

***Turnip crinkle virus* Coat Protein Suppresses the Hypersensitive Response  
in Plants**

**A Thesis**

**Submitted to the Faculty of the**

**WORCESTER POLYTECHNIC INSTITUTE**

**in partial fulfillment of the requirements for the**

**Degree of Master of Science**

**In Biochemistry**

**By**

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**January 2007**

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## ABSTRACT

*Turnip crinkle virus* (TCV) has been implicated in the suppression of the hypersensitive response (HR), a type of programmed cell death induced during active resistance in *Arabidopsis thaliana*. In order to investigate the involvement of individual viral components in mediating suppression, TCV genes were cloned for use in an *Agrobacterium tumefaciens* mediated transient expression in *Nicotiana benthamiana*. Agroinfiltration of the HR-inducing avrPto/Pto system in conjunction with individual TCV genes has identified the p38 gene, which encodes the viral coat protein, as the gene responsible for the cell death suppression phenotype. The extent of cell death suppression by coat protein was quantified and found to be equal to the level of suppression by the whole virus and AvrPtoB, another cell death inhibitor from bacteria. Thus, the coat protein alone is sufficient to inhibit the HR in plants.

Further, the effect of TCV on HR initiation by an avirulence factor from an unrelated bacterial pathogen was investigated. The presence of TCV does not affect the production, secretion or cellular processing of the bacterial avirulence factor.

## **ACKNOWLEDGEMENT**

This dissertation could not have been possible without Dr. K. K. Wobbe, who not only served as my advisor but also encouraged and inspired me throughout my academic programme at WPI. By giving me independence in planning the experimental strategies, she showed confidence in my abilities. For this I would always be grateful to her. My interest in research has strengthened after working with her. I would like to thank her and Dr. Jose Arguello for helping me in improving my technical skills.

I would like to thank the previous graduate students of our research group, Tom and Geetha. They did the initial work on the TCV project and gave me a great project to start with. I would also like to thank all the members of lab GH06, their friendship and help made my time here memorable and productive. Atin and Elif helped me a lot in learning molecular biology techniques. Majo and Ying always found time to answer my questions.

I would like to say special thanks to my parents and brother Arjun. My dream of higher education would not have been fulfilled without their love and support. Finally, I would like to thank Sachin for being a best friend and pillar of strength.

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# 1 INTRODUCTION

## 1.1 Hypersensitive response

The plant-pathogen interaction is a continuous warfare between the invading pathogen and the host plant. In this conflict both have evolved strategies to overcome each other's defense response. Understanding the host's defense and invader's counter defense mechanism can be used to enhance the plants inherent resistance against pathogens. In plants the waxy cuticles, anti-microbial compounds and cell wall are designed to prevent the invader's entry inside the cells. Though effective against most of the microbes, these barriers can be breached by some pathogens. For example, in Arabidopsis plants, *Pseudomonas syringae*, a gram negative phytopathogen, is capable of suppressing cell wall-based extracellular defense and causing cell death (Alfano *et al.* 1996, Paula Hauck *et al.* 2003).

Once the pathogen has gained entry to the plant cell, the plant's ability to differentiate between "self" and "non-self" forms the first line of defense against microbial invasion. When all the members of a plant species exhibit resistance to members of a given pathogen species, the host-pathogen interaction is termed as "non-host" resistance. In plants, the non-host defense network consists of a combination of passive and induced (basal) defense responses (Heath 1985, Thordal-Christensen 2003). Specific plant receptors interact with a variety of structural and secreted compounds commonly found in pathogens such as flagellin, lipopolysaccharides and chitin, known as pathogen associated molecular patterns (PAMPs) (Nurnberger *et al.* 2004). This interaction triggers the mitogen-activated protein kinase (MAPK) and salicylic acid (SA) signaling pathways which lead to a variety of defense responses like cell wall reinforcement and expression of pathogenesis related (PR) proteins. These basal defense responses are slowly induced and are non- specific.

To circumvent the plant's defense system, pathogens themselves have evolved by secreting effector proteins directly into the host's cell. The Avirulence factor (Avr) is a product of effector genes. Avr factors promote the pathogen's virulence and overcome the plant's defense. In response to this strategy plants have developed a specialized defense, the "resistance (*R*) gene". The *R* gene-mediated response is pathogen specific and is rapidly induced. The hypersensitive response (HR) is a hallmark of *R* gene-mediated defense; it occurs when the resistant (*R*) protein in the plant recognizes the avirulence (Avr) factor from the pathogen (Flor 1971). The HR results in complex signal transduction pathway, leading to rapid localized cell death at the site of infection to cut the pathogen's source of nutrients and prevent it from spreading further. The HR also aids the plant by sending a signal to the uninfected tissues to activate defense against secondary infection known as systemically acquired resistance (SAR) (Ross 1961, Ryals *et al.* 1994).

The R-Avr or gene-for gene hypothesis was first proposed by H. H. Flor in 1955; he observed (Flor 1946, 1947) that the outcome of the plant- pathogen interaction is determined by matched specificities at the pathogen's avirulence (*avr*) loci and corresponding dominant or semidominant resistance (*R*) loci in the plant. If genes at both of these loci are expressed, the plant is able to inhibit the pathogen by inducing the HR. (Dangl *et al.*, 1996; Hammond-Kosack and Jones, 1996). If either member of the gene pair is absent, there is no defense response and the pathogen successfully infects the plant.

It was shown recently that the *R*- gene products and the basal defense receptors share some downstream signaling pathways. This suggests an overlap between the two resistance responses (de Torres *et al.* 2006, Navarro *et al.* 2004).

## 1.2 Avirulence genes

Initially, the concept of *avr* genes in pathogens was not widely accepted. The opponents of the theory challenged it by stating that according to Darwin's "theory of natural selection" it is highly unlikely that nature will select a gene in a species that results in its own death (Person and Mayo 1974). The cloning of first *avr* gene in 1984 (Staskawicz *et al.* ) from bacteria *Pseudomonas syringae* pv. *glycinae* changed the scenario and the gene-for-gene concept was firmly established in bacteria-plant interactions. Since then approximately 50 *avr* genes have been cloned from various pathogens including fungi and bacteria.

The *avr* genes also help in growth and infection of pathogen in the absence of the *R* genes. For instance, the *avrPto* gene enhances the ability of *P. syringae* pv. *tomato* strains to cause necrosis on susceptible tomato lines lacking the *Pto* (*Pseudomonas syringae* pv. *tomato*) resistance gene (Chang *et al.* 2000). Similarly, the presence of the *avrRpt2* gene in *P. syringae* pv. *tomato* strains promoted bacterial growth up to 50-100 fold on *Arabidopsis thaliana* lacking the corresponding *RPS2* (resistance to *Pseudomonas syringae* pv. *tomato* 2) gene (Chen *et al.* 2000).

Recently a number of studies have showed another role of *avr* gene which helps in pathogenesis; the ability to suppress host defense. Abramovitch *et al.* (2003) has shown that AvrPtoB when transiently expressed in tomato can suppress programmed cell death (PCD) initiated by both Pto and Cf9 resistant proteins. In addition, AvrPtoB also suppress the heat and oxidative stress-induced cell death in yeasts. Similarly, effector proteins VirPphA, AvrPphC and AvrPphF from bean pathogen *P. syringae* pv. *phaseolicola* can block the induction of the HR response (Jackson *et al.* 1999; Tsiamis *et al.* 2000).

By enhancing the growth of the pathogen and suppressing host defenses the *avr* gene plays a dual role in pathogenesis. This explains why the *avr* gene is dominant in the pathogen, in spite of its role in limiting virulence.

In the last decade, cloning and sequencing of a variety of *avr* genes have identified motifs important for bacterial function. The structural analysis of *avr* genes has also given insight into their cellular localization. The activity of *avr* genes occurs within the plant cell. For example, the *avrBs3* gene family from xanthomonad species has found to contain C-terminal nuclear localization signals (NLS) (Yang and Gabriel 1995) and acidic transcriptional activation domains (AD) (Zhu *et al.* 1998); also the proteins have 90-97% amino acid identity (Gabriel 1999). In 2002, Szurek *et al.* provided the evidence that avirulence gene products enter the plant cell. They used antibody labeling to detect the AvrBs3 protein from *X. campestris* pv. *vesicatoria* inside the plant cell.

Another group of *avr* genes from pseudomonads were found to contain myristoylation motifs responsible for targeting the protein to the plasma membrane. AvrRpm1 and AvrB from *P. syringae* pv. *maculicola* contained amino N-terminal myristoylation sites which targeted these proteins to the plasma membrane of plant's cell (Nimchuk *et al.* 2000). Disrupting the NLS and myristoylation motifs resulted in abolishing the virulence function in both group of Avr proteins (Marois *et al.* 2002 & Nimchuk *et al.* 2000).

The AvrRpm1 and AvrB effector proteins enter the host's cell via the type III secretion system (TTSS). It was observed that mutations in a group of genes known as *hrp* (hypersensitive response and pathogenicity) and *hrc* (hypersensitive response and conserved) resulted in loss of *avr* gene function (Collmer *et al.* 2000, Salmeron and Staskawicz 1993). Together the *hrp* and *hrc* genes encode the TTSS which forms a transmembrane pore. The bacterium uses this system

to inject the effector proteins directly inside the cell. Type III effectors include Avr proteins and hrp-dependent proteins (hop) which are indicated as important for nutrient acquisition and successful bacterial colonization (Chang *et al.* 2004). Analysis of the Type III secretome and genome sequencing of *P. syringae* pv. *tomato* DC3000 (Buell *et al.* 2003) suggests that there are approximately 40 Type III effectors (Guttmann *et al.* 2002; Petnicki-Ocwieja *et al.* 2002).

The cellular activities of many Type III effectors from *P. syringae* strains have been identified. AvrPphB from *P. syringae* pv. *phaseolicola* exhibit similarity in sequence with the catalytic domains of YopT and Efa1 (Shao *et al.* 2002). The YopT and Efa1 effectors are members of cysteine protease family and are important virulence factors for mammalian pathogens, such as *Yersinia* and *Salmonella* (Shao *et al.* 2002, Bretz *et al.* 2004). Similar to these cysteine proteases, AvrPphB is also important for virulence in susceptible hosts (Bretz *et al.* 2004). Recently, AvrRpt2, one of the best characterized type III effector proteins was also identified as a cysteine protease. The secondary structure of the AvrRpt2 active domain was found to align with the secondary structure of staphopain, a cysteine protease from *Staphylococcus epidermidis* (Axtell *et al.* 2003).

HopPtoD2 from *Pseudomonas syringae* pv. *tomato* DC3000 is another effector translocated through the TTSS which modulates defense response in plants. It was shown to be a protein tyrosine phosphatase (PTP) and a conserved catalytic Cysteine residue (Cys<sub>378</sub>) was required for the PTP activity (Bretz *et al.* 2003, Espinosa *et al.* 2003). Interestingly, HopPtoD2 was capable of suppressing the HR induced by an avirulent *P. syringae* strain on *Nicotiana benthamiana* plants and the suppression was dependent on its PTP activity (Espinosa *et al.* 2003). The study also suggested that HopPtoD2 acted as a PCD suppressor by interfering with defense associated mitogen-activated protein kinase (MAPK) pathway.

### 1.3 Resistance genes

On the basis of their sequence, the *R* genes have been categorized into five main groups (Martin *et al.* 2003). The majority of the R proteins characterized so far fall into the category defined by the presence of a nucleotide binding site (NBS) and Leucine Rich Repeat (LRR) regions. This class can be further subdivided on the basis of their N-terminal structure. One subclass contains a coiled-coil (CC) domain at the N-terminal (CC-NBS-LRR) region; examples of R proteins from this class are RPS2 (Bent *et al.* 1994, Mindrinos *et al.* 1994, Whalen *et al.* 1991), RPM1 from *P. syringae* (Debener *et al.* 1991, Grant *et al.* 1995), and Pi-ta from *M. grisea* (Bryan *et al.* 2000). The other subclass is characterized by homology with the intracellular signaling domains of the *Drosophila* Toll and mammalian interleukin (IL)-1 receptors (TIR-NBS-LRR). RPS4 from *P. syringae* and the *N* gene from *Tobacco mosaic virus* (TMV) are examples from this class of *R* genes (Gassmann *et al.* 1999). Interestingly, so far the only reported function from this class of proteins is in disease resistance.

Another class of R protein encodes an intracellular serine/threonine kinase and has a myristylation motif on the N-terminus. However, Pto from tomato is the only protein belonging to this class (Loh *et al.* 1998, Martin *et al.* 1993, Ronald *et al.* 1992). The other classes of R proteins are also known to play a role in plant development and other cellular functions. One such class is known to have an extracellular LRR and it encodes small cytoplasmic regions; it's represented by all *Cf* genes from tomato (Dixon *et al.* 1998). Another class of protein encoding extracellular LRR also encodes for intracellular serine/threonine kinase activity. The *Xa21* gene from Rice is the only member from this class. (Song *et al.* 1995).

Not surprisingly, many of the R proteins do not fit any of the five classes mentioned above. The *RPW8* gene from Arabidopsis which confers wide-spectrum resistance to powdery mildew

contains a transmembrane (TM) domain connected to an intracellular CC domain (Xiao et al. 2001). Another gene which provides broad spectrum resistance to powdery mildew is the *Mlo* gene from barley and it's a membrane bound protein (Buschges *et al.* 1997). The *Rpg1* gene from barley, which controls stem rust resistance, has two tandem kinase domains and a potential transmembrane region (Brueggeman *et al.* 2002).

| Class | Structural motifs  | R gene example               | Plant  | Pathogen   | Effectors                       |
|-------|--|------------------------------|--|--|---------------------------------|
| I     | Encodes Serine/Threonine kinase, N-terminal myristylation motif                                | Pto                          | Tomato   | <i>P. syringae</i>   | AvrPto<br>AvrPtoB               |
| II    | LRR, NBS, N-terminus leucine zipper regions, CC domains  | RPM1<br>RPS2<br>Pi-ta        | <i>Arabidopsis</i><br><i>Arabidopsis</i><br>Rice | <i>P. syringae</i><br><i>P. syringae</i><br><i>M. grisea</i>                 | AvrRpm1,<br>AvrRpt2<br>AVR-Pita |
| III   | LRR, NBS, N-terminus leucine zipper regions, TIR domains homologous to animal resistance genes | N<br>RPS4                    | Tobacco<br><i>Arabidopsis</i>                    | TMV<br><i>P. syringae</i>  | Helicase<br>AvrRps4             |
| IV    | Extracellular LRR region, small cytoplasmic extensions   | Cf-2<br>Cf-4<br>Cf-5<br>Cf-9 | Tomato<br>Tomato<br>Tomato<br>Tomato             | <i>C. fulvum</i><br><i>C. fulvum</i><br><i>C. fulvum</i><br><i>C. fulvum</i> | Avr2<br>Avr4<br><br>Avr9        |
| V     | Extracellular LRR region with serine/threonine kinase activity                                 | Xa21                         | Rice   | <i>X. oryzae</i>   |                                 |

**Table1.1 Classification of R genes on the basis of structural features.** The table has been modified from Martin *et al.* 2003.

## 1.4 Mode of R- Avr interaction

The two popular theories that describe the R- Avr interaction at the molecular level are the guard theory and the receptor-elicitor theory.

