified, the software designs the primers by introducing artificial nucleotide mismatches in the 3' region of the primer based on the specific nucleotide differences between the SNP and the WT DNA sequences such that only one allele anneals perfectly to the 3'-5' primer thereby ensuring its efficient amplification over the alternative allele which only anneals partially to the 3'-5' primer (Drenkard et al., 2000). After 30 PCR cycles, both *ago1.25* and *ics1* SNP mutant plants can be distinguished from a WT plant based on the intensities of their band on a TAE agarose gel.

2.11 Aphid Experiments

2.11.1 *Myzus persicae* Stock

Virus-free clones of *Myzus persicae* were maintained on Chinese cabbage plants which were individually contained in microperforated plastic bags (Associated Packaging Ltd, Tonbridge, Kent, UK) and maintained inside a bench top fabric insect cage (Insect Cage Net, Carmarthen, Dyfed, UK) at the plant growth facility near the Cambridge University Garden, under long day conditions. After approximately every 2-4 weeks aphids are transferred onto fresh Chinese cabbage plants.

2.11.2 Aphid Mean Relative Growth Rate Assay

A cohort of wingless adult aphids were transferred onto healthy Chinese cabbage plants a day prior to the aphid mean relative growth experiment to produce 1-day old nymphs on the day of the experiment. A single 1-day old nymph was weighed on a microbalance (Mettler Toledo MX5, Columbus, OH, USA) and placed on the Arabidopsis rosette which was then contained in a microperforated bag. Aphid infested plants were kept in a rectangular insect cage (Insect Cage Net, Carmarthen, Dyfed, UK) at 21 ºC under long day conditions with a 16-hour photoperiod. At 6 days post-infestation, aphids were removed from the rosette and

weighed again to record the weight gained by the aphid. The relative growth rate of each aphid is then calculated using the following equation;

$$MRGR = \frac{\log_e W \text{ final} - \log_e W \text{ initial}}{\text{Time } (t)}$$

Where t= time in days between the initial and the final aphid measurements of the aphid fresh weight (W) (Leather and Dixon, 1984).

2.11.3 Aphid Colony Size Assay

After taking the 6th day aphid weight readings the aphids were placed back on their respective plants and allowed to remain for an additional 6 days to reproduce. At the end of the 12th day, the nymphs produced by each founding aphid were counted.

2.11.4 Aphid-induced loss of Plant Biomass Assay

One day old aphid nymphs were obtained as previously described and transferred onto fresh Chinese cabbage stock plant for 6 days. On the 6th day, a group of four 6-day old aphids were transferred from the Chinese cabbage plant onto each Arabidopsis rosette. The Arabidopsis plants were contained in a micro-perforated bag and kept under long day conditions for a period of 12 days for the aphids to produce colony. Un-infested plants were also included for each treatment where the plants undergo the same procedures but without aphid infestation. On the 12th day, the aphids were brushed off the Arabidopsis plants and the above-ground tissues of each plant was collected, put in a paper bag, labelled and then placed in an oven set at 55 ºC for a minimum of 4 days to dry. The dry weights of each of the un-infested and aphid infested plants were then recorded in grams using an A and D balance (A and D Instruments Limited, Abingdon, UK). The means of each treatment were then calculated for comparison and also for calculation of per cent loss in biomass due to aphid infestation. Per cent losses in plant biomass as a result of the aphid infestation were calculated as follows:

$$\text{Loss of biomass (\%)} = \left[\frac{\text{Average weight of un-infested plants} - \text{Average weight of infested plants}}{\text{Average weight of un-infested plants}} \right] \times 100$$

2.12 Statistical Analysis

All statistical analyses were performed using R version 3.4.0 (The R Foundation for Statistical Computing, Vienna, Austria). Significant differences between treatment means were tested using one-way Analysis of Variance (ANOVA). Tukey HSD test was then performed on datasets whose F-ratio associated probability values (p-values) were lesser than 0.05. Error bars on bar plots represent standard error of the treatment mean (SEM). Results from all the statistical tests and the R-codes used for the analysis are presented in appendices II-V.

Chapter 3

4-OH-ICN does not play significant role in the heightened resistance to aphids observed in *ago1.25* mutant plants

---

Introduction

3.1 Background to the study

Previous experiments showed that Arabidopsis *ago1.25* mutant responds to aphid infestation with heightened defence (Kettles et al., 2013; Westwood et al., 2013). Aphids reared on *ago1.25* mutant plants show reduced growth rate and colony size. Moreover, transferring aphids that have once fed on *ago1.25* mutant plants for a minimum of three days onto healthy WT plants does not ensue recovery of normal growth of the transferred aphids. It was therefore hypothesized that aphids on *ago1.25* mutant plants may be exposed to toxic metabolite(s) that permanently harms the physiology of the aphids (Westwood et al., 2013). The focus of this research therefore was to test whether the newly discovered Arabidopsis cyanogenic metabolite 4-OH-ICN plays a role in the heightened defence to aphids on *ago1.25* hypomorphic mutant plants.

Objective

The objective of these experiments was to assess the potential role of 4-OH-ICN in the heightened defence to aphids in Arabidopsis *ago1.25* mutant plants. *ago1/cyp82C2* and *fox1/ago1* double mutant plants were generated to assess aphid performance (aphid growth rate and colony size) on these double mutants.

3.3 Materials and Methods

3.3.1 Source of 4-OH-ICN Biosynthetic Gene Mutant Plants

Seeds for all four 4-OH-ICN biosynthetic pathway mutant lines were obtained from the Nottingham Arabidopsis Stock Centre. Further details on the specific mutant plants can be found in Section 2.2.2.

3.3.2 Designing of Primers for Genotyping 4-OH-ICN Biosynthetic Pathway Mutant Plants

Primers were designed using the NCBI Primer Blast primer design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). These primers amplified the plant's DNA flanking the T-DNA insertion. Figure 3.1 shows the proximate locations of the T-DNA insertions in each of the four 4-OH-ICN biosynthetic genes.

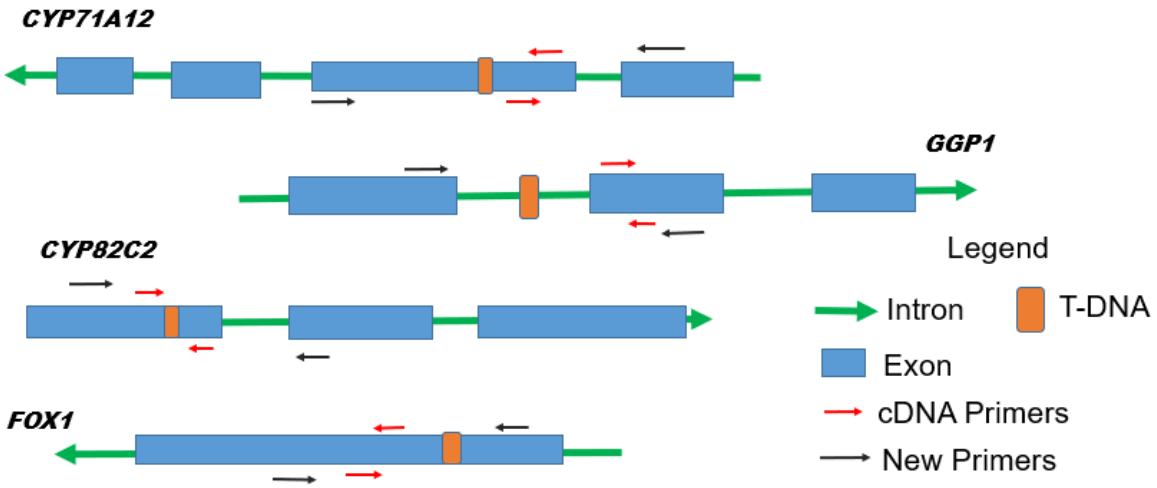


Figure 3.1: Proximate locations of T-DNA insertions in each of the four 4-OH-ICN biosynthetic genes

At least two different sets of primers were designed for each mutant plant. Primers that were used for RT-PCRs (Figure 3.1, red arrows) were designed to amplify a smaller DNA fragment compared to the primers designed for the genomic DNA based PCRs (Figure 3.1 black arrows). A T-DNA specific primer was also obtained to amplify the left border region of the T-DNA insertion.

The initial rounds of PCR for genotyping the plants generated from seeds obtained from the NASC were based on extracted genomic DNA from each of the sampled plants. A PCR for a selected plant included both the plant's gene-specific forward and reverse primers and the T-DNA-specific left border primer. In this way heterozygous mutant plants displayed two PCR product bands on an agarose gel; both the amplified WT gene fragment and the T-DNA fragment (Figure 3.2A). Homozygous mutant plants and recombinant WT plants showed a single band representing the amplified T-DNA fragment or the amplified WT gene fragment respectively. The absence of the WT gene in the homozygous mutant plants were again confirmed in a PCR that used only the WT gene primers (Figure 3.2B).

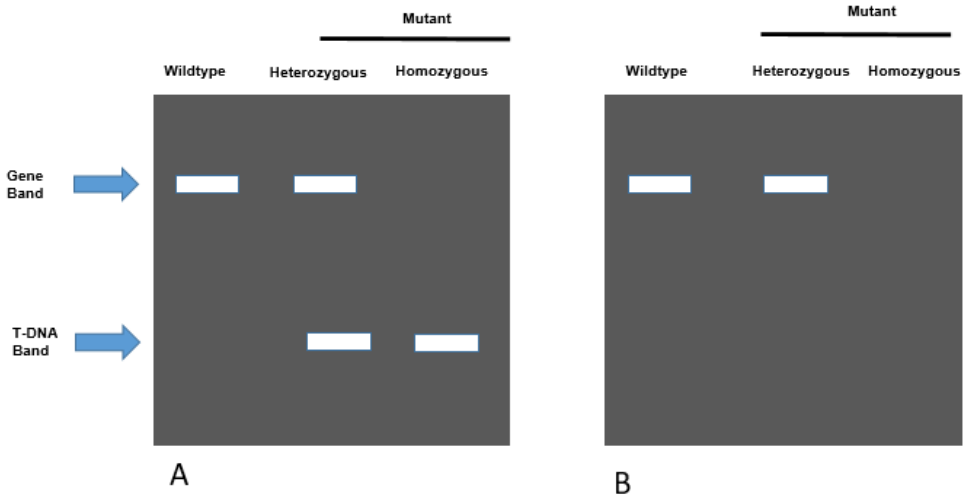


Figure 3.2: Expected bands for homozygous mutants, heterozygous mutants and WT transformants when (A) 3 primers or (B) 2 primers are used in a PCR reaction.

3.3.3 Generation of *ago1/cyp82C2* and *fox1/ago1* Double Mutant Plants

Detailed protocols for the *ago1* x *cyp82C2* cross and *fox1* x *ago1* cross are outlined in chapter 2. The resulting F_1 seeds were plated on MS agar without the addition of sulfadiazine. The seedlings were later pricked out into compost. At the onset of bolting each of the F_1 plants were contained in an Aracon tube and allowed to flower, self-pollinate and form siliques in the Aracon tubes.

Dried F_2 seeds were later collected and plated on a MS agar with sulfadiazine (Figure 3.3). Mutations in the *AGO1* gene and the specific 4-OH-ICN biosynthetic gene in the identified

double mutants were further confirmed by PCR and sequencing. Figure 3.4 gives a pictorial summary of how the double mutants were generated.

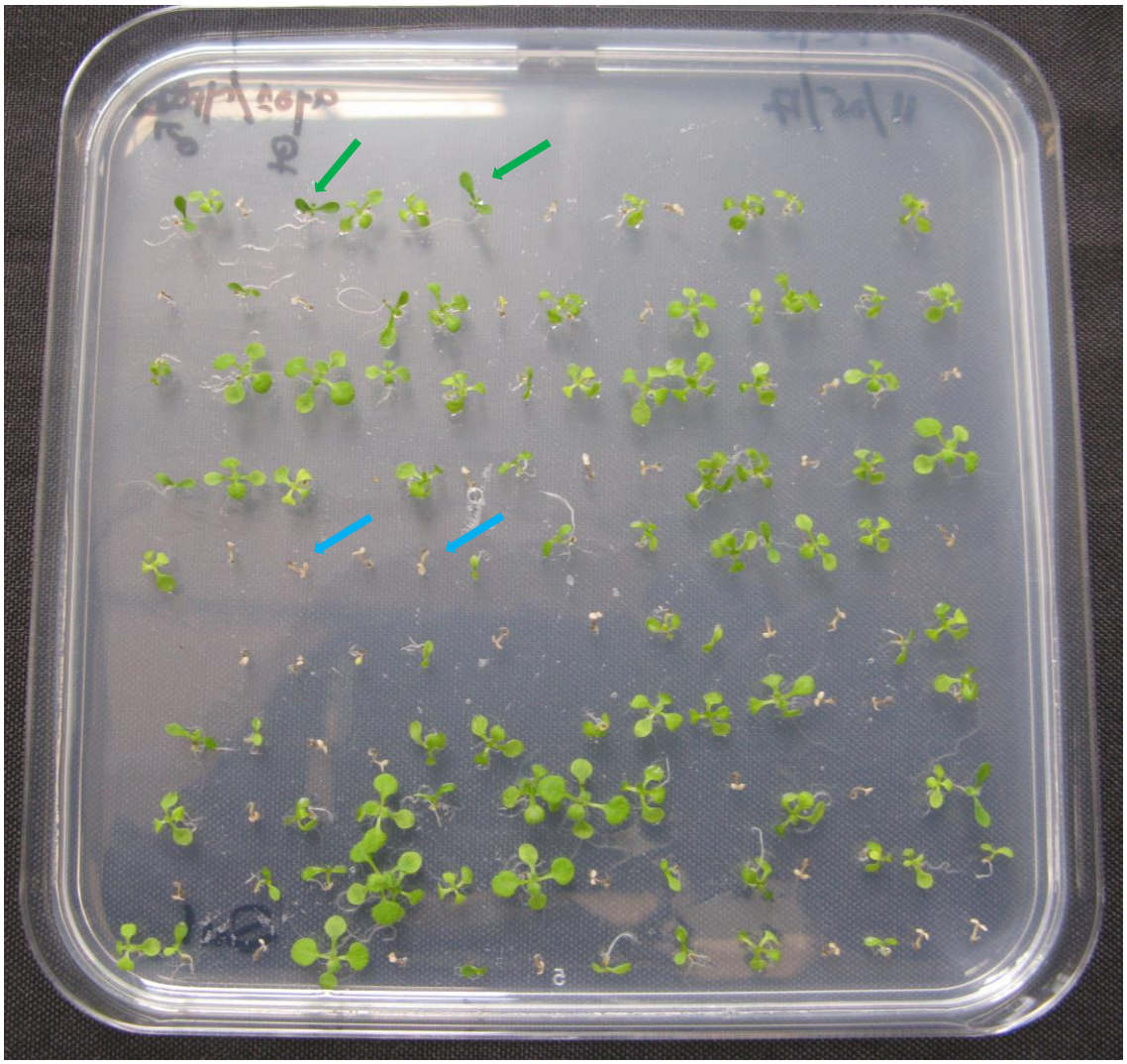


Figure 3.3 F_2 Seedling from *ago1/cyp82C2* cross growing on an MS-agar containing sulfadiazine. The segregants without the T-DNA insertion bleached out and died (light blue arrows). The *ago1/cyp82C2* double mutant plants were among the *ago1*-looking segregants that survived the sulfadiazine treatment (green arrows).

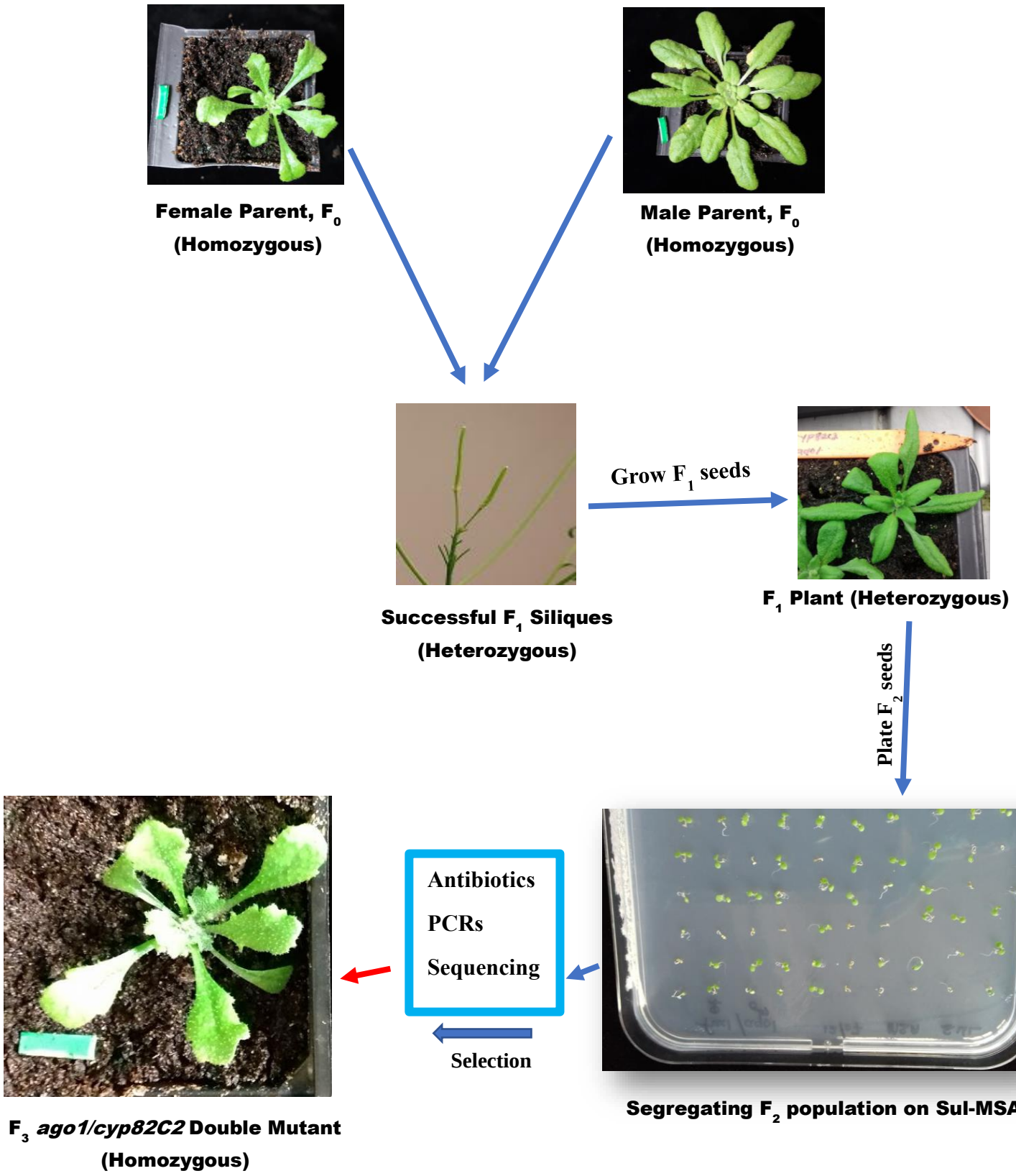


Figure: 3.4 Steps involved in the generation of the *ago1/cyp82C2* and *fox1/ago1* double mutant plants.

3.4 Results

3.4.1 Identification of 4-OH-ICN Pathway Homozygous Mutants

Segregating *cyp82C2*, *fox1*, *ggp1* and *cyp71A12* T-DNA insertional mutant seeds were germinated to generate plant tissues for genomic DNA extraction. Two sets of PCRs were performed on the extracted genomic DNA from each plant. First a PCR that included a primer to amplify the T-DNA together with primers that amplified the specified plant gene were performed (Figure 3.5A). Later, an additional PCR was performed using only primers specific to the gene of interest (Figure 3.5B). After the sets of PCRs, the majority of the genotyped plants were homozygous for the T-DNA insertion (such as plants 2, 5, 6, 10 and 11 in Figure 3.5). Table 3.1 lists the genotypes of the various mutants that were analysed.

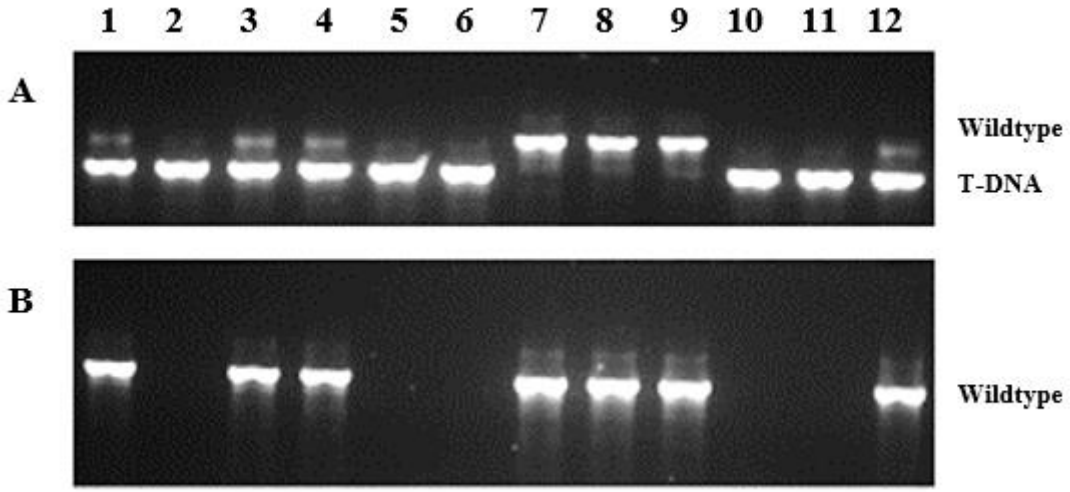


Figure 3.5: Amplified genomic DNA from segregating *fox1* mutant plants when (A) a T-DNA specific primer is used together with plants' gene specific primers. (B) when only the plant's gene specific primers are used in a reaction. (Product size for *Fox1* = 1,509 bp).

Table 3.1: Number of homozygous mutant plants identified for each of the 4-OH-ICN pathway genes.

Gene Mutated	Homozygous Mutants	Heterozygous Mutants	WT Recombinant	Total Number of Plants Tested
CYP82C2	65	3	6	79
FOX1	15	7	11	34
GGP1	35	5	11	52
CYP71A12	52	2	7	63

Gene-specific and T-DNA specific primers were designed for identifying the genotypes of the plants generated from the seeds obtained from NASC.

To test the nature of the identified 4-OH-ICN homozygous mutant plants, RT-PCR were performed using cDNA synthesized from total RNA extracted from a sample of plants from each of the four mutant plants. There were no expression products for both *fox1* and *cyp82C2* mutant plants, however, RT-PCR products were observed for *cyp71A12* and *ggp1* mutant plants (Figure3.6). The RT-PCR products from the *ggp1* and the *cyp71A12* mutant plants were purified and sequenced. The sequenced products of *ggp1* and *cyp71A12* which corresponded to the nucleotides flanking the T-DNA insertion in each of the two mutants were identical to their WT gene copies.

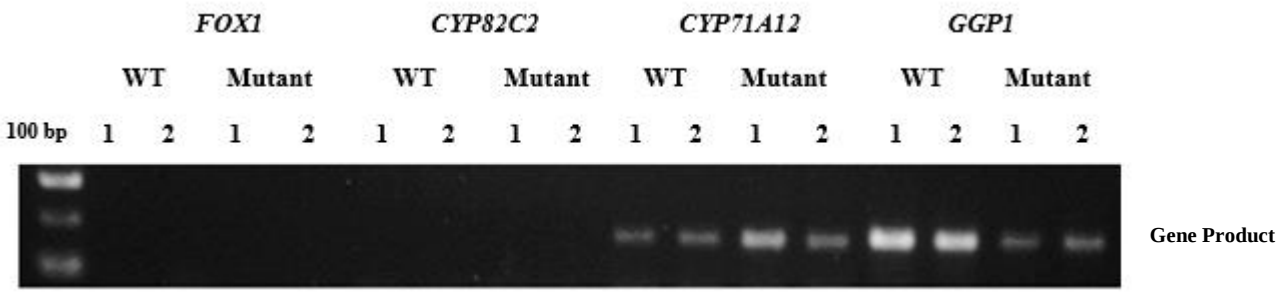


Figure 3.6: Constitutive expression of 4-OH-ICN biosynthetic genes in Arabidopsis. Both *Fox1* and *Cyp82C2* genes are not constitutively expressed. *Cyp71A12* and *GGP1* are constitutively expressed. Both of the identified *cyp71A12* and *ggp1* mutant plants with homozygous T-DNA insertions were not knock-out mutants (RT-PCR product sizes; *GGP1* 194 bp; *CYP71A12* 186 bp).

To verify whether the *cyp82C2* and *fox1* mutant plants were knock-out mutants or their expression is inducible, 21-day old mutant plants were infiltrated with 2.0 x 10^8 cfu/ml *Pseudomonas syringae DC3000 avrB* which is known to induce expression of both *Fox1* and *Cyp82C2* (Rajniak et al., 2015). Whilst there was no expression of *cyp82C2* in the *P. syringae* infiltrated *cyp82C2* mutant plants (Figure 3.7), *Fox1* expression was observed in both the mock-inoculated ($MgCl_2$-infiltrated) and *P. syringae* infiltrated *fox1* mutant plants (Figure 3.7).

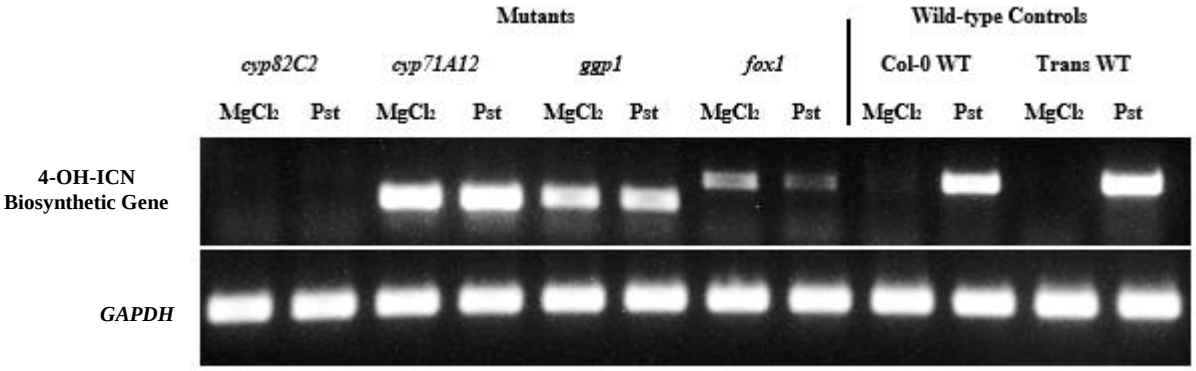
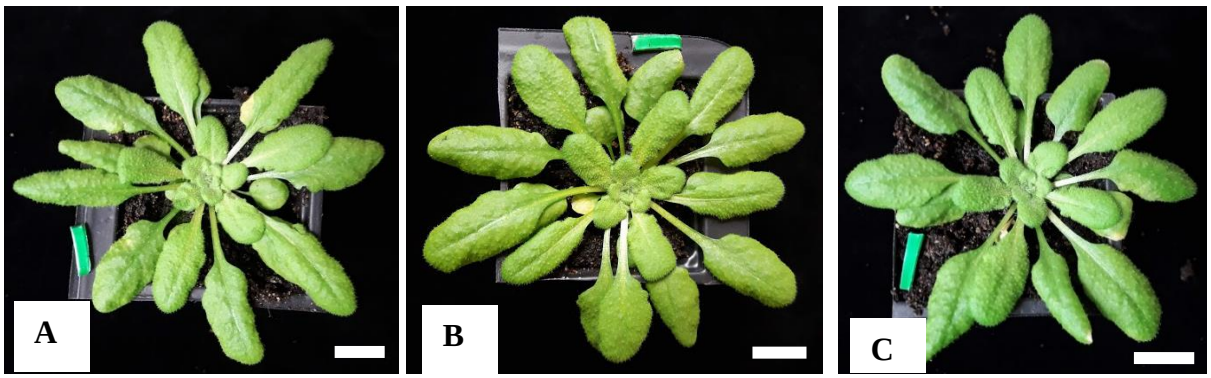


Figure 3.7: *P. syringae* induced expression of 4-OH-ICN biosynthetic genes. There is no expression of *Cyp82C2* in the *cyp82C2* mutant. Mock-inoculated plants were infiltrated with 10 mM $MgCl_2$ (infiltration buffer). Samples from plants infiltrated with *P. syringae* are labelled as Pst. The WT controls show amplified *CYP82C2* product. A reconstituted WT

GABI Kat plant (Trans WT) was included in the experiments. RT-PCR Product sizes: *Fox1* 346 bp; *GGP1* 194bp; *CYP71A12* 186 bp; CYP82C2 393bp.

The *cyp82C2* knockout mutant (Figure 3.8A) was the primary 4-OH-ICN biosynthetic pathway mutant used for the aphid experiments and also for the generation of the *ago1/cyp82C2* double mutant. The Cyp82C2 enzyme is the most important of all the enzymes that catalyze the biosynthesis of 4-OH-ICN in Arabidopsis. In the *cyp82C2* mutant plant there is no production of 4-0H-ICN. Interestingly, in the *cyp82C2* mutant, *cyp82C3,* a non-constitutively expressed cytochrome P450 encoding gene which is located next to *Cyp82C2* on Arabidopsis chromosome four becomes constitutive. The *fox1* mutant (Figure 3.8B) was also included in the generation of *fox1/ago1* double mutants and the aphid experiments as an additional mutant. The *cyp82C2* mutant plants and *fox1* mutant plants are not phenotypically distinct from a WT plant (Figure 3.8C).



cyp82C2 mutant *fox1* mutant Col-0 WT

Figure 3.8: *cyp82C2* and *fox1* mutant plants appear identical in developmental phenotype to a WT plant (Scale bar = 1cm).

3.4.2 *Cyp82C2* is induced by aphid infestation.

The first step in assessing whether 4-0H-ICN played a role in aphid defence was to check if aphid infestation induces *Cyp82C2* expression. Aphids were placed on plants with WT *Cyp82C2* gene to monitor the expression of *Cyp82C2*. Aphid infestation induced *Cyp82C2* expression (Figure 3.9). There was no *Cyp82C2* expression in non-infested plants nor aphid infested *cyp82C2* mutant plants.

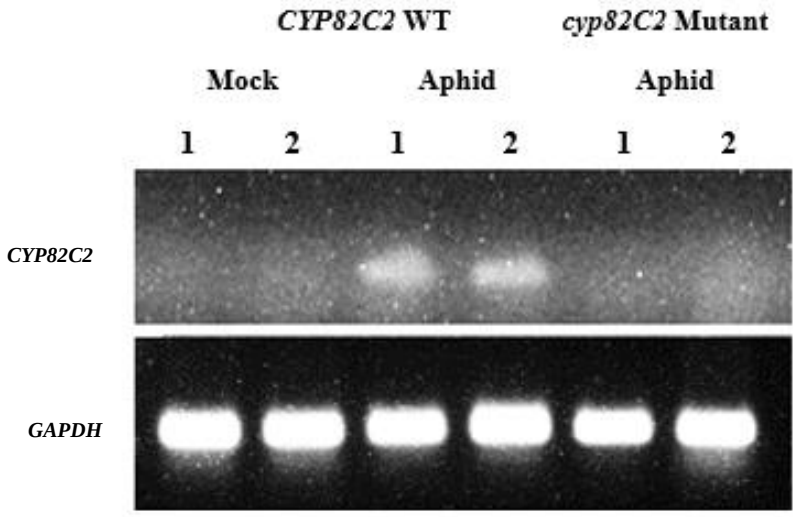


Figure 3.9: Induction of *CYP82C2* expression by aphids. In both non-infested plants and aphid infested *cyp82C2* mutant plants there is no expression of *CYP82C2* gene. (*Cyp82C2* product size: 393bp).

3.4.3 Mutation in *Cyp82C2* or *Fox1* does not affect aphid growth rate in Arabidopsis

Aphid growth rate experiments were performed on both *cyp82C2* mutant and *fox1* mutant plants. However, there was no significant differences in the growth rates of aphids placed on *cyp82C2* or *fox1* mutant plants in comparison to a WT plant (Figure 3.10). It was not clear whether the constitutive expression of *Cyp82C3* in *cyp82C2* mutant plants which is also aphid inducible was compensating for the loss of the *Cyp82C2* gene.

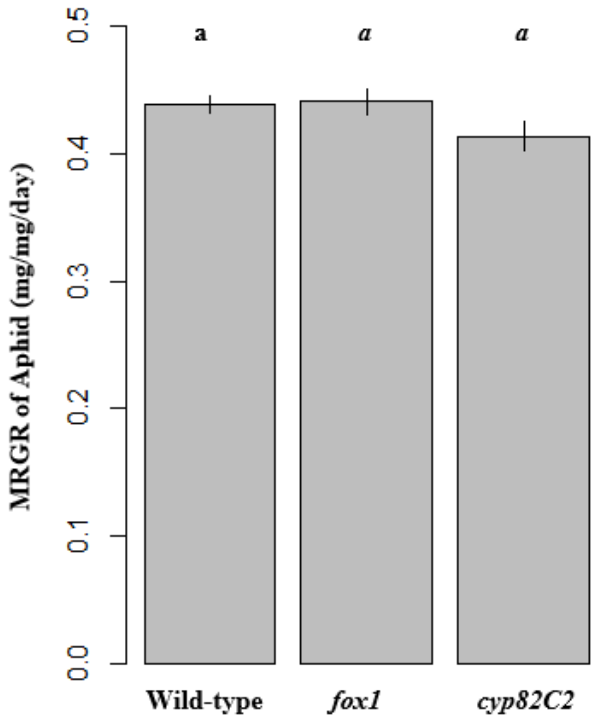


Figure 3.10: Mean relative growth rates (MRGR) of aphids on 4-0H-ICN biosynthetic pathway mutant plants. Mutation in either *Fox1* or *Cyp82C2* has no significant effect on aphid MRGR at $p < 0.05$ level. (ANOVA p-value = 0.112) Sample size (n): WT (11); *cyp82C2* mutant (12); *fox1* mutant (14). Error bars represent standard error of the mean. Treatment means (bar plots) denoted by the same letter are not significantly different from each other at $p < 0.05$.

