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ANALYSIS OF TURNIP CRINKLE VIRUS EFFECTS ON THE INNATE AND ADAPTIVE IMMUNITY IN ARABIDOPSIS THALIANA

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ANALYSIS OF TURNIP CRINKLE VIRUS EFFECTS ON THE INNATE AND ADAPTIVE IMMUNITY IN ARABIDOPSIS THALIANA

By

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ANALYSIS OF TURNIP CRINKLE VIRUS EFFECTS ON THE INNATE AND ADAPTIVE IMMUNITY IN ARABIDOPSIS THALIANA

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The ability to understand the interactions between plants and the variety of pathogens they encounter on a daily basis is an important area of research. In the following work presented in this dissertation, I sought to better understand the mechanisms that Turnip crinkle virus (TCV) employs to elude the defense responses of the host plant *Arabidopsis thaliana*. It was previously determined that TCV coat protein (CP) interacts with a transcription factor, TIP, within the about 10 amino acid region near the N-terminus of the CP called the R-domain. When this interaction was disrupted by making single amino acid substitutions through the R-domain region, I observed a marked variation in symptom severity and alterations in both basal and resistance gene mediated responses. To further explore the effect of the TCV CP-TIP interaction on virus invasiveness and the plant defense systems, I analyzed virus accumulation and defense gene expression from the susceptible (Col-0) and resistant (Di-17) lines throughout a time course of infection. I discovered that the wildtype TCV (wtTCV) had a transient replicative advantage over CP mutants that were not able to bind TIP. This effect occurred within the first 4 to 6 days of infection. Research reported here demonstrates that the ability of wtTCV to bind TIP

causes a suppression of the basal defense response that facilitates viral invasion of the systemic leaves in the susceptible ecotype Col-0.

Further experiments confirmed that TIP-CP binding also had an effect on the R-gene meditated defense conditioned by the *HRT* (Hypersensitive Response to TCV) gene in the resistant line Dijon-17. This was demonstrated by monitoring virus accumulation and symptom development between wtTCV and several CP mutants with altered TIP binding ability. I demonstrate that expression of the *TIP* gene in the presence or absence of the R-gene *HRT* altered development of disease symptoms and systemic spread of the virus.

A primary outcome of the research reported in this dissertation is the demonstration that the interaction of TCV CP with the TIP transcription factor modulates both major defense layers of the plant immune system. These are the basal defense layers referred to as Pathogen Triggered Immunity (PTI) defense and the development of systemic acquired resistance modulated by R-gene mediated defense referred to as the Effector Triggered Immunity (ETI) defense.

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LIST OF ABBREVIATIONS

aa	amino acid
ACC	1-aminocyclopropane-1-carboxylic-acid
ACS	ACC synthase
ACT	Actin
AGO	Argonaute
AP	alkaline phosphate
ARF	Auxin response factor
ATAF	Arabidopsis transcription activator/ transcription factor
ATP	Adenosine triphosphate
AVR	Avirulence protein
BAK1	BRI1-associated receptor kinas
BRI1	Brassinosteroid insensitive 1
CaMV	Cauliflower mosaic virus
CC	coiled coil
CHIB	Acidic endochitinase
CML38	Calmodulin like 38
CMV	Cucumber mosaic virus
COI1	Coronatine insensitive 1
Col-0	Columbia - 0
CP	coat protein
CUC2	Cup-shaped cotyledon 2
D13A	TCV non-TIP binding mutant

DA	Dalton
DAB	3,3-diaminobenzidine
DCL	Dicer-like protein
Di-17	Di-17
DNA	deoxyribonucleic acid
dpi	days post inoculation
DRB4	Double-stranded RNA binding protein 4
ECI	Enzyme conjugating buffer (Agdia)
EDS	Enhanced disease susceptibility
EDTA	Ethylenediaminetetraacetic acid
EFR	EF-Tu receptor
EF-Tu	Elongation factor-Tu
EIN2	Ethylene insensitive 2
ELISA	Enzyme-linked immunosorbent assay
ERF	Ethylene response factor
ET	Ethylene
ETI	Effector triggered immunity
flg22	flagella protein 22
FLS2	Flagellin sensitive 2
FRY1	Fiery1
G14A	TCV TIP binding mutant
GFP	Green flourescence protein
gRNA	Genomic RNA

GST	Glutathione S-transferase
GTP	Guanosine triphosphate
GW	Glycine/tryptophane
HEL	Hevein-like protein (also known as PR4)
HIN1	Harpin-induced gene1
HR	Hypersensitive response
hrpi	Hours post inoculation
HRT	Hypersensitive response to TCV
ICS	Isochorismate synthase
IL	Inoculated Leaves
IR	Inverted repeats
JA	Jasmonic acid
JAR1	Jasmonate resistance1
kDA	kilodalton
ko	knockout
LRR	Leucine rich repeats
MAMP	Microbe associated molecular patterns
MAP	Mitogen-activated protein
MEKK	Mitogen-activated protein kinase kinase
miRNA	micro-RNA
MPK4	Map kinase phosphatase
NAC	NAM, ATAF1,2, CUC2
NAM	No apical meristem

NBS	Nucleotide binding site
NDR1	Non-race specific disease resistance
NHL	Nicotiana HIN1 like (also known as YLS9)
NOD	Nucleotide-binding oligomerization domain
NPR1	Non-expressor of pathogenesis related genes 1
ORF	Open reading frame
P	phosphorus
PAD4	Phytoalexin deficient 4
PAMP	Pathogen associated molecular pattern
PBST	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PDF1.2	Plant Defensin-like protein
PEP	Phosphoenolpyruvate
PNP	p-nitrophenol
PR	Pathogenesis related gene
PRR	Pathogen recognition receptor
Pst	*Pseudomonas syringae* pv tomato
PTGS	Post transcriptional gene silencing
PTI	Pathogen triggered immunity
qRT-PCR	Real-time reverse transcription PCR
R	Resistance; R domain of CP; arginine
R6A	TCV non-TIP binding mutant
R8A	TCV non-TIP binding mutant

RAV2	Regulator of the ATPase of the vacuolar membrane
RDR6	RNA dependent RNA polymerase 6
RISC	RNA induced silencing complex
RLK	Receptor-like kinase
RNA	Ribonucleic acid
RRT	Regulates resistance to TCV
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulfate
sgRNA	Subgenomic RNA
SID2	Salicylic acid induction deficient 2
siRNA	small-interfering RNA
SL	Systemic Leaves
sqRT-PCR	semi-quantitative reverse transcription PCR
SSC	Saline-sodium citrate
TBE	Tris/Borate/EDTA
TCV	Turnip crinkle virus
Thl2.1	Thionin 1; toxin receptor binding
TIP	TCV interacting protein
TIR	Toll-like receptors
TMV	Tobacco mosaic virus
tRNA	Transfer RNA
TYMV	Turnip yellow mosaic virus

WRKY	WRKY domain binding protein
XRN	Exoribonucleases
YLS9	Yellow-leaf-specific gene 9 (also known as NHL10)

CHAPTER 1: INTRODUCTION

Understanding the specific molecular interactions between plants and their pathogens is an active area of research. Plants have evolved complex signaling networks intended to detect specific pathogens in order to trigger the appropriate defense responses (Koornneef and Pieterse, 2008). A growing body of evidence suggests that plants have also evolved intricate mechanisms to exert control over pathogen induced defense pathways. Many studies have shown that constitutive activation of defense signaling pathways compromises normal plant growth and overall fitness (Bostock, 2005; Koornneef and Pieterse, 2008; Pauwels, Inzé, and Goossens, 2009; Steppuhn and Baldwin, 2008; Stout, Thaler, and Thomma, 2006; Van Hulten et al., 2006). Therefore, it is hypothesized that the expression of the majority of defense associated genes are dampened by negative regulation and only activated upon pathogen infection (Bostock, 2005; Ge et al., 2007; Takken, Albrecht, and Tameling, 2006). The ability of a plant to perceive the invading pathogen and signal for the proper defense response involves two major layers of defense known as pathogen associated molecular pattern (PAMP) triggered immunity (PTI) and effector triggered immunity (ETI`; Jones and Dangl, 2006). An additional layer, known as the RNA silencing pathway, is a type of adaptive immunity that plays a major role in antiviral and other anti-microbial host related defense responses (reviewed in (Ruiz-Ferrer and Voinnet, 2009). RNA silencing will be reviewed further in Chapter 4.

Turnip crinkle virus (TCV) is a positive sense, RNA plant virus with a genome size of 4 kb. TCV systemically infects the susceptible *Arabidopsis thaliana* ecotype Columbia-0 (Col-0), and does not elicit a resistance response on inoculated leaves. In the resistant *A. thaliana* ecotype Dijon-17 (Di-17), TCV causes a hypersensitive response

(HR), which is analogous to programmed cell death in animals, at the site of infection. This is accompanied by a resistance response that sequesters the virus and prevents systemic invasion. It has been documented that Di-17 displays resistance to systemic infection in only about 70% of TCV infections (Dempsey, Wobbe, and Klessig, 1993). This is likely due to the fact that the TCV resistance response in Di-17 is environmentally modulated including being sensitive to photoperiod (Chandra-Shekara et al., 2006). HR and resistance to TCV are determined by the presence of the dominant gene *HRT* (hypersensitive *r*esponse to TCV) and the recessive allele *rrt* (regulates resistance to TCV; Kachroo et al., 2000). The coat protein (CP) is the elicitor of the resistance response although no direct interaction between HRT and TCV CP has been detected despite repeated attempts to demonstrate it (Oh et al., 1995; Wobbe and Zhao, 1998).

Previous work conducted by Ren *et al*. (2000), identified a NAC transcription factor protein TIP (TCV-Interacting Protein) that was shown to interact directly with TCV CP *in vitro* using a yeast two hybrid screen and with GST pull down experiments. TIP-CP interaction was also demonstrated *in vivo* in *N. benthamiana* using transiently expressed GFP-tagged TIP protein. This study further demonstrated that TIP-CP interaction appeared to affect the HRT mediated resistant defense response in Di-17. A TCV CP mutant R6A, with a single amino acid (aa) substitution in the CP, was created that lost its ability to bind to TIP. Interestingly, infections by R6A also caused more severe symptoms in the susceptible line Col-0 and broke resistance in Di-17. These phenotypes were shared by several other aa substitution mutants made in the same region near the N-terminus of the CP. In my studies described in chapter two, I report on the results of an in depth examination of infections of the R6A mutant virus. My data

demonstrates that loss of TIP interaction in the case of the TCV mutant R6A was primarily associated with loss of the ability of wt TCV to modify the basal defense response (PTI) in both the susceptible host Col-0 and the resistant host Di-17.

Pathogen triggered immunity

Pathogen Triggered Immunity (PTI) is the primary defense response in plants which recognizes common microbial patterns, known as pathogen associated molecular patterns (PAMPs), through plant-encoded pathogen recognition receptors (PRR). The recognition of PAMPs stimulates a signaling cascade through the MAP kinase network. This signaling results in the activation of transcription factors that leads to massive transcriptional reprogramming of defense related genes (Asai et al., 2002). PAMPs are generally indispensable for microbial metabolism or invasive growth and are thus broadly conserved among different pathogenic species. In general, the ability of a healthy host organism to recognize any one of the numerous PAMPs provokes an innate immune response that prevents most pathogens from invading and causing disease.

One of the first PRR-PAMP recognition events described was a leucine rich repeat (LRR)-receptor kinase known as FLAGELLIN SENSITIVE-2 (FLS2). FLS2 was first identified in *Arabidopsis thaliana* through screening of the bacterial flagella protein flg22 (Gómez-Gómez and Boller, 2000) and was later shown to bind and determine the specificity of flagella recognition response (Chinchilla et al., 2006). Following flg22 detection, FLS2 is internalized by a receptor-mediated endocytosis process that presumably has regulatory functions in conjunction with proteins such as BAK1, ERF, and MEKK (Chinchilla et al., 2007; Robatzek, Chinchilla, and Boller, 2006; Suarez-

Rodriguez et al., 2007; Xiang et al., 2008). FLS2 is believed to function early in infection because fls2 mutant lines have increased susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000 when applied as a topical spray, but not with internal infection using syringe agro-infiltration (Zipfel et al., 2004). Other Arabidopsis PRRs recognize different PAMPs such as elongation factor-Tu (EF-Tu) via the LRR-kinase called EFR (Kunze et al., 2004; Zipfel et al., 2006). EF-Tu is a conserved translational elongation factor in bacterial cells that mediates the entry of the amino-acylated (charged) tRNAs into a free site of the ribosome (Krab and Parmeggiani, 1998).

Effector triggered immunity

Successful pathogens have evolved ways to overcome PTI and evade plant recognition of their PAMPs in order to establish a suitable environment for growth and reproduction, and thus cause disease. Pathogens encode a range of proteins that can block the recognition of PAMPs and manipulate host machinery to favor pathogen invasion. Therefore, pathogen effectors often function as virulence factors during infection, and their presence results in a specific kind of disease response. In turn, plants have evolved surveillance systems to recognize the pathogen's effectors, thereby triggering another layer of the plant immune response known as effector triggered immunity (ETI). This second layer of defense, also referred to as resistance (R) protein based defense, is more specific and employs R proteins that recognize the presence of pathogen effector proteins, also known as avirulence (avr) proteins that can result in the hypersensitive response(HR; Jones and Dangl, 2006).

Specific physical characteristics or domains can be commonly found in plant R proteins which has allowed for the identification of many putative R genes in plants whose genomes have been sequenced such as *A. thaliana, Oryza sativa*, and *Populus trichocarpa deltoides*. These common domains are the coiled coil (CC), *Drosophila* Toll and mammalian interleukin like receptor (TIR), nucleotide binding site (NBS) domains, leucine-rich repeat (LRR) domains, and kinase domains. Five different classes of R proteins have been identified based on shared domains and their location within the cell (Figure 1-1). The CC and TIR are located at the N-terminus of the protein and are thought to play a role in transcription factor activation and cell death induction, respectively (Pan, Wendel, and Fluhr, 2000; Swiderski, Birker, and Jones, 2009). The NBS domain is a highly conserved region in R proteins that also contains blocks of sequence that are conserved in both plant and animal proteins (Takken, Albrecht, and Tameling, 2006; Van der Biezen and Jones, 1998a). The animal homologs of NBS domains are called NODs (nucleotide-binding oligomerization domain) which have been implicated in innate recognition of bacteria and the induction of inflammatory responses (Inohara and Nunez, 2003; Kanneganti, Lamkanfi, and Núñez, 2007). The NBS domain functions through the binding of ATP or GTP which results in the activation of a signal through the creation of binding sites for downstream signaling molecules and/or the conformational change for the formation of NBS-LRR protein multimers (DeYoung and Innes, 2006). LRR domains are found in various proteins and function as sites of protein-protein interactions, peptide-ligand binding, and protein-carbohydrate interactions (DeYoung and Innes, 2006). Kinase domains are responsible for transferring a phosphate from nucleotide triphosphates (often ATP) to one or more amino acid residues in a

protein substrate side chain, resulting in a conformational change affecting protein function. These enzymes typically are classified as either serine/threonine specific kinases or tyrosine specific kinases (Hanks, Quinn, and Hunter, 1988).

In order to explain the interaction and specificity between host - pathogen resistance and their effectors, Flor (1971) proposed the gene-for-gene model hypothesizing that pathogens encode a single gene whose product can be recognized by a specific plant encoded protein. Advances in research in plant-pathogen interaction since Flor's hypothesis have allowed for the realization of coevolutionary mechanisms between plants and their pathogens involving many interactions on the molecular level. Specific interactions are now recognized as being either direct or indirect interactions between plant encoded resistance (R) genes and pathogen encoded avirulence (avr) genes. The interaction between specific R - avr gene products would potentially result in dramatic changes in the infected cell that trigger the defense response, typically the HR, that then leads to resistance. For example, in tobacco (*Nicotiana tabacum*) the resistance gene *N* is a R gene that encodes a TIR receptor with both NBS and LRR domains (Whitham et al., 1994). The N gene encoded protein interacts with the helicase domain of the p50 from the tobacco mosaic virus (TMV (Padgett and Beachy, 1993). This N-p50 interaction was shown to be direct and confers resistance against TMV infections in tobacco lines that carry the N gene by inducing an HR at the site of infection (Erickson et al., 1999; Ueda, Yamaguchi, and Sano, 2006).

One case of R/Avr protein direct interaction during bacterial infection is demonstrated by the binding of the tomato Pto protein and the bacterial effector AvrPto. This interaction causes the HR which limits the growth of the pathogen, *P. syringae* pv.

tomato (Scofield et al., 1996). Another example of the R/Avr protein interaction is associated with one of the most aggressive fungal pathogens of maize, *Cochliobolus carbonum* race 1. *C. carbonum* race 1 pathogenicity is mapped to a single locus Tox2 (Scheffer and Ullstrup, 1965) that produces *Helminthosporium carbonum* (HC) toxin (Ullstrup, 1941). Most maize germplasm is resistant to infection due to the dominant gene, *Hm1* (*H. maydis1*), which inactivates HC toxin, and this result is sufficient to prevent infection (Johal and Briggs, 1992).

Though the gene-for-gene theory has been validated by the discovery of many specific plant *R* genes and their corresponding *avr* genes, it has been proposed that *R* genes can and do in a majority of cases play a more active role in the cell than just surveillance for one effector (Dangl and Jones, 2001). Therefore instead of direct interaction between R/avr proteins, it is now known that the majority of these interactions are indirect (Jones and Takemoto, 2004; Jones and Dangl, 2006). The guard hypothesis was proposed by van der Biezen and Jones in 1998 to account for the more common examples of indirect R/avr interactions. It predicted that R proteins activate resistance within the cell when they interact with other plant proteins that are targeted and modified by the invading pathogen effectors (Marathe and Dinesh-Kumar, 2003). This concept shifts the view of the possible roles of *R* genes and their products to a more active one in pathogen surveillance. Instead of viewing R proteins as passively waiting for a specific signal or interaction from a pathogen effector, the hypothesis predicts that R proteins act like a “guard”, to constantly monitor certain physiological and regulatory processes and proteins (“the guardee”) that pathogens generally target to benefit the pathogen’s own fitness.

One example supporting this view is the interaction of two plant factors, Pto and Prf, that govern the AvrPto-triggered resistance to the bacterial pathogen *P. syringae* pv. *tomato*. AvrPto, the pathogen effector, interacts with Pto, the guardee, and that interaction is recognized by Prf, the guard molecule. When Prf recognizes and binds to the Pto-AvrPto complex, it activates the defense response. Dangl and Jones (2001) reiterated the presence of direct interactions amongst these players and predicted two functional scenarios to define this interaction. One way involves the effector initially binding the guardee which results in a conformation change that increases the affinity of the complex for the R protein (guard), therefore activating resistance. The second scenario describes R proteins as constitutively forming complexes with their guardees which are disrupted by the guardee's interaction with the effector. When the guardee interacts with the effector, the result is the disassembly of the complex and subsequent activation of the R protein. Studies have also proposed that R proteins monitor the activities of multiple effectors by detecting physiological changes of a guardee caused by effectors instead of monitoring the complex that results from direct binding of effectors to the guardee (Chisholm et al., 2006). These physiological changes may involve covalent modification, such as phosphorylation or de-phosphorylation of a protein, or proteolytic cleavage of a protein. Another example of the guard hypothesis is the RPS2-mediated resistance to *P. syringae* DC3000 carrying AvrRpt2, a cysteine protease (Mackey et al., 2003). This resistance is activated by RPS2 when it detects the cleavage and elimination of RIN4 (guardee) induced by AvrRpt2 (effector) (Mackey et al., 2003).

Dangl and Jones (2001) suggested that the guard hypothesis was a useful model to explain the TIP-CP interaction associated with Arabidopsis-TCV resistance response

reported by Ren et al., 2000. No direct evidence for interaction has been described between CP, the effector of resistance and the mediator of defense HRT (Dempsey et al., 1997). However the identification of TIP provided the first example of a direct interaction between a viral pathogen avirulence factor and a putative guardee-like molecule that fit the guard model of plant host-pathogen interaction (Ren, Qu, and Morris, 2000).

Over the past few years, new data on the indirect recognition of effectors have emerged that are inconsistent with the original description of the Guard Model. It is now well documented that many pathogen effectors have multiple targets within the host (Zipfel and Rathjen, 2008), and that classical guardee proteins are often dispensable for the virulence activities of effectors in plants lacking the R protein (Van der Hoorn and Kamoun, 2008; Zhou and Chai, 2008). New data on additional targets of AvrPto and AvrBs3 gave rise to the idea that some host targets of effectors act as decoys to detect pathogen effectors via R proteins (Zhou and Chai, 2008; Zipfel and Rathjen, 2008). A new theory, called the Decoy Model, detailed by Van der Hoorn and Kamoun (2008), proposed that plants have evolved decoy proteins which act as targets of effectors to detect pathogens without disruption of important cell processes. The Decoy Model implies that the effector target monitored by the R protein functions as a decoy that mimics the operative effector target. The decoy, however, only functions in perception of pathogen effectors without contributing to pathogen fitness in the absence of its cognate R protein. Therefore, the major difference between the guard model and the decoy model is whether or not the pathogen is potentially benefited by the encoded protein that will interact with the guarded target (Block and Alfano, 2011).

Conceptually, decoys may be evolutionarily related to operative targets or may have evolved independently by target mimicry (Van der Hoorn and Kamoun, 2008). Both scenarios could be valid for Pto-related defense. As stated earlier, the effector AvrPto, from *P. syringae*, interacts with Pto but can also inhibit multiple defense-related kinases. Therefore, Pto could have directly evolved from one of these kinase targets, but lost its extracellular domains that are not required for AvrPto perception. This scenario is consistent with the observation that Pto is most closely related to the kinase domains of receptor-like kinases (Hardie, 1999; Van der Hoorn and Kamoun, 2008). However, Pto may have functioned in a kinase pathway that was not originally targeted by AvrPto but evolved to function in effector perception by mimicking the operative targets of Pto (target mimicry). Although the decoy model lacks the experimental evidence that the guard hypothesis has for validation, it is consistent with some recent studies (Schornack et al., 2008; Xiang et al., 2008; Xing et al., 2007) and presents a possible challenge to the current theory behind plant defense against biotic stresses (Van der Hoorn and Kamoun, 2008). Regardless of whether the guard or decoy model is supported by further studies, it is becoming clear that by monitoring pathogens which are seeking one or more host targets will be key in understanding the complexity of plant defense. Likewise, determining how R proteins can detect the effecter and/or the associated enzymatic activity of multiple pathogen effectors and signal appropriate defense responses will also be a great value in the analysis of plant innate immunity (Chisholm et al., 2006; Dangl and Jones, 2001; Van Der Biezen and Jones, 1998b). My research has sought to elucidate the proposed role of TIP as a potential guardee or decoy protein. My studies subsequently showed the binding of TCV CP to TIP was most likely an evolutionary

adaptation by TCV to alter the host innate immune response to permit more rapid systemic invasion by the virus.

Hypersensitive response

The HR is a complex early defense response that causes necrosis and cell death that can restrict the growth and spread of a pathogen, eventually leading to the development of broad spectrum resistance to the eliciting pathogen as well as other related pathogens (Hammond-Kosack and Jones, 1996). The specificity of this process is modulated by R proteins that associate in a race-specific manner with a pathogen encoded protein in a direct or indirect manner. This interaction leads to a change in the membrane potential and ion permeability of the host cell plasma membrane resulting in localized cell death (Heath, 2000). One of the first biological responses of the HR is an oxidative burst which includes the generation of superoxide anions (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (-OH) (Apostol, Heinstein, and Low, 1989). The oxidative burst has been shown to be a downstream consequence of some R and Avr protein interaction signal cascades leading to HR development (Wolfe et al., 2000). The HR is also linked to systemic plant responses by causing an increase in the number of secondary signal molecules such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and auxins in infected and systemic tissue (Heath, 2000). The HR precedes the secondary resistance response, referred to as systemic acquired resistance (SAR). SAR is a heightened state of resistance to a broad spectrum of microbial pathogens in tissues distal to the infection site that can last for an extended time period (Kombrink and Schmelzer, 2001). The resistance mechanisms that trigger SAR are still unclear.

In the case of the Arabidopsis-TCV interaction, HR development can be observed in the inoculated leaves of *A. thaliana* ecotype Di-17 infected with TCV. The onset of HR is elicited by the indirect recognition of the pathogen effector TCV CP by the R protein, HRT. The development of resistance to TCV is also dependent upon other proteins in addition to HRT and some abiotic factors as well. Another genetic locus *RRT* (Regulates Resistance to TCV) was identified that regulates resistance to TCV in Col-0, the susceptible host. To date, this gene and its putative function remain unresolved (Kachroo et al., 2000). In the resistant line Di-17, it is postulated that this gene must be present as a recessive locus (*rrt*) in order for HRT to induce ETI. The recessive locus *rrt*, and components of the SA pathway, including EDS1, EDS5, PAD4 and SID2, must all be present for TCV resistance to be manifested in Di-17 (Chandra-Shekara et al., 2004). Resistance is, however, independent of NPR1, and the JA and ET signaling pathways (Figure 1-2) (Kachroo et al., 2000). More recently, it has also been demonstrated that light intensity and duration of the photoperiod following TCV infection can be key modulators of TCV infection and resistance, and which components play a role downstream of the initial signaling following HRT recognition of CP (Figure 1-2) (Chandra-Shekara et al., 2006).

Systemic acquired resistance

In addition to the local resistance (HR) that protects host plants from pathogens, plants also signal to induce defense responses in systemic tissues after pathogen attack. SAR is induced in distal tissues following pathogen infection and confers a long-lasting resistance to secondary infections by a broad spectrum of pathogens, not just the

pathogen that initially infected the plant (Sticher, Mauch-Mani, and Métraux, 1997). Methyl salicylate (methyl-SA) has also been shown to be one of the signals that gets translocated to distal tissues inducing SAR (Park et al., 2007; Vlot, Klessig, and Park, 2008). Along with methyl-SA, other molecules like JA, glycerolipid-derived factors, and PEPs (Phosphoenolpyruvate) have been shown to be translocated as the signals for SAR depending on the type of microbial infection (Truman et al., 2007).

SAR development depends on pathogen recognition, translocation of the recognition signal, and the plant's ability to regulate which defense genes should be expressed upon pathogen invasion. There is sufficient evidence that shows constitutively expressed defense genes lead to a decrease in overall plant fitness and size, and therefore it is believed that plants have evolved the ability to keep the majority of their defense genes under negative regulation to prevent the waste of valuable resources (Felton and Korth, 2000). In our TCV-Arabidopsis model, we have hypothesized that TIP may be functioning as a negative regulator of defense and that the interaction of TCV CP with TIP stabilizes this negative regulation resulting in TCV being a more invasive pathogen. Studies reported in chapter two support this model in both susceptible and resistant lines of Arabidopsis. The mechanism underlying this resistant response is not understood and is the focus of this research.

Other factors, such as environmental conditions must be taken into account when studying SAR because abiotic factors like drought, temperature, and light will affect a plant's overall health and defense response (Vlot, Klessig, and Park, 2008). For instance, studies have shown that higher temperatures affect key components in a plant's defensive

response by modulating components such as the SA response which is essential for SAR development (Wang et al., 2009b).

Plant defense signaling

Three major plant hormones are responsible for regulating the major signaling networks activated by pathogen recognition: salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). SA signaling is important in establishing local and systemic resistance for biotrophic pathogens, while JA and ET signaling are generally stimulated in response to necrotrophic pathogens, herbivore predation, wounding, and abiotic factors. Each of these hormones can activate a specific defense signaling pathway which can act individually, synergistically, or antagonistically, depending upon the invading pathogen (Thomma et al., 2001). For example, the SA and JA defense pathways are mutually antagonistic, and pathogens have evolved ways to exploit this fact and overcome these defense responses (Bostock, 2005; Koornneef and Pieterse, 2008; Rojo, Solano, and Sanchez-Serrano, 2003). The resistance response to TCV infection activates the SA pathway dependent response, but is independent of NPR1, ET, and JA signaling (Figure 1-2).

Salicylic acid signaling pathway and some of its major components

Salicylic acid is a small phenolic plant compound that plays a vital role in the defense responses against many pathogens. Infections with biotrophic pathogens induce increased levels of SA, which in turn upregulate the expression of many defense-related genes (Malamy et al., 1990; Sticher, Mauch-Mani, and Metraux, 1997). Plants

dysfunctional for SA synthesis or defective SA signaling pathways exhibit enhanced susceptibility to pathogen infection generally (Glazebrook, 2001). In Arabidopsis and tobacco, SA is also crucial for the establishment of SAR (Durrant and Dong, 2004). SAR is also accompanied by the induction of a set of SA-dependent pathogenesis-related (*PR*) genes and senescence associate genes (Morris et al., 2000) in inoculated and systemic tissue (Ryals et al., 1996).

Many key parts of the SA pathway have been identified in Arabidopsis using the extensive Salk mutant library (Figure 1-3). The *SID2* (SA-induction deficient2) gene encodes isochorishmate synthase (ICS) and its inactivation (*sid2*) renders plants defective in SA synthesis and unable to activate the SAR (Wildermuth et al., 2001). *EDS* (enhanced disease susceptibility-5), *EDS1*, and *PAD4* (phytoalexin deficient-4) encode proteins that also contribute to SA production (Falk et al., 1999; Nawrath et al., 2002; Zhou et al., 1998). The *EDS1* and *PAD4* genes generally participate in defense signaling pathways triggered by R genes with TIR-NBS-LRR domains (Falk et al., 1999). The SA pathway also requires the function of a downstream component *NPR1* (non-expressor of PR genes) to trigger the expression of PR genes (Figure 1-3) (Kinkema, Fan, and Dong, 2000). A mutation in the *NPR1* gene abolishes SA-mediated induction of PR genes as well as SAR (Cao et al., 1994). Other proteins such as WRKY70 have also been shown to participate in the SA signal transduction pathway. The presence of SA was found to induce WRKY70 expression upon exogenous treatment or pathogen infection (Li, Brader, and Palva, 2004). Controlling the SA-dependent defense responses allows bacterial pathogens to inhabit the plant, either in the apoplast or the symplast, and multiply within host tissue for several days before causing plant cell death and tissue

damage. Hence, regulation of the SA response is a strategy employed by many pathogens to breech the basal defense layer.

asmonic Acid and Ethylene Pathways

JA, a fatty-acid-derived signaling molecule, is involved in several biotic and abiotic aspects of plant biology including pollen and seed development, and defense responses to wounding, ozone, insect pests, and microbial pathogens (Liechti and Farmer, 2006; Staswick, 2008). *A. thaliana* mutants that are impaired in JA production or perception exhibit enhanced susceptibility to a variety of pathogens, including the fungal pathogen *Pythium irregulare* (Staswick, Yuen, and Lehman, 1998), and bacterial pathogens like *Erwinia carotovora* (Norman-Setterblad, Vidal, and Palva, 2000). These pathogens employ a common necrotrophic virulence strategy that involves rapidly killing plant cells to obtain nutrients. Several mutants that exhibit enhanced or constitutive JA responses have been identified that exhibit enhanced resistance to necrotrophic pathogens, like *Botrytis cinerea* (reviewed in (Kachroo, 2006)). A point to note, JA induction has been shown to be involved in antiviral defense in only a few cases of virus infection such as in Cauliflower Mosaic Virus {CMV(Love et al., 2005)}.

The ET signaling pathway on the other hand, involves a relay of signals between multiple components, including cross-talk between several different pathways including the JA pathway. ET signaling can lead to root and hypocotyl growth, decreased growth in dark conditions, hypocotyl thickening with decreased light, and last but not least, a pathogen defense response (Alonso and Stepanova, 2004). The *EIN2* gene is a central component of the ethylene signaling pathway and is recognized as a molecular link

between previously distinct hormone response pathways (Alonso and Stepanova, 2004). ET itself is involved in the regulation of some of the key genes important for its own synthesis, including the ACC synthase (*ACS*), which controls the first catalytic step in the biosynthesis of ET (Kende, 1993; Liang et al., 1995). Much like the SA pathway, necrotrophic and herbivorous pathogens evolved proteins that can regulate or suppress the JA and/or the ET pathways in attempts to become more successful pathogens (See Figure 1-3 for working model of SA, JA and ET defense pathway).

Turnip Crinkle Virus

Turnip crinkle virus (TCV) is one of the most studied members of the genus Carmovirus in family *Tombusviridae*. It has a small single stranded positive sense 4,054 base genome that encodes five open reading frames (ORFs) which produce five proteins (Figure 1-4A). During replication, two subgenomic RNAs (sgRNA), 1.7kb and 1.45kb in size, are synthesized in addition to progeny genomic RNA. These RNAs have co-linear 3' termini and they lack poly (A) tails or tRNA-like structures (Carrington et al., 1989). The first gene located toward the 5' terminus encodes a protein of 28 kDa (p28). A read-through protein of 88 kDA (p88) is produced when the p28 amber termination codon is suppressed. The p28 and p88 proteins make up the replicase protein that is responsible for RNA replication and sgRNA transcription. Two smaller ORFs encoding polypeptides 8 kDa (p8) and 9 kDa (p9) are located in the middle of the genome and are translated from the 1.7 sgRNA. The p8 and p9 proteins are required for cell-to-cell movement in plants (Hacker et al., 1992). The ORF at the 3' terminus of the genome encodes a 38

kDA (p38) protein that is translated from the 1.45 kb sgRNA. This protein is the viral CP, and is the structural protein for the virus (Figure 1-4B).

TCV coat protein

The TCV virion shell is composed of 180 copies of the TCV CP. Each protein consists of 351 amino acids (aa) and is arranged into three distinct domains. The amino-terminal R domain consists of 52 aa and extends into the interior of the virus particle where it is predicted to interact with the viral RNA. The R domain is connected to the S domain via a 29 aa region called the arm. The S domain forms the shell of the virion and is connected to the P domain by a 5 aa residue hinge. The P domain is made up of 103 aa which are projected outward from the virion surface and exposed to the surrounding environment (Figure 1-4B) (Carrington et al., 1987; Hogle, Maeda, and Harrison, 1986). As mentioned earlier, the primary function of the CP is structural. The CP has been shown to be multifunctional as it is also necessary for movement (Cohen, Gisel, and Zambryski, 2000; Hacker et al., 1992; Lin and Heaton, 1999), serves as a virulence factor (Heaton et al., 1991; Wang and Simon, 2000; Zhao et al., 2000), and functions as a suppressor of RNA silencing (Qu, Ren, and Morris, 2003; Thomas et al., 2003), which will be further discussed in Chapter 4. Multifunctionality has also been observed for other plant virus CPs with icosahedral symmetry and single-stranded positive sense RNA genomes such as members of genera *Bromovirus* and *Cucumovirus* (Calhoun and Rao, 2008; Callaway et al., 2001; Lewsey et al., 2009; Sasaki et al., 2005). These genera along with members of the *Tombusvirus* have basic N-terminal domains that have been

predicted to play an important role in RNA recognition during encapsidation *in vivo* (Fox, Johnson, and Young, 1994) and as a virulence factor (Ren, Qu, and Morris, 2000).

The role of TCV CP as a modulator of host resistance was initially proposed from studies in the Morris lab in which a yeast two-hybrid screen using CP as bait was used to isolate and identify a putative component of resistance network called TIP (Ren, Qu, and Morris, 2000). TIP belongs to the NAC transcription factor family, members of this family have diverse roles in many aspects of plant growth, development, and defense. TIP was isolated because of its ability to bind to the N-terminal region of the R domain of the CP (Ren, Qu, and Morris, 2000). It was further established that TIP localized to the nucleus when expressed transiently in *N. benthamiana* (Ren, Qu, and Morris, 2005).

NAC genes and antiviral defense

In plants, the NAC [for NAM (no apical meristem), ATAF, CUC (cup-shaped cotyledon)] family represents a plant-specific group of transcription factors (Olsen et al., 2005). The genomes of Arabidopsis, tobacco, and rice all contain more than 100 genes encoding NAC domains (Ooka et al., 2003; Rushton et al., 2008), making it one of the largest transcription factor-gene families in plants. NAC genes were originally identified from forward genetic screens as key regulators of developmental processes and more recently have also been shown to be involved in the regulation of stress responses in both model plants and agronomically important crops (Kim et al., 2006; Olsen et al., 2005; Ren, Qu, and Morris, 2000).

Several NAC proteins have been identified due to their response to pathogen infection as either positive or negative regulators. In Arabidopsis, ATAF1 was

demonstrated to be a negative regulator of defense against necrotrophic fungal and bacterial pathogens (Wang et al., 2009a). Specifically, infections by *Botrytis cinerea*, *Pseudomonas syringae* pv. *tomato*, or treatments with SA, JA, and the precursor to ethylene biosynthesis (1-amino cycloproane-1-caroxylic acid) lead to a down regulation of *ATAF1* gene expression. Since ATAF1 is affected by multiple pathogen defense associated signaling pathways, it may not directly repress downstream targets but instead serve as a node of convergence for PTI by controlling the expression of other unknown negative regulators and transcription factors (Wang et al., 2009a).