## Receptor-elicitor theory

According to this theory the resistance gene product acts as a receptor that binds to the matching avirulence ligand and triggers the host defense system (Gabriel and Rolfe 1990). One such direct interaction was observed between the *avrPto* gene product from *P. syringae* pv. *tomato* and the *Pto* resistance gene product from tomato by using a yeast two-hybrid system (Scofield *et al.* 1996; Tang *et al.* 1996). The *Pto* gene encodes a serine/threonine kinase. It has been shown that *Pto* autophosphorylation is required for *Pto* and *AvrPto* interactions and mutations abolishing this activity resulted in an inability to form the HR. Interestingly, *Pto* when over-expressed, is capable of triggering immunity even in the absence of its corresponding avirulence factor (Tang *et al.* 1996).

The finding that majority of the *R* genes contain a LRR that is important for protein-protein interaction (Dangl and Jones, 2001; Martin *et al.*, 2003) also supports this theory. The *Pi-ta* resistance gene product from rice, a member of the NBS-LRR class of *R* genes (Bryan *et al.* 2000) interacts with *Avr-Pita* produced by *M. grisea* ( Jia *et al.* 2000). Loss of physical interaction in *AvrPita* mutants resulted in loss of *Pi-ta* mediated resistance in rice. Another example is the physical interaction between *RRS-1* from *Arabidopsis thaliana* and *PopP2*, a Type III effector from *Ralstonia solanacearum*. *RPS-1* has been shown to have a NBS-LRR sequence (Deslandes *et al.* 2002). In 2001, Luderer *et al.* reported that there is no direct evidence for interaction between *Cf-9* protein and its corresponding avirulence factor *avr9*. The lack of evidence suggesting direct binding between many *R* proteins and *Avr* proteins prompted the formation of a new model, the Guard hypothesis.

## Guard theory

This concept was proposed by Van der Biezen and Jones (1998) to explain the need for Prf (*Pseudomonas* resistance and fenthion sensitivity), an NBS-LRR protein, in avrPto-Pto signaling (Salmeron *et al.* 1996). According to this theory, R proteins interact with other defense-related proteins and act as a “guard”. When the avirulence factor from the pathogen interacts with another defense factor, the avirulence factor modifies the defense factor and interrupts its interaction with the guard. This modification leads the guard to activate the defense response. For AvrPto- Pto interaction, it was proposed that Prf acts as a guard for Pto. It activates plant defense when it detects avrPto-Pto complexes, thereby making *Prf* the true *R* gene.

In 2000, Leister and Katagiri used immunoprecipitation to show that the NBS-LRR R protein RPS2 from *Arabidopsis thaliana* formed a physical complex with its corresponding *P. syringae* Avr protein, AvrRpt2. Interestingly, RPS2 also formed a complex with another avirulence protein AvrB, which corresponds with Arabidopsis NBS-LRR R protein, RPM1 (resistance to *Pseudomonas syringae* pv. *maculicola* 1). The RPS2- AvrRpt2 and RPS2- AvrB complex had an additional common protein, later termed as RIN4 (RPM1 interacting 4). In 2002, Mackey *et al.* demonstrated that RIN4 interacted with RPM1 and two *P. syringae* effectors recognized by RPM1, which are AvrB and AvrRpm1. It was shown that AvrB and AvrRpm1 cause hyperphosphorylation of RIN4 and this hyperphosphorylation is required for virulence. The authors concluded that RPM1 guards RIN4 and upon its phosphorylation triggers cellular events leading to resistance. They also showed that RIN4 is a negative regulator of basal defense, since reduced RIN4 expression resulted in elevated defense response.

Subsequently, Axtell *et al.* (2003) and Mackey *et al.* (2003) reported that RIN4 also physically interacts with RPS2 and this interaction was required for resistance against AvrRpt2. Axtell *et al.* demonstrated that AvrRpt2 caused elimination of RIN4 even in the absence of RPS2. This explained previous reports of AvrRpt2 suppression of host's defense (Chen *et al.* 2000). Using *rin4* and *rps2* mutants they demonstrated that RPS2 initiates signaling defense upon perception of RIN4 degradation and it does not directly recognize AvrRpt2. This also explained why transgenic Arabidopsis expressing *avrRpt2* were interfering with the RPM1 mediated immune response when challenged with *avrRpm1* (Ritter and Dangl, 1996). When RPS2/RPM1 plants are infiltrated with AvrRpm1 and AvrRpt2, only RPS2-mediated resistance is exhibited. It's a possibility that since RIN4 plays a key role in both RPM1 and RPS2 mediated signaling pathway, when AvrRpt2 degrades RIN4, the ability of RPM1 to monitor RIN4 phosphorylation is inhibited.

The studies with RIN4 also showed that a single host resistance factor could be the target of multiple effectors and resistance genes. Producing a small number of *R* genes to monitor multiple Avr factors can be an efficient strategy for plants to cope against continuously evolving pathogens.

The direct interaction between RRS-1 and PopP2 also corroborates the guard model. The RRS-1 gene encodes a NLS and a C-terminal WRKY (tryptophan-arginine-lysine-tyrosine) domain. WRKY domains are found in plant transcription factors that bind to W-box domains in the promoter regions of certain genes, including PR genes. The fluorescent tagging of RRS-1 gene showed that NLS is non functional. Since the PopP2 contains a functional NLS, it's a possibility that RRS-1 aim is to misdirect PopP2 by providing its own WRKY domain as bait to

which PopP2 could bind. If so, it would appear that RRS-1 is still guarding certain proteins by incorporating some of their structure into itself (Deslandes *et al* 2003).

Most of the research done so far confirms the guard model and offers an insight into the complex signal transduction pathway involved in defense and diverse strategies employed by both host and invader.

## **1.5 Turnip crinkle virus**

*Turnip crinkle virus* (TCV) is a positive sense, single stranded, 4054 nucleotide long RNA virus composed of five open reading frames. It encodes two replicase proteins (p88 and p28), two movement proteins required for cell- to- cell movement (p8 and p9) and a coat protein (p38) (Hacker *et al.* 1992, Carrington *et al.* 1989).

The plant virus uses plasmodesmata, the cytoplasmic bridge, for intracellular movement. Earlier it was thought that virus attacks and modifies the plasmodesmata during an infection, for the movement of viral genome (Waigmann *et al.* 1998), but later it was shown that plasmodesmata are dynamic in nature and rapidly alter their dimensions to increase the transport capabilities for proteins important for development of the plant, as well as virus (Waigmann *et al.* 1998). The virus uses phloem for rapid, long distance movement in plants (Carrington *et al.*, 1996). As the virus spreads inside the plant, the region of the plant accessible for systemic infection reduces because the viruses always spreads from infected “source” tissue to young and new “sink” leaves. The mature leaves do not import virus from inoculated leaves (Leisner *et al.* 1993).

TCV has a broad host range which includes vegetative plants like bell pepper, tomato and the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana*. Different ecotypes of *A. thaliana* react differently to TCV, Columbia-0 (Col-0) is susceptible to TCV (Li *et al.* 1990)

and upon viral inoculation develop disease symptoms like vein striping, asymmetrical mid-vein formation, leaf chlorosis and discoloration whereas Di-17 which is derived from Dijon-0 is resistant (Dempsey *et al.*1993) and TCV inoculation results in HR and other classical defense responses including increase in salicylic acid levels, defense gene expression and strengthening of cell wall by synthesis of auto fluorescent compounds (Dempsey *et al.*1993, 1997).

It has been shown that the coat protein (CP) of TCV has multiple functions. In 1992, Hacker *et al.* showed that CP is required for long distance movement in Arabidopsis. The N-terminus of the coat protein was demonstrated to serve as the Avr factor in the TCV – Arabidopsis interaction (Oh *et al.* 2000, Ren *et al.* 2000, Zhao *et al.*2000, Cooley *et al.* 1995). Ren *et al.* reported that TCV CP physically interacts with an Arabidopsis protein known as TIP (TCV Interacting Protein) in yeast; this interaction was also reported to be important for the resistance response. The TIP has homology to the NAC family of proteins, which are transcription factors important for plant development. The role of TIP as a transcription factor in yeast was also observed (Ren *et al.* 2005). Using green fluorescence protein (GFP) tagged TIP the effect of CP on cellular localization of TIP was studied. The findings that CP interferes with the nuclear localization of TIP led the authors to propose that the CP interacts with Arabidopsis transcription factor TIP and blocks its nuclear localization (Ren *et al.* 2005).

In 2003, the same group of scientists reported that the C- terminus of the coat protein is capable of suppressing posttranscriptional gene silencing (PTGS) ( Qu *et al.* 2003). PTGS or RNA silencing is a fairly newly discovered mechanism of genetic regulation. It protects the eukaryotes against foreign RNA including viral and transposon RNA by detecting double stranded RNA and targeting it for sequence- specific degradation. In the past few years, many

viruses have been demonstrated to have the ability to suppress this phenomenon. The suppressors of PTGS have been shown to use a variety of different mechanisms. The potyvirus helper component proteinase HC-Pro reverses silencing and prevents accumulation of the invading nucleic acids (Anandalakshmi *et al.*, 1998; Vance and Vaucheret, 2001). The *Potato virus X* (Voinnet *et al.*, 2000) and *Cucumber mosaic virus* do not reverse silencing very strongly but suppress the silencing signal, thereby preventing intracellular spread. For investigating the role of CP in PTGS, it was expressed in *Nicotiana benthamiana* plants carrying a GFP transgene and the suppression of silencing was evaluated by the level of GFP fluorescence (Qu *et al.* 2003). The PTGS suppression activity of CP is a highly effective strategy employed by TCV to counteract a lethal defense response by the host plant.

It's not unusual for a viral protein to have multiple functions, the protein which encodes P1/HC-Pro viral polyprotein processing enzyme in *Tobacco etch virus* also suppresses the PTGS (Kasschau and Carrington, 1998). The possible effect of the TIP interaction on the RNA silencing effect was investigated for the TCV CP (Choi *et al* 2004). It was demonstrated that CP mutants that have lost their ability to bind TIP were still able to strongly suppress PTGS which confirmed that the PTGS function of CP is independent of its interaction with TIP. In this work, the possible role of TCV as a suppressor of HR is investigated.

## **1.6 Previous work**

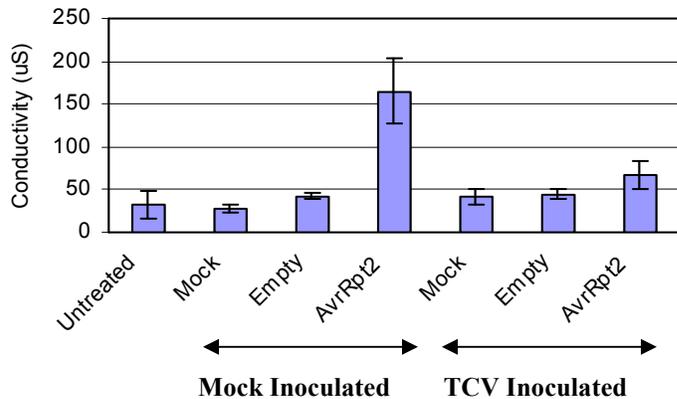
Previous work in our lab suggests that TCV is capable of suppressing the HR against itself, as well as against unrelated pathogens (Hammond 2001 and Mahadevan 2004; unpublished data). It was observed that upon TCV inoculation, the majority of resistant Di-17 plant developed the usual HR symptoms and remained disease free but a small number of inoculated plants developed systemic disease symptoms following HR reaction (Zhao *et al.* 2000). A possible

explanation for this could be that the virus is mutating inside the plants to become disease resistant and increase its range of pathogenesis. To test this hypothesis, total RNA from systemically infected Di-17 plant was extracted and reinoculated on Di-17 plants. This reinoculation largely resulted in HR on inoculated leaves and the plants were disease free, which ruled out this theory.

To investigate further, the symptomatic leaves from plants showing disease symptoms were tested for the presence of TCV. Interestingly, the virus was present in uninoculated leaves not showing the HR. This was confirmed by both RNA and protein analysis from the leaves (Hammond 2001).

*In-situ* hybridization and ds-RNA analysis showed that TCV is present and replicating in resistant plants without giving any HR response (Hammond 2001). This suggested that TCV is suppressing the HR. Suppressing the host defense response to win against host's defense system is a well documented strategy of bacterial avirulence proteins. Our data indicates that TCV is using this same strategy to successfully attack and invade the host.

TCV was also shown to suppress the HR initiated by bacterial avirulence factors AvrRpt2 and AvrRpm1. For comparing the extent of HR suppression, TCV and mock infected Col-0 plants were challenged with bacteria expressing avirulence factors. The extent of HR suppression was also quantified. The results confirmed the visual observation that the presence of TCV results in HR inhibition (Mahadevan 2004) (Figure 1.1).



**Figure 1.1 Statistical Analysis of *avrRpt2*-induced PCD in Col-0.** (Modified from Mahadevan 2004). The PCD induced by *AvrRpt2* in TCV and Mock-TCV inoculated plants. Bars represent the average of three independent trials consisting of nine total samples. Each group differs from the others at a confidence level of  $P = 0.05$ .

## 1.7 Proposed work

The suppression of host defense is a well known function of animal viruses (Hay *et al.* 2002). As mentioned earlier, many bacterial Avr factors have also been demonstrated to have this ability. But suppression of the HR by TCV is the first report of a plant virus suppressing the host defense. In this thesis, the TCV-plant interaction was studied further.

### Aim 1: Investigating Effect of TCV on HR initiation

One of the goals was to determine how TCV was affecting the HR response inside the host cell. An important question was whether the TCV is suppressing the HR by blocking the Avr factor from entering the plant cell.

*AvrRpt2* is a type III effector that is cleaved once it enters the plant cell (Mudgett *et al.* 1999). This N-terminal cleavage targets the Avr factor for subcellular localization and is important for recognition by the resistant factor RPS2 (Jin *et al.* 2003) which results in HR.

So, by monitoring the N-terminal cleavage of *AvrRpt2* in TCV infected plants, the effect of TCV on Avr's ability to penetrate the host cell can be demonstrated. If there is a change in the

AvrRpt2 expression or proteolysis in presence of TCV, it would establish that TCV is interfering with the bacteria's ability to trigger the HR in the plants. If there is no effect of TCV on Avr expression and proteolysis, it will show that the TCV is acting at a downstream step in defense associated signal transduction pathway to inhibit the HR.

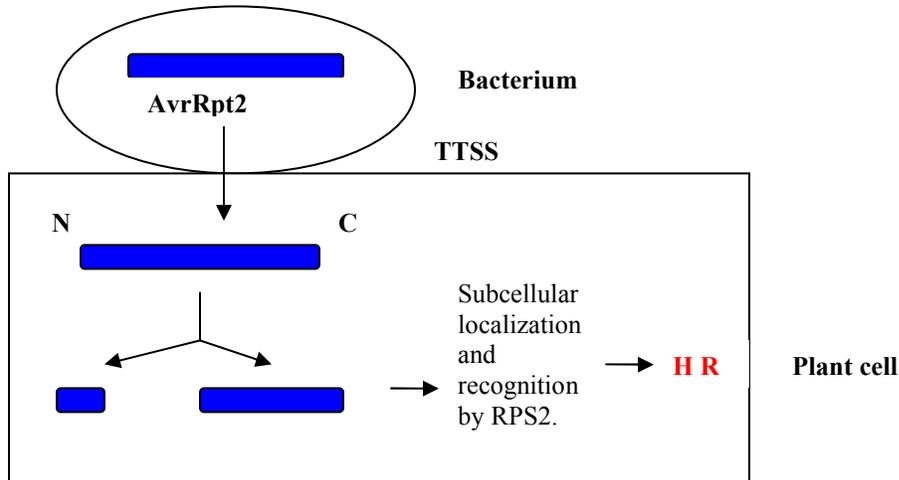


Figure 1.2 A simple model explaining the intracellular proteolysis of AvrRpt2

## Aim 2: Determine if TCV can suppress HR in *Nicotiana benthamiana*

We wanted to use an Agrobacterium-mediated transient expression assay in *Nicotiana benthamiana* for studying ability of the individual TCV genes to suppress the HR. Before testing the individual TCV genes it was necessary to ensure that TCV can suppress HR in *N. benthamiana*.