3.4.4 Mutation in *Cyp82C2* or *Fox1* genes in the *ago1.25* mutant background does not restore aphid susceptibility to WT levels

Aphid growth rates and colony sizes were assessed on *ago1/cyp82C2* and *fox1/ago1* double mutant plants. However, aphid performance on the *ago1/cyp82C2* (Figure 3.11A) and the *fox1/ ago1* (Figure 3.11B) double mutant plants did not differ significantly from aphid performance on the *ago1.25* mutant plants. Therefore, 4-OH-ICN does not play a significant role in aphid defence in Arabidopsis since mutation in either *Cyp82C2* or *Fox1* does not restore susceptibility to aphid infestation in the *ago1.25* mutant plants. On the contrary, aphid colony sizes tend to be slightly enhanced in *ago1/cyp82C2* and *fox1/ago1* double mutant plants although the differences were not statistically significant at $p < 0.05$ level (Figure 3.12).

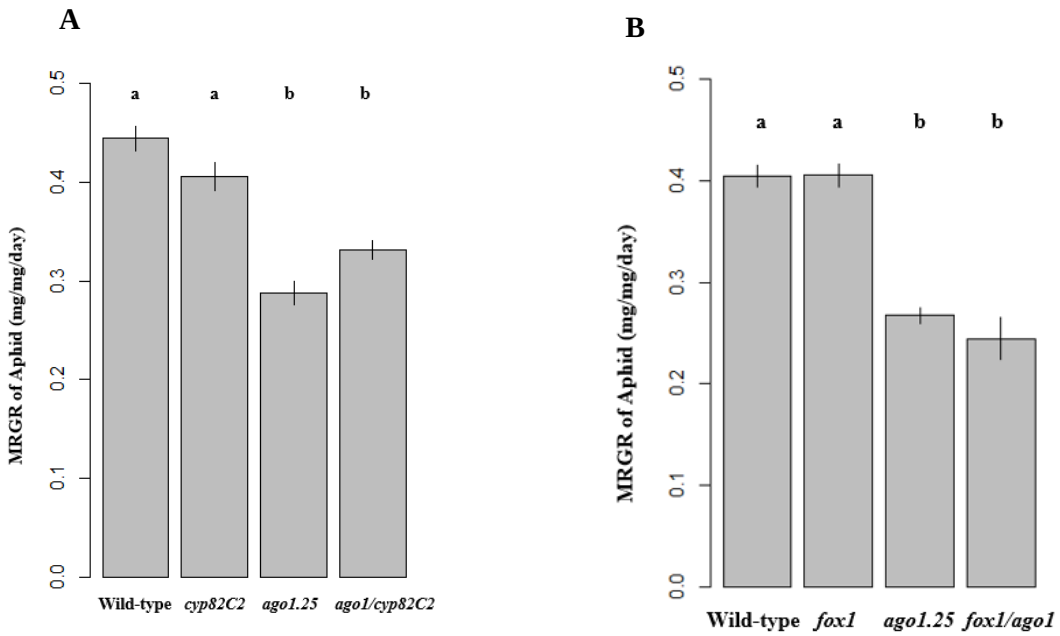


Figure 3.11: Mean relative growth rate (MRGR) of aphids on; A) *ago1/cyp82C2* double mutant plants. Sample sizes (n): WT (14); *cyp82C2* (14); *ago1.25* (12); *ago1/cyp82C2* (13) (ANOVA: 4.53 x 10 ^-12) and B) MRGR of aphids on *fox1/ago1* double mutant plants. Sample size (n): WT (12); *fox1* (13); *ago1.25* (14); *fox1/ago1* (7) (ANOVA: = 1.95 x 10^-14). Mutations in 4-OH-ICN biosynthetic genes *Cyp82C2* or *Fox1* in *ago1.25* mutant plants do not affect aphid growth rates. Error bars represent standard error of the mean. Treatment means (bar plots) denoted by the same letter are not significantly different from each other at $p < 0.05$.

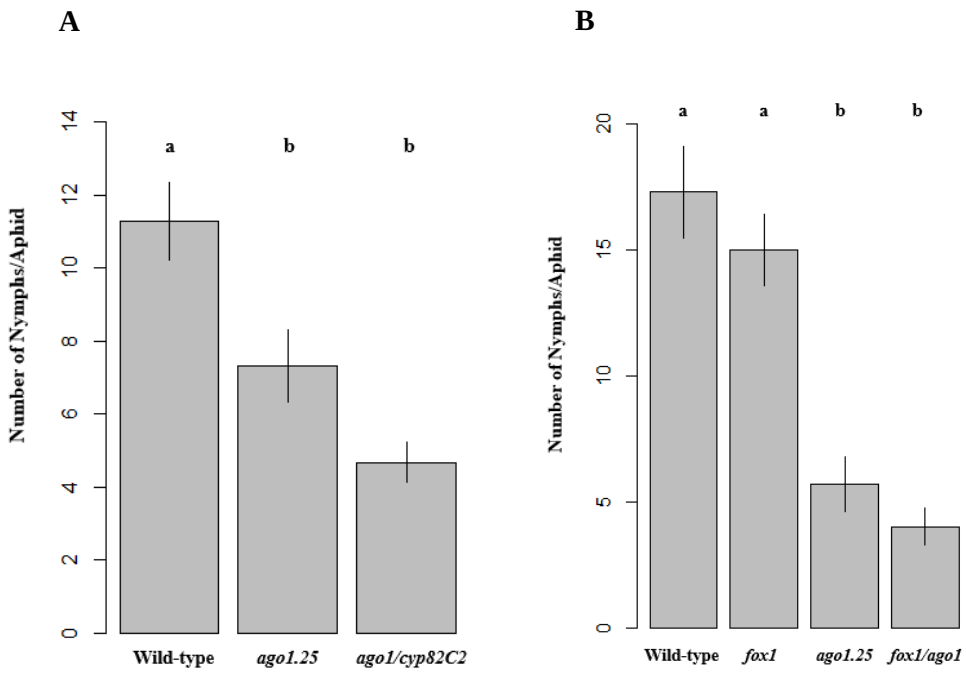


Figure 3.12: Aphid colony size on; A) *ago1/cyp82C2* and B) *fox1/ago1* double mutant plants. Aphids on *ago1/cyp82C2* or *fox1/ago1* double mutant plants do not have significantly different colony sizes from aphids on *ago1.25* mutant plants at $p < 0.05$ level. Error bars represent standard error of the mean. Treatment means (bar plots) denoted by the same letter are not significantly different from each other at $p < 0.05$.

3.4.5 Mutation in *Cyp82C3* does not affect aphid performance

Due to the constitutive expression of *cyp82C3* in *cyp82C2* mutant plants (Figure 3.13), the induction of *Cyp82C3* expression by aphid infestation and the realization that mutation in the *Cyp82C2* gene does not significantly affect aphid growth, *cyp82C3* mutant was obtained for aphid experiments to verify if *Cyp82C3* contributed to aphid defence in Arabidopsis. However, mutation in the *Cyp82C3* gene did not affect aphid growth rate. Growth rate of aphids on the *cyp82C3* mutant plants were similar to aphid growth rates on *cyp82C2* mutant plants and WT plants (Figure 3.14).

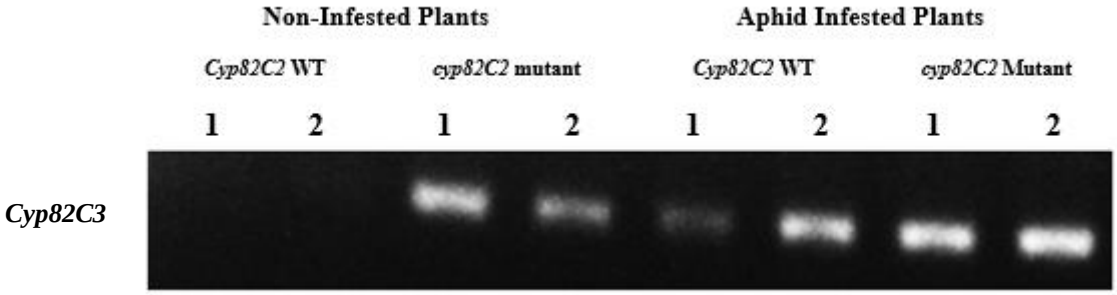


Figure 3.13. Constitutive expression of *Cyp82C3* in *cyp82C2* mutant plants. Infestation by aphids induces additional *Cyp82C3* expression in the *cyp82C2* mutant plants. (*Cyp82C3* product size: 305 bp).

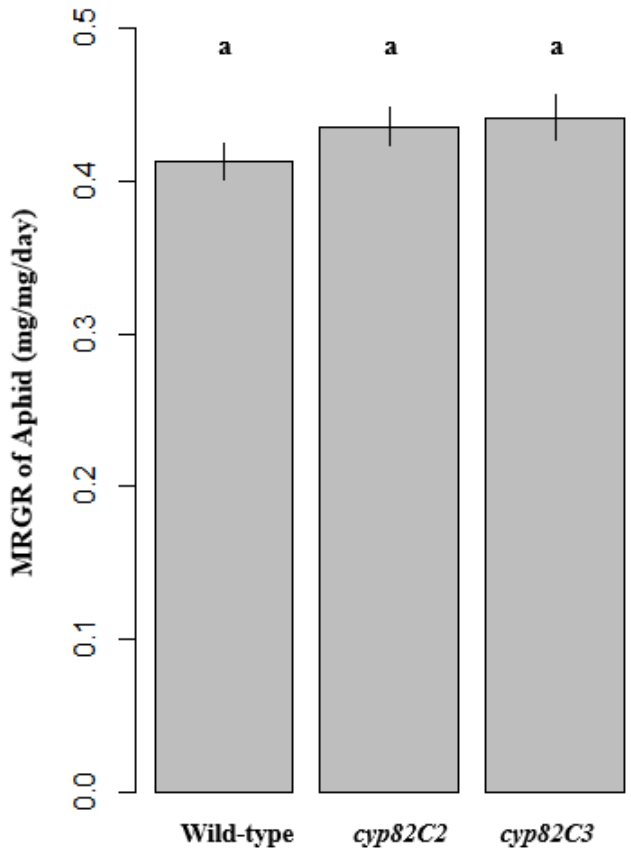


Figure 3.14: Mean relative growth rate (MRGR) of Aphids on *cyp82C3* mutant plants.

MRGR of aphids on *cyp82C3* mutant plants is comparable to MRGR of aphids on a WT plant or a *cyp82C2* mutant plant. (ANOVA p value = 0.268. Sample size (n); WT (13); *cyp82C2* (10); *cyp82C3* (14)). Error bars represent standard error of the mean. Treatment means (bar plots) denoted by the same letter are not significantly different from each other at $p < 0.05$.

3.5 Discussion

The ability to produce cyanogenic secondary metabolites is ancient and widespread among plant species (Chapter 1, subsection 1.7.1). Many species of plants including ferns produce and store cyanogenic glucosides in their vacuoles separate from the glucosidases and nitrile lyases required for their activation (Zagrobelny et al., 2004). The release of cyanide only occurs after significant tissue disruption that brings the two separated components together. Thus, cyanogenic glucosides may not be particularly effective against insect pests such as aphids which cause minimal tissue damage upon feeding and also feed almost exclusively from the phloem tissues (Zagrobelny et al., 2004; Pentzold et al., 2014).

Prior to 2015, Arabidopsis was not known to be cyanogenic therefore the biosynthetic pathway of the cyanogenic glucoside dhurrin from *Sorghum bicolor* was transgenically transferred from sorghum into Arabidopsis and was found to be particularly effective against the crucifer-specialist flea beetle *Phyllotreta nemorum* (Tatersall et al., 2001). When Arabidopsis was later found to be naturally cyanogenic and it was realised that 4-OH-ICN produced by Arabidopsis does not require activation by glucosidases I sought to investigate its effectiveness against the generalist *Myzus persicae* aphids particularly in *ago1.25* background.

However, knockout of genes required for 4-OH-ICN biosynthesis did not significantly affect aphid performance in the 4-OH-ICN biosynthetic mutant itself or in the *ago1.25* mutant plant background. Aphid growth rate and reproduction on the 4-OH-ICN biosynthetic pathway mutants were comparable to that of WT plants. On the contrary, aphids tended to reproduce less (although not to a statistically significant extent) on the *ago1/cyp82C2* and the *fox1/ago1* double mutant plants. Since aphids tend to feed much longer on *ago1.25* mutant plants but are not able to gain weight or reproduce as much as aphids on WT plants, it is possible that 4-OH-ICN is potentially serving as a phagostimulant to encourage feeding by aphids. In this case the absence of the 4-OH-ICN metabolite will limit phloem ingestion resulting in an even less growth rate and colony size on the *ago1/cyp82C2* and *fox1/ago1* double mutant plants. The role of cyanogenic metabolites serving as feeding stimulants especially among specialist insect herbivores is common. For instance, the larva of the southern armyworm *Spoloptera eridania* feed and grow better if there is cyanogenic compound in its diet (Benett and Wallsgrove, 1994). Other insect herbivores are also able to metabolise cyanogenic compounds into useful nutrients for their growth and development (Pentzold et al., 2014).

Also, the constitutive expression of *Cyp82C3* in the absence of the 4-OH-ICN biosynthetic gene *Cyp82C2* may potentially compensate for the loss of ability of the *cyp82C2* mutant plant to synthesize 4-OH-ICN. *Cyp82C3* which is physically located next to *Cyp82C2* on Arabidopsis chromosome four is co-expressed with the *R*-gene *Resistance methylated gene 1* (*RMG1*). *RMG1* encodes a nucleotide-binding leucine rich repeat (NB-LRR) protein with a Toll/interleukin-1 receptor domain involved in antibacterial defence in Arabidopsis (Yu et al., 2013). Although the level of *RMG1* expression increases after aphid infestation it is not known if it plays a possible role in aphid defence in the *cyp82C2* mutant plant.

3.6 Conclusions

The Arabidopsis cyanogenic metabolite 4-OH-ICN does not play a significant role in the heightened defence against aphids previously observed in Arabidopsis *ago1.25* mutant plants (Kettles et al., 2013; Westwood et al., 2013) and confirmed here. The constitutive expression of *cyp82C3* and also the increased expression of the *Cyp82C3* co-expressed *RMG1* transcripts upon aphid infestation may possibly compensate for the absence of the 4-OH-ICN in the *cyp82C2* mutant plant and the *ago1/cyp82C2* double mutant plants.

Chapter 4

Mutation of the *ICS1* gene in *ago1.25* mutant plants restores aphid susceptibility and decreases the extent of stunting in response to aphid infestation.

4.1 Introduction

SA accumulation and signalling are required for both the establishment of SAR and maintenance of basal resistance (Carr et al., 2010). As a result, plant lines defective in SA biosynthesis or signalling such as *eds1*, *eds5*, *ics1* or *phytoalexin deficient-4* (*pad4*) mutants are more susceptible to pathogen infection (Kunkel and Brooks, 2002). On the contrary, accumulation of SA upon insect infestation is mostly associated with enhanced susceptibility of the host to the infestation. Jasmonic acid and ethylene contribute more to defence against insect herbivory than SA (McConn et al., 1997; Onkokesung et al., 2010; Erb et al., 2012). However, there are instances where accumulation of SA is required for defence response against insect herbivory. For instance, the tomato *Mi-1* R gene that confers resistance to root knot nematodes and potato aphids requires the accumulation of SA to function. Survival rate of aphids on tomato plants transgenically expressing the *nahG* SA degrading bacterial gene is 10 % higher than aphids on WT tomato plants (Li et al., 2006). The increase in the survival of potato aphids on tomato plants that do not accumulate SA indicates that SA accumulation is required for potato aphid defence in tomato plants (Li et al., 2006). Similarly, SA is also required for resistance against *Bermisia tabaci* (whitefly) oviposition in tomato plants (Ueda et al., 2019). Whiteflies prefer laying eggs on healthy tomato plants in comparison to *Tomato mosaic virus*-infected tomato plants. However, whiteflies do not make distinction between a healthy tomato plant and a ToMV-infected tomato plant expressing *nahG*. In addition, whiteflies prefer to lay eggs on a healthy *nahG* tomato plant in comparison to a healthy WT tomato plant. These observations indicate that in tomato, resistance against whiteflies is also dependent on SA (Ueda et al., 2019).

The SA biosynthetic gene *ICS1* is upregulated upon aphid infestation (Morkunas et al., 2011). Therefore, it was worthy to investigate whether SA accumulation played a role in the heightened defence observed in *ago1.25* mutant plants.

4.2 Materials and Methods

Genotyping the *ics1*, *ago1.25* and *ago1.46* Single Nucleotide polymorphisms

Single nucleotide polymorphisms (SNP) constitute the largest set of naturally occurring gene mutations in the Arabidopsis genome. Since alleles of these mutations only differ in a single nucleotide from each other and do not have antibiotic selection markers, distinguishing between the alleles of an SNP can be challenging. However, there are a couple of simple but effective techniques that can accurately differentiate between the alleles of an SNP within a population. Among these are cleaved amplified polymorphism (CAPs), derived CAPs (dCAPs) and allele-specific (AS) PCR (Kwok, 2001). The first two methods rely on the presence of different restriction site(s) at the region of the SNP. After the amplification of the SNP alleles, the PCR products are digested with a restriction enzyme that is capable of cleaving one allele but not the other (Figure 4.1). The identities of the alleles are then determined through gel electrophoresis (Drenkard et al., 2000; Smith et al., 2009). The *ago1.46* mutant was identified using CAPs primers and digestion of *NheI* enzyme restriction site present in the mutant DNA sequence.

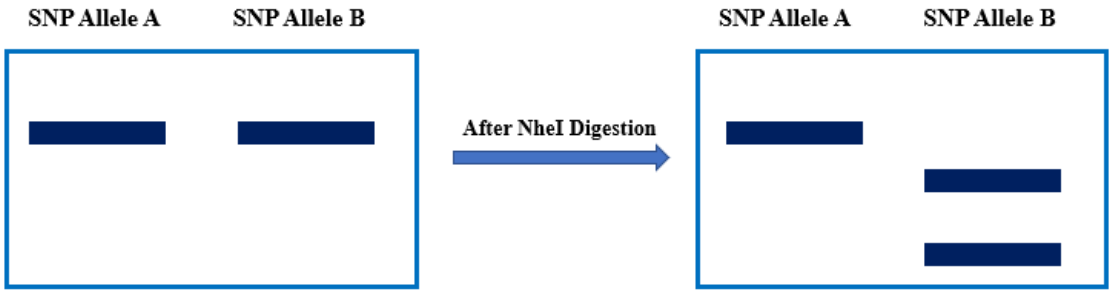


Figure 4.1: Principle of CAPs-based SNP genotyping. Differences in restriction sites at the region of the SNP help distinguish between the different alleles.

For SNP alleles with no or similar restriction sites at their region of the SNP, CAPs-based genotyping is not ideal. AS PCR, however, can effectively distinguish between alleles of an SNP irrespective of the presence of restriction sites using basic PCR procedures. This method is based on the principle that primer-template duplexes with a 3' terminal mismatch amplifies slowly compared with the amplification of a perfectly matched primer-template duplex.

Introduction of a 3' terminal mismatch in the forward primer expressively destabilizes the primer-template complex and significantly affect PCR amplification efficiency (Ugonzzoli and Wallace, 1991; Gaudet et al., 2009). Figure 4.2 shows the principle behind AS-PCR.

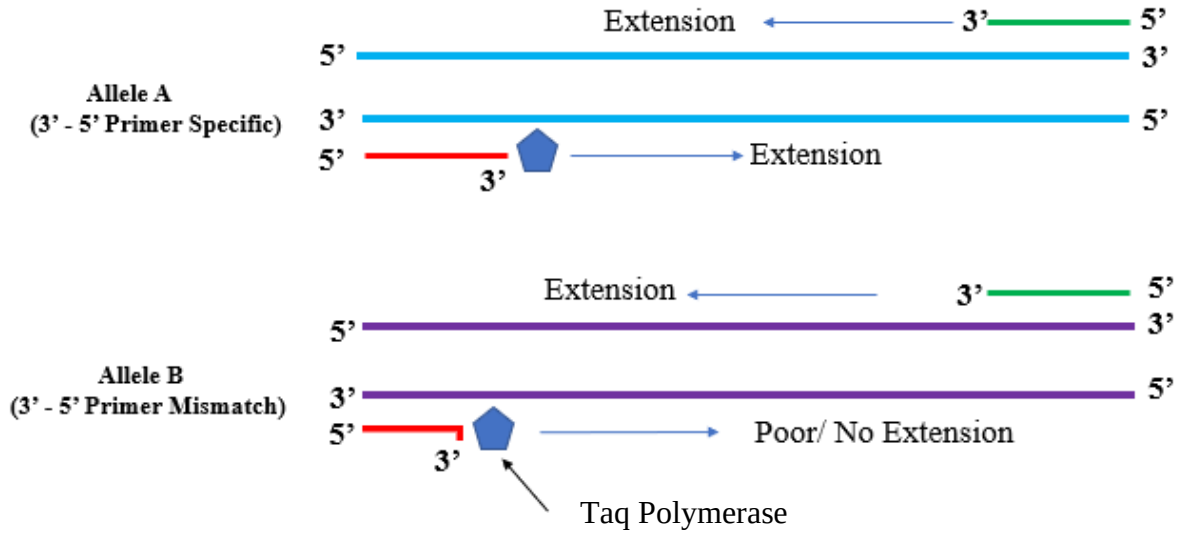


Figure 4.2: Principle of allele-specific PCR. The introduction of mismatches in the forward primer ensures that the primer is allele-specific and therefore the preferential amplification of the primer-specific allele.

The WebSnaper AS PCR primer design tool developed by researchers at Harvard University (https://pga.mgh.harvard.edu/cgi-bin/snap3/websnaper3.cgi) was used in generating allele-specific primers for the *ics1* and the *ago1.25* mutations. Aside from the 3' terminal nucleotide mismatches, the software introduces an additional mismatch within the four nucleotides closest to the 3' termini. The position of the additional nucleotide mismatch is determined based on incorporated empirical data in the WebSnapper software that assesses the effect of a second nucleotide mismatch on the amplification of the alleles and their PCR efficiency. The programme then generates a set of 32 possible allele specific primers together with a common reverse primer that is specific to both alleles of the SNP. The additional nucleotide mismatch in the 3' region of the primer significantly decreases the amplification of the primer non-specific allele with little effect on the amplification efficiency of the primer specific allele.

Therefore, with the appropriate PCR cycles both alleles can be distinguished on an agarose gel based on the intensities of the bands (Figure 4.3) (Drenkard et al., 2000).

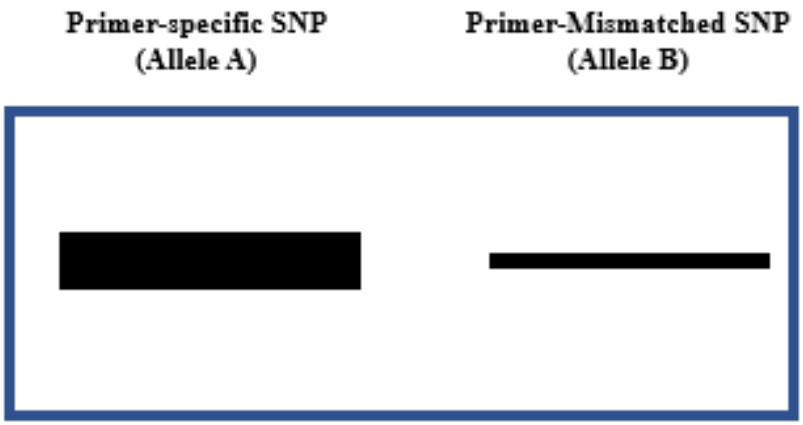


Figure 4.3: Diagrammatic presentation of results from Allele-Specific PCR Analysis. The alleles of an SNP can be distinguished on an ethidium bromide stained agarose gel based on the intensity of the bands due to differences in amplification efficiencies of the SNP alleles.

Primers designed using the WebSnaper programme were used in genotyping *ics1* and *ago1.25* mutations from the WT sequences. These primers which are listed in the Appendix I were also used in genotyping the F_2 population of *ago1/ics1* double mutant plants in addition to sequencing of products amplified by different set of normal primers.

4.3 Results

4.3.1 Decrease in biomass due to aphid infestation is more severe in *ago1.25* mutant plants than in *ics1* mutant plants

Despite the poor performance of aphids on *ago1.25* mutant plants (Kettles et al., 2013; Westwood et al., 2013), aphid infestation of the *ago1.25* mutant still results in severe stunting of the plants (Figure 4.4). A number of the *ago1.25* mutant plants also develop accelerated senescence of their older leaves. This aphid-induced loss of biomass in *ago1.25* mutant plants is more severe than those seen in *ics1* mutant plants, which have a mutation in the salicylic acid biosynthetic gene *ICS1* (Chapter 1, subsection 1.6.1). However, aphid growth rates and colony sizes on *ics1* mutant plants are comparable to that of a WT plant (Figure 4.16). Therefore, the emphasis of these experiments focused on aphid-induced stunting. Interestingly, the large colony size of aphids on the *ics1* mutant plant does not result in the extent of stunting observed in aphid infested *ago1.25* mutant plants which rather has smaller aphid colony sizes (Figure 4.5, Figure 4.16 and Figure 4.17). This observation prompted the idea that accumulation of SA upon aphid infestation could play a role in the greater decrease in growth in the *ago1.25* mutant plants.

4.3.2.1 Identification of the *ago1/ics1* double mutant plant

Since both parents used in the generation of the *ago1/ics1* double mutant plants were SNP mutants without any antibiotic resistance markers, identification of the double mutant was based on phenotypic appearances, allele-specific PCRs and sequencing of PCR products from the progeny to confirm the mutation in the *ICS1* and *Ago1* genes. After the germination of the F_2 seeds from the *ago1.25* x *ics1* cross, it was realized that at least 6 of the segregants had novel phenotypes different from the phenotypes of their parents or a WT plant. They were larger in size and were more vigorous in growth (Figure 4.6). These F_2 segregants with novel phenotypes were pricked out and grown under long day conditions.

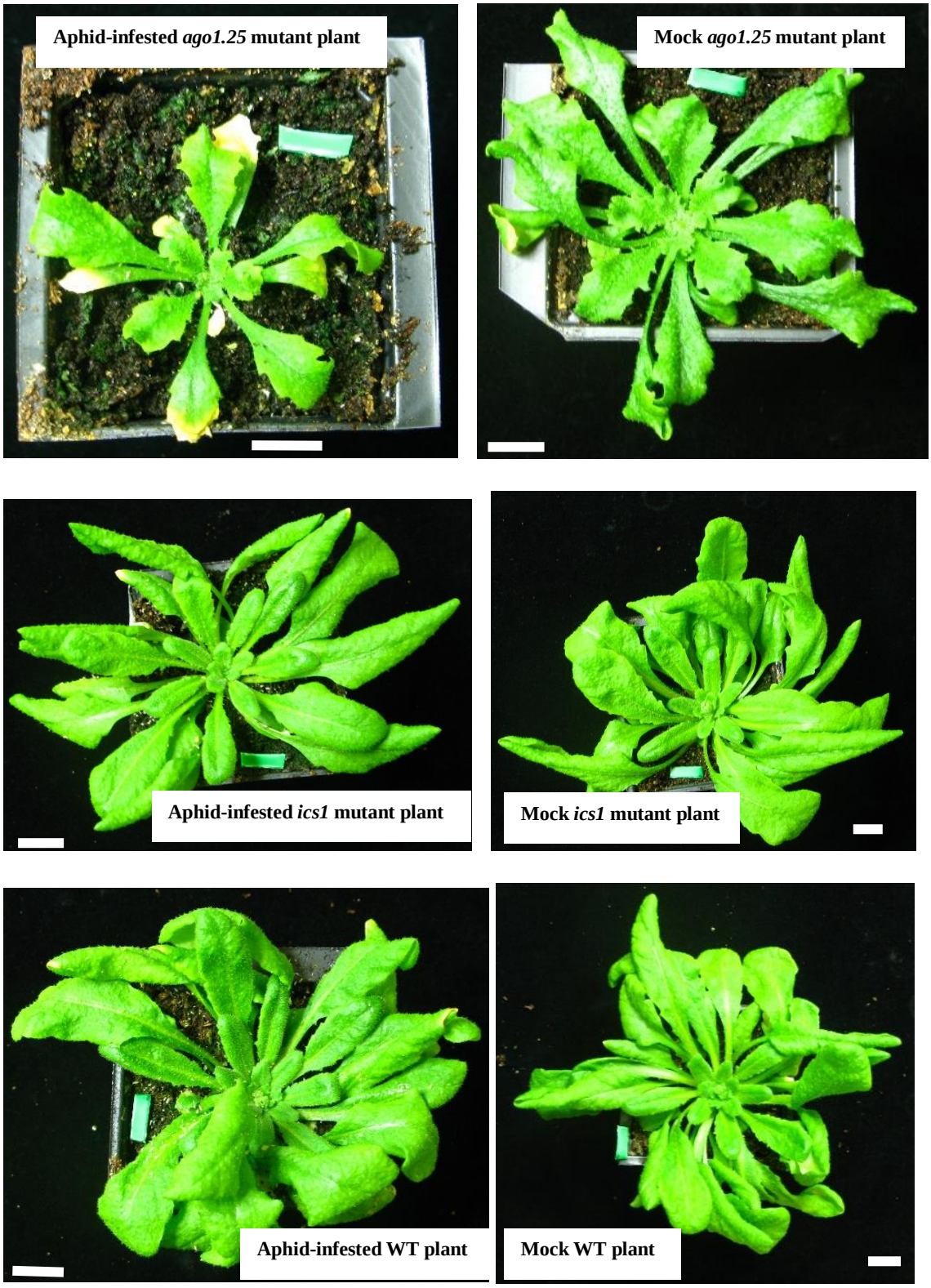


Figure 4.4: Aphid infestation of *ago1.25* mutant plant results in severe stunting in comparison to an aphid-infested *ics1* mutant plant or WT plant. Photos of the aphid non-infested *ago1*.25 mutant plant, *ics1* mutant plant and WT plant are intended for comparison with their aphid-infested counterparts (Scale bar = 1cm).

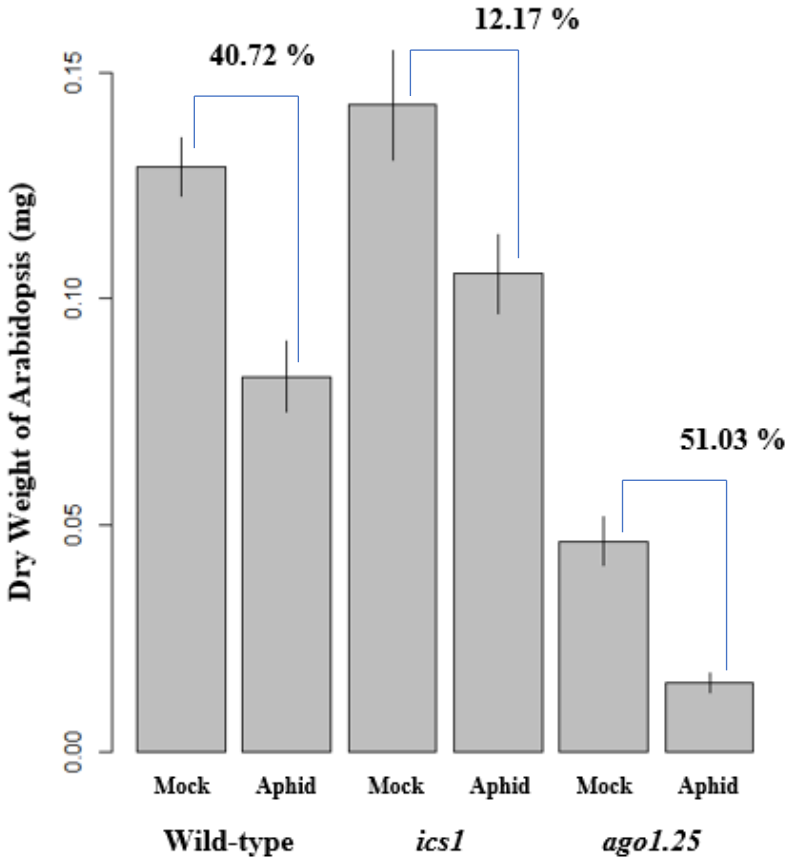


Figure 4.5: Aphid infestation-induced stunting is less severe in *ics1* mutant plants than in WT or *ago1.25* mutant plants. Mock plants were contained in transparent perforated plastic bags similar to the aphid infested plants except the addition of aphids. Error bars represent standard error of the mean.