Another Arabidopsis NAC gene, ATAF2, is a positive regulator of defense and was identified because of its ability to bind to the helicase domain of the TMV 126-/183-kDa replicase protein(s) (Wang, Goregaoker, and Culver, 2009). ATAF2 expression is induced by pathogen invasion which results in an enhanced basal defense response associated with SA-mediated defense signaling. The interaction with the TMV helicase also promotes the proteasome degradation of ATAF2 which leads to a reduced basal defense that promotes enhanced systemic invasion of TMV (Wang, Goregaoker, and Culver, 2009). Therefore, the ATAF2-replicase interaction represents a way to suppress basal defense and reduce SA's ability to transcriptionally activate defense-related genes in distal parts of the plant, thus promoting TMV invasion.

Similarly, the Arabidopsis NAC protein TIP was identified because of its ability to bind to TCV CP (Ren, Qu, and Morris, 2000). Specifically it was shown that TIP interacts with N-terminal region of the R-domain of TCV CP (Figure 1-4B). It was hypothesized that loss of TIP binding in a series of CP mutant viruses was correlated with the loss of the ability to induce an effective ETI response (Figure 1-5). This observation

suggested that CP-TIP interaction was needed for an effective ETI response to TCV infections. It was further determined that TIP has a membrane location signal (Kang personal communitcation), hence it is retained in the cytoplasm after translation where it is likely to interact with TCV CP inside the plant cell during infection. I have hypothesized that the interaction of TIP with TCV CP causes the release of a truncated TIP protein which then migrates to the nucleus where it subsequently represses or blocks the defense responses and/or signaling pathways (Kang, personal communication). It was originally thought that the R protein, HRT, might indirectly recognize the CP through this interaction causing repression of the PTI response and induction of the appropriate ETI response leading to the sequestration of the pathogen at the site of infection. This conclusion was supported by the observation of Ren et al. (2005) that each of the CP mutant viruses that were unable to induce an HR and move systemically in resistant Di-17 had also lost CP-TIP binding ability. Moreover, mutants in the same region of the CP in which TIP binding was restored induced HR and resistance. It was subsequently shown, using a TIP knockout (ko) line, that TIP was not essential for HR induction and ETI resistance to TCV (Jeong et al., 2008). This study further showed that TIP appeared to have a primary effect on the basal resistance response and the fine tuning of the defense response (Jeong et al., 2008). Recent data from our lab (Kang, personal communication) has shown that the same region of the CP associated with TIP binding also contains a nuclear localization signal. Hence, it appears that the R-domain region of the CP likely plays multiple roles as a virulence factor that affects both the PTI and ETI defense responses. This conclusion is supported by the data I will present in the next two chapters of this thesis which show that single aa changes in the R-domain of the

CP can have very marked and somewhat unpredictable affects on symptom severity and differential expression of numerous defense genes in both the susceptible and resistant lines of Arabidopsis.

To summarize, my initial efforts focused on understanding the changes in gene expression associated with infections caused by TCV mutants that disrupted TIP interaction as reported analyzed by Ren et al. (2000; Figure 1-5). I began by conducting an in depth study on one such mutant, R6A, which had a single aa change at the 6th position in the N-terminus of the coat protein where an arginine (R) was changed to an alanine (A). R6A infections in susceptible Col-0 typically caused more severe symptoms. Moreover, R6A also broke resistance in the resistant line carrying the HRT gene, (Di-17) and plants became systemically infected with the mutant virus. Together, these observations implicated TIP-CP interactions in both the basal (PTI) and R-gene (ETI) layers of the defense response. These results provoked the counter-intuitive question why does loss of interaction of TCV CP with TIP cause more severe symptoms in the susceptible Col-0 line? This question was initially addressed by analyzing gene expression differences in infections initiated by wt TCV and the mutant R6A in my MS thesis and in an unpublished microarray study. The Ren et al. (2000) study and the gene expression data prompted the formulation of the following hypothesis:

The regulation of the Arabidopsis basal defense against TCV is mediated by the transcription factor TIP. TCV has evolved an invasion strategy to repress the host basal defense system by altering the nuclear localization of TIP through CP-TIP interaction. The resistant ecotype Di-17 has evolved an R gene (HRT) based surveillance system that is also triggered by TCV CP, but not by the same R6A

mutant that fails to bind TIP. This suggests that the signaling pathway modified by TIP to induce the basal resistance response communicates with the R-gene based resistance pathway.

To test this hypothesis, I conducted an in-depth examination of infections by the non-TIP binding mutant R6A in susceptible Col-0 (Chapter 2). I sought to establish if CP-TIP binding had a direct role in the regulation of the basal defense response (PTI) by assessing if there were measurable differences in virus accumulation between wt TCV and R6A in both inoculated and systemic tissue of susceptible Col-0. In chapter three, I expanded these studies and assessed infections by TIP binding and non-TIP binding mutants in the resistant line Di-17 to assess if TIP binding had a measurable affect on ETI based defense. Finally in chapter four, I examined if there are any differential effects of TCV and TIP-binding mutant infections on the multiple genes associated with the RNA silencing pathways in Arabidopsis. I felt that this was necessary due to the universal importance of silencing based defense used against pathogens and the fact that the TCV CP functions as a silencing suppressor

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Figure legends

Figure 1-1. Visual representation of R proteins. This figure depicts five known classes of R proteins. The R proteins are further characterized based on domain regions found within the protein and their location within the cell (See text for domain function). HRT is a member of the CC-NBS-LRR class of R proteins and conditions the resistance response to TCV (Dempsey et al., 1997).

Figure 1-2. Model of the hypersensitive response and resistance to Turnip Crinkle Virus infection. Inoculation of TCV on resistant Arabidopsis ecotype Dijon-17 results in a hypersensitive response (HR) on inoculated tissue and the induction by systemic acquired resistance (SAR). HR development is mediated by CP recognition of HRT in the presence of recessive *rrt* allele. Signaling for TCV resistance requires a functional SA signaling pathway including proteins such as EDS1, PAD4, EDS5, and SID2. However resistance is independent of NPR1 and the JA and ET signaling pathway. The light regimen following TCV infection is critical for HR formation and resistance. Therefore the pathways required for TCV resistance are complex and differ from the resistance pathway induced by other biotrophic and necrotrophic pathogens {Adapted from (Chandra-Shekara et al., 2006; Kachroo et al., 2000)}.

Figure 1-3. A working model of the SA, A and ET pathogen defense pathways in *Arabidopsis thaliana*. In the salicylic acid (SA) pathway (in orange), the *SID2* and *EDS* genes appear to be directly involved in SA biosynthesis, whereas the *EDS1*, *EDS4* and *PAD4* genes regulate SA synthesis. The SA pathway can function independently of

NPR1 and still signal for the induction of *PR1* gene expression. In general, the SA pathway responds to biotrophic organisms. In the jasmonic acid (JA) pathway (in blue), the proteins, COI1 and MPK4, function early in the JA signaling pathway. JAR1 is tentatively placed downstream of COI1 and MPK4 because of the observations that JA signaling is only partially blocked in the *jar1* mutant and that *jar1* plants do not exhibit enhanced resistance to *P. syringae*. The ethylene (ET) response pathway (in purple) signals through many ET receptors (not shown) and EIN2. Positive regulatory interactions between these signaling pathways are indicated by green arrows, antagonistic interactions by red lines. The dashed green arrow indicates potential positive interactions between the ET and SA pathways. Putative positive interactions between the SA and JA pathways, and potential negative interactions between the ET and SA pathways, are not shown. (Diagram adapted from (Kunkel and Brooks, 2002).

Figure 1-4. Schematic diagram of TCV genome and TCV CP structure. (A) TCV genome consists of 5 ORFs that encode five proteins. The replicase protein is composed of p28 and p88. Systemic movement of the virus requires the function of p8 and p9. The CP of the virus, p38, is the structural protein. (B) TCV CP has 3 protein domains that are connected by arm and hinge regions as diagrammed above. The R domain is located within the virion shell and interacts with the viral genome. The S domain makes the shell of the virion and the P domain is located on its outside surface. 180 subunits of CP or 90 dimers, as shown next to the linear map of CP, make up the virion shell.

Figure 1-5. Connection between TCV-CP interaction and TCV resistance (Ren et al., 2000). The diagram shows the 25 amino acid (aa) sequence of the N-terminus of the R domain of the CP of wild TCV and its mutants. Mutant viruses with single aa substitutions in this region are shown on the left. Results of a yeast two-hybrid screen where TIP/TCV CP binding was evaluated are shown in the next column where a (-) depicts no TIP binding and a (+) depicts TIP-TCV CP interaction. On the right, results of infections by TCV and mutant viruses are shown. The ecotype Col-0 was susceptible (S) to all viruses, but susceptibility followed by increased disease symptoms (S+) was observed in the non-TIP binding mutants. The resistant ecotype, Di-17, was resistant (R) to TCV and the other TIP binding mutant G14A, but susceptible (S) to all non-TIP binding mutants. These data have been re-examined more thoroughly in Chapter 2.

Figure 1-1

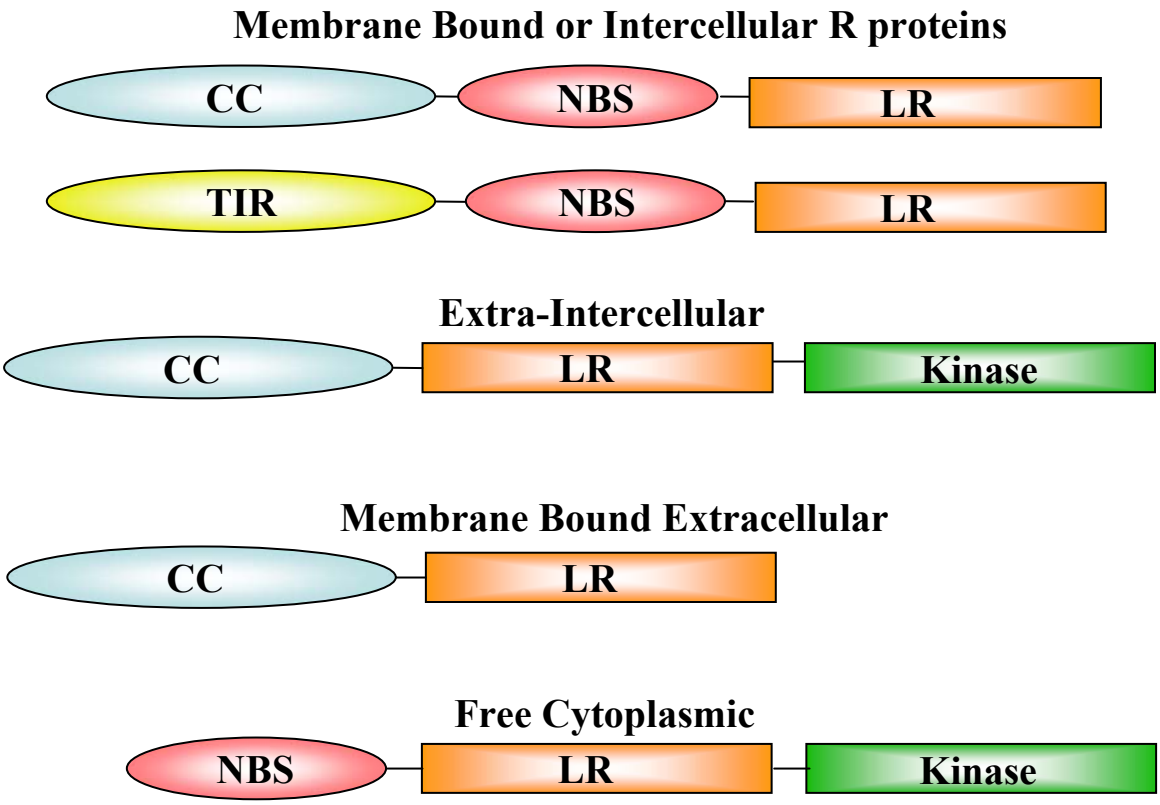

Membrane Bound or Intercellular R proteins
CC
NBS
LR
TIR
NBS
LR
Extra-Intercellular
CC
LR
Kinase
Membrane Bound Extracellular
CC
LR
Free Cytoplasmic
NBS
LR
Kinase

Figure 1-2

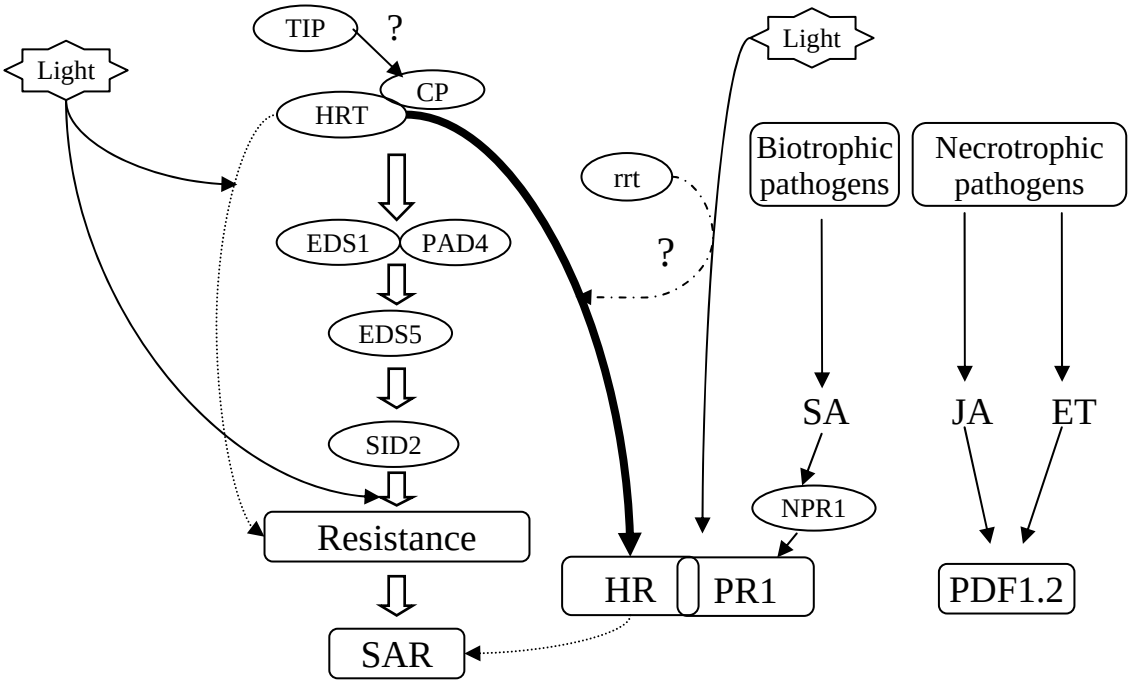

TIP
?
Light
CP
HRT
rrt
?
Light
Biotrophic pathogens
Necrotrophic pathogens
EDS1
PAD4
EDS5
SID2
SA
JA
ET
NPR1
Resistance
HR
PR1
PDF1.2
SAR

Figure 1-3

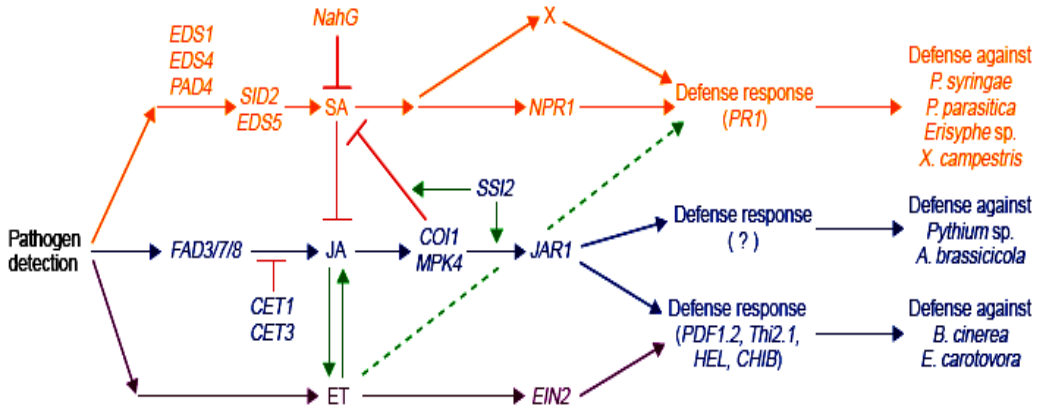
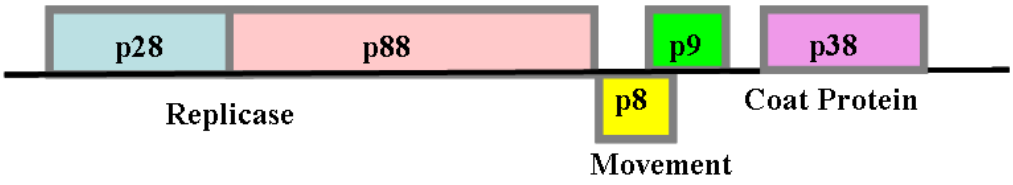

NahG
X
EDS1
EDS4
PAD4
SID2
EDS5
SA
NPR1
Defense response
(PR1)
Defense against
P. syringae
P. parasitica
Erisyphe sp.
X. campestris
SSI2
Pathogen
detection
FAD3/7/8
JA
COI1
MPK4
JAR1
Defense response
(?)
Defense against
Pythium sp.
A. brassicicola
CET1
CET3
Defense response
(PDF1.2, Thi2.1,
HEL, CHIB)
Defense against
B. cinerea
E. carotovora
ET
EIN2

Figure 1-4

A.


p28
p88
p9
p38
p8
Replicase
Coat Protein
Movement

B.

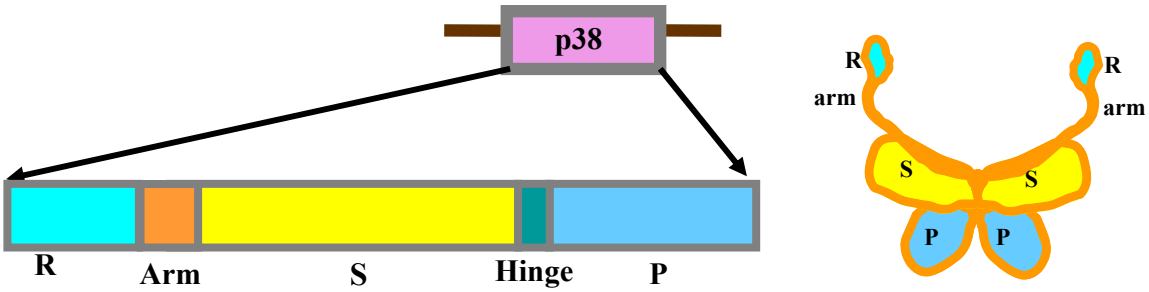

p38
R
Arm
S
Hinge
P
R
arm
R
arm
S
S
P
P

Figure 1-5

(Ren et al., 2000)

Chapter 2:

TCV coat protein mutants that fail to bind the NAC transcription factor TIP display altered virus accumulation and gene expression associated with innate immune system and systemic acquired resistance.

Abstract

Turnip crinkle virus (TCV) has previously been shown to interact with a member of the NAC family of transcription factors called TCV-interacting protein (TIP) via its coat protein (CP). A fully replication competent mutant virus (R6A) was constructed with a single amino acid replacement in the N-terminal region of TCV CP that failed to interact with TIP. R6A caused more severe symptoms in the susceptible Columbia-0 ecotype of *Arabidopsis thaliana* and broke resistance in the resistant Dijon-17 ecotype. Based on these observations, I hypothesized that the interaction of TCV CP with TIP was an evolutionary adaptation to suppress the host innate immune response to enhance a more rapid systemic invasion of the virus. I compared the rate of accumulation of TCV and R6A in inoculated and systemic leaves over a time course of infection. The accumulation of the R6A mutant was consistently slower relative to wt virus in the susceptible Col-0 ecotype. However, this difference disappeared in mutant plants that lacked a fully functional salicylic acid defense pathway. This result suggested that the primary defect in R6A was its inability to modulate the SA-associated innate defense response and signaling. Similar virus accumulation levels between TCV and R6A were observed in infections of Col-0 transgenic lines with constitutively expressed TIP under the control of the 35S promoter. These data support the conclusion that the TIP-CP interaction is important in modulating the innate immune response to virus infection in susceptible hosts.

Introduction

Plants employ multiple mechanisms to defend themselves against pathogens. A key element of plant defense is by the recognition of the pathogen encoded microbe-associated molecular patterns (MAMPs) and subsequent triggering of a MAP kinase signaling cascade that will induce the appropriate defense response. Recent reviews have described the plant innate immune system as consisting of two components: one component, referred to as pathogen triggered immunity (PTI), uses transmembrane pattern recognition receptors (PRRs) to recognize MAMPs, such as flagellin (Felix et al., 1999). The other component referred to as effector triggered immunity (ETI) uses the polymorphic NB-LRR protein products encoded by most *R* genes to induce a more intense defense response (reviewed in (Boller and Felix, 2009; Jones and Dangl, 2006). One such R gene, known as HRT, can mediate disease resistance against Turnip crinkle virus (TCV) by the recognition of its coat protein (CP) in *Arabidopsis thaliana* ecotype Dijon-17 (Dempsey et al., 1997). The HRT protein induces an ETI response which includes the hypersensitive response (HR) upon recognition of TCV CP. This response quarantines TCV to the site of infection and prevents systemic spread throughout the plant (Dempsey et al., 1997).

Along with MAMP recognition, plants also use antiviral RNA silencing defense to restrict virus replication and movement (Ding and Voinnet, 2007; Loake and Grant, 2007). The process of RNA silencing is an adaptive defense strategy wherein viral-associated RNAs are used as templates for making complementary siRNAs which in turn are used in RNA-induced silencing complexes (RISC) for sequence specific splicing of viral RNAs (Baulcombe, 2004; Brodersen and Voinnet, 2006). In addition to serving as

the elicitor of ETI, the TCV CP also serves as a strong suppressor of the RNA silencing pathway (Qu, Ren, and Morris, 2003). It has also recently been shown to interact with a key component of the RNA silencing pathway, ARGONAUTE1 (AGO1`;Azevedo et al., 2010). The CP-AGO1 interaction disrupts homeostatic interactions of the four dicer-like (DCL) proteins in Arabidopsis and prevents the proper RNA silencing-associated defense against TCV (Azevedo et al., 2010). RNA silencing and the role of TCV CP as a silencing suppressor will be covered further in Chapter 4.

Salicylic Acid (SA) is a small phenolic plant compound that plays a vital role in the defense responses against many pathogens in both branches of plant innate immunity. Infections by biotrophic pathogens induce increased levels of SA, which in turn upregulate the expression of many defense-related genes (Malamy et al., 1990; Sticher, Mauch-Mani, and Metraux, 1997). Plants with dysfunctional SA synthesis, signaling or accumulation pathways exhibit enhanced susceptibility to pathogen infection (Glazebrook, 2001). In Arabidopsis and tobacco, SA is also crucial for the establishment of systemic acquired resistance (SAR; Durrant and Dong, 2004). SAR is also accompanied by the induction of a set of SA dependent pathogenesis-related (*PR*) genes in inoculated and systemic tissue (Ryals et al., 1996) and senescence-associated genes (Morris et al., 2000). SA is also linked to parts of the senescence pathway like SEN1 (Schenk et al., 2005) which is one of the factors needed for regulating senescence (Morris et al., 2000).

Many key parts of the SA pathway important for PTI and ETI defense have been identified in Arabidopsis using the extensive Salk mutant library (Alonso et al., 2003) . *EDS1* (enhanced disease susceptibility-1) and *PAD4* (phytoalexin deficient-4) encode

proteins that also contribute to SA production (Falk et al., 1999; Zhou et al., 1998). The *EDS1* and *PAD4* genes generally participate in defense signaling pathways triggered by R genes with TIR-NBS-LRR domains (Falk et al., 1999). The SA pathway also requires the function of a downstream component *NPR1* (non-expressor of PR genes) to trigger the expression of pathogenesis-related (PR) genes (Kinkema, Fan, and Dong, 2000). A mutation in NPR1 abolishes SA-mediated induction of PR genes as well as SAR (Cao et al., 1994). Controlling the SA-dependent defense responses allows pathogens to inhabit the plant cell, either in the apoplast or the symplast, and multiply within host tissue for several days before causing plant cell death and tissue damage. Hence, regulation of the SA response is a strategy employed by many pathogens to overcome PTI.

NAM, ATAF, and CUC (NAC) transcription factors are a plant specific group of proteins, which contain a highly conserved N-terminal DNA-binding domain and a variable C-terminal domain (Olsen et al., 2005). Recent analyses has identified over 100 NAC encoding genes in the genomes of *Arabidopsis thaliana* and *Oryza sativa* that have tissue and stress response specific expression (Fang et al., 2008; Ooka et al., 2003). NAC proteins have been shown to be important components in different aspects of plant development, including formation of boundary cells of the meristem, cell division and expansion, lateral root development, senescence, secondary cell wall biosynthesis, and flowering time (Aida et al., 1997; Kim et al., 2006; Sablowski and Meyerowitz, 1998; Souer et al., 1996; Xie et al., 2000; Zhong, Demura, and Ye, 2006). One member of the NAC family, TIP (TCV-interacting protein), was shown to play a key role in binding to TCV CP. This interaction was key for ETI resistance in the resistant *A thaliana* ecotype *Di-17*. This binding was also correlated with the level of symptom severity in the

susceptible ecotype of Col-0 during infection (Ren, Qu, and Morris, 2000). Since then, a study by Jeong *et al*. (2008) demonstrated that TIP was not necessary for ETI resistance in Di-17 but hypothesized it may still play a role in the innate defense system (Jeong et al., 2008).

To further assess the role of TIP in PTI, I evaluated the differences between wt TCV infection and one of its non-TIP binding mutants R6A. Analyses of virus accumulation for both viruses were conducted to observe if there was any effect on their fitness with and without TIP interaction. In this work, I demonstrate that wt TCVôs ability to bind TIP gives it a clear advantage over R6A in its ability to down-regulate the SA pathway and accumulate to higher titers early in infection. I also show a correlation between TCV accumulation and TIP binding in the susceptible Col-0 ecotype.

Materials and Methods

Plant growth conditions

Plants lines of wt *A. thaliana* Col-0 and Di-17, and knockout (ko) lines in a Col-0 background of *npr1*, *pad4*, *jar1* and *ein2* were grown in growth chambers at 22°C with 12hr day cycles in Metro Mix 360 (Sun Gro; British Colombia, Canada). Transgenic lines of antisense TIP (asTIP) and a constitutively up-regulated TIP (UpTIP) line that had an additional TIP gene under the control of a 35S promoter were initially grown on selective media to verify the presence of inserts. Their were subsequently transplanted to Metro Mix 360 (Sun Gro), and placed in growth chambers under the same conditions as stated earlier for wt Col-0 and other previously mentioned lines.

Plant inoculations, tissue collection, and RNA isolation

Plants were consistently inoculated between the ages 22 to 24 days old. Three leaves were inoculated per plant as illustrated in Figure 2-1. The virus inocula consisted of a buffer solution containing 50 mM Na_2HPO_4 [pH 7.0] + 1% Celite 545 and purified virus transcript at a concentration of 1 ng/µl with a total of 10 ng of virus transcript or 10 µl of the virus transcript-buffer solution applied to each leaf. The virus inoculum was applied to each leaf by rub inoculation, allowed to stay on the leaf for five minutes, and then washed off with nanopure water. Five to six leaves (apx 0.3g) from different plants treated with the same inoculum buffer were collected at each time point and flash frozen in liquid nitrogen. RNA was extracted as previously described (Chomczynski and Sacchi, 1987) and RNA samples were subsequently purified using RNeasy columns (Qiagen; Valencia, CA, USA).

Virus detection and semi-quantitative RT-PCR

Detection of viral RNAs was conducted by analysis of 2 µg of total RNA isolated from infected plant tissue. The RNA was separated using electrophoresis in a 1.2% agarose/1.8% formaldehyde gel run at 100 mV/cm for 90 minutes at room temperature. Separated RNAs were then transferred to a Nylon membrane (Zeta probe blotting membranes; Bio-Rad, Hercules, CA, USA) at 4°C at 37mV/cm or 200mA. Hybridization was carried out at 40°C using ULTRAHyb-Oligo (Ambion; Foster City, CA, USA) solution according to manufacturerôs directions (Ambion). CP and PR1 were detected with the addition of ^{32}P-ɔ-ATP end-labeled probes (Table 2-2) to the hybridization buffer after one hour of pre-hybridization of the membranes with only the ULTRAHyb-Oligo

solution. Probes were generated using T4 polynucleotide kinase according to manufacturerôs directions (New England Biolabs; Ipswich, MA, USA). After overnight hybridization (minimum of 12 hours), the membrane was washed three times, 20 min each, with 2xSSC, 0.5% SDS at 40°C.

Reverse transcription PCR (RT-PCR) was conducted to evaluate gene expression. DNase treated RNA samples were used to synthesize first strand cDNA by using SuperScript III reverse transcriptase (Invitrogen; Carlsbad, CA, USA) and random primers according to the manufacturerôs protocol. The cDNA was then subjected to PCR amplification for semi-quantitative analysis with EconoTaq Plus Green 2X Master Mix according to the manufacturerôs protocol (Lucigen; Middleton, WI, USA). The following thermal cycling conditions used were: initial denaturation 95°C for 2 minutes, then cycles of denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and followed by an extension phase at 72°C for 1 min. The numbers of cycles were adjusted based on the transcript abundance and expression at a given time point and/or sample. The procedure was terminated with a final extension phase of 72°C for 5 minutes followed by a hold at 4°C. The PCR product was then subjected to electrophoresis and gene expression was analyzed based on band intensity of the transcripts relative to the control gene, *Actin2* (*ACT2*). The primers (Invitrogen) of the genes used for analysis for semi-quantitative PCR are listed in Table 2-1.

Real-time PCR

Real-time PCR was also used to evaluate gene expression. The qRT-PCR experiment was performed by using the ABI StepOne^{TM} Plus real time PCR machine

(Applied Biosystems; Foster City, CA), TaqMan® One-Step RT-PCR master mix, and the appropriate predesigned assay. A PCR master mix was prepared according to the manufacture's instructions along with 10εl TaqMan® RT-PCR Mix (2X; Applied Biosystems), 0.5εl TaqMan® RT enzyme mix (40X:Applied Biosystems), and enough RNase free water for a final volume of 50εl per well. The reaction plate was prepared by adding the PCR master mix, 1εl TaqMan® labeled probe (Applied Biosystems), 8.5εl total RNA at the concentration of 4.71ng/ εl to each well (total RNA per well was approximately 40ng). The final volume of each well was 20εl. Three wells were also assembled for each probe with 8.5εl water instead of RNA to rule out possible contamination. Thermal cycling conditions were 48°C for 30 min and 95°C for 10 min, followed by 95°C for 15 sec and 60°C for 1 min for 40 cycles. Relative expression was quantified by using the comparative Ct method with *ACT2* as the endogenous control. The qRT-PCR procedure was done in triplicate. See table 2-3 for a list of evaluated genes.

Enzyme-linked immunosorbent assay (ELISA)

Virus titers were evaluated using indirect enzyme-linked immunosorbent assay (ELISA). Total virions were isolated by grinding 0.3g of leaf tissue in 1ml of ddH_20 and centrifuging the samples at room temperature for 60 seconds at 12,000rpm. The supernatant was transferred to a new tube and virion levels in the crude extracts were determined by using indirect ELISA (Lommel, McCain, and Morris, 1982)

ELISA plates were coated by filling wells with 200µl 0.05M carbonate buffer, pH9.6 + 0.01% sodium azide and then 50µl crude extract was added to the well. A serial

dilution was done on all samples by taking 50µl from the initial well with the original crude extract sample and transferring it to a new well filled with 200µl carbonate buffer for a 1:5 dilution ratio. This was repeated three times, so the final dilution of the sample in the fourth well was 1/625 of the concentration of the original sample. A standard ELISA curve of virus concentrations was constructed to help estimate total virion concentration in the samples (Figure 2-2).

To allow for sufficient coating, the plates were incubated at 37°C for 60 minutes. Plates were then washed five times with 30 second incubations using 200 µl PBST (Phosphate Buffered Saline with Tween) wash buffer (Agdia; Elkhart, IN, USA). After the final wash, the rabbit anti-TCV antibody solution was added to each well at a concentration of 1:1000. The antibody was diluted in ECI buffer (Agdia). The plates were again incubated at 37°C for 60 minutes and then washed as before. Next the conjugated antibody (goat-anti rabbit with alkaline phosphate (AP) conjugate) was added to the wells at a concentration of 1:25,000 in the same ECI buffer (Agdia). Following another 60 minute incubation at 37°C, the plate was again washed before the p-nitrophenol (PNP) buffer (Agdia) was added to each well. The plate was incubated at room temperature in humid box covered in foil to prevent PNP from reacting with light. The plate was read at 405nm and could be read multiple times as long as the negative control wells remained below an absorbance reading of 0.200A.

Virus Gels

Virus concentrations were also validated using virus gels. TCV virions were isolated by grinding 0.5g of infected tissue in 1ml of 4°C sodium acetate extraction buffer. Extraction buffer consisted of 0.2M sodium acetate, pH 5.2 and 0.1% ß-

mercaptoethanol. Virus slurry was transferred to a 2ml tube and incubated on ice for 30 minutes. This was followed with a 10 minutes centrifugation at 13,200rpm. Supernatant was poured through miracloth funnel into a 1.5ml microcentrifuge tube. The virus was precipitated by adding 0.25 volumes of 40% polyethylene glycol solution for 30 minutes on ice. After precipitation, the solution was centrifuged for 10 minutes at 13,200 rpm. A 5µl sample of the virus extraction solution was then electrophoresed on a 1% agarose gel with 1X E buffer. A 20X stock of E buffer is made of 1.205% tris and 5.71% glycine. The gel was run at 60mV/cm for approximately two hours. Ethidium bromide was used to stain and observe the nucleic acid and coomassie blue was used to stain and observe the proteins.

Construction of Transgenic lines

The altered TIP expressing *A. thaliana* lines were constructed by Dr. Feng Qu. The p35-UpTIP construct was made by cloning the full length TIP cDNA into a plasmid pRTL2. This plasmid flanked the TIP cDNA insert with the CaMV 35S promoter and a polyA signal. The TIP cassette, including the 35S promoter, TIP cDNA, the 35S polyA signal (Figure 1-8) was cut out of pRTL2 and subcloned into the binary vector, pPZP212. The resulting construct was used to transform *Agrobacterium* strain C58C1 as previously described in (Bechtold, Ellis, and Pelletier, 1993). The asTIP down construct was made by cloning a partial fragment of the TIP cDNA (nt 1106 ï 1637), in reverse orientation, into pER8 (between *Xho*I and *Spe*I sites), as previously described in (Zuo, Niu, and Chua, 2000). The resulting construct (Figure 2-3) was then transformed into *Agrobacterium* and used for Arabidopsis transformation following the same protocol as above.

The estradiol promoter was induced by spraying the plant with a 30nM estradiol solution once the plants had reached their mature size (age 22 to 24 day). The estradiol solution was applied to plants in a fume hood which were then covered with a dome lid and placed back in the growth chamber. Dome lids were removed after 12 hours. This process was repeated every other day throughout the infection cycle.

Results

TCV mutant R6A accumulates slower than wt TCV

The non-TIP binding coat protein mutant, R6A, displayed the consistent phenotype of resistance breaking in Di-17 and more severe symptoms in susceptible Col-0 compared to wt TCV (Figure 2-4). This observation suggested that the single aa replacement mutation altered both the innate defense response (PTI) in the susceptible host and the ETI response in the resistant host. To test the PTI response, virus accumulation was measured by collecting leaf tissue from several plants at multiple time points after inoculation of Col-0 with either TCV or R6A.

Viral RNA transcript accumulation along with total virus titer were both assessed as a measurement of viral abundance in inoculated and systemic tissue over the time course of these infections. It was discovered that even though R6A infections caused more severe symptoms in Col-0, viral RNA and total virus accumulated more rapidly with TCV compared to R6A infections in both inoculated and systemic tissues (Figure 2-5). This is most evident by comparing levels of viral RNA accumulation between infections at 4 dpi and 6 dpi when TCV genomic and subgenomic RNAs are clearly visible while R6A RNAs are not yet present. By 8 dpi, R6A infected plants were able to

recover from the initial PTI response with the levels of genomic and subgenomic RNA approaching or exceeding that of wt TCV. These results were also supported by assessing virion accumulation by ELISA which showed that R6A accumulation was reduced at 4 and 6 dpi in systemic tissue compared to that of TCV virions (Figure 2-6).