The transient assay system was used by Abramovitch *et al.* (2003) to demonstrate the cell death suppression ability of bacterial Avr factor AvrPtoB from *Pseudomonas syringae* pv. *tomato*.

During the transient assay a foreign gene is transferred into plants using Agrobacterium as a biological vector. Agrobacterium was discovered in 1907, as a soil phytopathogen causing crown

tumors on dicotyledonous plants by transferring DNA into the plant's cell nucleus. The three components essential for these genetic transformations are the T-DNA (transferred DNA) region, which is transferred from bacteria to the plant; the *vir* region which encodes seven different proteins (*virA*, *virB*, *virC*, *virD*, *virE*, *virG* and *virH*) responsible for generating a copy of T-DNA region and mediating the cross kingdom transfer. The third component is the *chromosomal virulence (chv)* gene responsible for attachment to the wounded plant cell (reviewed in Zupan *et al.* 2000, Sheng and Citovsky 1996, Citovsky *et al.*, 1992). The proteins encoded by *vir* genes respond to specific compounds secreted by wounded plants. Acetosyringone, a phenolic compound is a potent inducer of these proteins. The presence of sugars and acidic pH also encourage the T-DNA transfer.

To induce the cell death, the AvrPto and Pto combination was used. The R and Avr genes were cloned into *Agrobacterium tumefaciens* compatible binary vector (a gift from G B Martin) and used in transient assay. The dual advantage of binary vector system is that with T-DNA on one plasmid, it can be easily manipulated in *E. coli* (because of its size) and later it can be transformed into *Agrobacterium* carrying the second plasmid with *vir* region (Hoekema *et al.* 1984, Hoekema *et al.* 1983).

After transient expression, the effect of TCV on cell death induced by R and Avr combination was quantified by ion leakage assay. During programmed cell death, the plasma membrane undergoes irreversible damage which results in leakage of electrolytes from the cells. For ion leakage assay, the time point at which the electrolyte leakage is at maximum was determined and extent of cell death in presence and absence of TCV was compared by measuring the conductivity of leaf samples undergoing cell death.

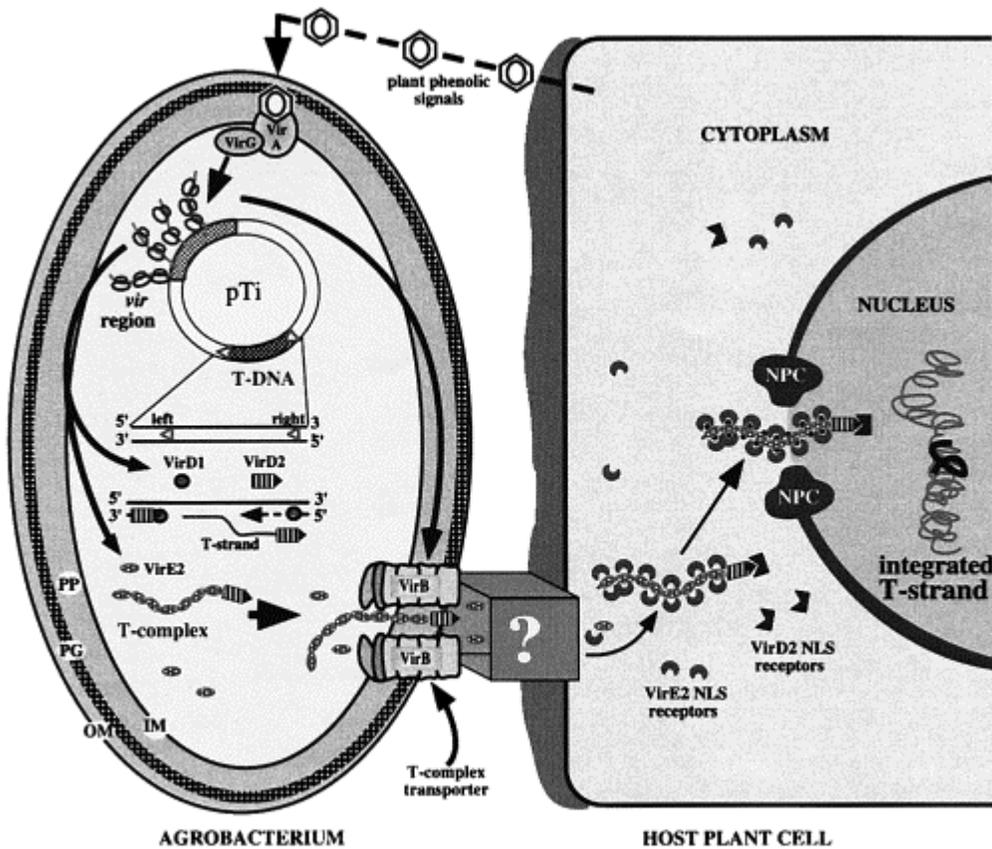


Figure 1.3 Schematic representation of *Agrobacterium* transformation in plants. The figure is taken from Zupan *et al.* 2000. OM=outer membrane, PG=peptidoglycan cell wall, PP= periplasm, IM= inner membrane, NPC= nuclear pore complex.

### Aim 3: Identifying TCV gene responsible for cell death suppression:

To identify the TCV gene responsible for suppressing the cell death, individual TCV proteins were coexpressed with AvrPto and Pto combination by transient assay. The extent of cell death was quantified and compared by ion leakage assay.

## 2 MATERIALS AND METHODS

### 2.1 Plants growth conditions

*Arabidopsis thaliana* Col-0 plants were grown on Metro Mix 360 (Sun grow horticulture distribution Inc., Bellevue, WA) in Percival scientific AR-60L growth chambers. The chambers were set for 16 hours of photoperiod at 23° C and 8 hr dark period at 21° C. Before adding the seeds to the soil, the soil was saturated with water. For planting the seeds in soil, seeds were suspended in water and added to the soil with a Pasteur pipette with the aim of having nine plants in each pot. After adding the seeds to the flats, it was covered with plastic sheet to enhance germination. The plastic was removed after the appearance of seedlings. Plants were watered as needed. After two weeks of growth, Miracle Grow at 0.35 g/L was added to tap water during watering. For watering, the flats carrying the plants were placed in trays filled with tap water. The plants were thinned before the appearance of four true leaves. Thinning ensured that the plants in each pot were equal in number and at equal stages of growth.

*Nicotiana benthamiana* plants were grown in similar conditions but at 28° C. The seedlings were transplanted to individual pots of bigger size. Axillary meristems were removed from the plants to ensure growth of leaves. Plants were watered as needed and supplemented with around 0.35g/L Miracle Grow about once every week after the first four weeks of growth. The seeds were collected by handpicking followed by sieving to remove dried flowers.

### 2.2 Viral inoculation

All TCV inoculations were performed with approximately 0.02 µg/µl total RNA in 1X inoculation buffer (Appendix C, modified from Dempsey *et al.*, 1993). In *Arabidopsis*, inoculations were performed on four older, fully expanded true leaves 16 days post planting. In

*N. benthamiana* a middle aged leaf on 4 weeks old plant was inoculated. 2-10  $\mu$ L aliquots of inoculum were pipetted onto a piece of parafilm and a sterile glass stirring rod was dipped into it and rubbed onto the adaxial side of true leaves while supporting the leaf with the index finger. Plants were left in the growth chamber to allow progression of TCV. 1X inoculation buffer was used as a negative control in all the experiments.

### **2.3 Bacterial infiltration in *A. thaliana***

For inducing HR in *A. thaliana*, bacterial strain Pst DC3000 carrying AvrRpt2 (gift from Ausubel lab) was used. It was grown overnight at 28° C, in NYGB medium (appendix C) with 25  $\mu$ g/ml Kan until it reached an  $A_{600}$  between 0.5-0.9. The bacterial culture was spun down at 4000 x g (Sorvall™ GSA rotor) for 10 min at 4°C. The bacterial pellet was then resuspended in 5 mL of sterile 10mM MgSO<sub>4</sub> and re-centrifuged. The resulting pellet of bacteria was dissolved in sterile 10mM MgSO<sub>4</sub> to a final  $A_{600}$  of 0.2. Bacterial samples were pressure infiltrated with needle-less 1 mL syringe into leaves, 10 days post viral inoculation. The bacterium was infiltration on both the sides of midvein as the midvein prevents the bacteria from spreading. The plants were moved back to the growth chamber and bacterially infiltrated leaves were collected 10 hours post infiltration for immunoblot analysis.

### **2.4 Soluble protein extraction and immunoblot analysis**

For AvrRpt2 expression from bacteria, Pst DC3000 was grown overnight at 28° C, in 50 ml NYGB medium and kanamycin (25  $\mu$ g/ml). For induction, cells were collected by centrifugation at 4000 x g and resuspended in minimal media (Appendix C) lacking antibiotics (Mudgett *et al.* 1999). It was grown in minimal media for 15 hours at 21°C in a rotary shaker.

For protein extraction cells were collected by centrifugation and resuspended in protein

extraction buffer (Appendix C)). It was sonicated 5 times for 30 seconds each and centrifuged at 14,000 rpm for 40 minutes.

To extract proteins from leaves, around 1.5-2 g of leaf tissue in liquid nitrogen was ground into a fine powder using a mortar and pestle. It was suspended in protein extraction buffer. SDS was added to a final concentration of 0.1% for further cell lysis and incubated for 15 min on ice with vortexing every 5 minutes. Differential centrifugation at 4° C was used to collect the soluble protein. It was centrifuged at 3000 x g for 10 minutes to remove unbroken cells and nuclei. After getting rid of the pellet, supernatant was centrifuged at 7000 x g for 20 minutes. It was centrifuged at 14000 x g for 40 minutes to remove chloroplast, mitochondria etc. The soluble protein was further concentrated up to 500 µL by amicon filters (10 KDa MWCO) (Catalog # UFC801008).

Protein gel electrophoresis and immunoblotting was carried out as in Sambrook *et al.* (1989). Protein samples were electrophoresed on 12.5 % polyacrylamide gels and transferred to PVDF membrane (Catalog # IPVH 304 FO, Millipore; sambrooks *et al.* 1989). AvrRpt2 and TCV CP were detected by using rabbit polyclonal anti-N-His6-AvrRpt2 sera (1: 1000 dilution) and anti-TCV coat protein (1:1000 dilution) respectively. Anti-rabbit Ig conjugated to horseradish peroxidase from donkey was used to detect the primary antibody. Detection of immunoblot was carried out with chemiluminescent kit (Catalog # 34080, Pierce)

## **2.5 RNA extraction and RT-PCR**

*N. benthamiana* leaves were collected 13 days post viral inoculation and total RNA was extracted using Purelink plant RNA reagent from Invitrogen ( Cat. # 12322-012) as per the manufacturer's protocol. The RNA pellet was dissolved in RNase free water (Qiagen) and

quantified at A<sub>260</sub> using a spectrophotometer. RNA gel electrophoresis was carried out as mentioned in Sambrook *et al.* (1989).

Reverse transcription (RT) was carried out with p38 (coat protein) using p38 gene specific primer (5'-AATTCTGAGTGCTTGCAATT5'TACCC-3') and Omniscript reverse transcriptase (Qiagen Cat. # 205113), as per the manufacturer's protocol. PCR was followed with *Taq* DNA polymerase with finished reverse transcription reaction as template. Volume of the template was 10 % of the final volume of PCR reaction. The forward primers used for the PCR reaction was (5'-ATGGAAAATGATCCTAGAGTCCGG-3') and the same reverse primer was used as in RT reaction.

## 2.6 Cloning Strategy

Individual ORFs were amplified by PCR using pT1d1ΔL as a template. Each gene was first cloned into pCRII vector (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol (Cat. # K2050-01). The primers used for cloning TCV ORF's into pCRII vector are:

| TCV ORF | Forward Primer                 | Reverse Primer               |
|---------|--------------------------------|------------------------------|
| p8      | 5'-ATGGATCCTGAACGAATTCCC-3'    | 5'-GCACTAGTTTTCCAGTCTAATG-3' |
| p38     | 5'-ATGGAAAATGATCCTAGAGTCCGG-3' | 5'-GAGGATCCACTATTACCGTAC-3'  |

**Table 2.1 List of Primers used for amplifying TCV genes**

For cloning into pBTEX, the pCRII clones and the pBTEX vector were digested with *Xba* I and *Kpn* I. The digested fragments were run on low melt agarose to check for linearity and purified from the gel using Spin-X columns (costar, Cat. # 8161). The vector and insert were ligated using T4 DNA ligase (NEB, Cat. # MO202T).

Colony screening for pCRII clones was performed by PCR using the forward primer for the insert and T7 promoter primer for the vector (5'-TAATACGACTCACTATAGGG-3'). For

pBTEX clones either restriction digestion or PCR with forward and reverse primer of the insert was used (Table 2.1). To check the result of PCR reaction and the progression of restriction digestion, DNA gel electrophoresis was carried out as in Sambrook *et al* (1989).

## 2.7 PCR

The PCR reaction was carried out in a 0.2 ml tube from USA scientific (Cat. # 1402-2900). The total volume of reaction was always 50  $\mu$ L. The reaction was set up as mentioned below:

|                              |                         |
|------------------------------|-------------------------|
| 50 $\mu$ M Forward Primer    | 1 $\mu$ L               |
| 50 $\mu$ M reverse Primer    | 1 $\mu$ L               |
| 10 mM dNTP                   | 1 $\mu$ L               |
| 10X Buffer                   | 5 $\mu$ L               |
| Taq polymerase (NEB, MS267S) | 0.2 $\mu$ L (5000 U/ml) |
| or                           |                         |
| Deep Vent( NEB, MO258S)      | 0.5 $\mu$ L (2000 U/ml) |
| DNA template                 | 40-80 ng                |
| Sterile water                | up to 50 $\mu$ L        |

Amplification was performed using Perkin PCR system 9600 with the following conditions:

1. 95° C for 5 minutes
2. 95° C for 15 seconds
3. 55° C for 30 seconds
4. 72° C for 3 minutes
5. Repeat steps 2 to 5 for 35 times
6. 72° C for 10 minutes
7. 4° C hold

## 2.8 Restriction Enzyme digestion

It was carried out as per NEB catalogue (2005-2006). For sequential digestion with *Kpn I* and

*Xba I*, the restriction digestion was first performed with *Kpn I*. After checking for linearity, the salt concentration of the reaction mixture was adjusted up to 50 mM with 500 mM NaCl stock solution. Then the *Xba I* enzyme was added and incubated at 37° C for the reaction to proceed further.