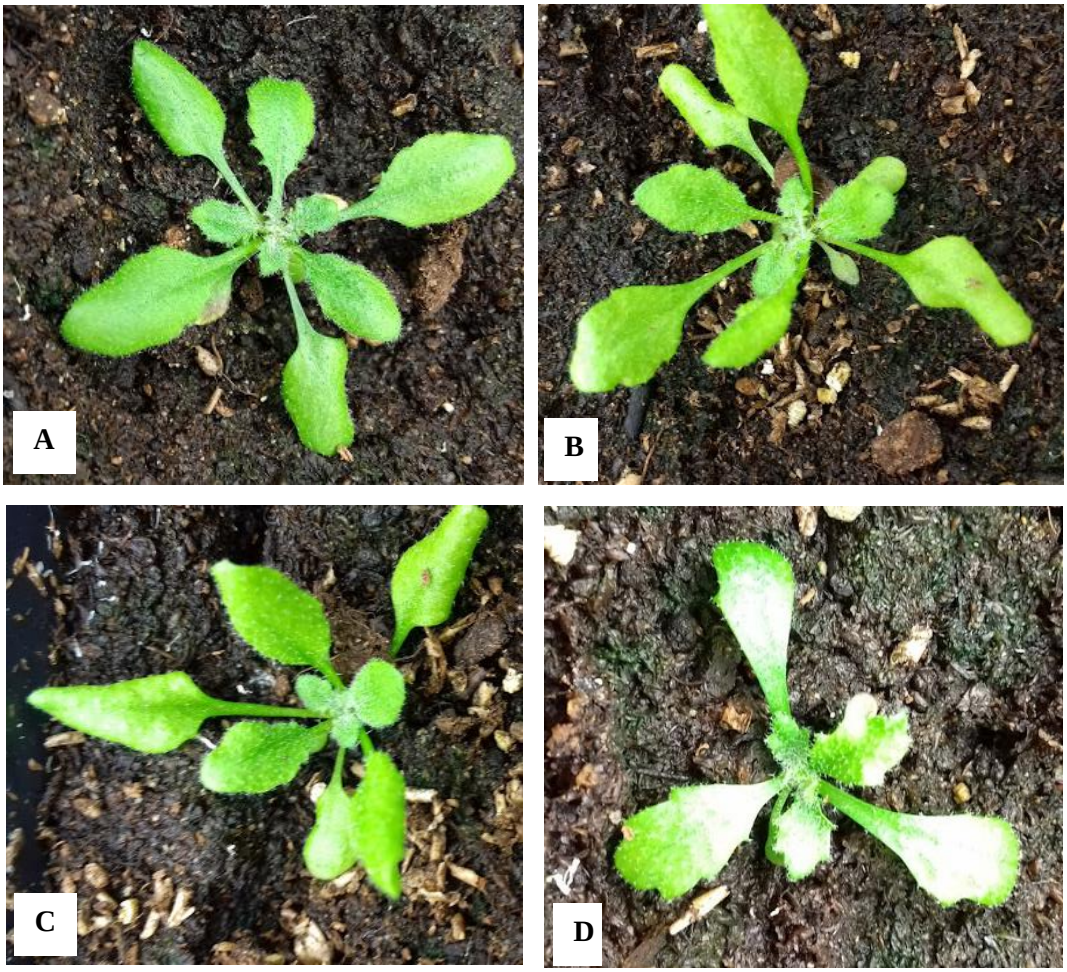
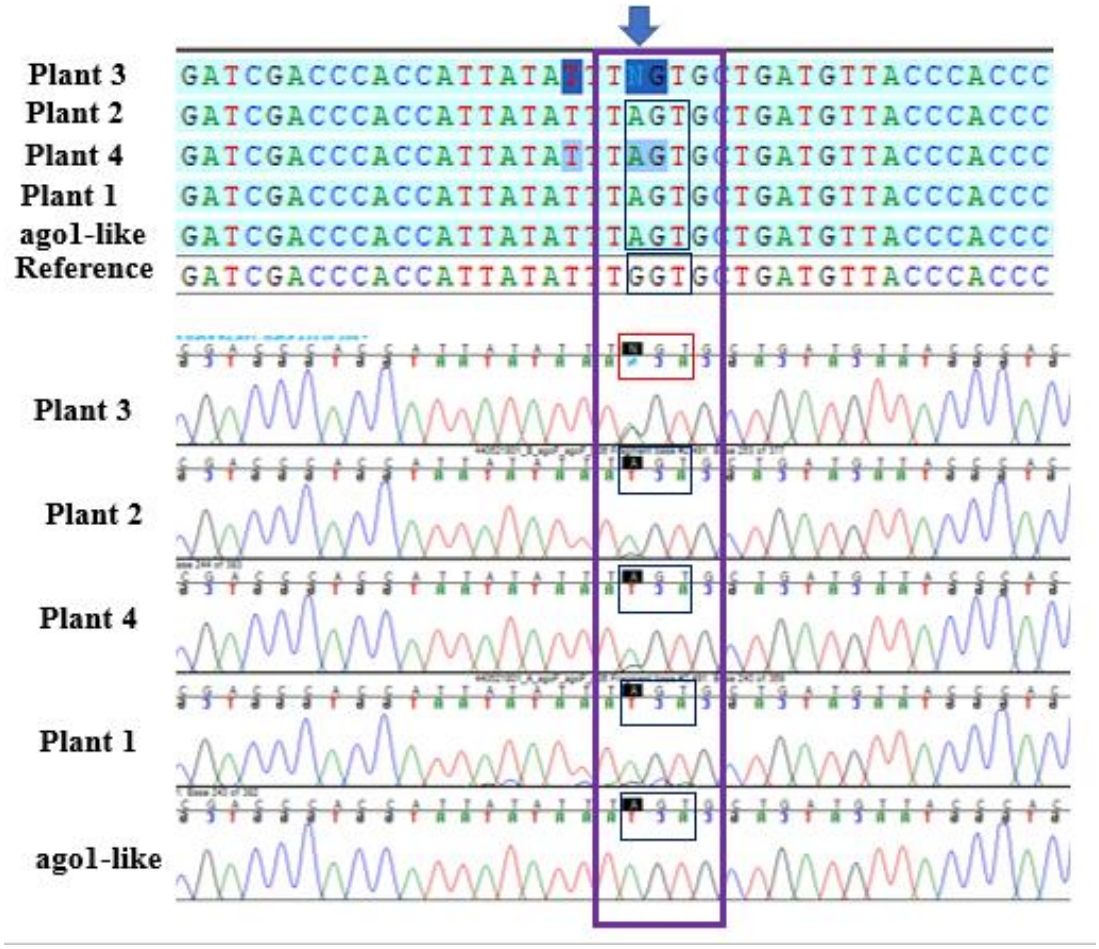


Figure 4.6: A selection of the *ago1* x *ics1* F_2 progeny with novel phenotypes (A, B and C) including one *ago1*-like progeny (D) which was later confirmed to be an *ago1* segregant.

Samples taken from four of these segregants for PCR and sequencing showed that they had a mutation in their *Ago1* and *ICS1* genes. However, a closer look at the sequencing chromatograms showed minor background peaks representing the WT base (G instead of A in terms of the *ago1* mutation or C instead of T in terms of the *ics1* mutation) in each of the three mutants (Figure 4.7). Seeds from one of the F_2 *ago1/ics1* plants were sown to generate an F_3 population. The majority of the F_3 population had similar phenotype as their F_2 parent however there were few of the F_3 plants that had varying degrees of *ago1-like* phenotypes. Samples from the F_3 population were analysed for mutation in their *Ago1* and *ICS1* genes. Three of the sampled F_3 *ago1/ics1* mutant plants were identified as pure *ago1* mutants without any traces of the WT base on their chromatograms (Figure 4.8). However, all these 3 pure *ago1* mutants had varying levels of the WT *ICS1* base appearing beneath the chromatograms of the point mutation (Figure 4.9). Progeny from *ago1/ics1* plants AS17 and AS20 (Figure 4.10) were primarily used for the aphid experiments (Section 4.3.4).

4.3.2.2 The presence of varying amounts of the WT bases in the *ago1/ics1* double mutant plants are unique to the *ago1* and *ics1* double mutation.

An interesting phenomenon observed among the *ago1/ics1* double mutant plants were the consistent presence of varying traces of the WT nucleotides in the double mutant plants (Figure 4.11 A). These observations were not seen among the *fox1/ago1(Figure 4.11B)* and *ago1/cyp82C2* double mutant plants which had similar phenotypes as their *ago1.25* parents. A cross involving an Fny-CMV *2b* transgenic plant and an *ago1* mutant plant also did not result in the reappearance of the WT *AGO1* base in the *2b/ago1* cross (Figure 4.11C). Progeny of the *ago1/ics1* double mutant plants showing trace amount of the WT bases also exhibited similar phenomenon (Figure 4.12). The presence of the WT base in the *ago1/ics1* double mutant plants also occurs in relation to the *ics1* mutation. Figure 4.12 shows the aligned *ICS1* DNA sequences of individual F_4 plants generated from selfing an F_3 AS17 *ago1/ics1* double mutant plant. The F_4 plants 8, 9 and 10 had WT-like phenotypes like their AS17 parent whilst plants 3 and 6 had *ago1-like* phenotypes despite all five plants having similar DNA sequences around the region of the *ics1* mutation. All five plants were *ics1* mutants (TAA) with traces of the WT *ICS1* base in their genome similar to the *ics1* mutation of their F_3 AS17 *ago1/ics1* parent (Figure 4.9).



GGT = Glycine/ *Ago1* WT codon

AGT = Serine/*ago1* mutant codon

Figure 4.7: Aligned chromatogram data and chromatograms from sequenced *AGO1* genes from a selection of the *ago1* x *ics1* F_2 progeny with novel phenotypes. One F_2 segregant with an *ago1*-like phenotype was also included in the experiment (G substituted to A in *ago1* mutant).

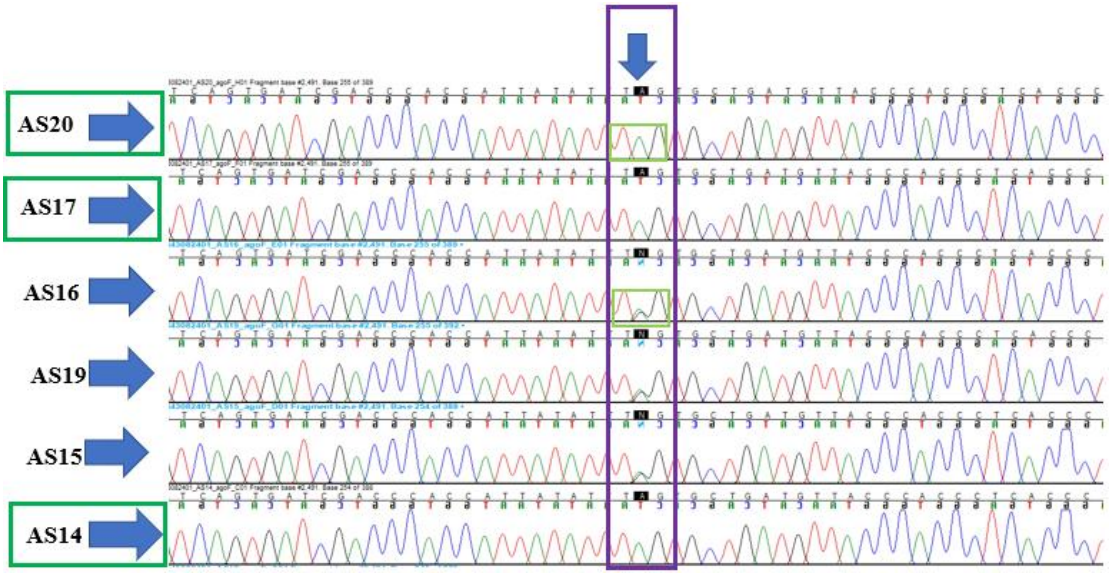


Figure 4.8: Aligned chromatograms from sequenced *AGO1* gene from a selection of the *ago1* x *ics1* F_3 progeny. Plants AS20, AS17 and AS14 were purely *ago1* mutant plants. Plants AS16, AS19 and AS15 are heterozygous for the *Ago1* mutation (G substituted to A in *ago1* mutant).

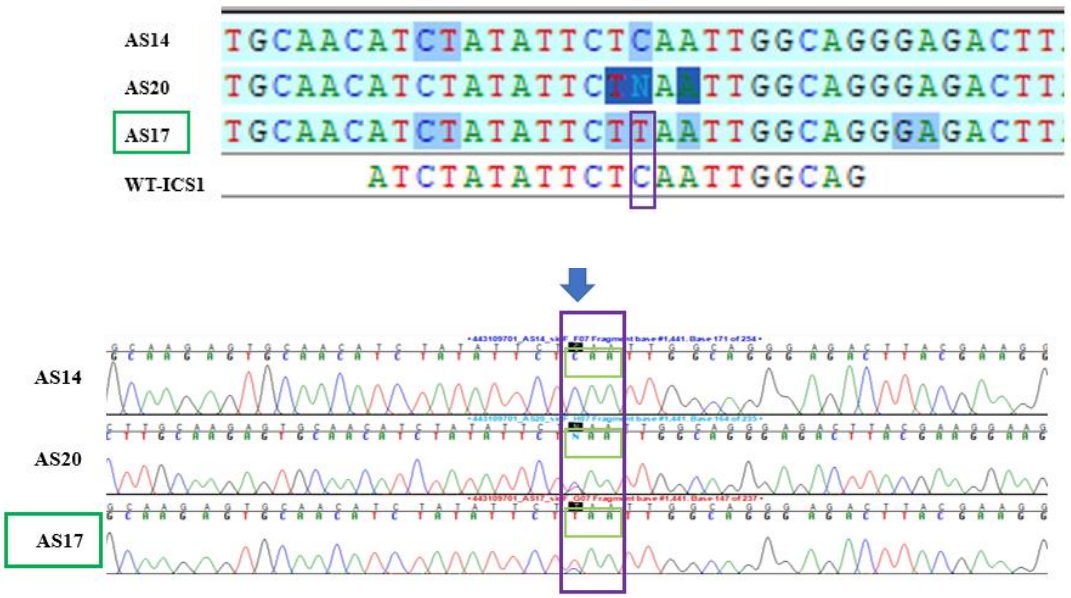


Figure 4.9: Aligned chromatogram data and chromatograms from sequenced *ICS1* gene from a selection of the *ago1* x *ics1* F_3 progeny. Although the sequencing base-caller assign AS17 *ICS1* sequence as a mutant but on the chromatograms, there was the re-emergence of the WT base beneath the mutation chromatogram peak (C substituted to T in the *ics1* mutant).

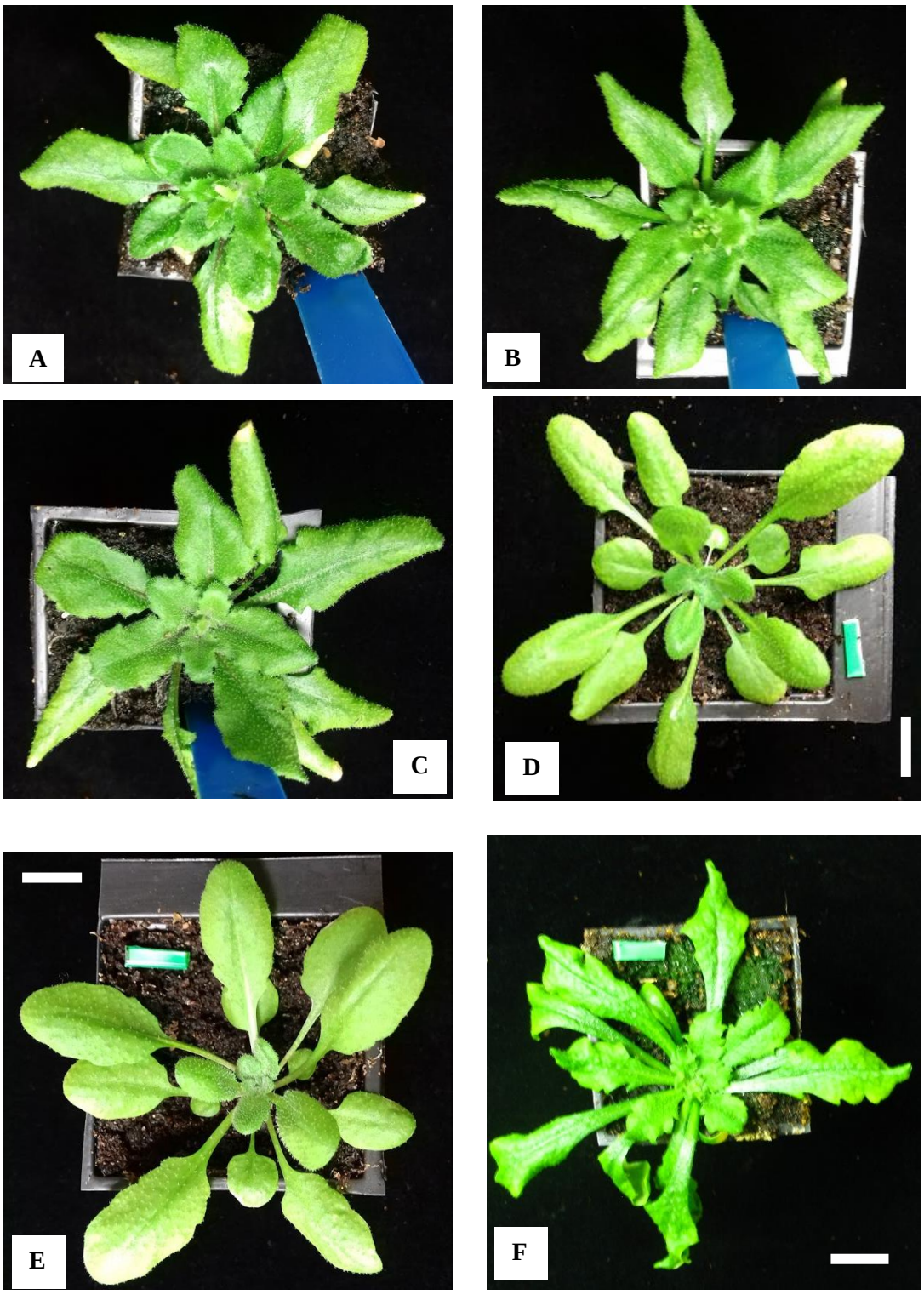


Figure 4.10: Phenotypes of *ago1/ics1* F_3 plants in comparison with a WT and an *ago1.25* mutant plant. A) AS14, B) AS20 and C) AS17, D) Col-0 WT, E) *ics1* mutant, F) *ago1.25* mutant. In certain cases, the extent of mutation in the *ICS1* gene have noticeable effect on the phenotype of the plants (Scale bar = 1cm).

A. Sequenced *AGO1* gene from an *ago1/ics1* double mutant plant.

The tiny blue (nucleotide C) peak underneath the red peak of the mutant base (T) (as shown by the black arrow) indicates the presence of a low amount of the WT base (nucleotide C) in the *ago1/ics1* double mutant plant.

TCA/AGT = Serine (*ago1* mutant).

B. Sequenced *AGO1* gene from a *fox1/ago1* double mutant plant.

There are no traces of the WT base in the *fox1/ago1* double mutant plant.

TCA/AGT = Serine (*ago1* mutant).

C. Sequenced *AGO1* gene from a *2ba/ago1* cross.

There are no traces of the WT base in the *2b/ago1* cross.

TCA/AGT = Serine (*ago1* mutant).

D. Sequenced *AGO1* gene from a *2b* transgenic Arabidopsis plant.

A *2b* transgenic Arabidopsis plant has a WT *AGO1* gene.

CCA/GGT = Glycine (*AGO1* WT).

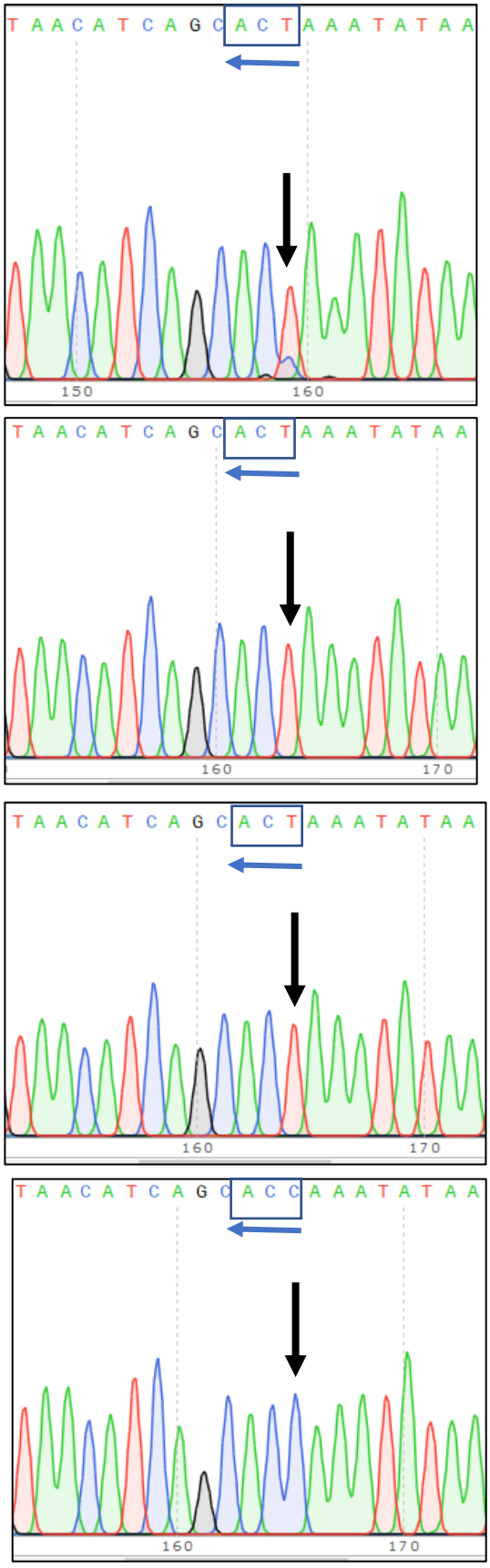


Figure 4.11: Aligned chromatograms from crosses involving an *ago1* mutation. The appearance of the WT *AGO1* base in the genome of the double mutant plants is specific to the *ago1/ics1* double mutation.

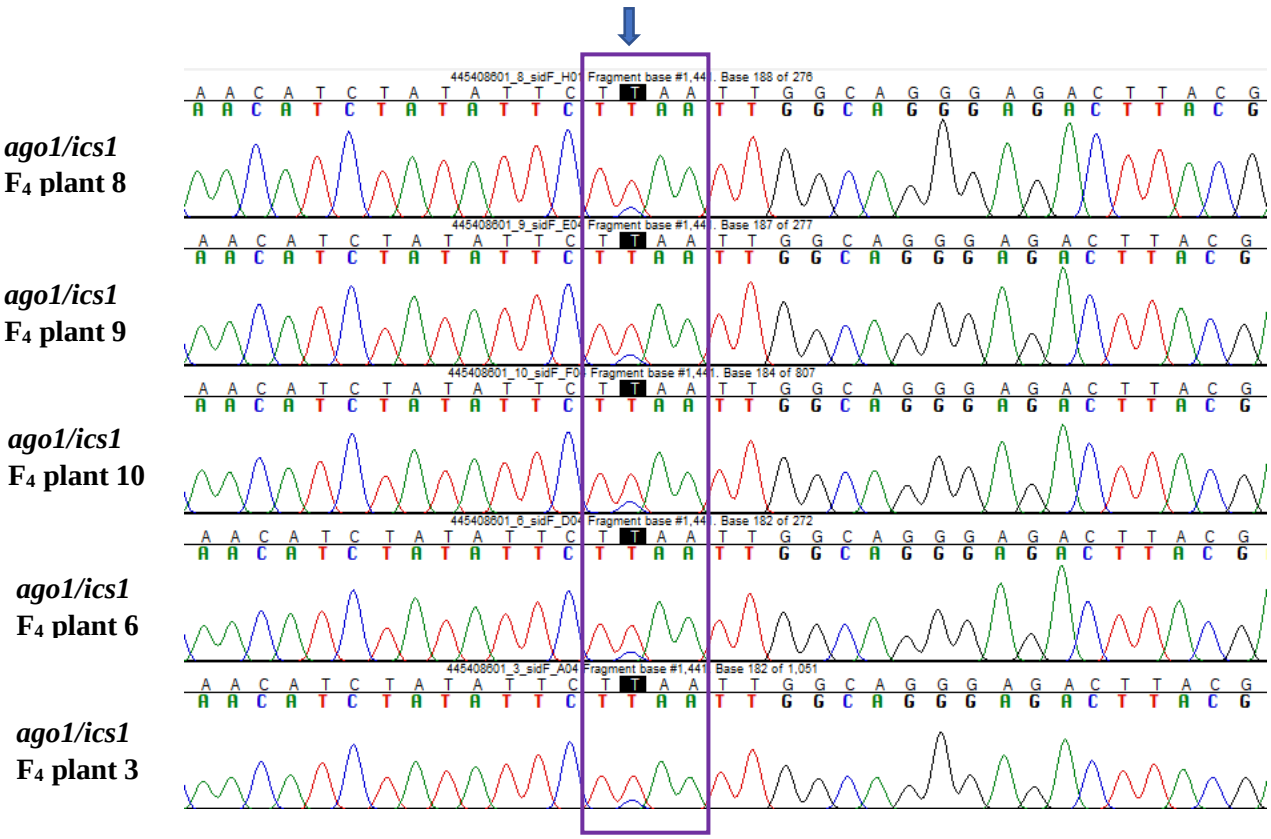


Figure 4.12: Aligned chromatograms from individual F_4 plants showing the region of DNA around the *ics1* mutation. All five plants show traces of the WT base in their genome irrespective of their phenotypes.

4.3.3 Mutation of *ICS1* gene in *ago1.25* mutant plant results in novel phenotypes.

The *ago1/ics1* seedlings are easily distinguishable from their *ics1* and *ago1.25* mutant parents and from a WT plant due to their relatively large cotyledons and longer hypocotyl. After pricking out into compost the *ago1/ics1* double mutant plants continue to outpace their parents in terms of growth and size (Figure 4.13A). Despite the WT looking phenotypes of *ago1/ics1* mutant seedlings, the *ago1/ics1* double mutant plants develops broader and narrow rosette leaves than WT plants (Figure 4.13 A and B) even at the juvenile stage of development. Juvenile rosette leaves of a WT plant are round in appearance (Figure 4.13B). The deeply serrated and broader leaves of the *ago1/ics1* mutant plants begin to curl up intensely particularly along the leaf margins as the plant advances into the adult vegetative stage.

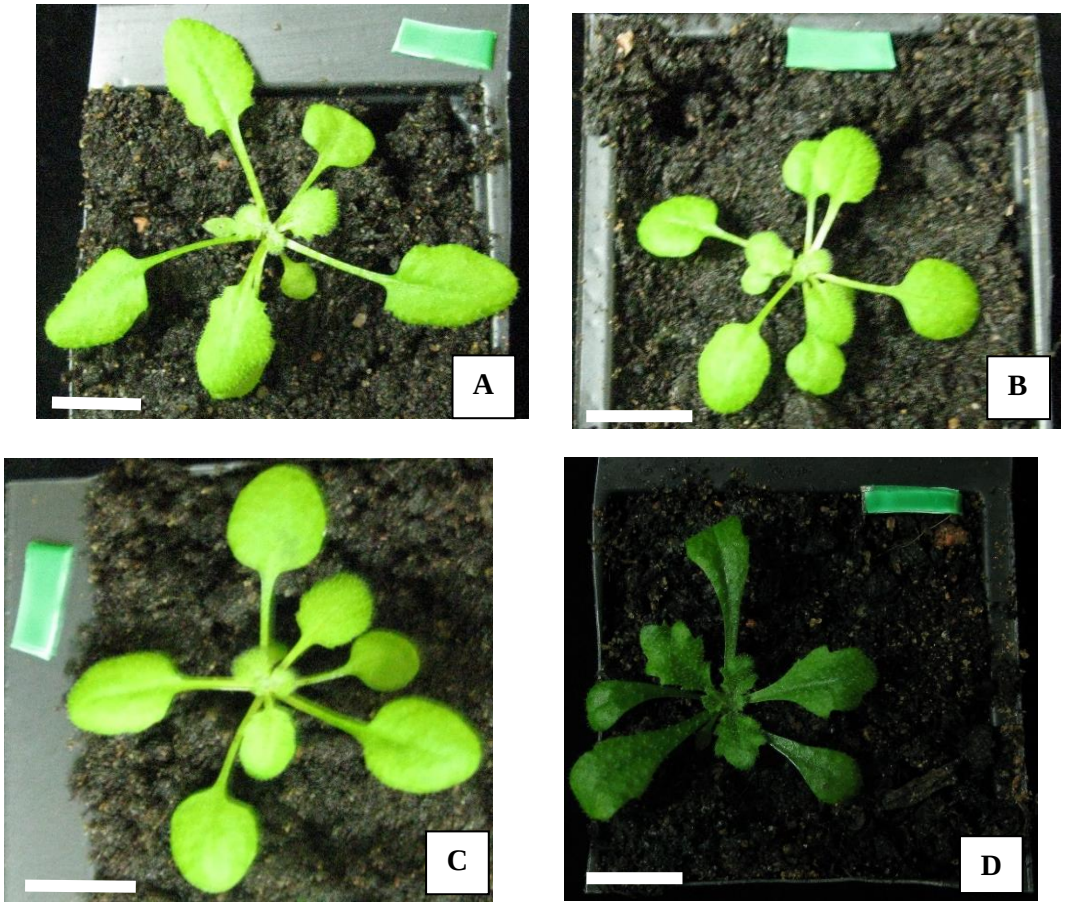


Figure 4.13: Comparison of 5 weeks old *ago1/ics1* double mutant plant (A) and WT plant (B). *ago1/ics1* mutant plants show precocious development in terms of leaf morphology. (C) 5 weeks old *ics1* mutant and (D) 6 weeks old *ago1* mutant plants are for comparison (Scale bar = 1cm).

However, the vigorous growth of the *ago1/ics1* double mutant plants is terminal. By the 8 weeks post planting the pace of *ago1/ics1* growth is slowed due to the spontaneous onset of pinpoint-like necrotic spots among the *ago1/ics1* progeny (Figure 4.14). The development of these necrotic spots largely starts from the leaf tip area and progresses toward the leaf petiole area as they widen over time. The necrotic lesions are mostly surrounded by chlorotic lesions especially among AS20 progeny where this phenomenon occurs early and is more severe. Subsequently, after 8 weeks post planting, the *ics1* mutant parent and the WT plants overtake the *ago1/ics1* progeny in growth and become larger than the *ago1/ics1* double mutants since they do not develop any spontaneous chlorotic lesions.

There was also the re-emergence of plants with *ago1-like* phenotypes among the progeny of the AS14, AS17 and AS20 mutant plants. For every 15 AS20 progeny one plant had an *ago1*-like phenotype. In terms of the AS17 progeny, one out of every 11 AS17 progeny had an *ago1*-like phenotype. These segregation ratios do not conform to classical Mendelian segregation ratios nor the segregation ratios of linked genes since both *Ago1* and *ICS1* are on the same arm of Arabidopsis chromosome 1. Interestingly, the few *ago1*-like phenotypes that appear among the progeny of the *ago1/ics1* progeny do not behave as *ago1.25* mutant plants but rather as their AS17-like and AS20-like counterparts. For instance, the *ago1/ics1* progeny with the *ago1-like* phenotypes also develop the necrotic lesions just as their AS17-like and AS20-like counterparts (Figure 4.15). Spontaneous necrotic spots were not developed by the WT plants or the *ics1* mutant but very old *ago1.25* mutant plants also begin to develop milder version of these spontaneous necrotic spots.

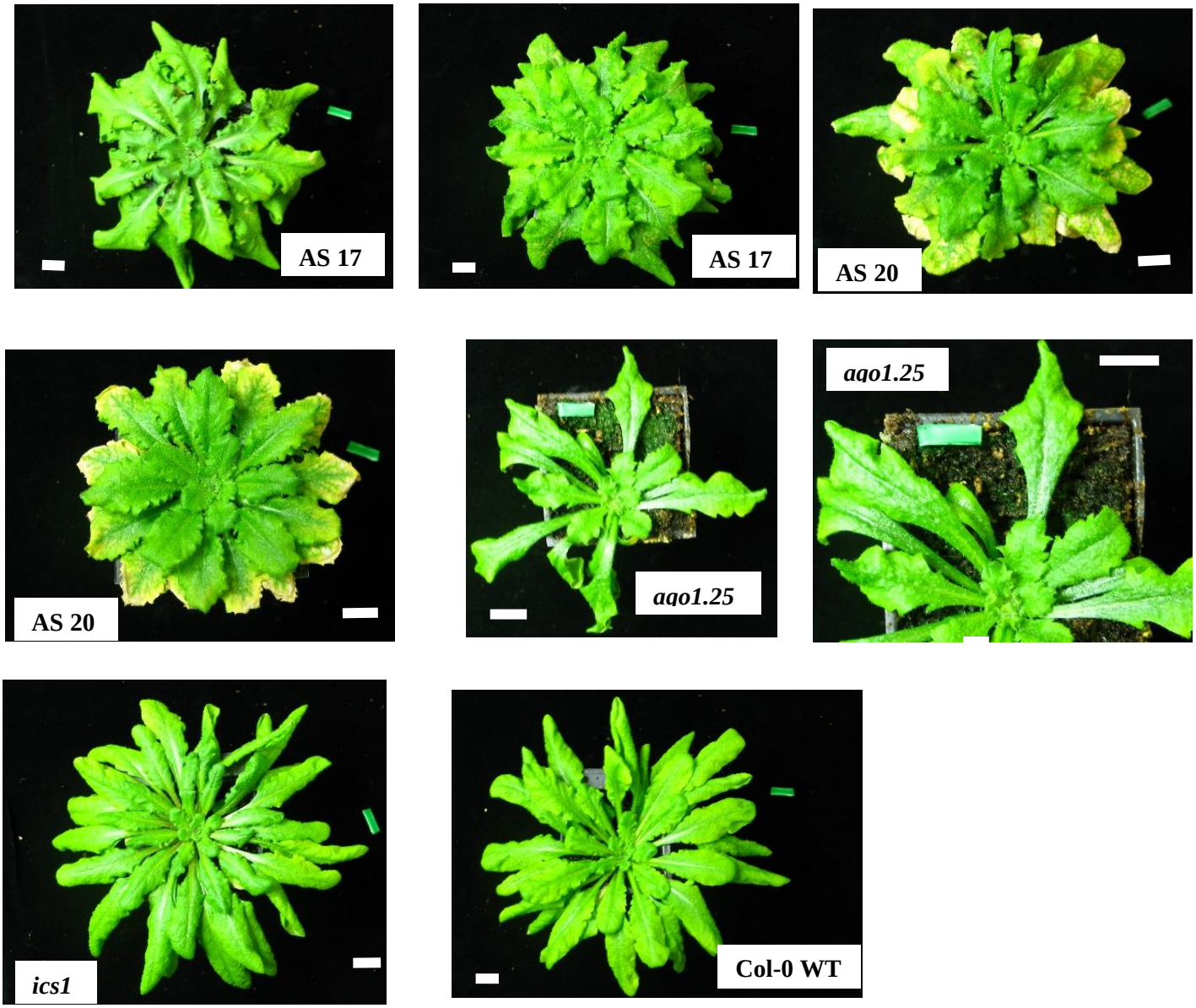


Figure 4.14: Ten weeks old *ago1/ics1* double mutant plants showing chlorotic/necrosis lesions on leaves. There was no necrosis on the leaves of the *ago1.25* and the *ics1* mutant parents nor the WT plant at ten weeks post planting (Scale bar = 1cm).

