Viral Suppression of Host Defenses

by

Geetha Mahadevan

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

April 2004

APPROVED:

_____

Dr. Kristin K. Wobbe, Major Advisor

_____

Dr. James Dittami, Head of Department

ABSTRACT

Upon detection of a pathogen, plants initiate specific signaling events designed to prevent host colonization and pathogen proliferation. Appearance of the hypersensitive response (HR), a type of programmed cell death signifies activation of active defenses in response to a one-to-one recognition of host, Resistance or *R* gene, and pathogen, avirulence or *avr* gene, encoded products. *Turnip crinkle virus* (*TCV*), however, has been shown to suppress the ability of Col-0 *Arabidopsis thaliana* plants to produce the HR in response to an avirulence factor. The extent of suppression was quantified by measuring cellular electrolyte leakage resulting from programmed cell death. Interestingly, cellular ion leakage levels were significantly lower in TCV-infected plants when challenged with bacteria expressing either of two bacterial effectors avrRpt2 or avrRpm1, suggesting that TCV can suppress the HR to a range of HR-inducing avirulence factors. In order to determine the viral component(s) responsible for mediating this suppression, each of the five TCV open reading frames (ORFs) was tested using an *Agrobacterium tumefaciens*-mediated transient expression assay in *Nicotiana benthamiana*. Though sequencing of the five TCV clones revealed mutations in the p28, p88, and p9 clones, *Agro* infiltration of an HR-inducing system in conjunction with individual TCV ORFs, or combinations of, was used to gather data to determine the role each may possess in the suppression phenotype. Full-length TCV was also expressed in the presence of AvrPto/Pto to establish suppression phenotype in *Nicotiana*. To assay for suppression of cell death in a heterologous system, both the mutant and wild-type clones were also tested in yeast for cell-death suppression induced by hydrogen peroxide exposure.

ACKNOWLEDGMENTS

First and foremost, I would like to express my sincerest gratitude to my Thesis Advisor Professor Kristin K. Wobbe. The amount of knowledge and experience that I have gained these past two years would not have been possible without an advisor like her. I had expected the transition from my mechanical engineering background into the world of molecular biology to be an uphill battle. This hardly was the case. Prof. Wobbe took every effort to clearly explain biological concepts and project details while being patient with my constant onslaught of questions. Whenever I faced experimental results that were not to my liking, she always gave me the encouragement to go on and moreover, alternative strategies to try. I would like to thank her once again for having the courage to take me on in her lab. I know the excellent work being undertaken in her lab will soon reveal novel findings in the world of plant pathogen interactions.

I would also like to thank Prof. Jose Arguello and Prof. Craig Fairchild for all their valuable advice regarding experimental protocols.

I would like to thank the lab of Greg Martin and Rob Abramovitch at the Boyce Thomson Institute at Cornell University for donating the pBTEX vector and the p423 vectors and their associated constructs. I also thank the lab of Andrew Bent for donating the *P. syringae* strains expressing avrRpt2 and avrRpm1.

I would like to thank Atin, Ana, Elif, Emine, Majo, Serdar, and Ying as well as past members Steve, Katrina, Jess, Jake, and Erin for helping me with my experiments and my millions of questions. Each and every one of these people took time from their busy schedules to make time to help me when I needed it. For this, I am eternally grateful.

Thanks to Ana and Majo specifically for being the best friends both inside and outside of the lab. I'm certain Majo will very soon make major achievements in her research now that I am leaving the lab.

I also express my greatest gratitude to Sumanth, Dad, Mom, Pritha, and Vinay for their support in all my wayward educational aspirations. I'm still in pursuit of that elusive perfect career!

And finally, a big thanks to the biggest inspiration in my life, the late Mrs. Mangalam Pattabiraman.

TABLE OF CONTENTS

LISTS OF FIGURES AND TABLES

1 INTRODUCTION

1.1 Plant-Pathogen Interactions

Understanding the molecular basis of plant-pathogen interactions and how plants can be made more resistant to infectious and deleterious pathogens can be derived from an understanding of the plant's inherent mode of disease resistance. Plants possess numerous mechanisms to cope with the harmful effects introduced by various biotic stressors. Upon pathogenic challenge, plants may typically invoke combinations of broad and specific defense responses to confine the pathogen to the entry site. These events may include increased levels of reactive oxygen species, upregulation of genes encoding cell-wall strengthening agents and anti-microbial phytoalexins, increased levels of benzoic and salicylic acid, and the mobilization of pathogenesis-related (PR) genes (Nimchuk et al., 2003). However, initiation of specific active defense responses requires molecular recognition between a host-encoded resistance gene (*R* gene) and a cognate pathogen-derived avirulence factor (*avr* gene) in a gene-for-gene specific manner (Flor, 1971). In the absence of either gene product, the plant becomes susceptible to the pathogen and its subsequent infestation. Avirulence genes may also serve as virulence factors in the absence of a cognate *R* gene. Examples of this duality include *avrRpt2* from *Pseudomonas syringae* pv. *tomato*, the causal agent of bacterial speck disease, which functions as an avirulence factor in resistant lines of Arabidopsis but as a virulence factor in susceptible lines (Chen et al., 2000). It is generally believed that pathogens first developed these genes to enhance their virulence on susceptible hosts, while plants

simultaneously evolved corresponding *R* genes as specific receptors to recognize and eliminate them (Lim and Kunkel, 2004).

To understand the molecular basis of plant-pathogen interactions, we use the plant model *Arabidopsis thaliana,* Figure 1. While *A. thaliana* is not in itself a crop plant, it is a member of the *Brassicaceae* family and is related to agriculturally significant plants such as broccoli, cabbage, and radish. *A. thaliana* provides an ideal model for research in the area of molecular genetics due to its short six-week life cycle along with the added benefit of a fully-sequenced genome. Other advantages of using *A. thaliana* include copious seed generation, making it a valuable tool in transgenic studies, and its relatively small size. With 26,000 genes on five chromosomes characterized to date, the system provides an excellent genomic resource for studying plant biology.

Figure 1: Model Plant: Wild-Type Arabidopsis Thaliana - Ecotype Columbia-0 (Col-0)

1.2 Molecular Recognition of Pathogens

To date, many cognate plant-pathogen interactions have been identified and characterized. Pto, one of the first *R*-gene encoded proteins to be characterized, recognizes the *Pseudomonas syringae* avrPto and avrPtoB proteins to initiate resistance responses in tomato (Scofield *et al*., 1996; Martin *et al*., 1993). The Col-0 disease

resistance locus *RPS2* mediates specific detection of the corresponding avirulence gene *avrRPT2* (Kunkel *et al*., 1993). Likewise, the *Cf-9* gene from tomato confers resistance to strains of the leaf mold fungus *Cladosporium fulvum* expressing the cognate *Avr9* gene (Jones *et al*., 1994). The R-protein Rpm1 recognizes two *Pst* pv. *tomato* derived effectors AvrRpm1 and AvrB (Grant *et al.,* 1995). Based on their sequences, *R* genes characterized to date fall into five main categories as shown in Table (Martin et al., 2003).

R genes falling into the classes I-III lack transmembrane segments and are thought to exert their activities from within the cytoplasmic space where they interact with avirulence factors employing the TTSS machinery for secretion into the cell. Bacterial effector proteins are typically secreted and injected through the plasma membrane into the host cell employing the Type III Secretion System (TTSS) (Hueck, 1998; Mudgett and Staskawicz, 1999). The TTSS machinery is encoded by the *hrp* (hypersensitive response and pathogenicity) locus and is required in many *R-avr* mediated interactions (Alfano and Collmer, 1996).

Table I: Classification of R genes involved in plant disease resistance (taken from Martin et al., 2003)

Class	Description	Examples
I	Serine/threonine kinase catalytic activity; N-terminal myristylation motif	Pto from tomato is the only member of this class
II	Contain LRR, NBS, N-terminal leucine zipper regions, and CC domains	RPM1, RPS2, Pi-ta
III	Contain LRR, NBS, N-terminal leucine zipper regions, and TIR domains homologous to animal resistance genes	TMV N gene, RPS4
IV	Extracellular LRR region; small cytoplasmic extensions	All *Cf* genes from the tomato fungus *Cladosporium fulvum*
V	Extracellular LRR region with a cytoplasmic region possessing serine/threonine kinase activity	Xa21 from Rice is the only member of this class

Approximately 150 *R* genes in the A. thaliana genome have been identified. The majority of *R* genes characterized encode products belonging to the Class II family of proteins. The specificity of R protein function is largely thought to lie within the LRR regions, the most variable of the domains in these proteins. The NBS (nucleotide binding site) and LRR (Leucine Rich Repeat) share homology with nucleotide binding sequences and leucine-rich repeat motifs essential for protein-protein interactions, respectively while the CC (coiled-coil domain) consists of a repeated heptad sequence with interspersed hydrophobic residues (Dangl and Jones, 2001; Martin et al., 2003).

Two popular mechanistic models to describe the interaction between plants and pathogens include the receptor-elicitor theory and the guard theory. The elicitor-receptor theory places the pathogen-secreted effector in the elicitor role, while the *R* gene product acts as its receptor as shown in Figure 2.

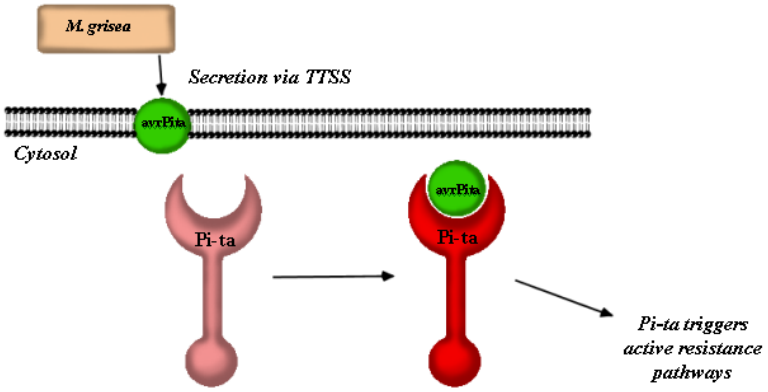


Figure 2: The Receptor-Elicitor Theory

To date however, only two direct R-avr interactions have been demonstrated. The C-terminal LRR region of the Pi- ta protein from rice specifically interacts with the rice blast fungus *Magnaporthe grisea* secreted AvrPita in yeast-two hybrid assays and in-vitro binding assays. Moreover, loss of physical interaction in AvrPita mutants segregated with loss of *Pi-ta* mediated resistance in resistant rice lines (Jia et al., 2000). Likewise, the Pto protein in tomato both recognizes and physically interacts with avrPto and avrPtoB as determined using the yeast-two hybrid screening method (Kim *et al*., 2002). The Pto-avrPto interaction is one the best characterized pathways in plant resistance. Pto possesses serine/threonine kinase activity to initiate immunity upon direct interaction with both avirulence factors and in fact is sufficient for triggering immunity in the absence of its cognate avirulence factor when overexpressed (Tang *et al.,* 1999). Pto autophosphorylation is required for Pto and avrPto interactions. Moreover, mutations abolishing Pto kinase activity showed an inability to form an HR (Tang *et al.,* 1999). However, direct physical interaction between Pto and its cognate avirulence factors is not sufficient to mediate immunity. Pto-mediated resistance requires the *Prf* gene and other associated factors for full immunity (Salmeron *et al.,* 1996). Though physical interaction

between the Pto and avrPto comprises a key signaling event in the host immune response, it is not in itself sufficient to mediate the entire process. To date, other factors have been identified as downstream phosphorylation targets by Pto including the protein kinase Pti1 and the Pti4, Pti5, and Pti6 family of transcription factors that play a role in the induction of pathogenesis-related genes (Zhou *et al.,* 1995).

Though the receptor-elicitor theory describes mechanisms for two well-characterized systems, there exists far more compelling evidence for indirect communication between *R* and *avr* proteins in the triggering of defense activation networks. Many *R* gene products have been postulated to function via the "guard" theory whereby modification of a different host factor by a foreign elicitor triggers active resistance as shown in Figure 3. The R-protein serves as a surveillance mechanism to "guard" or monitor the status of the cellular targets. Once modification of the guarded protein occurs, the *R* protein initiates various defense responses to isolate and eliminate the pathogen.

Recent data demonstrated that the presence of avrRpt2 caused elimination of a resistance-related protein RIN4 even in the absence of RPS2 and this ability segregated with activation of the RPS2 pathway (Mackey *et al*., 2003, Axtell and Staskawicz, 2003). Rin4 acts as the "guarded" component serving as a negative regulator for *RPS2*-mediated defense as shown in Figure 3. In 2003, Axtell and Staskawicz demonstrated that wild-type Col-0 plants infiltrated with bacteria expressing mutant avrRpt2 alleles were unable to induce any elimination of RIN4 and that knock-down mutants of *rin4* resulted in constitutive induction of active defense pathways in the absence of an avirulence factor. Interestingly, an unrelated avr, avrRpm1 also directly interacts with and modifies Rin4. AvrRpm1 causes hyperphosphorylation of Rin4. Further, Rin4 is required for Rpm1

mediated resistance (Mackey *et al.,* 2002). Thus it appears that RIN4 may be a convergence point in the signal transduction of activated defense initiated by both avrRpm1 and avrRpt2. This would explain why transgenic Col-0 plants expressing avrRpt2 were unable to elicit the RPM1 mediated immune response when challenged with avrRpm1 (Chen et al., 2000; Ritter and Dangl, 1996).

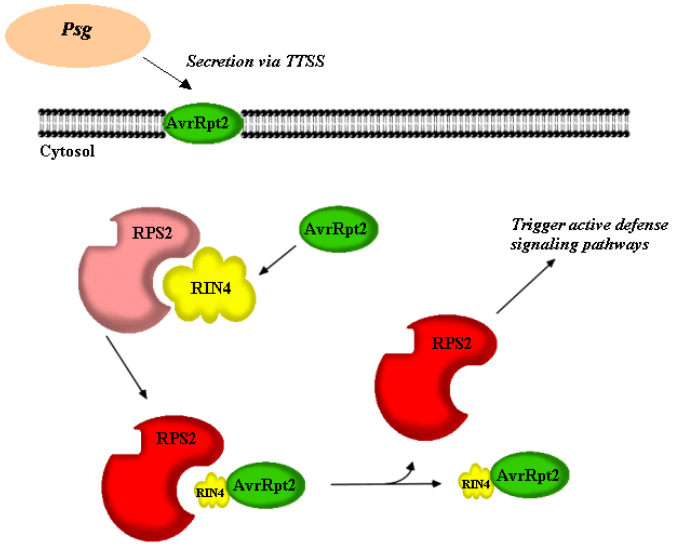


Figure 3: The Guard Theory

In further support of the guard theory, one set of researchers concluded, after extensive searching, that there was no evidence at all for any interactions between the Cf-9 protein and its cognate avirulence factor avr9 in elicitation of resistance, suggesting the existence of a factor that mediates interactions with both (Luderer et al., 2001).

1.3 Defense Responses and Pathogen Counter-defenses

Evidence for pathogen-mediated suppression of host cell death has been demonstrated in both plant and animal systems. The *Pseudomonas* type III effector AvrPtoB was shown to inhibit host-induced cell death in the presence of the HR-inducing disease resistance gene pairs Pto/AvrPto and Cf-9/Avr9 (Abramovitch *et al*., 2003). Interestingly, AvrPtoB was also able to suppress PCD in yeast undergoing death-inducing treatment.

The *Psg* pv. *Phaseolociola (Pph)* effector virPphA was able to suppress typical rapid HR induction, which ultimately lead to colonization of soybean by the bacteria (Jackson et al., 1999). The AvrPphF factor appeared to block HR induced by chromosomal avr effectors in *Pph* (Tsiamis et al, 2000). In mammalian cell lines, the TTSS effector YopJ from *Yersinia pestis,* the causal agent for bubonic plague disrupted phosphorylation-based activation of host mitogen-activated protein kinase kinases (MAPKKs) leading to suppression of host immunity (Orth et al., 1999). A number of YopJ-like proteins in other species of *Yersinia* as well as other plant and bacterial systems display similar interference with host defense signaling pathways, modulating the immune response and eventually contributing to virulence (refer to Orth et al., 1999). In 2002, Hay and Kannourakis compiled a comprehensive review citing evidence for viral-mediated suppression of host-initiated cell death allowing for longer viral replication cycles and increased virulence. According to the review, a number of viruses opt to disturb apoptotic-related processes by variously interfering with cellular TNF and Fas signaling, caspases involved in initiation cell death, the IFN pathway and Bcl-2-related proteins, and cell cycle and oxidative stress regulation. Likewise, expression of the baculovirus anti-apoptotic *p35* gene in tomato inhibits host induced programmed cell death by acting

as a strong caspase inhibitor and confers disease resistance to a broad spectrum of otherwise nectrotrophic pathogens (Lincoln *et al*., 2002; Zhou *et al*., 1998). Interestingly, there are no published examples of plant viruses suppressing PCD.

Establishing the mechanisms of inhibition of programmed cell death in plant systems could lead to greater understanding of the same processes in animal viruses employing similar tactics. Similarities between host initiation of programmed cell death in plant and animal systems have been identified. In 2003, Liang et al. demonstrated shared elements in the regulation and execution of ceramide-mediated PCD in plants and animals. The formation of reactive oxygen species such as superoxides and hydrogen peroxide was shared by both plant and animal systems undergoing host-induced cell death (Jabs, T., 1999). c-DNA obtained from *A. thaliana* was able to functionally complement a *dad1* (defender against apoptotic death 1) knock-out mutant cell line of hamster tsBN7 cells in preventing apoptosis, providing one of the earliest experimental justifications for functional apoptotic-related homologues across plant and vertebrate systems (Gallois et al., 1997). A more recent example was shown by Li and Dickman (2004) whereby transgenic tobacco plants expressing the human anti-apoptotic factor bcl-2 survived lethal treatments of severe heat and cold shock and chemical stress whereas wild-type tobacco plants did not.

1.4 Turnip crinkle virus as a model

Turnip crinkle virus, a member of the *Carmovirus* genus, is a small icosahedral, positive-strand RNA virus with five ORFs (Carrington et al., 1989). The genome, as shown in Figure 4, encodes two replicase proteins, p28 and its readthrough product p88 required for

in-planta replication, two intercellular movement proteins p8 and p9, and one coat protein p38 required for encapsidation of the infectious virion (Hacker *et al*., 1992).

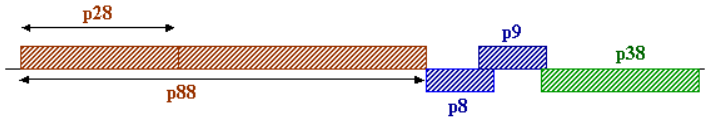


Figure 4: TCV Genome

Upon TCV inoculation, the TCV-susceptible line Col-0 becomes systemically infected and develops the characteristic disease symptoms of vein striping, asymmetrical mid-vein formation, leaf chlorosis and discoloration as shown in Figure 5.

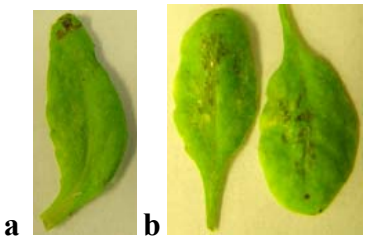


Figure 5: Leaves from TCV-inoculated Col-0 plants at 10 dpi. Uninoculated, systemically infected leaf (a); inoculated leaves (b).