## **2.9 Preparation of chemically competent cells**

The *E. coli* Top10F` cells were grown on LB plates (without any antibiotics) at 37° C. A single colony was used to inoculate 25 ml of LB media and grown overnight with vigorous shaking. Next day the culture was transferred to 250 ml LB in a 1L flask. It was grown until the  $A_{600}$  reached 0.6. The cells were collected by centrifugation at 4000 x g at 4° C. The pellet was suspended in 100 mL ice cold sterile  $CaCl_2$ . The cells were chilled on ice for 10 minutes and then spun for 5 minutes at 4000 x g at 4° C. The cells were resuspended in 20 ml ice cold 20% glycerol- 50 mM  $CaCl_2$ . 1 ml aliquots were distributed into sterile eppendorf tubes, flash frozen in liquid nitrogen and stored at - 80° C.

## **2.10 Transformation of chemically competent cells**

Transformation was carried out as per Invitrogen manual (cat. # K2050-01) with SOC Media (Appendix C).

## **2.11 Minipreparation**

Plasmid DNA was isolated from small scale (5 ml) *E. coli* cultures by alkaline lysis. The protocol was modified from Sambrook *et al.* (1989). If needed the minipreparation was quantified by running on agarose gel with DNA standards (25 ng/ $\mu$ L, 50 ng/ $\mu$ L and 100 ng/ $\mu$ L). The DNA gel electrophoresis was carried out as mentioned in Sambrook *et al* (1989).

## **2.12 Maxipreparation**

Plasmid DNA was isolated from large scale (100 ml) cultures by treatment with alkali and SDS. The resulting DNA preparation was purified by precipitation with polyethylene glycol (Sambrook *et al* 1989). The DNA was quantified at  $A_{260}$  using a spectrophotometer and checked for protein contamination at  $A_{280}$ . The DNA preparation was run on agarose gel to check the integrity of the samples.

## **2.13 Preparation of electrocompetent *Agrobacterium***

*Agrobacterium tumefaciens* strain GV2260 was grown overnight in 5 ml of LB media with 150 mg/L rifampicin at 28° C. Next day 500 ml of LB with rifampicin was inoculated with 400  $\mu$ l of overnight culture. The cells were grown to an absorbance of about 0.5 (16-18 hours). To harvest the cells, the flask was chilled on ice for 20 minutes and transferred to centrifuge bottle and centrifuged for 15 minutes at 4000 x g, 4° C in Sorvall RC-5B centrifuge. From this stage the cells were kept cold throughout the preparation. The supernatant was removed and cells were resuspended in 500 ml of 1 mM Hepes pH- 7.4 and centrifuged again. The cell pellet was resuspended in 250 ml of 1 mM Hepes and recentrifuged. This time the cells were resuspended in 10 ml Hepes and centrifuged at the same conditions. Finally, the cells were suspended in 2 ml ice-cold 10% glycerol and 40  $\mu$ l aliquots were distributed in 1.5 ml eppendorf tubes. It was flash frozen with liquid nitrogen and stored at - 80° C.

## **2.14 *Agrobacterium* transformation by electroporation**

Around 25  $\mu$ L of cells were gently mixed with 80 ng of DNA (not exceeding 2  $\mu$ L volume) in a sterile eppendorf tube. It was suspended between the electrodes of the electroporation chamber (Life Technologies, Rockville MD). After placing the chamber in the ice cold water bath, the

apparatus was charged to approximately 420 V and discharged (The final voltage usually reached ~2.4 kV due to voltage booster). The capacitance was adjusted at 300 mA and the resistance was set at 4k ohms. The cells were immediately removed to 1.5 ml eppendorf tube and 1 mL LB media was added without any antibiotics. They were incubated at room temperature (RT) for 1 hour without shaking. The cells were then plated on LB media with 50 mg/L kanamycin and 100 mg/L rifampicin and incubated at 28° C for 2 days. For starting the culture, a smear of colonies were used to inoculate 5 ml of LB (with kanamycin and rifampicin) and grown at 28° C. This culture was used to make 20% glycerol stock and stored at 80° C.

## **2.15 Transient expression assay**

For the transient expression assay, first the *Agrobacterium* strains was grown on LB plates with antibiotics and then multiple colonies were used to inoculate 5 ml of LB media (with 100 mg/L rifampicin and 50 mg/L kanamycin). It was grown on a rolling drum at 28°C overnight. The cultures were transferred in a 15 ml Falcon tube and spun down at 5000 x g for ten minutes in the Sorvall RC-5B centrifuge (Sorvall GSA rotor) and then resuspended in 5 mL of induction medium (Appendix C). The 200 mM acetosyringone in DMSO was prepared just prior to use and 500 ul from it was added and centrifuged again. The pellet was resuspended in 5 ml of induction media.

This culture was used to inoculate a 50 ml volume of induction media with 50 mg/L kanamycin (rifampicin is not required at this step), in a 250 ml flask and was then cultured overnight in the shaker at 28°C. The next day, the cultures were spun down in 50 ml Falcon tubes at 4000 x g for five minutes and resuspended in 40 ml of 10 mM MES with 200 uM acetosyringone. The cultures were spun down again at 4000 x g for five minutes and then resuspended in 30 ml of 10 mM MES with 200 uM acetosyringone. To find the absorbance of

the culture, a 1:10 dilution was made. The final absorbance at 600 nm was adjusted to 0.4, with 10 mM MES and 200  $\mu$ M acetosyringone as the diluting agent. For cell death suppression assay, agro cultures carrying individual AvrPto, Pto and the target TCV gene were mixed in 1:1:1 ratio.

For the transient expression assay, middle-aged leaves of about the width of a hand length were selected. Small circles, the size of a quarter were drawn on leaves. A needle was used to make a hole at the center of each circle and the agro mixture was delivered through the hole with a 1-ml needle-less syringe. During leaf infiltration, the center of the circle on leaf was supported with the index finger. Several duplicates of samples were made at different leaf positions. Plants were moved to 24 hour light and moderate temperature conditions and monitored daily for cell death suppression phenotypes. Leaves were scored 8 dpi for final analysis of cell death suppression.

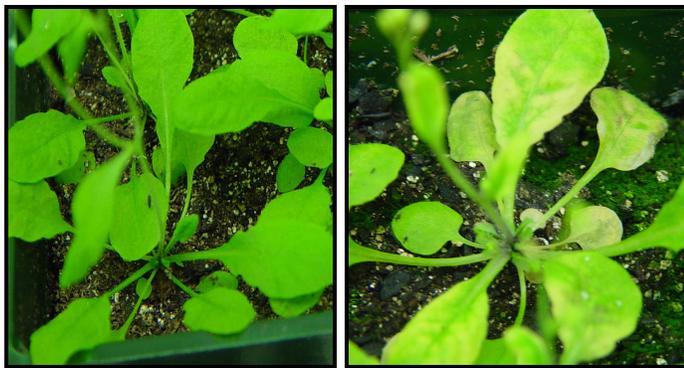
## **2.16 Ion leakage assay**

From *N. benthamiana*, leaf discs of 6-mm diameter were collected using a cork borer. The leaf samples were collected carefully to prevent excess damage to the leaf tissue. Leaf discs were floated on 2 ml ultra-pure water (high resistivity) with abaxial sides towards the solution. Samples were incubated at room temperature for 4 hours and conductivity was measured with a Cole-Parmer® 19815-00 Basic Conductivity Meter, calibrated with Traceable One-Shot™ Conductivity Calibration Standard (Control Company, Friendswood TX). Before taking the reading, the sample was stirred with the probe to create a homogenous sample.

## 3 RESULTS

### 3.1 TCV does not interfere with HR initiation

TCV has been shown to suppress the HR initiated by unrelated bacterial avirulence proteins which use the TTSS to attack and colonize the host (Hammond 2001 and Mahadevan 2004). There is a possibility that since TCV is already present in the host cell, it is blocking the ability of the bacteria to successfully attach the host cells. AvrRpt2, a type III effector protein undergoes N-terminal cleavage inside the plant cell and this processing is important for recognition by the resistant protein RPS2 and initiating signal transduction pathways resulting in HR (Jin *et al.* 2003). The N-terminal processing of the 28 kDa AvrRpt2 results in 20.7 kDa protein (Mudgett *et al.* 1999). To investigate whether TCV was inhibiting the HR by interfering with the expression or the N-terminal cleavage of AvrRpt2, expression and proteolysis of AvrRpt2 in TCV infected plants was analyzed. For this, 16 day old Col-0 Arabidopsis plants were inoculated with TCV or 1X inoculation buffer (mock-TCV inoculation).



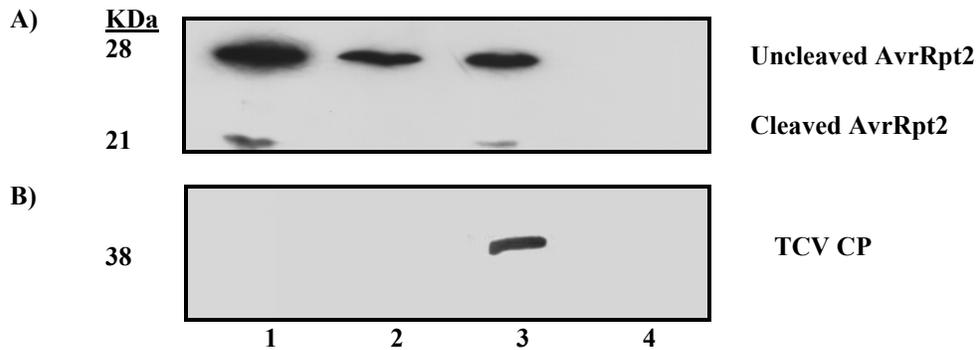
A)

B)

**Figure 3.1 Arabidopsis inoculated with inoculation buffer and TCV**  
16 days old Col-0 was inoculated with (A) 1X inoculation buffer and (B) TCV. The disease symptoms indicating successful viral attack like chlorosis and leaf deformation were visible one week post inoculation. The picture was taken 10 days post inoculation.

At 10 days post inoculation (dpi), when chlorosis, leaf deformation and other disease symptoms were visible (Figure 3.1 B), leaves were pressure infiltrated with *Pst* DC3000 (*AvrRpt2*) to induce the HR. Leaves were collected 10 hours post inoculation (hpi) and soluble protein from leaves sample were extracted and analyzed by immunoblot analysis with anti-*AvrRpt2* antibodies (Figure 3.2 A). As a negative control, soluble protein from uninfected Col-0 leaves was used (Figure 3.2 A- lane 4). Uncleaved *AvrRpt2* was extracted from induced *Pst* DC3000 for use as a positive control (Figure 3.2A- lane 2). The *AvrRpt2* expressed in bacteria was expected to be uncleaved, since the proteolysis occurs inside the plant cell (Mudgett *et al.* 1999). Both sets of bacterially infected leaves showed high level of uncleaved *AvrRpt2*, with only a small fraction cleaved to the smaller form (Figure 3.2 A- lane 1). However, the presence of TCV (Figure 3.2 A- lane 3) does not change the percentage of protein being cleaved. Densitometric analysis revealed virtually the same ratio of cleaved and uncleaved protein in both TCV-infected leaves (Figure 3.2 A- lane 3) and the virus free leaves (Figure 3.2 A- lane 1). The result of densitometric analysis has been summarized in Table 3.1. Thus, the infected plant cell retains its ability to cleave the avirulence factor. This suggests that TCV might be acting further downstream in signal transduction pathway to inhibit the HR.

To confirm the presence of virus, an identical gel was subjected to immunoblot analysis with anti-TCV CP antibodies (Figure 3.2 B). The 38 KDa TCV CP was detected in TCV and bacterially inoculated leaves sample (Figure 3.2 B- lane 3). Not surprisingly, CP was not present in the mock inoculated sample (Figure 3.2 B- lane 1). Again, protein samples from untreated leaves and induced *Pst* DC3000 (*avrRpt2*) were used as controls (Figure 3.2 B- lane 4 and 2).



**Fig. 3.2 Intracellular processing of AvrRpt2 in presence of TCV.**

TCV and mock inoculated Arabidopsis leaves were pressure infiltrated with *Pst* DC3000(*avrRpt2*) and leaves were collected 10 hpi. (A) Immunoblot analysis of protein samples from leaves and bacteria with AvrRpt2- specific antibodies. Lane 1- Proteins from Mock inoculated leaves (45 µg total protein), Lane 2- Protein from induced *Pst* DC3000 (*avrRpt2*), (40 µg total protein), Lane 3- Protein from TCV inoculated leaves (45 µg total protein), Lane 4- Protein fraction from untreated leaves, as a negative control (45 µg total protein). (B) To confirm the presence of TCV in leaves, the protein samples (same as in blot A) were probed with anti-TCV CP antibodies.

**Table 3.1 Summary of spot densitometry analysis.**

| TCV | Relative ratio<br>(uncleaved: cleaved) <i>avrRpt2</i> |
|-----|---|
| +   | 4.75  |
| -   | 4.62  |

### 3.2 TCV suppress the HR in *Nicotiana benthamiana*

With the eventual goal to use an agrobacterium-mediated transient assay to identify TCV genes responsible for HR suppression, the first step was to investigate whether TCV can suppress the HR in *Nicotiana benthamiana*. For this, plants were either inoculated with TCV or as a control with 1X inoculation buffer (mock-TCV inoculation). For viral inoculation, middle aged leaves were used. Since the virus spreads from infected to young tissues, the viral symptoms (leaf wrinkling, stunt growth, mottled appearance) were visible on middle and young aged leaves (Figure 3.3 B) and the old, uninoculated leaves showed no symptoms. To confirm the visual

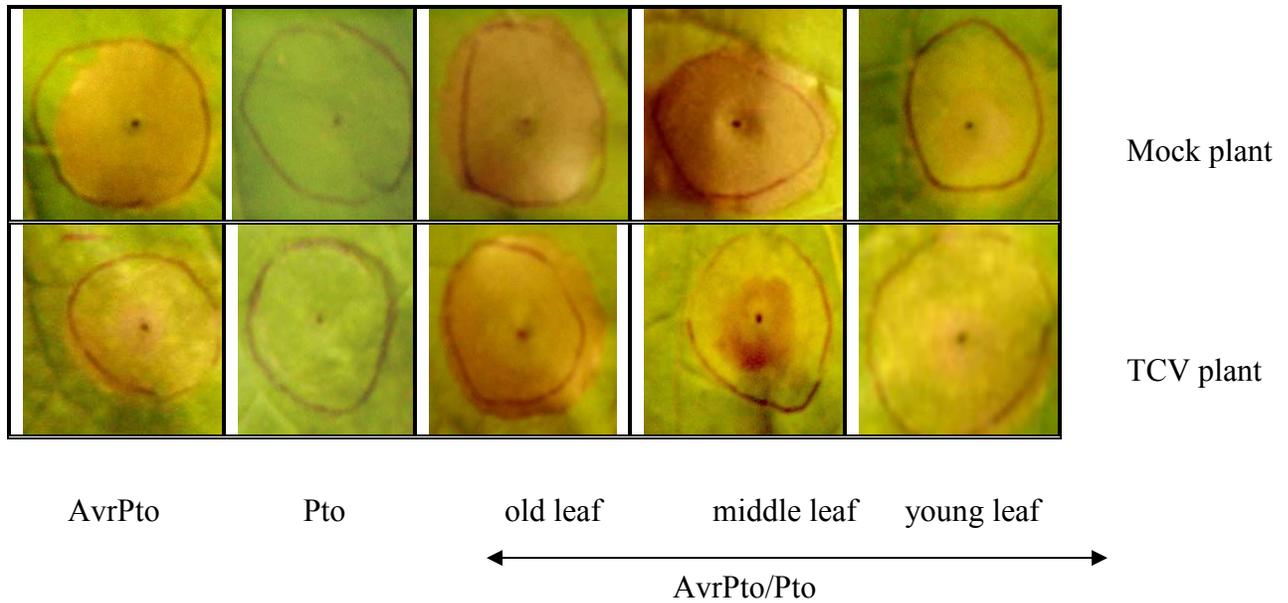
observation that the virus is present in only middle and young aged leaves, RT-PCR was used (Figure 3.4).