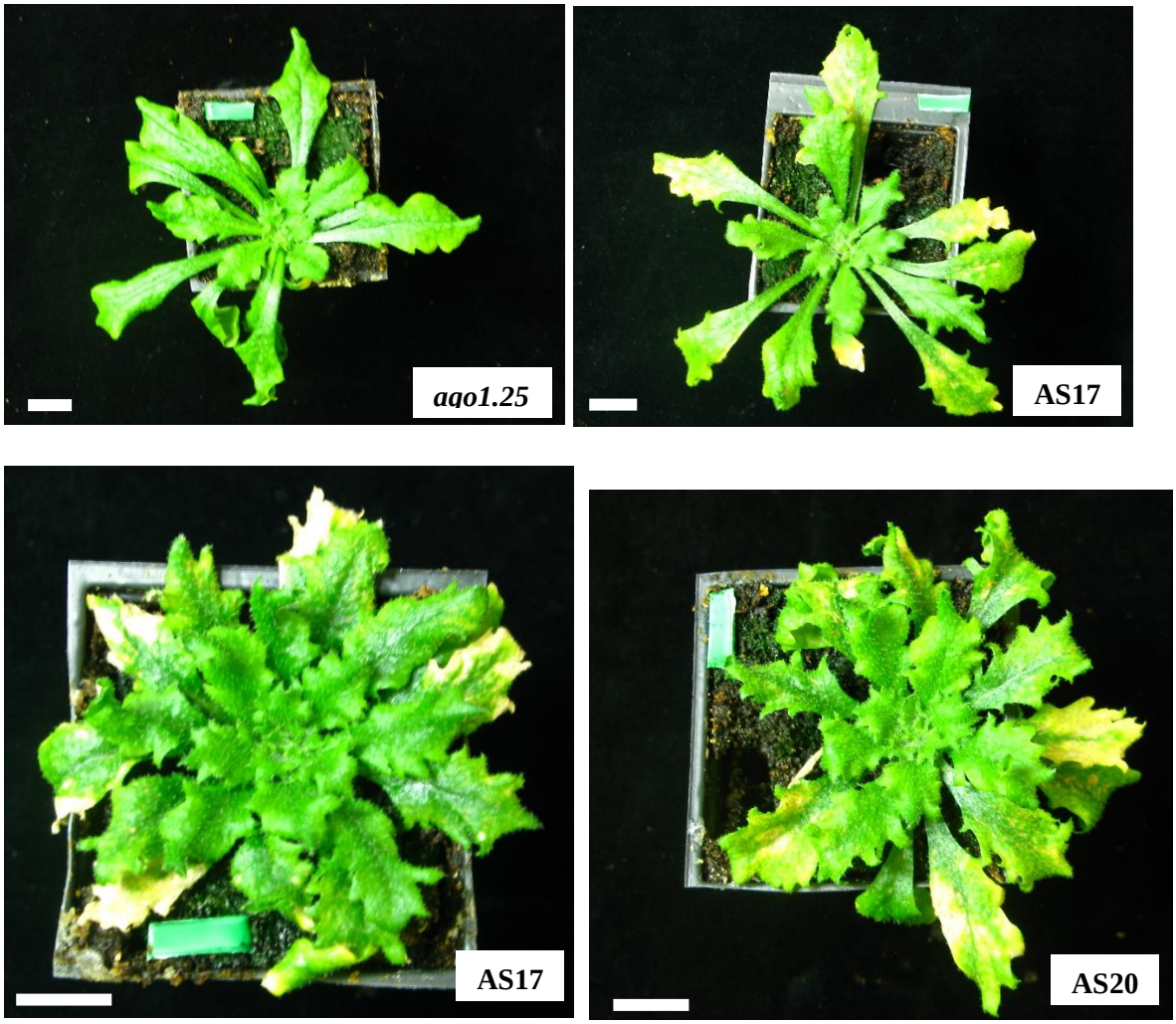


Figure 4.15: Ten weeks old AS17 and AS20 plants with ago1-like phenotypes showing necrosis similar to their AS17-like and AS20-like counterparts. There was no necrosis on the leaves of the *ago1.25* mutant parent at this stage (Scale bar = 1cm).

4.3.4 Mutation of the *ICS1* gene in the *ago1.25* mutant plant background restores susceptibility to aphid infestation

Aphid performance assays were primarily based on AS17 progeny with recovery-like phenotypes. The AS20 progeny with recovery-like phenotypes were also used in certain instances for the aphid performance assay. Mutation of the *ICS1* gene annuls the heightened resistance to aphid infestation observed in *ago1.25* mutant plants. Aphid growth rates on the *ago1/ics1* double mutants were significantly increased compared to the growth rates of aphids on an *ago1.25* mutant plant. Aphids growing on *ago1/ics1* mutant plants attained similar growth rates as aphids that grew on WT plants (Figure 4.16A).

Similarly, aphids reproduced significantly larger colony sizes on the *ago1/ics1* double mutant plants in comparison to the colony sizes of aphids on the *ago1.25* mutant plants (Figure 4.16B).

4.3.5 Mutation of the *ICS1* gene in the *ago1.25* mutant plant background reduces the extent of aphid-induced stunting observed in *ago1.25* mutant plants

Six-day-old aphids were placed on *ago1/ics1* double mutant plants for a 12-day duration to assess whether the aphid infestation will result in severe stunting of the *ago1/ics1* double mutant plants as observed in aphid-infested *ago1.25* mutant plants (Figure 4.17). Although aphid infestation results in greater loss of biomass in the *ago1/ics1* double mutant plant in comparison to a WT plant or its *ics1* parent, the loss in biomass was not as severe as observed in aphid-infested *ago1.25* mutant plants (Figure 4.17). There was approximately 19.69% decrease in loss of biomass when there was a mutation in the *ics1* gene in *ago1.25* mutant plants.

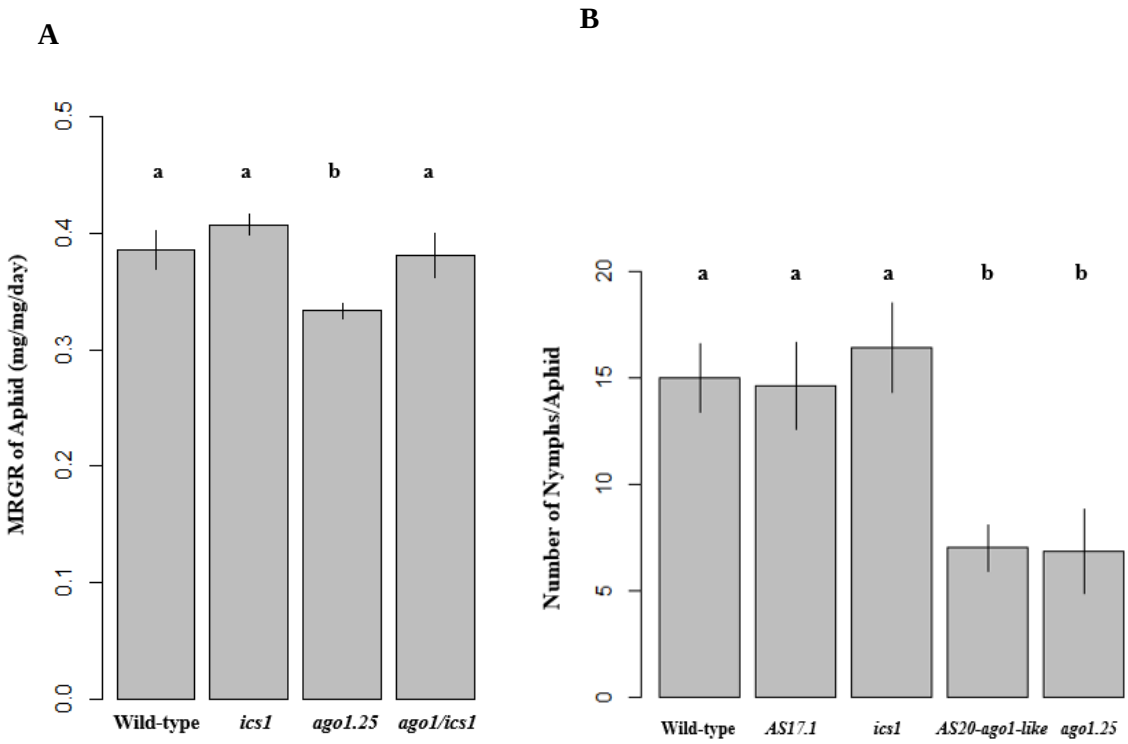


Figure 4.16: A) Mean relative growth rates (MRGR) of aphids at 6 days post infestation and B) colony size of aphids at 12 days post infestation on *ago1/ics1* double mutant plants. Mutation in *ICS1* significantly increases the growth rate and colony size of aphids on *ago1.25* mutant plants to levels comparable to a WT plant at $p < 0.05$ level. Error bars represent standard error of the mean. Treatment means (bar plots) denoted by the same letter are not significantly different from each other at $p < 0.05$.

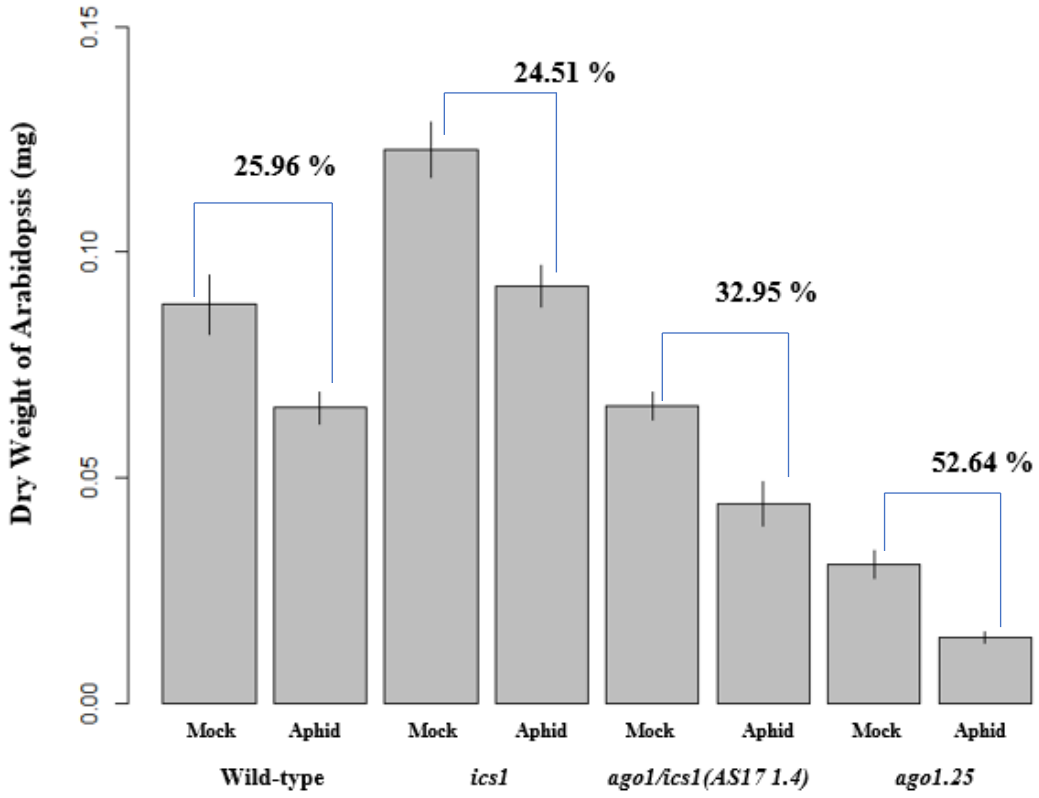


Figure 4.17: Loss of biomass as a result of 12-day aphid infestation. Mutation in *ICS1* gene in *ago1.25* mutant plants results in less severe loss in biomass in comparison to the *ago1.25* mutant parent when both plants are infested with aphids. Percent values represent decrease in biomass after 12 days of aphid infestation. Error bars represent standard error of the mean.

4.3.6 Expression of *nahG* in the *ago1.46* mutant background in certain instances alleviates the *ago1.46* developmental deformities.

To test if SA accumulation was responsible for the stunting and deformed phenotype of *ago1.25* mutant plants, a cross between *ago1.46*, another *ago1* allele with a comparable phenotype as *ago1.25* and a *nahG* transgenic plant was made. Interestingly, the *ago1.46/nahG* cross has a more WT-like phenotype than even the *ago1/ics1* double mutant plants (Figure 4.18). This indicates that SA plays an essential role in the developmental phenotype of *ago1* mutant plants.

Figure 4.18: Recovery-like phenotype of an *ago1.46/nahG* cross (left) in comparison to *ago1.46* segregant (centre) and WT (right) phenotypes. (Scale bars = 1cm).

4.3.7 Upregulation of *SPLs* may explain the precocious development of the *ago1/ics1* progeny with recovery-like phenotypes

In a quest to explain the precocious development of the *ago1/ics1* mutant plants with recovery-like phenotypes such as the early narrowing and enlargement of *ago1/ics1* double mutant leaves (Figure 4.13), the expression of *Squamosa promoter-binding-like-10* (*SPL10*) whose upregulation is associated with precocious development were tested (Yu et al., 2015a). *SPL10* levels were upregulated in the *ago1/ics1* double mutant plants in comparison to levels in WT plants. However, *SPL10* levels seemed higher in *ago1.25* mutant plants than the *ago1/ics1* double mutant plants (Figure 4.19). *SPLs* are regulated by miR159 guided AGO therefore it is expected that mutation in *AGO1* will result in their upregulation. However,

effects of *SPL10* upregulation seem more evident in the *ago1/ics1* than in the *ago1.25* mutant plants.

Figure 4.19: AGO-regulated *SPL10* is upregulated in *ago1.25* mutant and *ago1/ics1* double mutant plants. (*SPL10* product size: 183 bp).

4.4 Discussion

4.4.1 The enhanced susceptibility to aphids and increased growth rate of the *ago1/ics1* double mutant plants could be as a result of increased auxin signalling.

Auxins are central to regulation of plant growth and development. Accumulation of auxins is known to suppress SA defences. Hence in many cases plants with enhanced auxin signalling and increased accumulation of auxins are larger in size but more susceptible to infections due to prioritization of growth and development over host defence responses (Mutka et al., 2013). Similarly, plants actively accumulating SA with heightened SA signalling and responses are generally stunted in stature and more resistant to infections (Bowling et al., 1997; Wang et al., 2007). The stunted phenotype of most SA accumulating mutants may partly be due to repression of auxin signalling and responses. SA accumulation stabilizes the auxin response repressor AUX/IAA to repress auxin signalling and responses and also induces the expression of GH3 enzymes to inactivate free auxins (Zhang et al., 2007). Auxin response receptor genes *TIR* and *AFB1* that are required to degrade AUX/IAA repressors to activate auxin signalling and responses are also downregulated in SA over-accumulating mutant plants (Wang et al., 2007). Therefore, in the absence of SA accumulation, auxin signalling and responses are de-repressed. The longer hypocotyl, enhanced growth and the narrow cotyledons of *ago1/ics1* double mutant seedlings are reminiscent of an auxin over-accumulating mutant plant. Since auxins largely exert their role in regulating growth and development through DNA binding auxin response factors of which some are directly regulated by small RNA guided AGO proteins (Li et al., 2016), it is plausible that mutation in both *AGO1* and the SA biosynthetic gene *ICS1* may significantly disrupt the crosstalk between the growth-promoting auxin and the defence-promoting SA favouring growth and accumulation of auxins in the *ago1/ics1* double mutant plants. Furthermore, accumulation of auxins in the *ago1/ics1* double mutant plants may negatively affect the accumulation of the aphid-deterring glucosinolates and camalexin since these metabolites rely on tryptophan for their biosynthesis.

Interestingly, although auxin responsive genes were upregulated in the *ago1/ics1* double mutant plants, they were also upregulated in the stunted *ago1.25* mutant plants and in certain cases the levels of these auxin responsive genes were even higher in the *ago1.25* mutant than the double mutant plants. Expression of *PR-1* was tested in *ago1.25* mutant to verify if the stunting and enhanced defence to aphid infestation in *ago1.25* mutant was as a result of SA

accumulation and repressed auxin signalling. However, *PR-1* expression is more downregulated in *ago1.25* mutant plants compared to levels in WT plants and *ago1/ics1* double mutant plants. However, the possibility of accumulation of SA resulting in the stunting and enhanced defence to aphids in the *ago1.25* mutant plants could not be ruled out since there are instances where SA accumulation does not lead to increased expression of *PR-1* (Delaney et al., 1995). A cross between *ago1.46* mutant plant and *nahG* transgenic plant confirms that accumulation of SA is adequate to cause the *ago1* phenotypes, hence it may also be responsible for the heightened defence to aphids in *ago1* mutants. In the absence of SA accumulation, auxin signalling and responses are de-repressed resulting in enhanced growth and susceptibility of the *ago1/ics1* double mutant to aphid infestation.

4.4.2 Possible explanations for the consistent occurrence of the wild type sequences in the genetic pool of the *ago1/ics1* double mutant plants.

A. Sample Contamination

Contamination of *ago1/ics1* samples by extraneous nucleic acids with a WT *AGO1* or *ICS1* genotype during the nucleic acid extraction and purification process, or at the time of sequencing, could result in the observed phenomenon. However, the presence of the WT bases is restricted to *ago1/ics1* mutant plants and never observed among the other double mutant plants, which are also in the *ago1* background such as *fox1/ago1, ago1/cyp82C2* and *2b/ago1* (Figure 4.11). The chances that only the *ago1/ics1* double mutant plant samples will be contaminated whilst none of the samples from either *fox1/ago1, ago1/cyp82C2* or *2b/ago1* also become contaminated less likely although it remains a possibility.

B. Inability to identify a pure *ago1/ics1* double mutant plant

The initial F_2 *ago1/ics1* double mutant plants were sampled for further analysis based on their novel phenotypes among the segregating F_2 *ago1* x *ics1* population. Sequencing data for each of the selected F_2 plants with novel phenotypes indicated that they all had mutations in both their *ago1* and *ics1* genes. However, they also had varying levels of the WT bases in their genome. In the quest to investigate if a purely *ago1/ics1* double mutant plant will be identified, I allowed selfing of the F_2 plants to obtain an F_3 population. At one instance,

screening of the F_3 *ago1/ics1* progeny resulted in the identification of 3 plants that were purely *ago1* mutants (plants AS14, AS17 and AS20). However, in terms of their *ICS1* mutation, AS14 was purely *ICS1* WT, AS20 was *ICS1* heterozygous whilst AS17 was *ics1* mutant but with traces of the WT *ICS1* base in its genome. It is possible if I had screened a larger population of the F_3 progeny I may have identified a pure *ago1/ics1* double mutant plant without traces of the WT bases in its genome.

4.5 Conclusions

The loss of ability to accumulate SA is adequate to restore the *ago1* phenotype back into a WT-like phenotype. Accumulation of SA may also be responsible for the heightened defence to aphids observed in *ago1.25* mutant plants. SA accumulation and enhanced SA signalling in the *ago1.25* mutant plant may repress the accumulation and signalling of the susceptibility-enhancing auxin making the *ago1.25* mutant plants more resistant to aphid infestation.

Chapter 5

The Expression of Fny-CMV 2b protein further enhances the heightened resistance to aphids in *ago1.25* mutant plants

5.1 Introduction

PTGS is a mechanism through which a small RNA guided AGO protein as part of an effector complex referred to as RNA induced silencing complex (RISC) targets and degrades foreign or host nucleic acids of which they share complementarity (Vaucheret et al., 2001) Details on the mechanisms of PTGS can be found in Sections 1.8.1 and 1.8.2. PTGS therefore functions as an effective defence mechanism against plant virus infections. To promote infection, *Cucumoviruses* including CMV encode the 2b protein to suppress host PTGS (Chapter 1 subsection 1.8.4) (Goto et al., 2007; Jacquemond, 2012).

Transgenic expression of Fny-CMV 2b protein in Arabidopsis results in mild to severe developmental anomalies. In very severe cases the rosette leaves of *2b* transgenic plants become narrow, deeply serrated and curled upwards. These severe 2b transgenic Arabidopsis plant lines produce sterile flowers many of which remain unopened or produce anthers without pollen. However, mild or moderately severe *2b* transgenic lines are fertile despite the stunting, twisted petioles, intense serration and narrowing of the perianth (Figure 5.1) (Zhang et al., 2006; Lewsey et al., 2007).

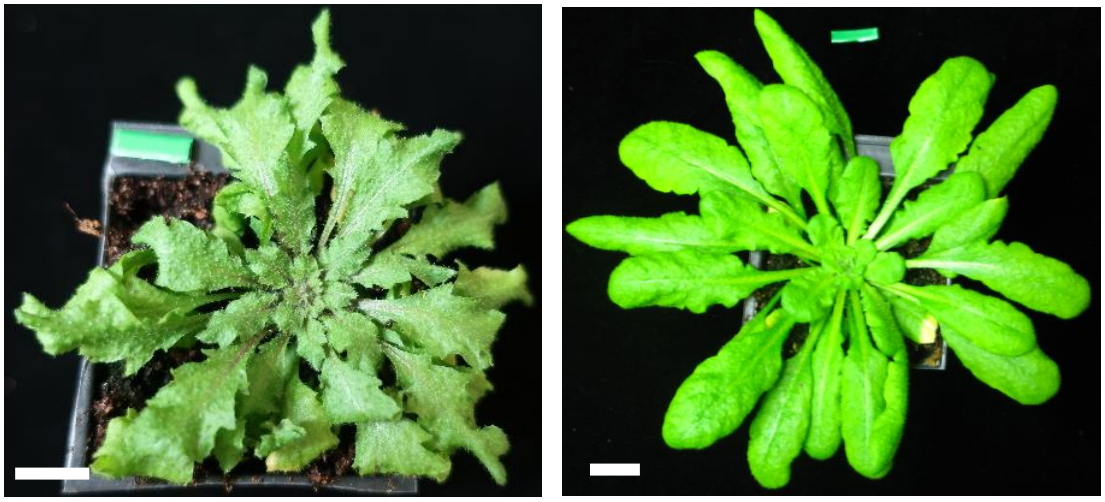


Figure 5.1: Fny-CMV *2b*-transgenic Arabidopsis plant (line 3.13F: Lewsey et al., 2007) in comparison to a WT Col-0 plant (Scale bars = 1cm).

Expression of viral proteins in host plants can alter the extent of callose deposition, the nutritional composition and also the volatile organic compound blends emitted by these transgenic plants which in turn affects the interaction of the host plants with insect vectors (Westwood et al., 2013; Casteel et al., 2014). The magnitude and direction of the host plant manipulation by viral proteins and the resulting effect on vector preference depend on the specific host involved in the interactions. For instance, in the tobacco-*Myzus persicae*-CMV 2b interactions, the 2b protein reduces the host defence responses against the aphid thereby encouraging the survival of the vector on infected tobacco plants. As a result, *Myzus persicae* prefer settling on CMV infected tobacco plants than on healthy tobacco plants. In line with the role of the 2b protein in the tobacco-*Myzus persicae*-CMV 2b pathosystem, tobacco plants infected with the CMVΔ2b virus are more resistant to aphid infestation than a healthy tobacco plant (Ziebell, et al., 2011).

On the contrary, in the Arabidopsis-*Myzus persicae*-CMV 2b pathosytem, the expression of the 2b protein rather enhances the resistance of the host to aphid infestation. Aphids therefore prefer settling on healthy Arabidopsis plants to settling on *2b* transgenic Arabidopsis plants (Westwood et al., 2013). Since the 2b protein directly interacts with AGO1 protein and that a mutation in the 2b protein affects 2b-mediated suppression of RNA silencing, I decided to examine whether or not expressing the 2b in *ago1.25* background affected aphid resistance.

5.2 Results

5.2.1 *2b/ago1* crosses plants have novel phenotypes and are largely sterile.

The *2b/ago1* crosses are severely deformed compared to either of their *ago1.25* mutant or *2b*-transgenic parental plants. They display developmental deformities ranging from moderately severe to very severe phenotypes (Figure 5.2). The moderately severe *2b/ago1* double mutant plants are slightly *ago1-like* in appearance but with much narrower and smaller rosette leaves. The phenotypes of the very severe *2b/ago1* progeny include plants with needle-like and slightly twisted rosette leaves to plants with significantly longer internodes and stems even at the vegetative stage with rosette-like leaves instead of cauline leaves emerging on their extended stems. These *2b/ago1* progeny produce inflorescence with open calices that are not capable of producing seeds. It is not certain whether these *2b/ago1* crosses plants are merely self-incompatible or they produce dysfunctional reproductive organs.

5.2.2 The 2b protein enhances the heightened resistance to aphids observed in *ago1.25* mutant plants

F_2 seeds from the heterozygous *2b/ago1* +/- F_1 plants were sown and the *2b/ago1* double mutant plants were selected based on developmental phenotype for aphid performance assays. Aphids performed very poorly on the *2b/ago1* crosses in comparison to its parents which in themselves have enhanced aphid resistance than a WT plant. The growth rate of aphids on the *2b/ago1* crosses were significantly lower than the growth rates of aphids on the *2b* transgenic, *ago1.25* mutant or the WT plants (Figure 5.3). Aphids also reproduce less on the *2b/ago1* crosses.

Figure 5.2: A selection of *2b x ago1* progeny in comparison to WT and mutant parental plants (Scale bar = 1cm).

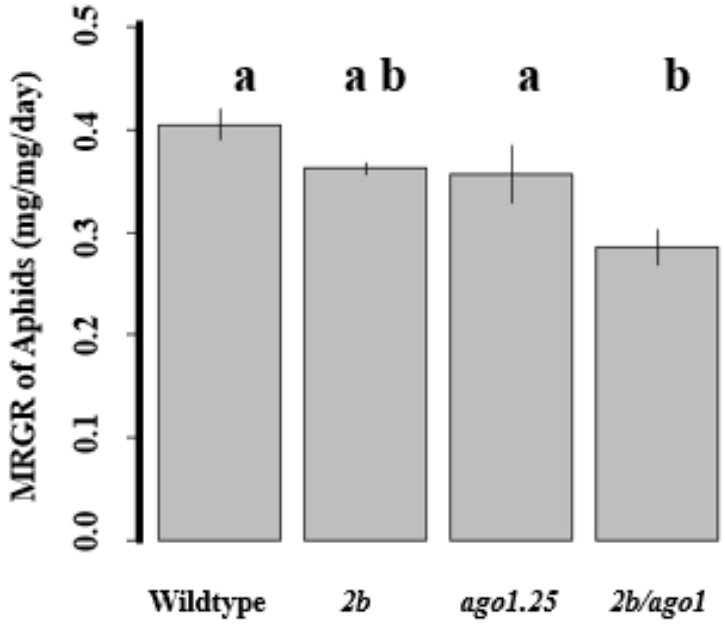


Figure 5.3: Mean relative growth rate (MRGR) of aphids on *2b/ago1* plants. Aphids significantly gain less weight on the *2b/ago1* cross in comparison with either the WT or the mutant parents (ANOVA p value = 0.000514). Sample size (n): WT (8), *2b* (5), *ago1.25* (8), *2b/ago1* (13). Error bars represent standard error of the mean. Treatment means (bar plots) denoted by the same letter are not significantly different from each other at $p < 0.05$.

5.3 Discussion

5.3.1 There is a deleterious synergistic interaction between the 2b protein and the AGO1 protein in relation to aphid resistance in Arabidopsis.

Mutation in *AGO1* alone or transgenic expression of the CMV 2b protein in Arabidopsis alone is adequate to increase the resistance to aphids in Arabidopsis plants. However, the combined mutations further enhance the extent of resistance against aphids in Arabidopsis beyond what would be expected if both mutations had independent effect on aphid resistance such that their combined deleterious interactions were additive. Mutation in the *AGO1* gene resulted in a 4.8% drop in aphid growth rate whilst the transgenic expression of the 2b protein resulted in a 4.2% drop in the rate of aphid growth. If the *ago1*-mediated resistance to aphids was independent of the 2b-mediated resistance to aphids in Arabidopsis plants then the combined mutations would have resulted in an approximately 9.03% drop in aphid growth rate in the *2b/ago1* plants. However, there was a 11.92% drop in aphid growth rate of the *2b/ago1* plants, which is a 2.89% more drop in aphid growth rate than expected if both the ago1 and the 2b proteins contributed independently to aphid resistance in Arabidopsis plants. This therefore suggests a negative deleterious epistatic interaction between the ago1 and the 2b protein in relation to aphid performance.

The epistatic interaction between the two proteins was expected as the 2b protein directly interacts with the AGO1 protein. However, this synergistic deleterious interaction between the 2b protein and the ago1 protein that resulted in more than expected drop in aphid growth rates may be due to an enhanced accumulation of an anti-aphid toxic metabolite in the *2b/ago1* plants. Previous experiments in our lab have shown that the transgenic expression of the 2b protein alone in Arabidopsis is able to induce an aphid resistance response which is not based on feeding deterrence but likely to be antibiosis in nature. The 2b-mediated antibiosis-type resistance to aphids was then proposed to be as a result of the inhibitory activity of the 2b protein on the regulatory role of the AGO1 protein. It was reasoned that *AGO1* downregulated this proposed anti-aphid toxic metabolite upon aphid infestation. However, the interaction of the 2b protein with the AGO1 protein hindered the effective downregulation of this toxic metabolite in the *2b*-transgenic Arabidopsis plants (Westwood et al., 2013). In line with this theory, a mutation in the AGO1 protein will further hinder the effective downregulation of the anti-aphid toxic metabolite in the *2b/ago1* plants resulting in

an even enhanced resistance to aphid performance on the 2b/ago1 crosses in comparison to the resistance to aphids observed in *2b* transgenic plants alone. This will therefore explain the negative deleterious synergistic interactions between the 2b protein and the ago1 protein in terms of aphid resistance in Arabidopsis.

5.3.2 The *2b/ago1* plants have more severe developmental deformities.

Both the *2b* transgenic Arabidopsis line and the *ago1* mutant line used in generating the *2b/ago1* plants are fertile, however, when the two mutations occur in the same genome, most of the resulting double mutant plants become sterile with flowers that are open even at the floral bud stage. Some of the *2b/ago1* plants also gain longer internodes and stems contrary to a WT CMV infected plant which usually have shortened internodes and may have flowers that remain unopened (Zhang et al., 2006). The severity of the *2b/ago1* developmental deformities may partly be due to the deleterious synergistic interactions between the 2b protein and the AGO1 protein. However, the absence of the CMV 1a protein could also contribute to the severity of the *2b/ago1* plants in comparison to a CMV infected plant. The 1a protein is thought to moderate the level of the 2b protein accumulation such that a *1a/2b* transgenic Arabidopsis plants recover from its characteristic *2b* phenotype (Westwood et al., 2013).

5.4 Conclusions

Transgenic expression of the CMV 2b protein in Arabidopsis *ago1.25* mutant plants results in a negative deleterious synergistic interaction between the two proteins such that the *2b/ago1* plants have more deformed phenotypes and increased resistance to aphid infestation than would have been expected if the two proteins acted independently in terms of Arabidopsis development and its defence response to aphid infestation.

Chapter 6

Exogenous application of salicylic acid induces resistance to *Cucumber mosaic virus* disease in sweet pepper plants (*Capsicum annuum* var. California Wonder)

Introduction

6.1 Background to the Study

Capsicum peppers are among the most important crops cultivated around the world. They have served as food and medicine for over 50 centuries. A 100 g of pepper fruit contains as high as 180 mg of Vitamin C and 12 mg calcium (Smith, 1951; Pickersgill, 1991; Hanif et al., 2006; Ortiz et al., 2010). In 2012, an estimated area of 539,688 hectares was planted with pepper (FAOStat, 2015). Despite the usefulness and the vast area of fields dedicated to pepper production worldwide, the pepper plant is susceptible to over 35 plant viruses, including CMV. CMV is considered the most destructive of all the viruses that infect pepper plants. It causes significant yield losses in pepper production, resulting in less profits for farmers (Green and Kim 1991). In the quest to mitigate the devastating effects of CMV disease in pepper production, a collaboration with the Rural Development Administration of the Republic of South Korea was established to investigate the feasibility of inducing resistance to CMV infection in pepper through the application of exogenous SA. The objective of these experiments was to assess whether the exogenous application of salicylic acid can induce resistance to *Cucumber mosaic virus* infection in pepper.