Similar experiments were conducted in the resistant ecotype Di-17. This confirmed previous results reported by Ren et al., (2000) that R6A was able to break resistance and move systemically in 100% of inoculated plants (Figure 2-7a). This differed from wt TCV which showed approximately 25% systemic infection of Di-17 plants with about 75% being resistant (Dempsey, Wobbe, and Klessig, 1993; Kachroo et al., 2000). This is consistent with the previous reports showing that resistance (ability to localize virus in inoculated leaves and prevent systemic infection) is associated with a second recessive allele designated rrt (regulates resistance to TCV; (Kachroo et al., 2000), and variations in growth conditions upon virus infection (Chandra-Shekara et al., 2006). Therefore the detection of wt TCV in the resistant Di-17 systemic leaves, as seen in Figure 2-7 a-b, can be explained by systemic infection of about 1 in 4 of the randomly selected leaves from the expected 25% escapes that are characteristic of Di-17 resistance response. Because of the random and unpredictable nature of virus invasiveness in Di-17, we could not make reliable estimates of TCV titers during the time course experiments in Di-17. Although our results (Figure 2-5 and 2-6) clearly show that R6A accumulates more slowly in the susceptible Col-0 line than wt TCV, we could not make a similar direct comparative assessment in Di-17 (Figure 2-7 a-b). However, the results in the resistant line Di-17 clearly show that R6A is unable to provoke any resistance response resulting in 100% systemic infection and maximal levels of virus accumulated

by 8 dpi as determine by indirect ELISA (Figure 2-7b). This phenotype appears to be linked to the inability of the R6A CP to bind to the TIP protein and hence modulate the PTI response. These studies also support the hypothesis that R6A has lost its ability to bind TIP and that this was somehow related to its ability to break resistance and invade Di-17 systemically.

R6A is as robust as wt TCV when the SA pathway has been compromised

The observation that R6A accumulated more slowly in wt Col-0 suggested that R6A was either an attenuated virus or that its slower accumulation was related to its inability to alter the PTI defense response stimulated upon infection. To evaluate this, knockout (ko) lines of Arabidopsis in the Col-0 background were acquired that lacked key components of the three major signaling pathways associated with plant defense. These included: the SA defective ko lines *npr1* and *pad4*, the JA defective ko line *jar1*, and the ET defective ko line *ein2*. The *npr1* mutant carries a single recessive mutation that abolishes the induction of SAR as well as the pathogen induced expression of other *PR* genes (Cao et al., 1994; Dong et al., 1991). The *pad4* ko line is unable to synthesize SA which is required for SAR and also shows a decrease in PR expression during pathogen infection (Zhou et al., 1998). The *jar1* ko line cannot make sufficient JA and the plants are more susceptible to necrotrophic pathogens (Staswick, Yuen, and Lehman, 1998). The *ein2* ko line is defective in ethylene signaling that reduces the overall plant size and development, but also causes the plant to be more susceptible to necrotrophic pathogens (Alonso and Stepanova, 2004; Bleecker and Schaller, 1996).

The mutant plants described above were reared under controlled conditions and PCR was conducted on all ecotypes to verify the lack of expression of the specific transcripts (Figure 2-8). The absence of detectable transcripts for *NPR1*, *PAD4*, *JAR1*, and *EIN2* verified the genotype of these ko lines. Upon confirmation, an infection time course was conducted by collecting systemic leaves from plants that had been inoculated with TCV or R6A under the same conditions as described in Materials and Methods. Virus accumulation was analyzed by monitoring viral RNA transcript levels by northern blot (Figure 2-9). In contrast to accumulation in wild type plants, R6A and TCV both accumulated at equivalent rates in the *npr1* ko line and *pad4* ko line (Figure 2-9). This is most evident by comparison of the 4 and 6 dpi time points in Col-0 and the ko lines of *npr1* and *pad4* (Figure 2-9). This result was confirmed by the indirect ELISA results measuring virion accumulation (Figure 2-10). Together these data reinforced the conclusion that at a fully functional SA pathway was the primary factor in the initial repression of virion accumulation in R6A infections in Col-0. The fact that no replicative advantage was evident in the *npr 1* and *pad4* ko lines suggests that R6A is as replicatively competent as TCV and that its primary defect is likely its inability to shut off the SA signaling pathway that leads to an elevated level of basal resistance (PTI). This is also supported by examination of the ethidium bromide stained gels which show that both TCV and R6A accumulated to significantly higher levels in the defense defective mutant lines compared to wild type lines (compare the TCV gRNA levels noted by arrows in Figure 2-9).

Similar time course experiments carried out in JA and ET defective lines showed that virus accumulation of both R6A and TCV followed similar patterns to wt Col-0

infections (Figure 2-9). However, in these infections virus accumulated more slowly overall compared to wt Col-0 levels at 6 dpi and 8 dpi, yet TCV still displayed a replication advantage over R6A (Figure 2-9). We observed the level of total virions in *jar1* follow the same trend as Col-0 at 6 dpi, but the total accumulation at 8 dpi appeared to be slightly lower which is likely a product of the randomized sampling. Taken together, these observations reinforce our contention that a functional SA pathway is important for defense against viral pathogens like TCV and the role of the JA and ET signaling pathway is not essential for defense against these viruses.

The importance of a functional SA pathway in modulating TCV invasion in susceptible Col-0 is emphasized by comparing virus accumulation rates in the various mutant plant lines. This is shown in the ELISA data which verified these differences in virus accumulation at 6 dpi in all of the Arabidopsis wt and ko lines (Figure 2-11). These data show that TCV has a significant replication advantage over R6A when inoculated onto plants with a functional SA defense system. R6A accumulation is six fold higher in the *pad4* mutant versus in Col-0. This is also evident in the *npr1* mutant for TCV and R6A accumulation where R6A and TCV are accumulating to equivalent levels as TCV in wt Col-0 plants. This data was also confirmed by virus gel analysis which verified that intact virions were accumulating faster in TCV infection compared to R6A infections (Figure 2-12) at 6 dpi in Col-0 and the *jar1* mutant. However virion accumulation in the *npr1* and *pad4* ko lines were comparable for wt TCV and its mutant R6A (Figure 2-12).

Defense pathway gene expression differences in TCV and R6A infections in susceptible Col-0 and resistant Di-17 plants

Gene expression levels during the different virus infections were also assessed in an effort to further understand the nature of the observed difference in virus accumulation. Initially, a microarray experiment was conducted to provide a global assessment of what genes and/or gene families might be differentially regulated during R6A and TCV infections compared to mock inoculated plants. We found that several hundred genes were differentially expressed in TCV and R6A infections at 24 and 48 hrpi in two different microarray studies (Donze, 2006) and unpublished data collected by Morris,T.J. and Qu, F. These studies revealed that 461 transcripts were responsive to infections of TCV and R6A at the 24 hr time point with 359 being induced and 102 being suppressed. From that data, we selected a subset of genes linked to pathogen resistance and followed their expression levels more carefully over a time course. I reported some of this data previously in the my MS thesis (Donze, 2006).

PR1, a key indicator of activation of the SA pathway, was found to be differentially regulated in the resistant and susceptible ecotypes infected with R6A and TCV by northern blots (Figure 2-13). These results show a differential induction of *PR1*. In the susceptible host Col-0, northern analysis revealed a moderate induction of *PR1* in wt TCV infections in inoculated leaves (IL) at 2 dpi which also appeared later in systemic leaves (SL) at 8 dpi. In the resistant Di-17, *PR1* was strongly induced by TCV in IL in contrast to a complete lack of *PR1* induction by R6A. This is consistent with previous studies for TCV and reflects the induction of HR and SAR in resistant Di-17. In contrast, R6A, caused no detectable *PR1* induction in IL, consistent with its inability to induce an HR response in inoculated tissue.

We further evaluated expression of *PR1* and additional defense related genes using the more sensitive semi-quantitative PCR as described in Material and Methods. In susceptible Col-0, *PR1* expression followed a similar pattern of gene expression as observed in the northern blots with moderate levels detected in IL at 2 dpi and SL at 8 dpi (Figure 2-14). We also analyzed levels of *isochorismate synthetase* (*ICS*) mRNA as a more direct indicator of SA synthesis (Wildermuth et al., 2001). Interestingly, it only showed significant accumulation in the SL of R6A infections at 8 dpi. *WRKY70* is involved in cross talk between the SA and JA pathway and it also appeared to be induced to slightly higher levels in R6A infections in the SL at the later time point. These results are consistent with the conclusion that R6A infections induce an enhanced level SA pathway associated defense responses which could well account for the slower accumulation of R6A in Col-0. In resistant Di-17, higher levels of both *PR1* and *ICS* expression are evident in the IL of TCV infections and absent in R6A infections. This again confirms that TCV strongly induces the SA pathway defense response compared to R6A. No evident differences in *WRKY70* expression were seen throughout the time course. An indicator of the JA pathway activation, *Pdf1.2*, showed a slight and somewhat variable increase in both virus infections in inoculated leaves.

Overall, we found that the expression patterns of the SA pathway marker genes, *PR1* and *ICS*, were consistent and supportive of our explanations for the differences observed in virus accumulation and in symptoms between the two infections (Figure 2-14). It is important to note here that a likely explanation for some of the experimental variability in the gene expression results seen in Di-17 TCV infections likely reflects that approximately 25% of samples analyzed that came from systemically infected escapes.

Differential effect of TCV and R6A infections in Col-0 on TIP expression.

In order to assess the effect that virus infections might have on the levels of *TIP*, we used the more sensitive quantitative real-time PCR assays to examine *TIP* expression during the time course. After 48 hrpi, we were unable to see any significant gene expression differences in IL among the infections relative to the control (Figure 2-15a). However, at 2 dpi and 4 dpi, we were consistently able to observe between 5-6 fold increases in levels of *TIP* in SL in both virus infections. This contrasted to the 2-3 fold increase in *TIP* levels in mock inoculated plants. This result suggests that *TIP* levels, although induced somewhat by a wound response, show enhanced induction in response to systemic virus infection by both viruses (Figure 2-15a). R6A infections induced *TIP* expression significantly more than wt TCV infections at 2 dpi and 4 dpi time points in SL and then declined at 6 dpi. The temporal pattern of TIP induction during virus infection was consistent in 3 separate experiments. Most interesting was the 8 dpi time point where it is evident that *TIP* levels declined in TCV infections but again increased in R6A infections. We hypothesize that the ability of TCV CP to bind TIP may allow TCV to more effectively control *TIP* expression and, as a consequence, more effectively regulate the PTI response.

R6A infections caused enhanced expression of senescence pathway genes.

One of the most characteristic features of R6A infections is the enhanced symptom severity compared to TCV (Figure 2-4). The systemic symptoms induced by R6A infections also resemble that of a prematurely senescent plant (Buchanan-Wollaston, 2008). We also noted in the array study that some genes associated with plant

senescence pathways were differentially expressed in TCV versus R6A infections. To further assess this possible link to increased symptom severity, we evaluated *SEN1* gene expression to see if the senescence pathway was induced more in R6A infections versus TCV. *SEN1* regulates signals that link plant defense and senescence responses and hence is a useful marker to study in crosstalk between the two responses (Schenk et al., 2005). Our results showed that *SEN1* gene expression was elevated 8 fold at 2 dpi and 6 fold at 4 dpi in systemic tissue compared to 2-2.5 fold for TCV infections at the same time points (Figure 2-15b). This temporal pattern of expression, which was consistent in three separate experiments, mirrored the pattern of *TIP* induction. Although our data does not directly link *TIP* and *SEN* gene expression differences to the differences observed in virus accumulation and symptom severity, it does support the idea that defense induction and senescence responses are connected. Therefore we propose that TCVôs ability to bind TIP not only allows it to suppress the SA response and hence evade PTI, but it also prevents the senescence pathway from being induced, leading to milder symptoms.

Differential induction of WRKY family transcription factors.

In addition to WRKY70 (Figure 2-14), we also evaluated *WRKY6* gene expression in response the different virus infections. *WRKY6* is a transcription factor that belongs to a plant specific transcription factor family that has a role as both an activator and repressor of defense responses. It negatively regulates itself and *WRKY42,* however it is also a positive regulator of *PR1*, *NPR1* and *SIRK,* which are also involved in the senescence pathway (Robatzek and Somssich, 2002). We did observe a slight induction of *WRKY6* in IL of R6A infections (1.5 fold compared to mock) compared to a 3 fold

induction in TCV IL. In TCV SL the levels varied between 1 and 3 fold over mock for the duration of the time course. Interestingly, WRKY6 levels were induced 6 fold in R6A over mock at 8 dpi (Figure 2-15c).

Making a connection between viral accumulation, TIP-CP interaction, and basal resistance

We next wanted to resolve if the higher level of stimulation of the innate defense pathway by R6A infection was specifically associated with the inability of R6A CP to interact with the TIP transcription factor. To determine if TIP was playing a direct role in basal defense, construction of a knockout line of TIP was attempted in the Col-0 background, but viable progeny were not recovered. It was hypothesized that eliminating TIP may have resulted in an embryonic lethal plant. At the time these experiments were conducted (2002 by F. Qu, unpublished), there were no TIP knockout lines available in the Salk seed source library. One such TIP ko line was later reported by Jeong et al., (2008) and will be discussed later. To overcome this, an antisense transcript of TIP was inserted into the genome under the control of an inducible estradiol promoter. This permitted us to reduce TIP expression levels once the plants reached an adult stage. To address the effect of TIP overexpression, Qu created another line with an additional copy of TIP inserted into the genome under the control of the constitutive 35S promoter (Figure 2-3). The quantity of TIP transcript synthesized in these lines was verified by semi-quantitative PCR (Figure 2-16). Over expression of TIP did not cause any dramatic alteration in plant phenotype. However, we did notice that excess TIP did cause plants to flower earlier and likewise antisense TIP plants showed a delay in flower development (unpublished data by Basnayake, V.; Figure 2-16).

A time course experiment was designed to compare rates of virus accumulation of TCV and R6A in these transgenic plants with altered levels of TIP. Interestingly, there was a significant and consistent effect of altered levels of TIP expression on the accumulation of the R6A mutant in both the TIP up-regulated and down-regulated transgenic plants. The data presented in Figure 2-17 are representative panels of three independent experiments. In the asTIP lines, in which TIP levels were reduced transiently, R6A accumulated to equivalent levels as TCV in inoculated leaves and to almost the same levels as TCV at the 4 dpi time point. This is in contrast to the results in Col-0 where R6A is barely detectable in IL and 4 and 6 dpi SL (compare panel 1 and 2 in Figure 2-17). This result demonstrates that absence of *TIP* appeared to eliminate the differential growth rate response between R6A and TCV in Col-0. This observation provides additional evidence that the binding of TCV CP to TIP was indeed responsible for the down regulation of basal defense that permitted the more rapid accumulation of TCV compared to the R6A mutant in Col-0 susceptible plants.

The results in the *TIP* overexpression plants (UpTIP line) showed a similar result as the asTIP line when virus accumulation levels were compared in the SL (Figure 2-17, panel 3). It is evident that TCV and R6A accumulated to equivalent levels at the 4 and 6 dpi time points suggesting that elevated TIP levels also eliminated the advantage that TCV displayed over R6A in Col-0 infections. A likely explanation is that under conditions of excess TIP, the ability of TCV CP to sequester a sufficient quantity of TIP to compromise the resistance response is negated. These results from the TIP upregulated and downregulated transgenic plants provide additional confirmation of the role of TIP in modulating basal defense. The data supports our hypothesis that TIP is

likely a negative regulator of anti-viral defense because its presence in excess would simply make the plants more susceptible to both viruses as we observed in the asTIP plants. Curiously, there was an interesting and reproducible difference in accumulation of the two viruses in inoculated leaves in the UpTIP line. R6A accumulation was lower than TCV, much as in Col-0 (compare lanes 1 &2 in Fig. 2-17). Although we currently cannot explain this observation, it may reflect the differential induction of genes in IL and SL such as TIP and WRKY6 as described previously in Figure 2-15a.

We also performed some limited analysis of defense gene expression in infections of plants with altered levels of TIP expression in an effort to identify if there was an altered defense pathway response (Figure 2-18). The PR1 responses were slightly elevated in infections of both R6A and TCV, suggesting a modulation of the SA pathway by TIP in response to virus infections in general. The response of WRK70 also appeared to be elevated in TIP-UP lines. Finally, the response of Pdf1.2, an indicator for the JA response, showed greater sensitivity in the elevated TIP expression line, perhaps in response to the wounding or the crosstalk/suppression of the SA pathway by the excess TIP suppressing the basal defense pathway against viruses.

Making the connection between TIP expression and HRT resistance

Recently, a knockout line of TIP was described that carried a tDNA insertion in the promoter of the gene (Jeong et al., 2008). Importantly, Jeong observed that TCV accumulated more rapidly in the inoculated leaves of the TIP ko plants (*tip*), as did the unrelated virus Cucumber mosaic virus (CMV), but not the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*). They concluded that absence of TIP affected

basal resistance, a conclusion consistent with our data. However, they erroneously concluded that the absence of TIP had no affect on systemic spread, primarily because they did not assay for virus in the systemic leaves until 7 dpi, a time point by which we have clearly shown that the PTI induced resistance has broken down. In this study, they also demonstrated that the absence of TIP did not prevent the SA-mediated induction of HR by TCV in Arabidopsis lines carrying HRT. Supportive results were also found by another member of the Morris Lab, Sung Hwan Kang, who demonstrated TIP is not needed for HR induction in *Nicotiana benthamania* leaves when co-infiltrated with TCV CP and the R-gene HRT in transient assays (data not published). These results suggested that TIP was not required for the ETI response, a conclusion contrary to our observation that the R6A mutant, that fails to bind TIP, also fails to induce HR in the resistant Di-17 line.

To address this question, we obtained transgenic seeds of the TIP ko plants with and without an introgressed HRT gene from the Kachroo lab (previous described in Jeong et al., 2008) and conducted a comparative infection time course with TCV and R6A to monitor virus accumulation and gene expression. TCV systemic accumulation occurred in Col-0 (*hrt TIP*) but not in Di-17 (*HRT TIP*) with the expected pattern of PR1 expression and about 25% escapes as described earlier (Fig. 2-19a, panels 1 & 2). Col-0 plants with the TIP ko (*hrt tip*) showed the expected pattern of systemic invasion and a complete absence of PR expression in the IL and SL. Surprisingly, the TIP ko Col-0 plants with HRT introgressed from Di-17 (*HRT tip*) displayed an unusual phenotype that differed from that described by Jeong et al. (2008). All plants displayed a systemic HR, became systemically infected with TCV and showed high levels of PR1 gene expression

in the SL. This result was unexpected because it was not described in the original Jeong paper. We cannot be sure if this is a consequence of the absence of the *TIP* gene or the presence of the *RRT* gene or some difference in environmental conditions in our experiments that affected the spread of the virus.

We also performed a similar experiment using the mutant R6A and monitored the virus transcript accumulation and PR1 transcripts across the same time course. We again saw that R6A was completely unaffected by the presence or absence of HRT and the lack of TIP did not affect R6A virus accumulation in wt Col-0 compared to the TIP ko line (Figure 2-19b). This data corroborates our previous findings shown in Figure 2-16. It appears that the lack of TIP in a susceptible plant has little effect on the amount of virus accumulating during a PTI event. However, although TIP is not needed for HR, it does affect PTI indirectly because it affects the rate at which the virus moves systemically. So when TIP is absent, TCV moves more quickly and in the presence of HRT causes the elevated PR1 response and the resulting systemic HR (Figure 2-19a). When TIP is absent, there is no reduction in the TCV induced PTI response, so TCV moves more slowly which permits more time of PR1 elevation and HR development.

Discussion

In this study, we analyzed the potential mechanisms associated with the differential ability of TCV and the mutant R6A to accumulate in susceptible and resistant ecotypes of *Arabidopsis thaliana*. In a previous study, Ren et al. (2000) described a series of single aa substitution mutants of TCV to access the role of TIP in the HR response. The ability of the R domain of TCV CP to bind to TIP, a NAC transcription

factor, was shown to correlate with the observed variability in disease symptom severity in the susceptible Col-0 and the ability to confer resistance in the resistant Di-17. It was hypothesized that the difference in disease symptoms and resistance breaking was a consequence of the inability of the mutant viruses to bind TIP. One of the mutants, R6A, was further evaluated in this chapter to help elucidate the role TCV CP-TIP binding has on the PTI response. Interestingly, although R6A caused an increase in disease symptoms, we showed it also accumulated more slowly in inoculated and systemic tissue of susceptible Col-0. The reduced rate of R6A virus accumulation was transient, being most evident early in the infection and disappearing at about 8 dpi. The recovery of the virus accumulation by the mutant was attributed to both wt TCV and R6A eliciting a similar level of silencing suppressor activity encoded in their CP (Choi et al., 2004). Therefore, we concluded that TIP-CP interaction was associated with an earlier PTI event such as the induction of SAR or the activation of the senescence pathway.

We were able to connect the observed phenotype of slower accumulation of R6A directly with the innate defense response of the host by identifying two knock out lines that had dysfunctional SA pathways. In *pad4* and *npr1* mutants, R6A accumulation recovered and/or exceeded wt TCV virus accumulation levels. This provided corroboration that not only was R6A as robust as wild type TCV, but also that the R6A deficiency was due to its inability to turn off or elude the SA defense pathway and subsequent SAR defense response that is characteristic of wt TCV infections.

These experiments support our contention that TCV has evolved a mechanism to specifically down regulate the SA defense pathway thus giving it a distinct advantage over non-TIP binding mutants. They support our hypothesis that TCV actively down

regulates this basal defense via the SA pathway through its interaction with TIP, and that R6A is unable to do so. This also raises the unexpected possibility that the increased symptom severity associated with R6A may be linked with over-activation of the SA defense response. This phenomenon would be analogous to an inflammation response in animal systems due to the over-stimulation of the innate immune response (Ausubel, 2005; Thorsten et al., 2004). Together these data also suggest that the SA signaling in association with the PTI response is important in partially repressing viral invasion.

The experiments in which we assessed accumulation of TCV and R6A in plants with reduced TIP (asTIP) and elevated TIP (UpTIP) provided additional evidence that linked TIP-CP interaction more directly with the differential accumulation. These experiments uncovered that the down regulation of *TIP* may not play as much of a role in the defense against TCV infection as previously hypothesized by Ren et al., 2000. This may be a consequence of genetic redundancy within the NAC transcription factor family which has over 100 members. Therefore eliminating one gene, *TIP*, might not necessarily abolish the resistance modulating function due to compensation by other related gene production (Briggs et al., 2006; Pickett and Meeks-Wagner, 1995).

These experiments showed constitutive overexpression of *TIP* did affect the virus accumulation (Figure 2-17). When we infected the over expressing *TIP* plants with TCV or R6A, we observed both viruses were able to accumulate to equivalent levels similar to what we observed in the ko lines with dysfunctional SA pathways (compare Figure 2-9 and Figure 2-17). This suggested that the increased fitness of TCV over the mutant R6A was less dependent on the complete absence of TIP in the cell, but more affected by the total amount of TIP present during an infection. These data suggested a working model

for TCV infection in the susceptible host (Figure 2-20). In this model, we propose that TCV CP binding to TIP would influence defense signaling in the cell after an infection. We further speculate that TIP is a negative regulator of defense signaling based on the previous array data. In addition, because TIP has a membrane localization signal along with a nuclear localization signal (Kang, unpublished data), we suggest that it is likely localized outside the nucleus in a cellular membrane from which it undergoes controlled released by cleavage when the plant is not infected to maintain negative regulation of the innate defense responses. Our model further proposes that TCV CP interaction with TIP increases its nuclear localization and subsequently down-regulates defense signaling to enhance TCV invasion. When the R6A mutant lost this ability to enhance TIPôs nuclear localization, defense signaling increased leading to a more robust PTI response (depicted in Figure 2-20).

Jeong *et al*. (2008) described a tip knockout line in Col-0 background that also was carrying the R gene, HRT. This transgenic line was found to be resistant to TCV infection. Therefore, they concluded that TIP did not have a direct role in the R-gene resistance to TCV infection (Jeong et al., 2008). This conclusion did not rule out possible genetic redundancy however, and seed lines that were obtained from the Kachroo lab still showed some escape lines. Therefore our results concerning TCV accumulation in the antisense TIP line in which we still saw that wt TCV had an accumulation advantage over R6A is still a valid point. Furthermore our results show that virus accumulation differences are still evident when less TIP is expressed.

It is well known that plants have the capacity to recognize pathogens and in many cases there is functional redundancy within multigene families that often complicates

genetic attempts to define the role of individual genes (Bouché and Bouchez, 2001). One example of this was found with a *wrky6* knockout mutation, which resulted in no obvious mutant phenotype yet overexpression of *WRKY6* caused a stunted phenotype and a significant increase in SA pathway associated genes like *PR1* and *NPR1* (Robatzek and Somssich, 2002). It is possible that we are observing a similar example. Excessive TIP levels appear to impact the plantôs defense system to a similar extent as seen in the SA pathway defective mutant knockout lines of *pad4* and *npr1* (compare Figures 2-9 and 2-17). Here we observe a defective defense signaling system that allows for more virus to accumulate at a faster rate than what is observed in wt Col-0. Yet, reduced amounts of the TIP transcription factor appears to have little affect on virus accumulation. Together these data support our conclusion that TIP is a negative regulator of PTI. If excess TIP was causing a reduction in PTI, then both TCV and its non-TIP binding mutant would accumulate to similar levels as observed in the npr1 and pad4 knockout line. Furthermore if the reduction of TIP presence in the cell has little to no affect on PTI then we would expect to see both viruses accumulating in a similar manner as we see in wt Col-0. The data presented here demonstrates the importance of the TCV CP binding TIP to suppress the PTI response and how parts of the SA signaling pathway are key components in TCV defense. This supports the hypothesis that TIP is affecting the level of the basal response (PTI).

Also our results provide some evidence for the fact that TIPôs role in an ETI response leading to HR may not be the key factor. Although our data rules out a direct role, TIP appears to be important in fine tuning the defense response (discussed more in Chapter 3). Figure 2-19 suggests that lack of TIP in the presence of the HRT during TCV

infections, can lead to a systemic HR like response. It is also important to note that R6A is unaffected by the presence or absence of either HRT or TIP. However, the symptom severity of R6A is elevated in the *tip* ko plants regardless of the presence of HRT. This supports previous gene expression data that TIP is having an effect primarily on the senescence pathway. This may also be shedding light on the balance between the PTI and ETI response. Perhaps the greater invasiveness in the absence of TIP permits TCV to invade systemically ahead of the HR response. This also supports data from Kang (personal communication) that the mutation not only effects TIP binding, but some other property of TCV, which we now know is nuclear localization (Kang, unpublished).

In summary, our data demonstrates that TIP expression is important in regulating the PTI response and impacts the rate of TCV accumulation in *A. thaliana*. We have shown that the level of TIP expression affects proper signaling of the SA pathway and other defense responses. Therefore we conclude that TIP is a key player in the PTI defense response against TCV. Further research needs to be done to look at what other proteins may have similar or redundant functions to TIP that are able to mask the effects of its absence in the ko lines (Jeong et al., 2008). This idea will be explored further in the Chapter 3 with emphasis on the resistant Di-17 line and ETI response.

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Figure 2-1. Diagram of inoculated and systemic leaves. The plant shown above depicts an example of which three leaves were inoculated through rub inoculation (red arrows) and the areas of the uninoculated leaves that were selected for analysis of systemically infected tissue (blue arrows). To be consistent on selection of systemic tissue, the newest leaf growth greater than one centimeter in width was selected for analysis in all experiments. The plant shown above is *A. thaliana* ecotype Col-0. The picture is a representative of typical symptoms of Col-0 infected with TCV at 10dpi grown in a growth chamber at 22°C with 12 hr day length.

Figure 2-2. Graph of the standard curve for virus concentration. A standard curve was prepared as a method of plotting virus concentration relative to absorbance. The data from the graph was used to determine the concentration of viruses in the indirect ELISA experiments preformed in this chapter. Starting with a TCV stock concentration of .1mg/ml or 100,000 ng/ml, a series of dilutions were made to accurately predict the standard curve line. The indirect ELISA plate was allowed to develop until the OD reading of the non-virus mock wells were 0.200. This value was subtracted from each value to account for background and the data points were used to make a standard equation.

Figure 2-3. Diagram of plasmids constructed for production of transgenic *A. thaliana*. Two transgenic lines of *A. thaliana* ecotype Col-0 were constructed to reduce or enhance the expression of the *TIP* gene by Dr. Feng Qu. The above plasmids were transformed into Col-0 by *Agrobacterium* infiltration (Bechtold, Ellis, and Pelletier,

1993) (described further in materials and methods). Shown here are the two constructs of the inserts used to transform Col-0 to alter levels of TIP expression. The asTIP construct has reduces the amount of TIP transcript available by taking advantage of plant encoded silencing mechanisms and the overexpressed TIP construct is constitutively expressed under the control of the CaMV 35S promoter (E35S).

Figure 2-4. Disease symptoms seen in *Arabidopsis thaliana* infected with TCV. Shown is the typical response to the susceptible Col-0 (top) and resistant Di-17 (bottom) plants infected with either wild-type TCV or mutant R6A 14 days after inoculation (dpi).

Figure 2-5. Comparison of temporal accumulation of TCV and R6A viral transcripts. A total of 10ng of virus transcript of wt TCV (T) or mutant R6A (R) were inoculated onto designated leaves. Inoculated leaves (IL) were collected at 48 hr post inoculation (hrpi) and systemic leaves (SL) were collected at 2, 4, 6, and 8 day post inoculation (dpi) as indicated. Viral RNA levels were monitored by northern blot using a probe for CP sequence (described in Materials and Methods section). Arrows identify location of viral genome (vRNA) and subgenomic RNAs (sgRNA). Panel below the blot shows ethidium bromide (EtBr) stained gel showing rRNA bands used as a loading control. This experiment was repeated three times with similar results.

Figure 2-6. Evaluation of total accumulation of TCV and R6A virions over a time course of infection. Accumulation of the total TCV and R6A virus particles were evaluated in both inoculated (IL) and systemic leaves (SL) of *A. thaliana* ecotype Col-0

over the indicated time course. A total of 10ng of virus transcript of wt TCV (T) or mutant R6A (R) were inoculated onto designated leaves. Virus titers were determined by ELISA as described in Materials and Methods section. Panel shows the average absorbance levels of three independent experiments as an indication of the amount of virion present in each sample. Error bars represent standard deviation from the absorbance levels of the three experiments. (*) denotes significant difference between R6A and TCV at a specific time. The OD reading of mock was 0.200 was subtracted from each value to account for background.

Figure 2-7. Viral RNA and virus particle accumulation in inoculated and systemic leaves of resistant Di-17 inoculated with either TCV or R6A. (a) Viral RNA accumulation evaluated by northern blot. Plants were inoculated following the same conditions as described in Figure 2-5. (b) Virus accumulation was evaluated by ELISA for inoculated leaves (IL) and systemic leaves (SL). Plants were inoculated under the same conditions as described in Figure 2-6. Results are an average of 3 independent experiments. Positive virus detection of TCV in both (a) and (b) is a consequence of the random sampling and likelihood of detection one in four plants that could have been systemically infected by TCV in Di-17. Error bars indicate standard deviation from the average absorbance levels. * denotes significant difference between R6A and TCV at a specific time point.

Figure 2-8. Verification of knockout lines of *Arabidopsis thaliana*. PCR amplification using primers specific for the genes indicated on left was used to verify absence of

transcript in the mutant line indicated on top of the figure. The number of PCR cycles for each gene is shown on the right. Primer sequences are shown in Table 2-1.

Figure 2-9. Comparison of viral RNA accumulation of TCV and R6A in defense gene knockout lines. Designated knockout lines of Arabidopsis thaliana ecotype Col-0 were inoculated with R6A (R) or TCV (T) as described in Figure 2-5. Samples were taken throughout the time course and viral RNA accumulation was monitored by northern blot. The probe used was specific for the coat protein transcript whose sequence in present in the viral genome and both sub-genomic RNAs. Ethidium bromide stained rRNAs used for loading control. TCV genome can also be seen on EtBr stained gel at later time points in systemic tissue. Note the much elevated levels of TCV genomic RNA that can be visually detected in the *npr1* and *pad4* ko lines compared to the wt Col-0, *ein2*, and *jar1*. This experiment was repeated 3 times with similar results.

Figure 2-10. Evaluation of total TCV and R6A mutant virion accumulation across a time course in defense signaling defective plants. Plants were inoculated and processed under the same conditions as described in Figure 2-6. The above graphs are absorbance levels obtained via indirect ELISA, used to evaluate the virus titer in the SA defective mutants. The virus titers in the SA defective mutants *pad4* and *npr1* are shown above in A and B respectively. In panel C, we see the absorbance levels in JA defective mutant *jar1*, which still has a functional SA/SAR defense response, across the predetermined time course. Results are an average of 3 independent experiments. Error bars indicate standard deviation from the average absorbance levels.

Figure 2-11. Comparison of total TCV and R6A mutant virion accumulation at 6 dpi in systemic tissue in wild-type and defense signaling defective *A. thaliana.* Plants were inoculated under the same conditions as described in Figure 2-6. Absorbance levels are used to determine virus titers in crude extract. Results shown are an average of 3 independent experiments. Error bars indicate standard deviation from the average absorbance levels. (*) denotes significant difference between R6A and TCV in a specific plant type.

Figure 2-12. Virus gel of TCV and R6A at 6 dpi in wt Col-0 and its mutants. Virus gels experiments, described in materials and methods, were conducted as another way to classify virus accumulation of TCV and R6A. Using wt Col-0 and the mutants *npr1*, *pad4* and *jar1*, we evaluated the amount of virions that accumulated at 6 dpi. We stained gels with ethidium bromide (EtBr) to stain nucleic acids and with coomassie blue (CB) to stain for proteins. The nucleic acid band and the protein band are located in the same spot on the gel when images were analyzed, which helps draw the conclusions that intact viruses were successfully recovered. The above panels are representative of two independent experiments.

Figure 2-13. PR1 expression levels in TCV and R6A infections. Resistant (Di-17) and susceptible (Col-0) were infected with TCV or R6A and maintained in a growth chamber throughout the duration of the infection. Approximately six randomly selected leaves from either inoculated leaves (IL) or systemic leaves (SL) were collected at 2 days post inoculation (dpi) or 2, 4, 6, and 8 dpi respectively. RNA levels were monitored by

northern blot using a PR1 probe (described in Materials and Methods). EtBr stained gels are shown for loading controls. These results show a differential induction of *PR1* a key indicator of an activated SA defense pathway.

Figure 2-14. Expression levels of defense related genes between TCV and R6A infections. Multiple genes were selected and evaluated using semi-quantitative RT-PCR. The genes that were chosen have previously been linked to defense related pathways in *A. thaliana*: *PR1* and *ICS* are SA pathway genes and *Pdf1.2* is a key indicator of JA pathway induction. PCR cycle numbers are shown to the right of each set of panels divided by the two ecotypes of Arabidopsis used in this study, Col-0 (susceptible) and Di-17 (resistant). *ACT2* expression was used as an endogenous control.

Figure 2-15. Evaluation of relative gene expression levels using Real-time PCR. *A. thaliana* ecotype Col-0 was inoculated with either TCV, R6A transcripts as described in Figure 2-5. Tissue samples were collected and RNA extracted for each time point. a) Expression levels genes were analyzed using One-step Real-time PCR as described in Materials and Methods. The relative fold change of three genes, (a) *TIP* (b) *SEN1* and (c) *WRKY6* are plotted. Fold change is calculated relative to the mock infection of each gene at 48hrpi. *ACT2* was used as the endogenous control. This graph is the average of results obtained for 2 independent experiments.

Figure 2-16. Phenotypes of altered TIP lines. (a) Semi-quantitative PCR evaluation of *TIP* transcript expression in wild type and *TIP* altered transgenic lines of Col-0. The

antisense TIP (asTIP) line has a reduced level of *TIP* transcripts compared to wild type Col-0. Constitutively upregulated TIP (UpTIP) has an increased level of TIP transcript compare to Col-0 and asTIP. *ACT2* was used as the endogenous control. PCR cycle number used is shown on the right side of the figure. (b) Phenotype of mature 6 week old transgenic *A. thaliana* with varying levels of TIP expression.

Figure 2-17. Differences in viral RNA accumulation in TIP transgenic lines. *Arabidopsis thaliana* Col-0 wt and transgenic lines were infected with either TCV (T) or R6A (R). Viral RNA accumulation was monitored over a time course by northern blot analysis of both inoculated (IL) and systemic leaves (SL) at times indicated above each panel and described in Figure 2-5. The probe used was specific for the coat protein transcript whose sequence is present in the viral genome and both sub-genomic RNAs. Arrows indicate viral RNA genome (vRNA) and subgenomic RNA (sgRNA). These are representative panels of three experiment which yielded consistent results.

Figure 2-18. Evaluation of gene expression between wt Col-0 and transgenic lines of Col-0 with altered levels of TIP expression. *A. thaliana* ecotype Col-0 wt and transgenic lines were infected with either TCV (T), R6A (R), or Mock (M). mRNA levels of several genes were evaluated at four days post infection in systemic tissue since virus accumulation differences were most obvious at that time. The above panel is represents one completed experiment. There were no obvious trends in any genes examined that helped explain the difference in virus accumulation at this time.