**Figure 3.3 Mock and TCV inoculated *Nicotiana*.** Four week old *N. benthamiana* were inoculated with (A) IX inoculation buffer and (B) TCV. The viral symptoms like stunt growth, mottled appearance, and leaf wrinkling and vein discoloration were visible two weeks post viral inoculation. The picture was taken 13 days post viral inoculation.

Young, middle and old leaf samples were collected from mock and TCV inoculated plants, 13 days post inoculation (dpi) and RNA was extracted. For RT-PCR, gene specific primers for CP were used. As a positive control (Figure 3.4- lane 8) RNA from TCV infected turnip leaves was used. The RT-PCR confirmed that the virus is actively replicating in the middle and young leaves in the TCV infected plant (Figure 3.4- lane 3 & 4) and absent in old, uninoculated leaves (Figure 3.4- lane 1).





**Figure 3.5 Transient expression assay to study the effect of TCV on PCD.** Four week old *N. benthamiana* plants were mock and TCV inoculated. 28 dpi leaves were infiltrated with AvrPto and Pto combination to induce the cell death. The leaves were observed for cell death symptoms up to 8 dpi and scored visually. The pictures were taken on 8 dpi.

As a control, leaves were infiltrated with individual Agro strains carrying AvrPto and Pto. The plants were moved to 24 hour light period at room temperature for observation. The leaves of TCV and mock plants were inspected visually at 12 hour intervals for difference in cell death phenotype up to 8 days post infiltration.

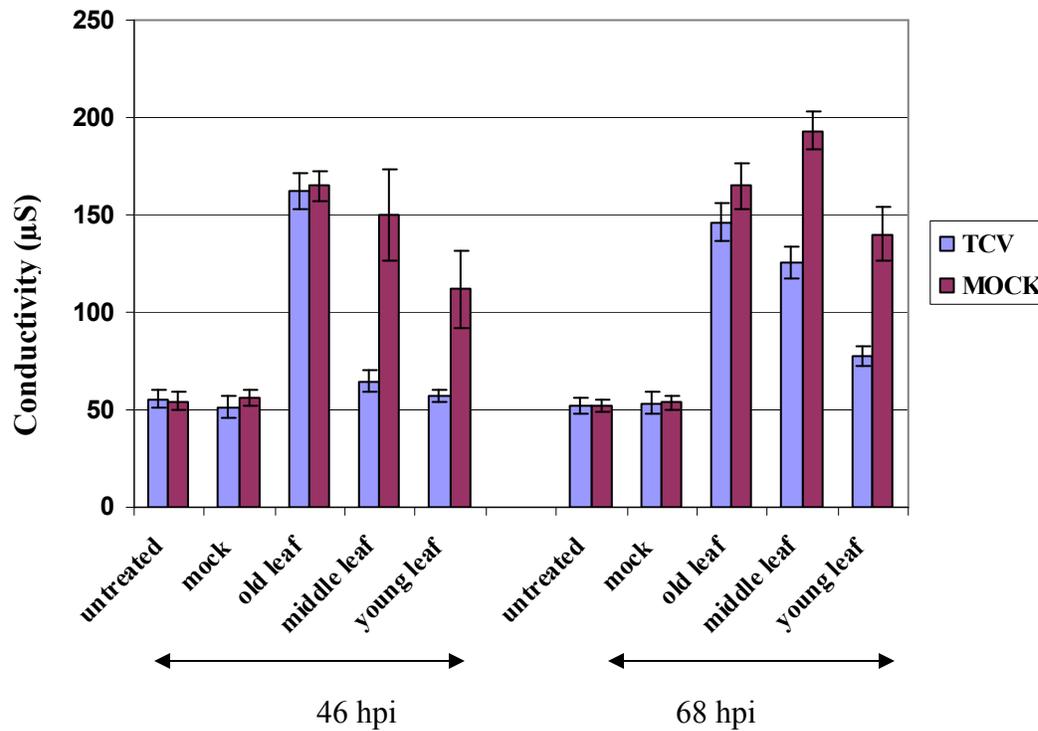
The maximum cell death was observed on 8 dpi on mock plants (Figure 3.5). As expected, the old leaves on both TCV infected and mock-TCV infected plants showed substantial cell death, since the virus was not present in either of the leaves. On young leaves, less cell death was visible on both TCV infected and mock-TCV infected plants. This suggests that the new leaf tissues were more resistant to bacterial invasion, which resulted in less PCD. The biggest difference was observed on middle aged leaves. The TCV-free leaves showed extensive cell death throughout the zone of infiltration. However, the TCV-infected leaf showed a small zone of cell death in the center and the remaining infiltrated zone appeared to be alive. Leaves were scored visually for cell death phenotype on 8 dpi.

## Quantitative analysis of HR suppression

To quantify the extent of PCD suppression by TCV, cell death was measured in terms of ion leakage. As cells die, the cell membranes break down allowing leakage of cellular contents to the surroundings. The ion leakage can be quantified by floating the leaf samples on high resistivity water and measuring the conductivity. Leaves undergoing greater cell death give a higher conductivity value.

From the visual scoring of cell death induced by AvrPto/Pto combination in *N. benthamiana* plants, it was observed that the cell death phenotype appears between 2<sup>nd</sup> and 3<sup>rd</sup> day on middle aged leaves, but since the cellular events will start earlier and electrolyte leakage precedes the physical symptoms of cell death, it was important to determine the time at which ion flux was at maximum. As the ion leakage was expected to happen between 2<sup>nd</sup> and 3<sup>rd</sup> day, the leaf samples were collected at 30 hpi, 46 hpi, 68 hpi, 80 hpi. (Appendix A.1). The peak ion fluxes were observed at 46 hpi and 68 hpi (Figure 3.6).

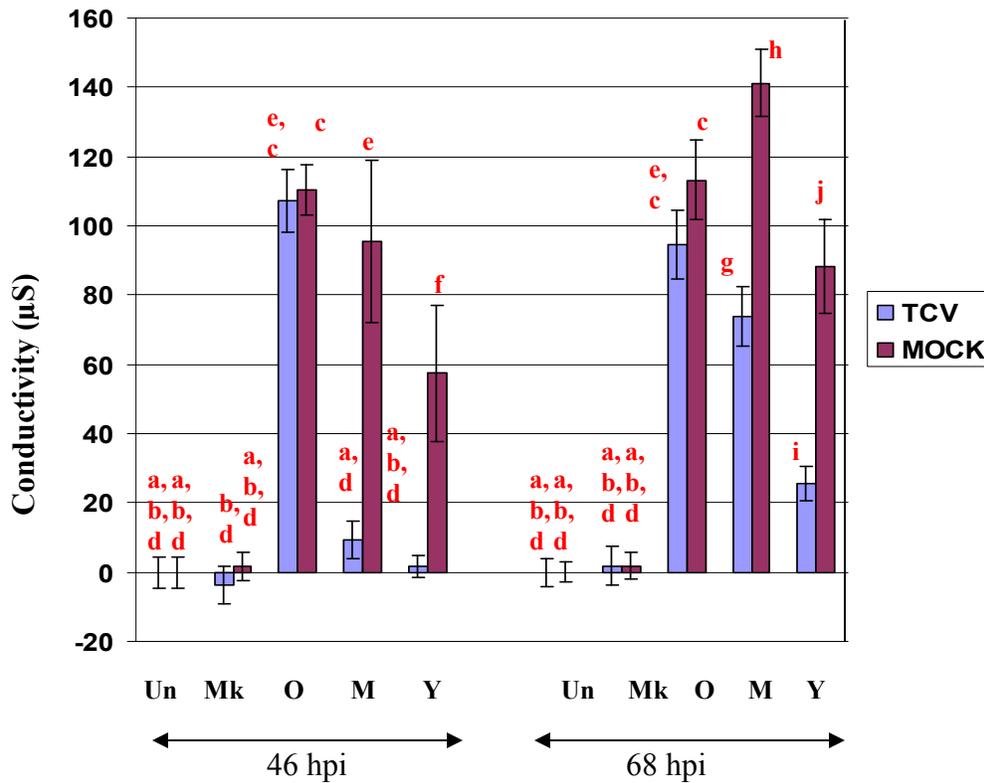
Since there is always mechanical damage to leaf tissues at the periphery of the leaf punches used for conductivity determination, even the virus and bacteria free leaf tissues showed some conductivity levels. Therefore, untreated leaf samples were used as a negative control for ion leakage assay. Leaves samples infiltrated with inoculation buffer (MES) were used as another negative control. The ion leakage data were collected from three independent trials with a total of nine samples from each category (Figure 3. 6, Appendix A.2). Each sample consisted of two leaf discs of 6-mm diameter each.



**Figure 3.6 Quantification of AvrPto/Pto induced PCD in TCV and mock inoculated plants by Ion Leakage.** *Nicotiana benthamiana* plants were either inoculated with TCV or mock inoculated with 1X inoculation buffer. On 28 dpi, leaves were agroinfiltrated with avrPto and Pto. Leaf punches were analyzed for conductivity after 46 hpi and 68 hpi. Bars represents mean of conductivity values from three independent trials consisting of nine samples. Error bar shows 95% confidence level.

To calculate the difference in cell death, the background ion leakage observed in untreated samples was subtracted from all the samples (Figure 3.7). Interestingly, the cell death suppression in young leaves was slightly greater than in comparison with middle aged leaves. At 46 hpi, the presence of TCV in middle aged leaf tissues resulted in a 9 fold decrease in conductivity, whereas in young aged tissues there was a around 12 fold decrease in cell death. A Significant decrease in cell death was also observed at 68 hpi. In young leaf tissues the presence of TCV resulted in a 4 fold decrease in cell death, whereas in middle aged leaves there was 2 times decrease in cell death by TCV.

Statistical analysis was performed to compare the difference in mean conductivity values of different samples (Figure 3.7). The statistical analysis confirmed the decrease in conductivity in presence of TCV. Thus, TCV suppresses the HR in *N. benthamiana* plants. This allows the use of *N. benthamiana* in transient assay to determine which TCV gene is responsible for cell death suppression.

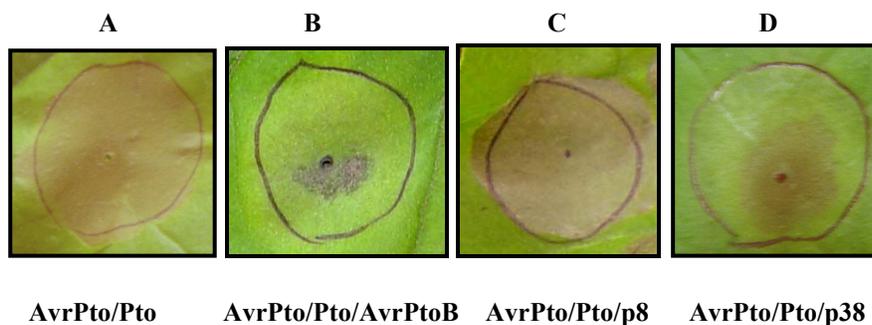


**Figure 3.7 Statistical analysis of PCD in TCV and mock-TCV inoculated plants.** Bars represent mean of conductivity values from three independent trials consisting of nine total samples. The mean values was plotted after deducting the background conductivity observed in untreated samples. Letters above bars represent distinct significance groups, determined by Tukey-Kramer method for multiple sample comparisons. Error bar shows 95% confidence level. The statistical analysis was performed with SAS statistical software. (Un- untreated leaves, Mk- Mock (MES) inoculated leaves, O-old aged leaves, M-middle aged leaves, Y-young aged leaves).

### 3.3 Identifying TCV gene responsible for HR suppression

To identify the TCV gene responsible for HR suppression, individual TCV genes, p8 (movement protein) and p38 (coat protein) were expressed with avrPto and Pto. AvrPtoB was used as a positive control as it is a known cell death suppressor and has been shown to inhibit AvrPto/Pto-mediated PCD in *N. benthamiana* plants (Abramovitch *et al.*, 2003). The assay was carried out in middle aged leaf tissues.

In this experiment the cell death inducing combination of AvrPto and Pto were mixed with Agrobacterium carrying individual TCV genes in 1:1:1 ratio. As the peak ion fluxes in the previous experiments were observed at 48 hpi and 68 hpi in middle aged leaves, same time points were used to quantify the cell death extent. As expected, the co-expression of AvrPto and Pto resulted in considerable cell death throughout the zone of infiltration (Figure 3.8 A) and AvrPtoB was successful in reducing the cell death (Figure 3.8 B) induced by them.



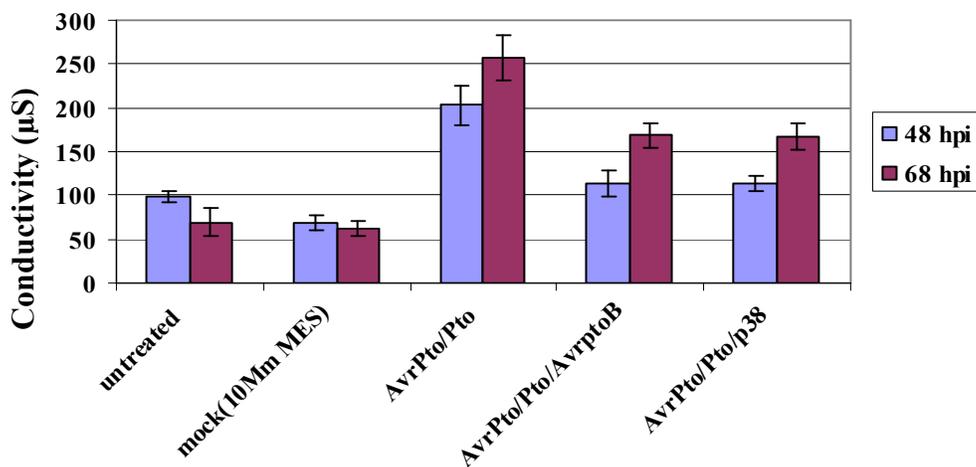
**Figure 3.8 Transient expression assay to identify the TCV gene responsible for cell death suppression.** To identify the TCV gene responsible for HR suppression, two of the TCV genes p8 and p38 were co-infiltrated with AvrPto and Pto combination. AvrPtoB was used as a positive control for cell death suppression. The p38 was identified as the gene suppressing the cell death. OD of the bacterial cultures expressing the *AvrPto*, *Pto* and target TCV genes was adjusted to 0.4 for leaf infiltration. The pictures were taken 8 dpi.