Results

6.2.1 Mode of SA Application and CMV Inoculations

In the majority of the experiments, SA was applied as foliar sprays and/or watered to plants at the cotyledonous to first true leaf stage. Other modes of SA application included imbibition of pepper seeds in SA solutions and submersion of whole pepper seedlings into SA solutions for a specified duration (Figure 6.1).

All pepper plants that received SA treatments through foliar sprays or through both foliar sprays and watering were inoculated a day after the last SA treatment. Thus, the plants were treated with SA for three consecutive days and on the fourth day, they were inoculated with CMV. These inoculated plants were largely at their first true leaf stage. Pepper plants that were treated with SA through drenching were pricked-out into trays and watered regularly with tap water until emergence of their first true leaf after which they were inoculated. SA imbibed seeds were pre-germinated in plates lined with filter paper moistened with SA solution. The pre-germinated seeds were then pricked-out into trays and watered with tap water until full development of cotyledonous leaves before inoculation.

6.2.2 Determination of SA Concentrations Adequate for the Induction of *PR-1* Expression in Sweet Pepper

SA concentrations ranging from 0.1 mM to 10 mM SA were tested on California Wonder sweet pepper plants to identify the optimum non-phytotoxic concentrations of SA required to induce the expression of *PR-1*, a marker for SA-induced resistance. The expression of *PR-1* was primarily used to verify if the SA were being absorbed by the treated pepper plants. It was observed that pepper seeds imbibed with as low as 0.1 mM SA solution for a three-day period and subsequent pre-germination on an SA-moistened filter paper was adequate for the induction of *PR-1* in the germinated seeds (Figure 6.2; plant 1).

Pepper plants sprayed or watered with as low as 0.3 mM SA solution for three consecutive days were also found to express the *PR-1* gene. Few of the pepper plants that were not treated with SA were also shown to have been expressing *PR-1*, particularly more evident with increasing PCR cycles. For instance, plants 2 and 7 in Figure 6.2 which were not treated with SA showed faint bands with a 30 cycle PCR but when the PCR cycles were increased to 40, these plants that were not treated with SA clearly showed distinct *PR-1* band. The absence of a *PR-1* band in the 30 cycle PCR for plant 8 whose seeds were imbibed in 0.1 mM SA for a

24-hour period could partly be due a low cDNA template since the corresponding *CaActin* band is also very faint. When the PCR cycles were increased to 40, plant 8 showed a distinct *PR-1* band. Plant 4 which was sprayed with 10 mM SA, the highest SA concentration used in this experiment, showed the boldest band in both cycles.

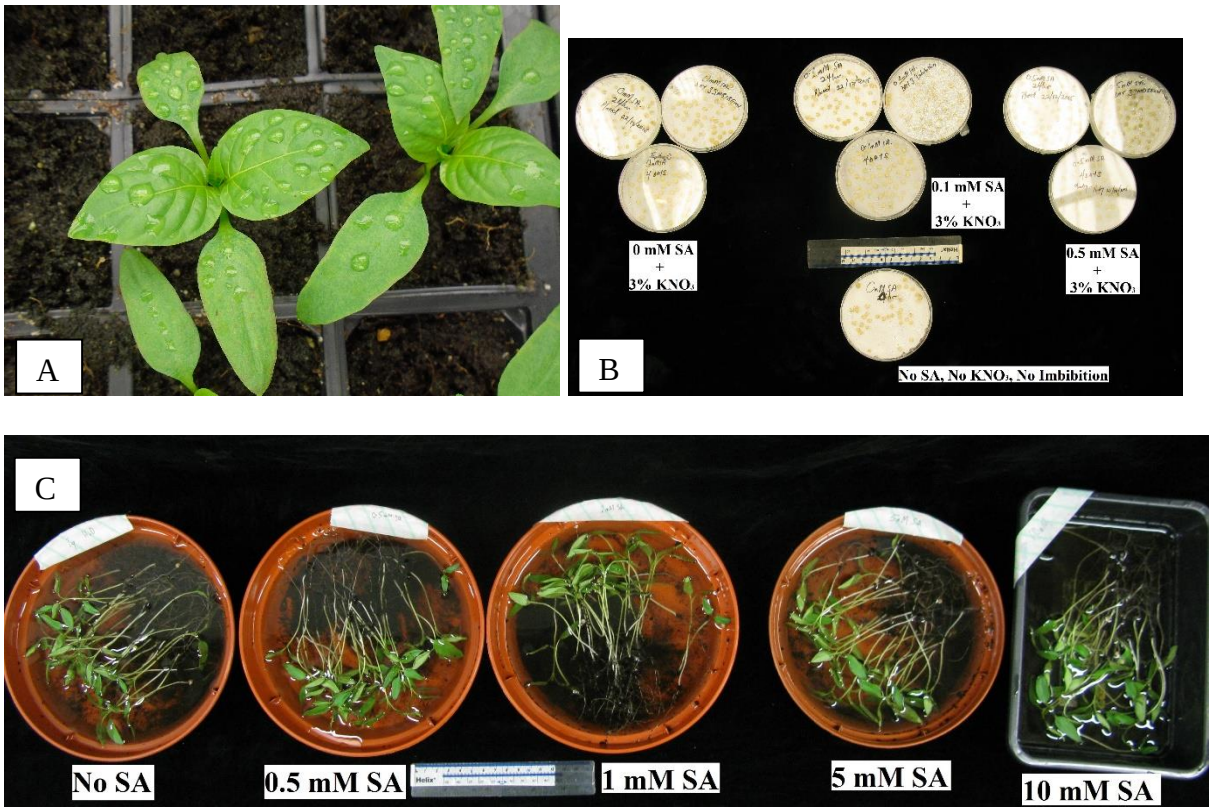


Figure 6.1: Mode of SA Application: Pepper plants were treated with SA through; A) foliar application (plants were sprayed with SA solution for 3 consecutive days); B) seed imbibition method (pepper seeds were imbibed with SA solution for either 1 day, 3 days or 4 days); and C) seedling drenching (whole pepper seedlings were drenched in SA solution for 1 hour, 30 minutes).

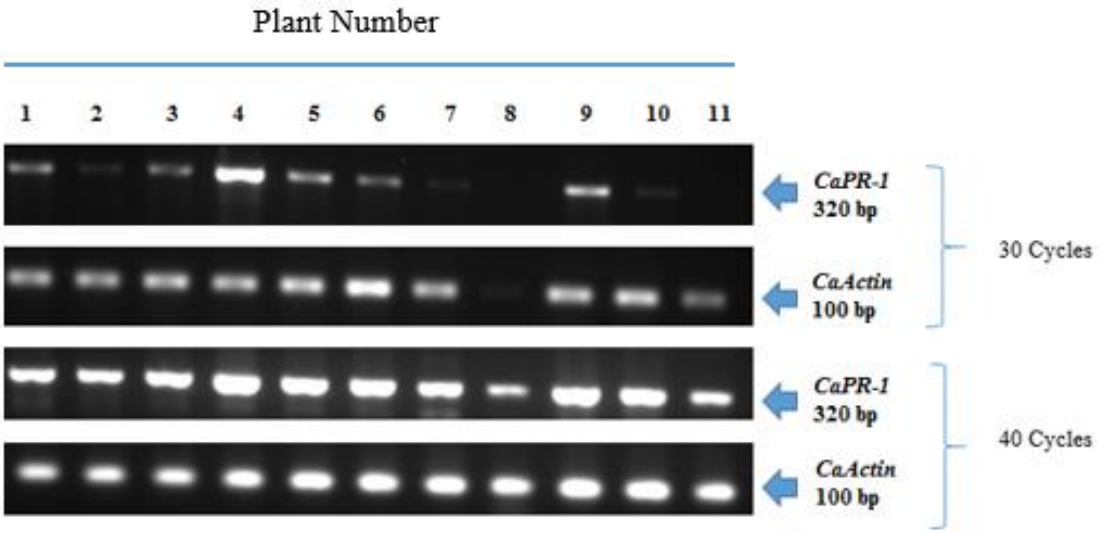


Figure 6.2: *PR-1* transcript accumulation in pepper after varying SA treatments (1) 0.1 mM SA, 17 days post 3-day seed imbibition in SA solution (2) no SA, 17 days post 3-day seed imbibition in tap water (3): 0.5 mM SA, 17 days post 3-day seed imbibition in SA solution. (4): 10 mM SA sprayed pepper plant (5) no SA treatment. (6): 0.3 mM SA spraying, 5µg/ml CMV inoculation, confirmed CMV infection. (7): no SA, 24-hour tap water imbibition. (8): 0.1 mM SA, 24-hour imbibition (9): no SA, no mock-inoculation (untouched plant). (10): 0.3 mM SA spraying, no CMV inoculation. (11): 0.5 mM SA, 24-hour seed imbibition. An RT-PCR with *CaPR1* primers The *Ca.Actin* gene was used as a control.

6.2.3 Phytoxic and Non-Phytotoxic Concentrations of SA in Sweet Pepper

The phytotoxicity of exogenously applied SA solution in sweet pepper increases significantly beyond 5 mM SA (Figure 6.3, Figure 6.5). Exogenously applied 10 mM SA solution is toxic to pepper plants, irrespective of the mode of application, whether by foliar sprays or seedling drenching. All 10 mM SA drenched seedlings withered-off a few days after drenching (Figure 6.6B). However, few 10 mM SA watered pepper plants survived and were expressing *PR-1* (Figure 6.6A and 6.2; plant 4) but they were severely stunted. The phytotoxicity of exogenously applied SA is more severe when applied as a seedling drench. Compared to the 87% survival rate of the 5 mM SA sprayed pepper plants 7 days post SA application, only 70% (69.96%) of the 5 mM SA drenched seedlings survived 9 days post drenching (Figure 6.6). However, the surviving 5 mM SA drenched seedlings were severely stunted in growth and showed strong purple coloration of leaves, possibly, a stress response manifested by accumulation of anthocyanins (Figure 6.3). Many of these surviving pepper plants withered-off afterwards.

Exogenously applied 1 mM SA is not phytotoxic to pepper plants no matter the method of application (Figure 6.4). Spraying or watering of pepper plants with 1 mM SA for 3 days before virus inoculation and even with an additional 3-day SA treatment after virus inoculations seem not to affect the growth of pepper plants. Drenching pepper seedlings in 1 mM SA also does not significantly affect growth, with the exception of slight crinkling of leaves and purple coloration on cotyledons. Few of the plants that were not treated with SA also had this purple coloration on their cotyledons as well. Exogenously applied 2 mM SA in the form of foliar sprays or through watering was also found not to be phytotoxic to pepper plants (Figure 6.4).

Figure 6.3: Pepper seedling showing stress symptoms 5 days after being drenched in 5 mM SA solution (A) in comparison to pepper seedlings drenched in tap water (B).

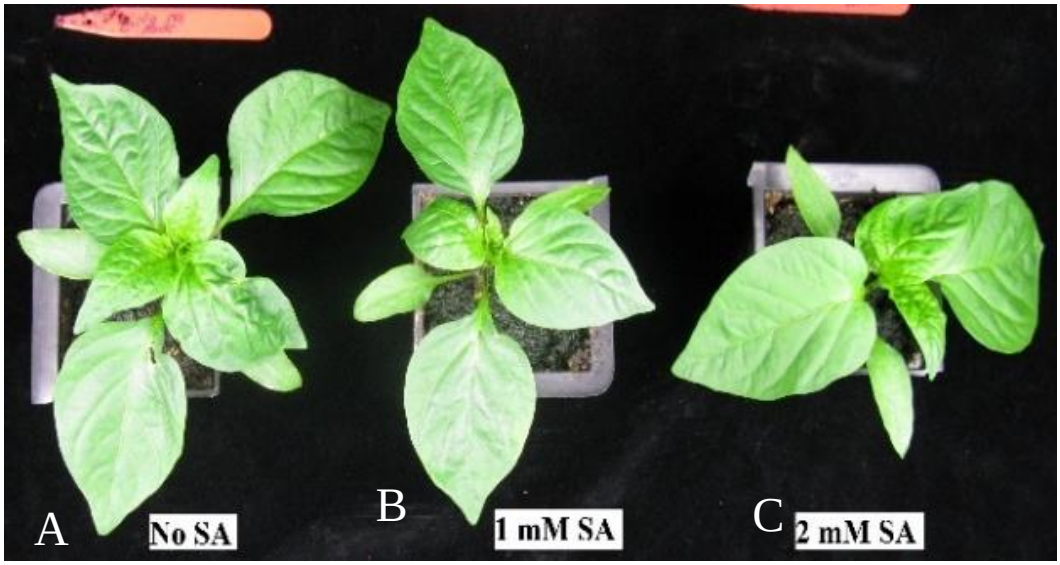
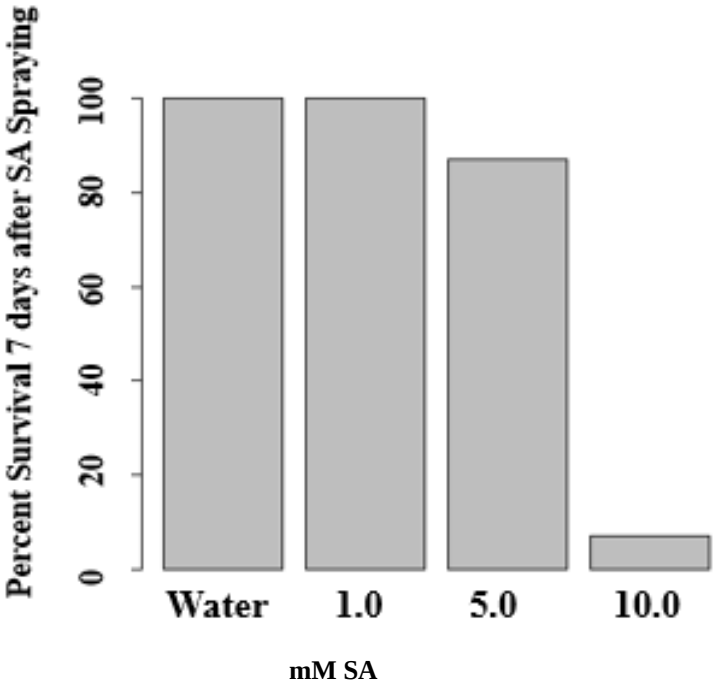


Figure 6.4: Pepper plants treated with (A) No SA, (B) 1 mM SA and (C) 2 mM SA. Exogenously applied 1 mM or 2 mM SA concentrations are not toxic to pepper plants.

Figure 6.5: Effects of salicylic acid on the morphology of pepper leaves 9 days post treatment. ((A) No SA, (B) 1 mM SA and (C) 5 mM SA). The phytotoxicity of exogenously applied SA increases significantly beyond the 5 mM concentration.

A



B

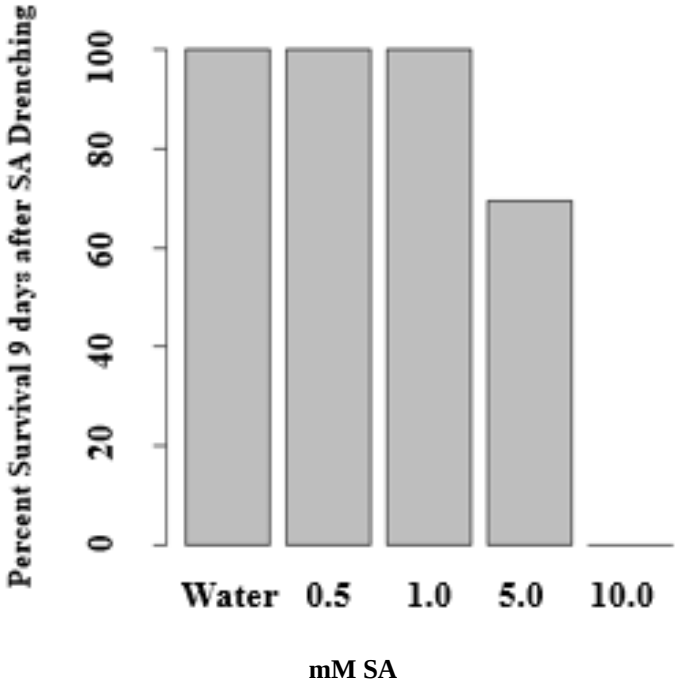


Figure 6.6: Per cent survival of pepper seedlings sprayed (A) or drenched (B) in different concentrations of SA solutions. All pepper plants that received 1 mM SA survived, irrespective of the method of application.

6.2.4 Optimization of CMV inoculum concentration

Different concentrations of purified CMV virions were inoculated on pepper seedlings to find out the minimum virus concentration needed to cause 100 % infection among pepper plants that were not treated with SA so as to attribute any differences in the level of CMV disease incidence in SA treated plants to the effect of SA. Initially, virus concentrations ranging from 0.25 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 5 µg/ml were tested.

However, there was never a 100 % CMV disease incidence in any of these virus concentrations tested. Therefore, the virus concentrations were increased to a range of 5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml. All the pepper plants that were inoculated with 25 µg/ml CMV but had not previously been treated with SA showed symptoms of CMV infection 5 days post inoculation (Table 6.1). The CMV incidence among the 1 mM SA and 2 mM SA treated plants inoculated with 25 µg/ml CMV were 57.14% and 50%, respectively at 5 days post-inoculation.

Therefore, 25 µg/ml CMV was enough to cause 100% CMV incidence among mock-inoculated plants in just 5 days post inoculations. To find out whether virus concentrations lower than 25 µg/ml CMV could also cause a 100% CMV disease incidence, virus concentrations of 10 µg/ml CMV and 15 µg/ml CMV were tested but none caused a 100 % CMV disease incidence among pepper plants that were not treated with SA. Out of the 12 plants inoculated with 15 µg/ml CMV, 8 showed CMV symptoms by 22 days post inoculation.

6.2.5 SA Induced Resistance to CMV Infection in Pepper

6.2.5.1 Comparison of CMV Disease Incidence among SA-Treated and SA-Non-Treated Plants

Spraying or watering pepper seedlings at the cotyledonous to first true leaf stage for 3 consecutive days with either 1 mM SA or 2 mM SA prior to challenge with CMV inoculations induce resistance to CMV infections even with a very high inoculum load of 100 µg/ml CMV.

Among the 15 µg/ml CMV inoculations, plants that were not pre-treated with SA before being inoculated started showing symptoms 5 days post inoculation (dpi), whilst the 1 mM

SA pre-treated plants only started showing symptoms 2 days later, thus, 7dpi when one plant became symptomatic. The number of symptomatic plants increased steadily among the plants that were not treated with SA, and by the 22 dpi, 8 out of these 12 plants inoculated with 15 µg/ml CMV were showing symptoms. Only 2 out of the 12 1 mM SA-treated plants that were inoculated with 15 µg/ml CMV showed symptoms by the 22 dpi (Figure 6.8).

In the 5 µg/ml - 200 µg/ml batch of the experiments, the level of CMV incidence recorded among the 2 mM SA:50 µg/ml CMV, 2 mM SA:25 µg/ml, 1 mM SA:25 µg/ml CMV inoculated plants were comparable to the level of incidence recorded among the plants that were not treated with SA but were inoculated with a lower CMV concentration of 5 µg/ml CMV. Apart from the 200 µg/ml inoculations where the level of CMV incidence in both the plants that were not SA-treated and the 2 mM SA pre-treated plants were similar, in all the other experiments, the plants that were not SA-treated recorded higher levels of CMV incidence than the SA treated plants by the last day (15 dpi) of CMV incidence assessment. Also, among the 5 µg/ml CMV – 200 µg/ml CMV inoculations, with the exception of the 1 mM SA: 50 µg/ml CMV inoculated plants that had a slight progression of CMV incidence throughout the course of the experiments, almost all the plants in this batch of the experiments were symptomatic between the 5 dpi to 7 dpi (Table 6.1). From the 8 dpi, an SA treated plant that had not developed symptoms were more likely not to develop symptoms by the 15 dpi, the last day of CMV incidence assessment.

6.2.5.2 Comparison of Symptom Expression Among SA-Treated and SA Non-Treated Plants

There were no major significant differences among symptomatic plants that were not SA-treated and their SA-treated counterparts. Both groups of symptomatic plants were stunted compared to their respective non-inoculated mocks (Figure 6.9.I). Both SA-treated plants and plants that were not treated with SA showed clear CMV symptoms. However, the severity of symptom expression seems to be quite moderate in some cases among SA-treated plants (Figure 6.9.II).

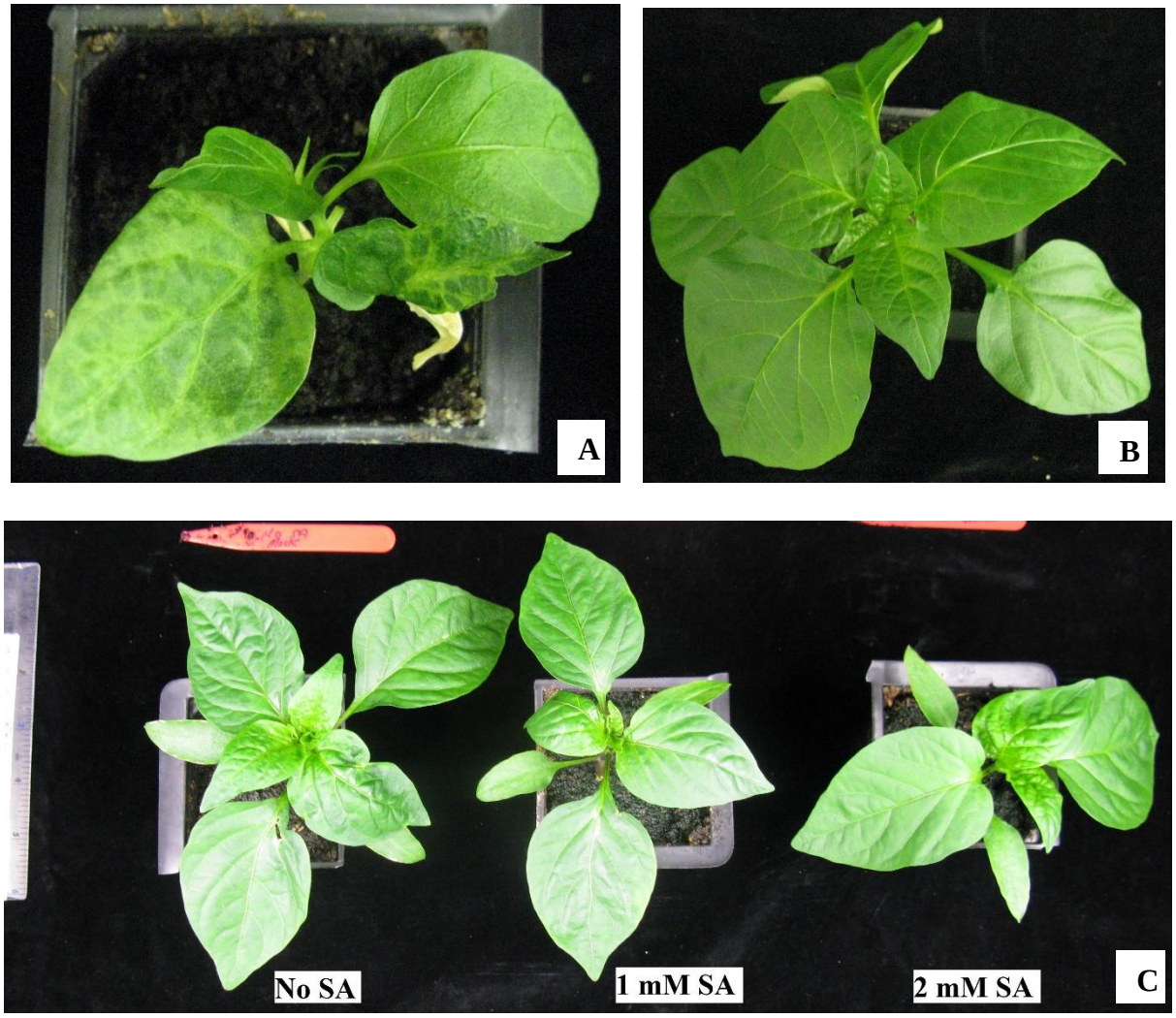


Figure 6.7: A) CMV infected 2 mM SA pre-treated plant showing slight recovery of symptoms 23 days post inoculation. B) Some of the CMV inoculated 2 mM SA pre-treated plants did not become infected or show symptoms 23 days post inoculation. C) Healthy plants with different SA-treatment regimes.

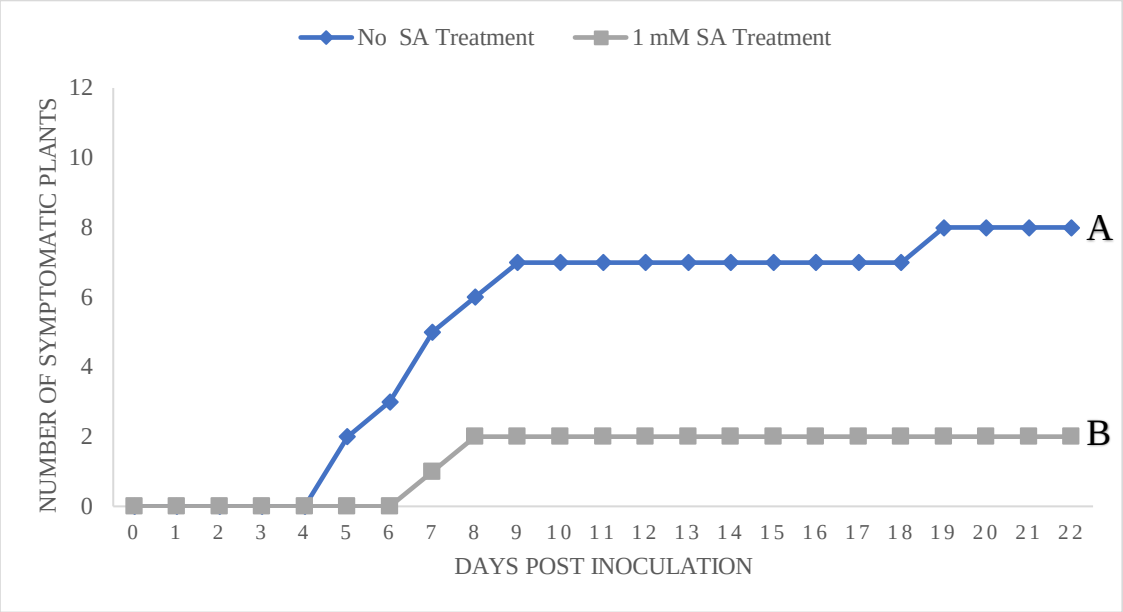


Figure 6.8: CMV-induced disease symptom incidence among (A) untreated pepper plants and (B) 1 mM SA treated pepper plants. Plants were scored as symptomatic when they displayed mosaic symptoms.

Table 6.1: CMV disease incidence among pepper plants inoculated with 5 µg/ml CMV-50 µg/ml CMV. Prior to inoculations, plants were treated with either no SA, 1mM SA or 2 mM SA

SA Concentration (mM)	Plant Total	Number of Symptomatic Plants (Days Post Inoculations)										
		5	6	7	8	9	10	11	12	13	14	15
	5µg/ml CMV											
0	**13**	7	7	7	7	7	7	7	7	7	7	7
1	**14**	3	4	4	4	4	4	4	4	4	4	4
2	**15**	1	1	1	1	1	1	1	1	1	1	1
	25 µg/ml CMV											
0	**13**	13	13	13	13	13	13	13	13	13	13	13
1	**14**	8	8	8	8	8	9	9	9	9	9	9
2	**14**	7	7	7	7	7	7	7	7	8	8	8
	50 µg/ml CMV											
0	**10**	10	10	10	10	10	10	10	10	10	10	10
1	**14**	7	7	8	9	10	11	11	11	11	11	11
2	**13**	8	8	8	8	8	8	8	8	8	8	8

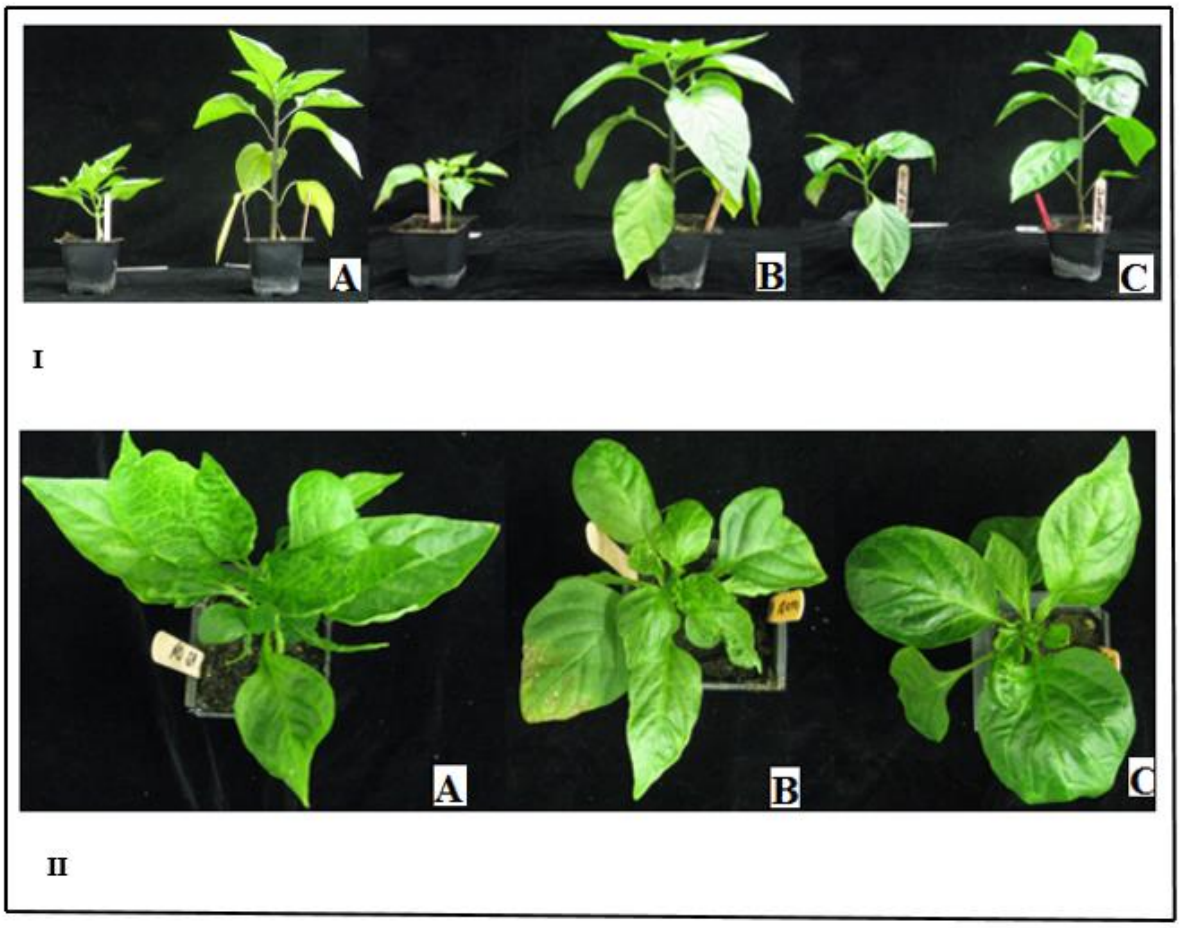


Figure 6.9: Comparison of CMV symptoms among SA-treated and SA-non-treated plants 27 dpi. I. (A) no SA, 10 µg/ml CMV inoculated plant in comparison to mock-inoculated, (B) 1 mM SA treated, 10 µg/ml CMV inoculated plant in comparison to mock-inoculated, (C) 2 mM SA treated, 10 µg/ml CMV inoculated plant in comparison to mock-inoculated. II. (A) up-close display of symptoms observed on no SA, 10 µg/ml CMV inoculated plant, (B) up-close display of symptoms observed on 1 mM SA, 10 µg/ml CMV inoculated plant, (C) up-close display of symptoms observed on 2 mM SA, 10 µg/ml CMV inoculated plant.

6.3 Discussion

Exogenously applied SA induces *PR-1* expression and resistance to CMV infection in pepper plants. The mere induction of *PR-1* proteins does not necessarily correlates to resistance to CMV disease in pepper as some of the CMV infected plants (Figure 6.2; plant 6) were also shown to have an induced *PR-1* gene expression despite being infected. Although, plant 6, in particular, was watered and sprayed with 0.3 mM SA prior to being inoculated which may be responsible for the *PR-1* expression, virus infections in themselves are known to elicit the induction of a key enzyme in the phenylpropanoid biosynthetic pathway of SA, benzoic acid 2-hydroxylase (Delaney, 2010). Therefore, it is possible for CMV infected plants to express *PR-1* even without prior SA treatment due to the ability of the virus to induce SA biosynthesis. However, exogenous application of SA which occurs prior to infection can enhance resistance to subsequent virus infection. CMV-elicited SA accumulation lags behind the systemic spread of the virus and hence may not be effective in inducing resistance to the virus infection (Carr et al., 2010). The timing and quantity of *PR-1* expression may be more critical to the level of SAR induced after exogenous SA application. Plants expressing SAR are known to respond swiftly to microbial or abiotic stressors compared to non-induced plants. For instance, cucumber plants expressing SAR lignifies more rapidly in response to wounding than non-SAR expressing plants (Kessmann et al., 1994; Hammerschmidt, 1999).