Viral nucleic acids are transported through intercellular conduits called plasmodesmata that are formed during incomplete cytoplasmic dissociation when plant cells undergo cytokinesis (McLean et al., 1997). Though plasmodesmata are typically used for intercellular communication and for the transport of biomolecules, viruses commonly use them for cell-to-cell movement while employing the phloem for long distance movement (Carrington et al., 1996; Samuel G., 1934). Viral translocation typically originates from older, fully expanded "source" leaves to younger, developing "sink" leaves (Leisner *et al.,* 1993)

A common feature displayed by viruses undergoing a double-stranded RNA intermediate is their ability to induce host-mediated RNA silencing as shown in Figure 6. This phenomenon has been observed and documented for a broad range of eukaryotic systems. Upon cytoplasmic replication of the virus, host detection of long double-stranded RNA triggers a pathway that results in degradation of these molecules by ribonucleases into small 21-25 nt RNAs termed small interfering RNAs or siRNAs. These shorter oligonucleotides associate with what is known as the RNA Induced Silencing Complex, or RISC, to silence complementary strand mRNA transcribed from the viral genome in a sequence specific manner. Interestingly enough, many of these same viruses that induce RNA silencing also have mechanisms to silence them. The coat protein of TCV has been shown to possess suppression of RNA silencing capabilities (Qu *et al*., 2003).

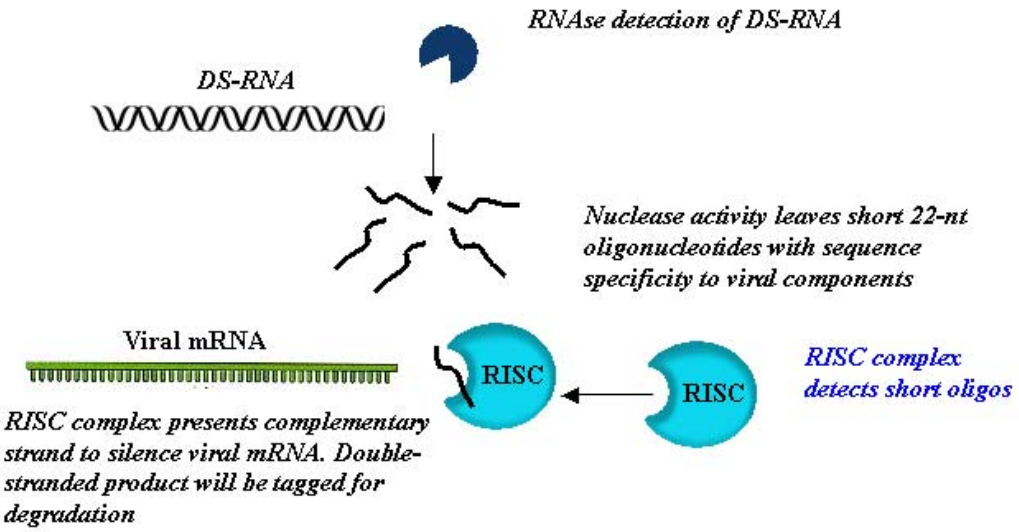


Figure 6: Simplified schematic of RNA silencing

In *Drosophila*, the B2 protein from flock house virus is a potent inducer of host-induced gene silencing while simultaneously suppressing it (Li *et al*., 2002). The potyvirus helper component proteinase HC-Pro is a strong suppressor of RNA silencing and prevents accumulation of the invading nucleic acids (Anandalakshmi et al., 1998; Vance and Vaucheret, 2001). *Potato virus* X also displays suppression of RNA silencing capabilities but to a much lesser extent than HC-Pro as the viral p25 protein was shown only to suppress the mobile silencing signals (Voinnet et al., 2000).

1.5 TCV suppresses the HR

Previous work (Hammond 2001) suggested that TCV was capable of reducing the host plant's innate ability to induce the HR not only to itself but also to unrelated pathogens. Infiltrating with bacteria expressing *avrRpt2* in systemically TCV-infected Col-0 plants produced a milder tissue collapse suggesting a reduced resistance response. Likewise, TCV-resistant Di-17 plants displayed attenuated HR on symptomatic leaves after TCV challenge in plants previously inoculated with TCV. This suppression was seen though both TCV RNA and the viral coat protein were detected in the suppressed tissue. As TCV coat protein serves as the avirulence determinant conferring resistance in Di-17 plants (Zhao et al., 2000), this suggested that the HR response was suppressed in these tissues. The same uninoculated symptomatic leaves also showed high levels of PR-1 induction. It was therefore concluded that the presence of TCV in systemically infected tissues correlated with suppression of HR induction. As this is the first documentation of a viral mediated suppression of cell death in plants, we sought to further extend previous findings that HR-lesions were visibly diminished in systemic TCV Col-0 plants.

1.6 Proposed Work

In this thesis, we show quantitative evidence that the presence of TCV interferes with the induction of the HR in the presence of the avirulent factor avrRpt2. By measuring ion leakage from leaves challenged with bacteria expressing the avirulence factors in the absence and presence of TCV, we were able to quantify the extent of cell death at equivalent time points. Moreover, we were able to extend this observation to a completely different avirulence factor, avrRpm1. avrRpm1 possesses faster kinetics of PCD induction allowing detection of peak ion fluxes much sooner than leaves infiltrated with avrRpt2.

In order to determine which viral component mediates this observed suppression, we isolated and cloned each of the five individual TCV open reading frames as well as the TCV genome into the *Agrobacterium tumefaciens* compatible pBTEX vector and transfected each construct into the Agro strain GV2260. Each of the TCV ORFs was cloned into the pBTex expression vector for use in the transient assay. Sequencing of the five TCV clones revealed mutations in the p28, p88, and p9 clones. The p28MUT contained a single amino acid change from tyrosine to cysteine due to an A $\rightarrow$ G mutation at position 59 in the sequence. The p88MUT sequence contained over five mutations while the p9MUT had two mutations resulting in significantly different residues in the amino acid sequence. As sequencing data was reviewed after performing transient assays with each construct, all experiments performed with both mutant and wild-type clones, are included. As future cloning efforts to reconstruct p28, p88 and p9 could result in evidence for suppression, the data obtained from experiments with the

mutant proteins could be valuable in determining structure-function requirements. Both the p38 and p8 clones had full consensus with the TCV coding sequence.

Each of the mutant and wild-type genes were expressed in *Nicotiana benthamiana* - a plant commonly used for transient transgene expression – in the presence of the PCD-inducing combination of avrPto and Pto. Evidence and extent of suppression was determined by scoring leaves based on the percent of infiltrated area showing necrosis.

To test for activity in a heterologous system, both the mutant and wild-type TCV ORFs were cloned into the yeast compatible vector p423 to test for anti-apoptotic activity in yeast treated with H_2O_2. Yeast strain INVSc1 was transformed with each of the TCV constructs and exposed to hydrogen peroxide treatment to induce oxidative stress. Yeast survival was determined by survival of colony forming units (cfus) upon plating.

2 MATERIALS AND METHODS

2.1 Plant Material and Growth Conditions

Arabidopsis thaliana Col-0 plants were grown in Pro Mix BX soil (Premiere Horticulture Inc., Red Hill, PA) in Percival Scientific AR-60L growth chambers with a photoperiod of 16 hours and a dark period of 8 hours. Growth chamber temperature was set to 23.0°C and 21.0°C during light and dark periods, respectively. Col-0 seeds were planted in flats and subsequently covered with plastic wrap to maintain high humidity conditions through germination and before the appearance of four true leaves. Plants were watered as needed by immersing the flats in 1-L of tap water. After the first two weeks of growth, water was supplemented with 0.35g/L Miracle-Gro®. *Nicotiana benthamiana* were grown in similar soil, humidity and light/dark conditions but at a constant temperature of 28°C. Upon germination in high humidity conditions, seedlings were transplanted to individual pots. Auxiliary meristem sections were removed from developing plants to encourage enhanced growth of leaf tissue area. Plants were watered as needed and supplemented with 0.35g/L Miracle-Gro® once every two weeks after the initial four weeks of growth.

2.2 Polymerase Chain Reaction (PCR)

PCR was performed to isolate each of the five TCV open reading frames (ORFs) using the pT1D1ΔL (Heaton *et al*., 1989; Akgoz *et al*., 2001) clone as template.

Table II lists the primer pairs used to amplify each of the individual ORFs. PCR reaction conditions were 1X RedTaq Polymerase Buffer (Sigma-Aldrich, St. Louis MO), 1 μM each forward and reverse primer, 2.5 mM dNTPs, 1 unit RedTaq Polymerase (Sigma-

Aldrich, St. Louis MO), and 10 – 50 ng template DNA. All reactions were carried out in 50 μL volumes. Amplification was performed using the following thermocycler (Perkin Elmer Cetus Gene Amp PCR System 9600) conditions:

1. 95ºC for 5 minutes (Initial Denaturation)
2. 95ºC for 2 minutes (Denaturation)
3. 68ºC for 1 minutes (Annealing)
4. 72ºC for 1 minutes (Elongation)
5. Repeat Steps 2 – 5 for 35 cycles
6. 72ºC for 10 minutes (Final Elongation)
7. 4ºC Hold

Table II: Primers for the Amplification of Individual TCV Open Reading Frames

Target	Forward Primer	Reverse Primer
p28MUT	5'-ATGCCTCTTCTACACACACTCAAC-3'	5'-CTAGCGGACAAAAGAGATCGC-3'
p88MUT	5'-ATGCCTCTTCTACACACACTCAAC-3'	5'-TTAGAGAGTTGTAGGGAATTCG-3'
p8	5'-ATGGATCCTGAACGAATTCCC-3'	5'-GCACTAGTTTTCCAGTCTAATG-3'
p9MUT	5'-ATGAAGGTTCTGCTAGTCACGG-3'	5'-GCACTAGTTTTCCAGTCTAATG-3'
p38	5'-ATGGAAAATGATCCTAGAGTCCGG-3'	5'-GACCAGCCCTTCTTCTG-3'

As full-length p88 encodes both p28 and p88, the p88ΔSTOP was created to produce only p88. The megaprimer method was used to change the 814G→C for removal of the p28 leaky stop codon and to incorporate a silent mutation at 832G→C by introducing a Sac I restriction site to test for incorporation of the mutation. The following mutagenic pair of primers was used: 5'-GTCCGCTACGGGTGCTTGCGGGAGCTCGTCGGGAGGGAGACTC-3' and 5'-CCCGACGAGCTCCCGCAAGCACCCGTAGCGGACAAAAGAGATCG-3.

Two rounds of PCR were performed – one using the p88 forward primer and the reverse mutagenic primer and the other using the p88 reverse primer and the forward mutagenic primer – to generate the "megaprimers". Thermocycler conditions were as previously

mentioned. One final round of PCR was used to create the mutant using the following reaction conditions: 1X RedTaq Polymerase Buffer (Sigma-Aldrich, St. Louis MO), 1 μM p88 forward primer, 1 μM p88 reverse primer, 2.5 mM dNTPs, 1 unit RedTaq Polymerase (Sigma-Aldrich, St. Louis MO), 25 ng PCR product 1, and 25 ng PCR product 2. All reactions were carried out in a 50 uL volume. Thermocycler conditions were as listed before.

2.3 Cloning

All PCR products were cloned into the pCR® 2.1 cloning vector (Invitrogen, Carlsbad CA) and screened for desired directionality using the universal SP6 promoter forward primer and the reverse primer of the ORF or through restriction digestion analysis when appropriate. For cloning the ORFs into the agro-compatible binary vector pBTEX, the pCR® 2.1 construct was digested with *Xba*I and *Kpn*I, to excise the desired fragment and ligated into compatible sites between the cauliflower mosaic virus (CaMV) 35S promoter and a downstream NOS terminator sequence. For cloning into the yeast-compatible vector p423, the pCR® 2.1 construct was digested with *SpeI* and *XhoI*, to excise the desired fragment and ligated into compatible sites downstream of the GAL1 promoter. The yeast vector contains a histidine synthesis and ampicillin resistance marker for selection in yeast and E. *coli*, respectively. The resultant recombinant plasmids were propagated in *E. coli* Top 10 cells and isolated using standard plasmid isolation procedures.

Each construct was sequenced by Macrogen, Inc. (Korea). Only two clones, p38 and p8, were verified as accurate. Results of each sequencing reaction are listed in Appendix B.

2.4 Preparation of Chemically Competent E. Coli

Fresh DH5α cells were plated from glycerol stocks onto LB plates (see Table) containing no antibiotics. Cells were grown overnight in 37°C. A single colony was used to inoculate 25 ml of LB liquid media (Table) without antibiotics. Cells were grown overnight in a shaker at 37°C at 200 RPM. The next day, the culture was used to inoculate a fresh 500 ml volume of LB without antibiotics in a 1 L flask. Cells were agitated at 37°C until the A_{600} was about 0.6 (after approximately 2 hours). The cells were then centrifuged at 4,000 RPM at 4°C for five minutes. The pellet was resuspended in 200 ml of ice-cold 50 mM $CaCl_2$ and placed on ice for 10 minutes followed by centrifugation at 4,000 RPM for five minutes at 4°C. The pellet was resuspended in 40 ml ice-cold 20% glycerol-50 mM $CaCl_2$. Aliquots of 1ml each were distributed to sterile microcentrifuge tubes and flash frozen in liquid nitrogen. All cells were stored in –80°C.

2.5 Transformation of E. Coli – Heat Shock

Stocks of chemically competent cells were taken from –80°C and thawed on ice. DNA was added to a 100 ul aliquot of competent cells, gently mixed with the end of a pipette tip, and kept on ice for 30 minutes. The cells were then placed in a 37°C water bath for 3 minutes for transformation. Immediately thereafter, 1 ml of LB (see Table) was added to the cells. After incubation at 37°C on a rolling drum for one hour, the cells were then spun down and plated on media containing the appropriate antibiotic for selection of transformants.

2.6 Transformation of E. Coli – Electroporation

Stocks of previously made electro competent cells were taken from –80°C and thawed on ice. Approximately 100 ng of DNA in water was added to a 20-ul aliquot of competent cells and gently mixed with the end of a pipette tip. The mixture was then pipetted into a sterile electroporation cuvette and placed into the chilled electroporation chamber. The Cell-Porator® (Life Technologies, Carlsbad CA) was set to the following conditions: voltage booster = 4 kΩ, capacitance = 330 μF, and DC volts = low. A pulse of 2 kV was delivered to the sample. The sample was left in the cuvette for ten minutes and then transferred to 1 ml of LB media (see Table). The sample was placed in a rolling drum at 37°C for one hour. The cells were then spun down and plated on media containing the appropriate antibiotic for selection of transformants.

2.7 DNA Mini Preparation

A single colony was used to inoculate a 5-ml volume of LB media (see Table) containing the appropriate antibiotic in a loosely capped 15-ml tube. The tube was placed in a rolling drum in a 37°C incubator for 16-18 hours. Approximately 1.5 ml of the culture was placed in a microcentrifuge tube and centrifuged at 14,000 RPM in a benchtop centrifuge for 30 seconds. The supernatant was removed leaving the bacterial pellet as dry as possible. The pellet was resuspended in 100 ul of ice-cold GTE solution (50mM Glucose, 25 mM Tris-HCl - pH 8.0 and 10 mM EDTA - pH 8.0) by vigorous vortexing. To lyse the cells, 200 ul of a freshly prepared solution of 0.2 M NaOH and 1% SDS was added to the tube and the contents subsequently mixed by inverting the tube rapidly several times without any vortexing. To the lysed cells, 150 ul of ice-cold 5M

KoOAC (5 M KOAC and 3 M Glacial Acetic Acid) was added and gently vortexed in an inverted position for approximately 10 seconds. The tube was placed on ice for 5 minutes to facilitate precipitation of the detergents. After centrifugation for 5 minutes, the resulting supernatant was transferred to a new tube. Two volumes of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the supernatant and vortexed and then centrifuged for two minutes. The supernatant was transferred to a new tube without disturbing the organic layer. Double-stranded plasmid DNA was precipitated by adding two volumes of ice-cold ethanol. The mixture was then vortexed and placed in –20°C for a minimum of one hour, after which the samples were centrifuged for 30 minutes. The supernatant was removed and the pellet resuspended in 30 ul of ultra-distilled sterile water. A 1-ul aliquot of RNAse enzyme was added to the nucleic acid solution and incubated for 37°C for 30 minutes to degrade RNA in the sample. For simple DNA preparations purposes, agarose gel electrophoresis was performed to verify plasmid integrity and yield. For post-ligation screening of colonies, samples were analyzed using restriction digestion analysis using 2.5 ul of DNA in a 15 ul total reaction volume.

2.8 DNA Maxi Preparation

A single colony was used to inoculate 100 ml of LB media (see Table) in a 500 ml Erlenmeyer flask. The culture was incubated in a 37°C shaker for 16-18 hours with vigorous shaking. Cells were poured into a large centrifuge tube and were spun down at 3,500 RPM for 15 minutes using the Sorvall RC-5B centrifuge (Sorvall GSA rotor). After removal of the supernatant, cells were resuspended in 6 ml of GTE buffer (50mM

Glucose, 25 mM Tris-HCl - pH 8.0 and 10 mM EDTA - pH 8.0). To lyse the cells, 12 ml of a freshly prepared solution of 0.2 M NaOH and 1% SDS was added and gently mixed. The cells were placed on ice for 5 minutes after which 8 ml of ice-cold KOAC (pH 4.8) (5 M KOAC and 3 M Glacial Acetic Acid) was added to precipitate the detergent. The cells were then iced for 20 minutes and centrifuged at 3,500 RPM for 15 minutes. The supernatant was poured through a 3-ply layer of cheesecloth into a new centrifuge tube. To precipitate DNA, 14 ml of ice-cold isopropanol was added. Immediately thereafter, the sample was centrifuged at 10,000 RPM for five minutes. After careful removal of the supernatant, the resulting pellet was dissolved in 200 ul TE buffer and transferred to a microcentrifuge tube. To the tube, 10M NH_4OAc was added to make a final concentration of 2M NH_4OAc. The sample was placed on ice for 10 minutes and then spun down at 14,000 RPM in a benchtop centrifuge for 10 minutes. The supernatant was transferred to a fresh tube. After adding two volumes of ethanol, the sample was placed in –20°C for one hour to overnight to precipitate double-stranded DNA. The solution was then spun down in a microcentrifuge for 10 minutes with resuspension of the pellet in 100 ul TE buffer. 1 ul of a 10 ug/ml solution of RNAse A was added to the DNA and incubated for 37°C for 30 minutes. After incubation, a 1/10 volume of 3M NaOAc pH 5.2 and two volumes of ice-cold ethanol were added to precipitate DNA. The solution was placed in –20°C for 30 minutes after which 42.7 ul of 5M NaCl and 37 ul of 30% polyethylene glycol (PEG)/1.5 M NaCl was added. The sample was vortexed and placed on ice for 30 minutes, and then spun down for 10 minutes. The pellet was resuspended in 100 ul of ultra-filtered water and 100 ul of 2x proteinase K (PK) buffer and placed in a 37°C water bath for 30 minutes. To complete the DNA prep, one volume of

phenol/chloroform/isoamyl alcohol (25:24:1) was added to the solution, vigorously vortexed and then spun down for two minutes. The aqueous phase was then transferred to a new tube to which two volumes of ice-cold ethanol were added. The solution was placed in –20°C for a minimum of one hour. The sample was spun down for five minutes and the pellet resuspended in 100 ul of ultra-pure water. DNA was quantified at A_{260} using a spectrophotometer and also checked for protein contamination at A_{280}. Minimal protein contamination was ensured at a A_{260}/A_{280} ratio of 1.8 – 2.0.