The transient assay showed that the co-expression of movement protein p8 with the AvrPto and Pto combination resulted in no visible change in the cell death (Figure 3.8 C). The level of

visible cell death induced by AvrPto and Pto was similar in the presence and absence of p8 throughout the zone of infiltration. However, the coat protein p38 was able to limit the AvrPto/Pto-mediated cell death to a greater extent (Figure 3.8 D). The cell death was observed only around the center of zone of infiltration and stopped after that. The leaves were scored visually on 8 dpi and pictures were taken.

### Quantitative evidence of PCD suppression by TCV CP

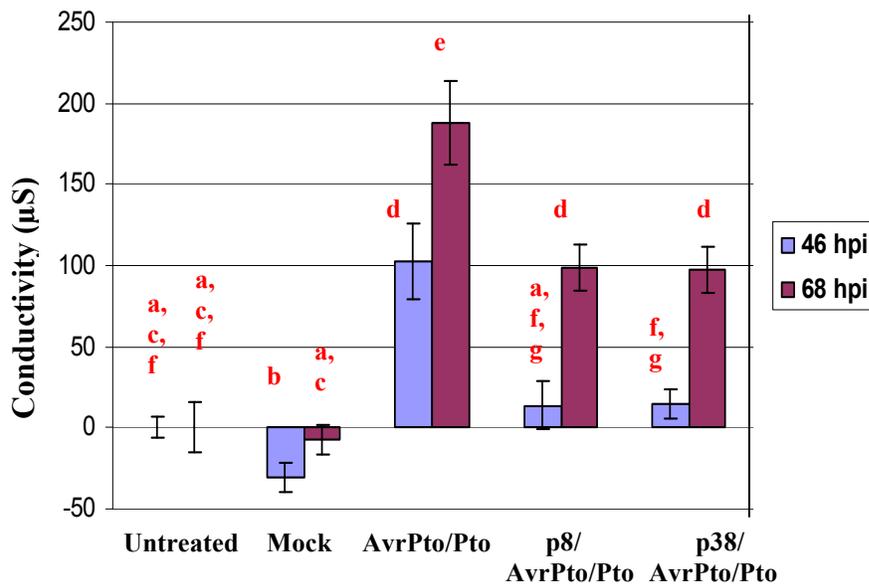
The ion leakage assay further confirmed the visual observation that TCV CP was suppressing the cell death. Leaf samples co-infiltrated with the AvrPto and Pto combination gave higher conductivity values consistent with the broad cell death induced by AvrPto/Pto (Figure 3.9). The co-expression of p38 with AvrPto/Pto combination resulted in decreased conductivity. In fact, the decrease in conductivity in the presence of p38 was similar to the decrease in the presence of AvrPtoB, the known suppressor of cell death (Figure 3.9). As expected, the co-expression of p8 with AvrPto/Pto combination did not decrease conductivity (Appendix A.3).



**Figure 3.9 Quantification of AvrPto/Pto induced PCD in presence of TCV genes.** Individual TCV genes were transiently expressed in *Nicotiana benthamiana* leaves in combination with *avrPto* and *Pt. avrPtoB* was used as a positive control for cell death suppression. Leaf punches were analyzed for conductivity after 46 hpi and 68 hpi. Bars represents mean of conductivity values of six samples. Error bar shows 95% confidence level.

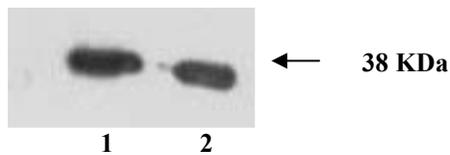
After subtracting the background conductivity from untreated leaves sample (Figure 3.10), it was observed that the level of cell death suppression by CP in middle aged leaves was similar to the level of cell death suppression by whole TCV. The presence of CP resulted in around a 7 fold decrease in cell death at 46 hpi, which is similar to the decrease by whole TCV. At 68 hpi, there was about a 2 fold decrease in cell death, which is equal to the cell death suppression by TCV. This shows that the CP alone is sufficient for HR suppression.

Statistical analysis was performed to calculate the difference in mean conductivity values of the samples. The results are shown in Figure 3.10. The statistical analysis confirmed that the CP is sufficient to suppress the HR in plants. Further, the level of suppression by CP is equal to the cell death suppression by a known cell death inhibitor.



**Figure 3.10 Statistical analysis of PCD with individual TCV genes.** Bars represent mean of conductivity values after deducting the background seen in untreated leaves sample. The mean was obtained from six different samples. Error bar shows 95% confidence level. Letters above bars represent distinct significance groups, determined by Tukey-Kramer method for multiple sample comparisons. The statistical analysis was performed with SAS statistical software.

The expression of CP was confirmed by immunoblot analysis (Figure 3.11). Leaf samples were collected at 2 dpi and soluble protein was extracted. Anti-CP antibodies were used to detect the CP (Figure 3.11- lane1). As a positive control, CP from induced *E. coli* was used (Figure 3.11- lane 2).



**Figure 3.11 Expression of TCV CP in *N. benthamiana* leaves.**

To confirm the delivery and expression of TCV CP in leaves by transient assay, leaf samples were collected 2dpi. Protein was extracted and subjected to immunoblot analysis with anti-CP antibodies. 45 µg of total protein was loaded in each lane. Lane 1- Expression of CP from leaf sample infiltrated with *Agrobacterium* carrying p38 , Lane 2- positive control, expression of CP from induced Top10F<sup>+</sup> ( pBAD:p38).

## 4 DISCUSSION

The previous work (Hammond 2001, Mahadevan 2004) demonstrated that TCV is able to counteract the plant's resistance by suppressing the HR, the defense-associated cell death. In TCV resistant Di-17 Arabidopsis plants, the virus was capable of suppressing the HR in a small population of plants and was able to induce systemic disease symptoms (Hammond 2001). Further investigation showed that this HR suppression phenomenon was not due to mutation of the virus inside the host. In fact, the virus was replicating actively without giving any HR symptoms in uninoculated leaves.

In TCV susceptible Col-0, the virus was demonstrated to successfully suppress the HR induced by *Psg* strains carrying *avr* factors (Mahadevan 2004). But in this case, there are possibilities that, since the virus is already present in leaves, it is preventing the bacterial infection by blocking directly or indirectly the Avr factors from entering the host cell. If this was the case and the virus was not letting the bacteria to establish a successful attack, the HR suppression symptoms will turn out to be the result of an unsuccessful bacterial infection. For example, the effector proteins VirPphA, AvrPphC and AvrPphF from *P. syringae* pv. *Phaseolicola* have also been shown to block the HR phenotype conferred by other *avr* genes (Jackson *et al* 1999, Tsiamis *et al* 2000). Although the exact molecular mechanism by which these effector proteins escape the host's resistance is not known, several hypotheses have been proposed to explain it. One of the hypotheses is that the interaction between Avr proteins prevents the host from detecting the pathogen. These interactions prevent either the *avr* gene expression or block the Avr protein secretion and translocation, thereby inhibiting the HR. We have shown in this work that HR suppression by TCV is clearly not due to suppressing the *avr* gene expression or its intracellular secretion.

To prove this, the effect of TCV on the bacteria's ability to enter and induce HR in the host was studied. By demonstrating the translocation and intracellular processing of AvrRpt2 in presence of TCV, it was confirmed that TCV's ability to inhibit the HR is not simply by stopping the avirulence protein from entering the plant cell or from being processed once the avirulence protein is in the cell. However, it might be possible that TCV is interfering with R-Avr recognition inside the plant cell or interfering with the HR signal transduction pathway downstream of Avr recognition.

The work also demonstrates that the virus can suppress cell death induced by an unrelated avirulence factor in *N. benthamiana*. The extent of cell death was quantified by an ion leakage assay. It showed that the presence of TCV in leaves results in substantial decrease in cell death. After the HR suppression capability of TCV in *N. benthamiana* was established, a transient expression assay was used to determine which of the TCV genes/ proteins were responsible for cell death suppression phenotype. Two TCV genes, p8 (movement protein) and p38 (coat protein), were tested and only p38 showed the cell death suppression phenotype. The co-expression of p8 with AvrPto and Pto showed no change in cell death, in comparison with cell death induced by AvrPto/Pto. But the co-expression of CP with AvrPto and Pto showed significant decrease in cell death. Though the other TCV genes were not tested, the cell death suppression by CP was comparable with a known suppressor of cell death, AvrPtoB. Further, the level of suppression seen with the CP alone was equivalent to the level of suppression seen in the presence of intact virus. This suggests that, even though the other TCV genes may contribute, CP alone is sufficient to achieve the level of suppression seen by whole virus.

Numerous animal viruses are known to suppress the PCD but this is the first evidence showing anti-PCD ability of a plant virus. Recently, it was reported that AvrPtoB, an avirulence

protein from tomato pathogen *Pseudomonas syringae* pv. *tomato* suppresses the PCD in plants by acting as E3 ubiquitin (Ub) ligase and has a structure homologous to RING-finger/U-box E3 Ub ligases (Abramovitch *et al.* 2003, Abramovitch *et al.* 2006, Janjusevic *et al.* 2006).

Possessing E3 Ubiquitin ligase activity will enable the pathogen to target host proteins for degradation and interfere with the HR. But, the CP primary structure doesn't show any similarity with RING-finger or U-box family of Ubiquitin ligase.

Espinosa *et al.* (2003) showed that HopPtoD2, another Avr protein from *P. s. pv. tomato* is capable of suppressing the PCD in plants. Similar to AvrPtoB, HopPtoD2 is also delivered into the host's cell by TTSS. It was shown to suppress HR in *N. benthamiana* induced by avirulent *P. syringae* strain. The study by Espinosa *et al.* (2003) suggested that the HR suppression capability in HopPtoD2 was due to its PTP activity. They demonstrated that it was targeting defense-associated mitogen-activated protein kinase (MAPK) cascades by acting downstream of NtMEK2, a MAPK kinase that plays an important role in induction of defense response. Using PTP activity to suppress the host's defense can be an effective strategy as there are several homologues of MAPK in plants which use tyrosine-phosphorylated proteins. In addition, many other type III effector proteins from plant pathogens have been demonstrated to have cysteine protease, ubiquitin-like protein protease, and tyrosine phosphatase activities, but to date no enzymatic activity has been reported for CP. The possibility that CP has a similar mode of action as the bacterial type III effector is unlikely.

Many animal viruses have been shown to target the transcription process in the host cell to win over the host's defense system. Regulating transcription can be a powerful tool to control the cell death. It has already been reported that human virus proteins are capable of controlling the PCD by binding with p53, a transcription factor. The E6 protein from *Human papilloma*

*virus* (HPVs) (Pan & Griep 1995, Thomas & Banks 1998, 1999) and the E1B protein from *Adenovirus* (Teodoro & Branton 1997, White *et al.* 1992, Yew *et al.* 1994) binds and functionally inactivate p53. The IE2 protein from *Human cytomegalovirus* is also reported to interact with the p53 tumor suppressor protein and block its ability to activate transcription (Speir *et al.* 1994).

It has been demonstrated that TCV CP interacts with TIP (TCV Interacting Protein), a transcription factor belonging to the NAC family of proteins (Ren *et al.* 2000, 2005). NAC proteins play an important role in response to viral infections and stress (Xie *et al.* 1999, Ren *et al.* 2000, Collinge and Boller 2001). They have also been implicated in transcriptional regulation of a variety of plant processes like flowering and development of the shoot apical meristem and in the formation of lateral roots (Souer *et al.* 1996; Aida *et al.* 1997, Xie *et al.* 2000).

A 25-amino acid (aa) region of the CP N-terminus interacts with TIP and mutations in this region lead to not only the loss in CP-TIP interaction but also in the failure to induce the HR in resistant *Arabidopsis* plants (Ren *et al.* 2000). This shows the correlation between TIP-CP interaction and HR in *Arabidopsis*. Furthermore, nuclear localization of TIP is blocked in the presence of CP (Ren *et al.* 2005). This evidence suggests that CP is acting as a transcriptional regulator by interacting with TIP and blocking the HR.

To confirm the role of CP as a transcriptional regulator, CP mutants incapable of interacting with TIP can be used. Five such mutants N3A, D4N, P5S, R6A and D13A with a single amino acid change in 25-aa region have been reported (Ren *et al.* 2000). There are two possible ways to test the role of CP in transcriptional regulation. We can compare the HR induced by unrelated bacterial pathogen, in *Arabidopsis* plants inoculated with TCV wild type (WT) and CP mutant virus. We would expect that WT virus will suppress the HR. If the CP

mutant does not suppress the HR, it is likely that the interaction with TIP is a significant factor in the HR suppression.

Alternatively, the transient expression assay in *N. benthamiana* could be used. For this, first the CP mutants have to be cloned in the Agro compatible vector. Co-infiltration of Agro strains carrying a combination of AvrPto, Pto and WT will result in cell death suppression. The extent of cell death can be quantified with ion leakage assay and compared with the cell death induced by co-expression of AvrPto, Pto and CP mutant. If the presence of mutant does not result in cell death suppression, it will show that the TIP-CP interaction is required for HR suppression. Hence, it would establish the role of CP as a transcriptional regulator.

Further, cell death assay in yeast can be used to check whether HR inhibition capability of CP is common across various kingdoms. For this the CP has to be cloned and expressed in yeast. The PCD can be induced by oxidative stress and the anti-apoptotic activity of CP and AvrPtoB can be compared.