Figure 2-19. Wt TCV and R6A accumulation and PR1 expression in HRT and TIP ko lines. Col-0 and Di-17 plants along with transgenic Col-0 (tip ko), which has a tDNA insertion in the TIP promoter, and HRT tip, which was a cross between the tip ko and Di-17 (as described in Jeong et al., 2008), were inoculated with (a) TCV and (b) R6A. Samples were collected and analyzed as described in Figure 2-5. PR1 transcripts were evaluated using northern blots as described in Figure 2-13. The lower panel shows plants at 8 dpi.

Figure 2-20. Proposed model of the role of TIP-TCV CP interaction during TCV infection in the susceptible host Col-0 during a PTI event. We propose that TCV CP (or some other as yet undefined PAMP) is recognized by an as yet uncharacterized toll-like receptor (an RLK that could be in the plasma membrane or an internal endosomal membrane) that provokes SA defense signaling. This would then lead to a MAPK cascade and enhanced basal defense. Infection by virus with wild type CP results in an interaction with the negative regulator TIP that is tethered in an endosomal membrane in the cytoplasm, enhancing the rate of its release for migration into the nucleus leading to the suppression of basal defense genes. Infection by the TCV mutant R6A is unable to repress the basal defense response because it can't interact with TIP and enhance its release. This difference in basal defense regulation in the susceptible host gives a selective advantage to TCV early in infection.

Figure 2-1.

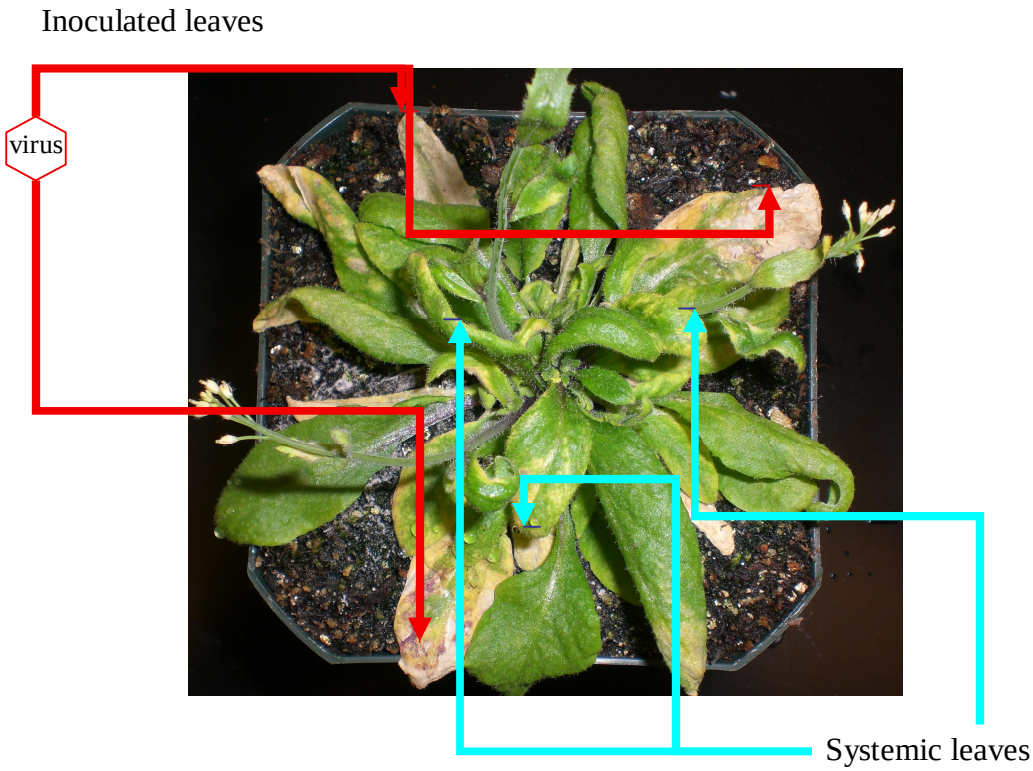


Figure 2-2.

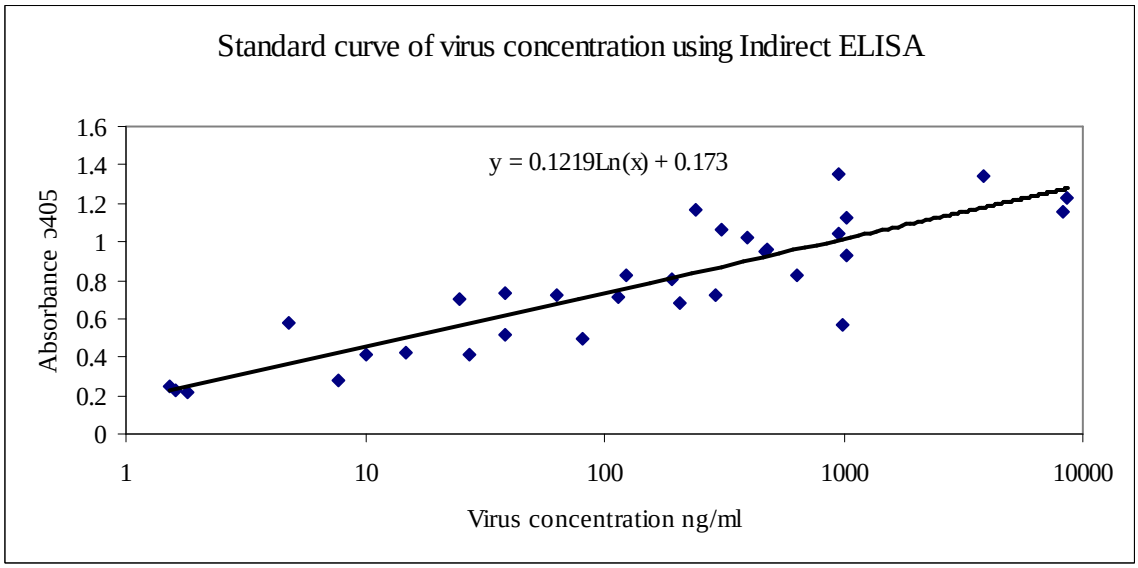

Standard curve of virus concentration using Indirect ELISA
y = 0.1219Ln(x) + 0.173
Absorbance 405
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
1000
10000
Virus concentration ng/ml

Figure 2-3.

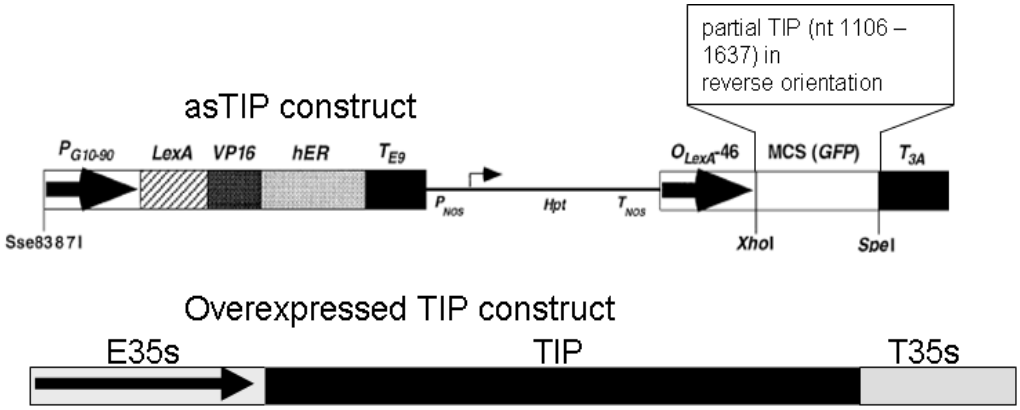

partial TIP (nt 1106 – 1637) in reverse orientation
asTIP construct
$P_{G10\text{-}90}$
LexA
VP16
hER
T_{E9}
P_{NOS}
Hpt
T_{NOS}
O_{LexA}-46
MCS (GFP)
T_{3A}
Sse8387I
XhoI
SpeI
Overexpressed TIP construct
E35s
TIP
T35s

Figure 2-4.

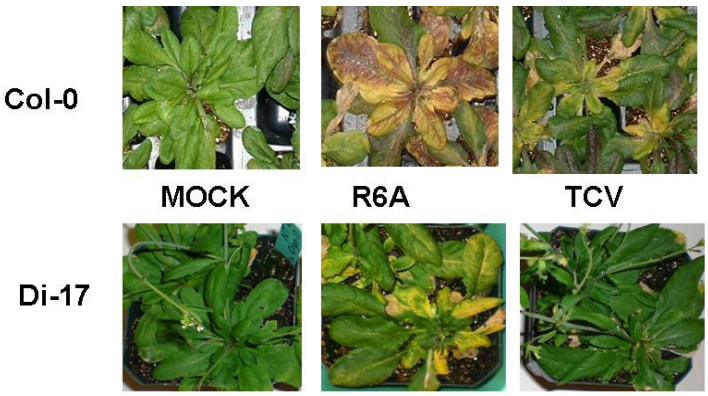


Figure 2-5.

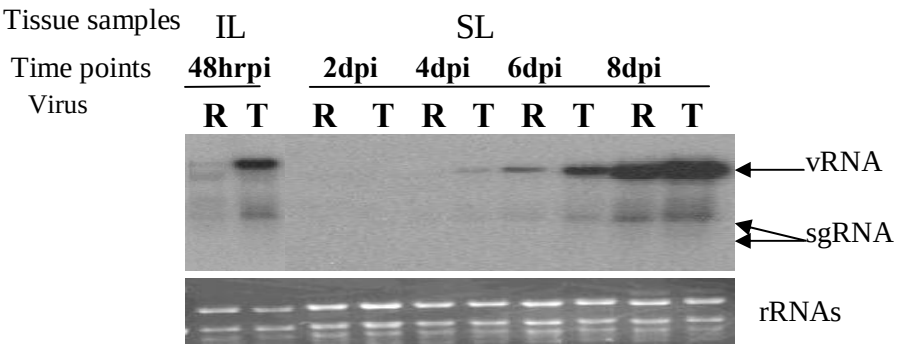

Tissue samples
IL
SL
Time points
48hrpi
2dpi
4dpi
6dpi
8dpi
Virus
R T R T R T R T R T
vRNA
sgRNA
rRNAs

Figure 2-6.

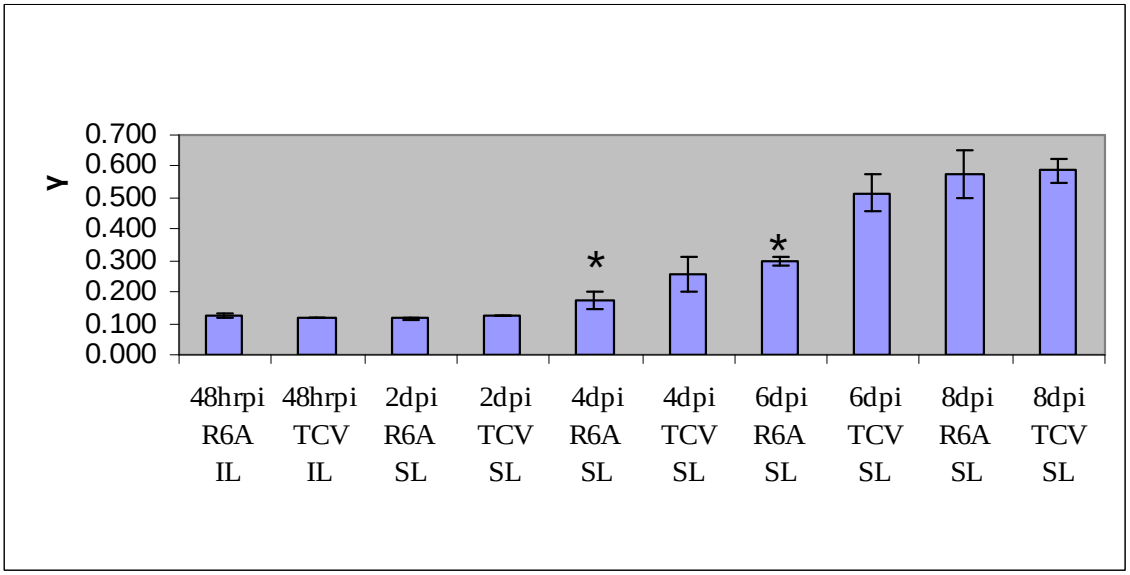
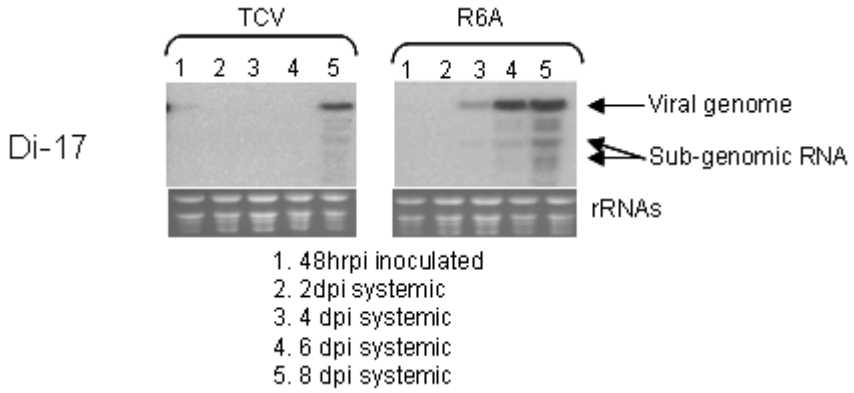

0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
y
*
*
48hrpi R6A IL
48hrpi TCV IL
2dpi R6A SL
2dpi TCV SL
4dpi R6A SL
4dpi TCV SL
6dpi R6A SL
6dpi TCV SL
8dpi R6A SL
8dpi TCV SL

Figure 2-7.

a.


TCV
R6A
1 2 3 4 5
1 2 3 4 5
Di-17
Viral genome
Sub-genomic RNA
rRNAs
1. 48hrpi inoculated
2. 2dpi systemic
3. 4 dpi systemic
4. 6 dpi systemic
5. 8 dpi systemic

.

b.

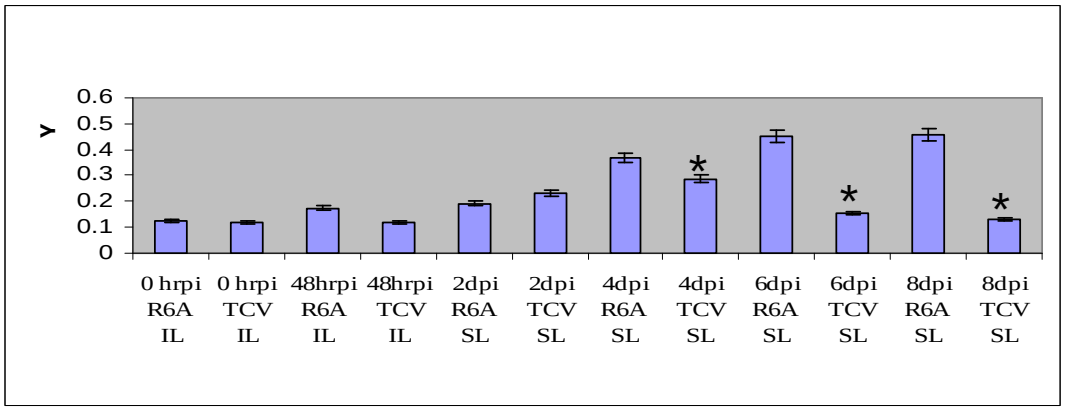

0.6
0.5
0.4
0.3
0.2
0.1
0
0 hrpi R6A IL
0 hrpi TCV IL
48hrpi R6A IL
48hrpi TCV IL
2dpi R6A SL
2dpi TCV SL
4dpi R6A SL
4dpi TCV SL
6dpi R6A SL
6dpi TCV SL
8dpi R6A SL
8dpi TCV SL
*
*
*

Figure 2-8.

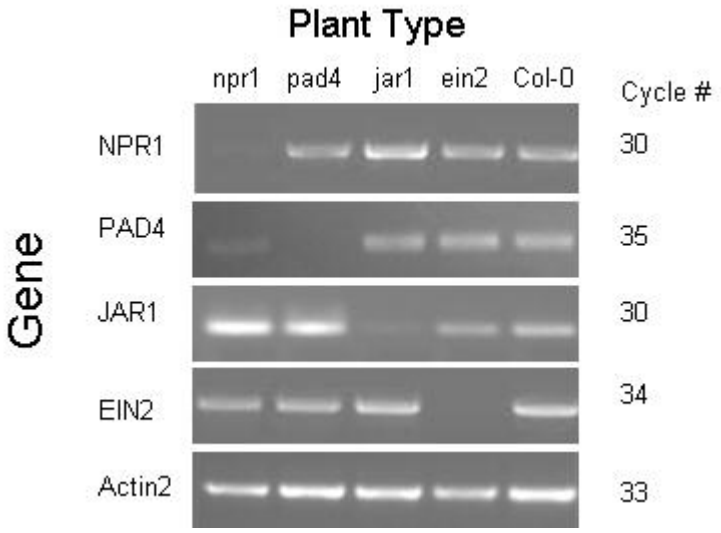

Plant Type
npr1
pad4
jar1
ein2
Col-0
Cycle #
Gene
NPR1
30
PAD4
35
JAR1
30
EIN2
34
Actin2
33

Figure 2-9.

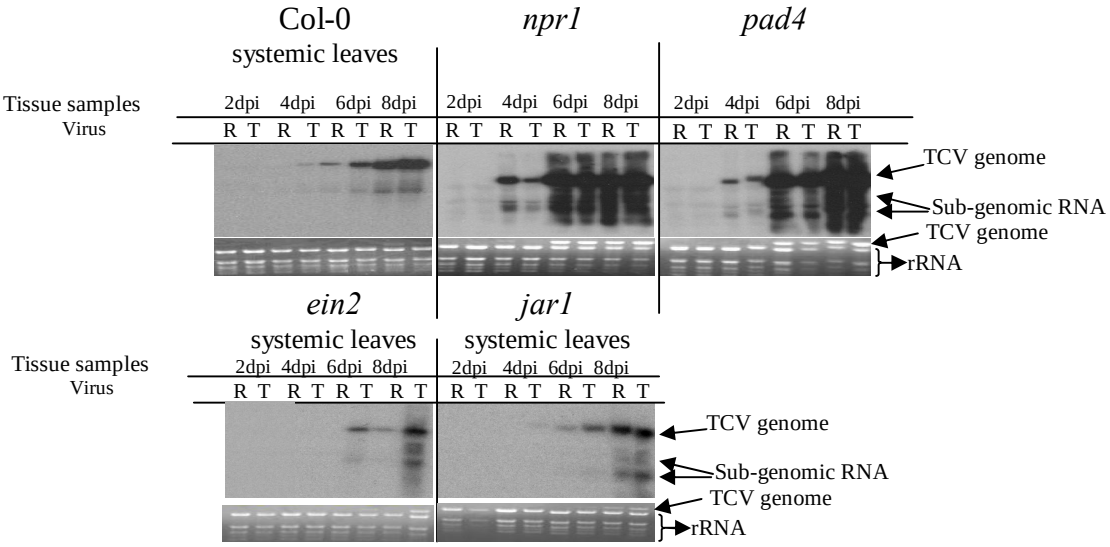
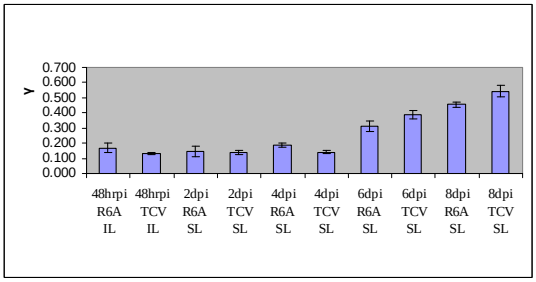
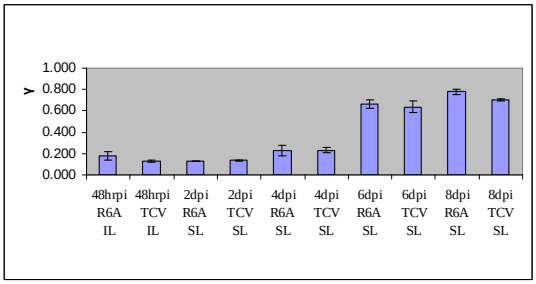

Col-0
npr1
pad4
systemic leaves
Tissue samples
Virus
2dpi 4dpi 6dpi 8dpi
R T R T R T R T
TCV genome
Sub-genomic RNA
TCV genome
rRNA
ein2
jar1
systemic leaves
Tissue samples
Virus
2dpi 4dpi 6dpi 8dpi
R T R T R T R T
TCV genome
Sub-genomic RNA
TCV genome
rRNA

Figure 2-10.

a.



b.



c.

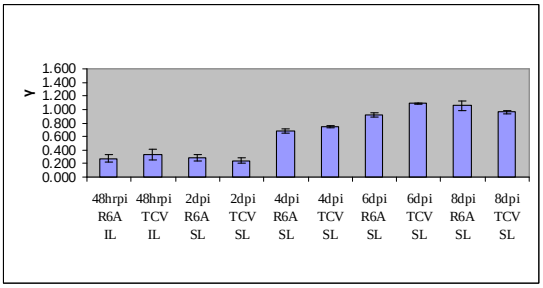


Figure 2-11.

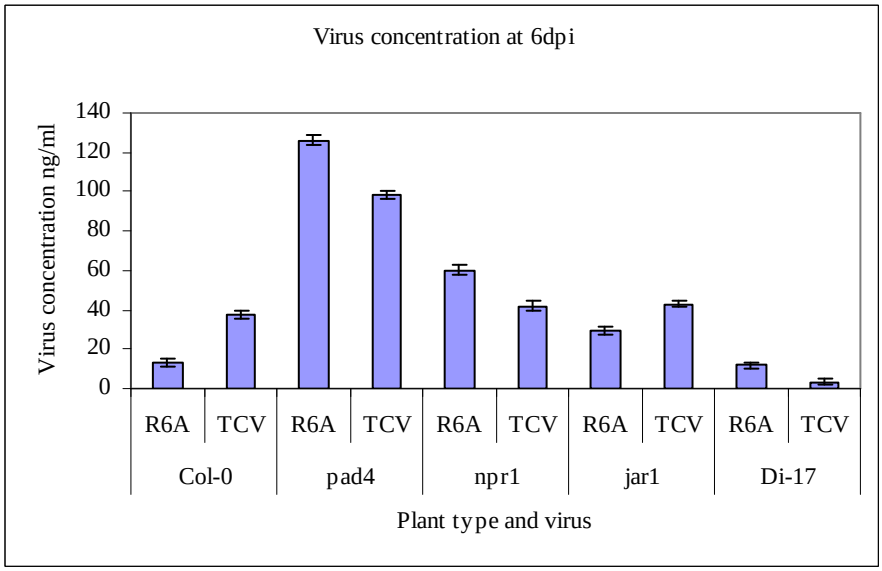

Virus concentration at 6dpi
Virus concentration ng/ml
140
120
100
80
60
40
20
0
R6A
TCV
R6A
TCV
R6A
TCV
R6A
TCV
R6A
TCV
Col-0
pad4
npr1
jar1
Di-17
Plant type and virus

Figure 2-12.

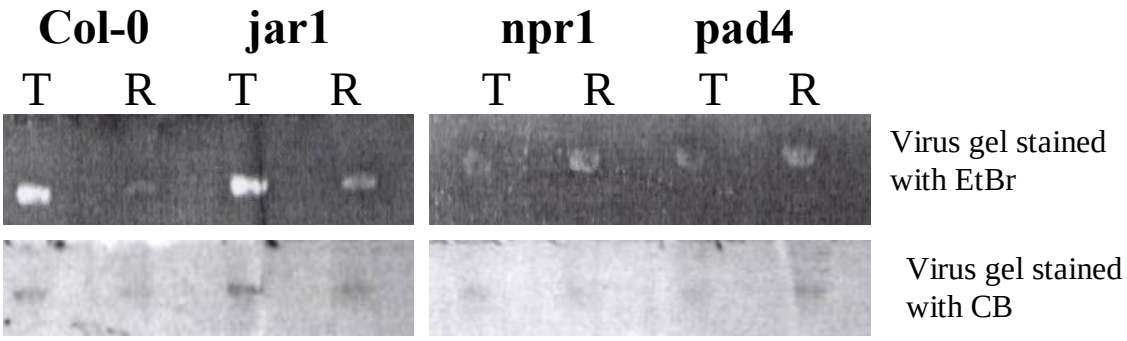

Col-0
jar1
npr1
pad4
T R T R T R T R
Virus gel stained with EtBr
Virus gel stained with CB

Figure 2-13.

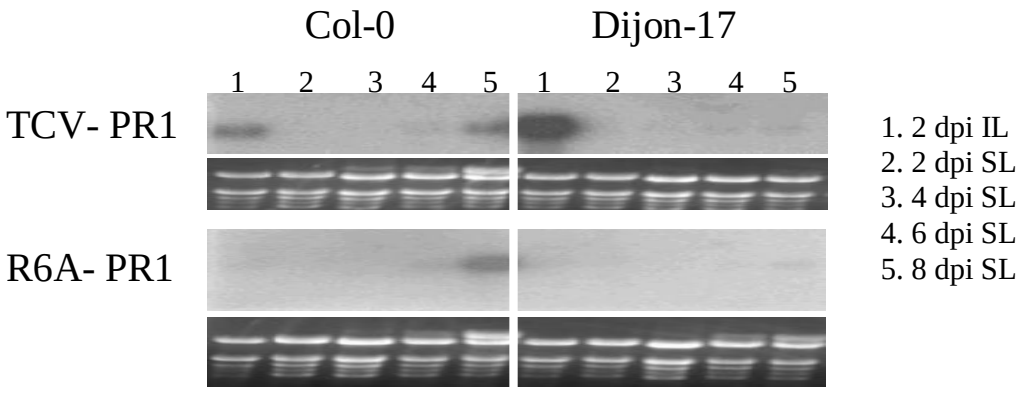

Col-0
Dijon-17
1 2 3 4 5 1 2 3 4 5
TCV- PR1
R6A- PR1
1. 2 dpi IL
2. 2 dpi SL
3. 4 dpi SL
4. 6 dpi SL
5. 8 dpi SL

Figure 2-14.

1. 48 hrpi IL
2. 2 dpi SL
3. 4 dpi SL
4. 6 dpi SL
5. 8 dpi SL

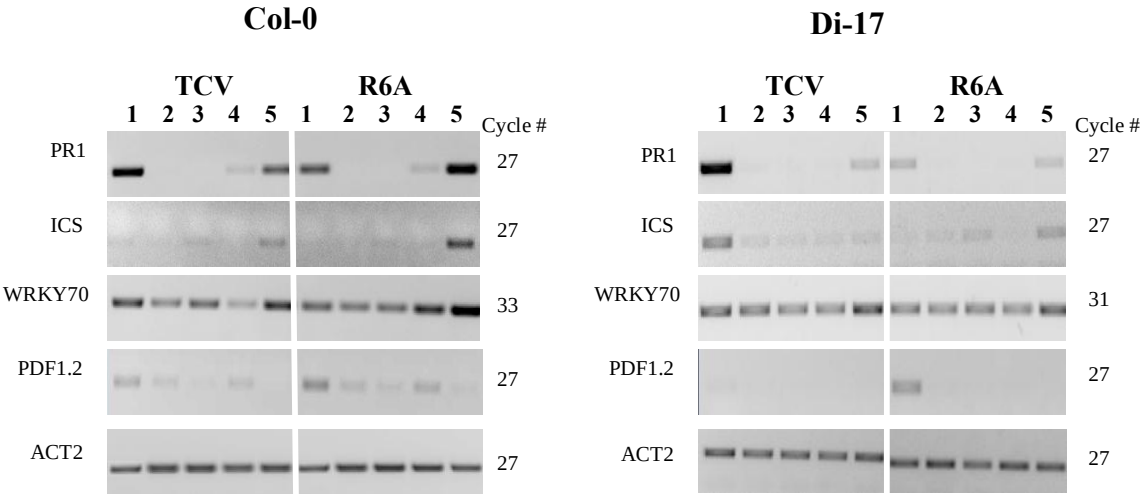
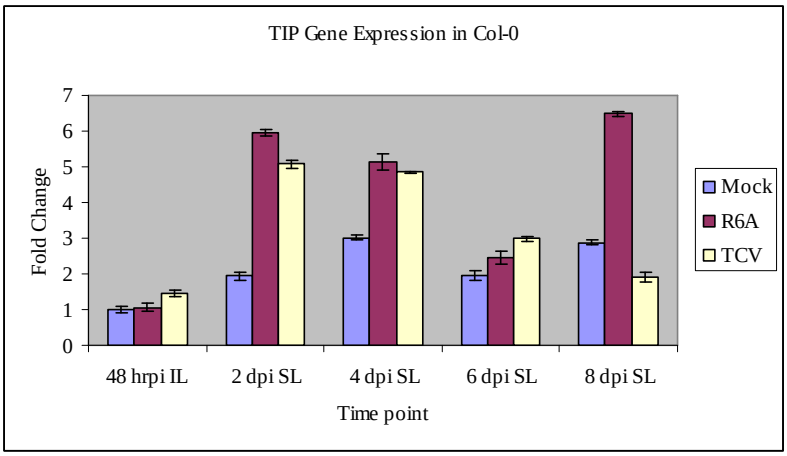
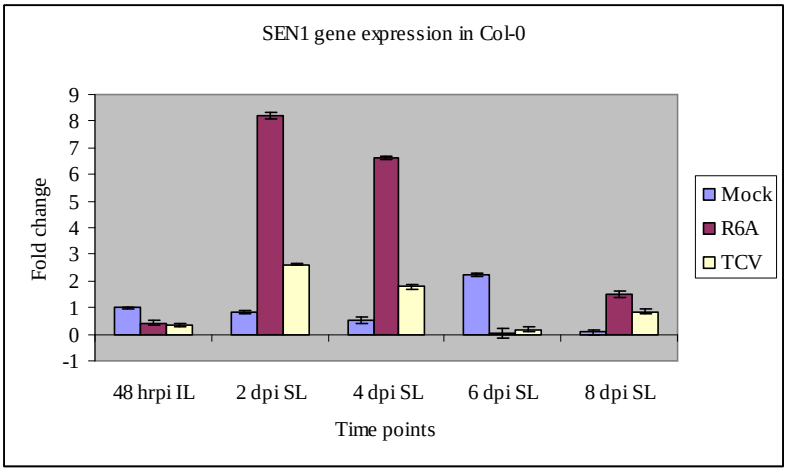


Figure 2-15.

a.



b.



c.

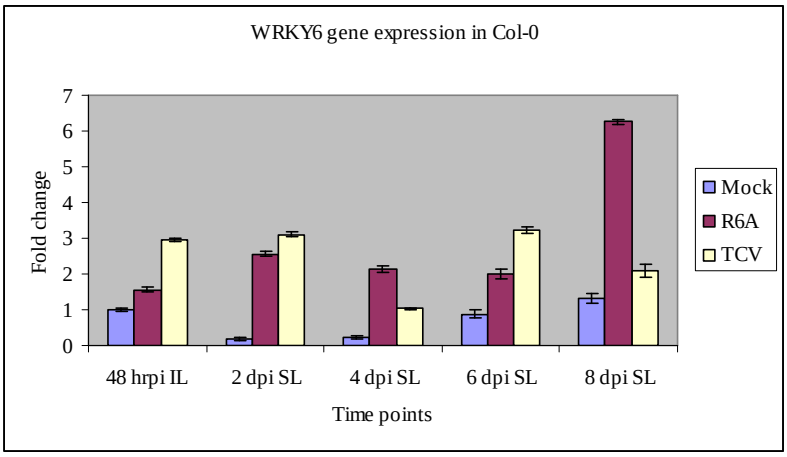
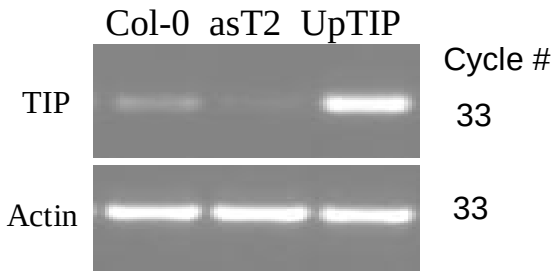


Figure 2-16.

a.



b.

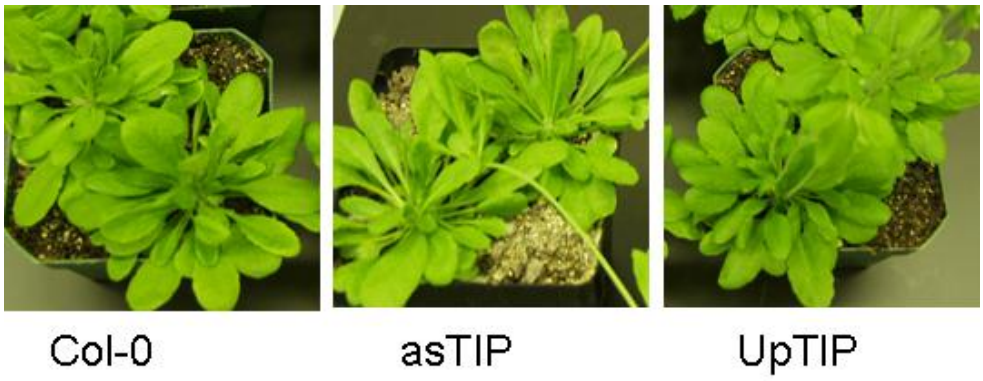


Figure 2-17.

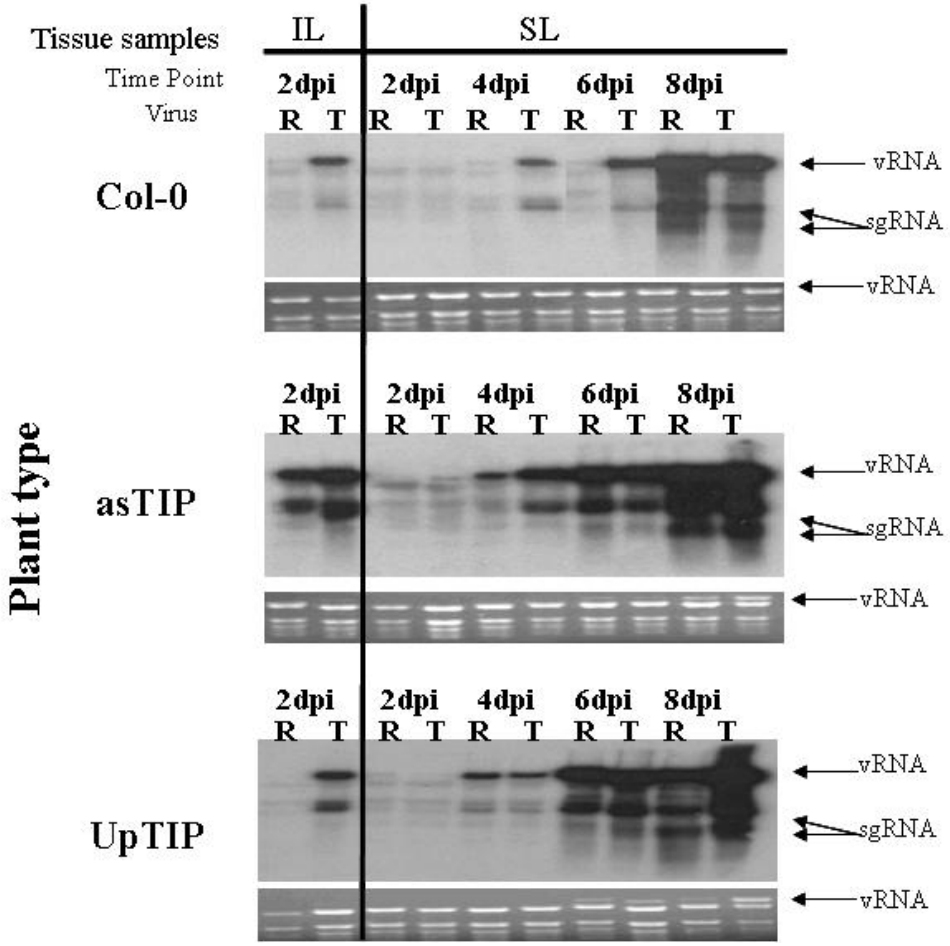

Tissue samples
IL
SL
Time Point
Virus
2dpi
2dpi
4dpi
6dpi
8dpi
R T R T R T R T R T
Col-0
asTIP
UpTIP
Plant type
vRNA
sgRNA
vRNA

Figure 2-18.