2.9 In-vitro transcription

TCV-B was transcribed from pT1d1Δl. 12 ug of plasmid was linearized with *XbaI*. Two volumes of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the restriction digest, vortexed and centrifuged at 14,000 RPM for two minutes. The supernatant was transferred to a new tube where 1/10 volume of 3 M NaOAC was added with vigorous vortexing, followed by two volumes of ice-cold ethanol. The DNA was placed in –20°C for one hour to precipitate DNA. The DNA was then centrifuged at 14,000 RPM for 30 minutes. The pellet was resuspended in 15 ul of DEPC-treated water. Transcription was executed using 50 units of T7 RNA Polymerase (N.E. Biolabs, Beverly MA), 100 units of RNasin Ribonuclease Inhibitor (Promega, Madison WI), and 1mM of each ribonucleotide in 100 uL of 1x transcription buffer (N.E. Biolabs, Beverly MA) at 37 C for 2.5 hours. Afterwards, 15 units of RQ1 RNAse-free DNAse (Promega, Madison WI) was added. The reaction was incubated at 37°C for 30 minutes. To retrieve the RNA, phenol/chloroform/isoamyl alcohol (25:24:1) extraction was performed followed by

ethanol precipitation. After recovery of the pellet, RNA was resuspended in 50 ul DEPC-treated water and quantified using the spectrophotometer.

2.10 Viral Inoculation of TCV

Infectious BBM-TCV, consisting of total RNA extracted form TCV-infected turnip, was resuspended in 1X inoculation buffer (0.05M glycine, 0.03 M K_2HPO_4 and 1% celite) to a final inoculum concentration of 0.2 µg/µl. Inoculations were performed at 24-26 dpp on older, fully expanded leaves. A sterile glass stirring rod was briefly dipped into 1-µl aliquots of the viral suspension and rubbed onto the adaxial side of several oldest leaves while using a stiff platform for abaxial support to provide adequate inoculum penetration. Plants were left in the growth chamber for 10 days to allow progression of TCV.

N. benthamiana plants were inoculated in a similar manner. However, instead of inoculating with BBM virus, the plant was inoculated with in-vitro transcribed RNA.

2.11 Bacterial Infiltration

Fresh plates of *Pseudomonas syringae* pv. *glycinea* were streaked from glycerol stocks. A single colony was used to inoculate a 5-ml culture of fresh NYG media (see Table III) supplemented with 50 µg/ml kanamycin and allowed to grow overnight at 28°C. A 200-ul aliquot was used inoculated to a fresh sub-culture to an A_{600} of 0.5-0.9. Samples were centrifuged at 3,000 RPM (Sorvall GSA rotor) for 10 minutes at 4°C. The pellet was resuspended in 10 ml of 10mM $MgSO_4$ and re-centrifuged. The pellet was finally resuspended in 10 mM $MgSO_4$ and samples were diluted to an A_{600} of 0.2. Bacterial

samples were syringe- infiltrated into symptomatic leaves of TCV and mock-inoculated plants at 10 days post inoculation.

2.12 Ion Leakage Assays

Four-6mm diameter leaf punches were obtained from symptomatic leaves harvested 18 hours post infiltration and 5 hours post infiltration for avrRPT2 and avrRPM1, respectively. Leaf discs were subsequently floated on 2 ml ultra-pure water with abaxial sides towards the solution. Samples were incubated at room temperature for 4 hours and measurements obtained with a Cole-Parmer® 19815-00 Basic Conductivity Meter recently calibrated with Traceable One-Shot™ Conductivity Calibration Standard (Control Company, Friendswood TX).

2.13 Preparation of Electro-competent Agrobacterium

GV2260 cells were plated and incubated at 30°C for two days. Several colonies were then used to inoculate a 5 ml volume of LB (see Table) supplemented with 100 mg/l rifampicin and 50 mg/l kanamycin. The culture was left to grow overnight on a rolling drum at 30°C. The next day, a 200-400 ul aliquot of the culture was used to inoculate 500 ml of LB supplemented with 50 mg/l rifampicin. The flask was placed in a 28-30°C shaker at approximately 400 RPM until an A_{600} of about 0.5 was attained (usually 16-18 hours). To harvest the cells, the flask was chilled on ice for 30 minutes and then transferred to centrifuge bottles and centrifuged in the Sorvall RC-5B centrifuge (Sorvall GSA rotor) at 4,000 RPM for 15 minutes and 4°C. The supernatant was discarded and cells resuspended in 500 ml of 1 mM Hepes pH 7.4 and spun again at the same

conditions. The cells were the resuspended in 250 ml 1mM Hepes pH 7.4 and spun again at the same conditions. The cells were once again resuspended in 10 ml of 1 mM Hepes pH 7.4 and spun down one final time at the same conditions. The cells were resuspended in 2 ml of ice-cold 10% glycerol. The competent cells were distributed into 40 ul aliquots per microcentrifuge tube, placed in liquid nitrogen, and then stored in –80°C until needed.

2.14 Agrobacterium Transformation

Cells were thawed at room temperature and then immediately placed on ice. About 25 ul of the cells was transferred to a sterile microcentrifuge tube. Approximately 80 ng of DNA was added to the cells in a volume not exceeding 2 ul. The cells and DNA were gently mixed and then pipetted into a sterile electroporation cuvette. The Cell-Porator® (Life Technologies, Carlsbad CA) was set to the following conditions: voltage booster = 4 kΩ, capacitance = 330 μF, and DC volts = low. The cuvette was placed into the chilled electroporation chamber and pulsed at 1.4 kV. 1 mL of LB (Table) was immediately added to the cells. The cells were left to incubate at room temperature, with no agitation, for one hour. The cells were then plated on LB/kan/rif plates and incubated for 2-3 days in 30°C. After isolated colonies appeared, transformants were used to inoculate 5 ml cultures of LB/rif/kan. Cultures were grown overnight at 30°C to make glycerol stocks. Transformants were verified using colony screening via PCR and then grown overnight in 5 ml of LB supplemented with the appropriate antibiotic. Cultures were used to make 20% glycerol stocks to be stored in -80°C.

2.15 Agrobacterium-Mediated Transient Expression Assay

Agro strains were streaked onto LB plates (see Table) containing 50 mg/l kanamycin and 100 mg/l rifampicin and grown for two days in 30°C. Plates were kept for no more than two weeks at a time. New plates were always streaked from glycerol stocks. To start the cultures, a 5 ml volume of LB supplemented with 100 mg/l rifampicin and 50 mg/l kanamycin was inoculated with a smear of colonies and grown overnight in a rolling drum at 28-30°C. The cultures were then placed in a 15 ml Falcon tube and spun down at 4,000 RPM for five minutes in the Sorvall RC-5B centrifuge (Sorvall GSA rotor) and then resuspended in 5 mL of induction medium (400 ml water, 4.88g MES, 2.5 g glucose, and 0.12 g NaH_2PO_4 and 25 mL AB salts (20 g NH_4Cl, 6g $MgSO_4$-$7H_2O$, 3g KCl, 0.2g $CaCl_2$, and 0.05g $FeSO_4$-$7H_2O$); 500 ul of 200mM fresh acetosyringone in DMSO prepared JUST prior to use) and spun again. The pellet was resuspended in 5 ml of induction media. The culture was used to inoculate a 50 ml volume of induction media, 50 mg/l kanamycin (rifampicin is not required at this step), in a 250 ml flask and was then cultured overnight in the large shaker at 30°C by simply turning the shaker heater switch off. The next day, the cultures were spun down in 50 ml Falcon tubes at 4,000 RPM for five minutes and resuspended in 40 ml of 10 mM MES + 200 uM fresh acetosyringone in DMSO. The cultures were spun down again at 4,000 RPM for five minutes and then resuspended in 30 ml of 10 mM MES + 200 uM acetosyringone. To find the absorbance of the culture, a 1:10 dilution was made and used to make a final cell suspension absorbance of 0.3 using 10 mM MES + 200 uM acetosyringone as the diluting agent. For cell death suppression assays, HR-inducing agents such as the Pto and avrPto combination were mixed at a 1:1 ratio, while the candidate or known suppressor

or empty vector was mixed at a final ratio of 1:1 to the R-avr volume. Cases requiring coinfiltrations of more than one TCV ORF were mixed at equal ratios. For example, 3ml of 0.3 OD agro containing p28MUT and 3 ml of 0.3 OD agro containing p88MUT would be added to 3 ml of 0.3 OD agro containing avrPto and 3 ml of 0.3 OD agro containing Pto for a final solution volume of 12 ml and a final OD of 0.3

For the transient expression assay, middle-aged leaves about the width of a hand length were selected. Small circles about the size of a quarter were made followed by a needle poke to the center of each circle. A 1-ml syringe was used to pressure infiltrate the Agro into the hole so as to cover the tissue demarcated by the marker. Several duplicates of samples were made at varying leaf positions as leaf position does tend to affect the transient assay. Plants were moved to 24 hour light and moderate temperature conditions and monitored daily for transient expression phenotypes. For RNA extraction, leaves were harvested 36 hpi. Leaves were scored 7 dpi for final analysis of cell death suppression or progression.

2.16 RNA Isolation from Plant Tissue

Leaves were harvested from plants, weighed, and flash frozen in liquid nitrogen and placed in –80°C until preparation. Concert Plant RNA Reagent (Invitrogen) was used for RNA isolation from *Arabidopsis thaliana* and *Nicotiana benthamiana* leaves. After isolation, RNA was resuspended in DEPC-water with A_{260} quantification of yield using the spectrophotometer. RNA samples were always stored at –80°C.

DEPC water was prepared by adding 200 ul of DEPC to 100 ml water. After vigorous shaking, the solution was autoclaved. All solutions used in the RNA prep were made

from autoclaved DEPC water. Note: Only autoclaved DEPC water may be used to prepare buffers to prevent ethylation of titratable groups and loss of buffer conditions.

2.17 RNA Gel Electrophoresis

RNA samples were run on a 1.2% agarose denaturing gel. To prepare the gel, agarose was first melted in distilled water. After cooling the superheated mixture, 1X gel running buffer (0.2M MOPS, 50 mM NaOAc and 1 mM EDTA) and 2.2 M 37% formaldehyde were added to the agarose solution. The gel was poured and allowed to solidify. After solidification, the gel was placed into an electrophoresis chamber containing1X gel running buffer. Approximately 10 ug of RNA was used per lane. 15 ul of denaturation buffer (1V 10X gel running buffer, 1.75V 37% formaldehyde and 5V 99% formamide) was added per 5 ul of RNA. The solution was mixed well and incubated at 55°C for 30 minutes. After denaturing the sample, 1/10V loading buffer (50% glycerol, 1mM EDTA and 0.4% bromophenol blue) was added to the solution. The samples were loaded onto the gel and run until the bromophenol blue marker migrated 90% of the full length of the gel.

To visualize RNA integrity, the gel was placed in a 0.1% Ehidium Bromide/DEPC water solution and gently agitated for 10 minutes. The gel was then placed under UV light, with minimal exposure, using the UV transilluminator to check for intact ribosomal RNA bands. The gel was destained by gentle agitation in DEPC water for 10 minutes. Occasionally the gel was saved for no more than 24 hours before blotting.

2.18 Northern Blots

After gel electrophoresis, the gel was rinsed with several washes of DEPC water. The gel was then allowed to soak in alkaline transfer buffer (0.01M NaOH and 3M NaCl) for 10 minutes. While soaking the gel, the membrane was prepared for subsequent RNA transfer. A section of positively charged nylon membrane was cut to a size approximately 1 mm larger than the gel in both dimensions. The membrane was then carefully placed onto a container of DEPC water. After the membrane was fully wetted, the membrane was pushed into the water and allowed to soak for five minutes.

After retrieving the gel from the alkaline transfer buffer, the gel was moved to a clean surface where a sharp scalpel was used to cut away unused portions of agarose and to cut one corner of the gel as a positional marker. A similar cut was made into the nylon membrane after soaking to mark orientation of the gel.

To assemble the capillary transfer system, a 3 cm stack of lab grade folded-paper towels was constructed. Sections of paper towel were cut approximately one inch greater than the gel in both dimensions. On the side, five pieces of filter paper cut to the size of the gel were set to soak in alkaline transfer buffer. To construct the second layer, two pieces of the wetted filter paper were placed on top of the first layer. The nylon membrane was then added as the third layer. The trimmed gel was then placed on top of the membrane as the fourth layer taking care to align the cut corners of the membrane and gel. Four 0.5 inch-strips of parafilm were cut and each placed underneath the four sides of the perimeter of the gel to make a seal between the gel and membrane. At this point, care was taken to ensure that no air bubbles resided between the membrane and the gel.

The top of the gel was then wetted with transfer buffer and the remaining three wetted sections of filter paper were added on top of the gel. Two long 2-in X 24-in strips of filter paper were wetted in transfer buffer and placed on top of the gel with either end of the strip immersed in transfer buffer (see Figure 7). A thin glass plate was placed on top of the strips to prevent evaporation of buffer. A 100-g weight was finally placed on top of the entire assembly. One hour was typically sufficient for transfer of RNA to the membrane. After one hour the system was dismantled and the membrane placed in 5X SSC (22g NaCl, 11g sodium citrate in 400 mL water, pH to 7.0, top to 500 ml) for five minutes with gentle agitation.

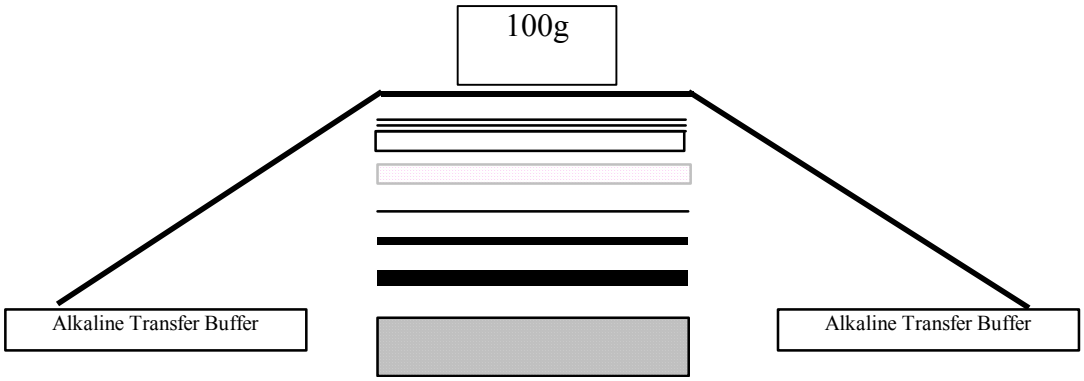


Figure 7: Capillary Transfer System

After soaking in 5X SSC, the membrane was transferred to a piece of 3 mm filter paper to dry. Upon complete drying, the membrane was placed onto a new piece of filter paper and placed into the FB-UVXL-1000 UV crosslinker (Fisher Scientific, Hampton NH), to fix the sample to the membrane, and set to "Optimal Crosslink". After irradiation, the membrane was placed into a box containing 3 ul of a 400 ug/ml ethidium bromide solution for staining. The membrane was gently agitated for five minutes and then visualized using the UV transilluminator with minimal UV exposure. After observation

of the ribosomal bands, the membrane was destained by immersion in DEPC water and gentle agitation for 20 minutes. The membrane was then allowed to dry on a piece of 3 mm filter paper and was then wrapped in plastic wrap and stored in 4°C.

2.19 Northern Hybridization

To prepare a probe for hybridization, 25 ng of purified DNA fragment was placed in a microcentrifuge tube. To the DNA, 24 ul of sterile water was added. The DNA was then randomly primed following using the Prime-it II kit (Stratagene, La Jolla CA). The reaction was incubated at 37°C for 30 minutes. After incubation, 150 ul of water was added to the reaction and the probe was ready for spin column purification. NucAway™ Spin Columns (Ambion, Austin TX) were used to recover purified probe DNA. After spin column purification, the probe was pipetted to a fresh microcentrifuge tube. Two scintillation vials were partially filled with scintillation fluid. A 2 ul-aliquot of purified probe was added to one vial and both vials were placed into the scintillation counter for measurement of radioactivity. Typical acceptable counts for a functional probe were around 20,000 cpm/ul. Probes possessing counts less than 10,000 were typically deemed inefficient and were not used.

In preparation for hybridization, 1% BSA was freshly added to 10 ml of hybridization solution (0.5 M NaPO4, 1 mM EDTA and 7% SDS). The solution was poured into a glass hybridization tube followed by the membrane, RNA side facing into the tube, ensuring that the membrane was fully covered by solution. The tube was placed into the micro hybridization chamber and rotated for two hours at 65°C. The purified probe was kept on ice behind a polycarbonate shield in the interim.

After pre-hybridization of the membrane, the tube was opened and the solution discarded. A fresh 10 ml volume of hybridization buffer was prepared as described above and added into the tube. The probe was then boiled for 5 minutes in a water bath and then pipetted into the tube. The amount of probe to add should yield a final concentration of approximately 1 x 10^6 cpm solution. The tube was placed into the hybridization chamber and left to rotate for 18 hours at 65°C.

After hybridization, the membrane was retrieved from the tube and immersed into Wash Solution #1 (1X SSC and 0.1% SDS) for ten minutes, with gentle agitation, at room temperature. After Wash #1, the membrane was returned to the tube along with Wash Solution #2 (0.5 SSC and 0.1% SDS). The tube was placed back into the chamber and rotated at 60 C for ten minutes. The membrane was then left to dry on plastic wrap. A Geiger Counter was used after each wash step to ensure removal of unhybridized probe.

The dried membrane was placed into a cassette and exposed to a panel from the BAS 1000 (Fujix, Kyoto Japan) phosphorimager for two hours. The panel was then developed in the phosphorimager for image capture and analysis.

2.20 RT-PCR

Using RNA harvested from leaves agro-infiltrated with TCV ORFs, 1 uL 50 uM oligo dT, 2 ug total RNA and 1 uL10 mM dNTPs were added to a microcentrifuge tube and brought to 13 uL with RNAse-free water or DEPC water (for samples containing the agro-infiltrated full-length TCV construct, Spe I primer was used in lieu of oligo dT). The tube was then placed in 65° C for 5 minutes and then spun down and placed on ice. To the tube, 4 uL of 5X First Stand Buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15

mM $MgCl_2$], 1 uL 0.1 mM DTT, 1 uL RNASEOUT™ Ribonuclease Inhibitor (Invitrogen, Carlsbad CA) and 1 uL SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad CA) was added. The tube was placed in a 50° C water bath for one hour and then spun down and placed on ice. The enzyme was deactivated by placing the tube in 70° C for 15 minutes. For amplification of the resulting cDNA, standard PCR was performed using an internal primer pair and the RT reaction as template. Typically 1 uL of the RT reaction in a 50-uL total PCR reaction volume was sufficient for amplification. Analysis was performed using standard gel electrophoresis.