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## APPENDIX A

### ION LEAKAGE ASSAY DATA

#### **A.1 Determining the time point for peak ion fluxes.**

The leaves of four weeks old *N. benthamiana* were infiltrated with Agro carrying AvrPto and Pto combination and leaf punches were collected at 30, 46, 68 and 80 hpi to determine the time point at which the ion fluxes was at peak. From each category two samples were collected. For each sample two leaf discs of 6 mm diameter were used. The conductivity was measured in microsiemens. The peak in cell death was observed at 46 hpi and 68 hpi.

| <b>Mean</b>       | <b>30 hpi</b> | <b>46 hpi</b> | <b>68 hpi</b> | <b>80 hpi</b> |
|-------------------|---------------|---------------|---------------|---------------|
| <b>untreated</b>  | 58.8          | 52.05         | 64.95         | 50.05         |
| <b>mock(MES)</b>  | 45.15         | 57.15         | 41.95         | 54.85         |
| <b>AvrPto</b>     | 58.35         | 116.95        | 159.75        | 190.5         |
| <b>Pto</b>        | 50.2          | 71.7          | 65.8          | 51.4          |
| <b>AvrPto/Pto</b> | 47.35         | 142.8         | 241           | 252.5         |

**Table A.1 Conductivity values measured at four different time points**

## **A.2 Quantitative analysis of HR suppression in TCV and mock –TCV inoculated *N.***

### ***benthamiana.***

Cell death was induced in TCV inoculated and control (mock-TCV inoculated) *N. benthamiana* plants by infiltrating with Agro carrying AvrPto and Pto combination. Cell death was compared in leaves of similar age and size from TCV and control plants. The leaf punches of 6-mm diameter were collected at 46 hpi and 68 hpi. For each sample two leaf discs of 6 mm diameter were used. For the purpose of statistical analysis, the ion leakage data was collected from three independent trials. From each category, total number of nine samples were collected from these trials. Sample # 1 is from the first trial, sample # 2-4 are from the second trial and sample # 5-9 are from the third trial.

| sample #        | 1     | 2     | 3    | 4     | 5     | 6     | 7     | 8     | 9     | mean  | sd   | cl   |
|-----------------|-------|-------|------|-------|-------|-------|-------|-------|-------|-------|------|------|
| <b>TCV</b>      |       |       |      |       |       |       |       |       |       |       |      |      |
| untreated       | 53.5  | 65.2  | 50.2 | 45.6  | 56.2  | 48.1  | 58.4  | 65    | 55.7  | 55.32 | 6.87 | 4.49 |
| Mock (MES)      | 39    | 59    | 42.5 | 51.2  | 43.8  | 57.5  | 55    | 63.7  | 51    | 51.41 | 8.29 | 5.42 |
| old leaf        | 150.7 | 149.3 | 163  | 158.6 | 148.5 | 164.2 | 192.5 | 173.2 | 162.2 | 162.5 | 13.9 | 9.07 |
| middle leaf     | 60.5  | 70.6  | 62.1 | 68.1  | 63.8  | 52.7  | 82.1  | 60    | 63.2  | 64.79 | 8.24 | 5.38 |
| young leaf      | 52.5  | 57.2  | 48.9 | 58.9  | 65.2  | 54.5  | 58.7  | 61    | 56.4  | 57.03 | 4.77 | 3.12 |
| <b>MOCK-TCV</b> |       |       |      |       |       |       |       |       |       |       |      |      |
| untreated       | 55.7  | 55.7  | 42.7 | 57.1  | 66.7  | 60    | 54.2  | 50.8  | 47.7  | 54.51 | 6.96 | 4.55 |
| Mock (MES)      | 49.8  | 48.7  | 53.2 | 61.8  | 66    | 58.8  | 62.8  | 54.9  | 50.6  | 56.29 | 6.29 | 4.11 |
| old leaf        | 138.2 | 158.2 | 172  | 169.3 | 165.8 | 168.2 | 164.8 | 171   | 176.6 | 164.9 | 11.3 | 7.37 |
| middle leaf     | 76.2  | 150.2 | 145  | 148.3 | 131.2 | 175.7 | 198.4 | 139.3 | 187.2 | 150.2 | 35.9 | 23.5 |
| young leaf      | 53.3  | 138.6 | 152  | 143.2 | 100.1 | 110.8 | 107.8 | 105   | 96.3  | 111.9 | 30   | 19.6 |

**Table A.2.1 TCV v/s Mock: conductivity values measured at 46 hpi. The confidence level (cl) was calculated with significance level (alpha) of 0.05 (95 percent confidence level) and sd (standard deviation).**

| sample #        | 1     | 2     | 3    | 4     | 5     | 6     | 7     | 8     | 9     | mean  | sd   | cl   |
|-----------------|-------|-------|------|-------|-------|-------|-------|-------|-------|-------|------|------|
| <b>TCV</b>      |       |       |      |       |       |       |       |       |       |       |      |      |
| untreated       | 45.8  | 45.8  | 52.5 | 57.7  | 48.5  | 55    | 43    | 57.2  | 60    | 51.72 | 6.15 | 4.02 |
| Mock (MES)      | 50.3  | 50.3  | 61.3 | 65.3  | 52.6  | 56.5  | 62.8  | 42.5  | 40.2  | 53.53 | 8.77 | 5.73 |
| old leaf        | 131.3 | 134   | 126  | 142.3 | 148.3 | 145.6 | 170   | 166.1 | 154.2 | 146.4 | 15.2 | 9.91 |
| middle leaf     | 118.7 | 128.3 | 115  | 115.9 | 133.3 | 135.5 | 139.2 | 140.2 | 102.2 | 125.4 | 13   | 8.51 |
| young leaf      | 67.4  | 74.2  | 65.5 | 79.6  | 82.4  | 79.9  | 82.2  | 76.2  | 89.4  | 77.42 | 7.55 | 4.93 |
| <b>MOCK-TCV</b> |       |       |      |       |       |       |       |       |       |       |      |      |
| untreated       | 52.8  | 52.8  | 55.1 | 42.8  | 55.6  | 48.2  | 52.6  | 48.2  | 58.2  | 51.81 | 4.69 | 3.06 |
| Mock (MES)      | 44.5  | 44.5  | 52.7 | 52.8  | 62.2  | 54.1  | 55.8  | 56.3  | 60    | 53.66 | 6.07 | 3.96 |
| old leaf        | 136.5 | 141.3 | 155  | 160.2 | 181.2 | 184.2 | 172.4 | 176.2 | 178   | 165   | 17.6 | 11.5 |
| middle leaf     | 163.3 | 210   | 197  | 205   | 187.2 | 191.1 | 180   | 201   | 203   | 193.1 | 14.6 | 9.53 |
| young leaf      | 97.5  | 149.3 | 168  | 148.6 | 150.2 | 156   | 128.6 | 137.1 | 125.6 | 140.1 | 20.8 | 13.6 |

**Table A.2.2 TCV v/s Mock: conductivity values measured at 68 hpi.** The confidence level was calculated with significance level (alpha) of 0.05 (95 percent confidence level) and sd (standard deviation).

### **A.3 Quantitative analysis of HR suppression by individual TCV ORF's**

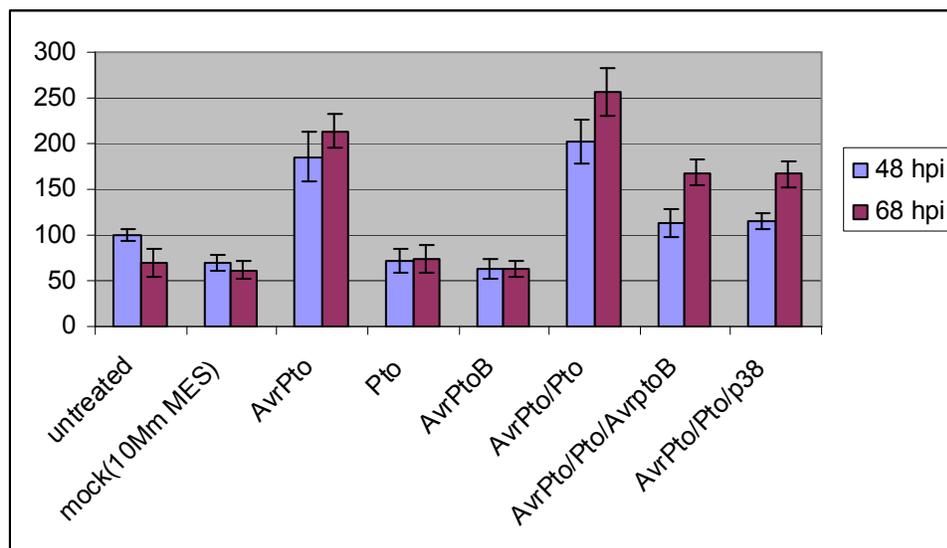
To identify the individual TCV gene responsible for HR suppression, the AvrPto/Pto combination was co-expressed with target TCV genes (p8 or p38). AvrPtoB, a cell death inhibitor was used as a positive control. The leaf punches of 6-mm diameter were collected at 46 hpi and 68 hpi. For each sample two leaf discs of 6 mm diameter were used. From each category six samples were collected.

| sample #           | 1     | 2     | 3     | 4     | 5    | 6     | mean  | sd   | cl    |
|--------------------|-------|-------|-------|-------|------|-------|-------|------|-------|
| untreated          | 97.4  | 97.7  | 96    | 105.9 | 88.8 | 111.3 | 99.52 | 7.93 | 6.34  |
| mock (MES)         | 67.7  | 64.2  | 72.1  | 58.9  | 89   | 61.5  | 68.9  | 10.9 | 8.71  |
| AvrPto             | 196   | 151.1 | 245   | 169   | 189  | 164.2 | 185.7 | 33.4 | 26.71 |
| Pto                | 76.2  | 84.1  | 50.2  | 88.1  | 53.1 | 81.3  | 72.17 | 16.4 | 13.11 |
| AvrPtoB            | 78.3  | 66.5  | 54.2  | 52.8  | 50.7 | 80.4  | 63.82 | 13.3 | 10.6  |
| p38                | 74.2  | 59.2  | 61.2  | 53.3  | 72.4 | 74.4  | 65.78 | 9.04 | 7.23  |
| AvrPto/Pto         | 192.3 | 172.6 | 179.8 | 210   | 254  | 207   | 202.6 | 29.1 | 23.31 |
| AvrPto/Pto/AvrptoB | 90.4  | 113.8 | 129.2 | 110.7 | 97.7 | 138.9 | 113.5 | 18.3 | 14.68 |
| AvrPto/Pto/p38     | 107.4 | 123.8 | 116.1 | 110.9 | 99.2 | 129.1 | 114.4 | 11   | 8.76  |

**Table A.3.1 Ion leakage assay with p38.** Conductivity was measured at 46 hpi. The confidence level was calculated with significance level (alpha) of 0.05, sd= standard deviation.

| sample #           | 1     | 2     | 3     | 4     | 5     | 6     | mean  | sd   | cl    |
|--------------------|-------|-------|-------|-------|-------|-------|-------|------|-------|
| untreated          | 78.2  | 58.1  | 81.9  | 52.5  | 47.9  | 97.1  | 69.28 | 19.4 | 15.5  |
| mock (MES)         | 48.1  | 53.1  | 79.1  | 71.5  | 59.3  | 60    | 61.85 | 11.5 | 9.23  |
| AvrPto             | 213   | 183   | 192.8 | 233   | 225   | 237   | 214   | 22   | 17.61 |
| Pto                | 71.1  | 71.9  | 93.3  | 50.1  | 97.1  | 57.9  | 73.57 | 18.7 | 14.96 |
| AvrPtoB            | 62    | 63.9  | 79.6  | 48.7  | 67.2  | 52    | 62.23 | 11.1 | 8.88  |
| p38                | 94.1  | 72.5  | 62.7  | 48.1  | 73.2  | 81    | 71.93 | 15.7 | 12.54 |
| AvrPto/Pto         | 216   | 220   | 288   | 274   | 287   | 259   | 257.3 | 32.3 | 25.81 |
| AvrPto/Pto/AvrptoB | 148.6 | 184   | 192   | 172.1 | 165.2 | 148.3 | 168.4 | 18   | 14.4  |
| AvrPto/Pto/p38     | 168.2 | 196.1 | 147.3 | 159.5 | 178.1 | 153.4 | 167.1 | 17.9 | 14.32 |

**Table A.3.2 Ion leakage assay with p38.** Conductivity was measured at 68 hpi. The confidence level was calculated with significance level (alpha) of 0.05 and sd (standard deviation).



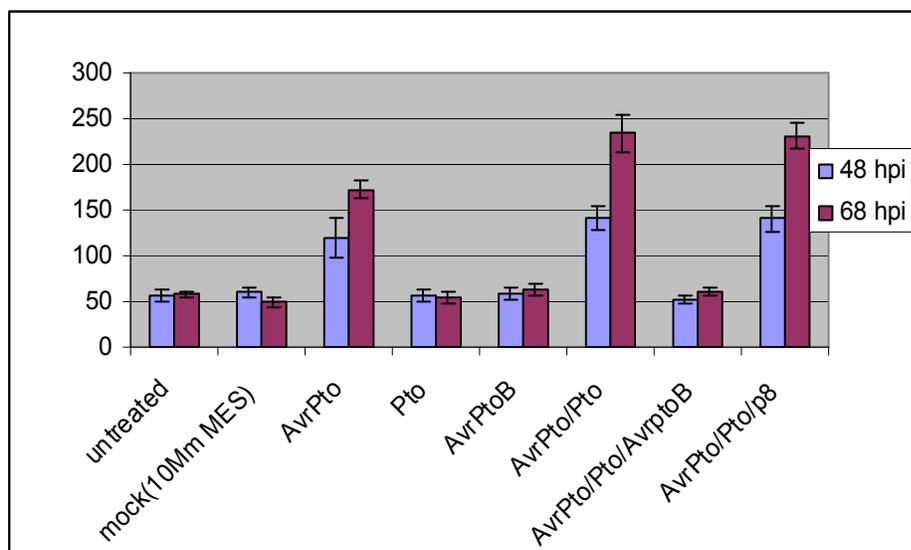
**Figure A.1 Ion leakage assay with p38.** Bars represent the mean conductivity values from six different samples. Error bar shows 95% confidence level.

| sample #           | 1     | 2     | 3     | 4     | 5     | 6     | mean  | sd   | c l   |
|--------------------|-------|-------|-------|-------|-------|-------|-------|------|-------|
| untreated          | 51.3  | 68.2  | 51.6  | 45.9  | 62.5  | 58.2  | 56.28 | 8.23 | 6.588 |
| mock (MES)         | 58.2  | 57    | 65.1  | 55.6  | 55.2  | 70.7  | 60.3  | 6.24 | 4.992 |
| AvrPto             | 154.1 | 151.4 | 98.2  | 89.5  | 108.2 | 120.2 | 120.3 | 27.2 | 21.75 |
| Pto                | 64    | 66.5  | 53.7  | 51.4  | 48.2  | 56.3  | 56.68 | 7.19 | 5.757 |
| AvrPtoB            | 62.8  | 60.5  | 71.4  | 49    | 57.2  | 53.7  | 59.1  | 7.77 | 6.216 |
| p8                 | 67.3  | 54.6  | 62.5  | 59.9  | 62.4  | 50.6  | 59.55 | 6.03 | 4.822 |
| AvrPto/Pto         | 128.6 | 142.2 | 165.3 | 151.1 | 120.9 | 135.6 | 140.6 | 16   | 12.8  |
| AvrPto/Pto/AvrptoB | 55.7  | 45.5  | 61.2  | 52.6  | 48.9  | 50    | 52.32 | 5.55 | 4.44  |
| AvrPto/Pto/p8      | 109.1 | 148.3 | 157.8 | 152.1 | 132.6 | 142.8 | 140.5 | 17.6 | 14.08 |

**Table A.3.3 Ion leakage assay with p8.** Conductivity was measured at 46 hpi. The confidence level was calculated with significance level (alpha) of 0.05 and sd( standard deviation).

| sample #           | 1     | 2    | 3     | 4     | 5     | 6     | mean  | sd   | c l  |
|--------------------|-------|------|-------|-------|-------|-------|-------|------|------|
| untreated          | 60.7  | 58.6 | 62.9  | 52    | 56.2  | 57.6  | 58    | 2.61 | 2.09 |
| mock (MES)         | 39.2  | 46.2 | 49.8  | 48.5  | 53.5  | 58.4  | 49.27 | 11.1 | 8.88 |
| AvrPto             | 191.2 | 182  | 156.1 | 167.5 | 172.2 | 166.6 | 172.6 | 35.2 | 28.2 |
| Pto                | 45.7  | 58.8 | 57.5  | 63.5  | 43.9  | 56.9  | 54.38 | 5.48 | 4.39 |
| AvrPtoB            | 75.2  | 57.1 | 58.7  | 62.1  | 55.5  | 67.3  | 62.65 | 7.61 | 6.09 |
| p8                 | 58.3  | 52.1 | 60    | 57.7  | 56.9  | 52.1  | 56.18 | 3.32 | 2.66 |
| AvrPto/Pto         | 269   | 211  | 206   | 259   | 226   | 233   | 234   | 25.4 | 20.3 |
| AvrPto/Pto/AvrptoB | 63.2  | 65.1 | 55.7  | 54.3  | 59.6  | 70.1  | 61.33 | 5.98 | 4.78 |
| AvrPto/Pto/p8      | 209   | 245  | 201   | 241   | 229   | 225   | 225   | 17.3 | 13.9 |

**Table A.3.4 Ion leakage assay with p8.** Conductivity was measured at 68 hpi. The confidence level was calculated with significance level (alpha) of 0.05 and sd( standard deviation).