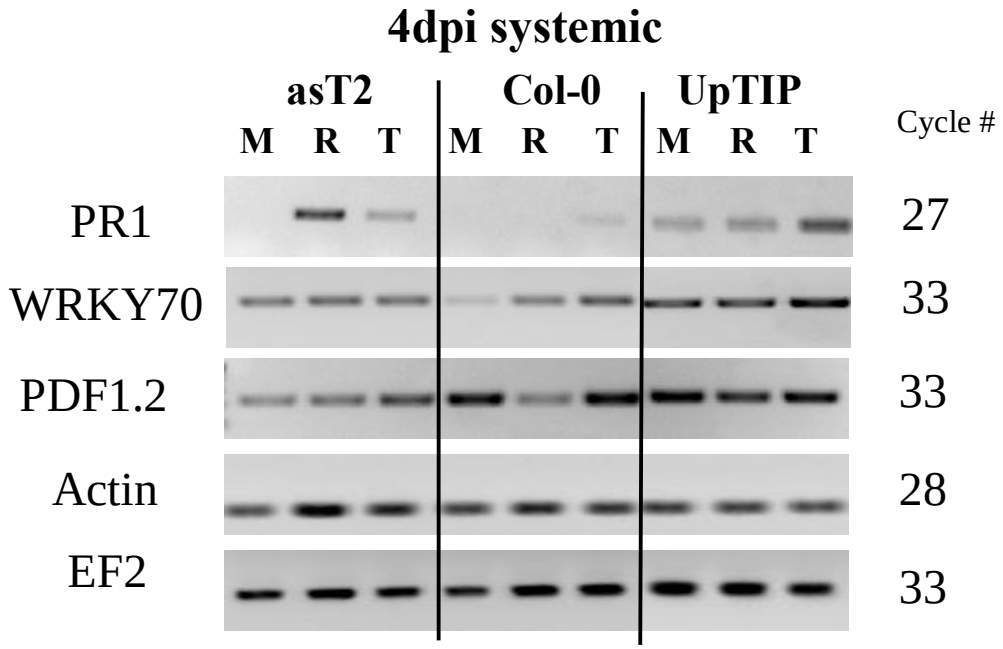
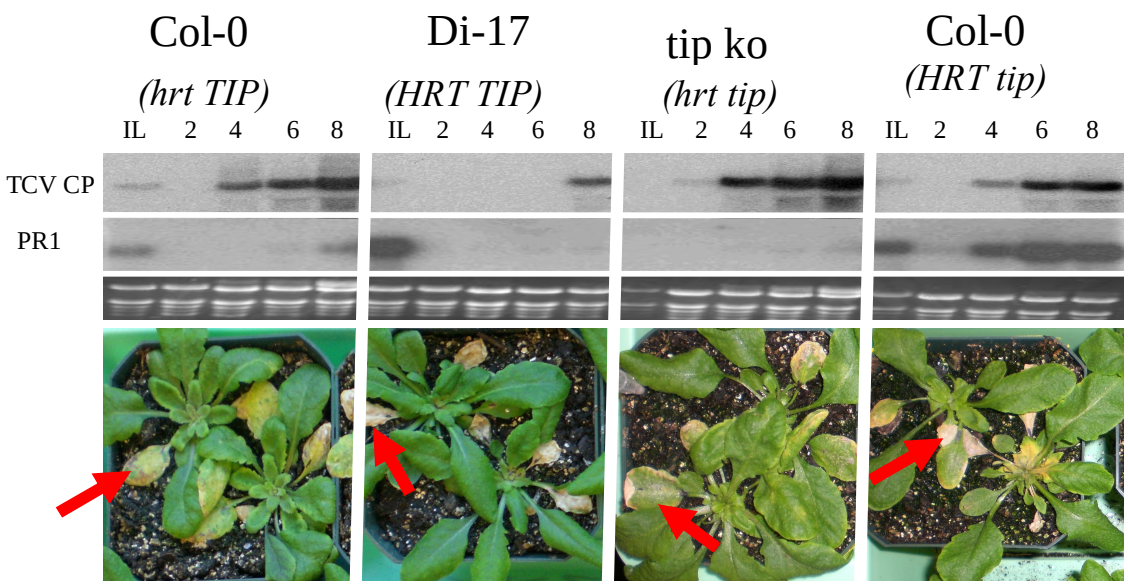

4dpi systemic
asT2
Col-0
UpTIP
M R T M R T M R T
Cycle #
PR1
27
WRKY70
33
PDF1.2
33
Actin
28
EF2
33

Figure 2-19.

a.



b.

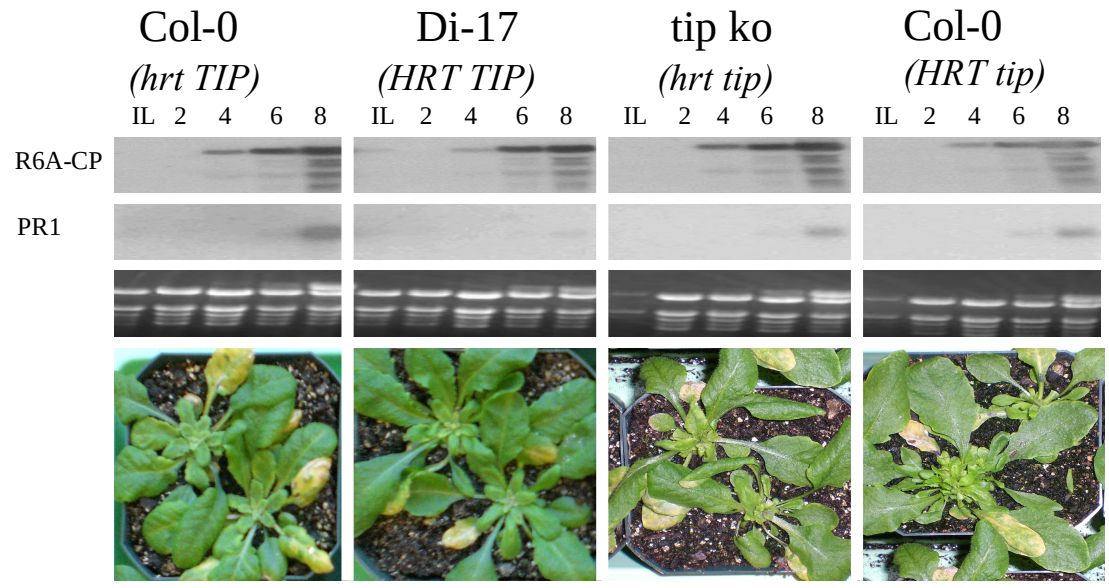


Figure 2-20.

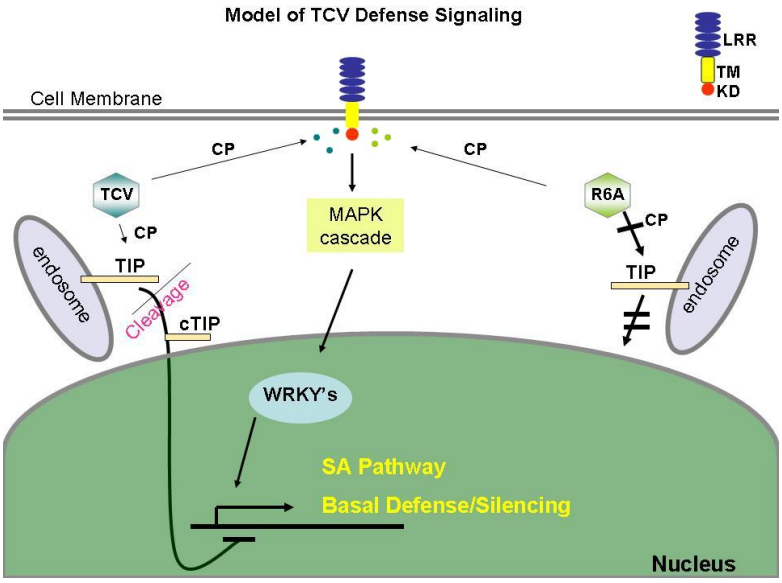

Model of TCV Defense Signaling
LRR
TM
KD
Cell Membrane
CP
CP
TCV
CP
MAPK
cascade
R6A
CP
endosome
TIP
Cleavage
cTIP
TIP
endosome
WRKY's
SA Pathway
Basal Defense/Silencing
Nucleus

Table 2-1. Sequences of probes used for semi-quantitative PCR

Gene	Sequence
ACT2	Fwd: 5'-GTCTGAGATTTCTCCTGCCG-3'
	Rev: 5'-CACGGTTAGCCTTTGGGTTA-3'
EIN2	Fwd: 5ô-CTTGGCTTCATCGTGCTACA
	Rev: 5ô-CTTAAGCTGCGGAATGAAGG
JAR1	Fwd: 5'-ACTAGCGCAGGATGTTGGAG-3'
	Rev: 5'-AGCGTTTCCATTGAGACCAC-3'
NPR1	Fwd: TGCATCAGAAGCAACTTTGG
	Rev: GAGGCAAGAGTCTCACCGAC
PAD4	Fwd: 5'-TTGTCGATTCGAGACGAGTG-3'
	Rev: 5'-TTTTTAAATCACTTGGGCGG-3'
TIP	Fwd: 5'-CCGGCTCAAGATCAACGGTCACG-3'
	Rev: 5'- CTGCTCAGCACAACCCGGGG -3'
PR1	Fwd: 5ô- AACCAGGCACGAGGAGCGGT
	Rev: 5ô-GTTCACGGCGGAGACGCCAG
ICS	Fwd: 5ô-TTCCTCCGGCGTCGTTCGGT
	Rev: 5ô-CCCAAGCAATAGCTGCAGCCAAC
PDF1.2	Fwd: 5ô- TGCTTTCGACGCACCGGCAA
	Rev: 5ô- CCGCAAACCCCTGACCATGTCC
WRKY70	Fwd: 5ô-TGAACCAACTCGTTGAAGGCCATGA
	Rev: 5ô-CAACGGCGGCGAGGGATGAG

Table 2-2. Probes for Northern Analysis

TCV-CP	Rev: 5'-CAGGACCGAGAAGTCAGAGG-3'
	Rev: 5'-GGCCCACCCGACACCACTGG-3'
	Rev: 5'-CTTGTCTTGACCGAGTTGGT-3'
PR1	Rev: 5ô-GTTCACGGCGGAGACGCCAG

Table 2-3. Genes used for Time Course qRT-PCR

	Gene Name/Description	Reference Sequence	Transcript ID	Assay ID
1	TIP	NM_122367.3	AT5G24590	At02185798_s1
2	SEN1	NM_119743.3	AT4G35770	At02255940_g1
3	WRKY6	NM_104910.2	AT1G62300	At02216109_gH
4	ACT2	NM_112764	AT3G18780	At02335270_gH

CHAPTER 3

Turnip crinkle virus coat protein mutants that fail to bind the NAC transcription factor TIP display altered hypersensitive response induction and systemic infection.

Abstract

The coat protein (CP) of turnip crinkle virus (TCV) has previously been shown to interact with a member of the NAC family of transcription factors called TCV-interacting protein (TIP). We have shown that this interaction is important in modulating innate immune defense (PTI) in susceptible *Arabidopsis thaliana* ecotype *Columbia-0*. Several TCV CP mutant viruses were made with a single amino acid replacement across the N-terminal region shown previously to interact with TIP to evaluate the importance of this interaction. All mutants that failed to bind TIP also broke resistance in the resistant *A. thaliana* ecotype *Dijon-17* and invaded systemically. We hypothesized that a positive interaction of TCV CP with TIP was likely required for induction of the resistance response that prevented systemic invasion by wild type TCV in Di-17. To assess this we monitored viral RNA accumulation by northern blot analysis and expression levels of select host defense-related genes were analyzed using semi-quantitative and real-time PCR in wild type TCV and CP mutant infections. Expression of defense genes varied greatly in wild type and mutant virus infections. This was particularly apparent for one of the mutant viruses (R8A) which broke resistance and induced a systemic hypersensitive-like response in Di-17. Another mutant (G14A) which restored the ability for CP to bind TIP induced a micro-hypersensitive response in inoculated leaves and caused complete resistance to systemic invasion. These data support the conclusion that the R-domain region of the CP and its ability to bind TIP is important in modulating the effector triggered immunity responses to virus infection.

Introduction

Plants have evolved elaborate regulatory networks of genes to defend themselves against potential pathogens which are involved in complex signaling based on the type of infection. The defense mechanisms directed against most microbial pathogens involve two branches of innate immunity which constitute the plant immune system (Chisholm et al., 2006; Jones and Dangl, 2006; Liu and Coaker, 2008). The first branch involves the use of pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs) and initiate a signaling cascade leading to PAMP-triggered immunity (PTI). This typically results in a basal defense response that may or may not effectively attenuate pathogen invasion. PTI is triggered by virulent pathogens on susceptible hosts. Successful pathogens have evolved virulence factors (or effectors) that can overcome basal resistance and in turn, plants have evolved a second branch, referred to as effector triggered immunity (ETI; (Jones and Dangl, 2006). ETI involves race specific resistance mediated by distinct nucleotide binding-leucine rich repeat (NB-LRR) proteins encoded by resistance genes (R genes). This involves recognition of a pathogen effector, or its activity, and typically results in a hypersensitive response (HR) at the site of infection, followed by the activation of systemic acquired resistance (SAR).

Turnip crinkle virus (TCV) is a single stranded positive sense RNA virus that induces an HR in *Arabidopsis thaliana* ecotype *Dijon-17* (Di-17). The resistance (R) gene HRT is responsible for HR induction in Di-17 after TCV infection due to the indirect recognition of TCV coat protein (Dempsey et al., 1997). Previous work (Ren, Qu, and Morris, 2000) identified a NAC transcription factor (TIP; TCV Interacting Protein) that was believed to play a role in defense against TCV infection. NAC genes

are a plant specific group of transcription factors that play important roles in growth, development, and defense against both abiotic and biotic stresses (Olsen et al., 2005; Ooka et al., 2003). TIP was identified using a yeast-two hybrid screen where TCV CP was used as bait. It was further discovered that disruption of the N-terminal region of the R-domain of the CP and consequently TIPôs ability to bind TCV CP resulted in altered phenotypes and resistance to TCV in Di-17. More recently it was shown that TIP is not required for HR induction but was important in modulating the basal defense (Jeong et al., 2008). My results reported in Chapter 2 confirmed the role of TIP in PTI defense.

To investigate this phenomenon further, we monitored defense gene expression in wt TCV infections compared to several additional TCV CP mutants with single amino acid substitutions in the TIP binding region of the CP (in addition to R6A and described previously in Figure 1-5). These results supported our hypothesis that the N-terminal region on the R-domain of the CP was playing an active role in HR induction that was somehow associated with its interaction with TIP.

Materials and Methods

Plant growth conditions

Plant lines of wt *A. thaliana* ecotypes *Columbia-0* (Col-0) and Di-17 were grown in growth chambers at 22°C with 12 hr day cycles in Metro Mix 360 (Sun Gro; British Colombia, Canada). Transgenic Col-0 lines of tip ko (tip) and HRT tip (HRT tip) were obtained from the Kachroo lab and described in Jeong *et al*., 2008. These transgenic lines were grown under the same conditions as the wild type lines.

Plant inoculations, tissue collection, and RNA isolation

Plants were consistently inoculated between the ages 22 to 24 days old. Three leaves were inoculated per plant as described in Chapter 2 (Figure 2-1). The virus inocula consisted of a buffer solution containing 50 mM Na_2HPO_4 [pH 7.0] + 1% Celite 545 and purified virus transcript at a concentration of 1 ng/µl with a total of 10 ng of virus transcript or 10µl of the virus transcript-buffer solution applied to each leaf. The virus inoculum was applied to each leaf by rub inoculation, allowed to stay on the leaf for five minutes, and then washed off with nanopure water. Five to six leaves (apx 0.3g) from different plants treated with the same inoculum buffer were collected at each time point and flash frozen in liquid nitrogen. RNA was extracted as previously described (Chomczynski and Sacchi, 1987) and RNA samples were subsequently purified using RNeasy columns (Qiagen; Valencia, CA, USA).

Virus detection and RT-PCR

Virus RNA transcript detection was conducted by analysis of 2 µg of total RNA isolated from infected plant tissue. The RNA was separated using electrophoresis in a 1.2% agarose/1.8% formaldehyde gel run at 100 mV/cm for 90 minutes at room temperature. Separated RNAs were then transferred to a nylon membrane (Zeta probe blotting membranes; Bio-Rad, Hercules, CA, USA) at 4°C at 37mV/cm. Hybridization was carried out at 40°C using ULTRAHyb-Oligo (Ambion; Foster City, CA, USA) solution according to manufacturerôs directions (Ambion). *CP* and *PR1* transcripts were detected with the addition of ^{32}P-ɔ-ATP end-labeled probes (Table 3-2) to the

hybridization buffer after one hour of pre-hybridization of the membranes with only the ULTRAHyb-Oligo solution. Probes were generated using T4 polynucleotide kinase according to manufacturerôs directions (New England Biolabs; Ipswich, MA, USA). After overnight hybridization (minimum of 12 hours), the membrane was washed three times, 20 min each, with 2xSSC, 0.5% SDS at 40°C.

Reverse transcription PCR (RT-PCR) was conducted to evaluate gene expression. DNase treated RNA samples were used to synthesize first strand cDNA by using SuperScript III reverse transcriptase (Invitrogen; Carlsbad, CA, USA) and random primers according to the manufacturerôs protocol. The cDNA was then subjected to PCR amplification for semi-quantitative analysis with EconoTaq Plus Green 2X Master Mix according to the manufacturerôs protocol (Lucigen; Middleton, WI, USA). The following thermal cycling conditions were used: initial denaturation 95°C for 2 minutes, then cycles of denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and followed by an extension phase at 72°C for 1 min. The numbers of cycles was adjusted based on the transcript abundance and expression at a given time point and/or sample. The procedure was terminated with a final extension phase of 72°C for 5 minutes and then being held at 4°C. The PCR product was then subjected to electrophoresis and gene expression was analyzed based on band intensity of the transcripts relative to the control gene, Actin2 (ACT2). The primers (Invitrogen) of the genes used for analysis for semi-quantitative PCR are listed in Table 3-1.

DAB staining

HR was visualized by monitoring H_2O_2 *in situ* by staining Arabidopsis leaves with 3,3-diaminobenzidine (DAB) that had been inoculated with TCV and the mutant viruses. Leaves were collected 48 hr after rub inoculation with 10 µl of virus at a concentration of 1ng/µl. Three to four leaves per virus treatment were vacuum-infiltrated with the DAB solution for 16 minutes. Leaves then were placed in a plastic box under high humidity until brown precipitate was observed. The DAB staining was then fixed with 70% ethanol (warmed). HR lesions were visualized by light microscopy.

Real time PCR

Real-time PCR was also used to evaluate gene expression levels. The qRT-PCR experiments were performed by using the ABI StepOne™ Plus real time PCR machine (Applied Biosystems; Foster City, CA), TaqMan® One-Step RT-PCR master mix, and the appropriate predesigned assay. A PCR master mix was prepared according to the manufacture's instructions along with 10µl TaqMan® RT-PCR Mix (2X; Applied Biosystems), 0.5µl TaqMan® RT enzyme mix (40X:Applied Biosystems), and enough RNase free water for a final volume of 50µl per well. The reaction plate was prepared by adding the PCR master mix, 1µl TaqMan® labeled probe (Applied Biosystems), 8.5µl total RNA at the concentration of 4.71ng/ µl to each well (total RNA per well was approximately 40ng). The final volume of each well was 20µl. Three wells were also assembled for each probe with 8.5µl water instead of RNA to rule out possible contamination. Thermal cycling conditions were 48°C for 30 min and 95°C for 10 min, followed by 95°C for 15 sec and 60°C for 1 min for 40 cycles. Relative expression was quantified by using the comparative Ct method with *ACT2* as the endogenous controls.

The qRT-PCR procedure was performed in triplicate. See table 3-3 for a list of evaluated genes.

Results

Single amino acid substitution mutations in the TIP binding region of the TCV CP R-domain caused major differences in symptom severity and virus accumulation in Di-17. To further assess the effect of mutations in the R-domain of the TCV CP on the resistance response in Di-17, we selected a set of mutant viruses that showed significant differences in symptom severity and systemic invasiveness for a more in depth comparative analysis of gene expression differences. More specifically, we compared gene expression differences between wtTCV and the R8A CP mutant virus that failed to bind TIP and which induced a systemic HR in Di-17. We also analyzed R6A in addition to another non-TIP binding mutant (D13A) that was similar to R6A in that it systemically invaded Di-17 without inducing systemic HR. The fifth mutant analyzed,G14A, was selected because the mutation did not affect TIP binding ability (similar to wt TCV) and G14A infections in Di-17 elicited a micro HR on inoculated leaves and displayed 100% resistance to systematic spread of the virus.

The replication competency of each of the mutants was initially assessed by inoculation of virus transcripts onto the susceptible host, Col-0. Each of the mutants caused robust infection in the Col-0 as expected (Fig 3-1a). As described in detail in Chapter 2 for R6A, the other non-TIP binding mutants (R8A and D13A) also displayed more severe symptoms after prolonged infections. This is not as evident in the 10 dpi plants shown in Fig. 3-1a. We also examined accumulation levels of virus transcript over

a time course by northern blot analysis and each of the mutants accumulated to equivalent levels by 8 dpi (Fig. 3-2a) as expected. We did not, however, assess each of the non-TIP mutants in sufficient detail to confirm if their effect on the PTI response was similar to that described for R6A.

The primary purpose of this study was to explore the basis for the very marked symptom differences of the mutants in the resistant line Di-17. We therefore also infected Di-17 plants with virus transcripts and monitored disease progression (Fig. 3-1b and c) and virus accumulation (Fig. 3-2b) over a time course. Wild type TCV infection developed visible HR lesions on inoculated leaves at 4-6dpi and approximately 70% of plants showed resistance to systemic spread of the virus, a result consistent with previous literature (Dempsey, Wobbe, and Klessig, 1993). The detectable TCV gRNA in Figure 3-2b at 8 dpi reflects the fact that we sampled six leaves randomly from six different plants for RNA extractions prior to symptom development. Thus it is likely that some of the sampled leaves would include tissue from some of the 30% systemically infected escapes. Importantly, the virus accumulation data, shown in Fig. 3-2b, shows very clearly that each of the non-TIP binding mutants (R6A, R8A and D13A) accumulated in the systemic leaves of Di-17 beginning at 4 dpi and achieved maximal levels of virus replication by 8 dpi. These results provide conclusive evidence that all 3 mutants broke the normally resistant phenotype displayed by Di-17 when inoculated with wt TCV. Interestingly, the one mutant, in which TIP binding was not affected, failed to accumulate at detectable levels in systemic leaves. Indeed, the G14A inoculated plants showed no evidence for systemic invasion in 100% of the inoculated plants at 24 dpi (Fig. 3-1c) and resembled the TCV resistant plants at that time point. The one significant difference

between the TCV inoculated Di-17 plants and those inoculated with G14A was the absence of a strong PR1 signal in the inoculated leaves that was most evident in the TCV inoculated leaves (Figure 3-2b). Careful inspection of these inoculated leaves revealed pinpoint local lesions that we interpreted as a microHR response (Figure 3-3). We also observed a detectable PR1 signal in the inoculated leaves of both R8A and R13A that was not evident in the R6A systemic leaves. We interpret this result as evidence for induction of an HR-like response in the systemic leaves which was most visibly evident in the R8A infections (Fig. 3-1b).

Further evaluation of R8A and G14A in the presence and absence of and

We next sought to assess if the two extreme disease phenotypes displayed by R8A and G14A, systemic HR and localized micro HR, might be correlated with the presence of *TIP* and/or *HRT* alone or together in similar genetic backgrounds. To address this question, we again used the transgenic seeds of the TIP ko plants with and without an introgressed HRT gene from the Kachroo lab and conducted a comparative infection time course with R8A and G14A similar to what was described for TCV and R6A infections in Chapter 2. In this experiment, we assessed virus accumulation and PR1 expression in wt Di-17 (*TIP HRT*), wt Col-0 (*TIP hrt*), transgenic Col-0 *tip* ko (*tip hrt*) and transgenic Col-0 crossed with Di-17 (*HRT tip*). In R8A infections, we observed that the presence or absence of *TIP* and/or *HRT* singly or together did not appreciably affect R8A virus accumulation (Figure 3-4a). However the severity of symptoms was drastically increased when *HRT* and *TIP* were both expressed in Di-17 as evidenced by the development of systemic HR (Fig. 3-4a, panel 2) and enhanced accumulation of PR1. Unexpectedly,

systemic HR failed to develop in both of the *tip* ko lines whether or not *HRT* was present or absent (Figure 3-4a, panels 3 & 4). These results clearly suggest that presence of the TIP gene along with the HRT gene was necessary for the systemic HR to develop. The presence of both HRT and TIP genes in each of these plant lines was confirmed by PCR.

Analyses of G14A infections in the four previously described plant types also revealed some interesting insights. First, infections in Col-0 (*hrt TIP*) showed maximum accumulation with almost a complete absence of symptoms (Fig. 3-4b, panel 1). This is consistent with infection by a TIP binding mutant that has the capacity to reduce the PTI response as demonstrated for the wt TCV in Chapter 2. This conclusion is corroborated by the observed slower accumulation and increased symptom severity in the absence of the TIP gene whether or not HRT is absent (Col-0, *hrt tip*) or present (Col-0, *HRT tip*; Fig. 3-4b, panels 3 and 4). Importantly, G14A infections induced rapid containment of the virus when both HRT and TIP were present but systemic infection escapes when HRT was present and TIP was absent.

To summarize, these experiments demonstrate that the presence of TIP appears to be necessary in plants for the induction of systemic HR in infections by the non-TIP binding mutant R8A. Moreover, in infections with the TIP binding mutant G14A, the presence of the TIP gene also appears to be necessary for increased anti-virus resistance because in its absence, G14A invaded systemically. Taken together these results provide evidence that TIP is playing a role in both the PTI and the ETI defense responses against TCV infections.

Comparison of NHL10 gene expression in Di-17 during TCV and CP mutant virus infections

In order to better understand the causes of the markedly different symptom and resistance phenotypes displayed by the CP mutants in Di-17, I sought to determine which defense genes might be being differentially induced during these infections. To approach this, I began by analyzing a select set of defense genes using semi-quantitative RT-PCR (sqRT-PCR) prior to initiating more in depth analysis by real-time PCR (qRTPCR). I selected a time course to collect samples that coincided with the earliest visible HR formation on inoculated tissue (3dpi) and the earliest appearance of systemic symptoms (6 dpi). To capture expression of potentially induced genes in systemic leaves, I sampled systemic tissue at 3 dpi prior to the time we would expect to be able to detect virus in systemic tissue and again at 10dpi, which coincided with the appearance of the severe systemic HR associated with R8A infections.

NHL10 was selected for analysis because it is the Arabidopsis ortholog to *HIN1* from *Nicotiana benthamiana* that has been shown to be induced by bacteria that elicit an HR response (Gopalan, Wei, and He, 1996). It has been shown that *NHL10* is upregulated in Arabidopsis infected with avirulent strains of Cucumber mosaic virus (Zheng et al., 2004). It has also previously been recognized as one of the yellow-leaf-specific clones (*YLS9*) (Yoshida et al., 2001). Hence we chose *NHL10* as a primary monitor of HR induction in the TCV and mutant infections. *NHL10* showed the most variable pattern of gene expression in the TCV and mutant CP infections over the time course (Figure 3-5 and 3-6). It was most highly induced in inoculated leaves of TCV and R8A at 3 dpi in inoculated tissue, an observation consistent with the appearance of HR

like lesions on the inoculated leaves of these infections (Figure 3-5a). Although we did not observe a substantial increase in NHL levels on the inoculated leaves of G14A, we did see slight induction in systemic leaves of plants infected with TCV at 3 dpi (Figure 3-5b). The highest level of NHL10 induction appeared to occur in systemic leaves of R8A infections at 6dpi and 10 dpi, a result consistent with the observation of the development of systemic HR. We also observed significant induction of this gene in the 10dpi infections of TCV and less but still detectable induction in D13A infections. (Figure 3-6a and b). We also saw some increase in *NHL10* expression in R6A infections at the late time point. We think that this was possibly associated with the onset of more severe symptom development and induction of senescence associated cell death rather then the strict HR-like response observed in R8A infections (Figure 3-6b). It is interesting to note that the extreme resistance associated with the G14A mutant didnôt cause a significant increase in *NHL10* transcript at any time point in Di-17 other than in the 3dpi systemic leaves (Figure 3-5 and 3-6). We attribute this to the fact that this mutant failed to substantially invade either the inoculated or systemic leaves.

We also monitored several other orthologs of *HIN1* expressed in Arabidopsis (*NHL1*, *NHL2*, *NHL3* and *NDR1*) to assess if the *NHL10* induction was specifically correlated with HR induction in Arabidopsis, and that there was not a general trend of induction of the NHL gene family. The other NHL genes did not show any consistent differences in the gene expression patterns among the mock and virus infected plants at any time point (Figure 3-5 and 3-6). This supports the conclusion that TCV induces the HR associated gene *NHL10* and that this was correlated in R8A infections with the induction of an HR-like response in systemic tissue.

We confirmed the pattern of differential *NHL10* expression in the mutant infections using the more sensitive qRTPCR method. We hypothesized that this method might detect more sensitive changes in *NHL10* expression in G14A infections, but we were unsuccessful in demonstrating this. However, the qRTPCR results did confirm that there was a 60 fold induction over mock of this gene in inoculated leaves of TCV at 3dpi and 10 dpi systemic leaves (Figure 3-7a). Interestingly, in R8A infections, inoculated leaves showed about a 20 fold induction at 3 dpi and a remarkable 500 ï 1000 fold induction in the 6 and 10 dpi infections, respectively. Taken together, these data suggest that there was a strong correlation between the level of expression of *NHL10* and the amount of tissue displaying visible HR. This conclusion is consistent with our inability to detect elevated expression of this gene in G14A infections which appeared to be limited to very small micro lesions on the inoculated leaves.

Comparison of PR1 gene expression in Di-17 during TCV and CP mutant virus infections

PR1 is a key indicator of activation of the SA pathway which is associated with the development of resistance to TCV in Di-17 and many other host pathogen interactions. We consistently found that it was also differentially regulated among the resistant ecotypes infected with TCV and the CP mutants by northern blots (Figure 3-2). In the resistant Di-17, *PR1* was strongly induced by TCV in inoculated leaves in contrast to a complete lack of *PR1* induction in the inoculated tissue by R6A, R8A, D13A and G14A. This is consistent with previous studies for TCV and reflects the induction of HR and SAR in resistant Di-17. We also noted some induction in systemic leaves in R8A and

D13A infections in these initial studies that appeared to coincide with the onset of the severe symptoms (Figure 3-1a, 3-1b, and 3-2). Hence we felt more careful analysis of this gene was also warranted.

We further evaluated expression of *PR1* using the more sensitive sqRT-PCR (Figure 3-5 and 3-6) and real-time PCR (Figure 3-7b). In Di-17, *PR1* expression followed a similar pattern of gene expression as NHL 10 (Fig. 3-5 and 3-6). TCV induction of PR1 was most evident in inoculated leaves of TCV and R8A and subsequently induced in TCV, R8A and R13A infections at 6dpi and 10 dpi in systemic tissue. However, the qRT-PCR analysis revealed some interesting quantitative differences in the pattern of PR-1 expression in the mutant virus infections. Induction of PR1 over the mock control in the inoculated leaves of TCV and R8A approached 50 and 20 fold respectively. Interestingly, PR1 was also induced in the systemically infected leaves of TCV and all of the mutant infections by several hundred to several thousand fold. The only exception was G14A infections in which it showed only a 10 fold increase at 3 dpi in systemic leaves. PR1 expression approached more than a 10,000 fold increase in systemic leaves of R8A infections at 10 dpi. The absence of detectable PR1 in G14A infections and the extreme level of accumulation in R8A infections prompt speculation that its induction may be less associated with the onset of resistance and more so with the onset of symptom severity and concomitant cell death accompanied by the onset of HR.

Differential effect of TCV and CP mutant infections on WRKY expression in Di-17

With the finding that *PR1* expression was drastically induced in the non-TIP binding mutants, we next examined other transcription factors associated with the SA

pathway and antiviral defense. *WRKY70* has been shown to be involved in cross talk between the SA and JA pathway and to upregulate the SA pathway while down regulating the JA pathway (Li, Brader, and Palva, 2004). It has also been associated with plant senescence (Ülker, Shahid Mukhtar, and Somssich, 2007). Our results showed that *WRKY70* was induced in TCV and R8A infections at 3dpi in inoculated tissue (Figure 3-5a) but no differences were seen between mock and the virus infections at 3dpi in systemic tissue. The *WRKY70* increased expression at 3dpi on inoculated tissue coincides with the increase in *PR1* gene expression in TCV and R8A infections, however there is no evident difference at 3dpi in systemic tissue (Figure 3-5a and b). There was a mild induction of *WRKY70* in R8A at 6dpi which is not apparent in the other virus infections (Figure 3-6a). We also saw a reduction in *WRKY70* expression in D13A infections at 10dpi that we can not currently explain (Figure 3-6b). These results taken together again confirm that TCV strongly induces the SA pathway defense response in inoculated leaves compared to R6A and D13A. It also shows that R8A can activate the SA pathway without inducing a localized HR.

In addition to *WRKY70*, we also evaluated *WRKY6* gene expression in response the different virus infections. As previously described in Chapter 2, *WRKY6* encodes a transcription factor that belongs to a plant specific transcription factor family that is induced during biotrophic infections (Robatzek and Somssich, 2002). We observed a slight induction of *WRKY6* in inoculated leaves in TCV and R8A infections at 3dpi (Figure 3-5a and 3-7d). At 10 dpi in systemic leaves, the levels of *WRKY6* were induced the most, approximately 30 fold higher during R8A infections (Figure 3-6a and b, Figure 3-7d). These results suggest that *WRKY6* activation correlates more with the induction of

systemic HR and less with the onset of the resistance response that leads to virus containment in the IL.

Differential effect of TCV and CP mutant infections on TIP expression in Di-17.

With the finding that the presence of TIP affected both symptom severity and systemic spread of the mutant viruses R8A and G14A in Di-17, we sought to assess the effect that virus infections might have on the induction of *TIP* during an ETI response. We used both sqRT-PCR and qRT-PCR assays to look at *TIP* expression over the time course previously described in Figure 3-5 and 3-6 in Di-17. The sqPCR did not reveal any significant difference in TIP expression at any time point (Figure 3-5 and 3-6). To confirm this, we also evaluated the expression using qRTP-CR. The results showed that TIP did not appear to be significantly induced over mock in any of the virus infections except for a modest 3 fold induction in the SL of the R8A infections (Figures 3-7c). These results differ from the results obtained in susceptible Col-0 infected with TCV or R6A where TIP was induced to several fold higher levels in systemic leaves of the R6A infections. These results suggest that TIP induction may be more important in the PTI response than in the ETI response.

Discussion

In recent studies, effectors elicited from a bacterial pathogen, have been shown to target many parts of the plant immune response network, and in return, plants refine and expand their immune system to defend against pathogens (Boller and He, 2009). In this study, we took a closer look at the ETI response against viral pathogens by investigating

TCV infections in resistant *A. thaliana* ecotype *Di-17* to determine if we could establish any links between the presence and absence of the NAC family member and PTI associated protein, TIP (Chapter 2), and the HR associated resistance responses linked to HRT expression. NAC genes have previously been shown to be important players in the defense against abiotic and biotic stresses (Jensen et al., 2010; Olsen et al., 2005; Wang, Goregaoker, and Culver, 2009) and previous work has shown that disrupting NAC genes associated with a virus can alter the resistance of the plant (Yoshii et al., 2009). The results obtained here confirmed those of Ren et al. (2000), by demonstrating that mutations in the TIP binding region of TCV CP that affected TIP binding markedly affected the disease response in Di-17.

We further demonstrated that by comparing wt TCV to four CP mutants with single aa substitutions, we were able to observe a variable induction of HR and systemic defense gene signaling responses. Previous work on other plant virus pathogens have demonstrated that by mutating a single nucleotide caused differences in symptoms and HR development when observed (Kim and Palukaitis, 1997). We were not only able to demonstrate the variable defense responses with the TCV CP mutants but we also revealed a possible role for TIP in modulating the ETI response to TCV infections. The strongest evidence for this conclusion was the marked differences in disease symptom development by the non-TIP binding mutant R8A and the TIP binding mutant G14A. When both *TIP* and *HRT* were expressed in the ecotype Di-17, we saw an unregulated defense response which leads to severe necrosis and accelerated death of the plant during R8A infections. However when HRT was present and TIP was absent, R8A still spread

systemically but was unable to induce systemic HR and hence caused a milder systemic infection.