2.21 Yeast Transformation

Fresh INVSc1 (Invitrogen, Carlsbad CA) yeast strains were streaked onto YPD plates (see Table) from glycerol stocks and allowed to grow for 2-3 days at 30°C. A single colony was then used to inoculate 10 ml of YPD media in a 25-50 ml flask. The culture was grown overnight in a 30°C shaker at 400 RPM. The 10 ml culture was used to inoculate 25 ml of fresh YPD in a 100 ml flask. The flask was placed in a 30°C shaker for 1-2 hours until A_{600} as about 1. The culture was then transferred to a 50 ml falcon and spun down at 3,000 RPM in the Sorvall RC-5B (Sorvall GSA rotor) at 4°C for 5 minutes. The cells were then resuspended in 40 ml of ice-cold sterile water and spun at 2,500 RPM at 4°C for 5 minutes. The wash was repeated with 20 ml sterile ice-cold water at 2,500 RPM for 5 minutes. The pellet was resuspended in 5 ml ice-cold 1 M glycerol. The culture was then centrifuged a final time at 2,000 RPM at 4°C for five minutes. The cells were resuspended in 150 ul of ice-cold 1 M glycerol and transferred to a sterile 1.5 ml microcentrifuge tube and kept on ice until ready for transformation. A 20 ul aliquot of

yeast cells were transferred to a sterile microcentrifuge. Approximately 5 ug of DNA in distilled water was added to the cells in a volume no more than 5 ul. The mixture was carefully pipetted into a sterile electroporation cuvette and placed into the chilled Cell-Porator® (Life Technologies, Carlsbad CA) chamber and set to the following conditions: voltage booster = 4 kO, capacitance = 330 μF, and DC volts = low. Cells were electroporated at 1.5 kV. Immediately thereafter, 1 ml of 1M ice-cold glycerol was added and the cells left to incubate at room temperature for one hour without agitation. The cells were then plated on SD plates and incubated at 30°C.

2.22 Yeast Cell Death Assays

Wild-type INVSc1 cells were transformed with the appropriate constructs just prior to performing each experiment. Transformants were then selected and grown in 10 mL SD media, lacking histidine (see Table I), and 2% glucose and grown overnight to select for the presence of the plasmid. The cells were then centrifuged at 3,500 RPM for five minutes and resuspended in SD medium, lacking histidine, containing 2% galactose and 1% raffinose. The cells were centrifuged again at 3,500 RPM for five minutes and resuspended in 10 ml of SD/gal/raff/-his media to induce expression from the GAL1 promoter in p423.

The yeast cells were induced for six hours with agitation at 30°C. After six hours, cells were diluted to an A_{600} of 0.05 in a total volume of 10 ml of SD/gal/raff/-his and were stressed by either chemical treatment or heat shock treatment. For chemical treatment, cells were treated with 3 mM H_2O_2 final concentration in the medium and incubated at 30°C with vigorous shaking at 800 RPM for 6 hours. For heat stress, yeast cells were

incubated at 37°C for 30 minutes with vigorous shaking at 800 RPM, then transferred to a stationary water bath at 50°C for 30 minutes, and then returned to 30°C with vigorous shaking at 800 RPM for 6 hours. Following treatments, cell viability was determined by plating five serial ten-fold dilutions, using SD-/gluc media as the diluting agent, of each sample on SD/-his plates. To ensure cell viability, both wild-type and transformed cells were grown at 30°C for six hours with agitation at 400 RPM. Wild-type yeast cells were also subject to heat treatment and chemical treatment to ensure cell susceptibly to stressful conditions.

Table III: Media Recipes - Based on 1L total volume

Media	Liquid	Solid
LB	10.0 g tryptone 10.0 g sodium chloride 5.0 g yeast extract	10.0 g tryptone 10.0 g sodium chloride 5.0 g yeast extract 20.0 g agar
NYG	5.0 g Bacto™ Proteose Peptone #3 (BD, Sparks MD) 3.0 g yeast extract 80.0 mL 25% glycerol	5.0 g Bacto™ Proteose Peptone #3 (BD, Sparks MD) 3.0 g yeast extract 80.0 mL 25% glycerol 15.0 g agar
YPD	10.0 g yeast extract 20.0 g Bacto™ Proteose Peptone #3 (BD, Sparks MD) 20.0 g dextrose	10.0 g yeast extract 20.0 g Bacto™ Proteose Peptone #3 (BD, Sparks MD) 20.0 g dextrose 20.0 g agar
SD	26.7 g Minimal SD Base (Clontech, Palo Alto CA) 0.77 g -His DO Supplement (Clontech, Palo Alto CA)	26.7 g Minimal SD Base (Clontech, Palo Alto CA) 0.77 g -His DO Supplement (Clontech, Palo Alto CA) 20.0g agar

3 RESULTS

3.1 TCV suppresses formation of HR to avrRpt2

Col-0 plants infiltrated with *P. syringae* strains expressing either endogenous or plasmid-encoded avrRpt2 typically form visual HR lesions within 24 hours (Shapiro et al., 2001). In previous experiments, TCV-infected Col-0 plants showed a marked reduction in lesion size or severity. In order to quantify the apparent suppression of the HR, an ion leakage assay approach was used, as electrolyte leakage is a common hallmark of PCD (Rate and Greenberg, 2001).

Before commencing experiments investigating TCV-mediated suppression of avrRpt2 induced PCD, it was necessary to establish the time of peak ion flux in avrRpt2-induced PCD in the absence of TCV. Accordingly, Col-0 plants were infiltrated with *Psg* carrying the avrRpt2-expressing vector. Leaf punches were harvested at 14, 16, 18 and 20 hpi. Maximum conductivity for each of the three samples was apparent at 18 hours post infilatration, as shown in Table IV. Leaves from all subsequent ion leakage assays involving avrRpt2 mediated PCD were scored at 18 hpi consistent with the time-trace experiment detailed in Table IV.

In order to assay the effect of TCV on ion leakage, three-week old Col-0 plants were inoculated with TCV. Upon appearance of viral symptoms, the uninoculated but symptomatic leaves were challenged with one of the following: buffer, *Psg* carrying an empty vector, or *Psg* carrying a vector encoding avrRpt2. Equivalent size and age leaves were infiltrated on all mock-TCV inoculated plants. Samples representing negative controls were mock-bacterial and mock-viral inoculated with 10 mM $MgSO_4$ infiltration

buffer and inoculation buffer, respectively. After bacterial infiltration, leaf punches were taken from infiltrated tissue and analyzed for conductivity.

Table IV: Time course data for determination of peak ion flux upon avrRpt2-induced PCD. Three-week old Col-0 plants were either mock-infiltrated with 10 mM MgSO4, bacterially-infiltrated with Psg harboring an empty vector, or bacterially-infiltrated with Psg carrying a plasmid expressing avrRpt2. All infiltrations were done with 10^7 cfu/mL. Four 6-mm leaf punches were taken at the indicated time points and assayed for conductivity.

Time (hpi)	Mock Infiltrated		Psg (Empty)		Psg (avrRpt2)	
14	42.6	43.3	85.2	73.9	74.4	32.7
16	49.3	46.6	32.2	38.5	89.5	108.5
18	44.5	47.2	43	48.6	140.7	101.2
20	43.7	33.7	28.5	32.1	156.3	30.2

Figure 8 shows the results of three independent trials. Untreated leaves show a low level of ion leakage due to the mechanical damage to cells at the cut site. Mock bacterial infiltration with bacteria carrying an empty vector show a level of ion leakage not significantly different than the untreated leaves. This is true for both the TCV infected plants and the plants not inoculated with virus. A large increase in conductivity was seen in the leaves infiltrated with the *avr* and that were not infected with TCV. This large increase is characteristic of tissue undergoing programmed cell death. In contrast, TCV-infected leaves leaf samples challenged with *avrRpt2* showed a significant decrease in conductivity providing evidence of inhibition of cell death in leaves harboring the virus.

Averaging the data from the three independent experimental trials, the conductivity for HR suppressed samples was consistently half that of its virus free counterpart as shown

in Figure 9. In fact, after subtracting the background seen in untreated tissue, the TCV-infected tissue showed a four-fold decrease conductivity.

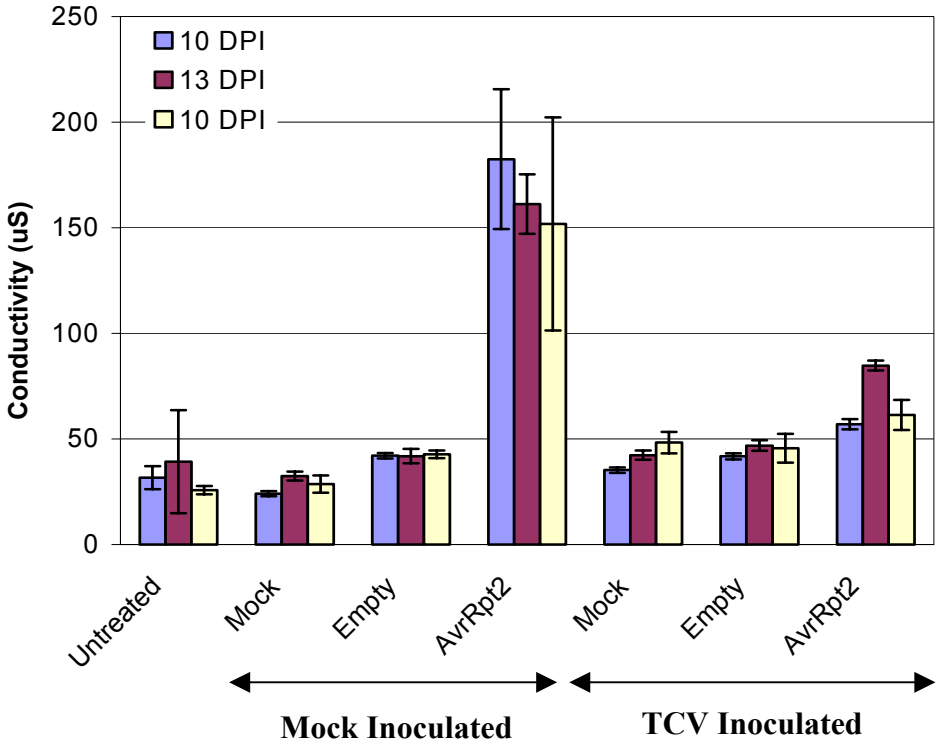


Figure 8: Quantification of AvrRpt2-induced Programmed Cell Death by Ion Leakage. Three week-old Col-0 plants were inoculated with 0.2 ug/uL TCV. After 10 days, plants were either mock-infiltrated or Psg infiltrated with 10mM $MgSO_4$ or 10^7 cfu/ml, respectively. Four 6-mm leaf discs were taken 18 hours post infiltration and analyzed for conductivity. Plot shows conductivity values obtained from three independent trials. Each trial consisted of three samples of four leaf discs each. Blue and yellow bars show ion leakage data for leaves infiltrated 10 days post-viral inoculation, while red bars shows data for leaves infiltrated 13 days post-viral inoculation.

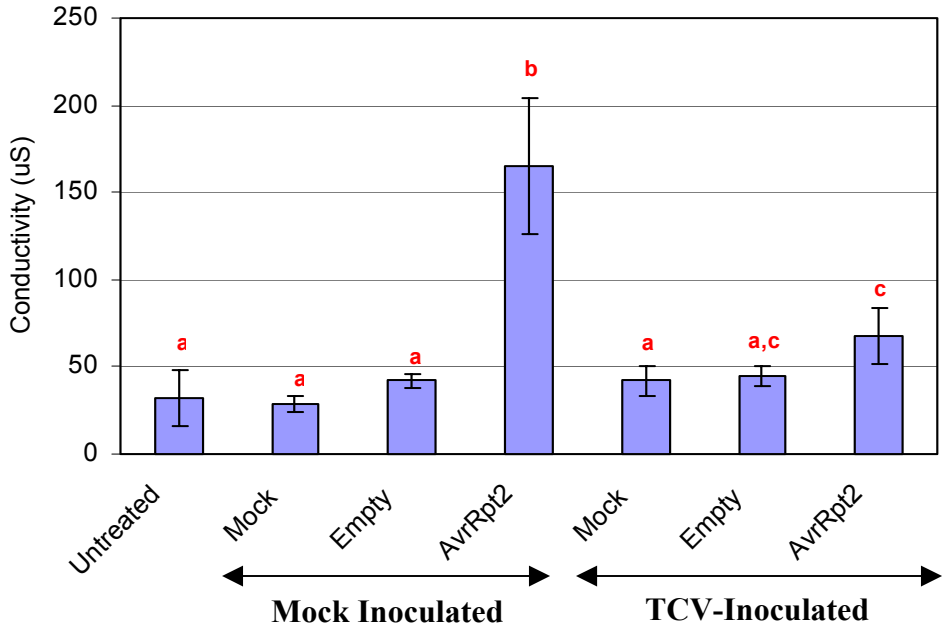


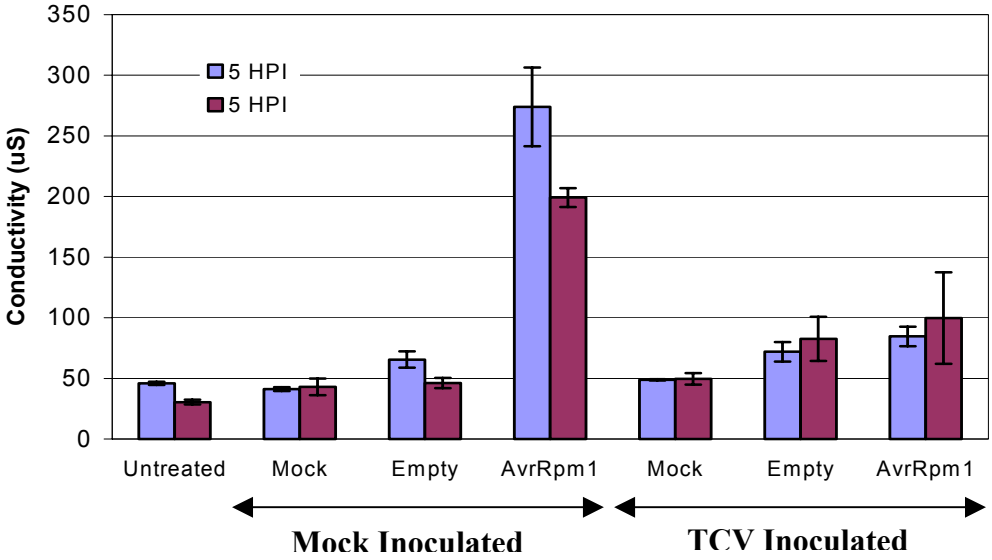
Figure 9: Statistical Analysis of avrRpt2-induced PCD Quantification in Col-0. Bars represent the average of three independent trials consisting of nine total samples. Letters above bars represent distinct significance groups as determined by the Tukey-Kramer Method for multiple sample comparisons. Each group differs from the others at a confidence level of P = 0.05. All statistics were performed using NCSS statistical software.

3.2 TCV suppresses formation of HR to avrRpm1

In order to determine if TCV could suppress the HR to a different avr, a similar set of experiments were carried out - the only difference being the avr carried on the plasmid in these experiments was avrRpm1. To establish HR suppression, Col-0 plants were either mock inoculated or inoculated with TCV. Ten days post viral inoculation, the symptomatic leaves and equivalent sized leaves on mock-inoculated plants, were infiltrated with one of three samples: 10 mM MgSO4, *Psg* carrying an empty vector, or *Psg* carrying a vector encoding avrRpm1. Since the kinetics of HR to avrRpm1 is much

quicker than avrRpt2, conductivity and tissue collapse was monitored at 5 hpi rather than 18 hpi (Shapiro et al., 2001).

As predicted, leaf samples subjected to *P. syringae* strains expressing *avrRpm1* displayed the highest levels of conductivity as shown in Figure 10.



a

b

c

Figure 10: Quantification of AvrRpm1-induced Programmed Cell Death by Ion Leakage. Three week-old Col-0 plants were inoculated with 0.2 ug/uL TCV. At 10 dpi, plants were then either infiltrated with 10mM MgSO4 or infiltrated with or 10^7 cfu/ml of *Psg*. Four 6-mm leaf discs were taken 5 hours post infiltration and analyzed for conductivity. Plot shows conductivity values obtained from two independent trials. Each trial consisted of three samples of four leaf discs each (a). Pictures of *Psg* avr infiltrated leaves at 5 hpi from plants mock-inoculated (b) or virus infected (c).

In contrast, the leaves that harbored virus showed a much smaller increase in conductivity levels upon infiltration with *Psg* carrying the *avr,* and in fact, showed values similar to all the other groups. Averaging the data from the two independent experimental trials, the conductivity for HR suppressed samples was consistently 2.5 times less that of its virus free counterpart as shown in

Figure 11. After subtracting the background seen in untreated tissue, the difference is four-fold.

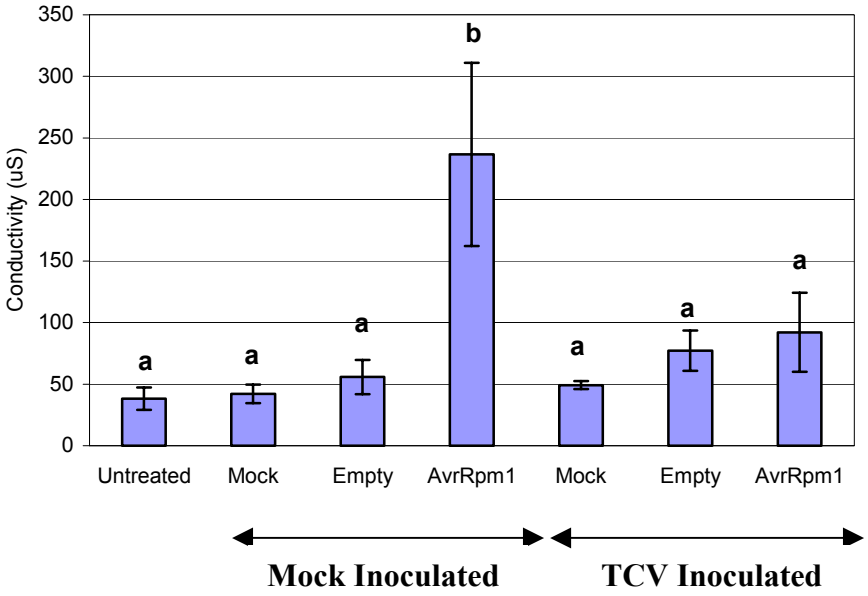


Figure 11: Statistical Analysis of avrRpm1-induced PCD Quantification in Col-0. Bars represent the average of two independent trials consisting of six total samples. Letters above bars represent unique significance groups as determined by the Tukey-Kramer Method for multiple sample comparisons. Each group differs from the others at a confidence level of P = 0.05. All statistics were performed using NCSS statistical software.