The induction of *PR-1* in plants that were not treated with SA may also be as a result of the close proximity among the experimental plants. Pathogen infected plants or plants expressing SAR are known to release the volatile organic compound methyl-SA. This airborne volatile organic compounds could be perceived by neighbouring plants, where the endogenous methyl-SA transferase in the receiving plants catalyses the conversion of the methyl-SA back to free SA (Durner et al., 1997; Hayat et al., 2010). Hence, potential methyl-SA emitted by the SA-treated or CMV-infected plants may be responsible for the induction of *PR-1* in the neighbouring SA-non-treated plants. The faint *PR-1* bands in the non-SA treated plants (plants 2 and 7) compared to the very bright and bold *PR-1* band of the 10 mM SA treated plant (plant 4) especially with the 30 cycle PCR may suggest that *PR-1* induction in pepper is SA concentration dependent. The higher the concentration of SA applied to a pepper plant, the more *PR-1* the plant expresses.

Based on the various SA/CMV treatment combinations, it could be stated that SA-induced resistance to CMV infection in pepper possibly occurs in two possible ways. Firstly, by

causing a delay in the appearance of symptoms in the induced plants. In the 15 µg/ml CMV:1 mM SA experiment, SA treatment clearly delayed the appearance of first symptoms by 2 days. Also, among the 50 µg/ml inoculated plants, whilst all 10 SA-non-treated plants were showing symptoms by 5 dpi, only 7 out of the 14 plants that were treated with 1 mM SA were showing symptoms by the 5 dpi. The number of symptomatic plants among the 1 mM SA treated plants that were inoculated with 50 µg/ml CMV gradually increased from 7 plants by 5 dpi to 11 plants by the 10 dpi, showing a clear delay in symptom appearance in comparison with the plants that were not treated with SA.

A similar delay in the development of symptoms among SA-treated plants has also been observed in a compatible CMV-*Nicotiana tabacum* (tobacco) pathosystem (Naylor et al., 1998). In the CMV-tobacco pathosystem, the delay in the appearance of symptoms among the SA-treated plants was found to be as a result of a delay in the movement of the virus out of the inoculated leaf. Whilst the CP of CMV was detectable in all tissues of tobacco plants that were not treated with SA by the 8 dpi, the CMV CP only became detectable systemically in SA-treated plants 2 days later (Naylor et al., 1998).

A second possible way SA treatment may have caused resistance to CMV infection is by enhancing the ability of the induced plants to totally avoid infection (immunity) or symptom development. With the exception of the 200 µg/ml inoculations where the extremely high viral load might have overwhelmed the SA-induced resistance, in all other inoculations, the SA-treated plants recorded lower CMV incidence compared to the plants that were not treated with SA. This shows that the treatment of pepper plants with SA in some cases induces resistance strong enough to resist or tolerate CMV infection. Compared to an approximately 54% CMV incidence among plants inoculated with 5 µg/ml CMV without prior SA treatment, only 29% and 7% of the plants that were treated with 1 mM SA and 2 mM SA, respectively, were symptomatic 15 dpi. There were also similar observations among the 25 µg/ml CMV, 50 µg/ml CMV and 100 µg/ml CMV inoculated plants as well. During the previous study in my research group on the feasibility of exogenously applied SA in CMV pathosystem in pepper production, it was observed that floating California Wonder pepper leaf discs in 1 mM SA solutions for 3 consecutive days prior to CMV inoculations reduces the accumulation of CMV RNA in the SA-treated leaf discs during subsequent

inoculations with CMV, suggesting an effect on replication or cell-to-cell spread (Mayers, 1999).

6.4 Conclusions

SA treatment of pepper plants induces *PR-1* expression and also enhances the ability of pepper plants to resist CMV infection. However, SA treatment or the expression of *PR-1* in SA-treated plants does not always correlate with resistance to CMV infection, which is perhaps not surprising since PR-1 is not an antiviral factor (Carr et al., 2010). SA concentrations beyond the 5 mM threshold are phytotoxic to pepper plants. Expression of SAR in pepper is still slightly noticeable among 1 mM SA and 2 mM SA treated plants inoculated with 100 µg/ml but not with 200 µg/ml CMV inoculations. The absence of significant SAR manifestation among 200 µg/ml CMV inoculations could be due to a possible breakdown of the resistance due to the enormous viral load. Optimum resistance induction to CMV infection in pepper is only achieved after attaining the right SA concentration-CMV inoculum load treatment combinations.

Chapter 7

Final Discussion

7.1 Overall Conclusion

Plant virus diseases are a major threat to global food security. Two of the most efficient ways to mitigate crop losses caused by virus diseases are through enhancement of the plant's ability to resist the virus infection and secondly controlling the vectors that transmit these viruses to crop plants. The role of SA application in inducing resistance to CMV infection in pepper plants and the basis of the heightened resistance to aphid infestations in Arabidopsis *ago1.25* mutant plants were examined in this study. Exogenous application of 1 mM SA on pepper plants prior to CMV inoculation was found to delay the onset of CMV symptoms and in general the level of CMV disease incidence in pepper plants. SA but not 4-OH-ICN contributed to the heightened resistance to *Myzus persicae* in Arabidopsis *ago1.25* mutant plants.

7.2 Mutation in 4-OH-ICN biosynthetic genes *Cyp82C2* or *Fox1* does not affect the heightened resistance to aphids observed in Arabidopsis *ago1.25* mutant plants

The use of cyanogenic metabolites among plants to deter insect herbivory is widespread. More than 2,600 species of plants can synthesize various forms of cyanogenic glucosides that require activation by specific glucosidases to be potent against insect herbivory (Ganjewala et al., 2010). Since the feeding behaviour of aphids does not cause significant tissue disruption needed to efficiently activate many cyanogenic glycosides, these metabolites are not particularly effective against aphid infestation (Gleadow and Woodrow, 2002). In 2015 when the first cyanogenic metabolite in the *Brassicaceae* family was discovered in Arabidopsis and was found to spontaneously release HCN without the need for its activation by glycosidases, its role in the heightened resistance to aphids in Arabidopsis *ago1.25* mutant plants was investigated.

Mutation in the cytochrome P450 gene *Cyp82C2*, which encodes an indolic hydroxylase that catalyses the hydroxylation of ICN into 4-OH-ICN, did not result in significant changes in

either aphid growth rates or colony sizes. A similar observation was also observed with *fox1* mutant plants, an additional 4-OH-ICN biosynthetic pathway mutant plant. Details of these observations can be found in Chapter three. Three possible reasons may explain why mutation in the 4-OH-ICN biosynthetic genes *cyp82C2* or *fox1* did not significantly affect aphid performance.

Firstly, mutation in *Cyp82C2* may be compensated by other isoforms of the Cyp82C subfamily of cytochrome P450s present in the Arabidopsis genome. The *cyp82C2* mutant line used in this study is devoid of any 4-OH-ICN biosynthesis after *P. syringae* infection (Rajniak et al., 2015). However, this experiment was based on aphid infestation of Arabidopsis plants and not *P. syringae* infection. *Myzus persicae* infestation-induced transcriptome in *cyp82C2* mutant plants may differ from *P. syringae* infection-induced transcriptome in *cyp82C2* mutant plants. In all, there are over 245 genes for cytochrome P450 enzymes in the Arabidopsis genome. They catalyse a diverse range of biochemical processes ranging from the synthesis of signalling molecules such as JA, gibberellic acid (GA), abscisic acid (ABA) to the production of defence metabolites such as terpenes, glucosinolates and cyanogenic glucosides. Among the defence roles played by cytochrome P450s is their involvement in the biosynthesis of metabolites directed against insect herbivory. For instance, the conversion of S-dihydrocamalexic acid into camalexin relies on the cytochrome P450 Cyp71b15 (Schuler et al., 2006). The emission of the aphid repellent and parasitic mite attractant (E)- 4, 8- dimethyl- 1, 3, 7, -nonatriene volatile organic compound upon tissue damage requires the cytochrome P450 Cyp82G1 (Lee et al., 2010). The biosynthesis of many aliphatic and indolic glucosinolates also rely on specific subfamilies of cytochrome P450s of which usually one isoform of the enzyme may be substituted by another in the biosynthesis of the metabolite (Schuler et al., 2006; Rajniak et al., 2015).

Both Cyp82C2 and Cyp82C3 are isoforms of cytochrome P450 Cyp82C subfamily which are members of the dicots only cyp82 family of cytochrome P450s (Werck-Reichhart et al., 2002; Lee et al., 2010). Cyp82C2 and Cyp82C3 share 86% identity in their amino acid sequences and therefore it is plausible, in the absence of Cyp82C2, Cyp82C3 could catalyse the conversion of ICN into 4-OH-ICN.

In this study it was observed that mutation in *Cyp82C2* results in the constitutive expression of the normally inducible *Cyp82C3* gene. Both *Cyp82C2* and *Cyp82C3* are inducible by aphids. Since *Cyp82C3* is already constitutive in a non-infested *cyp82C2* mutant plant, aphid

infestation of *cyp82C2* mutant plants results in even higher expression of *Cyp82C3* in the *cyp82C2* mutant plants (Chapter 3 subsection 3.4.5). Therefore, the enhanced expression of *Cyp82C3* in the absence of *Cyp82C2* could compensate for the loss of CYP82C2 if both isoforms of CYP82C can equally hydroxylate ICN at the fourth carbon atom to produce 4-OH-ICN. Alternatively, Cyp82C4 which also share 88% identity to Cyp82C2 in terms of amino acid sequences could also compensate for the mutation in *Cyp82C2*. In these scenarios, mutation in *Cyp82C2* will not abolish aphid induced biosynthesis of 4-OH-ICN and therefore aphid resistance in the *cyp82C2* mutant plants will not differ from resistance to aphids in WT plants. A quantitative measurement of 4-OH-ICN metabolite by high performance liquid chromatography in an aphid infested *cyp82C2* mutant plants will be conclusive in deciphering whether other Cyp82C isoforms compensate for the mutation in Cyp82C2 in the aphid-4-OH-ICN system or 4-OH-ICN as a metabolite does not play a significant role in aphid resistance.

Secondly, since Arabidopsis is a natural host of *Myzus persicae* it is possible that years of infestations and co-evolution of the host with the insect vector may have resulted in the tolerance of the aphid to the 4-OH-ICN metabolite. The aphid could potentially eliminate any ingested 4-OH-ICN metabolite through its honeydew without metabolising it probably due to unfavourable midgut pH required for the release of the HCN or the absence of appropriate enzymes needed for the recognition of the 4-OH-ICN catabolic products. The ability to selectively eliminate specific compounds from their diet is known among various aphid species. For instance, *Myzus persicae* can excrete ingested polar cardenolides from their body through their honeydew (Zust and Agrawal, 2016).

Finally, the amount of 4-OH-ICN produced by Arabidopsis upon aphid infestation may not be high enough to cause any significant changes in the performance of the aphid. Therefore, its absence may also cause subtle or no change in the growth or reproduction of the aphid. Assessing the performance of aphids on *Cyp82C2* over-expressing lines may help decipher whether changes in the level of 4-OH-ICN biosynthesis may affect aphid performance in Arabidopsis.

7.3.1 Mutation of the *ICS1* gene in *ago1.25* mutant plants restores aphid susceptibility and lessens the extent of aphid infestation-induced stunting in the *ago1/ics1* double mutant plants.

Infestation of *ago1.25* mutant plants results in severe stunting, accelerated senescence of the aphid infested older leaves and poor aphid performance in comparison to a WT plant or an *ics1* mutant plant. Despite the large aphid colony development on WT plants or *ics1* mutant plants, aphid infestation-induced loss of biomass in these plants is moderate. The role of the SA biosynthetic gene *ICS1* in aphid-induced loss of biomass was then investigated. Aphid performance assays and the assessment of aphid induced loss of biomass in the *ago1/ics1* indicated that *ICS1* is required for both the stunting and the heightened resistance to aphids in *ago1.25* mutant plants (Chapter 4; subsections 4.3.4, 4.3.5). Interestingly, the *ICS1* gene was also required for the characteristic phenotype of *ago1* mutant plants (Chapter 4 subsections 4.3.3). Some of the *ago1/ics1* plants were vigorous in growth and were larger in size. However, not all the progeny of *ago1/ics1* plants had a recovery-like or a complete recovery of their *ago1* phenotypes. The *ago1/ics1* progeny that retained *their ago1-like* phenotypes also retained their heightened resistance to aphid infestation. Sequencing of a selection of the *ago1/ics1* progeny indicated that the phenotype of an *ago1/ics1* plant could not always be explained by their *ago1* and *ics1* genotypes. A cross between *nahG* transgenic plant and *ago1.46* mutant plant confirmed that SA accumulation may be important in the *ago1* phenotype. However, expression levels of the SA accumulation marker *PR-1* in *ago1.25* mutant plants were lesser than the expression levels of *PR-1* in a WT plant. On the contrary, genes involved in auxin response and signalling were rather upregulated in the *ago1* mutant plants and *ago1/ics1* progeny (Chapter 4 subsection 4.4.1).

7.3.2 The *ago1/ics1* mutant plants phenocopy *jaw 1 D* mutant plants and may have similar genetics

Striking similarities were found between the *ago1/ics1* progeny with enhanced growth and larger plant size (recovery-like phenotypes) and the phenotype of the *jagged and wavy dominant* (*jaw 1D*) mutant plants. The heavy serration, wavy margins and the rolling of the leaves of adult *ago1/ics1* double mutant plants phenocopy the *jaw 1D* mutant plant (Chapter 4; subsection 4.3.3). The mutant *jaw 1D* is a dominant T-DNA insertion mutant that accumulates over 90-fold more *mir319* in comparison to a WT plant (Liu et al., 2010). *mir319* regulates auxin response factors such as *ARF16* and a group of five *Cincinnata-like*

Teosinte Brandeal/Cicadoidea/Proliferating cell factors (*TCPs*) that repress the cell proliferation activities of *Knotted-like homeobox*-1 (*Knox1*) genes particularly at the marginal regions of the leaf (Shani et al., 2009).

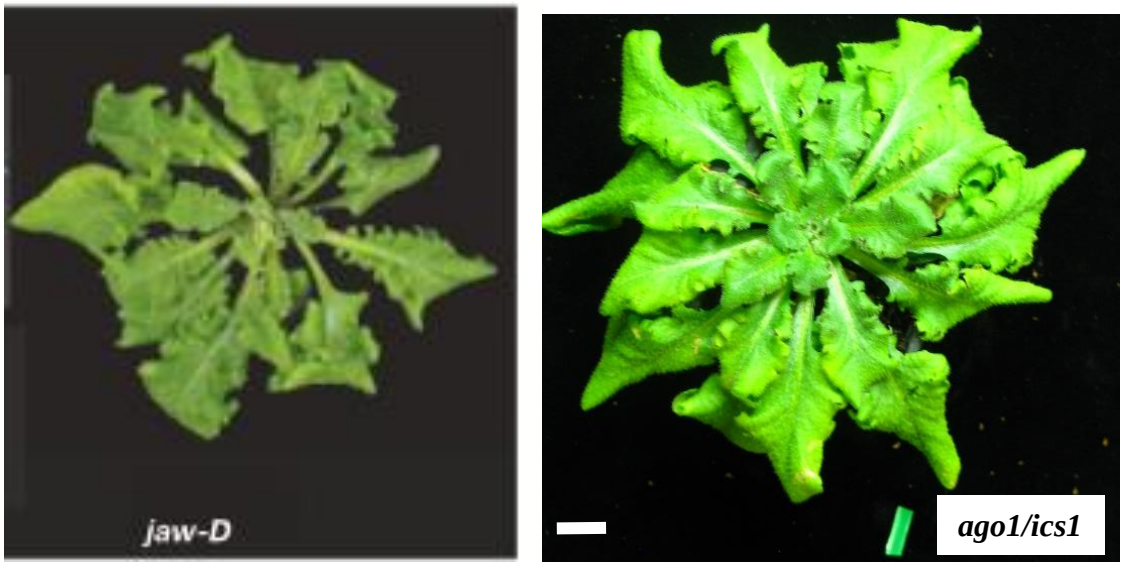


Figure 7.1: The *miR319* overexpressing mutant *jaw 1D* has similar phenotype as the majority of the *ago1/ics1* double mutant plants (*jaw-1D* photo from Palatnik et al., 2003) (Scale bar = 1cm).

There are four *Cin-like* TCPs in Arabidopsis namely; *Brevipedicellus* (*BP*), *Knotted-like from Arabidopsis thaliana 2* (*KNAT2*), *KNAT6* and *Shoot meristemless* (*STM*). The role of these *Cin-like* TCPs is to precondition cells particularly at the marginal regions of the leaf to be more sensitive to the cell arrest front in the developing leaf. In the *jaw 1D*, the enhanced upregulation of *mir319* results in the depletion of *Cin-like* TCPs therefore cells particularly at the marginal regions where intense activity of TCPs occurs proliferate to levels that cannot be accommodated by the basic structure of the leaf resulting in larger leaf with buckled and wavy leaf margins as a mechanism to contain the extra mediolateral cell proliferations (Figure 7.1) (Nath et al., 2003; Shani et al., 2009; Jiang et al., 2018). The *ago1/ics1* progeny with *jaw 1D-like* phenotype are initially very vigorous in growth and larger in size than WT plants. This vigorous growth could possibly result in excessive proliferation of cells in both the mediolateral and proximal-distal axes of the *ago1/ics1* progeny leaf laminar beyond the limit that could be accommodated by the basic structure of the leaf leading to the formation of larger and wavy leaves (Chapter 4; subsection 4.3.3).

Another parallel between the *jaw 1D* mutant and the *ago1/ics1* progeny is their non-Mendelian inheritance patterns and the discrepancies between their genotypes and expressed phenotypes. A cross between *jaw 1D* and a mutant of the polycomb group protein *Curly leaf* (*CLF)* encoding gene, which also represses *Knox1* genes results in F_1 progeny that are all WT-looking. Selfing the heterozygous *jaw ID* +/- *clf-29* +/- F_1 plants results in a segregating F_2 population of plants that are also all WT-looking irrespective of their genotypes. Selfing the WT-like F_2 *jaw 1D* mutants (denoted as *jaw 1D**) for four consecutive generations still results in progeny that are all WT looking despite the intact *jaw 1D* mutation (Figure 7.2.). The WT-like phenotype of the *jaw 1D** progeny is as a result of *clf-29* induced epigenetic silencing of the *jaw 1D* mutation such that *mir319* levels in the *jaw 1D** plants are comparable to that of a WT plant hence the WT-like phenotype of *jaw 1D** plants despite their *jaw 1D* genotype. The *clf-29* induced silencing of the *jaw 1D* mutation is stable enough to be inherited in subsequent generations even in the absence of the *clf-29* mutation (Jiang et al., 2018).

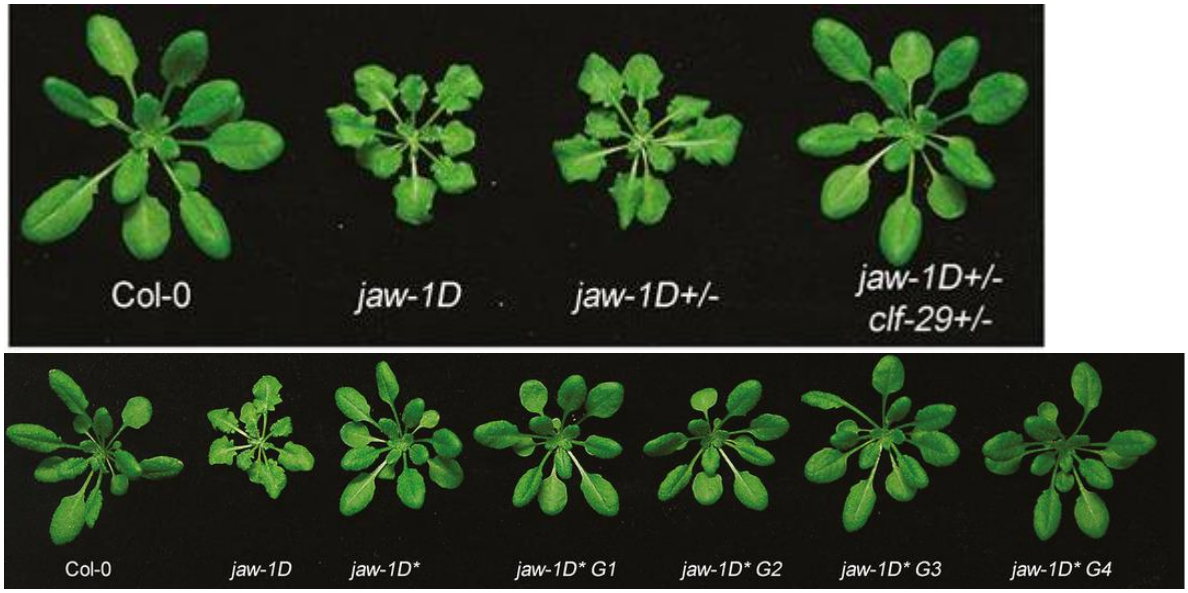
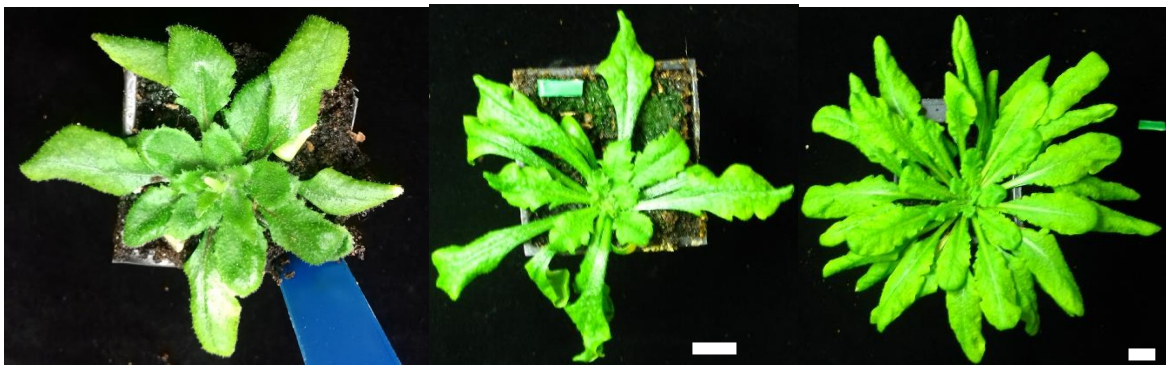


Figure 7.2: Mutation in the *CLF* gene silences the *jaw-1D* mutation. The silenced *jaw-1D* mutation (*jaw 1D**) is heritable irrespective of the presence of the *clf* mutation (From Jiang et al., 2018).

In the *ago1* x *ics1* cross there were progeny whose phenotypes could not be explained by their genotypes. For instance, the F_3 AS14 *ago1/ics1* plant had a WT-like phenotype (Figure 7.3) despite having the exact genetic identity as *ago1.25* mutant plant in terms of the *AGO 1* and *ICS*1 gene (Figure 7.3). Since the AS14 plant has a mutated *AGO1* gene and a WT *ICS1* gene, it was expected to resemble *ago1.25* mutant in phenotype but rather it was more WT-like than *ago1-like* in appearance. Selfing the AS14 plants resulted in progeny the majority of which were WT-like and few of the progeny having *ago1*-like phenotypes (Figure 7.4). The progeny of AS14 plants were not used in the aphid experiments.



AS 14 ***ago1.25*** **Col-0 WT**

Figure 7.3: Both *ago1/ics1* progeny AS14 and *ago1.25* mutant plant have the same mutation in their *Ago1* gene and a WT *ICS1* gene but they differ in their phenotypes. (Scale bar = 1cm).

Figure 7.4: Progeny of *ago1/ics1* plant AS14. The majority of AS14 progeny have WT-like phenotypes with few *ago1-like* individuals among the progeny (Scale bar = 1cm).

However, among the AS17 and AS20 progeny that were used in the aphid performance assay there were also plants whose phenotypes could not be explained by their genotypes. It is possible the *ics1* allele is paramutagenic to the *ago1* allele such that when the two mutations occurred together in the F_1 *ago1/ics1* +/- the *ics1* mutation induced epigenetic modifications of the *ago1* allele which boosted the residual activity of expressed *ago1* epiallele in the *ago1/ics1* background. However, the newly generated *ago1* epiallele was stable enough to be inherited in subsequent generations irrespective of the mutation status of the paramutagenic *ics1* allele. The reappearance of *ago1/ics1* progeny with *ago1-like* phenotypes indicate that in certain instances the *ago1* epiallele loses it epigenetic modification and revert to its unmodified mutated state. Variations in epigenetic modifications are known to contribute to a diversity of phenotypes and nutritional qualities of plants with the same genotypes (Pignatta et al., 2014; Quadrana et al., 2014). Also, the presence of both *ago1* and *ics1* on the same arm of Arabidopsis chromosome 1 may contribute to the complexities of their genetics and the disparities in their phenotypes since all the F_2 *ago1/ics1* plants were products of two independent recombination events.

7.4 Exogenous application of salicylic acid induces resistance to *Cucumber mosaic virus* disease in pepper plants (*Capsicum annuum* var. California Wonder)

SA is an important secondary metabolite essential for plant growth and defence against pests and pathogens (Malamy and Klessig, 1992; Hayat et al., 2010) In many incompatible host-pathogen interactions, such as in TMV resistant-tobacco and TMV pathosystem, endogenous SA levels increase by 20-fold upon perception of the virus (Malamy et al., 1990). Increased resistance to pathogens is mostly associate with enhanced accumulation of SA (Malamy and Klessig 1992). Therefore, the effect of exogenously applied SA on the ability of pepper plants to resist CMV infection was investigated. Exogenous application of SA on pepper plants prior to CMV infection induced the expression of *PR-1* and enhanced the resistance of the pepper plants to the CMV infection (Chapter 6; subsection 6.2.5). However, SA treatment or the expression of *PR-1* in SA-treated plants does not always correlates with resistance to CMV infection. Exogenously applied 1 mM - 2 mM SA is adequate to induce resistance to CMV infection in pepper. However, 2 mM SA tend to be associated with slight crinkling of the pepper leaves. SA concentrations beyond the 5 mM concentration threshold is phytotoxic to pepper plants (Chapter 6: Section 6.2.3). The resistance to CMV infection observed among

the SA treated plants occurred through delay of symptom induction and in few cases avoidance of symptoms or infection by the induced pepper plants.

7.5 Mutation in *AGO1* gene enhances the resistance to aphid infestations observed in Arabidopsis *2b* transgenic plants

The CMV 2b protein directly interacts with and inhibits AGO1-mediated RNA cleavage in Arabidopsis (Gonzalez et al., 2010; Duan et al., 2012; Gonzalez et al., 2012). Mutation in the 2b protein affects its efficiency to suppress RNA silencing (Goto et al., 2007). Therefore, I sought to investigate whether mutant AGO1 protein will also affect the resistance to aphids observed in *2b* transgenic Arabidopsis plants. Resistance to aphids in the *2b/ago1* cross was higher than would have been expected if both mutations acted in independent pathways of aphid resistance. There was a deleterious synergistic interaction between the AGO1 and 2b proteins in relations to aphid resistance indicating that both proteins play a role in a common pathway of induced resistance to aphid infestations. Previous experiments in our lab indicated that AGO1 may potentially downregulate a 2b induced toxic metabolite against aphid infestation (Westwood et al., 2013). In line with this, a hypomorphic AGO1 will even be less effective in downregulating the 2b induced toxic metabolite against aphid infestation hence resulting in the observed heightened resistance to aphids in the progeny of the *2b/ago1* cross.

The *2b/ago1* crosses were also severely deformed in comparison to either of their *ago1.25* mutant or *2b*-transgenic parental plants. The deleterious epistatic interactions between the 2b protein and the ago1 protein may have contributed to the severity of the *2b/ago1* developmental deformities.

7.6 Future work

7.6.1 Measuring 4-OH-ICN levels in *cyp82C2* mutant plants

Measuring levels of 4-OH-ICN in the *cyp82C2* mutant plants and also in WT plants will indicate whether the *cyp82C2* mutant plant is completely devoid of all 4-OH-ICN metabolite after aphid infestation. Quantitative levels of 4-OH-ICN in the *cyp82C2* mutant plants will then clarify whether the observed performance of aphids on the *cyp82C2*, *fox1*, *ago1/cyp82C2* and *fox1/ago1* were due to non-involvement of 4-OH-ICN metabolite in aphid resistance or rather the biosynthesis of 4-OH-ICN was compensated by enzymes similar to

the mutated or knock-out 4-OH-ICN biosynthetic enzymes. Aphids could then be fed on synthesized 4-OH-ICN to assess the effect the artificial diet will have on the performance of the aphids.

7.6.2 Does *Cyp82C3* compensate for the loss of *Cy82C2?*

Mutation in *Cyp82C3* alone does not affect aphid growth or reproduction possibly due to compensation of the absence of *Cyp82C3* by *Cyp82C2*. The cytochrome P450 encoded by both genes share 86% identity in their amino acid sequences and may potentially act as substitute enzymes in the absence of the other. Generating a *cyp82C2/cyp82C3* double mutant plant to assess the performance of aphids on this double mutant plant and possibly introducing the mutations into *ago1* background will aid in deciphering whether both enzymes can compensate for the absence of the other.

7.6.3 Is accumulation of SA directly responsible for the *ago1* phenotype?

Accumulation of SA in most cases results in enhanced expression of *PR-1* transcripts. The *ago1/nahG* cross indicates that SA accumulation plays a role in the phenotype of *ago1* mutant plants. However, *PR-1* expression is lower in *ago1* mutant plants than a WT plant. Therefore, it will be prudent to quantitatively measure the levels of SA accumulation in *ago1.25* mutant plants to indicate the extent of its SA accumulation and whether accumulation of SA in the *ago1* mutant plant does not result in the expression of *PR-1*.

7.6.4 Does exogeneous SA application reverts *ago1/ics1* progeny with recovery-like phenotypes into *ago1-like* phenotypes?

Another method of assessing if accumulation of SA is responsible for the *ago1* phenotype is to spray and/or water *ago1/ics1* seedlings that have recovery-like phenotypes with SA and observe if there will be any significant changes in the phenotypes of the *ago1/ics1* plants. A reversion of phenotype will be a good indication that SA accumulation is important to the Arabidopsis *ago1* phenotype.

7.6.5 Basis of the spontaneous necrotic lesion formation in *ago1/ics1* plants

The vigorous growth of *ago1/ics1* plants is halted by the 8 weeks post planting due to the spontaneous onset of necrotic lesions on the leaves of the *ago1/ics1* plants. All progeny of *ago1/ics1* plants including plants with recovery-like phenotypes and those with *ago1-like* phenotypes undergo varying degrees of this spontaneous necrotic lesion formation. It will be interesting to investigate the mechanism behind the sudden onset of necrotic lesions on the *ago1/ics1* progeny and how they reduce plant growth vigour.

7.6.6 Basis for the disparities in the phenotypes of *ago1/ics1* plants

Epigenetic modifications and DNA damage repair mechanisms have been proposed to partly account for the observed disparities between the phenotypes and genotypes of *ago1/ics1* progeny. Examining the expression levels of DNA damage repair markers such as *At.MSH2* and *At.ATM* and the use of appropriate DNA methylation assays to assess the methylated statuses of the *ago1* and *ics1* alleles will be beneficial in deciphering whether epigenetics or DNA damage repair contribute to the genetics of the *ago1/ics1* progeny.

7.6.7 Is the accumulation of auxins responsible for *ago1/ics1* phenotypes?

The enhanced expression of auxin response genes involved in auxin signalling indicates that de-repressed auxin signalling and auxin accumulation in the *ago1/ics1* progeny could directly be responsible for the vigorous growth observed in *ago1/ics1* seedlings and juvenile vegetative stage plants. Although auxin response factors and genes involved in auxin signalling are also upregulated in the *ago1.25* mutant plants they do not result in enhanced growth of the mutant. A preliminary quantitative measure of free indole acetic acid in both the *ago1/ics1* and *ago1* mutant plants will indicate if auxin levels in these mutants are higher than levels of auxin in WT or *ics1* mutant plants.

List of References

Abercrombie, J.M., Halfhill, M.D., Ranjan, P., Rao, M.R., Saxton, AM., Yuan, J.S., Stewart, C.N. (2008). Transcriptional responses of *Arabidopsis thaliana* plants to As (V) stress. *BMC Plant Biology*, **8**:87.

Adams, R.G.J., Lilly J.H., Gentile, A.G. (1976). Evaluation of Some Insecticides in Controlling Aphids and Reducing Flower Losses from Virus Disease in Gladiolus Plantings. *Journal of Economic Entomology*, **69**(2): 171-172.

Agrios, G.N., Walker, M.E., Ferro, D.N. (1985). Effects of *Cucumber mosaic virus* inoculation at successive weekly intervals on growth and yield of pepper (*Capsicum annuum*) plants. *Plant Disease*, **69**:52-55.

Ali, A., Kobayashi, M. (2010). Seed transmission of *Cucumber mosaic virus* in pepper. *Journal of Virological Methods*, **163**:234–237.

Alonso J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H, Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby W.L., Berry, C.C., Ecker, J.R. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**(5633): 653-7.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*. **5**;215(3):403-10.

Anderson, P.K., Cunningham, A.A., Patel, N.G., Morales, F.J., Epstein, P.R., Daszak, P. (2004). Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology. *Trends in Ecology and Evolution*, **19**(10): 535-544.

Auclair, J.L. (1963). Aphid Feeding and Nutrition. *Annual Review of Entomology*, **8**:439-490.

Avilla, C., Collar, J.L., Duque, M., Fereres, A. (1997). Yield of bell pepper (*Capsicum annuum*) inoculated with CMV and/or PVY at different time intervals. *Journal of Plant Diseases and Protection*, **104**(1): 1-8.