The presence of the *TIP* gene also appeared to be necessary for the resistance to the TCV mutant G14A. We consistently saw an increase in virus resistance that restricted G14A to the site of inoculation in the *HRT TIP* (Di-17) plants and the development of a microHR. The lack of TIP permitted systemic invasion of G14A plants in either the presence or absence of *HRT*. These results taken together provide evidence that TIP is indeed playing a role in the ETI defense responses against TCV infections, in contrast to the demonstration by Jeong *et al.*, (2008) that TIP is not required for the induction of HR. Our results show that the extreme resistance elicited by G14A is lost when TIP is eliminated and argues in favor of an important role for TIP in the resistance to systemic invasiveness against the virus. This data allowed us to draw the conclusion that HRT alone is unable to prevent systemic invasion of TIP binding TCV mutants. However, it further confirms HRTôs role in the formation of the HR (Dempsey et al., 1997) since we were unable to detect HR necrotic lesions or elevated *NHL10* gene expression without *HRT* expression.

NHL10 is a protein associated with both HR and late senescence (Zheng et al., 2005). In this study, it was used to monitor the HR progression between wt TCV and the CP mutants. The comparison of the effects of different mutant virus infections on expression of *NHL10* failed to reveal a clear understanding of signal transduction pathway leading to the resistance response or the altered symptom phenotypes induced by the mutants. We were, however, able to verify the TCV mutants that produced the most extensive HR, such as the systemic HR associated with R8A, showed the highest levels

of *NHL10* expression in systemic leaves which confirmed a link between induction of this gene and the HR response in Di-17. Taken together, these data suggest that there was a strong correlation between the level of expression of *NHL10* and the amount of tissue displaying visible HR. This conclusion is consistent with our inability to detect elevated expression of this gene in G14A infections which appeared to be limited to very small microHR lesions on the inoculated leaves.

The WRKY gene family consists of 74 members in *A. thaliana* (Eulgem et al., 2000), many of which are associated with defense gene responses. We examined *WRKY6* and *WRKY70* in some detail for their possible role in the defense against TCV infection since both have been linked to pathogen defense signaling (Robatzek and Somssich, 2001; Ülker, Shahid Mukhtar, and Somssich, 2007) and both were elevated in susceptible Col-0 in the non-TIP mutant infection by R6A (Chapter 2). We observed a similar result in Di-17 infections with both *WRKY6 and WRKY70* showing slightly higher induction over background levels early in infections that induced HR as opposed to those that did not. However, the most substantial difference was the elevated induction (30 fold) of *WRKY6* by the mutant R8A later in infections at the onset of severe systemic necrosis. Currently, we do not know how much of a role the elevated *WRKY6* expression is playing in the more severe disease symptoms observed in R8A infections. This would be an interesting candidate gene to monitor disease symptoms and HR induction in its absence during viral infections.

We were not able to reliably demonstrate a significant induction of TIP over mock inoculated leaves in any of the virus infections over the time course in Di-17, except for a modest 3 fold induction in the SL of R8A infections at 10dpi. We concluded

that TIP is not induced during an ETI response or HR induction. This differs from what was observed in the susceptible Col-0 line (Chapter 2, Figure 2-15) to R6A infections, where we saw a six fold induction of TIP at 8dpi in R6A infections. This supports our conclusion that the primary role of TIP is in the regulation of PTI and SAR but not HR.

In conclusion, we have shown that TIP does indeed have a role in the ETI response. Its presence is also necessary for controlling spread of the TCV mutant G14A and regulating defense in the presence of HRT during R8A infections. However, although we have solid evidence that CP-TIP binding can lead to a reduced basal defense response in Col-0, our data does not permit a clear interpretation of the role of TIP in modulating the defense response activated in the presence of the HRT gene. Moreover, we could not clearly identify an interpretable pattern of defense gene responses that explains all of our results.

To summarize briefly, we know TCV CP binds TIP and that this leads to a lowered basal defense in Col-0 and likely in Di-17 early in infection. TCV CP also promotes HR in concert with HRT and this leads to systemic resistance, albeit somewhat leaky. The TIP binding mutant G14A induces a rapid HR and complete resistance to systemic spread. In contrast, the three mutants that were unable to bind TIP (R6A, R8A and D13A) all broke resistance and moved systemically. Interestingly, R6A was unable to induce HR while R8A induced extreme HR in systemic leaves. Recent results from our lab by Sung-Hwan Kang (PhD student) provides a partial explanation for this conundrum. He has shown definitively that TIP is not required for HR induction in assays where both *TCV CP* and *HRT* are transiently expressed in *N. benthamiana* leaves. More importantly, he has identified a nuclear localization signal that overlaps the TIP

binding region in which we made the mutations. His results have revealed that those CPs that induce HR (TCV, R8A, and G14A) localize to the nucleus in the presence of HRT where they form cajal-like bodies, while the R6A mutant that fails to induce HR does not. Taken together with our results, it appears that the TIP binding region has two functional activities: 1) TIP binding activity that leads to the regulation of basal defense responses, and 2) Nuclear localization activity that is necessary for elicitation of HR in the presence of the HRT.

These two sets of results prompt us to formulate the following model for how TCV CP might be regulating both the PTI and ETI defense responses (Figure 3-8). In this model, we propose that TCV CP binding to TIP would decrease defense signaling in the cell after infection by promoting release of TIP from the endosomal membrane so it can traffic to the nucleus where it functions as a negative regulator of the innate defense response. This would promote more rapid invasion in both Col-0 and Di-17 by TCV and the TIP binding mutant G14A. We also now know that TCV CP will cause HR in the presence of HRT and, by some as yet unknown mechanism, cause nuclear localization of CP into cajal-like bodies. This event triggers HR and in some cases, resistance to systemic spread. Importantly, the presence of TIP was required for elicitation of systemic HR by R8A and for the containment of G14A (ie. onset of systemic resistance) in Di-17. This observation supports the conclusion TIP is also modulating the enhanced ETI defense response associated with HRT. At this stage, we do not have a clear understanding of the defense gene responses that promote this effect.

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Figure 3-1. Variable symptoms and HR development to TCV infections. *Arabidopsis thaliana* ecotypes Col-0 (susceptible) at 10dpi(a) and Di-17 (resistant) at 10dpi (b) and 24dpi (c) infected with TCV and mutants R6A, R8A, D13A, and G14A.

Figure 3-2. Comparison of temporal accumulation of TCV and CP mutant transcripts. Northern analysis of CP transcripts accumulation of TCV and CP mutants in *A. thaliana* ecotype Col-0 (a) and Di-17 (b) in both inoculated and systemic tissue. A total of 10ng of virus transcript of wt TCV or one of the CP mutants R6A , R8A, D13A, and G14A were inoculated onto designated leaves. Inoculated leaves were collected at 48 hr post inoculation (hrpi) and systemic leaves were collected at 2, 4, 6, and 8 day post inoculation (dpi) as indicated. Viral RNA levels were monitored by northern blot using three probes for CP sequence (described in Materials and Methods section) and one probe for PR1. Panel below the blot shows ethidium bromide (EtBr) stained gel showing rRNA bands used as a loading control. This experiment was repeated three times with similar results.

Figure 3-3. Variable HR induction associated with TCV infections and CP mutants. *In situ* detection of peroxides using DAB staining on wt Di-17 to determine the extent of HR induction upon infection of TCV and its mutants. Brown pigment is an indication of HR induction.

Figure 3-4. TCV mutants R8A and G14A virus accumulation and PR1 expression in HRT and TIP ko lines. Col-0 (hrt TIP) and Di-17 (HRT TIP), transgenic Col-0 (tip ko)

with a tDNA insertion in the TIP promoter and a hybrid (HRT tip) which was a cross between the tip ko and Di-17 (as described in Jeong et al., 2008) were each inoculated with R8A (a) and G14A (b). Samples were collected and analyzed as described in Figure 3-2. PR1 transcripts were evaluated using northern blots as described in Materials and methods. The lower panel shows plants at 8dpi. Results from similar experiments done with wt TCV and R6A can be seen in Figure 2-19.

Figure 3-5. Expression levels of defense related genes comparing TCV and CP mutant infections in Di-17 at 3dpi in inoculated and systemic tissue. Multiple genes were selected and evaluated using semi-quantitative RT-PCR from samples taken at 3dpi from (a) inoculated tissue and (b) systemic tissue. The genes chosen were previously shown to be linked to defense related pathways in *A. thaliana*: *PR1* is an SA pathway gene. WRKY6 is a transcription factor associated with viral defense. WKRY70 is another transcription factor that acts as a regulator between the JA and SA pathway during infection. *NHL10* is an ortholog to HIN1 in *Nicotiana benthamiana* and is an indicator of HR. *NHL1*, *NHL2*, *NHL3* and *NDR1* are homologs of *NHL10* but are not induced during HR or senescence. PCR cycle numbers are shown to the right of each panel. *ACT2* expression was used as an endogenous control. This experiment was repeated twice with similar results.

Figure 3-6. Expression levels of defense related genes comparing TCV and CP mutant infections in Di-17 at 6dpi and 10dpi in systemic tissue. Multiple genes were selected and evaluated using semi-quantitative RT-PCR from systemic leaves taken at (a)

6dpi and (b) 10dpi. See fig. 3-5 for details. This experiment was repeated twice with similar results.

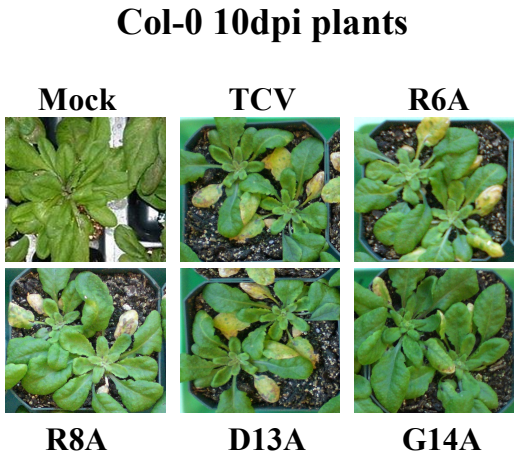
Figure 3-7. Evaluation of relative gene expression levels using Real-time PCR. *A. thaliana* ecotype Di-17 was inoculated with TCV, R6A, R8A, D13A or G14A transcripts as described in Figure 3-2. Tissue samples were collected and RNA extracted for each time point. a) Expression levels genes were analyzed using One-step Real-time PCR as described in Materials and Methods. The relative fold change of four genes, (a) *NHL10*, (b) *PR1*, (c) *TIP* and (d) *WRKY6* are plotted here. Fold change is calculated relative to the mock infection of each gene at 48hrpi. *ACT2* was used as the endogenous control. This graph is the average of results obtained for two independent experiments.

Figure 3-8. Proposed model of the role of TIP-TCV CP interaction during TCV infection in the resistant host Di-17 during a ETI event. We propose that TCV CP (or some other as yet undefined PAMP) is recognized by an as yet uncharacterized toll-like receptor (an RLK that could be in the plasma membrane or an internal endosomal membrane) that provokes SA defense signaling. This would then lead to a MAPK cascade and enhanced basal defense. The left side of the diagram depicts the proposed PTI response: Infection by virus with wild type CP results in an interaction with the negative regulator TIP that is tethered in an endosomal membrane in the cytoplasm, enhancing the rate of its release and increasing suppression of basal defense. The right side of the diagram depicts the proposed ETI response: the R protein HRT is able to indirectly recognize TCV CP and subsequently induce an HR and SAR response through

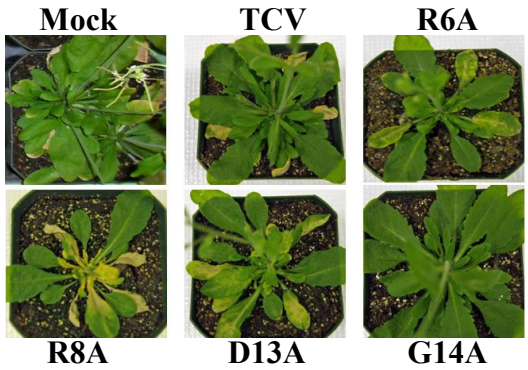
a nuclear localization event. The proposed involvement of TIP in the ETI is shown by the yellow arrow which depicts modulation of the SAR defense response induced by HR. This interaction would provide a rational for why infections by the TCV non-TIP binding mutants are able to evade HRT recognition and therefore are able to spread systemically with no localized HR. This difference in ETI defense regulation in the resistant host gives a selective advantage to TCV non-TIP binding mutants (R6A, R8A, and G14A) during infection.

Figure 3-1.

a)



b)



c)

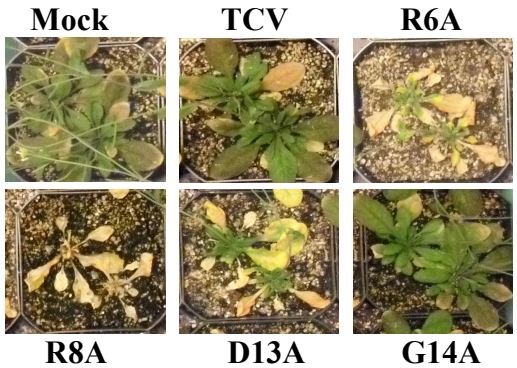


Figure 3-2.

1. 48 hrpi inoculated leaves
2. 2 dpi systemic leaves
3. 4 dpi systemic leaves
4. 6 dpi systemic leaves
5. 8 dpi systemic leaves

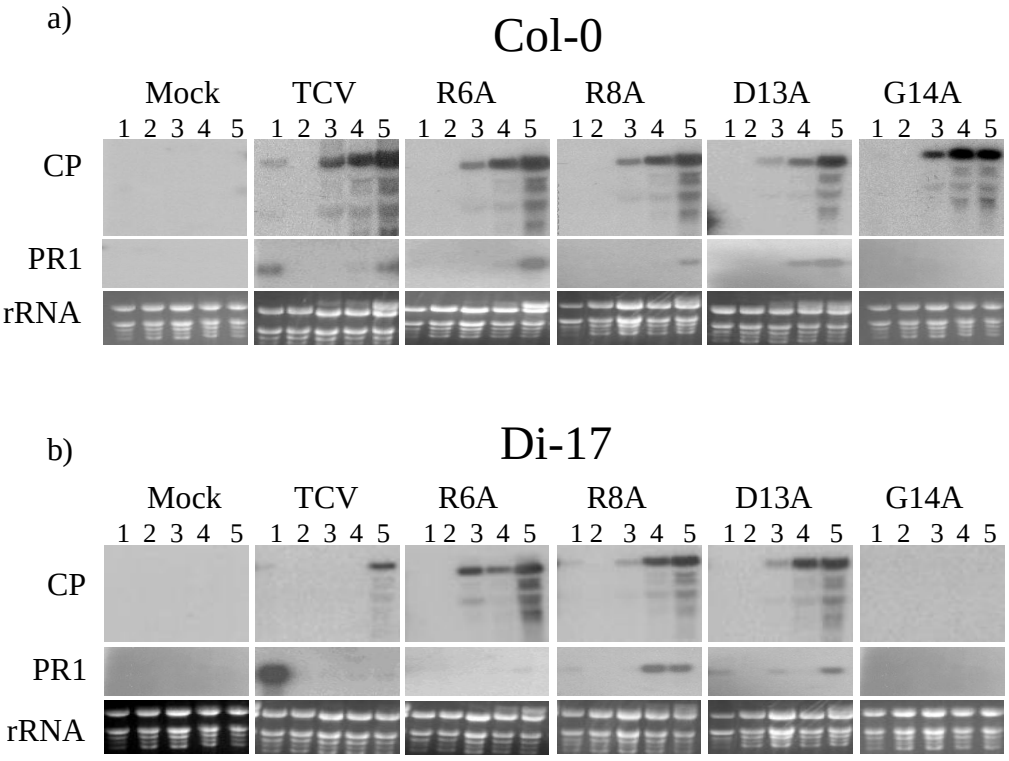
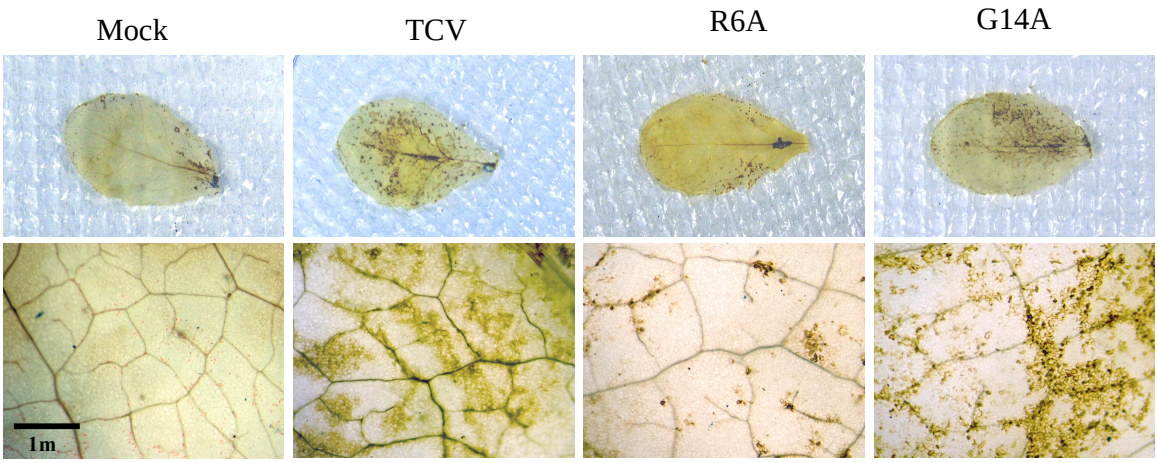


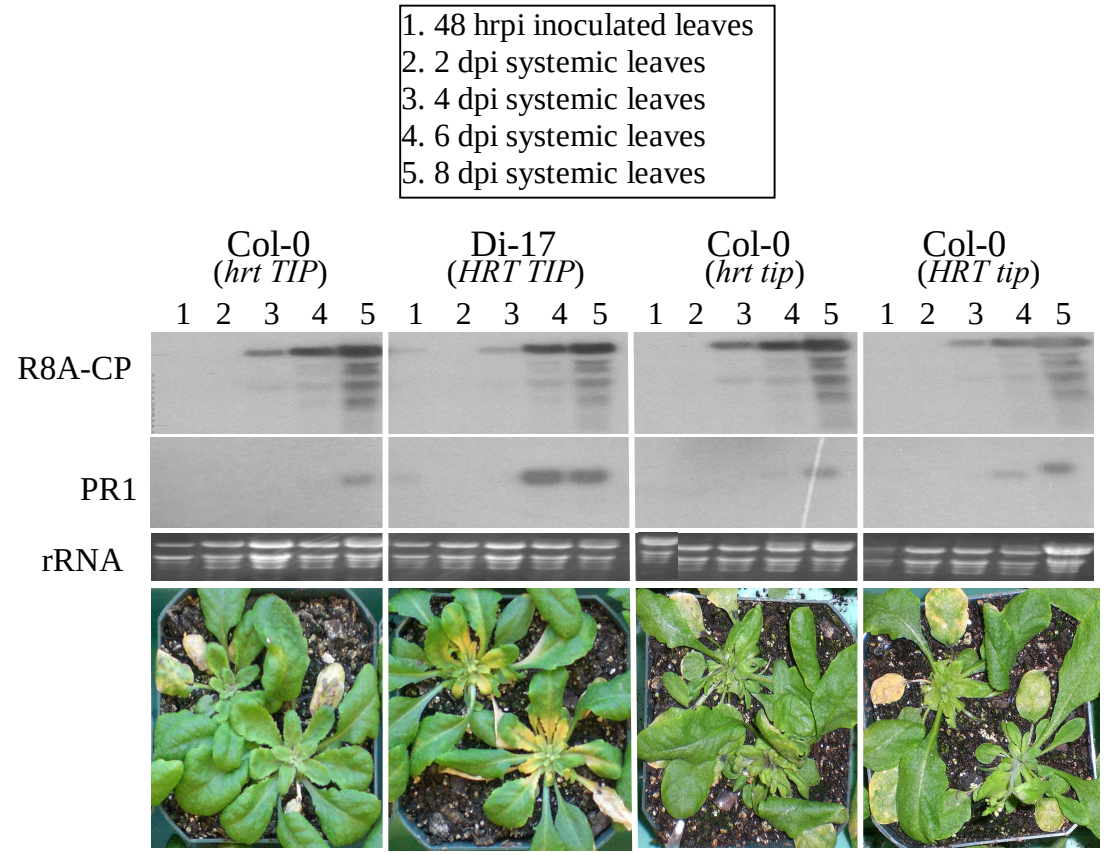
Figure 3-3.



(Unpublished data from Basnayake,V)

Figure 3-4.

(a)


1. 48 hrpi inoculated leaves
2. 2 dpi systemic leaves
3. 4 dpi systemic leaves
4. 6 dpi systemic leaves
5. 8 dpi systemic leaves
Col-0 (hrt TIP)
Di-17 (HRT TIP)
Col-0 (hrt tip)
Col-0 (HRT tip)
1 2 3 4 5
R8A-CP
PR1
rRNA

(b)

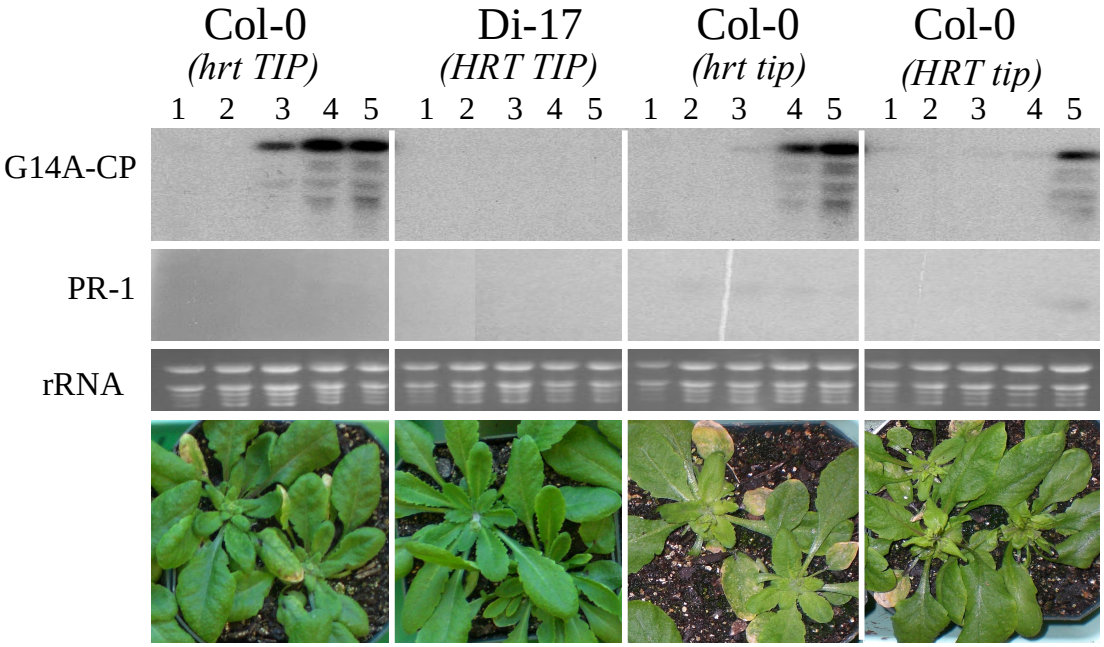

Col-0 (hrt TIP)
Di-17 (HRT TIP)
Col-0 (hrt tip)
Col-0 (HRT tip)
1 2 3 4 5
G14A-CP
PR-1
rRNA

Figure 3-5.

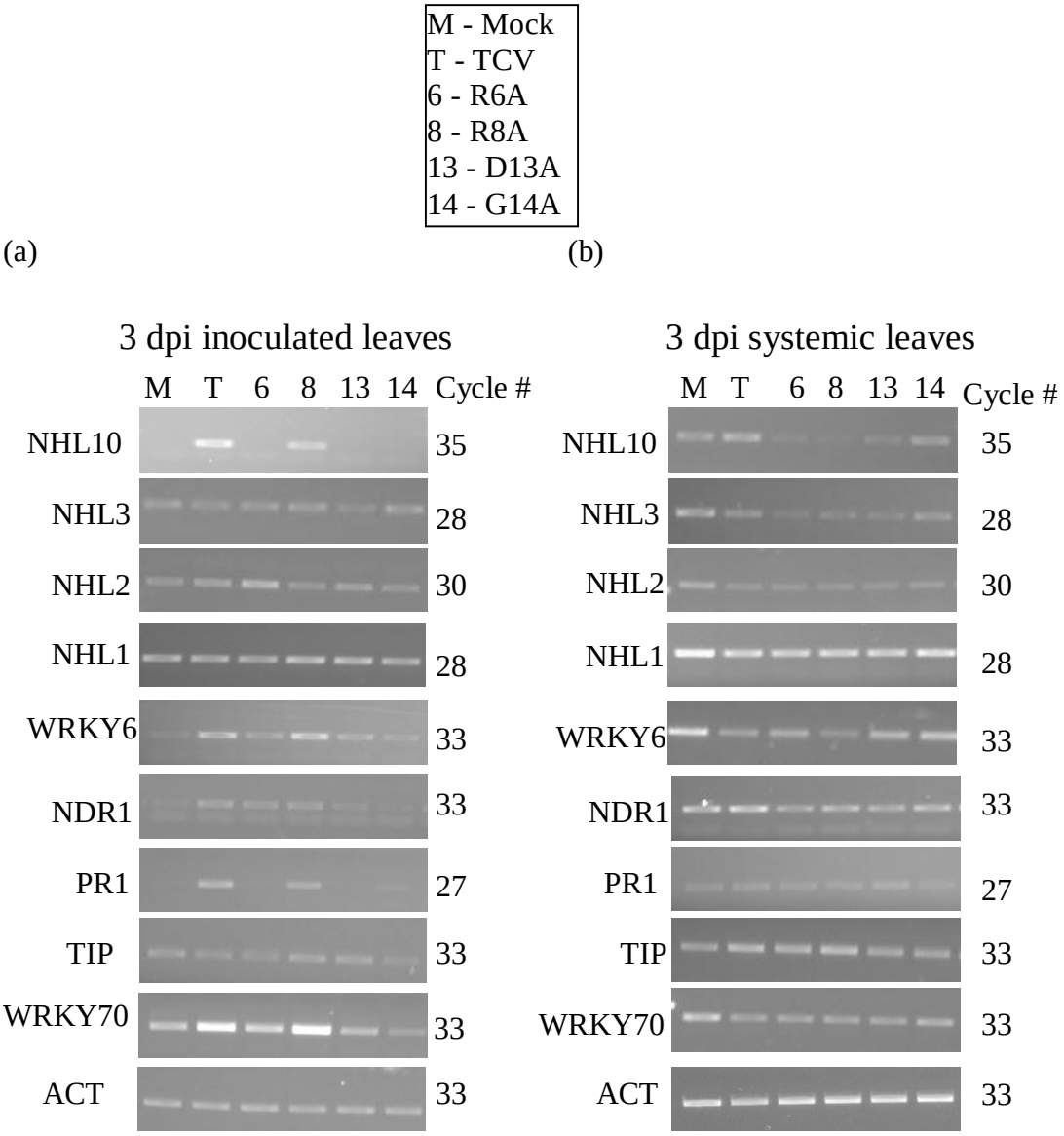

M - Mock
T - TCV
6 - R6A
8 - R8A
13 - D13A
14 - G14A
(a)
(b)
3 dpi inoculated leaves
3 dpi systemic leaves
M T 6 8 13 14 Cycle #
NHL10 35
NHL3 28
NHL2 30
NHL1 28
WRKY6 33
NDR1 33
PR1 27
TIP 33
WRKY70 33
ACT 33

Figure 3-6.

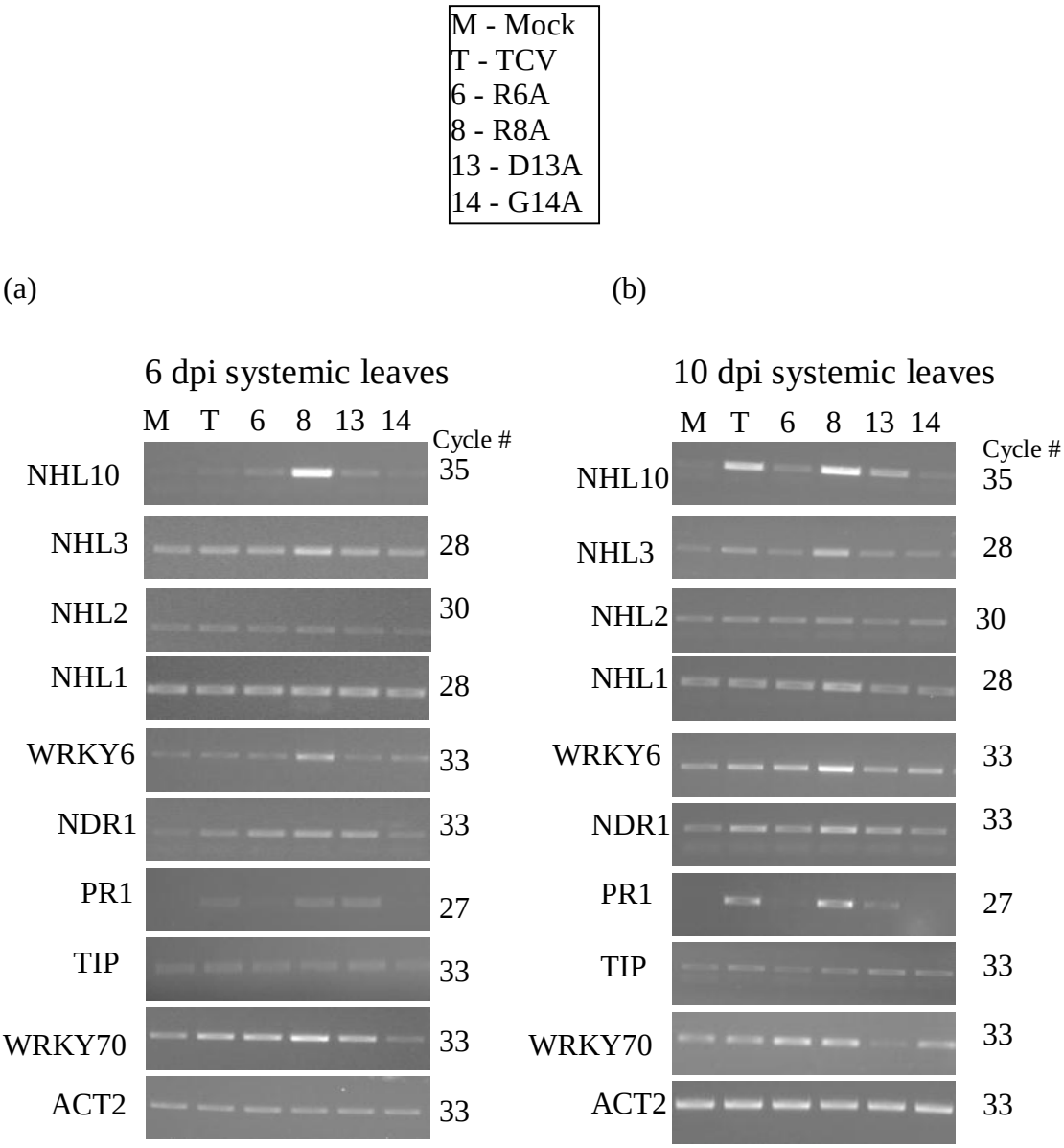

M - Mock
T - TCV
6 - R6A
8 - R8A
13 - D13A
14 - G14A
(a)
(b)
6 dpi systemic leaves
10 dpi systemic leaves
M T 6 8 13 14
Cycle #
NHL10 35
NHL3 28
NHL2 30
NHL1 28
WRKY6 33
NDR1 33
PR1 27
TIP 33
WRKY70 33
ACT2 33

Figure 3-7.

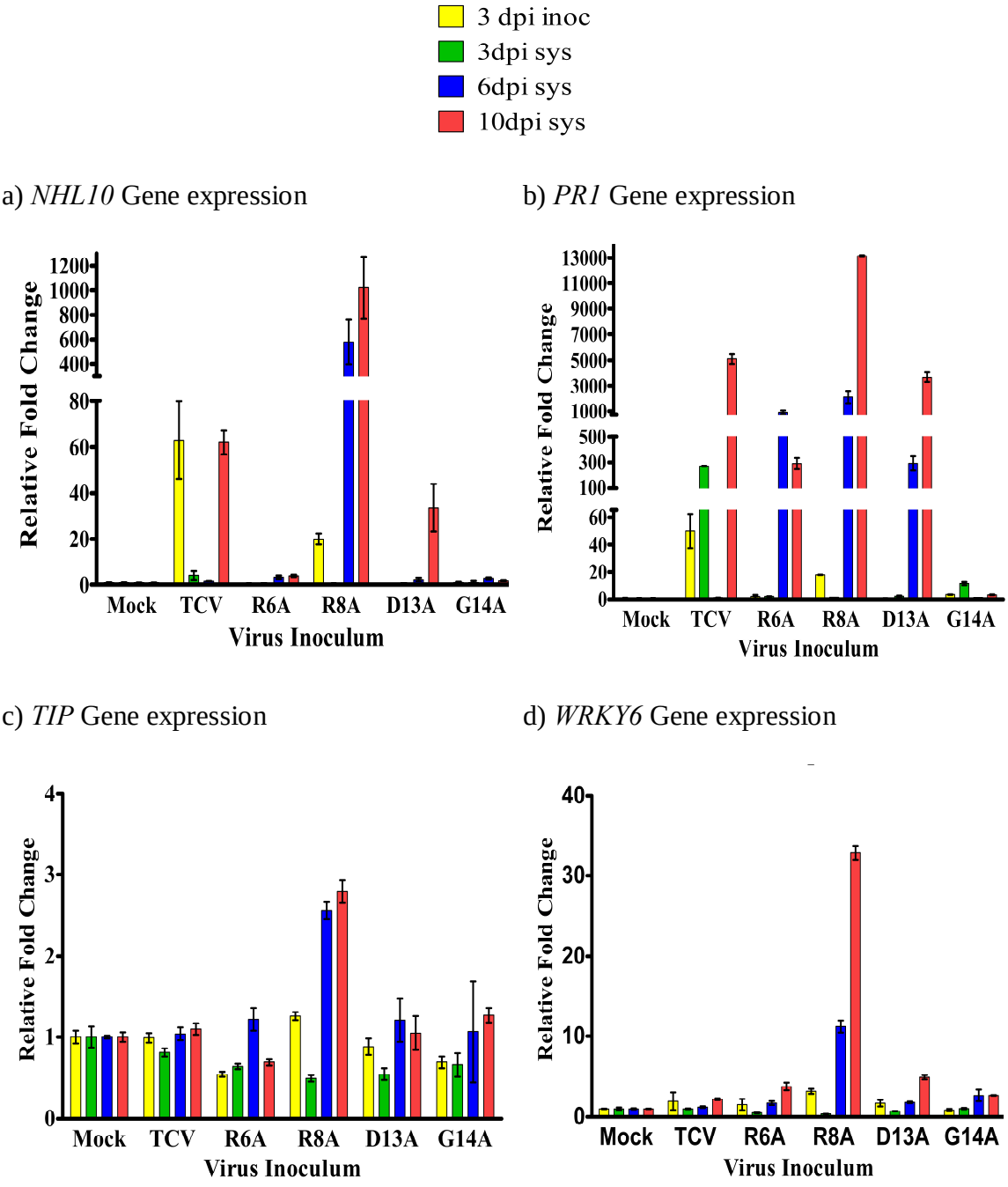

3 dpi inoc
3dpi sys
6dpi sys
10dpi sys
a) *NHL10* Gene expression
b) *PR1* Gene expression
Relative Fold Change
Mock
TCV
R6A
R8A
D13A
G14A
Virus Inoculum
c) *TIP* Gene expression
d) *WRKY6* Gene expression

Figure 3-8.