3.3 Pto/AvrPto Challenge of TCV infected *Nicotiana benthamiana*

With the eventual goal to determine the viral component responsible for reducing or suppressing the HR, we began developing a new system for monitoring the TCV suppression of HR. A transient assay in *Nicotiana benthamiana* was recently used to

demonstrate the ability of avrPtoB to suppress the HR (Abramovitch et al, 2003). In order to utilize this system effectively, it was first necessary to determine if TCV could suppress the HR in these plants. As it had previously been shown that TCV could systemically infect *N. Benthamiana* (Lin and Heaton, 1999), four-week old plants were inoculated with in vitro transcribed TCV genomic RNA. At 22 dpi, viral symptoms were clearly visible on younger leaves. Symptoms of infections included leaf wrinkling, stunted growth, rough texture and vein coloring causing a mottled appearance.

Figure 12: TCV infection in *Nicotiana benthamiana* at 22 dpi. Four week-old plants were either mock inoculated or TCV inoculated with 0.2 ug/uL RNA. Mock-Inoculated plants (a) and (b) TCV inoculated plants (c) and (d).

Symptomatic and asymptomatic leaves of TCV-infected and control plants were then infiltrated with a mixture of *Agrobacterium tumefaciens* carrying two different plasmids. One encoded for avrPto while the other encoded for Pto. Under normal conditions, co-expression of these two proteins results in a visible HR throughout the infiltrated area (Abramovitch et al., 2003)

Three different types of leaf tissue were infiltrated – symptomatic young leaves and middle-aged leaves, and older, uninoculated leaves. Older leaves were included as a control since virus was not anticipated to be present in these tissues allowing, measurement of PCD activity in the absence of virus. Symptomatic young and middle-aged leaves were infiltrated to assay for HR suppression as these leaves harbor active virus.

These experiments were evaluated two different ways – electrolyte leakage measurements taken within two days following infiltration and visual inspection of the tissue after several days. For the ion leakage test, leaf punches were taken at 30 hpi and 48 hpi. These time points were selected because older leaves were showing visible signs of tissue collapse.

Figure 13 shows conductivity readings for the two time points.

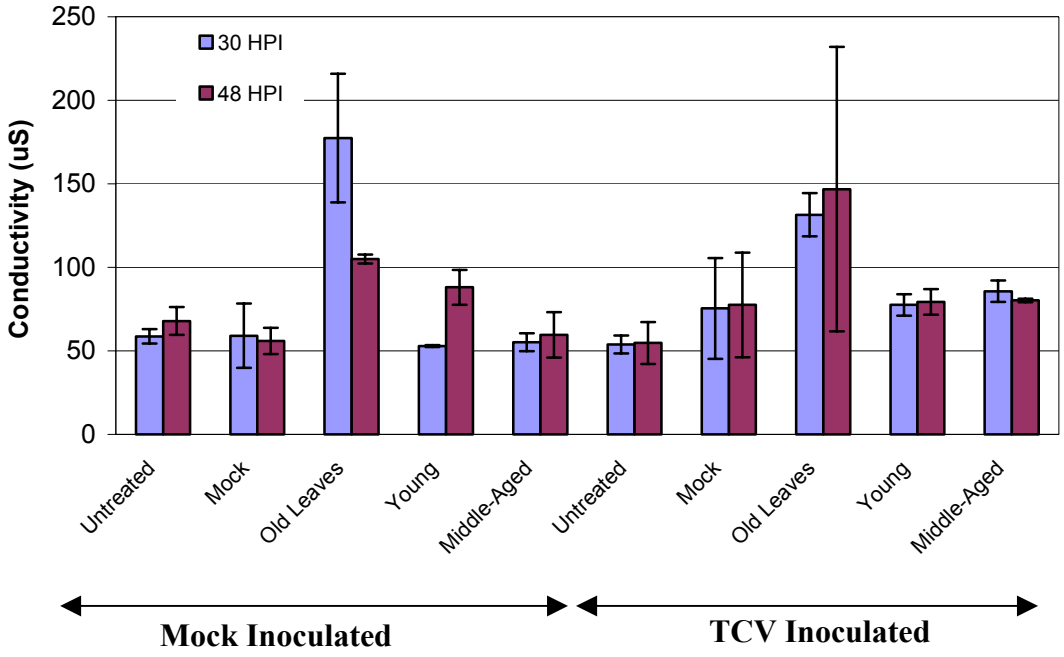


Figure 13: Quantification of AvrPto/Pto induced Programmed Cell Death in *N. benthamiana* plants. Four week-old plants were inoculated with 0.2 ug/uL in vitro transcribed TCV. Plants were then either mock-infiltrated or Agro infiltrated with 10mM MES or with bacteria at A_{600} = 0.3, respectively. Four 6-mm leaf discs were taken analyzed for conductivity. Plot shows conductivity values obtained at 30 hours (blue bars) and 48 hours (red bars) post infiltration.

As expected, older leaves revealed the highest conductivity levels in both TCV and control plants. In the virus free plant, the peak of conductivity is at 30 hpi and decreases considerably at 48 hpi. The TCV-infected older leaves show a different pattern with a higher level of ion leakage at 48 hpi but the difference is not so great between the time points. Interestingly, the younger and middle-aged leaves of the virus-free plant showed little ion leakage at either time point. However, by 4 dpi visual inspection showed significant tissue collapse. Isolating the time point at which peak ion flux occurs in these younger mock-inoculated tissues is crucial before extending the analyses to include TCV. Overall, the range of conductivity values in the TCV-infected plant was

higher and much broader indicating that the virus may variably affect the PCD pathway as visual inspection of younger and middle-aged TCV-infected leaves showed variable levels of lesion formation, from 5 – 100%, when scored 7 dpi.

3.4 Transient Expression of TCV in *Nicotiana Benthamiana*

The entire TCV-genome was cloned into the *Agrobacterium* compatible plant expression vector pBTex for use in the transient assay system. The clone was sequenced and checked. The p28, p9, and p38 ORFs had full consensus. The p88 ORF contains a single amino acid change from glutamic acid to glutamine. Moreover, p88 and p8 will need to be resequenced as their sequence data was incomplete.

The full-length TCV clone was then tested to check for recapitulation of a viral infection. After infiltration of *N. benthamiana* plants with *Agro* carrying the vector, samples were harvested 2, 4, and 6 dpi from Agro-infiltrated leaves. To determine whether the expressed virus was spreading from infiltrated tissue to uninfiltrated tissue, sections of leaves proximal to infiltration sites were harvested at 4 and 6 dpi. RT-PCR was performed on RNA extracted from these samples to detect for presence of TCV. Figure 14 shows the results of the RT-PCR analysis.

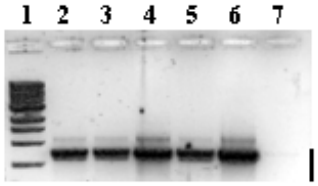


Figure 14: RT-PCR of Total RNA extracted from tissue infiltrated with TCV carrying the full-length TCV expressing vector. 1-kB DNA Ladder (Lane 1); Infiltrated leaf 2 dpi (Lane 2); Uninfiltrated and infiltrated leaf 4 dpi (Lanes 3 and 4); Uninfiltrated and infiltrated leaf 6 dpi (Lanes 5 and 6); negative PCR control (Lane 7).

As indicated by the figure, TCV transcript was detectable at 2 dpi. Movement of TCV to uninfiltrated sections of tissue was detected at 4 and 6 dpi. Active replication of the virion in infiltrated tissue was also evident as intensity of amplified cDNA increased from 4 to 6 dpi.

3.5 Detection of Individual TCV ORFs in N. Benthamiana

Each construct was transfected into *Agrobacterium* and infiltrated into *N. benthamiana* leaves. To determine if the constructs were being properly transcribed in-planta, RNA gel blot analysis and RT-PCR were performed on total RNA extracted from infiltrated tissue. As the p28MUT:pBTEX clone was the first construct made, a gel blot analysis was used to detect p28MUT transcript. Figure 15 shows detection of p28MUT transcript in infiltrated leaves.

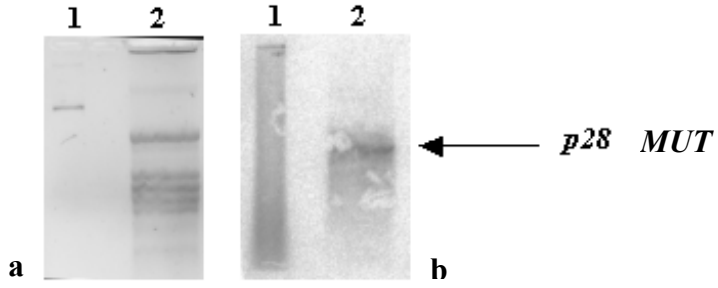


Figure 15: RNA gel blot detection of p28MUT transcript in total RNA harvested from N. benthamiana leaves 36 hpi. Ethidium bromide stained RNA denaturing gel showing 50 ng pT1D1ΔL DNA (Lane 1) and 10 ug total RNA from leaves infiltrated with agro carrying pBTEX expressing p28MUT (Lane 2) (a); RNA gel blot of the same gel blotted and hybridized with a p28 probe (Lane 2) (b).

To test for the presence of p8, p9MUT, p38 and p88MUT transcript, RT-PCR was used. Total RNA was extracted from *N. benthamiana Agro*-infiltrated with each of the

TCV ORFs. The RNA was then used as template for first strand synthesis by reverse transcription. The resulting cDNA was used as template for amplification using traditional PCR and primer pairs specific for each transcript as shown in Figure 16.

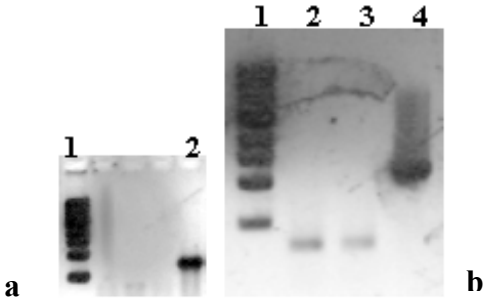


Figure 16: RT-PCR detection of TCV ORF transcripts in total RNA harvested from *N. benthamiana* leaves 36 hpi. 1-kB DNA Ladder (Lane 1) and amplified cDNA from leaves infiltrated with Agro carrying pBTEX expressing p88MUT (Lane 2) (a); 1-kB DNA Ladder (Lane 1), amplification of cDNA from leaves infiltrated with Agro carrying pBTEX expressing p8 (Lane 2), p9MUT (Lane 3) and p38 (Lane 4) (b).

3.6 Transient Expression of Individual TCV ORFs in *N. benthamiana*

As previously discussed, *N. benthamiana* plants infiltrated with *Agro* expressing AvrPto and Pto display localized necrotic lesions – evidence of the HR and a functional R-avr interaction. Each of the TCV constructs was evaluated for anti-PCD activity in the presence of AvrPto/Pto-mediated PCD. Additionally, combinations of each of the constructs were also tested to see if the suppression phenotype required the presence of more than one component. For almost all of the systems tested, a variable extent of lesion formation was observed in the transient assay system, as shown in Figure 17-Figure 19.

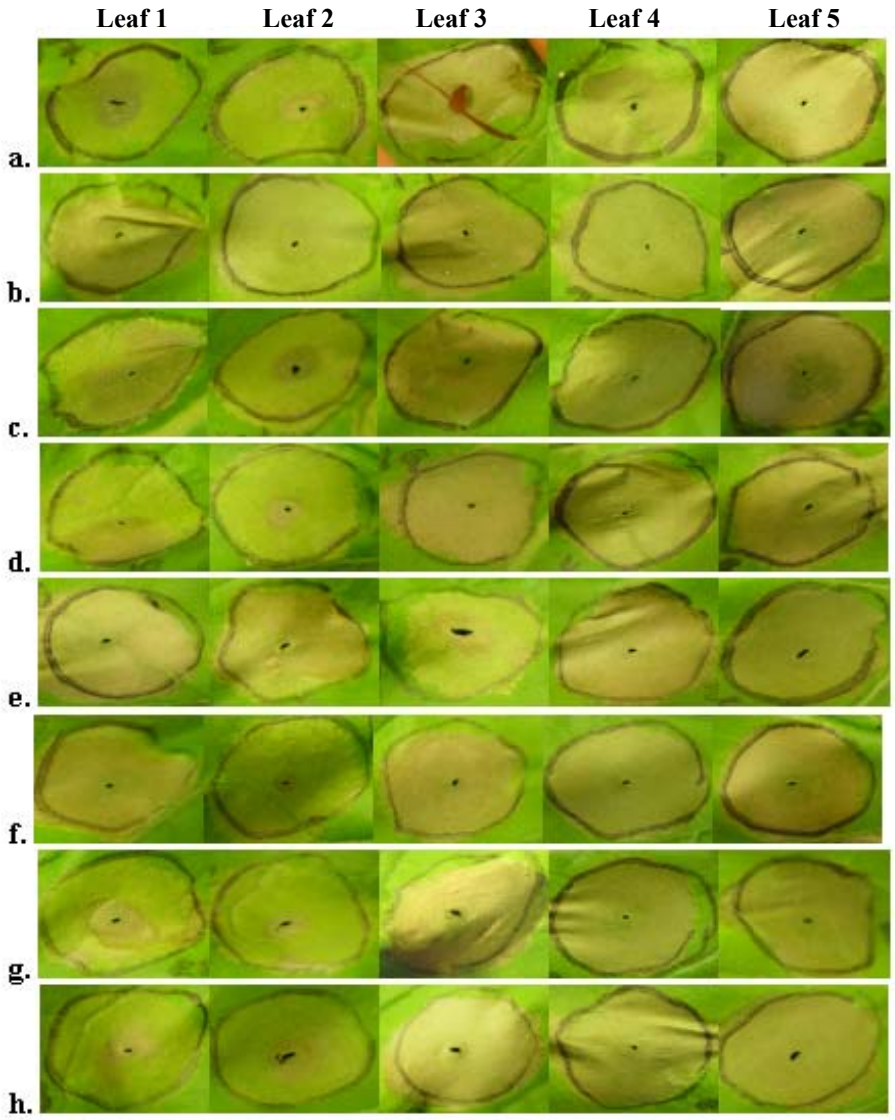


Figure 17: Transient Expression Assay in *N. benthamiana*. Eight-week old *N. Benthamiana* plants were infiltrated with *Agrobacterium* carrying a vector expressing each of the TCV ORFs at an A_{600} = 0.3 in combination with *Agrobacterium* carrying vectors expressing AvrPto and Pto, each at an A_{600} = 0.3. Leaves were scored for suppression phenotypes 7 dpi. p28MUT(a) p88MUT(b) p8(c) p9MUT(d) p38(e) p28MUT,p88MUT(f) p28MUT,p8(g) p28MUT,p9MUT(h). Three different plants were tested. Leaves 1 and 2 were taken from one plant, while leaf 3 was taken from a second plant and leaves 4 and 5 were taken from a third plant.

The percentage of infiltrated area that resulted in tissue collapse varied from 5% to 100% in the five distinct samples expressing the same construct or combination of

constructs. The only combinations that showed consistency in observable phenotype was the p88MUT and p9MUT pair, panel (j) of Figure 18, and the p88MUT and p38 pair, panel (k) of Figure 18, which resulted in 90-100% tissue collapse in all five leaf samples. Tables B-1 through B3 in Appendix B show progression of necrosis from 4 dpi – 7 dpi.

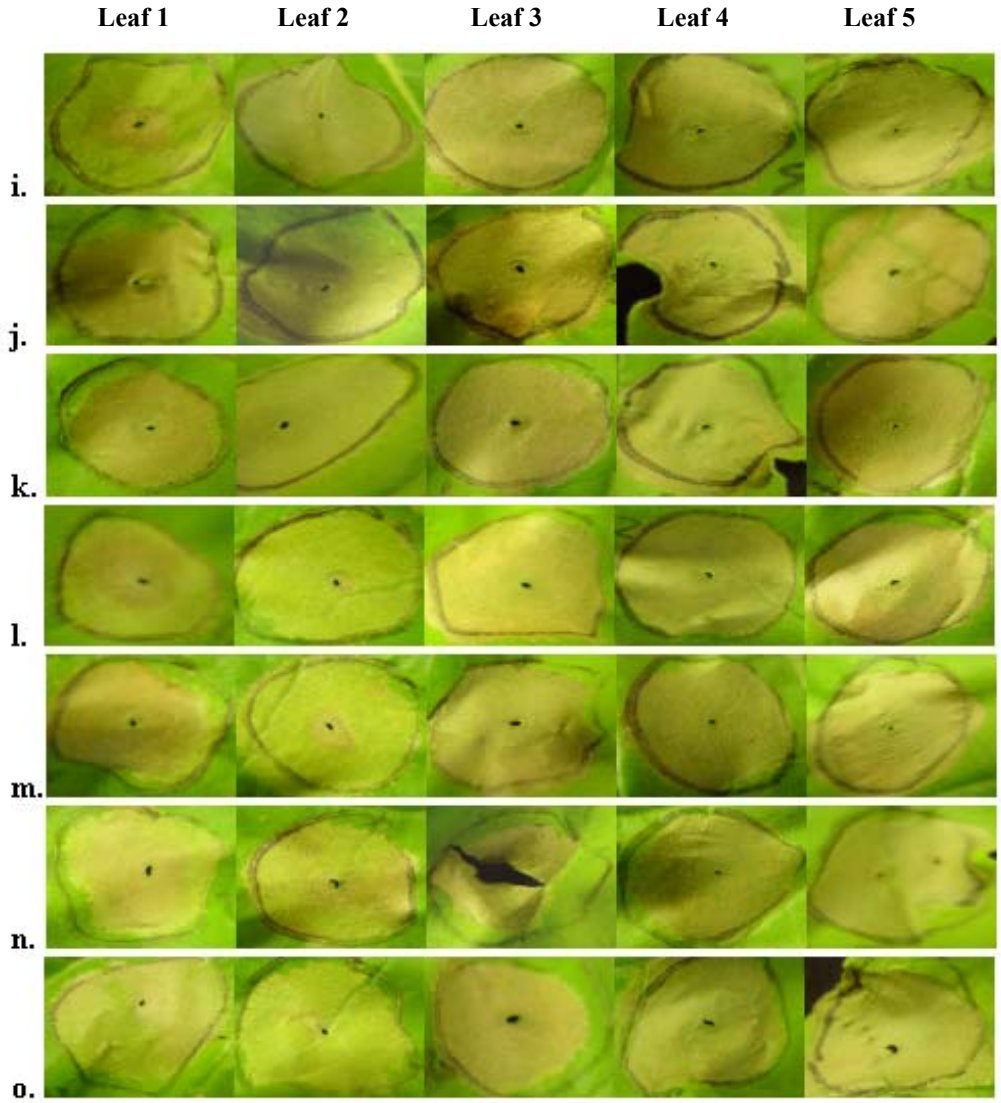


Figure 18: Transient Expression Assay in *N. benthamiana*. Eight-week old *N. Benthamiana* plants were infiltrated with combinations of *Agrobacterium* carrying a vector expressing each of the TCV ORFs at an A_{600} = 0.3 with *Agrobacterium* carrying vectors expressing AvrPto and Pto, each at an A_{600} = 0.3. p88MUT, p8(i) p88MUT, p9MUT(j) p88MUT, p38(k) p28MUT, p38(l) p8MUT, p9MUT(m) p8, p38(n) p9MUT, p38(o). Three different plants were tested. Leaves 1 and 2 were taken from one plant,

while leaf 3 was taken from a second plant and leaves 4 and 5 were taken from a third plant.