Baebler, Š., Stare, K., Kovač, M., Blejec, A., Prezelj, N., Stare, T., Kogovšek, P., Pompe-Novak, M., Rosahl, S., Ravnikar, M., Gruden K. (2011) Dynamics of responses in compatible potato-*Potato virus Y* interaction are modulated by salicylic acid. *PLoS One*, **6**(12): e29009.

Baebler, Š., Witek, K., Petek, M., Stare, K., Tušek-Žnidarič, M., Pompe-Novak, M., Renaut, J., Szajko, K., Strzelczyk-Żyta, D., Marczewski, W., Morgiewicz, K., Gruden, K., Hennig, J. (2014). Salicylic acid is an indispensable component of the *Ny-1* resistance-gene-mediated response against *Potato virus Y* infection in potato. *Journal of Experimental Botany*, **65**(4):1095–1109.

Bennette R.N., Kiddle G., Wallsgrove R.M. (1997). Biosynthesis of benzylglucosinolate, cyanogenic glucosides and phenylpropanoids in *Carica papaya*. *Phytochemistry*. **45**(1): 59-66.

Blackman, L.M., Boevink, P., Santa Cruz, S., Palukaitis, P., Oparka, K.J. (1998). The movement protein of *Cucumber mosaic virus* traffics into sieve elements in minor veins of *Nicotiana clevelandii*. *The Plant Cell*, **10**:525–538.

Blackman, R.L. (1974). Life-cycle variation of *Myzus persicae* (Sulz.) (Hom., Aphididae) in different parts of the world, in relation to genotype and environment. *Bulletin of Entomological Research*, **63**(4): 595-607.

Bohmert, K., Camus, I., Bellini, C., Bouchez, D., Caboche, M., Benning, C. (1998). AGO1 defines a novel locus of Arabidopsis controlling leaf development. *The EMBO Journal*, **17**(1): 170-180.

Bowling, S.A., Clarke, J.D., Liu, Y., Klessig, D.F., Dong, X. (1997). The *cpr5* mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. *The Plant Cell*, **9**(9):1573-84.

Canto, T., Prior, D.A.M. Hellwald, K-H., Oparka, K.J., Palukaitis, P. (1997). Characterization of *Cucumber mosaic virus*. *Virology*, **237**(2): 237–248.

Cao, H-H., Zhang, Z-F., Wang, X-F., Liu, T-X. (2018). Nutrition versus defense: Why *Myzus persicae* (green peach aphid) prefers and performs better on young leaves of cabbage, *PLoS ONE* **13**(4): e0196219.

Caplan, J. L., Mamillapalli, P., Burch-Smith, T. M., & Czymmek, K. (2008). Chloroplastic protein NRIP1 mediates innate immune receptor recognition of a viral effector. *Cell* **132**: 449–462.

Cardon, G.H., Hohmann, S., Nettesheim, K. Saedler, H., Huijser, P. (1997). Functional analysis of the *Arabidopsis thaliana* SBP-box gene SPL3: a novel gene involved in the floral transition. *Plant Journal*, **12**(2):367-77.

Carr, J.P., Donnelly, R., Tungadi, T. Murphy, A.M., Jiang, S., Bravo-Cazar, A., Yoon, J-Y., Cunniffe, N.J., Glover, B.J., Gilligan, C.A. (2018). Viral Manipulation of Plant Stress Responses and Host Interactions with Insects. *Advances in Virus Research*, **102**:177-197.

Carr, J.P., Lewsey, M.G., Palukaitis, P. (2010). Signaling in induced resistance. *Advances in Virus Research*, **76**:57-121.

Casteel, C.L., Yang, C., Nanduri, A.C., De Jong, H.N., Whitham, S.A., Jander, G. (2014). The NIa-Pro protein of *Turnip mosaic virus* improves growth and reproduction of the aphid vector, *Myzus persicae* (green peach aphid). *The Plant Journal*, **77**(4): 653-663.

Chaim, A.B., Grube, R.C., Lapidot, M., Jahn, M., Paran, I. (2001). Identification of quantitative trait loci associated with resistance to *Cucumber mosaic virus* in *Capsicum annuum*. *Theoretical and Applied Genetics*, **102**(8):1213–1220.

Chen, B., Francki, R.I.B. (1990). *Cucumovirus* transmission by the aphid *Myzus persicae* is determined solely by the viral coat protein. *Journal of General Virology*, **71**: 939-944.

Chen, X. (2004). A microRNA as a translational repressor of the APETALA2 in Arabidopsis flower development. *Science*, **303**(5666): 2022-5.

Chen, Z., Malamy, J., Henning, J., Conrath, U., Sanchez-Casas, P., Silva, H., Ricigliano, J. (1995). Induction, modification, and transduction of the salicylic acid signal in plant defense responses. *Proceedings of the National Academy of Sciences of the USA*, **92**(10): 4134–4137.

Chivasa, S., Murphy, A. M., Naylor, M., Carr, J. P. (1997). Salicylic Acid Interferes with *Tobacco mosaic virus* Replication via a Nove1 Salicylhydroxamic Acid-Sensitive Mechanism. *The Plant Cell* **9**:547-557.

Clements, K.M., Wiegmann, B.M., Sorenson, C.E., Smith, C.F., Neese, P.A., Roe, R.M. (2000). Genetic Variation in the *Myzus persicae* Complex (Homoptera: Aphididae): Evidence for a Single Species. *Annals of Entomological Society of America*, **93**(1):31-46.

Coleman, A.D., Wouters, R.H.M., Mugford, S.T., Hogenhout S.A. (2015). Persistence and transgenerational effect of plant-mediated RNAi in aphids. *Journal of Experimental Botany* **66**(2): 541–548.

Daskalov, S., Poulos, J.M. (1994). Updated Capsicum gene list. *Capsicum and Eggplant Newsletter*, **13**: 15-26.

de Vos, M. Jander, G. (2010). Volatile communication in plant-aphid interactions. *Current Opinion in Plant Biology*, **13**(4):366-371.

Delaney, T.P. (2010). Salicylic Acid. In: Davies P.J. (eds) *Plant Hormones*. Springer, Dordrecht, 681-699.

Delaney, T.P., Friedrich, L., Ryals, J.A. (1995). Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. *Proceedings of the National Academy of Sciences of the United States of America*, **92**(14):6602-6606.

Detzer, A., Engel, C., Wunsche, W., Sczakiel, G. (2011). Cell stress is related to the re-localization of AGO 2 and to decreased RNA interference in human cells. *Nucleic Acid Research*, **39**(7): 2727-2741.

Dincher, S., Staub, T., Uknes, S., Metraux J-P., Kessmann, H., Ryals, J. (1996). A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *The Plant Journal*, **10**(1): 61-70.

Dixon, R. A. 2001. Natural products and plant disease resistance. *Nature*, **411**(6839): 843–847.

Drenkard, E., Richter, B.G., Rozen, S., Stutius, L.M., Angell, N.A., Mindrinos, M., Cho, R.J., Oefner, P.J., Davis R.W., Ausubel, M. (2000). A simple procedure for the analysis of single nucleotide polymorphisms facilitates map-based cloning in Arabidopsis. *Plant Physiology* **124:** 1483-1492.

Duan, C-G., Fang, Y-Y., Zhou, B-J., Zhao, J.-H., Hou, W-N., Zhu, H., Ding, S-W., Guoa, H-S. (2012) Suppression of Arabidopsis ARGONAUTE1-Mediated Slicing, Transgene-Induced RNA Silencing, and DNA Methylation by Distinct Domains of the *Cucumber mosaic virus* 2b Protein. *The Plant Cell*, **24**:259–274.

Durner, J., Shah, J., Klessig, D.F. (1997). Salicylic acid and disease resistance in plants. *Trends in Plant Science*, **2**(7): 266–274.

Earley, K., Smith, M.R., Weber, R., Gregory, B.D., Poethig, R.S. (2010). An endogenous F-box protein regulates AGO1 in *Arabidopsis thaliana*. *Silence*, **1**:15.

Elkayam, E., Kuhn, C.D., Tocilj, A., Haase, A.D., Greene, E.M., Hannon, G.J., Joshua-Tor, L. (2012). The structure of human AGO-2 in complex with miR-20a. *Cell*, **150**(1): 100-10.

Ender, C., Meister, G. (2010). AGO proteins at a glance. *Journal of Cell Biology*, **123**(11), 1819-23.

Erb, M., Meldau, S., Howe, G.A. (2012). Role of phytohormones in insect-specific plant reactions. *Trends in Plant Science*, **17**(5):250-259.

Fahlgren, N., Montgomery T.A, Howell, M.D., Allen, E., Dvorak, S.K., Alexander, A.L., Carrington, J.C. (2006). Regulation of the AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA affects developmental timing and patterning in Arabidopsis. *Current Biology*, **16**(9): 939-44.

Falcioni, T., Ferrio, J.P., Cueto, A.I., Giné, J., Achón, M.Á., Medina, V. (2014). Effect of salicylic acid treatment on tomato plant physiology and tolerance to *Potato virus X* infection. *European Journal of Plant Pathology*, **138**(2):331–345.

Farkas, G.L., Solymosy, F. (1965). Host Metabolism and Symptom Production in Virus-Infected Plants. *Journal of Phytopathology*, **53**(1):85-93.

Fereres, A., Kampmeier, G.E., Irwin, M.E. (1999). Aphid Attraction and Preference for Soybean and Pepper Plants Infected with *Potyviridae*. *Annals of the Entomological Society of America*, **92**(4):542–548.

Feys, B.J., Parker, J.E. (2000). Interplay of signaling pathways in plant disease resistance. *Trends in Genetics*, **16**(10): 449–455.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M.G., Meier, B., Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. *Science*, 261(5122): 754-6.

Gallego-Giraldo, L., Escamilla-Trevino, L., Jackson, A.A., Dixon, R.A. (2011). Salicylic acid mediates the reduced growth of lignin down-regulated plants. *Proceedings of the National Academy of the United states of America* **108**(51): 20814-20819.

Ganjewala, D., Kumar, S., Devi, A.S., Ambika, K. (2010). Advances in cyanogenic glycosides biosynthesis and analyses in plants: A review. *Acta Biologica Szegediensis*, **54**(1): 1–14.

Gaudet M., Fara, A.G., Beritognolo, I., Sabatti, M. (2009). Allele-Specific PCR in SNP Genotyping. In: Komar, A. (eds) Single Nucleotide Polymorphisms. *Methods in Molecular Biology (Methods and Protocols)*, **578**: 415-424.

Geu-Flores, F., Nielsen, M.T., Nafisi, M., Moldrup, M.E., Olsen, C.E., Montawia, M.S., Halkier, B.A. (2009). Glucosinolate engineering identifies a gamma-glutamyl peptidase. *Nature Chemical Biology*, **5**(8): 575–577.

Girousse, C., Moulia, B., Silk, W., Bonnemain, J-L. (2005). Aphid Infestation Causes Different Changes in Carbon and Nitrogen Allocation in Alfalfa Stems as Well as Different Inhibitions of Longitudinal and Radial Expansion. *Plant Physiology*, **137**:1474-1484.

Gish, M., Inbar, M. (2006). Host Location by Apterous Aphids After Escape Dropping from the Plant. *Journal of Insect Behavior*, **19**:143.

Gleadow, R.M., Woodrow, I.E. (2002). Constraints on effectiveness of cyanogenic glycosides in herbivore defense. *Journal of Chemical Ecology*, **28**(7): 1301–1313.

Goggin, F.L. (2007). Plant-aphid interactions: molecular and ecological perspectives. *Current Opinion in Plant Biology*, **10**(4): 399-408.

González, I., Martínez, L., Rakitina, D.V., Lewsey, M.G., Atencio, F.A., Llave, C., Kalinina, N.O., Carr, J.P., Palukaitis, P., Canto, T. (2010). *Cucumber mosaic virus* 2b Protein Subcellular Targets and Interactions: Their Significance to RNA Silencing Suppressor Activity. *Molecular Plant-Microbe Interactions*, **23**(3):294 – 303.

González, I., Rakitina, D., Semashko, M., Taliansky, M., Praveen, S., Palukaitis, P., Carr, J.P., Kalinina, N., Canto, T. (2012). RNA binding is more critical to the suppression of silencing function of *Cucumber mosaic virus* 2b protein than nuclear localization. *RNA*, **18**(4): 771–782.

Goto, K., Kobori, T., Kosaka, Y., Natsuaki, T., Masuta, C. (2007). Characterization of Silencing Suppressor 2b of *Cucumber mosaic virus* Based on Examination of its Small RNA-Binding Abilities. *Plant and Cell Physiology* **48**(7): 1050–1060.

Green, S. K., Kim, J.S., (1991). Characteristics and control of viruses infecting peppers: A literature review. Asian Vegetable Research and Development Center.

Groen, S.C., Jiang, S., Murphy, A.M., Cunniffe, N.J., Westwood, J.H., Davey, M.P., Bruce, T.J.A., Caulfield, J.C., Furzer, O.J., Reed, A., Robinson, S.I., Miller, E., Davis, C.N., Pickett, J.A., Whitney, H.M., Glover, B.J., Carr, J.P. (2016). Virus Infection of Plants Alters Pollinator Preference: A Payback for Susceptible Hosts? *PLoS Pathogens*, **12**(8): e1005790.

Groen, S.C., Wamonje, F.O., Murphy, A.M., Carr, J.P. (2017). Engineering resistance to virus transmission. *Current Opinion in Virology*, **26**: 20-27.

Grube, R.C., Zhang, Y., Murphy, J.F., Loaiza-Fiqueroa, F., Lackney, V.K., Provvidenti, R., Jahn, M.K. (2000). New source of resistance to *Cucumber mosaic virus* in *Capsicum frutescens*. *Plant Disease*, **84**(8): 885–891.

Gurr, S.J., Rushton, P.J. (2005). Engineering plants with increased disease resistance: What are we going to express? *Trends in Biotechnology*, **23**(6): 275–282.

Hamilton, W.D., Brown S.P. (2001). Autumn tree colours as a handicap signal. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **268**(1475).

Hammerschmidt, R. (1999). Induced disease resistance: how do induced plants stop pathogens? *Physiological and Molecular Plant Pathology*, **55**: 77–84.

Hanif, R., Iqbal, Z., Iqbal, M. (2006). Use of vegetables as nutritional food: role in human health. *Journal of Agricultural and Biological Science*, **1**(1): 18–22.

Hayat, Q., Hayat, S., Irfan, M., Ahmad, A. (2010). Effect of exogenous salicylic acid under changing environment: A review. *Environmental and Experimental Botany*, **68**(1):14–25.

He, X-F., Fang, Y-Y., Feng L., Guo, H-S. (2008). Characterization of conserved and novel microRNAs and their targets, including a TuMV-induced TIR–NBS–LRR class R gene-derived novel miRNA in Brassica. *FEBS Letters*, **582**(16): 2445-2452.

Hock, J., Meister, G. (2008). The Argonaute protein family. *Genome Biology*, **9**(2):210.

Hodge, S., Powell G. (2008). Do Plant Viruses Facilitate Their Aphid Vectors by Inducing Symptoms that Alter Behavior and Performance? *Environmental Entomology*, **37**(6):1573–1581.

Holopainen, J.K., Peltonen, P. (2002). Bright autumn colours of deciduous trees attract aphids: nutrient retranslocation hypothesis. *OIKOS*, **99**:184-188.

Howard, C.R., Fletcher, F.N. (2012). Emerging virus diseases: can we ever expect the unexpected? *Emerging Microbes Infections*, 1(12): e46.

Huang, Z., Yeakley, J.M., Garcia, E.W., Holdridge, J.D., Fan, J-B., Whitham, S.A. (2005). Salicylic Acid-Dependent Expression of Host Genes in Compatible Arabidopsis-Virus Interactions. *Plant Physiology*, **137**(3): 1147–1159.

Hutvagner, G., Simard, M.J. (2014). Argonaute proteins: key players in RNA silencing. *Nature Reviews Molecular Cell Biology*, **9**: 22-32.

Ince, A.G., Karaca, M., Onus, A.N. (2010). Genetic relationships within and between capsicum species. *Biochemical Genetics*, **48**(1-2):83–95.

Ingwell, L.L., Eigenbrode, S.D., Bosque-Perez, N. (2012). Plant viruses alter insect behavior to enhance their spread. *Scientific Reports*, **2**(578).

Inzé, A., Vanderauwera, S., Hoeberichts, F.A., Vandorpe, M., Van Gaever, T., Van Breusegem, F. (2012). A subcellular localization compendium of hydrogen peroxide-induced proteins. *Plant, Cell and Environment*, **35**(2):308–320.

Jacquemond, M. (2012). Chapter 13 - *Cucumber mosaic virus*. *Advances in Virus Research*, **84**: 439-504.

Jiang, W., Li, Z., Yao, X., Zheng, B., Shen W.H., Dong, A. (2018). *jaw-ID*: a gain-of-function mutation responsive to paramutation-like induction of epigenetic silencing. *Journal of Experimental Botany*, 70(2):459-468.

Jones, J. D. G., Dangl, J. L. (2006). The plant immune system. *Nature Reviews*, **444**: 323–329.

Jung, J.H., Lee, S., Yun, J., Lee, M., Park, C.M. (2014). The miR172 target TOE3 represses AGAMOUS expression during Arabidopsis floral patterning. *Plant Science*, **215-216**: 29-38.

Kang, W.H., Hoang, N.H., Yang, H.B., Kwon, J.K., Jo, S.H., Seo, J.K., Kim, K.H., Choi, D., Kang, B.C. (2010). Molecular mapping and characterization of a single dominant gene controlling CMV resistance in peppers (*Capsicum annuum* L.). *Theoretical and Applied Genetics*, **120**(8): 1587–1596.

Kang, W.H., Seo, J-K., Chung, B.N., Kim, K-H, Kang, B-C. (2012). Helicase domain encoded by *Cucumber mosaic virus* RNA1 determines systemic infection of Cmr1 in pepper. *PLoS ONE*, **7(8): e43136.**

Kessmann, H., Staub, T., Hofmann, C., Maetske T., Herzog, J., Ward, E., Uknes, S., Ryals, J. (1994). Induction of systemic acquired disease resistance in plants by chemicals. *Annual Review of Phytopathology*, **32**: 439–459.

Kettles, G. J., Drurey, C., Schoonbeek, H-j., Maule A. J., Hogenhout, S. A. (2013). Resistance of *Arabidopsis thaliana* to the green peach aphid, *Myzus persicae*, involves camalexin and is regulated by microRNAs. *New Phytologist*, **198**(4):1178-1190.

Kleinboelting, N., Huep, G., Kloetgen, A., Viehoever, P., Weisshaar, B. (2012). GABI-Kat SimpleSearch: new features of the *Arabidopsis thaliana* T-DNA mutant database. *Nucleic Acids Research*, **40**(Database issue): D1211-5.

Kola, V.S.R., Renuka, P., Madhav, M.S., Mangrauthia, S.K. (2015). Key enzymes and proteins of crop insects as candidate for RNAi based gene silencing. *Frontiers in Physiology* **6**(119).

Kozera, B., Rapacz, M. (2013). Reference genes in real-time PCR. *Journal of Applied Genetics*, **54**(4): 391-406.

Kruse, T., Ho, K., Yoo, H.D., Park, J.H., Flavell, R., Bobzin, S. (2008). In Planta Biocatalysis Screen of P450s Identifies 8-Methoxypsoralen as a Substrate for the CYP82C Subfamily, Yielding Original Chemical Structures. *Chemistry and Biology*, **15**:149–156.

Kunkel, B.N., Brooks, D.M. (2002). Cross talk between signaling pathways in pathogen defense. *Current Opinion in Plant Biology*, **5**:325–331.

Kwok, P-Y. (2001). Methods for Genotyping Single Nucleotide Polymorphisms. *Annual Review of Genomics and Human Genetics*, **2**:235-58.

Larson, K.C., Whitham, T.G. (1991). Manipulation of food resources by gall-forming aphid: the physiology of sink-source interactions. *Oecologia*, **88** (1):15-21.

Leather, S. R., Dixon, A.F.G. (1984). Aphid growth and reproductive rates. *Entomologia Experimentalis et Applicata* **35**: 137-140.

Lee, S., Badieyan, S., Bevan, D.R., Herde, M., Gatz, C., Tholl, D. (2010). Herbivore-induced and floral homoterpene volatiles are biosynthesized by a single P450 enzyme (CYP82G1) in

Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **107**:21205-21210.

Lee, W.S., Fu, S.F., Li, Z., Murphy, A.M., Dobson E.A., Garland, L., Chaluvadi, S.R., Lewsey, M.G., Nelson, R.S., Carr J.P. (2016). Salicylic acid treatment and expression of an RNA-dependent RNA polymerase 1 transgene inhibit lethal symptoms and meristem invasion during *Tobacco mosaic virus* infection in *Nicotiana benthamiana*. *BMC Plant Biology*, **16**(15).

Lemarié, S., Robert-Seilaniantz, A., Lariangon, C., Lemoine, J., Marnet, N., Levrel, A., Jubault, M., Manzanares-Dauleux, M.J., Gravot, A. (2015). Camalexin contributes to the partial resistance of *Arabidopsis thaliana* to the biotrophic soilborne protist *Plasmodiophora brassicae*. *Frontiers in Plant Science*, **6**:539.

Li, Q., Ryu, K.H. & Palukaitis, P. (2001). *Cucumber mosaic virus*-plant interactions: identification of 3a protein sequences affecting infectivity, cell-to-cell movement, and long-distance movement. Molecular plant-microbe interactions. *Molecular Plant-Microbe Interactions*, **14**(3):378–385.

Li, Q., Xie, Q-G., Smith-Becker, J., Navarre, D.A., Kaloshian, I. (2006). *Mi-1*-Mediated Aphid Resistance Involves Salicylic Acid and Mitogen-Activated Protein Kinase Signaling Cascade. *Molecular-Plant Microbe Interactions* **19**(6): 655-664.

Li, S-B., Xie, Z-Z., Hu, C-G., Zhang, J-Z. (2016). A Review of Auxin Response Factors (ARFs) in Plants. *Frontiers in Plant Science*, **7**(47).

Liu, F., Jiang, H., Ye, S., Chen, W.P., Liang, W., Xu, Y., Sun, J., Wang, Q., Cohen, J.D., Li, C. (2010). The Arabidopsis P450 protein CYP82C2 modulates jasmonate-induced root growth inhibition, defense gene expression and indole glucosinolate biosynthesis. *Cell Research*, **20**(5): 539–552.

Lot, H., Kaper, J.M. (1976). Physical and chemical differentiation of three strains of *Cucumber mosaic virus* and *Peanut stunt virus*. *Virology*, **74**(1):209-222.

Love, A. J., Laird, J., Holt, J., Hamilton, A. J., Sadanandom, A., Milner, J. J. (2007). *Cauliflower mosaic virus* protein P6 is a suppressor of RNA silencing. *Journal of General Virology*, **88**: 3439-3444.

Love, A. J., Yun, B. W., Laval, V., Loake, G. J., Milner, J. J. (2005). *Cauliflower mosaic virus*, a Compatible Pathogen of Arabidopsis, Engages Three Distinct Defense-Signaling Pathways and Activates Rapid Systemic Generation of Reactive Oxygen Species. *Plant Physiology*, **139**:935–948.

Love, A.J., Laval, V., Geri, C., Laird, J., Tomos, A.D., Hooks, M.A. and Milner, J.J. (2007) Components of Arabidopsis defense- and ethylene-signaling pathways regulate susceptibility to *Cauliflower mosaic virus* by restricting long-distance movement. *Molecular Plant-Microbe Interactions*, **20**: 659-670.

Malamy, J., Carr, J.P., Klessig, D.F., Raskin, I. (1990). Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. *Science* **250**: 1002-1004.

Malamy, J., Klessig, D.F., (1992). Salicylic acid and plant disease resistance. *Plant Journal*, **2**, pp.643–654.

Mandadi, K. K., Scholthof, K. G. (2013). Plant immune responses against viruses: How does a virus cause disease? *The Plant Cell* **25**:1489–1505.

Martin, G. B., Bogdanove, A. J., & Sessa, G. (2003). Understanding the functions of plant disease resistance proteins. *Annual Review of Plant Biology*, **54**:23–61.

Mattson, Jr, W. J. (1980). Herbivory in Relation to Plant Nitrogen Content. *Annual Review of Ecology and Systematics*, **11**:119-161.

Mauck, K.E., De Moraes, C.M., Mescher, M.C. (2010). Effects of *Cucumber mosaic virus* infection on vector and non-vector herbivores of squash. *Communicative & Integrative Biology*, **3**(6): 579–82.

Maule, A.J., Wang, D. (1996). Seed transmission of plant viruses: A lesson in biological complexity. *Trends in Microbiology*, **4**(4):153–158.

Maxwell, D.J., Partridge, J.C., Roberts, N.W., Boonham, N., Foster, G.D. (2016). The Effects of Plant Virus Infection on Polarization Reflection from Leaves. *PLoS ONE*, **11**(4): e0152836.

Mayers N.C. (1999). *Cucumber mosaic virus*: defence and counter-defence. PhD Dissertation. Department of Plant Sciences, University of Cambridge, UK.

Mayers, C.N., Lee, K-C., Moore, C. A., Wong, S-M, Carr, J. P. (2005). Salicylic Acid-Induced Resistance to *Cucumber mosaic virus* in Squash and Arabidopsis thaliana: Contrasting Mechanisms of Induction and Antiviral Action. *Molecular Plant-Microbe Interactions*, **18**(5): 428 – 434.

McConn, M., Reelman, A.C.R., Bell, E., Mullet, J.E., Browse, J. (1997). Jasmonate is essential for insect defense in Arabidopsis. *Proceedings of National Academy of Sciences of the United States of America*, **94**: 5473-5477.

McDowell, J.M., Woffenden, B.J. (2003). Plant disease resistance genes: Recent insights and potential applications. *Trends in Biotechnology*, **21**(4):178–183.

Moldrup, M.E., Salomonsen, B., Geu-Flores, F., Olsen, C.E., Halkier, B.A. (2013). De novo genetic engineering of the camalexin biosynthetic pathway. *Journal of Biotechnology*, **167**:296–301.

Møller, B.L. (2010). Functional diversifications of cyanogenic glucosides. *Current Opinion in Plant Biology*, **13**(3):338–347.

Montasser, M.S., Tousignant, M.E., Kaper, J.M. (1998). Viral satellite RNAs for the prevention of *Cucumber mosaic virus* (CMV) disease in field-grown pepper and melon plants. *Plant Disease*, **82**: 1298-1303.

Morel, J-B., Godon, C., Mourrain, P., Beclin, C., Boutet, S., Feuerbach, F., Proux, F., Vaucheret, H. (2002). Fertile Hypomorphic *ARGONAUTE (ago1)* Mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell*, **14**(3): 629-639.

Morkunas, I., Mai, V. C., Gabrys, B. (2011). Phytohormonal signalling in plant responses to aphid feeding. *Acta Physiologiae Plantarum*, **33**(6): 2057-2073.

Murphy, A.M., Carr, J. P. (2002). Salicylic Acid Has Cell-Specific Effects on *Tobacco mosaic virus* Replication and Cell-to-Cell Movement. *Plant Physiology*, **128**(2):552–563.

Murphy, J.F., Masiri, J., Hadi, B.A.R., Dute, R.R. (2016). Recovery from *Cucumber mosaic virus* infection for 'Calwonder' bell pepper plants does not counter negative impacts on plant growth. *Journal of Phytopathology*, **164:** 840-846.

Mutka A.M., Fawley, S., Tsao, T., Kunkel, B.N. (2013). Auxins promotes susceptibility to *Pseudomonas syringae* via a mechanism independent of suppression of salicylic acid-mediated defences. *Plant Journal*, **74**(5): 746-54.

Mutti, N. S., Park, Y., Reese, J. C., Reeck, G.R. (2006). RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. *Journal of Insect Science*, **6**:1-7.

Nalam, V., Louis, J., Shah, J. (2019). Plant defense against aphids, the pest extraordinaire. *Plant Science*, **279**: 96-107.

Nath, U., Crawford, B.C., Carpenter, R., Coen, E. (2003). Genetic control of surface curvature. *Science*, 299:1404-7.

Nault, L.R. (1997). Arthropod transmission of plant viruses: a new synthesis. *Annals of the Entomological Society of America*, **90**(5):521-541.

Naylor, M., Murphy, A.M., Berry, J.O., Carr, J.P. (1998). Salicylic Acid Can Induce Resistance to Plant Virus Movement. *Molecular Plant-Microbe Interactions*, **11**(9): 860 – 868.

Ng, J.C., Perry, K.L. (2004). Transmission of plant viruses by aphid vectors. *Molecular Plant Pathology*, **1**;5(5): 505-11.

Ng, J.C.K., Falk, B.W. (2006). Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. *Annual Review of Phytopathology*, **44**:183-212.

Nicaise, V. (2014). Crop immunity against viruses: outcomes and future challenges, *Frontiers in Plant Science*. **5**:1–18.

Nodine, M.D., Bartel, D.P. (2010). MicroRNAs prevent precocious gene expression and enable pattern formation during plant embryogenesis. *Genes and Development*, **24**(23):2678-92.

Nouri, S., Arevalo, R., Falk, B.W., Groves, R.L. (2014). Genetic structure and molecular variability of *Cucumber mosaic virus* isolates in the United States. *PLoS ONE*, **9(5): e96582.**

Olmedo-Monfil, V., Duran-Figueroa, N., Arteaga-Vazquez, M., Demesa-Arevalo, E., Autran, D., Grimanelli, D., Slotkin, R.K., Martienssen, R.A., Vielle-Calzada, J.P. (2010). Control of female gamete formation by the small RNA pathway in Arabidopsis. *Nature*, **464**: 628-632.

Onkokesung, N., Baldwin, I.T., Gailis, I. (2010). The role of jasmonic acid and ethylene crosstalk in direct defense of *Nicotiana attenuata* plants against chewing herbivores. *Plant Signaling and Behavior*, **5**(10): 1305-1307.

Oostendorp, M., Kunz, W., Dietrich, B., Staub, T. (2001). Induced Disease Resistance in Plants by Chemicals. *European Journal of Plant Pathology*, **107**(1): 19-28.

Ortiz, R., de la Flor, D.F., Alvarado, G., Crossa, J. (2010). Classifying vegetable genetic resources-A case study with domesticated *Capsicum* spp. *Scientia Horticulturae*, **126(**2):186–191.

Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Scwab, R., Carrington, J.C., Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. *Nature*, **18:425**(6955):257-63.

Palukaitis, P., Carr, J. P. (2008). Plant resistance responses to viruses. *Journal of Plant Pathology*, **90**:153-172.

Palukaitis, P., Yoon, J.Y., Choi, S.K., Carr, J.P. (2017). Manipulation of induced resistance to viruses. *Current Opinion in Virology*, **26**: 141-148.

Paran, I., Van Der Knaap, E. (2007). Genetic and molecular regulation of fruit and plant domestication traits in tomato and pepper. *Journal of Experimental Botany*, **58**(14): 3841–3852.

Parker, J.S., Barford, D. (2006). Argonaute: a scaffold for the function of short regulatory RNAs. *Trends in Biochemical Sciences*, **31**(11): 622-630.

Peden, K.W.C., Symons, R.H. (1973). *Cucumber mosaic virus* contains a functionally divided genome. *Virology*, **53**(2): 487–492.

Pentzold, S., Zagrobelny, M., Roelsgaard, P.S., Moller, B.L., Bak, S. (2014). The multiple strategies of an insect herbivore to overcome plant cyanogenic glucoside defence. *PLOS One* **9(3): e91337.**

Perring, T.M., Gruenhagen, N.M., Farrar, C.A. (1999). Management of plant viral diseases through chemical control of insect vectors. *Annual Review of Entomology*, **44**: 457-81

Perry, K.L., Zhang, L., Palukaitis, P. (1998). Amino acid changes in the coat protein of *Cucumber mosaic virus* differentially affect transmission by the aphids *Myzus persicae* and *Aphis gossypii*. *Virology*, **242**(1):204-10.

Peter, L., Meister, G. (2007). Argonaute proteins: Mediators of RNA silencing. *Molecular Cell*, **26**(5): 611-623.

Pickersgill, B. (1991). 8 - Cytogenetics and Evolution of Capsicum L. *Developments in Plant Genetics and Breeding*, **2(B)**: 139-160.

Pickersgill, B., (1997). Genetic resources and breeding of *Capsicum* spp. *Euphytica*, **96**(1): 29–133.

Pignatta, D., Erdmann, R.M., Scheer, E., Picard, C.L., Bell, G.W., Gehring, M. (2014). Natural epigenetic polymorphisms lead to intraspecific variation in Arabidopsis gene imprinting. *eLife*, **3**: e03198.

Pirone, T.P., Harris, K.F. (1977). Nonpersistent Transmission of Plant Viruses by Aphids. *Annual Review of Phytopathology*, **15**:55-73.

Pitino, M., Coleman, A.D., Maffei, M.E., Ridout, C.J., Hogenhout, S. A. (2011). Silencing of Aphid Genes by dsRNA Feeding from Plants. *PLOS One*. **6**(10): e25709.

Powell, G., Colin, R.T., Hardie, J. (2006). Host Plant Selection by Aphids: Behavioral, Evolutionary, and Applied Perspectives. *Annual Review of Entomology*, **51**:309-330.

Price, D.R., Gatehouse, J.A. (2008). RNAi-mediated crop protection against insects. *Trends in Biotechnology* **26**(7):393-400.

Prokopy, R. J., Owens, E. D. (1983). Visual Detection of Plants by Herbivorous Insects. *Annual Review of Entomology*, **28**:337-364.