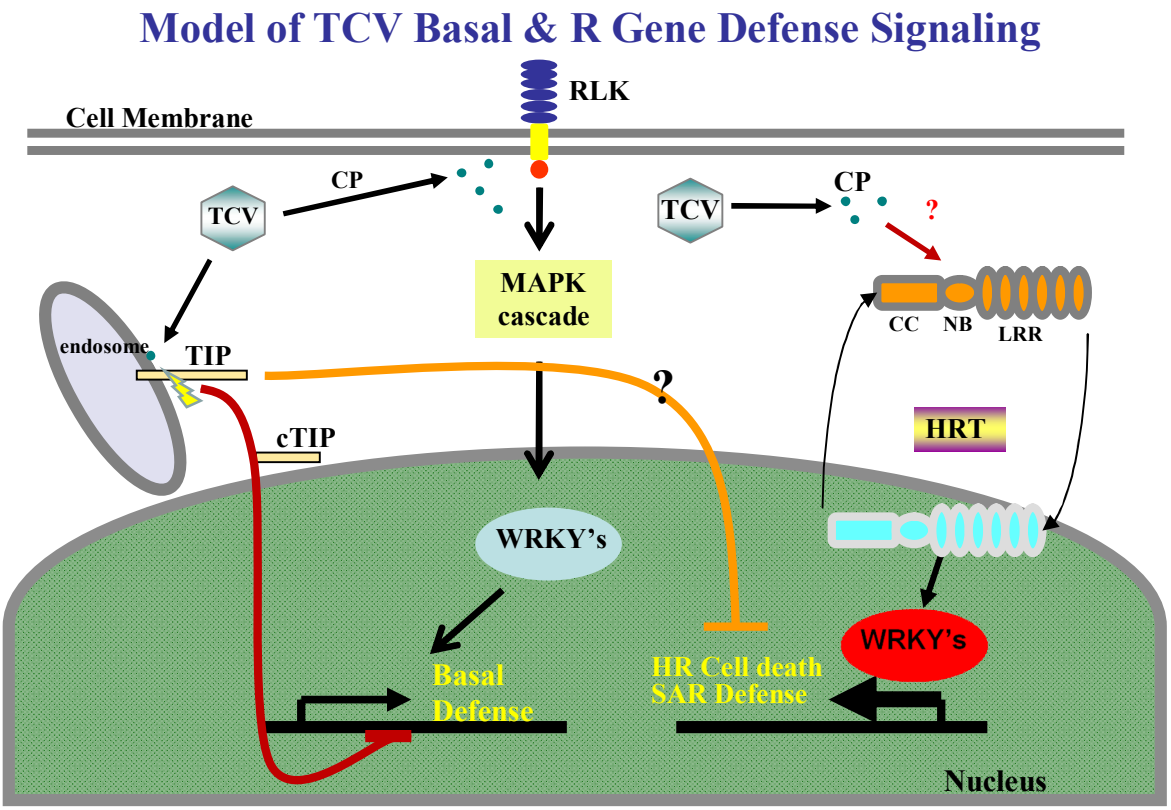

Model of TCV Basal & R Gene Defense Signaling
RLK
Cell Membrane
CP
TCV
MAPK cascade
TCV
CP
?
CC
NB
LRR
endosome
TIP
cTIP
?
HRT
WRKY's
WRKY's
Basal Defense
HR Cell death SAR Defense
Nucleus

Table 3-1. List of semi-quantitative PCR primers

Gene	Sequence
ACT2	Fwd: 5'-GTCTGAGATTTCTCCTGCCG-3'
	Rev: 5'-CACGGTTAGCCTTTGGGTTA-3'
NHL3	Fwd: 5ô-CGGCGGTGGATGCGGTTGTT-3ô
	Rev: 5ô-TCTCCGCCGTCAAGCAGCAC-3ô
NHL10	Fwd: 5ô-AGTCCCACCACCAGCTCCCA-3ô
	Rev: 5ô-GCGGGTAAGGGACGCATCGG-3ô
NHL2	Fwd: 5ô-TCCTCGGAGTCGCCGCTCTT
	Rev: 5ô-GTCGCCGAGCACCACCAGAT
NHL1	Fwd: 5ô-TCCCCACCACCACCACTCACC
	Rev: 5ô-TGCCGGAGACGTTGAAGGCG
WRKY6	Fwd: 5ô-CCGTGTCTCCGTTCGTGCCC-3ô
	Rev: 5ô-TTCGCCGTCGTGGTGGTTCG-3ô
NDR1	Fwd: 5ô-GTCTCCGTGCGGACAAACCCA
	Rev: 5ô-ACCGTCTGGTTGTTTAGCGGCTT
PR1	Fwd: 5ô- AACCAGGCACGAGGAGCGGT
	Rev: 5ô-GTTCACGGCGGAGACGCCAG
WRKY70	Fwd: 5ô-TGAACCAACTCGTTGAAGGCCATGA
	Rev: 5ô-CAACGGCGGCGAGGGATGAG
TIP	Fwd: 5'-CCGGCTCAAGATCAACGGTCACG-3'
	Rev: 5'- CTGCTCAGCACAACCCGGGG -3'

Table 3-2. Probes for Northern Analysis

TCV CP	Rev: 5'-CAGGACCGAGAAGTCAGAGG-3
	Rev: 5'-GGCCCACCCGACACCACTGG-3'
	Rev: 5'-CTTGTCTTGACCGAGTTGGT-3'
PR1	Rev: 5ô-GTTCACGGCGGAGACGCCAG

Table 3-3. Genes used for Time Course qRT-PCR

	Gene Name/Description	Reference Sequence	Transcript ID	Assay ID
1	TIP	NM_122367.3	AT5G24590	At02185798_s1
2	NHL10	NM_129157.2	AT2G35980	At02322550_s1
3	WRKY6	NM_104910.2	AT1G62300	At02216109_gH
4	PR1	NM_127025.2	AT2G14610	At02170748_s1
5	ACT2	NM_112764.3	AT3G18780	At02335270_gH

CHAPTER 4

Evaluation of silencing pathway gene expression and miRNA accumulation in Turnip crinkle virus infections of *Arabidopsis thaliana*.

Abstract

RNA silencing pathways in Arabidopsis is involved in sequence specific inhibition of gene expression. Arabidopsis encodes four Dicer-like proteins (DCL) that are responsible for cleaving specific miRNAs, mRNAs or aberrant RNAs for incorporation into host RISC complexes and the subsequent slicing of its target. The coat protein (CP) of Turnip crinkle virus (TCV) has previously been shown to be a strong suppressor of RNA slicing. In this chapter, we sought to establish a connection between TCVs ability to suppress the endogenous RNA silencing pathways and its ability to bind the NAC transcription factor, TIP. We evaluated the gene expression of key players in the RNA silencing pathway between TCV and the fully replication competent mutant viruses that don't interact with TIP. We hypothesized, based on these observations that the interaction of TCV CP with TIP evolved to reduce the host innate immune response and favoring a more rapid systemic invasion. To test whether the mutation and associated inability to bind TIP was a factor in effecting the RNA silencing pathway, we compared the expression level of select host silencing-related genes by semi-quantitative PCR in TCV and mutant R6A infections. We also compared the rate of virus accumulation of wt TCV and mutant R6A as well as other non-TIP binding mutants in both inoculated and systemic leaves of an infection up to 8 dpi. Though we were unable to establish a connection between the RNA silencing pathway and TIP biding with these experiments, we did obtain valuable information on gene expression of DCLs and other silencing pathway genes during TCV infection

Introduction

RNA silencing, including post-transcriptional gene silencing in plants, RNA interference in animals, and gene quelling in fungi, represents a sequence-specific RNA degradation mechanism directed against down regulate gene expression and as a defense against invasive nucleic acid molecules (Baulcombe, 2004; Dunoyer and Voinnet, 2005; Ghildiyal and Zamore, 2009; Voinnet, 2005). Analyzing the anti-defense strategies employed by viruses to overcome this defense system has recently been the focus of much research. Silencing suppressors encoded by viral pathogens have been linked to developmental abnormalities when expressed in plants, suggesting their direct involvement in symptom development (Voinnet, 2005). Viral silencing suppressors are able to abolish RNA silencing mediated through small interfering RNAs (siRNA), however disruption of plant development has been correlated with the ability of silencing suppressors to prevent the cleavage and degradation of endogenous siRNAs and microRNAs (Baulcombe, 2004; Chapman et al., 2004; Dunoyer et al., 2004). The silencing strategies that are employed by different viral proteins can interfere with steps that are common to plant silencing and endogenous silencing pathways which can lead to variations in developmental defects that contribute to symptoms and eventually yield losses. A good example of this was shown for the tobamoviruses, Tobacco mosaic virus and Tomato mosaic virus, and the potyviruses, Tobacco etch virus and Potato virus Y, where it was demonstrated that silencing suppressors were able to alter miRNA accumulation and cause abnormalities in plant growth and development (Bazzini et al., 2007). Although much work has been done to provide evidence that shows viruses and their specific proteins or RNA components can alter the expression of host genes, the

extent to which host genome expression is altered and the mechanisms responsible for these alterations during viral infections remain largely unknown.

Turnip crinkle virus (TCV) is a small positive sense RNA virus with a genome of 4054nt (Carrington et al., 1989). TCV coat protein (CP) is a multifunctional protein that is needed for structure and movement but is also a strong silencing suppressor (Qu, Ren, and Morris, 2003; Thomas et al., 2003). It also contributes significantly to the severity of TCV symptoms upon infection in the compatible plant-pathogen interactions and is essential for resistance in the incompatible interactions (Ren, Qu, and Morris, 2000). It was proposed by Ren et al. (2000), that the variation in resistance and symptoms was the result of TCV CP ability to bind to a NAC transcription factor named TIP (TCV interacting protein). This was further confirmed by demonstrating a direct correlation between the inability of the R domain of TCV CP mutant viruses to bind TIP that cause a break in the resistance in the resistant line Di-17 and cause an increase in symptom severity in the susceptible line, Col-0 (Ren, Qu, and Morris, 2000). More recently, work published Jeong et al., 2008, described a tip knockout line of Col-0 into which the resistance gene HRT was introgressed, was also resistant to TCV infection. They concluded in this study that the mutant TCV viruses that showed a break in resistance in the Ren et al., study was likely due to another as yet uncharacterized defense system component and not a direct result of the loss of ability to bind TIP (Jeong et al., 2008).

As presented in Chapters 2 and 3, our work has shown that the presence of TIP does indeed having an effect on TCV infection at both the PTI and ETI levels of the plant immune system. Other major factors affecting the ability for virulent viruses to successfully infect their host are suppression of the RNA silencing pathway and

manipulating the ability to control the expression of endogenous genes which regulate pathogen invasion. Recently, it was discovered that the TCV CP mimics host-encoded glycine/tryptophan (GW)-containing proteins normally required for RNA induced silencing complex (RISC) assembly and function (Azevedo et al., 2010). TCV coat protein GW residues bind directly and specifically to Arabidopsis AGO1, which has been identified as a major effector of TCV-derived siRNAs (Azevedo et al., 2010). The observation that one of the two functional GW motifs in the CP is in close physical proximity (N terminal aa 25-26) to the TIP binding domain (N-terminal aa 1-25) raised the possibility of a connection between TIP-CP interaction and silencing suppressor function.

The objective of the studies described in this chapter was to assess any possible role of the TCV CP binding TIP in the defense response in Arabidopsis and possible association with the RNA silencing defense pathway. We have shown in the previous chapters that TIP is a negative regulator of defense and that the ability of the TCV CP to bind TIP enhances this negative regulation which leads to a more successful defense by altering basal viral defense components of the PTI response and modulates the ETI response in the presence of the resistance gene HRT. The nature of the resistance mechanism that is induced as a consequence of the ability of TCV CP to bind TIP is not known. It has, however, been demonstrated that the silencing suppressor activity of TCV CP is not altered in the non-TIP binding mutants R6A and R8A (Choi et al., 2004). However, it remains an open question if the basal defense response modulated by TIP-CP binding might not be a consequence of altering the expression of one to several components of the RNA silencing pathway. To address this possibility, I examined for

any differential effects that TCV and the TIP binding mutants might have on the expression of silencing pathway associated genes, endogenous silencing suppressors, and two miRNAs during infection.

Materials and Methods

Plant growth conditions

Plant lines of wt *A. thaliana* ecotypes *Columbia-0* (Col-0) and Di-17 were grown in growth chambers at 22°C with 12hr day cycles in Metro Mix 360 (Sun Gro; British Colombia, Canada). Mutant plants dcl2ï 1, dcl3ï 1, dcl4ï 2 have been described previously (Hammond, 2005; Qu, Ye, and Morris, 2008) and were provided to the Morris lab by Dr. James C. Carrington. The mutant rdr6ï 11 was ordered from the Arabidopsis Biological Resource Center. All mutants were verified through genotyping by Dr. Feng Qu and grown under the same conditions as wt Col-0. The transgenic lines of Col-0 asTIP and UpTIP were previously described in the material and methods section of Chapter 2. The transgenic GFP line was constructed similar to the asTIP line with a GFP insert instead of the antisense TIP sequence under the control of the inducible estradiol promoter.

Plant inoculations, tissue collection, and RNA isolation

Plants were consistently inoculated between the ages 22 to 24 days old. Three leaves were inoculated per plant as described in Chapter 2 (Figure 2-1). The virus inocula consisted of a buffer solution containing 50 mM Na_2HPO_4 [pH 7.0] + 1% Celite

545 and purified virus transcript at a concentration of 1 ng/µl with a total of 10ng of virus transcript or 10µl of the virus transcript-buffer solution applied to each leaf. The virus inoculum was applied to each leaf by rub inoculation, allowed to stay on the leaf for five minutes, and then washed off with nanopure water. Five to six leaves (apx 0.3g) from different plants treated with the same inoculum buffer were collected at each time point and flash frozen in liquid nitrogen. RNA was extracted as previously described (Chomczynski and Sacchi, 1987) and RNA samples were subsequently purified using RNeasy columns (Qiagen; Valencia, CA, USA).

Virus detection and RT-PCR

Virus RNA transcript detection was conducted by analysis of 2µg of total RNA isolated from infected plant tissue. The RNA was separated using electrophoresis in a 1.2% agarose/1.8% formaldehyde gel run at 100 mV/cm for 90 minutes at room temperature. Separated RNAs were then transferred to a nylon membrane (Zeta probe blotting membranes; Bio-Rad, Hercules, CA, USA) at 4°C at 37mV or 200mA. Hybridization was carried out at 40°C using ULTRAHyb-Oligo (Ambion; Foster City, CA, USA) solution according to manufacturerôs directions (Ambion). *CP* and *PR1* were detected with the addition of ^{32}P-ɔ-ATP end-labeled probes (Table 3-2) to the hybridization buffer after one hour of pre-hybridization of the membranes with only the ULTRAHyb-Oligo solution. Probes were generated using T4 polynucleotide kinase according to manufacturerôs directions (New England Biolabs; Ipswich, MA, USA). After overnight hybridization (minimum of 12 hours), the membrane was washed three times, 20 min each, with 2xSSC, 0.5% SDS at 40°C.

Reverse transcription PCR (RT-PCR) was conducted to evaluate gene expression. DNase treated RNA samples were used to synthesize first strand cDNA by using SuperScript III reverse transcriptase (Invitrogen; Carlsbad, CA, USA) and random primers according to the manufacturerôs protocol. The cDNA was then subjected to PCR amplification for semi-quantitative analysis with EconoTaq Plus Green 2X Master Mix according to the manufacturerôs protocol (Lucigen; Middleton, WI, USA). The following thermal cycling conditions were used: initial denaturation 95°C for 2 minutes, then cycles of denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and followed by an extension phase at 72°C for 1 min. The numbers of cycles was adjusted based on the transcript abundance and expression at a given time point and/or sample. The procedure was terminated with a final extension phase of 72°C for 5 minutes and a hold at 4°C. The PCR product was then subjected to electrophoresis and gene expression was analyzed based on band intensity of the transcripts relative to the control gene, *Actin2* (*ACT2*). The primers (Invitrogen) for the genes used for analysis for semi-quantitative PCR are listed in Table 4-1.

siRNA detection

For siRNA analysis, 5ï 15 µg total RNA was loaded onto a 0.1x TBE, 8 M urea, 16% polyacrylamide gel and run until the bromophenol blue dye migrated out. The separated RNAs were then transferred to a nylon membrane and hybridized with ^{32}P-labeled oligonucleotides obtained from Dr. Bin Yu. The hybridization buffer was UltraHyb Oligo from Ambion, and the hybridization temperature was 40°C. After

overnight hybridization, the membrane was washed three times, 20 min each, with 2x SSC, 0.5% SDS; at 50°C.

Results and Discussion

TCV CP TIP non-binding mutant R6A altered silencing associated gene expression in susceptible *A. thaliana* ecotype Col-0

The Dicer gene family in *A. thaliana* has four members with varying functions. Dicer-like(DCL)1 is required for miRNA biogenesis (Finnegan, Margis, and Waterhouse, 2003; Papp et al., 2003) and was shown to repress antiviral RNA silencing through negative regulation of the expression of DCL4 and DCL3 in TCV infections (Qu, Ye, and Morris, 2008). The role of DCL2 is not as well understood but analysis of dcl1, dcl2, and dcl3 single mutants suggested that DCL2 was involved in TCV siRNA production and was the first DCL shown to play a role in defense against TCV (Xie et al., 2004). It was also shown that dcl2 mutant are more susceptible to TCV infection (Xie et al., 2004) and the 22nt TCV siRNAs are reduced in dcl2 (Bouche et al., 2006). DCL3 produces retroelement and transposon siRNAs and is required for chromatin silencing (Xie et al., 2004). These siRNAs are longer (24nt) compared to the DCL1 (21nt) products (Hamilton et al., 2002; Tang et al., 2003). Neither DCL1 or DCL3 are involved with making TCV siRNAs (Bouche et al., 2006). DCL4 has been associated with antiviral defense and it generates 21nt siRNA (Gasciolli et al., 2005; Xie et al., 2005). Previous work has shown that DCL4-dependent siRNA generation from a transgenic inverted-repeat (IR) locus is suppressed in TCV-infected plants or in transgenic plants expressing

p38 (Deleris et al., 2006). DCL2 and DCL4 have been found to have hierarchal and partially redundant roles and were found to be the major and minor contributors of TCV siRNAs respectively (Bouche et al., 2006).

To assess if the TIP-non binding mutants had any effect on *DCL* expression, we initially analyzed an infection time course in the susceptible (Col-0) *A. thaliana* lines infected with either wt TCV or non-TIP binding mutant R6A. R6A differs from TCV in one aa in the TIP binding region of the CP and it invades Col-0 more slowly but causes more severe disease symptoms and alterations in defense gene expression (Chapter 2). Initial results using semi-quantitative PCR showed some differential expression of the *DCL* family of genes in TCV and R6A infections over the time course. No evident differences were observed in IL at 2 dpi. *DCL1*, which regulates the miRNA pathway, appeared to be more elevated in the mock SL at 4 and 6 dpi. At 8 dpi, both TCV and R6A infections had higher levels of *DCL1* and *DCL2* than mock. Interestingly, R6A infections had higher levels of expression of *DCL2, DCL3*, and *DCL4* than did TCV infections. This could be indicative of an elevated silencing response in R6A infections which could account for the decreased accumulation associated with this mutant as previously described in Chapter 2.

This initial experiment prompted us to next examine temporal expression of the *DCL* genes in response to infections in the transgenic lines in which the levels of TIP expression were altered. We infected the transgenic anti-sense TIP Col-0 line (asTIP) under the control of the inducible estradiol promoter and the constitutively up-regulated TIP (UpTIP) line (Detailed in Chapter 2). We used a transgenic line of Col-0 that had a *GFP* transgene under the control of the inducible estradiol promoter as a control for the

effect of spraying plants with estradiol on gene expression. As shown in Figure 4-2, *DCL1* appeared to be induced later in TCV infections than in R6A infections achieving its highest levels at 8 dpi. This same pattern was most evident for *DCL2*, and *DCL4* in the UpTIP infected plants, suggesting a possible role of TIP in modulating *DCL* expression during virus infections. This was supported by the lack of expression differences of any the *DCL*s in the asTIP infections.

We also examined *DRB4* (Double-Stranded-RNA-Binding Protein 4) expression. This protein has been shown to assist DCL4 in the biogenesis of at least one transacting siRNA (Adenot et al., 2006), and more recently studies were done that suggested DRB4 could stabilize the 21-nt viral siRNAs and deliver them to the RISC complex rather than being directly involved in siRNA production (Qu, Ye, and Morris, 2008). With this information in hand, we sought to find if any difference in *DRB4* gene expression could be observed that were related to TCV or its mutant infections. Interestingly, *DRB4* appeared to be induced throughout the time course in both TCV and R6A infections in the asTIP plants. However, induction of *DRB4* was not sustained at the same level in the R6A infections compared to TCV infections at 6 and 8 dpi in the UpTIP plants. These results indicate that TIP levels did have some effect on *DRB4* induction during the course of infection, possibly implicating induction of the silencing pathway genes in the differential accumulation of the two viruses. However, the finding that virus infections do indeed cause induction of this gene is supported by recent studies that showed *DRB4* was induced in systemic infections of Turnip yellow mosaic virus (TYMV) in Arabidopsis (Jakubiec, Yang, and Chua, 2011).

Differential expression of DCL genes in *A. thaliana* resistant ecotype Di-17

We extended our study of *DCL* expression by assessing induction of the *DCL* genes in TCV and R6A infections of Di-17 over a time course. We examined infections at earlier time points since TCV induces an HR on inoculated tissues that is visible three days after infection. All the *DCL's* appeared to be induced in systemic tissue at 2 dpi and this response decreased at 4 dpi (Figure 4-3). The expression levels were similar in the mock and virus infections suggesting that the induction may have been as a result of wounding caused by rub inoculation. However, the expression levels in R6A infections at these time points were reduced in comparison to mock and TCV infection samples (Figure 4-3). This trend was the opposite of *DCL4* expression, where we saw the highest induction of this gene in R6A infections rather than mock or wtTCV (Figure 4-3). Taken together, we were unable to interpret these data at this time since as previously reported, DCL1 and DCL3 are not associated with TCV antiviral defense (Bouche et al., 2006). However, the induction of *DCL4* in R6A infections reveals that the TIP non-binding mutant may be inducing the antiviral pathway while the wtTCV does not. However, since TCV also induces the HR in Di-17, this may be a moot point.

Comparison of DCL Gene induction by TCV and other CP mutants in susceptible (Col-0), resistant (Di-17) and tip ko plants.

We next performed a more comprehensive analysis of *DCL* family genes in response to infections by each of the CP mutants analyzed in the previous chapters (see chapter 1 Figure 1-5). Because these mutants caused a range of disease symptoms and resistance responses associated with both TIP binding and HR mediated resistance, we

chose to assess *DCL1* because of its association with miRNA processing and *DCL4* because of its direct involvement in TCV associated antiviral silencing. In all of the infections, the *DCLs* appear to be induced only in the systemic leaves. In the susceptible Col-0 line, we observed that TCV, R6A, and R8A all had a similar pattern of *DCL1* and *DCL4* expression (Figure 4-4a). *DCL1* was most evidently induced in systemic tissue at 2 dpi and 4 dpi for all virus infections. Curiously, the level of *DCL1* decreased at 6 dpi in some but not all infections and then were restored at 8 dpi (Figure 4-4a). A similar effect was seen in *DCL4* expression but the overall expression was again much lower. We also observed that *DCL4* expression in D13A and G14A infections mirrored the expression we saw in mock infections more closely than in the other viral infections (Figure 4-4a). We know from the appearance of disease symptoms (Figure 3-1a) and Northern blots (Figure 3-2a) that the plants analyzed in this experiment were all infected. However the pattern of *DCL* expression observed does not lend itself to an obvious interpretation.

In the resistant line of Di-17, the pattern of *DCL1* expression in all the infections was similar for the CP mutants being highest at 4 dpi. Curiously, the wtTCV infection did not show evident *DCL1* induction until 8 dpi in this experiment. This was different from what was observed previously in Figure 4-3a where *DCL1* expression was highest at 2 dpi and decreased at 8 dpi in TCV infections. We were only able to detect modest expression of *DCL4* in this experiment and there did not appear to be any notable differences between the infections.

We also examined induction of *DCL1* and *DCL4* expression in the Col-0 tip ko line previously described in Chapter 3. We saw some evident differences in DCL1 gene expression in comparison to infections in wt Col-0 in all the virus and mock infections,

suggesting that TIP may have had an effect on expression of silencing pathway genes. The only consistent data points between the two experiments of *DCL1* expression was in TCV infections at 4 dpi and R6A and R8A at 8 dpi (Figure 4-4c). *DCL4* expression was significantly reduced with no noticeable difference among the viruses (Figure 4-4c).

Taken together, we could not consistently identify a coherent and reproducible pattern that would connect any of the observed phenotypes of the mutants with DCL gene expression. The patterns of expression of the various *DCL* genes did seem to respond differently to virus infections and the presence or absence of the TIP gene. However, the results were too variable between experiments to draw any concrete conclusions about a connection between TIP expression and a silencing-based defense response.

Virus accumulation differences in silencing pathway ko lines

In addition to assessing the effect of virus infection on silencing pathway genes, we also compared the accumulation of the CP mutants relative to TCV accumulation over a time course in Arabidopsis lines deficient in the key antiviral silencing associated genes *DCL2*, *DCL3*, *DCL4* and *RDR6*. We reasoned that if the mutantsô symptoms reverted to wt in silencing defective plants then it would potentially expose which antiviral pathway was most affected by the single aa mutation in the CP. We grew three *dcl* ko lines, previously described by Qu et al. (2008) and monitored virus accumulation. We again saw the overall trend that TCV accumulated to higher levels earlier in infection than the mutant viruses. These results were consistent with our data on infection in Col-0 described in chapter 2 showing that TCV had a replicative advantage over the R6A. This

data also showed that removing just one component of the silencing pathway was not sufficient to restore the replicative advantage of wt TCV.

RNA-dependent RNA polymerase 6 (RDR6) is one of 6 putative RDR genes in the *A. thaliana* genome. RDR6 has previously been implicated in antiviral defense (Baulcombe, 2004) and since then it has been shown that RDR6 is one of the key components in plant post-transcriptional gene silencing (PTGS), and is likely to function with other silencing components in a genetic pathway regulating leaf patterning (Xu et al., 2006). In *N. benthamiana*, RDR6 plays a role in limiting virus particles from entering shoot apical meristems, likely through the promotion of viral siRNA production in systemic tissues (Qu and Morris, 2005; Schwach et al., 2005). With this in mind we compared the accumulation of TCV and the CP mutants in the *rdr6* ko line. We observed similar results to the *dcl* ko lines, with TCV maintaining a replicative advantage. Since these experiments were performed, it has become clear that there is significant genetic redundancy in antiviral silencing pathways (Deleris et al., 2006; Gasciolli et al., 2005). Hence our results cannot completely rule out the possibility of the involvement of the silencing pathway genes in the absence of testing multiple knock out lines as recently reported by Cao et al., (2010). This more extensive analysis was deemed outside the scope of this dissertation.

The Hunt for Endogenous Silencing Suppressor Genes

Previous work demonstrated that the two silencing suppressors of TCV CP (p38) and Turnip mosaic virus (TuMV) HC-Pro required RAV2 for the suppression of target degradation via the activity of primary siRNAs (Endres et al., 2010). *RAV2* is part of a

gene family that comprises six members and encodes an ethylene-inducible transcription factor that appears to be a control point for viral suppression of silencing (Endres et al., 2010). Therefore we wanted to determine if the non-TIP binding CP mutant, R6A, had any effect on *RAV2* expression. We hypothesized that if TCV CP does interact with RAV2, then an alteration in the TIP binding region might have an effect on the expression of *RAV2* which could account for the differences in virus accumulation and symptom expression. We did observe reduced expression of *RAV2* at 2 dpi compared to both mock and TCV infections but the differences were less evident at 4, 6 and 8 dpi between TCV and R6A infections, suggesting that this was not likely a promising explanation for the accumulation differences (Figure 4-6).

We also looked for possible differences in other endogenous silencing suppressors. FRY1 and CML38 (At1g76650) have both been classified as endogenous silencing suppressors (Anandalakshmi et al., 2000; Gy et al., 2007) and were shown to have induced expression by TuMV HC-Pro in a RAV2 dependent manner in *A. thaliana* (Endres et al., 2010). In the case of TCV infections, we did not observe a consistent pattern of induction of these two genes until 8 dpi in systemic tissue. Interestingly, at 2 dpi and 6 dpi we did see reduced levels of *FRY1* in TCV infections relative to R6A and mock. One additional note to add is that *CML38* was significantly upregulated in R6A infections versus TCV infections at 24 hrpi (data not shown). We had another calmodulin binding protein (At4g27280) in our microarray data that was consistently upregulated in R6A versus TCV infections. The time course analysis of this calcium-binding EF hand family protein was very similar to *CML38* with a reduction in expression relative to mock infections at 2 dpi and an increase in expression in R6A

infections at 8 dpi (Figure 4-6). These results suggest that it would be interesting to do a more thorough study on calcium binding proteins to see if other mutants cause altered expression of this gene family.

I expanded my search of endogenous silencing suppressors that might be affected by TCV infections to the 5ô to 3ô exoribonucleases XRN2, XRN3 and XRN4. Previous worked showed that XRN2 and XRN3, along with FRY1, can individually act as post transcriptional gene suppressors (Gy et al., 2007). Other work also demonstrated that XRN4 degrades microRNA (miRNA)-guided mRNA cleavage products and also likely acts as an endogenous silencing suppressor by degrading RdRp templates (Gazzani et al., 2004; Souret, Kastenmayer, and Green, 2004). We saw a similar effect among these three genes throughout the time course of the R6A and TCV infections (Figure 4-6). There was no altered expression at 2 and 4 dpi and only a minor decrease in expression levels at 6 dpi in R6A and TCV infections. At 8 dpi, we did see an induction of *XRN2*, *XRN3* and *XRN4* in R6A infections, but we cannot interpret what affect that may be having on the differential disease development at this time.

Differences in mi RNA accumulation during TCV infections

MicroRNAs (miRNAs) are small encoded RNAs that act as regulators of eukaryotic gene expression at the post transcriptional gene level. Many developmental processes are regulated by miRNAs including hormone signaling. Since we previously showed in Chapter 2 that the ability to alter SA pathway signaling through interaction with TIP was the main functional difference between R6A and TCV, and that *DCL1* induction was differentially affected when TIP levels were altered (Fig. 4-2), we reasoned

that R6A infection might be having an effect on global miRNA levels. To test this, we obtained miRNA probes from Dr. Bin Yu, and tested TCV and R6A infections for differential levels of several miRNAs. The first miRNA accumulation we evaluated was miRNA167, which has been shown to oscillate with the diurnal cycle (Sire et al., 2009). It also controls patterns of *ARF6* and *ARF8* expression and regulates both female and male reproduction (Wu, Tian, and Reed, 2006). We found that in the susceptible line (Col-0) and in the tip ko line, there was a steady decrease in miRNA167 over time but there was no significant difference between wild, mutant or mock infections. Interestingly, this effect was less marked in the Di-17 resistant line, but the pattern did not indicate any differential effects.

We also looked that miRNA 171 to determine if the trend we saw in miRNA 167 was consistent. The miRNA171 also oscillates diurnally like miRNA167 (Sire et al., 2009) and it was also shown to have increased accumulation in TMV infected plants (Bazzini et al., 2007). We observed no significant differences in virus infected plants compared to mock. These data taken together permit us to conclude that there was no obvious global differences in miRNA levels that would account for the susceptibility or symptom differences between TCV and R6A. It is possible that there might be some specific differences in miRNA accumulation which could only be revealed with a more extensive miRNA microarray study.

Conclusions

TCV CP is a multifunctional protein that is involved in both PTI and ETI layered defense, as well as being a strong silencing suppressor that prevents the endogenous RNA

silencing pathways from degrading TCVs genome. However, TCV CP mutants that are unable to bind to the NAC transcription factor TIP, were shown to have an altered range of disease symptoms in the susceptible host and were also able to break resistance in the resistant host. Recently, it was shown that TCV CP mimics host encoded GW containing proteins required for RISC assembly and function. More specifically, a set of two GW motifs in the TCV coat protein were shown to be necessary for binding of the CP to AGO1 and thus disrupting the antiviral silencing pathway (Azevedo et al., 2010). What makes this interesting in terms of the TCV CP mutants studied in this project, is that the GW sequence of TCV CP is located at aa 25-26, in close proximity to the TIP binding region of the CP which was mapped with aa #1-25. Therefore we wanted to see if the non-TIP binding mutants had any effect on the silencing pathway induction that could shed light on the variability of disease symptoms we observed during infection by the CP mutant virus.

In this chapter, we examined expression in both inoculated and systemic tissue in plants with altered levels of *TIP* gene expression and sought to make a connection between the silencing pathway and the role of TIP in the defense responses. Our results were not conclusive and we were therefore not able to draw any definitive conclusions as to whether silencing pathway associated genes were being differentially affected by wtTCV and the non-TIP binding mutants. We were however able to demonstrate that the differential viral accumulation observed in Col-0 was not due to the altering of the major and minor contributors of TCV siRNAs (DCL4 and DCL2, respectively).

Though much of this work was intended to connect TCV CP ability to bind TIP and alterations with the silencing pathway, unfortunately no definitive connections were

found at this time. We did observe some differential gene expression correlating to varying levels of TIP expression and altered gene expression in the presence of the different mutant viruses that could be a potential area of examination but are currently outside the scope of this project.

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Figure 4-1. Analysis of DCL gene expression differences during TCV and mutant R6A infections in the susceptible host Col-0. Semi-quantitative RT-PCR analysis of the expression of 4 DCL genes in the *Arabidopsis thaliana* ecotype *Col-0* infected with TCV (T), R6A (R), or Mock (M). Samples were collected from systemic tissue at 2 dpi, 4 dpi, 6 dpi, and 8 dpi. PCR cycle numbers are shown to the right of each panel. ACT2 expression was used as an endogenous control.

Figure 4-2. Evaluation of silencing associated pathway gene expression in TCV and R6A infections in Col-0 plants with altered levels of TIP expression. Semi-quantitative RT-PCR analysis of the expression of 3 DCL genes and DRB4 in transgenic lines of *A. thaliana* ecotype *Col-0* with varying levels of TIP expression and infected with TCV (T), R6A (R), or Mock (M). Samples were collected from systemic tissue at 4 dpi, 6 dpi, and 8 dpi. Reduced TIP expression (asTIP) and the control (GFP) both had an inducible estradiol promoter. The up-regulated TIP (UpTIP) had an additional copy of TIP under the control of the constitutively expressed p35 promoter. PCR cycle numbers are shown to the right of each panel. EF1Ŭexpression was used as an endogenous control.

Figure 4-3. DCL expression in the resistant ecotype Di-17 infected with TCV and R6A. Semi-quantitative RT-PCR analysis of DCLŏs expression in wt and transgenic lines of *A. thaliana* ecotype Di-17. Samples were collected from inoculated tissue at 6 hrpi and 48 hrpi and from systemic tissue at 2 dpi, 4 dpi, and 8 dpi. PCR cycle numbers are shown to the right of each panel. *EF1α* expression was used as an endogenous control.

Figure 4-4. Assessment of TCV and CP mutant viruses' infections on DCL1 and DCL4 expression levels in both susceptible (Col-0) and resistant (Di-17) ecotypes of *A. thaliana*. Semi-quantitative RT-PCR analysis of *DCL1* and *DCL4* expression in (a)wt Col-0 and (b)tip ko lines and (c)Di-17. Tissues samples inoculated with TCV and the CP mutants indicated above each panel were analyzed from inoculated leaves (IL) at 48 hrpi (lane 1) and systemic leaves (SL at 2 dpi (lane 2), 4 dpi (lane 3), 6 dpi (lane 4), and 8 dpi (lane 5). *DCL1* was monitored to assess the effect on miRNA biogenesis and *DCL4* was monitored to assess the effect on antiviral defense. PCR cycle numbers are shown to the right of each panel. *EF1α* expression was used as an endogenous control.

Figure 4-5. Comparison of temporal accumulation of TCV and CP mutant transcripts in silencing pathway defective mutant plants. Northern analysis of CP transcripts accumulation of TCV and CP mutants in *A. thaliana* ecotype Col-0 ko lines that were deficient in key roles in the silencing pathways. A total of 10ng of virus transcript of wt TCV or one of the CP mutants R6A, R8A, D13A, and G14A were inoculated onto designated leaves. Inoculated leaves were collected at 48 hr post inoculation (hrpi) and systemic leaves were collected at 2, 4, 6, and 8 day post inoculation (dpi) as indicated. Total RNA (2µg) of each sample was used to evaluate viral RNA transcripts levels (described in Materials and Methods section).

Figure 4-6. Evaluation of expression of endogenous silencing suppressor genes during TCV and R6A infections in susceptible Col-0. Semi-quantitative RT-PCR analysis was performed on systemically infected tissue to monitor if either TCV (T) or

mutant R6A (R) was having a differential effect on the induction of endogenous silencing suppressors relative to mock (M) infections. Samples were collected at 2 dpi, 4 dpi, 6 dpi, and 8 dpi. PCR cycle numbers are shown to the right of each panel. *ACT2* expression was used as an endogenous control.

Figure 4-7. Evaluation of TCV and R6A infections on miRNA167. MiRNA167 was evaluated to see if TCV infection was having an effect on miRNA accumulation across a time course looking at both inoculated and systemic tissue. Samples were collected from inoculated tissue at 6 hrpi and 48 hrpi and from systemic tissue at 2 dpi, 4 dpi, and 8 dpi from three different plant types (a) wt Col-0, (b) wt Di-17, (c) Col-0 *tip ko*. 15µg of total RNA was used for this analysis with P^{32} end labeled probes.

Figure 4-8. Evaluation of TCV and R6A infections on miRNA171. MiRNA171 was evaluated to see if TCV infection was having an effect on miRNA accumulation across a time course looking at both inoculated and systemic tissue. Samples were collected from inoculated tissue at 6 hrpi and 48 hrpi and from systemic tissue at 2 dpi and 4 dpi from two different plant types (a) Col-0 and (b) Di-17. Unless otherwise designated, 15µg of total RNA was used for this analysis with P^{32} end labeled probes.