Minimal cell death was also observed for the p28MUT, p38 and p88MUT combination, panel (d) in Figure 19. Leaves scored at 7 dpi from this combination showed no more than a 10% total collapse of infiltrated tissue across both samples. However, samples containing these three components in addition to a fourth construct, showed a more variable percentage of collapse. The p28MUT, p38, p88MUT and p8 combination, as shown in Panel (k) showed almost 75–100% lesion formation in the infiltrated sites. However, the p28MUT, p38, p88MUT and p9MUT sample, Panel (l) showed reduced cell death phenotype with only 15-30% of the infiltrated area showing lesions. Table B-4 in Appendix B shows progression of necrosis from 4 dpi – 7 dpi.

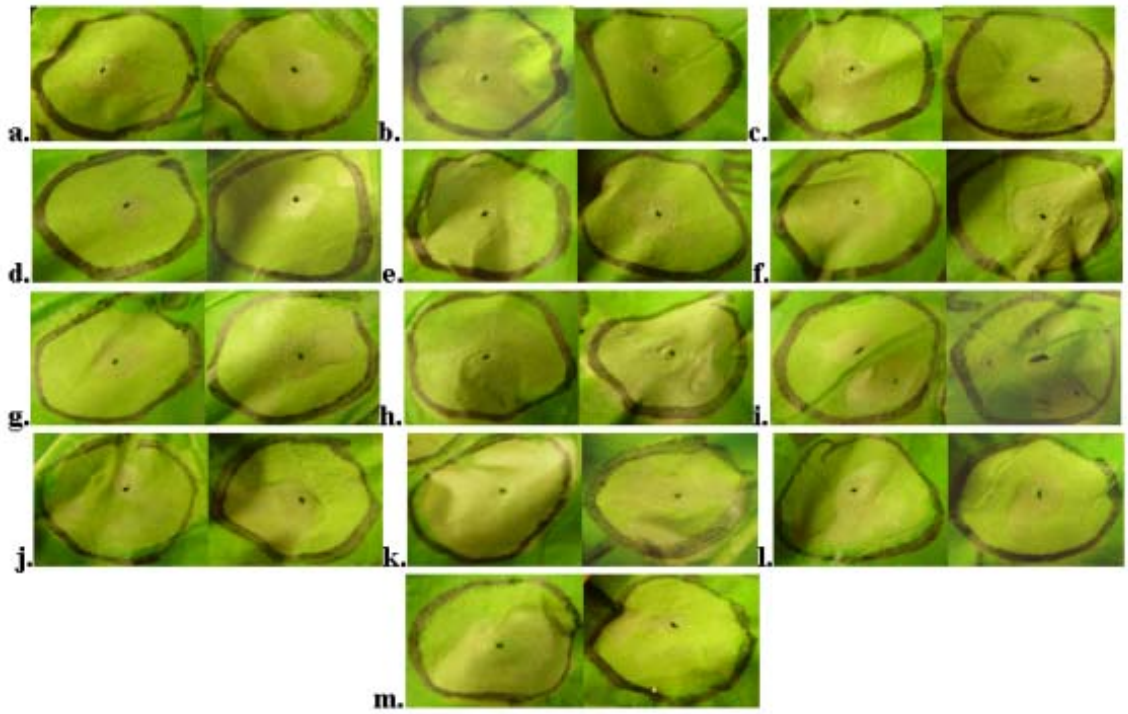


Figure 19: Transient Expression Assay in *N. benthamiana*. Eight-week old *N. Benthamiana* plants were infiltrated with combinations of *Agrobacterium* carrying a vector expressing each of the TCV ORFs at an A_{600} = 0.3 with *Agrobacterium* carrying vectors expressing AvrPto and Pto, each at an A_{600} = 0.3.

p8,p9MUT,p38(a) p28MUT,p88MUT,p8(b) p28MUT,p88MUT,p9MUT(c) p28MUT,p88MUT,p38 (d) p28MUT,p8,p38(e) p28MUT,p9MUT,p38(f) p88MUT,p8,p38(g) p88MUT,p9MUT,p38MUT(h) p88MUT,p8,p9MUT,p38(i) p28MUT,p8,p9MUT,p38MUT (j) p28MUT,p88MUT,p8,p38 (k) p28MUT,p88MUT,p9MUT,p38 (l) and p28MUT,p88MUT,p8,p9MUT (m). All samples were scored 7 dpi. All leaves were taken from the same plant.

Controls for the transient expression assays are shown in Figure 20-Figure 22 shows the results from ten separate co-infiltrations of *Agrobacterium* carrying the vector expressing avrPto and Pto on a single leaf from the same plant containing leaves 1 and 2 in Figure 17 and Figure 18. At 7 dpi, only mild tissue collapse could be observed in each of the ten samples where full tissue collapse was expected. This is consistent with data from these same leaves infiltrated with TCV ORFs and displayed a much reduced severity of necrosis when compared with leaves from different plants. Plant to plant variation in induction of PCD-pathways may be accountable for this observation.

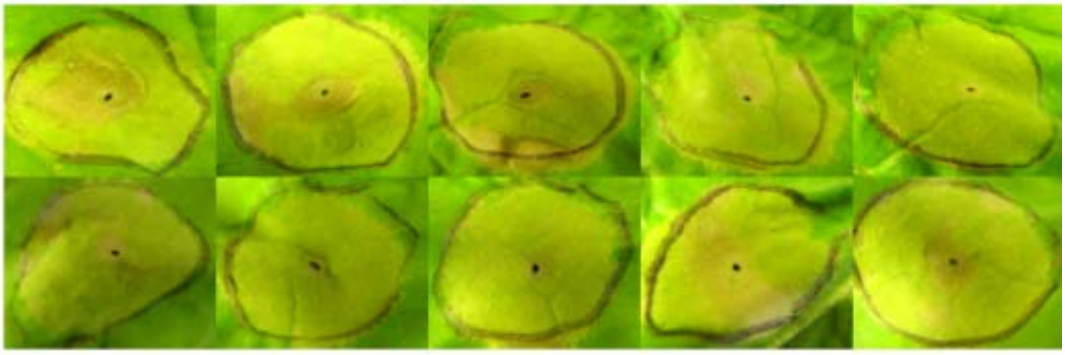


Figure 20.: Controls for Transient Expression Assays of TCV ORFs *N. benthamiana*. Eight-week old *N. Benthamiana* plants were infiltrated with *Agrobacterium* carrying vectors expressing AvrPto and Pto, each at an A_{600} = 0.3. The leaf was scored 7 dpi for cell death phenotypes. All samples are from the same leaf.

Figure 21 shows the results from twelve separate co-infiltrations of *Agrobacterium* carrying the vector expressing avrPto and Pto in combination with the anti-PCD effector

avrPtoB on a single leaf from the same plant containing leaves 4 and 5 from Figure 17-Figure 18. As expected, leaf tissue was completely suppressed for cell death in each of the twelve samples at 7 dpi.

Figure 22 shows each of the TCV ORF constructs along with avrPtoB, avrPto, and Pto constructs individually expressed in N. benthamiana plants. As expected, only tissue infiltrated with *Agrobacterium* carrying the vector expressing avrPto results in induction of an HR – consistent with previous findings (Abramovitch et al, 2003).

Figure 21: Controls for Transient Expression Assays of TCV ORFs *N. benthamiana*. Eight-week old *N. Benthamiana* plants were infiltrated with *Agrobacterium* carrying vectors expressing AvrPto and Pto, each at an A_{600} = 0.3 in combination with Agrobacterium carrying the vector expressing avrPtoB at A_{600} = 0.3. The leaf was scored 7 dpi for cell death phenotypes. All samples are from the same leaf.

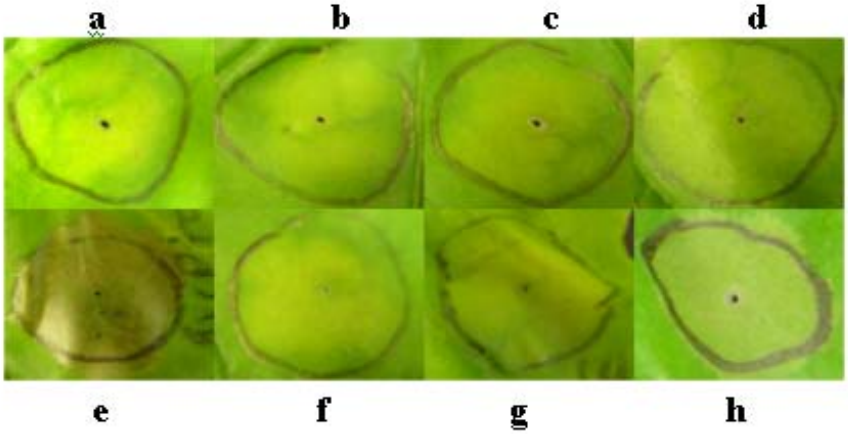


Figure 22: Controls for Transient Expression Assays of TCV ORFs *N. benthamiana*. Eight-week old *N. Benthamiana* plants were infiltrated with *Agrobacterium* carrying vectors expressing p8(a) p9MUT(b) p28MUT(c)

avrPtoB(d) avrPto(e) Pto(f) p38(g) and p88MUT(h) at A_{600} = 0.3. The leaf was scored 7 dpi for cell death phenotypes. All samples are from the same leaf.

3.7 Expression of TCV ORFs in *Sacharomyces cerevisiae*

To check for PCD suppression in a heterologous system, both the mutant and wild-type TCV ORFs were cloned into the yeast-compatible vector p423. The vector p423 contains a histidine selection marker along with a GAL1 galactose-inducible promoter. Prior to each yeast cell death assay, the constructs were transfected into competent INVSC1 yeast cells and selected using histidine-free media.

Transformants were cultured overnight, induced and then subjected to stress by including 3 mM H_2O_2 in their growth media after induction. After the six-hour H_2O_2 exposure, 20-uL aliquots of culture and each of 5 ten-fold serial dilutions of each of the samples were plated on His-free media to determine yeast survival. Untreated samples were also plated to compare viability with the treated cells.

Yeast transformed with p38 showed the greatest colony growth in dilution 3 of H_2O_2 treated cells when compared with the positive control avrPtoB. Likewise, yeast carrying the empty vector, along with the yeast samples carrying each of the TCV ORFs, showed a reduced extent of yeast survival for the same dilution. Untreated cells showed very similar concentrations as all constructs produced several colonies at the 5^{th} dilution. However, the H_2O_2-treated cells showed a wide range of survival depending upon the construct it contained. Empty vector and vector encoding p28MUT and p88MUT showed 10 or so cfu by the 3^{rd} dilution. In contrast, avrPtoB and p38 had about ten times for cfus.

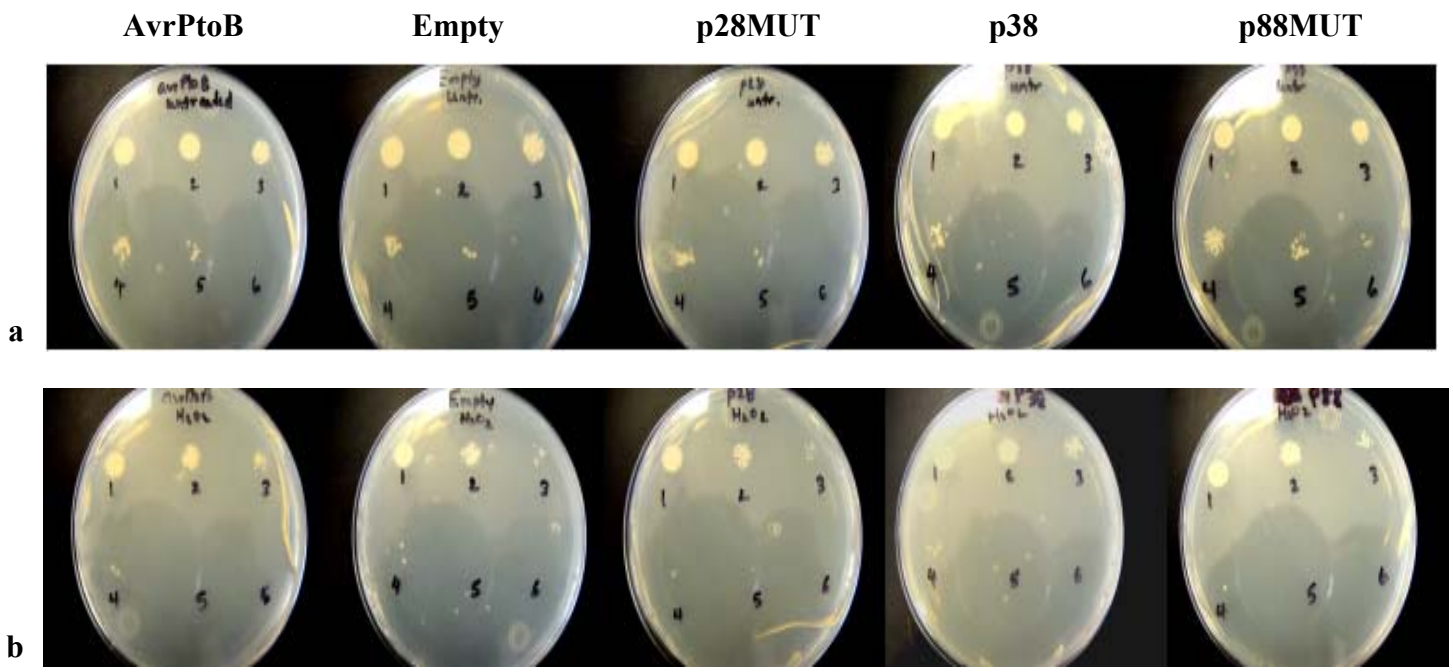


Figure 23: Survival of *S. cerevisiae* strain INVSC1 expressing TCV ORFs undergoing oxidative stress. Untreated samples (a) and 3 mM H_2O_2 (b). Transformants were grown overnight and induced with galactose for six hours. Cells were then diluted to A_{600} = 0.05 and incubated for six hours at 30°C in SD/-HIS or SD/-HIS supplemented with 3 mM H_2O_2. Samples were then plated along with five ten-fold dilutions to test for yeast survival.

4 DISCUSSION

In this thesis, we have demonstrated that *A. thaliana* plants systemically infected with *Turnip crinkle virus* are suppressed in their ability to initiate a PCD-response when challenged with the cognate avirulence factor avrRpt2. Moreover, we were able to extend these findings to an entirely different avirulence factor, avrRpm1, which displays faster kinetics in the induction of the PCD pathway. Infiltration of TCV-infected leaves with either *avr* resulted in a significant four-fold difference in conductivity levels when compared with non-TCV infected leaves undergoing equivalent treatments.

We propose that TCV may be involved in the modification of a host factor that is common between both the Rps2 and Rpm1 mediated PCD pathways as both Rps2 and Rpm1 initiate active defense in a Rin4 dependent manner. In fact, it has been shown that RPS2 knockout Col-0 plants overexpressing avrRpt2 were unable to mount an HR to avrRpm1 due most likely to avrRpt2-mediated elimination of Rin4 (Chen et al., 2000). The fact that Rps2 and Rpm1 both associate with Rin4 leads to the assertion that these two disease resistance proteins evolved out of necessity to monitor different pathogen-induced modifications of Rin4 (Mackey et al., 2003). Interaction between plant virus proteins and host factors is not uncommon as the p8 movement protein was shown to directly interact with the Arabidopsis protein Atp8 in both yeast-two hybrid and in-vitro binding assays. As Atp8 was shown to possess two putative transmembrane fragments, TCV has the capability to exploit host factors to promote its own virulence (Lin and Heaton, 2001).

We have also demonstrated that both the mutant and wild-type TCV constructs was transcribed in-planta. Moreover, full-length TCV expressed by agrobacterium was

not only expressed, but was able to reproduce endogenous viral movement as it was detected in uninfiltrated tissue.

The results of the ion leakage assay in N. benthamiana plants challenged with avrPto and Pto were inconclusive. Though an increase in conductivity levels in older leaves was evident at 24 hpi with respect to untreated and mock tissue, peak ion flux for younger and middle-aged leaves was not determined. Before repeating the experiment with TCV infected plants, it is recommended that a time trace analysis be performed on healthy N. Benthamiana plants challenged with the AvrPto and Pto scheme to isolate the time point for maximum electrolyte leakage. The infiltrated leaves should be monitored for appearance of tissue collapse every twelve hours as lesions on these tissues were detectable by 4 dpi. Tracking of lesion formation using tables similar to those in Appendix B would reveal the time span in which these younger leaves invoke their PCD programs. Once that time point is established, leaves can be then be assayed for comparative assessment of ion leakage from both TCV and mock-TCV plants undergoing PCD.

The p28-WT, p88-WT, and p9-WT clones will need to be reconstructed as soon as possible. If future experiments with these clones reveal HR suppression, data from the mutant constructs could provide some insight into key residues required for function. I wish the next person that constructs these clones the best of luck in showing that one of these ORFs plays a role in HR suppression.

The ORFs from the full-length TCV were mostly fine except for a single glutamic acid to glutamine change in the p88 ORF. Moreover, p8 and p88 sequencing reactions

were incomplete, so it is recommended that these two ORFs be resent for sequencing to ensure full consensus.

In the meantime, transient assays involving the p38 and p8 constructs will need to be repeated with more precise experimental parameters. As the plant-to-plant response in the HR severity under similar treatments was quite variable, there may be a concentration threshold for the agrobacterium to efficiently translocate T-DNA to the host genome. When testing three-way and four-way expression of TCV components, the overall concentration of the individual factors may need to be optimized to ensure sufficient delivery of each.

Incorporating one of the R-avr schemes that we used for quantification of HR suppression may offer some new revelations in the use of the transient expression system. If TCV is operating through modification of a signaling component essential to both the RPS2 and RPM1 pathways, then we may not be able to observe any visible affect on HR induction in completely different systems such as avrPto-Pto and Avr9-Cf9. This seems to be a very promising alternative as we now have solid evidence that TCV interferes with both Rps2 and Rpm1 mediated immunity.

As Abramovitch et al. (2003) were able to show anti-apoptotic activity of avrPtoB in both plants and yeast, we tried expressing each of the TCV ORFs in yeast undergoing oxidative stress. Though we were only able to try the assay once, yeast expressing p38 showed stronger survival rates with respect to avrPtoB. Performing the yeast apoptosis assay to duplicate the suppression phenotype of avrPtoB may help in optimizing the assay protocol for use with the TCV constructs. Inclusion of p8 and p9-WT will also need to be performed in future experiments.