Qin, C. Qin, C., Yu, C., Shen, Y., Fang, X., Chen, L., Min, J., Cheng, J., Zhao, S., Xu, M., Luo, Y., Yang, Y., Wu, Z., Mao, L., Wu, H., Ling-Hu, C., Zhou, H., Lin, H., González-Morales, S., Trejo-Saavedra, D.L., Tian, H., Tang, X., Zhao, M., Huang, Z., Zhou, A., Yao, X., Cui, J., Li, W., Chen, Z., Feng, Y., Niu, Y., Bi, S., Yang, X., Li, W., Cai, H., Luo, X., Montes-Hernández, S., Leyva-González, M.A., Xiong, Z., He, X., Bai, L., Tan, S., Tang, X., Liu, D., Liu, J., Zhang, S., Chen, M., Zhang, L., Zhang, L., Zhang, Y., Liao, W., Zhang, Y., Wang, M., Lv, X., Wen, B., Liu, H., Luan, H., Zhang, Y., Yang, S., Wang, X., Xu, J., Li, X., Li, S., Wang, J., Palloix, A., Bosland, P.W., Li, Y., Krogh, A., Rivera-Bustamante, R.F., Herrera-Estrella, L., Yin, Y., Yu, J., Hu, K., Zhang, Z. (2014). Whole-genome sequencing of cultivated and wild peppers provides insights into *Capsicum* domestication and specialization. *Proceedings of the National Academy of Sciences of the United States of America*, **111**(14):5135–40.

Quadrana, L., Almeida, J., Asis, R., Duffy, T., Dominguez, P.G., Bermudez, L., Conti, G., Correa da Silva, J., Peralta, I.E., Colot, V., Asurmendi, S., Fernie, A.R., Rossi, M., Carrari, F. (2014). Natural occurring epialleles determine vitamin E accumulation in tomato fruits. *Nature Communications*, **5:** 4027.

R Core Team. (2017). R: A Language and Environment for Statistical Computing. Vienna, Austria. www.R-project.org.

Radcliffe E.B., Ragsdale, D.W. (2002). Aphid-transmitted potato viruses: The importance of understanding vector biology. *American Journal of Potato Research*, **79**(5): 353-386.

Rajniak, J., Barco B., Clay, N.K., Satterly, E. (2015). A new cyanogenic metabolite in Arabidopsis required for inducible pathogen defence. *Nature*, **525**: 375–379.

Rashid, U.J., Paterok, D., Koglin, A., Gohlke, H., Piehler, J., Chen, J.C-H. (2007). Structure of *Aquifex aeolicus* Argonaute highlights conformational flexibility of the PAZ domain as a potential regulator of the RNA-induced silencing complex function. *The Journal of Biological Chemistry*, **282**(18):13824-13832.

Rausher, M.D. (2001). Co-evolution and plant resistance to natural enemies. *Nature*, **411**(6839): 857–864.

Regnault-Roger, C. (1997). The potential of botanical essential oils for insect pest control. *Integrated Pest Management Reviews*, **2**(1): 25-34.

Rizzo, T.M., Palukaitis, P. (1990). Construction of full-length cDNA clones of *Cucumber mosaic virus* RNAs 1, 2 and 3: Generation of infectious RNA transcripts. *Molecular & General Genetics*, **222**: 249–256.

Rizzo, T.M., Palukaitis, P., (1988). Nucleotide-sequence and evolutionary relationships of *Cucumber mosaic virus* (CMV) strains: CMV RNA 2. *Journal of General Virology*, **69**: 1777–1787.

Robert-Seilaniantz, A., MacLean, D., Jikumaru, Y., Hill, L., Yamaguchi, S., Kamiya Y., Jones1, J. D. G. (2011) The microRNA miR393 re-directs secondary metabolite biosynthesis away from camalexin and towards glucosinolates. *The Plant Journal* **67**, 218–231.

Roossinck, M.J., White, P.S. (1998). *Cucumovirus* Isolation and RNA Extraction. *Methods in Molecular Biology*, **81**:189-196.

Sandström, J. (2000). Nutritional quality of phloem sap in relation to host plant-alternation in the bird cherry-oat aphid. *Chemoecology*, **10**(1):17–24.

Sattar, S., Addo-Quaye, C., Thompson, G.A. (2016). miRNA-mediated auxin signalling repression during *Vat*-mediated aphid resistance in *Cucumis melo. Plant, Cell and Environment*, **39**(6):1216-1227.

Sattler, S.E., Mene-Saffraine, L., Farmer, E.E., Krischke, M., Mueller, M.J., DellaPenna, D. (2006). Nonenzymatic Lipid Peroxidation Reprograms Gene Expression and Activates Defense Markers in Arabidopsis Tocopherol-Deficient Mutants. *The Plant Cell*, **18**(12):3706–3720.

Schöb, H., Kunz, C., Meins Jr, F. (1997). Silencing of transgenes introduced into leaves by agroinfiltration: a simple, rapid method for investigating sequence requirements for gene silencing. *Molecular and General Genetics.* **256**(5):581–585.

Scholthof, K.B., Adkins, S., Czosnek, H., Palukaitis, P., Jacquot, E., Hohn, T., Hohn, B., Saunders, K., Candresse, T., Ahlquist, P., Hemenway, C., Foster, GD. (2011). Top 10 plant viruses in molecular plant pathology. *Molecular Plant Pathology*, **12**(9):938-54.

Schröder, M.L., Glinwood, R., Ignell, R., Krüger, K. (2014). Visual cues and host-plant preference of the bird cherry-oat aphid, *Rhopalosiphum padi* (Hemiptera: Aphididae). *African Entomology* **22**(2):428–436.

Schuler, M.A., Duan, H., Metin, B., Ali, S. (2006). Arabidopsis cytochrome P450s through the looking glass: a window on plant biochemistry. *Phytochemistry Reviews*, **5**(2-3):205-237.

Sels, J., Mathys, J., De Coninck, B. M. A., Cammue, B. P. A., De Bolle, M. F. C. (2008). Plant pathogenesis-related (PR) proteins: A focus on PR peptides. *Plant Physiology and Biochemistry*, **46**(11):941–950.

Shang, J., Xi, D-H., Huang, Q-R., Xu, M.Y., Yuan, S., Wang, S-D., Jia, S-D., Cao, S., Zhou, Z-L., Lin, H-H. (2009). Effect of two satellite RNAs on *Nicotiana glutinosa* infected with

Cucumber mosaic virus (CMV). *Physiological and Molecular Plant Pathology*, **74**(2):184–190.

Shani, E., Burko, Y., Ben-Yaakov, L., Berger, Y., Amsellem, Z, Goldsmidt, A., Sharon, E., Oro, N. (2009). Stage-specific regulation of *Solanum lycopersicum* leaf maturation by the class 1 KNOTTED1-LIKE HOMEOBOX proteins. *Plant Cell* **21**(10): 3078-92.

Shi, B.-J., Palukaitis, P., Symons, R.H. (2002). Differential virulence by strains of *Cucumber mosaic virus* is mediated by the 2b gene. Molecular plant-microbe interactions: *Molecular Plant-Microbe Interactions*, **15**(9):947–955.

Shivaprasad, P.V., Chen, H.-M., Patel, K., Bond, D. M., Santos, B.A.C.M., Baulcombe, D.C. (2012). A MicroRNA Superfamily Regulates Nucleotide Binding Site–Leucine-Rich Repeats and Other mRNAs. *Plant Cell*. **24**(3):859–874.

Singh, R.K., Gase, K., Baldwin, I.T., Pandey, S.P. (2015). Molecular evolution and diversity of the Argonaute family of proteins in plants. *BMC Plant Biology*, **15**: 23.

Smith, L.M., Sanders, J.Z., Kaiser, R.J., Hughes, P., Dodd, C., Connell, C.R., Heiner, C., Kent, S.B.H., Hood, L.E. (1986). Fluorescence detection in automated DNA sequence analysis. *Nature*, **321**: 674-679.

Smith, M.R., Willmann, M.R., Wu, G., Berardini, T.Z., Moller, B., Weiiers, D., Poethig, R.S. (2009). Cyclophilin 40 is required for microRNA activity in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, **106**(13):5424-9.

Smith, P.G. and Heisner Jr., C.B. (1951). Taxonomic and genetic studies on the cultivated peppers of *Capsicum annuum* and *frutescens*. *American Journal of Botany*, **38**(5): 362–368.

Song, G.C., Choi, H.K., Ryu, C.M. (2013). The folate precursor para-aminobenzoic acid elicits induced resistance against *Cucumber mosaic virus and Xanthomonas axonopodis*. *Annals of Botany*, **111**(5):925–934.

Song, J-J., Smith, S.K., Hannon, G.J., Joshua-Tor, L. (2004). Crystal Structure of Argonaute and its implications for RISC slicer activity. *Science*, **305**(5689): 1434-1437.

Spoel, S. H., Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nature Reviews Immunology*, **12**(2):89–100.

Staskawicz, B.J., Ausubel, F.M., Baker, B.J., Ellis, J.G., Jones, J.D. (1995). Molecular Genetics of Plant Disease Resistance. *Science*, **268**(6): 661–667.

Stutz, R.S., Croak, B.M., Proschogo, N., Banks, P.B., McArthur, C. (2017). Olfactory and visual plant cues as drivers of selective herbivory. *OIKOS*,**126**(2).

Suzuki, M., Kuwata, S., Kataoka, J., Masuta, C., Nitta, N., Takanami, Y. (1991). Functional analysis of deletion mutants of *Cucumber mosaic virus* RNA3 using an *in vitro* transcription system. *Virology*, **183**(1): 106–113.

Swarts, D.C., Makarova, K., Wang, Y., Nakanishi, K., Ketting, R.F., Koonin, E.V., Patel, D.J., van der Oost, J. (2014). The evolutionary journey of Argonaute proteins. *Nature Structural and Molecular Biology*. **21**(9): 743-53.

Takahashi, H., Kai, A., Yamashita, M., Ando, S., Sekine, K., Kanayama, Y., & Tomita, H. (2012). Cyclic nucleotide-gated ion channel-mediated cell death may not be critical for *R* gene-conferred resistance to *Cucumber mosaic virus* in *Arabidopsis thaliana*. *Physiological and Molecular Plant Pathology*, **79**:40–48.

Tang, S., Wang, Y., Li, Z., Gui, Y., Xiao, B., Xie, J., Zhu, Q-H., Fan, L. (2012). Identification of wounding and topping responsive small RNAs in tobacco (*Nicotiana tabacum*) *BMC Plant Biology* **12**(28).

Tattersall D.B., Bak, S., Jones, P.R., Olsen, C.E., Nielsen, J.K., Hansen, M.L., Hoj, P.B., Moller, B.L. (2001). Resistance to an herbivore through engineered cyanogenic glucoside synthesis. *Science* **293**(5536):1826-1828.

Taylor, T.H.C. (1955). Biological Control of Insect Pests. *Annals of Applied Biology*, **42**(1): 190-196.

Terry, L.A., Joyce, D.C. (2004). Elicitors of induced disease resistance in postharvest horticultural crops: A brief review. *Postharvest Biology and Technology*, **32**(1):1–13.

Trujillo, M., Shirasu, K. (2010). Ubiquitination in plant immunity. *Current Opinion in Plant Biology*, **13**(4):402–408.

Tsuda, K., Katagiri, F. (2010). Comparing signalling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Current Opinion in Plant Biology*, **13**(4):459–465.

Ueda, H., Kugimiya, S., Tabata, J., Kitamoto, H., Mitsuhara, I. (2019) Accumulation of salicylic acid in tomato plant under biological stress affects oviposition preference of *Bemisia tabaci*. *Journal of Plant Interactions*, **14**(1):73-78.

Ugozzoli, L.R., and Wallace, B. (1991). Allele-specific polymerase chain reaction. *Methods*, **2**(1): 42-48.

van Emdem, H.F. (1969). Plant resistance to aphids induced by chemicals. *Journal of the Science of Food and Agriculture*, **20**(7):385-387.

van't Slot, K.A.E., Knogge, W. (2010). A Dual Role for Microbial Pathogen-Derived Effector Proteins in Plant Disease and Resistance. *Critical Reviews in Plant Sciences*, **21, 2002** (3):229-271.

Varma, A., Malathi, V.G. (2003). Emerging *Geminivirus* problems: A serious threat to crop production. *Annals of Applied Biology*, **142**(2): 145-164.

Vaucheret, H. (2008). Plant ARGONAUTES. *Trends in Plant Science*, **13**(7): 350-358.

Vaucheret, H., Béclin, C., Fagard M. (2001). Post-transcriptional gene silencing in plants. *Journal of Cell Science,* **114**: 3083-3091.

Villada, E.S., González, E.G., López-Sesé, A.I., Castiel, A.F., Gómez-Guillamón, M.L. (2009). Hypersensitive response to *Aphis gossypii* Glover in melon genotypes carrying the *Vat* gene. *Journal of Experimental Botany*, **60**(11):3269–3277.

Votava, E.J., Bosland, P.W. (2002). A cultivar by any other name: Genetic variability in heirloom bell pepper "California Wonder." *HortScience*, **37**(7):1100–1102.

Wang, D., Bosland, P.W., (2006). The genes of *Capsicum*. *HortScience*, **41**(5):1169–1187.

Wang, D., Pajerowska-Mukhtar, K., Culler, A.H., Dong, X. (2007). Salicylic Acid Inhibits Pathogen Growth in Plants through Repression of the Auxin Signaling Pathway. *Current Biology*, **17**:1784–1790.

Werck-Reichhart, D., Bak, S., Paquette, S. (2002). Cytochrome P450. *Arabidopsis Book* **1: e0028.**

Westwood, J.H., Groen, S.C., Du, Z., Murphy, A.M., Anggaro, D.T., Tugandi, T., Luang-In, V., Lewsey, M.G., Rossiter, J.T., Powell, G., Smith, A.G., Carr, J.P. (2013). A trio of viral proteins tunes aphid-plant interactions in *Arabidopsis thaliana*. *PLoS ONE*, **8(12): e83066.**

Whitham, S.A., Quan, S., Chang, H-S., Cooper, B., Estes, B., Zhu, T., Wang X., Hou, Y-M. (2003). Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. *The Plant Journal* **33**:271–283.

Wildermuth, M.C., Dewdney, J., Wu, G. Ausubel, F.M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature*, **414**: 562-565.

Windels, D., Bielewicz, D., Ebneter, M., Jarmolowski, A., Szweykowska-Kulinska, Z., Vazquez, F. (2014) miR393 Is Required for Production of Proper Auxin Signalling Outputs *PLoS One*.; **9**(4): e95972.

Wollmann, H., Mica, E., Todesco, M., Long, J.A., Weigel, D. (2010). On reconciling the interactions between APETALA2, miR172 and AGAMOUS with the ABC model of flower development. *Development*, **137**(21): 3633-3642.

Xia, X., Shao, Y., Jiang, J. Du, X., Sheng, L., Chen, F., Fang, W., Guan, Z., Chen, S. (2015). MicroRNA Expression Profile during Aphid Feeding in Chrysanthemum (*Chrysanthemum morifolium*) *PLoS One*.; **10**(12): e0143720.

Xie, Z., Allen, E., Fahlgren, N., Calamar, A., Givan, S.A., Carrington, J.C. (2005). Expression of *Arabidopsis* miRNA genes. *Plant Physiology*, **138**:2145-2154.

Xie, Z., Fan, B., Chen, C., Chen, Z. (2001). An important role of an inducible RNA-dependent RNA polymerase in plant antiviral defense. *Proceedings of the National Academy of Sciences of the U S A.* **98**(11):6516-21.

Yalpani, N., Leon, J., Lawton, M.A., Raskin, I. (1993). Pathway of salicylic acid biosynthesis in healthy and virus-inoculated tobacco. *Plant Physiology*, **103**(2):315-321.

Yang, G., You, M., Vasseur, L., Zhao Y., Liu C. (2011). Chapter 2: Development of RNAi in Insects and RNAi-Based Pest Control. In: Pesticides in the Modern World - Pests Control and Pesticides Exposure and Toxicity Assessment, Dr. Margarita Stoytcheva (Ed.). ISBN: 978-953-307-457-3.

Yao, M., Li, N., Wang, F., Ye, Z. (2013). Genetic analysis and identification of QTLs for resistance to *Cucumber mosaic virus* in chili pepper (*Capsicum annuum* L.). *Euphytica*, **193**(2):135–145.

Yu A., Lepere, G., Jay, F., Wang, J., Bapaume, L., Wang, Y., Abraham, A-L., Penterman, J., Fischer, R.L., Voinnet, O., Navarro, L. (2013) Dynamics and biological relevance of DNA demethylation in Arabidopsis antibacterial defense. *Proceedings of the National Academy of Sciences* **5**: 2389-2394.

Yu, D., Fan, B., MacFarlane, S.A., Chen, Z. (2003). Analysis of the involvement of an inducible Arabidopsis RNA-dependent RNA polymerase in antiviral defense. *Molecular Plant-Microbe Interactions*, **16**(3):206-16.

Yu, N., Christiaens, O., Liu, J., Niu, J., Cappelle, K., Caccia, S., Huvenne, H. Smagghe G. (2013). Delivery of dsRNA for RNAi in insects: an overview and future directions. *Insect Science*, **20**(1):4-14.

Yu, S., Lian, H., Wang, J.W. (2015a). Plant development transitions: the role of microRNAs and sugars. *Current Opinion in Plant Biology*, **27**: 1-7.

Yu, Z-X., Wang, L-J., Zhao, B., Shan., C-M., Zhang, Y-H., Chen, D-F., Chen X-Y. (2015b). Progressive regulation of the sesquiterpene biosynthesis in Arabidopsis and Patchouli (*Pogostemon cablin*) by the mir156-Targeted SPL Transcription Factors. *Molecular Plant*, **8**(1): 98-110.

Zagrobelny, M., Bak., S., Rasmussen A.V., Jorgensen, B., Naumann, C.M., Moller, B.L. (2004). Cyanogenic glucosides and plant-insect interactions. *Phytochemistry*, **65**(3):293-306.

Zaitlin, M., Hull, R. (1987). Plant Virus-Host Interactions. *Annual Review of Plant Physiology*, **38**:291-315.

Zhai, J., Jeong, D-H., Paoli, E.D., Park, S., Rosen, B.D., Li, Y., Gonza´ lez, A. J., Yan, Z., Kitto, S.L., Grusak, M.A., Jackson, S.A., Stacey, G., Cook, D.R., Green, P.J., Sherrier, D.J., Meyers, B.C. (2011). MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs. *Genes and Development*, **25**:2540–2553.

Zhang, H., Li, H-C., Miao, X-X. (2013). Feasibility, limitation and possible solutions of RNAi-based technology for insect pest control. *Insect Science*, **20**(1):15-30.

Zhang, X., Yuan, Y.R., Pei, Y., Lin, S.S., Tuschl, T., Patel, D.J., Chua, N.H. (2006). *Cucumber mosaic virus*-encoded 2b suppressor inhibits Arabidopsis Argonaute 1 cleavage activity to counter plant defense. *Genes and Development*, **20**(23):3255–3268.

Zhang, Z., Li, Q., Li, Z., Staswick, P. E., Wang, M., Zhu, Y., He, Z. (2007). Dual Regulation Role of GH3.5 in Salicylic Acid and Auxin Signaling during *Arabidopsis-Pseudomonas syringae* Interaction. *Plant Physiology*, **145**(2): 450–464.

Ziebell, H., Murphy, A.M., Groen, S.C., Tungadi, T., Westwood, J.H., Lewsey, M.G., Moulin, M., Kleczkowski, A., Smith, A.G., Stevens, M., Powell, G., Carr, J.P. (2011). *Cucumber mosaic virus* and its 2b RNA silencing suppressor modify plant-aphid interactions in tobacco. *Scientific Reports*, **1**(187).

Zust, T., Agrawal, A.A. (2016). Mechanisms and evolution of plant resistance to aphids. *Nature Plants*, **2**:15206.

Appendix I: List of Primers Used in this Study

At. = *Arabidopsis thaliana*

Ca. = *Capsicum annuum*

Primer ID	Target Gene	Sequence	Reference
nahG	*nahG*	CACCGGGCGGATTTCAT	Gallego-Giraldo et al., 2011
		CCCGAATTGGGCGATACC	
ago1.46	*At.AGO1*	TGCAAGATGCACACGCTCAGTTTC	Earley et al., 2010
		TGATGTCTCTGGCTCCATGTAGAAGCTAG	
SPL10	*At.SPL10*	TGTGGAATGGGTTGTCCCTTA	
		CCACCAGATGTTTGAAACGCA	
ARF8	*At.ARF8*	TAGAACCCGGAAATCCGCAG	
		GCCTAGAACAAACCAGCACC	
AUX1	*At.AUX1*	GCACTTCTCGACCACTCCAA	
		AGATGTTATACCTGACGAGCG	
GH3.5	*At.GH3.5*	TGGATGTGATTGTGACCGGG	
		AGTTTTGTTTTCTTACCGGCGT	
ARF2	*At.ARF2*	TGTTAATGACAGCAGGATGGG	
		CCGATACCAGCCTCCGACTA	
SID SNP C	*At.ICS1*	CCTTCGTAAGTCTCCCTGCCAATCG	
		TGCAGCTTCAATGCTTCATTTCTTGGATA	
AGO SNP C	*At.AGO1*	GGTGAGGGTGGGTAACATCAGCAGC	
		GGTGATTTGAAACGCATATGTGAGACTGAAC	
SLK CYP82C3N	*At.CYP82C3*	TGGGAGCAGTTTTGAGGTGG	
		TGTGTAGATAAAGTGTACCAGGCA	
SLK FOX1	*At.FOX1*	TGAGTGCTGAAGGTTACTCCG	
		ATAAAACCATGCAGGGCCGA	
AGO1.25N	*At.AGO1*	TCAGGCCAATCCTGAGATGC	
		TGCTTAGGAACACACTCTCAACT	
SID2N	*At.ICS1*	ACGACCTCGAGTTCTCTATCGT	

		TCGAAGAAATGAAGAGCTTGGAAA	
LBb 1.3	T-DNA (Salk line)	ATTTTGCCGATTTCGGAAC	Salk iSect Primers
RGM1	*At.RGM1*	AAGCTAGGTCAGAGAGCCCA	
		AAGCTCCCATTCCGTTGGAG	
CYP71A12 cDNA	*At.CYP71A12*	AAGTCCTCTTTATGATTACCTGCC	
		GCTCAAGAGGTATTGAAAACACAC	
FOX1 cDNA	*At.FOX1*	CAACATTGTCTTCCAAATCTTCTC	
		GTAGGAGCTGGAGGACACATAAG	
CYP82C2 cDNA	*At.CYP82C2*	AACAGCTTCTTTACCGAACCTTAG	
		AACCAGCTCTTTAGATCAACCATT	
GGP1 cDNA	*At.GGP1*	ATGATGATTGGATCCTTAAGCTCT	
		TCTTGGAATACGCAAGCACTTTAG	
Ca.Actin	*Ca.Actin*	CACTGAAGCACCCTTGAACCC	
		GAGACAACACCGCCTGAATAGC	
CMV CP	CMV *CP*	CGTTGCCGCTATCTCTGCTAT	
		GGATGCTGCATACTGACAAACC	
At.82C2 gDNA	*At.CYP82C2*	CCAAACACACATCTCTTTTGCAC	
		GCATCTTCAGGGGATAACGAG	
At.Fox1 gDNA	*At.FOX1*	CCTCACCCTTGATAAAAG	
		TTTATTGAATGCCTTCGG	
At.GGP1 gDNA	*At.GGP1*	AGATCTGGAGAAATACGATGGCT	
		ATAAAACCAACCTTGACGTAGCC	
CYP71A12 gDNA	*At.CYP71A12*	CCATGGAGGCTTCCGTTGAT	
		TGTCTATCCATGCCAAAGCC	
LB_o8474	T-DNA (GABI Kat)	ATAATAACGCTGCGGACATCTACATTTT	Kleinboelting et al., 2012

Appendix II: Statistical Tests for Data Presented in Chapter 3

Figure	**Test**	**F Ratio**	**Degree of freedom**	**p-value**	**Interpretation**
Figure 3.10	ANOVA	2.337	2	0.112	Not Significant
Figure 3.11A	ANOVA	34.2	3	4.53 x 10^-12	Significant
	Tukey				
ago1/cyp82C2 vs. *ago1.25*				0.0677574	Not Significant
WT vs. *ago1.25*				0.0000000	Significant
cyp82C2 vs. *ago1.25*				0.0000001	Significant
WT vs. *ago1/cyp82C2*				0.0000001	Significant
cyp82C2 vs. *ago1/cyp82C2*				0.0003413	Significant
cyp82C2 vs. WT				0.1017521	Not Significant
Figure 3.11B	ANOVA	53.77	3	1.95 x 10^-14	Significant
	Tukey				
WT vs. *ago1.25*				0.0000000	Significant
fox1 vs. *ago1.25*				0.0000000	Significant
fox1/ago1 vs. *ago1.25*				0.5963932	Not Significant
fox1 vs. WT				0.9998589	Not Significant
fox1/ago1 vs. WT				0.0000000	Significant
fox1/ago1 vs. *fox1*				0.0000000	Significant
Figure 3.12A	ANOVA	16.62	2	6.73 x 10^-5	Significant
	Tukey				
ago1/cyp82C2 vs. *ago1.25*				0.0934412	Not Significant
WT vs. *ago1.25*				0.0149896	Significant
WT vs. *ago1/cyp82C2*				0.0000428	Significant
Figure 3.12B	ANOVA	19.95	3	1.78 x 10^-7	Significant
	Tukey				
WT vs. *ago1.25*				0.0000069	Significant
fox1 vs. *ago1.25*				0.0002079	Significant
fox1/ago1 vs. *ago1.25*				0.8727300	Not Significant

fox1 vs. WT				0.6413878	Not Significant
fox1/ago1.25 vs. WT				0.0000077	Significant
fox1/ago1.25 vrs-*fox1*				0.0001489	Significant
Figure 3.14	ANOVA	1.368	2	0.268	Not Significant

Appendix III: Statistical Tests for Data Presented in Chapter 4

Figure	**Statistical Test**	**F Ratio**	**Degree of Freedom**	**p-value**	**Interpretation**
Figure 4.5	ANOVA	40.74	5	5.85 x 10^-3	Significant
Figure 4.16A	ANOVA	7.853	3	0.00041	Significant
	Tukey				
ago1/ics1 vs. *ago1.25*				0.0292524	Significant
WT vs. *ago1.25*				0.0384863	Significant
ics1 vs. *ago1.25*				0.0003435	Significant
WT vs. *ago1/ics1*				0.9956114	Not Significant
ics1 vs. *ago1/ics1*				0.4559289	Not Significant
ics1 vs. WT				0.6918716	Not Significant
Figure 4.16B	ANOVA	6.772	4	0.000456	Significant
	Tukey				
ago1/ics1 (AS17 1.1) vs. *ago1.25*				0.0315292	Significant
ago1/ics1(AS20) ago1like vs. *ago1.25*				0.9999975	Not Significant
WT vs. *ago1.25*				0.0282195	Significant
ics1 vs. *ago1.25*				0.0070406	Significant
ago1/ics1 (AS20 ago1-like) vs. *ago1/ics1 (AS17 1.1)*				0.0279951	Significant
WT vs. *ago1/ics1 (AS17 1.1)*				0.9998826	Not Significant
ics1 vs. *ago1/ics1* (AS17 1.1)				0.9511048	Not Significant
WT vs. *ago1/ics1* (AS20 *ago1-like*)				0.0252484	Significant
ics1 vs. *ago1/ics1 (AS20 ago1-like)*				0.0059467	Significant
ics1 vs. WT				0.9811939	Not Significant
Figure 4.17	ANOVA	66.87	7	2.0 x 10^-16	Significant

Appendix IV: Statistical Tests for Data Presented in Chapter 5

Figure	**Statistical Test**	**F Ratio**	**Degree of freedom**	**p-value**	**Interpretation**
Figure 5.3	ANOVA	7.86	3	0.000514	Significant
Figure 5.3	Tukey				
2bago vs. *2b*				0.0702717	Not Significant
ago1.25 vs. *2b*				0.9979490	Not Significant
WT vs. *2b*				0.5708688	Not Significant
ago1.25 vs. *2bago*				0.0440319	Significant
WT vs. *2bago*				0.0003482	Significant
WT vs. *ago1.25*				0.3496302	Not Significant

Appendix V: R-Codes Used for Data Analyses

I. R-code for Analysing Aphid Mean Relative Growth Rate Data

```
mrgr<-read.csv(file.choose(),header = T)
aggregate(aphid~plant,data = mrgr,summary)
boxplot(aphid~plant,data = mrgr)
ANOVA.mrgr<-aov(aphid~plant,data = mrgr)
summary(ANOVA.mrgr)
TukeyHSD(ANOVA.mrgr)
gpmean<-aggregate(aphid~plant,data = mrgr,mean)
newdf<-as.data.frame(gpmean)
newdf[1,]<-gpmean[4,]
newdf[2,]<-gpmean[1,]
newdf[3,]<-gpmean[3,]
newdf[4,]<-gpmean[2,]
semean<-aggregate(aphid~plant,data = mrgr,FUN = function(x) sd(x)/sqrt(length(x)))
newdfse<-as.data.frame(semean)
newdfse[1,]<-semean[4,]
newdfse[2,]<-semean[1,]
newdfse[3,]<-semean[3,]
newdfse[4,]<-semean[2,]
barplot(newdf$aphid,names.arg = c("Col", "2b", "ago1", "2bago"),ylim = c(0,0.50), ylab
="MRGR")
segments(0.7,newdf[1,2]+newdfse[1,2],0.7,newdf[1,2]-newdfse[1,2])
segments(1.9,newdf[2,2]+newdfse[2,2],1.9,newdf[2,2]-newdfse[2,2])
segments(3.1,newdf[3,2]+newdfse[3,2],3.1,newdf[3,2]-newdfse[3,2])
segments(4.3,newdf[4,2]+newdfse[4,2],4.3,newdf[4,2]-newdfse[4,2])
```

II. R-code for Analysing Aphid Colony Size Data

```
col<-read.csv(file.choose(),header = T)
aggregate(colony~plant,data = col,summary)
boxplot(colony~plant,data = col)
ANOVA.col<-aov(colony~plant,data = col)
summary(ANOVA.col)
TukeyHSD(ANOVA.col)
gpmean<-aggregate(colony~plant,data = col,mean)
newdf<-as.data.frame(gpmean)
newdf[1,]<-gpmean[4,]
newdf[2,]<-gpmean[1,]
newdf[3,]<-gpmean[3,]
newdf[4,]<-gpmean[2,]
semean<-aggregate(colony~plant,data = col,FUN = function(x) sd(x)/sqrt(length(x)))
newdfse<-as.data.frame(semean)
newdfse[1,]<-semean[4,]
newdfse[2,]<-semean[1,]
newdfse[3,]<-semean[3,]
newdfse[4,]<-semean[2,]
barplot(newdf$colony,names.arg = c("Col", "2b", "ago1", "2bago"),ylim = c(0,25), ylab
="Colony Size")
segments(0.7,newdf[1,2]+newdfse[1,2],0.7,newdf[1,2]-newdfse[1,2])
segments(1.9,newdf[2,2]+newdfse[2,2],1.9,newdf[2,2]-newdfse[2,2])
segments(3.1,newdf[3,2]+newdfse[3,2],3.1,newdf[3,2]-newdfse[3,2])
segments(4.3,newdf[4,2]+newdfse[4,2],4.3,newdf[4,2]-newdfse[4,2])
```

III. R-code for Analysing Aphid-Induced Loss of Plant Biomass Data

```
Arabwt<-read.csv(file.choose(),header = T)
aggregate(weight~plant,data = Arabwt,summary)
boxplot(weight~plant,data = Arabwt)
ANOVA.weight<-aov(weight~plant,data = Arabwt)
summary(ANOVA.weight)
TukeyHSD(ANOVA.weight)
gpmean<-aggregate(weight~plant,data = Arabwt,mean)
newdf<-as.data.frame(gpmean)
newdf[1,]<-gpmean[6,]
newdf[2,]<-gpmean[5,]
newdf[3,]<-gpmean[8,]
newdf[4,]<-gpmean[7,]
newdf[5,]<-gpmean[4,]
newdf[6,]<-gpmean[3,]
newdf[7,]<-gpmean[2,]
newdf[8,]<-gpmean[1,]
semean<-aggregate(aphid~plant,data = mrgr,FUN = function(x) sd(x)/sqrt(length(x)))
newdfse<-as.data.frame(semean)
newdfse[1,]<-semean[6,]
newdfse[2,]<-semean[5,]
newdfse[3,]<-semean[8,]
newdfse[4,]<-semean[7,]
newdfse[5,]<-semean[4,]
newdfse[6,]<-semean[3,]
newdfse[7,]<-semean[2,]
newdfse[8,]<-semean[1,]
barplot(newdf$weight,names.arg = c("Col.M","Col.A", "ICS1.M", "ICS1.A", "AS17.1.4M",
"AS17.1.4A", "Ago.M", "Ago.A"),ylim = c(0,0.16), ylab ="Dry Weight")
```

```
segments(0.7,newdf[1,2]+newdfse[1,2],0.7,newdf[1,2]-newdfse[1,2])
segments(1.9,newdf[2,2]+newdfse[2,2],1.9,newdf[2,2]-newdfse[2,2])
segments(3.1,newdf[3,2]+newdfse[3,2],3.1,newdf[3,2]-newdfse[3,2])
segments(4.3,newdf[4,2]+newdfse[4,2],4.3,newdf[4,2]-newdfse[4,2])
segments(5.5,newdf[5,2]+newdfse[5,2],5.5,newdf[5,2]-newdfse[5,2])
segments(6.7,newdf[6,2]+newdfse[6,2],6.7,newdf[6,2]-newdfse[6,2])
segments(7.9,newdf[7,2]+newdfse[7,2],7.9,newdf[7,2]-newdfse[7,2])
segments(9.1,newdf[8,2]+newdfse[8,2],9.1,newdf[8,2]-newdfse[8,2])
```