Figure 4-1.

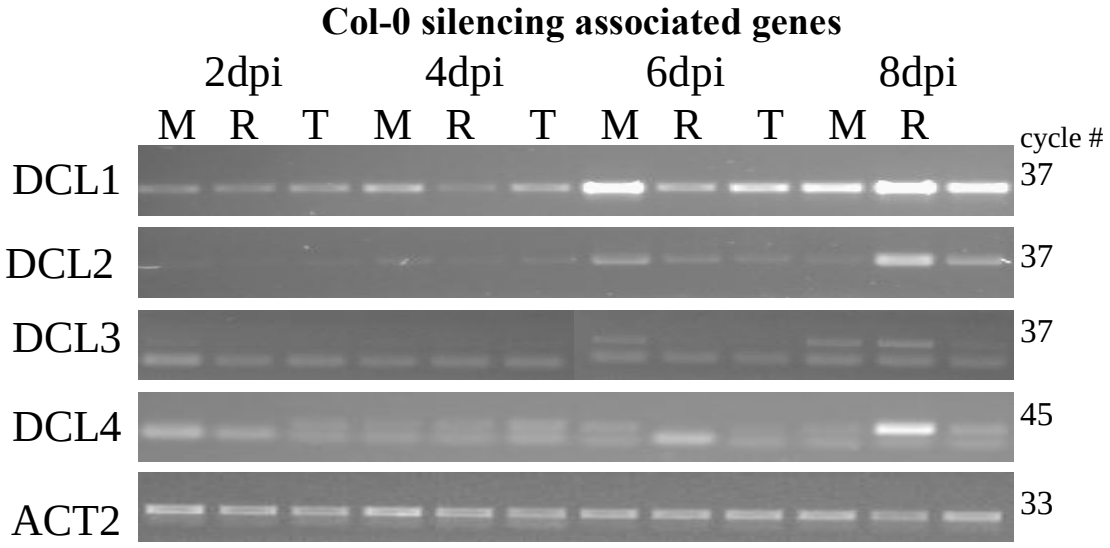

Col-0 silencing associated genes
2dpi
4dpi
6dpi
8dpi
M R T M R T M R T M R
cycle #
DCL1
37
DCL2
37
DCL3
37
DCL4
45
ACT2
33

Figure 4-2.

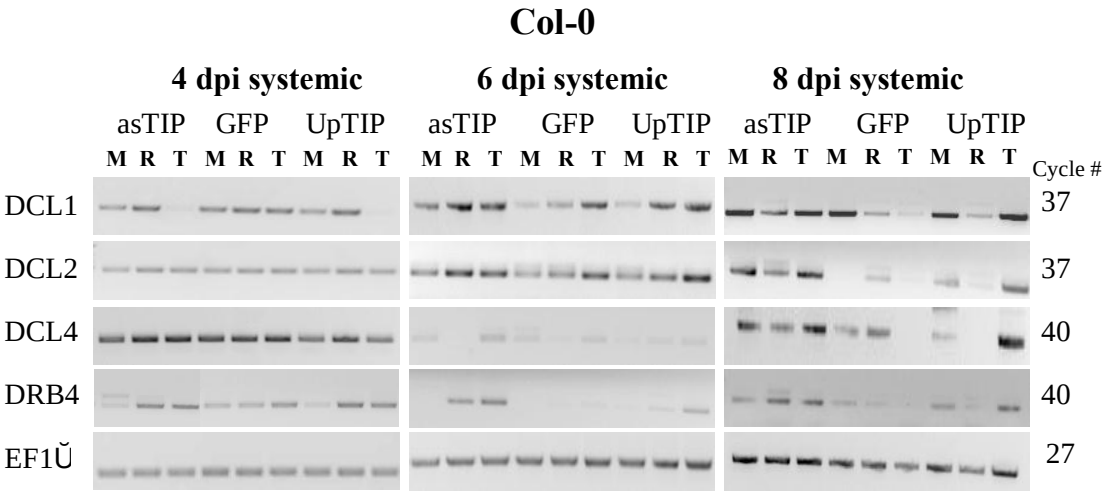

Col-0
4 dpi systemic
6 dpi systemic
8 dpi systemic
asTIP GFP UpTIP asTIP GFP UpTIP asTIP GFP UpTIP
M R T M R T M R T M R T M R T M R T M R T M R T
Cycle #
DCL1
37
DCL2
37
DCL4
40
DRB4
40
EF1Ŭ
27

Figure 4-3.

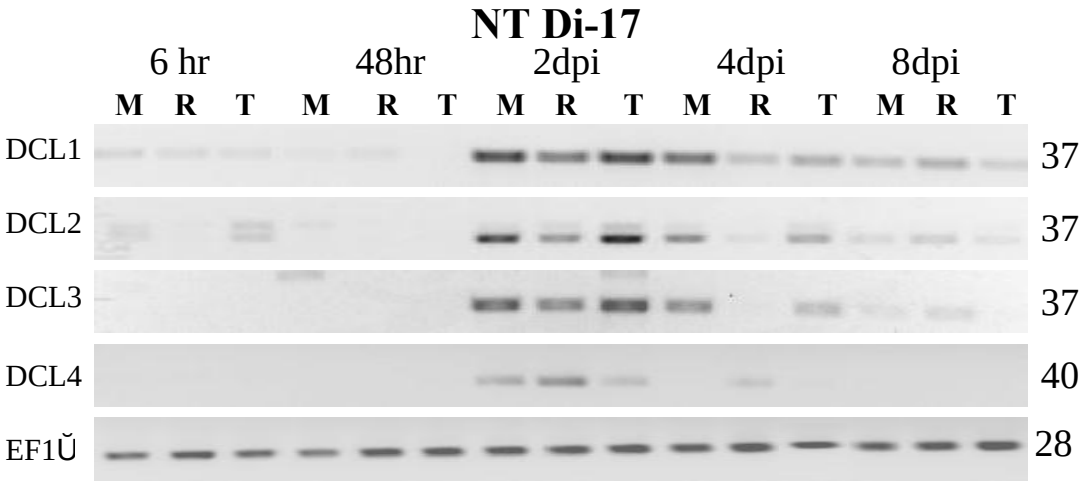
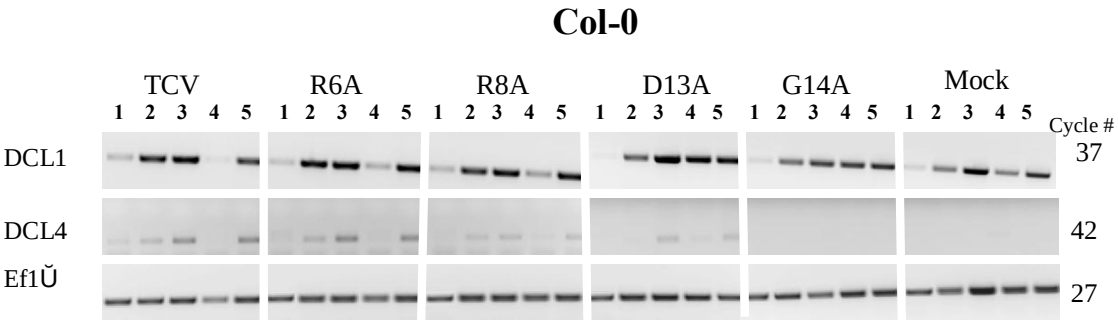

NT Di-17
6 hr
48hr
2dpi
4dpi
8dpi
M R T M R T M R T M R T M R T
DCL1
DCL2
DCL3
DCL4
EF1Ŭ
37
37
37
40
28

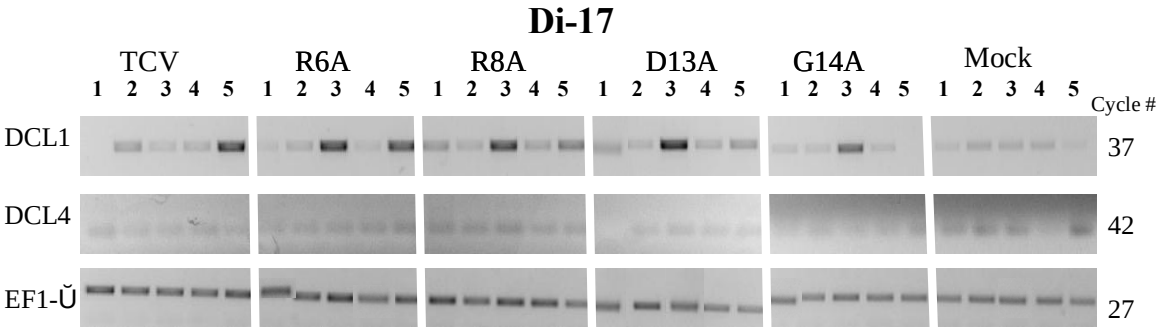
Figure 4-4.

1. 48 hrpi IL
2. 2 dpi SL
3. 4 dpi SL
4. 6 dpi SL
5. 8 dpi SL

A)



B)



C)

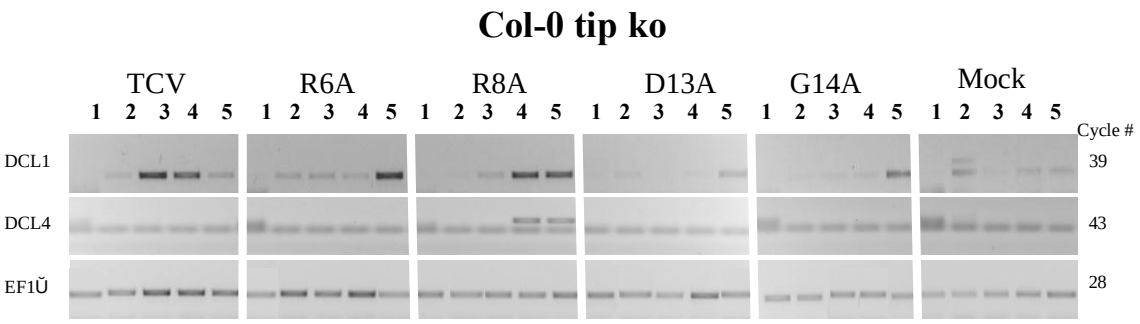


Figure 4-5.

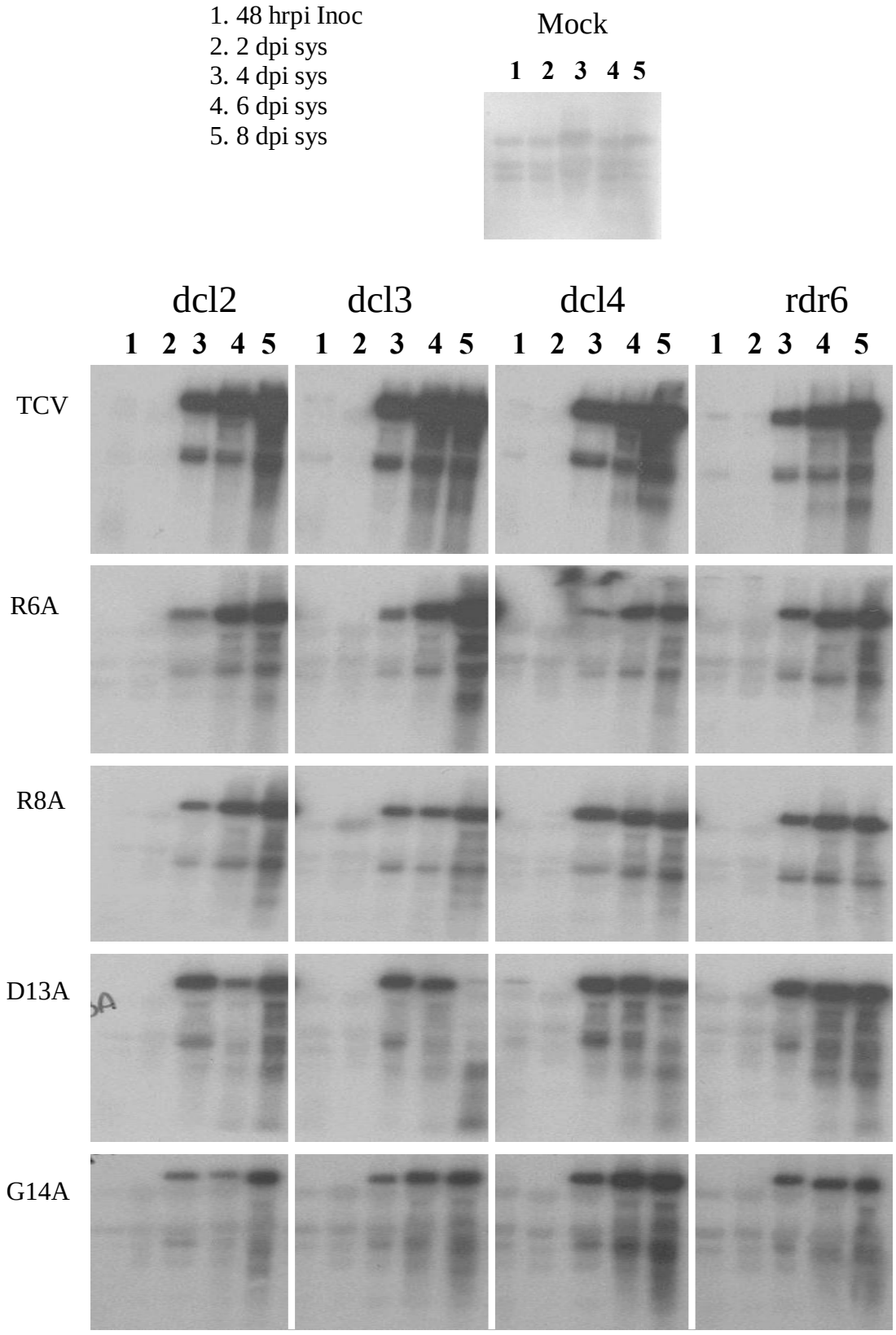

1. 48 hrpi Inoc
2. 2 dpi sys
3. 4 dpi sys
4. 6 dpi sys
5. 8 dpi sys
Mock
1 2 3 4 5
dcl2
dcl3
dcl4
rdr6
1 2 3 4 5
TCV
R6A
R8A
D13A
G14A

Figure 4-6

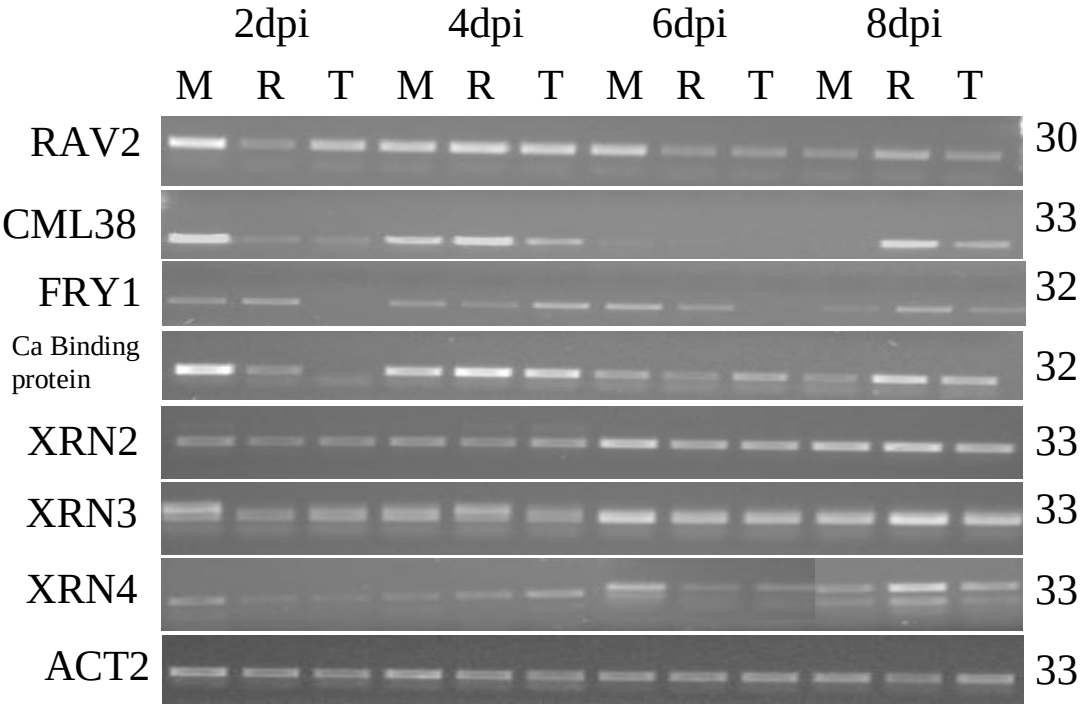

2dpi
4dpi
6dpi
8dpi
M R T M R T M R T M R T
RAV2
30
CML38
33
FRY1
32
Ca Binding protein
32
XRN2
33
XRN3
33
XRN4
33
ACT2
33

Figure 4-7.

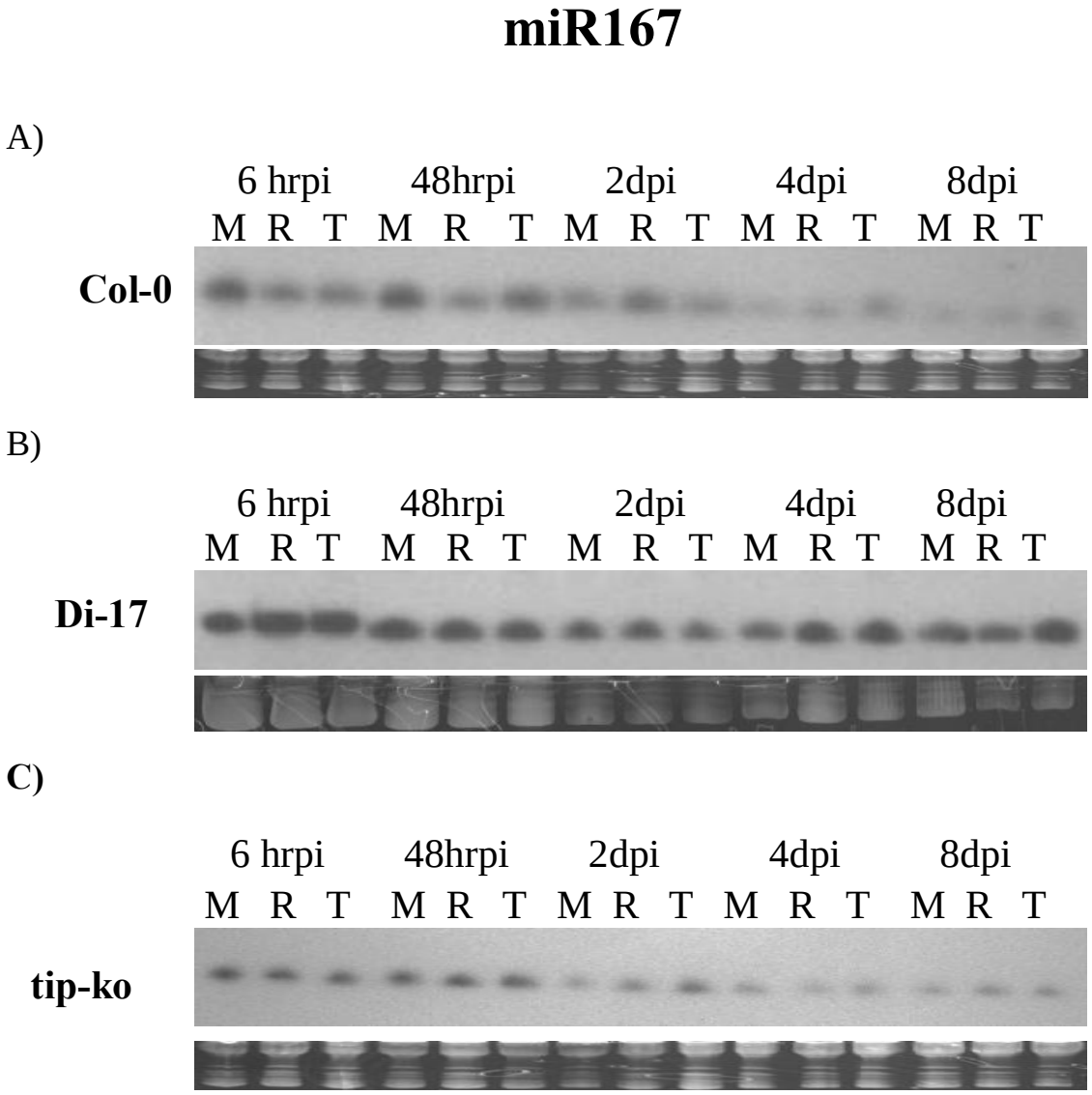

miR167
A)
6 hrpi
48hrpi
2dpi
4dpi
8dpi
M R T M R T M R T M R T M R T
Col-0
B)
6 hrpi
48hrpi
2dpi
4dpi
8dpi
M R T M R T M R T M R T M R T
Di-17
C)
6 hrpi
48hrpi
2dpi
4dpi
8dpi
M R T M R T M R T M R T M R T
tip-ko

Figure 4-8.

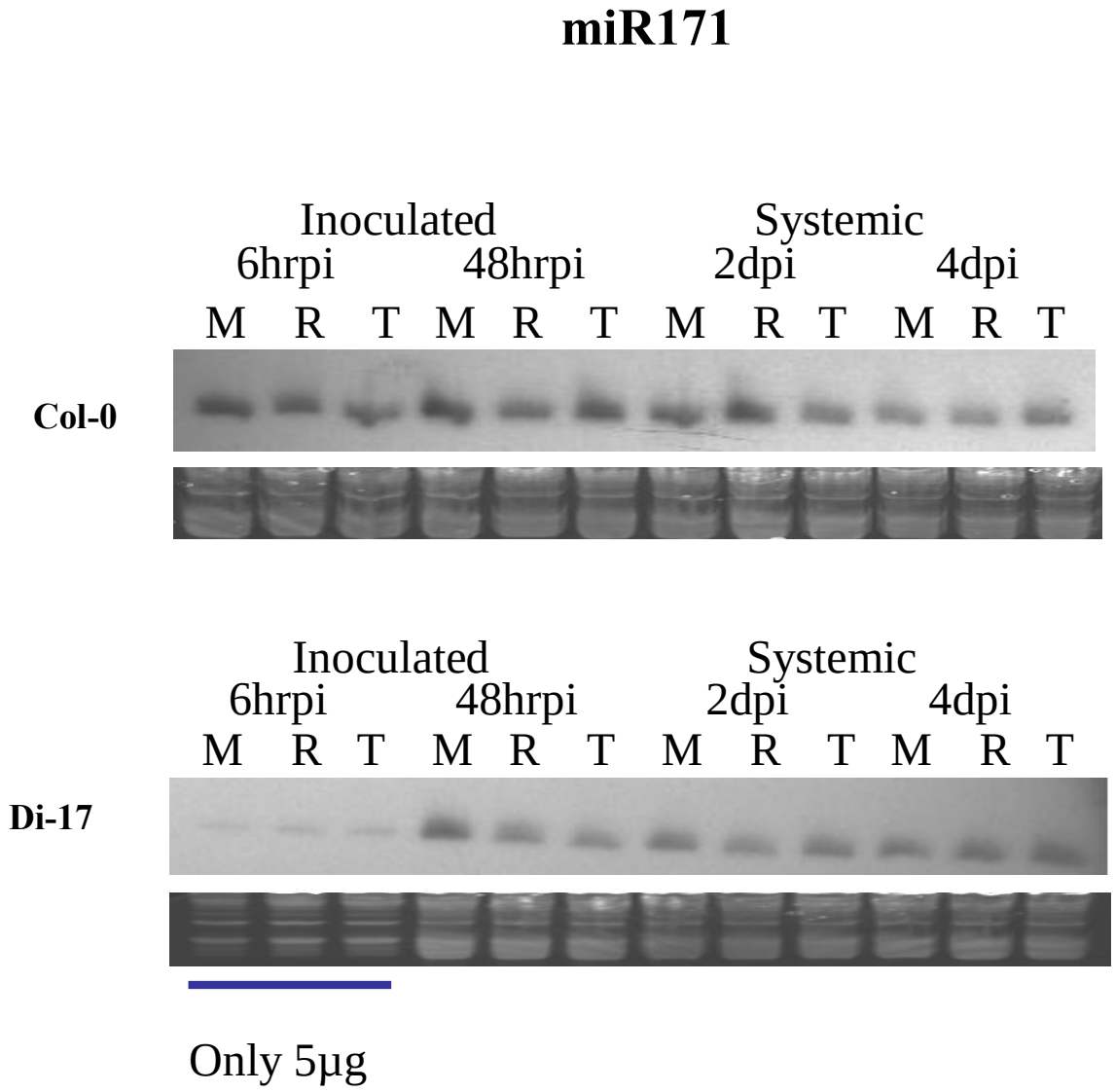

miR171
Inoculated
Systemic
6hrpi
48hrpi
2dpi
4dpi
M R T M R T M R T M R T
Col-0
Inoculated
Systemic
6hrpi
48hrpi
2dpi
4dpi
M R T M R T M R T M R T
Di-17
Only 5µg

Table 4-1. List of semi-quantitative PCR primers

Gene	Sequence
ACT2	Fwd: 5'-GTCTGAGATTTCTCCTGCCG-3'
	Rev: 5'-CACGGTTAGCCTTTGGGTTA-3'
DCL1	Fwd: 5'-AGTGGTCTCTAGGGTTTTGCTTGCT-3'
	Rev: 5'-TCGCATTCGCGGTTTCCACCA-3'
DCL2	Fwd: 5'-AGACCTCTGCAGAATGCCTGTGGT-3'
	Rev: 5'-CGCGTCGAGCAAAATGGCAGG-3'
DCL3	Fwd: 5'-GCACCCGAACCAGCCGTTGA-3'
	Rev: 5'-CCAGGTGCAGTCCAGCGACG-3'
DCL4	Fwd: 5'-AGATTGCAGCGAATGAGGTTCTTGT-3'
	Rev: 5'-AGAGAGGGCCTCAGCAGCCA-3'
DRB4	Fwd: 5'-TCGAACCAGACCGGATCGCCT-3'
	Rev: 5'-TCGGGGTTCCATGGGCGACA-3'
EF1Ŭ	Fwd: 5'-GCCGAGCGTGAGCGTGGTAT-3'
	Rev: 5'-TGGCGGCACCCTTAGCTGGA-3'
RAV2	Fwd: 5'-CTACCGGATGGGAAGCGGCG-3'
	Rev: 5'-CACTGCCGGTGACGGTGACG-3'
FRY1	Fwd: 5'-GCTGCTTCACTCGCTGCTCGT-3'
	Rev: 5'-GCACCAGCGACATGGTCCCAA-3'
CML38	Fwd: 5'-AGAGGGAAGATTCAGCCGGAGAGA-3'
	Rev: 5'-TCCAACATCCCATCTCCGTCCGT-3'
At4g27280	Fwd: 5'-TAACGCTGCGGCGGTTCTGG-3'
	Rev: 5'-TGTCAATGCCGGCGCGTGAA-3'
XRN2	Fwd: 5'-TCCTGGCGAGGGGGAACACA-3'
	Rev: 5'-TGCTGCTCGGTGCCCCTCTT-3'
XRN3	Fwd: 5'-CCTGGACAGCAGGAGAGGTGCT-3'
	Rev: 5'-TGGTGGGTTGGGAATCCTCATCT-3'
XRN4	Fwd: 5'-CGCTTCGCCTGGCAGGTTCA-3'
	Rev: 5'-GCTGGCCCAGTGGATGAGCG-3'

Table 4-2. Probes for Northern Analysis

TCV CP	Rev: 5'-CAGGACCGAGAAGTCAGAGG-3
	Rev: 5'-GGCCCACCCGACACCACTGG-3'
	Rev: 5'-CTTGTCTTGACCGAGTTGGT-3'
miRNA167	5ô-ACUUUGACGCUUGUACUAGAU-3ô
miRNA171	5ô-ACUAACUCGGCGCGGUUAUAG-3ô

CONCLUDING REMARKS

CONCLUDING REMARKS

Major conclusions

In this dissertation, I sought to elucidate the role of the NAC transcription factor, TIP, in pathogen trigger immunity (PTI) and effector triggered immunity (ETI) directed against Turnip crinkle virus (TCV) infection in *Arabidopsis thaliana*. As a starting point, I obtained TCV coat protein mutants, previously constructed by Dr. Feng Qu, with single amino acid replacement mutations in the region of the CP previously established to interact with the TIP protein *in vitro*. Several of the mutants I selected for analysis had lost the ability to interact with TIP. One of these mutants that I studied extensively (R6A) produced more severe disease in the susceptible Col-0 ecotype and broke resistance in the resistant Di-17 ecotype. Another mutant that had lost ability to bind TIP (R8A) also broke resistance but had a systemic hypersensitive response. Other mutant viruses with intermediate phenotypes were also examined including one in which TIP binding had been restored (G14A) along with an extreme resistance response in the resistant host. To assess the effect of the mutant on the PTI response, I compared total virus concentration of wt and mutant viruses in multiple lines of *Arabidopsis thaliana* to determine the main factors responsible for any differences in the rate of virus accumulation. I also evaluated multiple genes identified as key players in the host defense pathways to see if any observed differential gene expression could shed light on what was causing the mutant viruses to develop such diverse resistance responses and symptom differences. The results of these studies are detailed in this dissertation and are summarized here.

1). TCV coat protein mutant R6A, that lost ability to bind the NAC transcription factor TIP, accumulated more slowly than wtTCV and displayed altered defense gene expression associated with the innate immune response (PTI) . Using both Northern analysis and ELISA data, I monitored virus accumulation of TCV and the non-TIP binding mutant R6A to assess if the loss of the ability to bind TIP had an effect on replicative efficiency in *A. thaliana* ecotype Col-0. Previous work had shown that R6A was unable to bind TIP, caused more severe disease in Col-0 and broke resistance in Di-17. I demonstrated that TCV was able to accumulate to higher levels early in infection in both inoculated and systemic tissue than the mutant R6A. I also showed that this replicative advantage of TCV over R6A was eliminated in plants with either compromised salicylic acid (SA) signaling or defective systemic acquired resistance (SAR) signaling. This provided evidence that the inability of R6A to suppress SA signaling was likely associated with the loss of its ability to bind TIP. This conclusion was reaffirmed by demonstrating that the TCV and R6A accumulation in plants was also not significantly altered in infections of Col-0 plants that constitutively over-expressed TIP or in plants in which TIP was transiently down-regulated. Therefore, my research was able to convincingly demonstrate that TCV-CP interaction with the transcription factor TIP provided an evolutionary selective advantage to TCV by permitting the virus to partially evade the PTI resistance layer by suppressing SA signaling in the susceptible host to give it a replicative advantage early in infection.

2). Turnip crinkle virus coat protein mutants that fail to bind the NAC transcription factor TIP also display altered hypersensitive response induction and

systemic infection associated with the ETI defense layer. I further evaluated the resistance response observed in Arabidopsis ecotype Di-17 to TCV and the TIP binding mutants to determine the possible role of TIP in the ETI response. Each of the CP TIP non-binding mutants examined in this study was shown to be able to invade systemic leaves and hence break resistance in Di-17. However one mutant, R8A, had an unusual phenotype in that it caused a systemic hypersensitive response (HR) that is usually associated with localization of the infection to the inoculated leaves. The systemic HR response was verified in this study by the demonstration of increased expression of *NHL10* in systemic leaves. I also examined another mutant, G14A, in which TIP binding activity was restored. This mutant developed a microHR on inoculated leaves and was completely confined to the inoculated leaves. An article appeared during the course of my studies that showed that TIP was not required for the HR response (Jeong et al., 2008). My data, however, conflicted with this conclusion in that the results suggested that TIP-CP interaction seemed to be important in the onset of the ETI based resistance. I completed a thorough analysis of infections of plants in which the R-gene, *HRT*, has been introgressed into a Col-0 tip ko line. In these experiments I showed that R8A was still able to move systemically but it was not able to induce an HR response in the absence of the TIP gene. I was also able to demonstrate that the G14A was not able to induce a microHR and was capable of moving systemically in the presence of HRT when the TIP gene was absent. These results demonstrate quite convincingly that TIP is indeed playing a role in modulating the ETI defense directed against TCV.

3). Altered TIP binding by TCV mutants leads to differences in expression patterns of multiple genes involved in defense. Previous analysis established TIP to be a NAC transcription factor with a nuclear localizing signal. Additional work in our lab showed that it is likely sequestered in the cytoplasm by a membrane localization signal and that release from the cytoplasm permited nuclear localization and negative regulation of defense genes. To confirm the proposed role of TIP in defense genes regulation, I conducted a comparison of gene expression differences of key defense genes in infections of TCV and TIP non binding mutant viruses. A key indicator of the SA pathway, PR1, was shown to be differentially induced between TCV and R6A infections in both the susceptible and resistant host lines. I also evaluated WRKY70, which is involved in fine-tuning the SA-JA response, and WRKY6, which is linked to anti-viral defense. I consistently observed that both of these defense related genes were elevated in R6A infections, especially at the later time point of 8 dpi in systemic tissue. Another significantly different finding involved *SEN1*, which is associated with senescence. This gene was found to be upregulated in R6A infections early in systemic tissue. I surmise that this could account for the increased symptom severity associated with the TIP non-binding mutants and speculate that it might reflect the outcome of an unregulated innate defense response analogous to the inflammatory response in vertebrates.

4). A working model for the role of TIP in the PTI and ETI response against TCV infections. From the data reported in this project, I was able to propose some working models for how both the PTI and ETI pathways differentially respond to TCV and non-TIP binding mutant infections. I propose that wtTCV infection is facilitated by TCV CP-

TIP binding due to enhanced release of the TIP transcription factor and subsequent suppression of SA signaling leading to a slower development of SAR. Because the non-TIP binding mutants fail to interact with TIP upon initial infection, TIP is not released and the PTI induced up-regulation is not suppressed leading to a more rapid and robust increase in the initial defense responses resulting in decreased in virus accumulation. The increase in disease symptoms is associated with this elevated defense signaling causing an earlier onset of virus associated senescence. We know, however, that the role of TIP in modulating the ETI response is less clear. This is because a previously published a report (Jeong et al., 2008) and work from our lab (Kang, personal communication) has shown that it is not required for the HR response mediated by HRT. This would suggest that the role of TIP in modulating the basal defense response is in the specific defense response that controls the rate of spread of the virus in the infected host. I attempted to address this possibility by exploring a connection between TIP mediated defense responses and the silencing pathway, unfortunately I was not able to identify any obviously coherent relationship.

Suggestions for future research

1). To confirm which genes are being regulated by TIP. If TIP is a key player in the PTI and ETI in Arabidopsis against TCV, then knowing which genes are under its control would contribute greatly to understanding the reason for TCV evolving the ability to bind TIP. This was examined in the presence and absence of virus infection by conducting a microarray analysis of wt Col-0 vs tip ko line and wt Col-0 vs constitutively expressed

UpTIP. Experiments like these were attempted prior to obtaining the tip ko line, therefore the re-analysis of these lines should prove to be much more informative than previous microarray experiments.

2). Determination of the underlying mechanism of how G14A is able to induce a microHR and how R8A is able to induce a systemic HR. In order to understand the complex signaling of HR development and virus resistance more comprehensively, it would be beneficial to understand how these two phenotypes, extreme resistance vs extreme susceptibility, are established. I have shown that in R8A infection the HR is not contained to the inoculated tissue but instead spreads systemically when TIP is expressed. And contrary to that extreme HR development, G14A induces a microHR and the virus is quarantined to the site of infection with no systemic spread. However, both R8A and G14A act similarly to R6A (as described in chapter 2) when HRT is introgressed into a Col-0 *tip* ko tDNA insertion line. It would be intriguing to see how the combination of TIP, HRT and TCV mutants influences this process.

3). Determine if TIP is affecting the viral accumulation and gene expression during infections by viruses other than TCV. It would be intriguing to know what effect, if any, TIP is having on other viruses during infection. As of now, TCV and CMV are the only two viruses known whose viral accumulation increases in the absence of TIP (*tip ko*) at day three post infection. We know that TIP is a negative regulator of defense and its presence and location within the cell during TCV infection has an impact on the defense

response and virus accumulation. Therefore determining how TIP is a key component to anti-viral defense would help shed light on the role of this NAC gene.

4). Evaluate if TIP non-binding mutants are still able to interact with AG 1. As mentioned earlier, TCV CP is able to bind to AGO1 via its N-terminal 25-26 GW amino acids and disrupt the RISC formation. This region also overlaps the region that TIP binds to on TCV CP (N-terminal aa# 1-25). Therefore, this could be a contributing cause to the differential symptoms that develop between wtTCV and its TIP-non binding mutants. If the binding of AGO1 is affected by TIP binding of the TCV CP then we could demonstrate that TIP is indeed having an effect on the silencing pathway.