5 REFERENCES

1. Abramovitch, R.B., Young-Jin, K., Shaorong, C., Dickman, M.B. and Martin, G. (2003) *EMBO* 22 60-69

2. Alfano, F.R. and Collmer, A. (1996) *Plant Cell* 8 1683-1698

3. Anandalakshmi, R., Pruss G.J., Ge, X., Marathe R., Mallory, A.C., Smith, T.H. and Vance, V.B. (1998) *PNAS* 95 13079-84

4. Axtell, M.J. and Staskawicz, B.J. (2003) *Cell* 112, 369-377

5. Carrington, J.C., Heaton, L.A., Zuidema, D., Hillman, B.I. and Morris, T.J. (1989) *Virology* 170 219-226

6. Carrington, J.C., Kasschau, K.D., Mahajan, S.K. and Schaad, M.C. (1996) *Plant Cell* 8 1669-1681

7. Chen, Z., Kloek, A.P., Boch, J., Katagiri, F. and Kunkel, B.N. (2000) *MPMI* 13 1312-1321

8. Dangl, J.L. and Jones, J.D. (2001) *Nature* 411 826-833

9. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C. (1998) *Nature* 391 806-811

10. Flor, H.H. (1971) *Annu. Rev. Phytopathol.* 9 275-296

11. Gallois, P., Makishima, T., Hecht, V., Despres, B., Laudie, M., Nishimoto, T. and Cooke, R. (1997) *Plant J* 11 1325-31

12. Grant, M.R., Godiard, L., Straube, E., Ashfield, T., Lewald, J., Sattler, A., Innes, R.W. and Dangl, J.L. 1995 Science 269 843-46

13. Hacker D.L., Petty I.T., Wei, N. and Morris T.J. (1992) *Virology* 186 1-8

14. Hammond-Kosack, K.E. and Jones, D.G. (1996) *Plant Cell* 8 1773-1791

15. Hueck, C.J. (1998) *Microbiol. Mol. Biol.* 62 379-433

16. Jabs, T. (1999) *Biochem. Pharmacol*. 57 231-245

17. Jackson, R.W., Athanassopoulos, E, Tsiamis, G., Mansfield, J.W., Sesma, A., Arnold, D.L., Gibbon, M.J., Murillo, J., Tayolor, J.D. and Vivian, A. (1999) *PNAS* 96 10875-10880

18. Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P. and Valent, B (2000) *EMBO* 19 4004-4014

19. Jones, D.A., Thomas, C.M., Hammond-Koscak, K.E., Balint-Kurti, P.J., and Jones, J.D. (1994) *Science* 266 789-793

20. Kim Y.J., Lin N.C. and Martin G.B. (2002) *Cell* 109 589-598

21. Kunkel, B.N., Bent, A.F., Dahlbeck, D., Innes, R.W. and Staskawicz, B.J. (1993) *Plant Cell* 5 865-875

22. Leisner, S.M., Turgeon, R. and Howell, S.H. (1993) *Plant Cell* **5** 191-202

23. Li, W. and Dickman, M.B. (2004) *Biotechnol Lett* 26 87-95

24. Liang, H., Yao, N., Song, J.T., Luo, S., Lu, H. and Greenberg J.T. (2003) *Genes & Development* 17 2636-2641

25. Lim, M.S. and Kunkel, B. (2004) *MPMI* 17 313-321

26. Lin, B and Heaton, L.A. (2001) Virology 259 34-42

27. Lincoln, J.E., Richael, C., Overduin, B., Smith, K., Bostock, R. and Gilchrist, D.G. (2002) *Proc Natl Acad Sci USA* 99 15217-15221

28. Luderer, R., Rivas, S., Nurnberger, T., Mattei, B., Van den Hooven HW, Van der Hoorn, R.A., Romeis, T., Wehrfritz, J.M., Blume, B., Nennstiel, D., Zuidem D., Vervoort, J., De Lorenzo, G., Jones, J.D., De Wit, P.J. and Joosten, M.H. (2001) *MPMI* 12 867-876

29. Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R. and Dangl, J.L. (2003) *Cell* 112, 379-389

30. Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D. and Tanksley, S.D. (1993) *Science* 262, 1432-1436

31. McLean, B.G., Hempel, F.D. and Zambryski, P.C. (1997) *Plant Cell* 9 1043-4054

32. Mudgett, M., and Staskawicz, B.J. (1999) *Molecular Microbiology* 32 927-941

33. Nimchuk, Z., Eulgem, T., Holt, B.F. and Dangl, J.L. (2003) *Annu Rev Genet* 37 579-609

34. Orth, K., Palmer, L.E., Bao, Z.Q., Stewart, S., Rudolph, A.E., Bliska, J.B. and Dixon, J.E. (1999) *Science* 285 1920-1923

35. Qu, F., Ren, T. and Morris, T.J. (2003) Journal of Virology 77 511-522

36. Rate, D.N. and Greenberg, J.T. (2001) *Plant J.* 27 203-11

37. Ritter, C. and Dangl, J.L. (1996) *Plant Cell* 8 251-257

38. Samuel, G. (1934) *Ann. Appl.* Biol. 21 90-111

39. Scofield, S.R., Tobias, C.M., Rathjen, J.P., Chang, J.H., Lavelle, D.T., Michelmore, W. and Staskawicz, B.J. (1996) *Science* 274 2063-2065

40. Tang,X., Xie,M., Kim,Y.J., Zhou,J., Klessig,D.F. and Martin,G.B. (1999) *Plant Cell* 11 15–30

41. Tsiamas, G., Mansfield, J.W., Hockenhull, R., Jackson, R.W., Sesma, A., Athanassopoulos, E., Bennett, M.A., Stevens, C., Vivian, A., Taylor, J.D. and Murillo, J. (2000) *EMBO* 19 3204-3214

42. Vance, V. and Vaucheret, H. (2001) *Science* 292 2277-2280

43. Voinnet, O., Lederer, C., and Baulcombe, D.C. (2000) *Cell* 103 157-167

44. White, K.A., Skuzeski, J.M., Li, W., Wei, N., and Morris, T.J. (1995) *Virology* 211 525-534

45. Zhao Y., DelGrosso, L., Yigit. E., Dempsey, D.A., Klessig, D.F. and Wobbe, K.K. (2000) *MPMI* 9 1015-1018

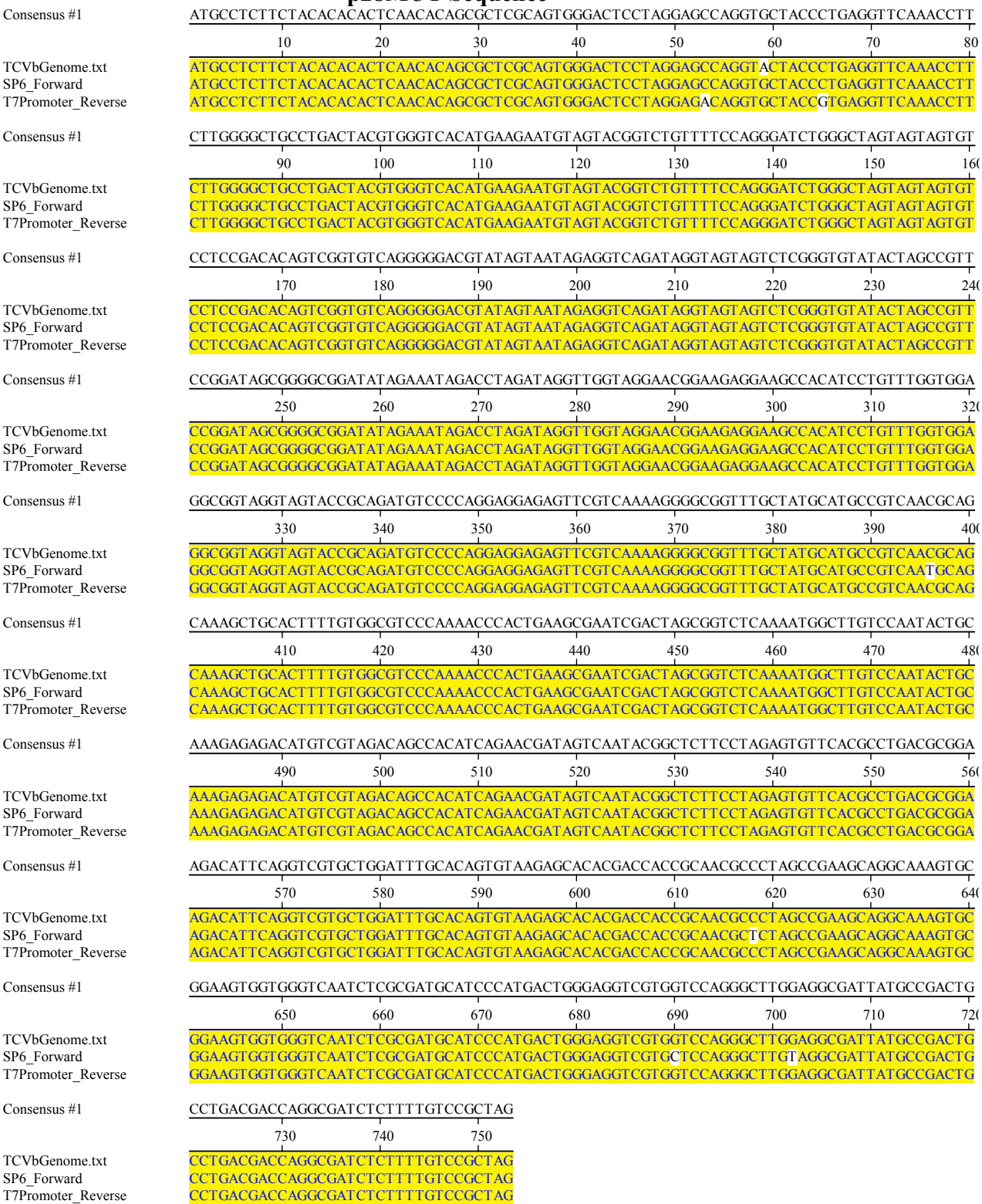
46. Zhou, Q., Krebs, J.F., Snipas, S.J., Price, A., Alnemri, E.S., Tomaselli, K.J. and Salvesen, G.S. (1998) *Biochemistry* 37 10757-10765