**Figure A.2 Ion leakage assay with p38.** Bars represent the mean conductivity values from six different samples. Error bar shows 95% confidence level.

#### **A.4 Ion leakage assay with p38 at three different time points.**

To determine the maximum change in ion flux with p38, the ion leakage assay was carried out at three different time points. The leaves of *N. benthamiana* were infiltrated with Agro carrying AvrPto/Pto/p38 combination and leaf punches were collected at 30, 46, 68 and 80 hpi.

| sample #       | 1     | 2    | 3    | 4     | mean | sd   | cl   |
|----------------|-------|------|------|-------|------|------|------|
| untreated      | 59.7  | 59.7 | 58.7 | 52.2  | 57.6 | 3.61 | 3.54 |
| mock(MES)      | 55.2  | 54   | 52.2 | 57.3  | 54.7 | 2.14 | 2.1  |
| AvrPto         | 152.9 | 158  | 143  | 142.6 | 149  | 7.56 | 7.41 |
| Pto            | 67    | 66.3 | 55.2 | 56.7  | 61.3 | 6.21 | 6.09 |
| p38            | 61.7  | 63.8 | 58.6 | 57.6  | 60.4 | 2.85 | 2.79 |
| AvrPto/Pto     | 163.9 | 162  | 159  | 155.8 | 160  | 3.44 | 3.37 |
| AvrPto/Pto/p38 | 135.2 | 130  | 141  | 138.7 | 136  | 4.89 | 4.79 |

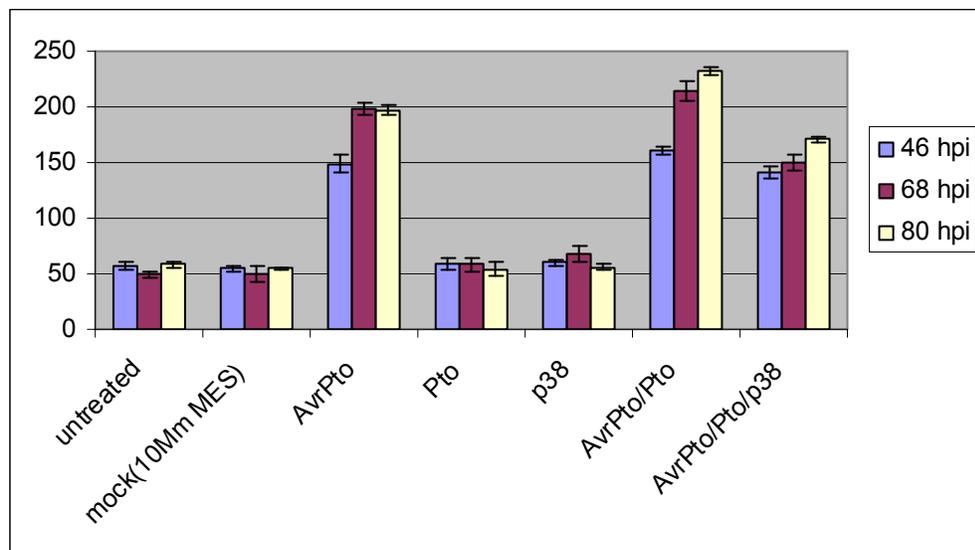
**Table A.4.1 Ion leakage assay with p38 (3 time points).** Conductivity was measured at 46 hpi. The confidence level was calculated with significance level (alpha) of 0.05 and sd( standard deviation).

| sample #       | 1     | 2     | 3    | 4    | mean    | sd   | cl    |
|----------------|-------|-------|------|------|---------|------|-------|
| untreated      | 48.5  | 49.3  | 53.7 | 47.8 | 49.825  | 2.66 | 2.602 |
| mock(MES)      | 58.4  | 53.8  | 42.3 | 45.6 | 50.025  | 7.39 | 7.238 |
| AvrPto         | 203   | 201   | 191  | 196  | 197.725 | 5.42 | 5.311 |
| Pto            | 55.8  | 50.7  | 62.8 | 65.3 | 58.65   | 6.65 | 6.519 |
| p38            | 65.7  | 59.1  | 75.2 | 72.8 | 68.2    | 7.29 | 7.139 |
| AvrPto/Pto     | 203   | 209   | 223  | 221  | 214     | 9.59 | 9.4   |
| AvrPto/Pto/p38 | 158.4 | 154.3 | 148  | 142  | 150.65  | 7.32 | 7.175 |

**Table A.4.2 Ion leakage assay with p38 (3 time points).** Conductivity was measured at 68 hpi. The confidence level was calculated with significance level (alpha) of 0.05 and sd( standard deviation).

| sample #       | 1     | 2    | 3    | 4     | mean | sd   | cl   |
|----------------|-------|------|------|-------|------|------|------|
| untreated      | 60.4  | 55.5 | 57.7 | 59.6  | 58.3 | 2.18 | 2.14 |
| mock( MES)     | 56.6  | 55.2 | 53.6 | 54.6  | 55   | 1.25 | 1.23 |
| AvrPto         | 197.6 | 190  | 198  | 202   | 197  | 4.99 | 4.89 |
| Pto            | 56.4  | 61   | 51.8 | 47.9  | 54.3 | 5.67 | 5.56 |
| p38            | 57.5  | 53.4 | 59.6 | 54.1  | 56.2 | 2.91 | 2.86 |
| AvrPto/Pto     | 235   | 236  | 232  | 228   | 233  | 3.59 | 3.52 |
| AvrPto/Pto/p38 | 168.1 | 173  | 174  | 169.7 | 171  | 2.67 | 2.61 |

**Table A.4.3 Ion leakage assay with p38 (3 time points).** Conductivity was measured at 80 hpi. The confidence level was calculated with significance level (alpha) of 0.05 and sd( standard deviation).



**Figure A.1 Ion leakage assay with p38 (3 time points).** Bars represent the mean conductivity values from four different samples. Error bar shows 95% confidence level.

## APPENDIX B

### Alignment report of cloned TCV genes

#### p8 gene

```
p8 ATGGATCCTGAACGAATTCCCTACAACCTCTCTAAGCGACAGCGACGCAACAGGAAAACGG 60
T7_p8 ATGGATCCTGAACGAATTCCCTACAACCTCTCTAAGCGACAGCGACGCAACAGGAAAACGG 60

p8 AAGAAAGGCGGAGAGAAAAGTGCGAAGAAGAGATTGGTAGCTAGCCACGCGGCTAGCTCT 120
T7_p8 AAGAAAGGCGGAGAGAAAAGTGCGAAGAAGAGATTGGTAGCTAGCCACGCGGCTAGCTCT 120

p8 GTTTTAAACAAGAAAAGAAATGAAGGTTCTGCTAGTCACGGGGGTACTTGGGTTATTGTT 180
T7_p8 GTTTTAAACAAGAAAAGAAATGAAGGTTCTGCTAGTCACGGGGGTACTTGGGTTATTGTT 180

p8 GCTGATAAAGTGAAGTCTCAATCAACTTCAACTTCTAA 219
T7_p8 GCTGATAAAGTGAAGTCTCAATCAACTTCAACTTCTAA 219
```

#### p38 gene

```
p38 ATGGAAAATGATCCTAGAGTCCGGAAGTTCGCATCTGATGGCGCCCAATGGGCGATAAAG 60
SP6_p38 ATGGAAAATGATCCTAGAGTCCGGAAGTTCGCATCTGATGGCGCCCAATGGGCGATAAAG 60

p38 TGGCAGAAGAAGGGCTGGTCAACCCTAACCAGCAGACAGAAACAGACCGCCCGCGCAGCG 120
SP6_p38 TGGCAGAAGAAGGGCTGGTCAACCCTAACCAGCAGACAGAAACAGACCGCCCGCGCAGCG 120

p38 ATGGGGATCAAGCTCTCTCCTGTGGCGCAACCTGTGCAGAAAGTGAAGTCAAGTCAAGTCAAGT 180
SP6_p38 ATGGGGATCAAGCTCTCTCCTGTGGCGCAACCTGTGCAGAAAGTGAAGTCAAGTCAAGTCAAGT 180

p38 CCGGTGGCCCTTGCCCTACCGCGAGGTTTCCACCCAGCCTCGGGTCTCTACTGCCAGGGAC 240
SP6_p38 CCGGTGGCCCTTGCCCTACCGCGAGGTTTCCACCCAGCCTCGGGTCTCTACTGCCAGGGAC 240

p38 GGCATAAACCAGAAGCGGTTCTGAACTGATCACAACCTTGAAGAAGAACAAGTCAAGTCAAGT 300
SP6_p38 GGCATAAACCAGAAGCGGTTCTGAACTGATCACAACCTTGAAGAAGAACAAGTCAAGTCAAGT 300

p38 CCTAAGTACACCACAGCTGTGCTTAACCCAAAGCGAACCAGGAAACATTCAACCAGCTCATT 360
SP6_p38 CCTAAGTACACCACAGCTGTGCTTAACCCAAAGCGAACCAGGAAACATTCAACCAGCTCATT 360

p38 AAGGAGGCGGCCAGTATGAAAAATACCGATTACGTCACCTCAGATTTAGGTACTCCCCC 420
SP6_p38 AAGGAGGCGGCCAGTATGAAAAATACCGATTACGTCACCTCAGATTTAGGTACTCCCCC 420

p38 ATGAGCCCTTCAACCACCGAGGCAAGGTGGCTCTGGCATTGACCGAGATGCAGCCAAA 480
SP6_p38 ATGAGCCCTTCAACCACCGAGGCAAGGTGGCTCTGGCATTGACCGAGATGCAGCCAAA 480
```

p38 TCAGATTTAGGTACTCCCCATGAGCCCTTCAACCACCGGAGGCAAGGTGGCTCTGGCAT 460  
 T7\_p38 TCAGATTTAGGTACTCCCCATGAGCCCTTCAACCACCGGAGGCAAGGTGGCTCTGGCAT 460

p38 TCGACCGAGATGCAGCCAAACCTCCGCCAAACGACCTCGCTTCCCTCTACAACATAGAGG 520  
 T7\_p38 TCGACCGAGATGCAGCCAAACCCCGCCAAACGACCTCGCTTCCCTCTACAACATAGAGG 520

\*

p38 GTTGTGTATCTAGCGTGCCCTGGACAGGGTTTATTTTGACCGTCCCAACAGATTCTACTG 580  
 T7\_p38 GTTGTGTATCTAGCGTGCCCTGGACAGGGTTTATTTTGACCGTCCCAACAGATTCTACTG 580

p38 ACCGCTTTGTGGCGGATGGTATCAGCGATCCAAAGCTTGTTCGATTTTCGGCAAGCTCATCA 640  
 T7\_p38 ACCGCTTTGTGGCGGATGGTATCAGCGATCCAAAGCTTGTTCGATTTTCGGCAAGCTCATCA 640

p38 TGGCCACCTACGGCCAAGGAGCCAATGATGCCGCCAACTCGGTGAAGTGCAGTCGAGT 700  
 T7\_p38 TGGCCACCTACGGCCAAGGAGCCAATGATGCCGCCAACTCGGTGAAGTGCAGTCGAGT 700

p38 ACACCGTGCAGCTCAAGAACAGAAGTGGCTCAACCAGCGACGCCAGATTGGGGACTTCG 760  
 T7\_p38 ACACCGTGCAGCTCAAGAACAGAAGTGGCTCAACCAGCGACGCCAGATTGGGGACTTCG 760

p38 CAGGTGTTAAGGACGGACCCAGGCTGGTTTCATGGTCCAAGACCAAGGGGACAGCTGGGT 820  
 T7\_p38 CAGGTGTTAAGGACGGACCCAGGCTGGTTTCATGGTCCAAGACCAAGGGGACAGCTGGGT 820

p38 GGGAGCACGATTGTCAATTTTCTCGGAACCGGAACTTCTCGTTGACATTGTTCTACGAGA 880  
 T7\_p38 GGGAGCACGATTGTCAATTTTCTCGGAACCGGAACTTCTCGTTGACATTGTTCTACGAGA 880

p38 AGGCGCCGGTCTCGGGGCTAGAAAACGCAGACGCCTCTGACTTCTCGGTCTGGGAGAAG 940  
 T7\_p38 AGGCGCCGGTCTCGGGGCTAGAAAACGCAGACGCCTCTGACTTCTCGGTCTGGGAGAAG 940

p38 CCGCAGCAGGTAGTGTCCAATGGGCAGGAGTGAAGGTAGCAGAAAGGGGACAAGGCGTGA 1000  
 T7\_p38 CCGCAGCAGGTAGTGTCCAATGGGCAGGAGTGAAGGTAGCAGAAAGGGGACAAGGCGTGA 1000

p38 AAATGGTCACAACTGAGGAGCAGCCAAAGGGTAAATGGCAAGCACTCAGAATTTAG---- 1056  
 T7\_p38 AAATGGTCACAACTGAGGAGCAGCCAAAGGGTAAATGGCAAGCACTCAGAATTTAGTACG 1060

Note: \* - Silent mutation at 483 position.

## APPENDIX C

### Media and Buffers

#### NYG Media (1L)

| Reagents            | NYGB (Liquid media)   | NYGA (Solid Media)    |
|---------------------|-----------------------|-----------------------|
| Proteose Peptone #3 | 5g                    | 5 g                   |
| Yeast Extract       | 3 g                   | 3 g                   |
| Glycerol            | 80 ml of 25% glycerol | 80 ml of 25% glycerol |
| Agar                |                       | 15 g                  |

#### LB media (1L)

| Reagents        | Liquid media | Solid Media |
|-----------------|--------------|-------------|
| Tryptone        | 10 g         | 10 g        |
| Sodium chloride | 10 g         | 10 g        |
| Yeast Extract   | 5 g          | 5 g         |
| Agar            |              | 20 g        |

#### SOC Media

2 % Tryptone  
0.5 % Yeast Extract  
10 mM NaCl  
2.5 mM KCl  
10 mM MgCl<sub>2</sub> · 6H<sub>2</sub>O  
20 mM glucose

#### Minimal Media

50 mM KH<sub>2</sub>PO<sub>4</sub> (pH-7.4)  
7.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>  
1.7 mM MgCl<sub>2</sub>  
1.7 mM NaCl  
10 mM fructose  
10 mM mannitol

### **Induction medium**

50 mM MES, pH-5.6  
27 mM glucose  
2 mM NaH<sub>2</sub>PO<sub>4</sub>  
20X AB salts  
500 ul of 200 mM acetosyringone (freshly prepared)

(200 mM acetosyringone= 1.6 mg in 0.5 ml DMSO)

### **20X AB salts**

0.37 M NH<sub>4</sub>Cl,  
24 mM MgSO<sub>4</sub>-7H<sub>2</sub>O  
40 mM KCl  
1.8 mM CaCl<sub>2</sub>  
0.18 mM FeSO<sub>4</sub>-7H<sub>2</sub>O

### **Virus inoculation buffer (2X)**

0.1M glycine  
0.06M K<sub>2</sub>HPO<sub>4</sub>  
2 % Celite

### **Protein extraction buffer**

20 mM Tris, pH-7  
1 mM EDTA  
1 mM PMSF.