47. Zhou, J., Loh,Y.T., Bressan, R.A. and Martin, G.B. (1995) *Cell* 83 925-935

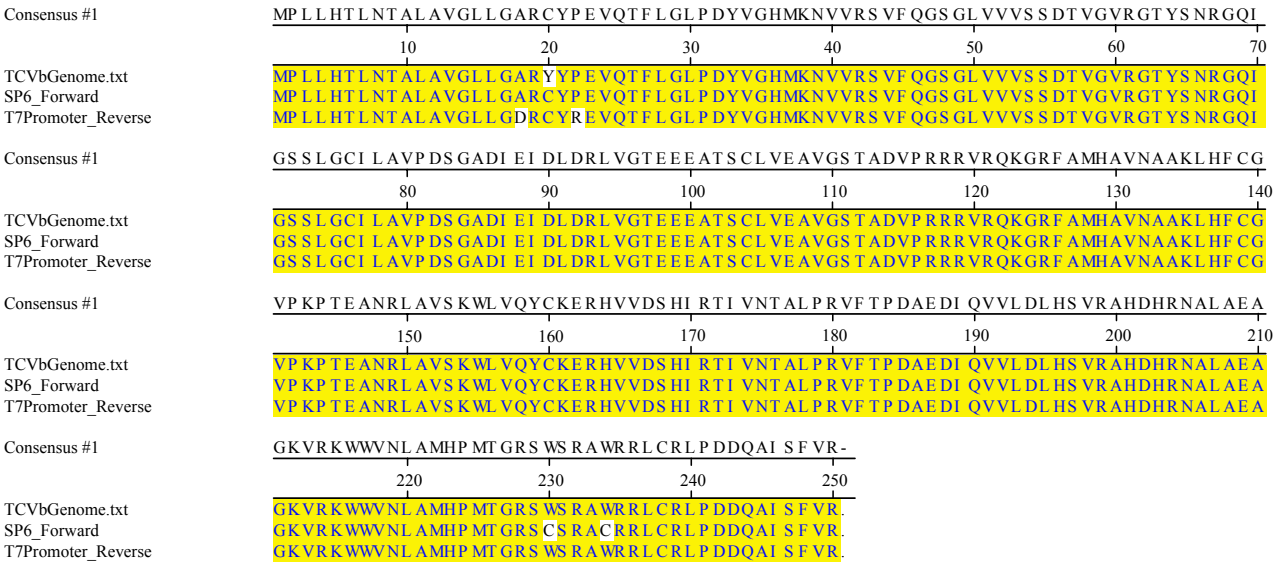
APPENDIX A

SEQUENCES OF TCV CLONES

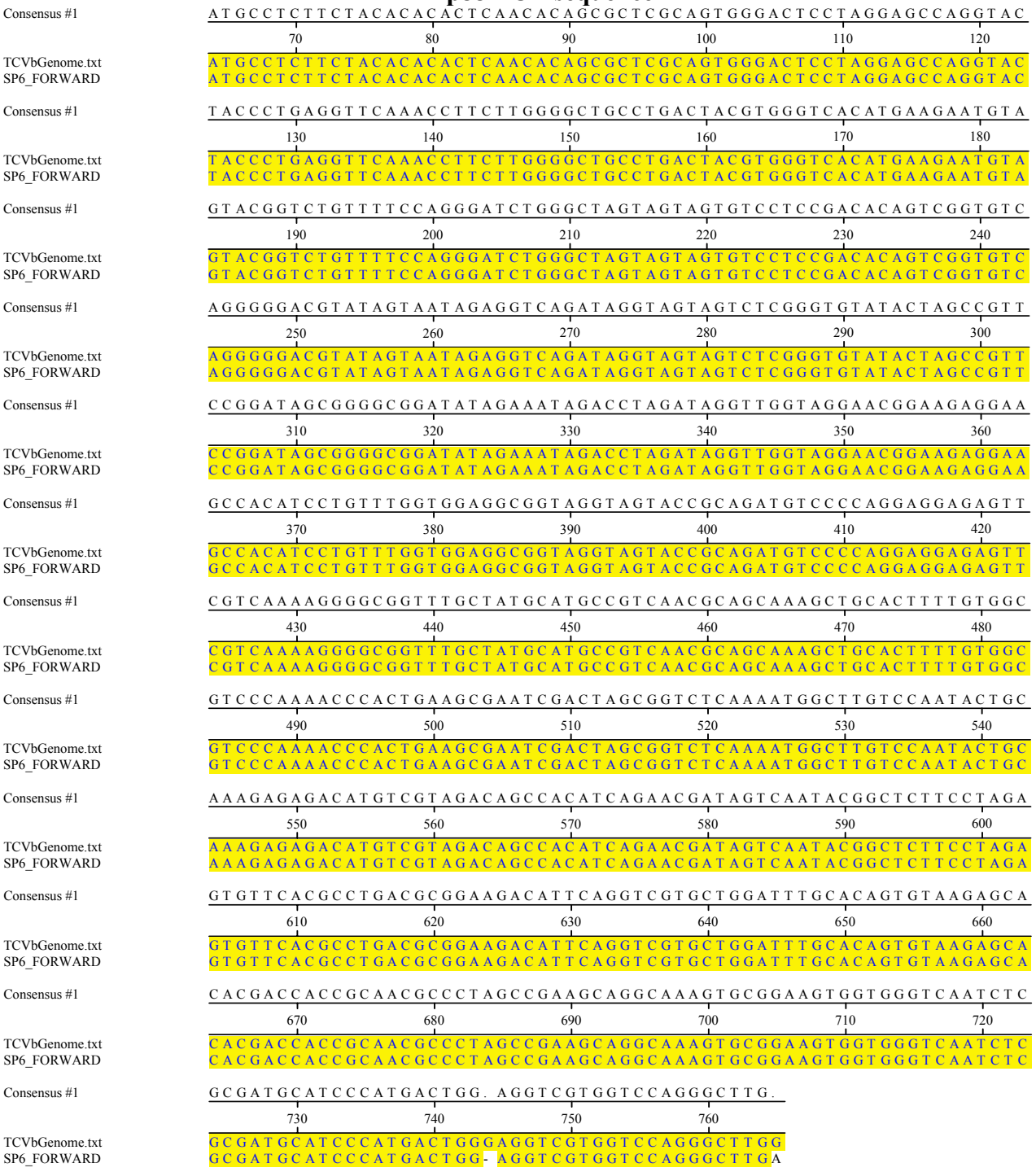
p28MUT Sequence

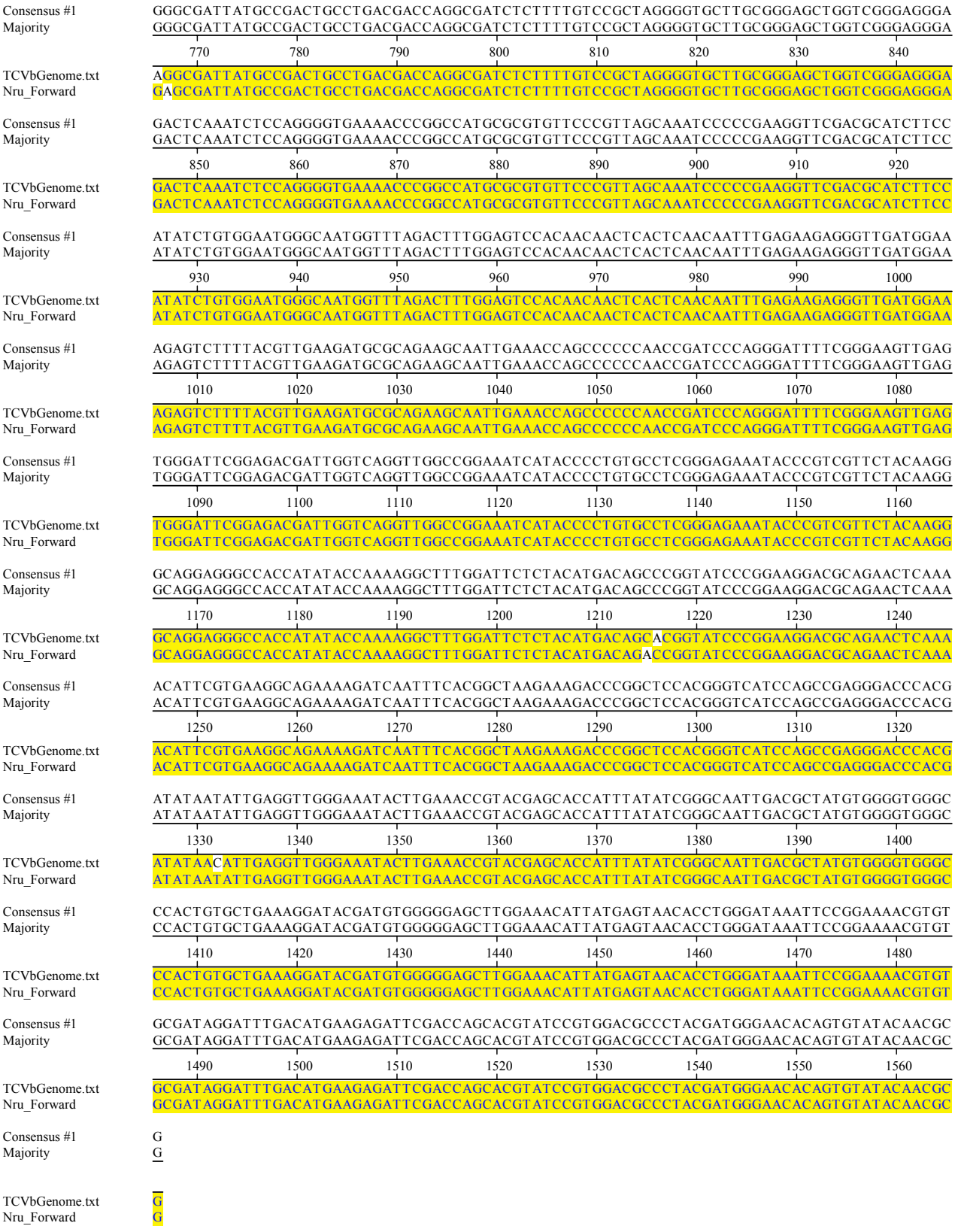


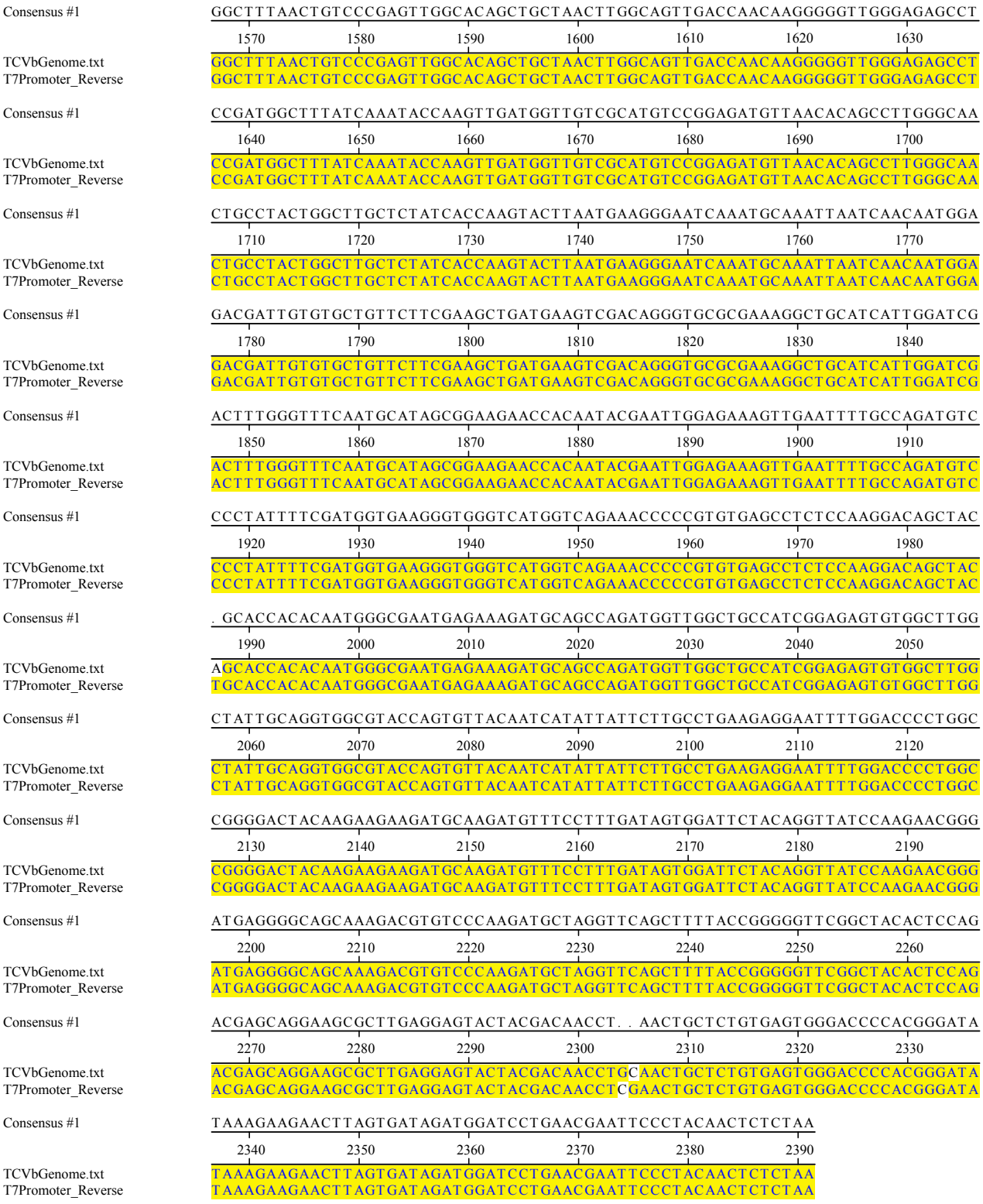
p28MUT Translated

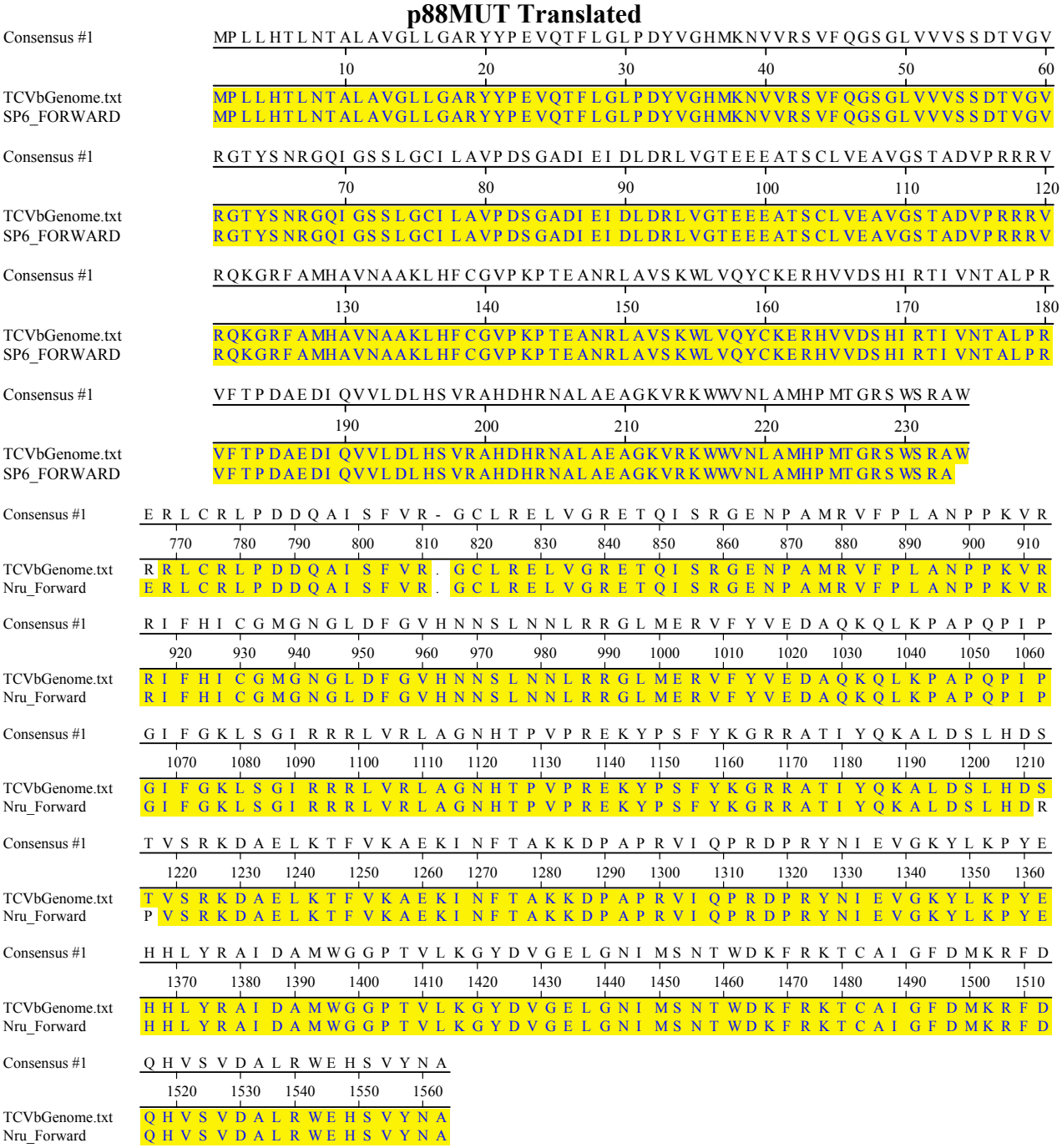


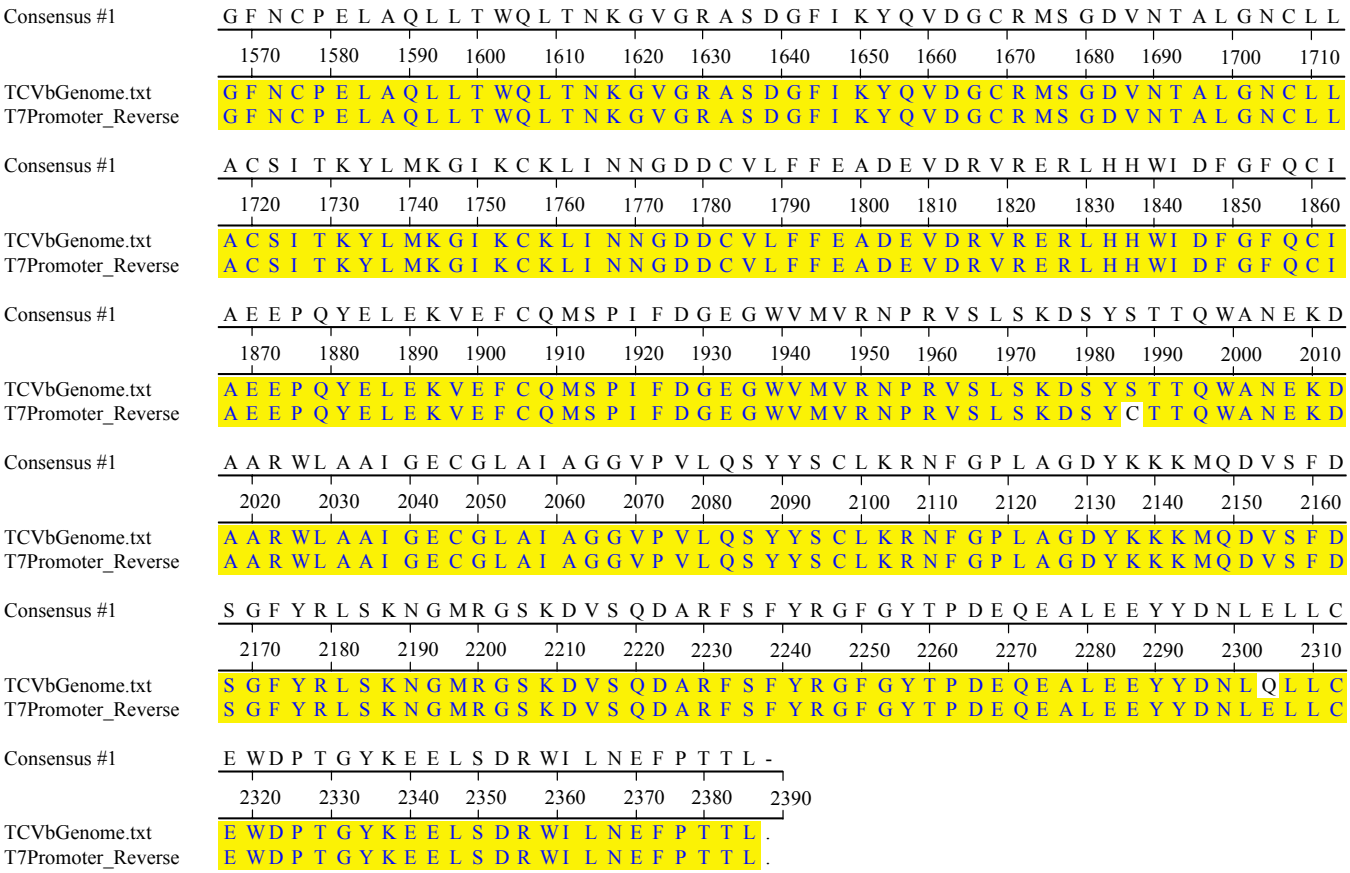
p88MUT sequence



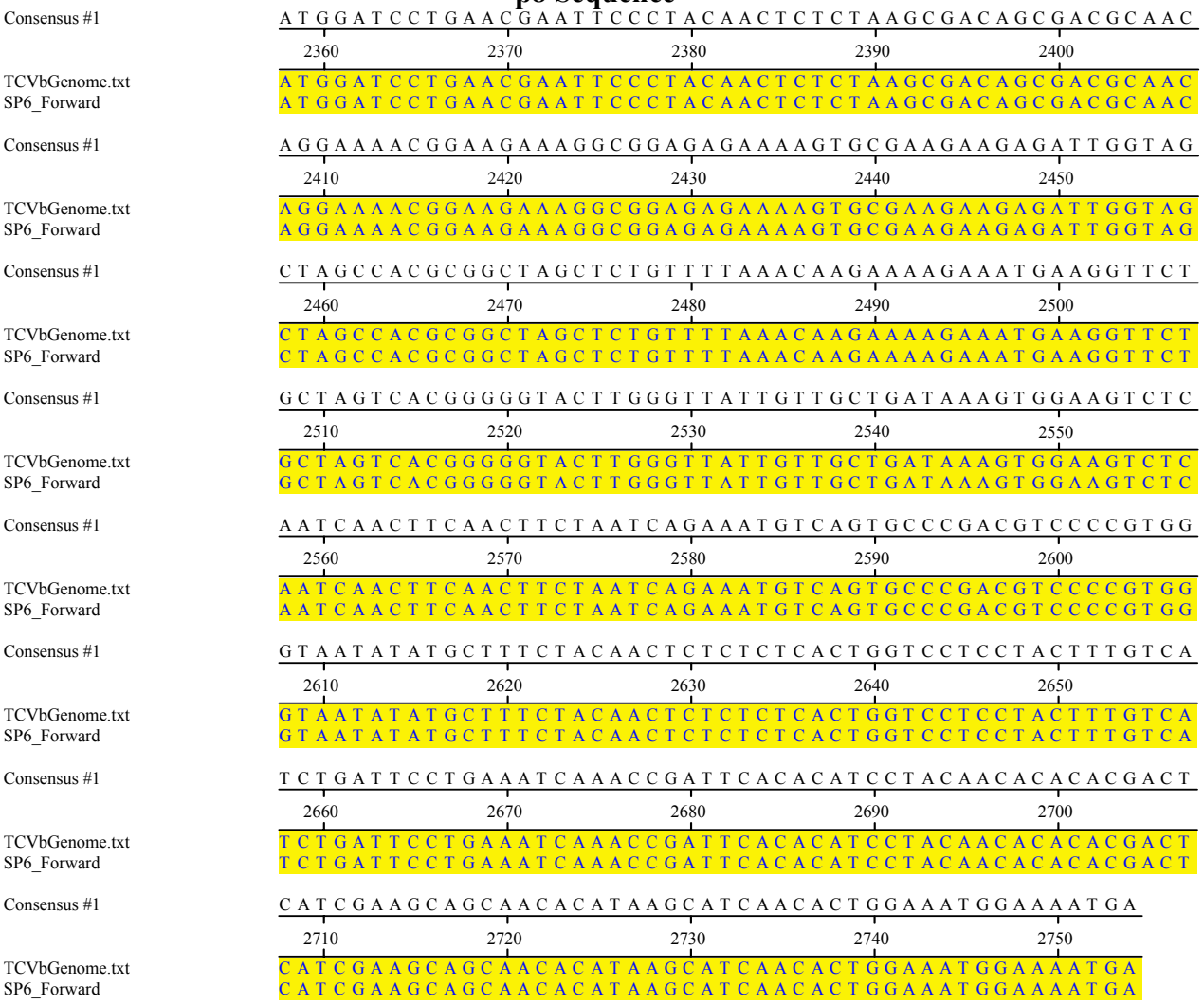

Consensus #1
Majority
770 780 790 800 810 820 830 840
TCVbGenome.txt
Nru_Forward
Consensus #1
Majority
850 860 870 880 890 900 910 920
TCVbGenome.txt
Nru_Forward
Consensus #1
Majority
930 940 950 960 970 980 990 1000
TCVbGenome.txt
Nru_Forward
Consensus #1
Majority
1010 1020 1030 1040 1050 1060 1070 1080
TCVbGenome.txt
Nru_Forward
Consensus #1
Majority
1090 1100 1110 1120 1130 1140 1150 1160
TCVbGenome.txt
Nru_Forward
Consensus #1
Majority
1170 1180 1190 1200 1210 1220 1230 1240
TCVbGenome.txt
Nru_Forward
Consensus #1
Majority
1250 1260 1270 1280 1290 1300 1310 1320
TCVbGenome.txt
Nru_Forward
Consensus #1
Majority
1330 1340 1350 1360 1370 1380 1390 1400
TCVbGenome.txt
Nru_Forward
Consensus #1
Majority
1410 1420 1430 1440 1450 1460 1470 1480
TCVbGenome.txt
Nru_Forward
Consensus #1
Majority
1490 1500 1510 1520 1530 1540 1550 1560
TCVbGenome.txt
Nru_Forward
Consensus #1
Majority
TCVbGenome.txt
Nru_Forward


Consensus #1
GGCTTTAACTGTCCCGAGTTGGCACAGCTGCTAACTTGGCAGTTGACCAACAAGGGGGTTGGGAGAGCCT
1570 1580 1590 1600 1610 1620 1630
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
CCGATGGCTTTATCAAATACCAAGTTGATGGTTGTCGCATGTCCGGAGATGTTAACACAGCCTTGGGCAA
1640 1650 1660 1670 1680 1690 1700
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
CTGCCTACTGGCTTGCTCTATCACCAAGTACTTAATGAAGGGAATCAAATGCAAATTAATCAACAATGGA
1710 1720 1730 1740 1750 1760 1770
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
GACGATTGTGTGCTGTTCTTCGAAGCTGATGAAGTCGACAGGGTGCGCGAAAGGCTGCATCATTGGATCG
1780 1790 1800 1810 1820 1830 1840
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
ACTTTGGGTTTCAATGCATAGCGGAAGAACCACAATACGAATTGGAGAAAGTTGAATTTTGCCAGATGTC
1850 1860 1870 1880 1890 1900 1910
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
CCCTATTTTCGATGGTGAAGGGTGGGTCATGGTCAGAAACCCCCGTGTGAGCCTCTCCAAGGACAGCTAC
1920 1930 1940 1950 1960 1970 1980
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
. GCACCACACAATGGGCGAATGAGAAAGATGCAGCCAGATGGTTGGCTGCCATCGGAGAGTGTGGCTTGG
1990 2000 2010 2020 2030 2040 2050
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
CTATTGCAGGTGGCGTACCAGTGTTACAATCATATTATTCTTGCCTGAAGAGGAATTTTGGACCCCTGGC
2060 2070 2080 2090 2100 2110 2120
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
CGGGGACTACAAGAAGAAGATGCAAGATGTTTCCTTTGATAGTGGATTCTACAGGTTATCCAAGAACGGG
2130 2140 2150 2160 2170 2180 2190
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
ATGAGGGGCAGCAAAGACGTGTCCCAAGATGCTAGGTTCAGCTTTTACCGGGGGTTCGGCTACACTCCAG
2200 2210 2220 2230 2240 2250 2260
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
ACGAGCAGGAAGCGCTTGAGGAGTACTACGACAACCT. . AACTGCTCTGTGAGTGGGACCCCACGGGATA
2270 2280 2290 2300 2310 2320 2330
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
TAAAGAAGAACTTAGTGATAGATGGATCCTGAACGAATTCCCTACAACTCTCTAA
2340 2350 2360 2370 2380 2390
TCVbGenome.txt
T7Promoter_Reverse

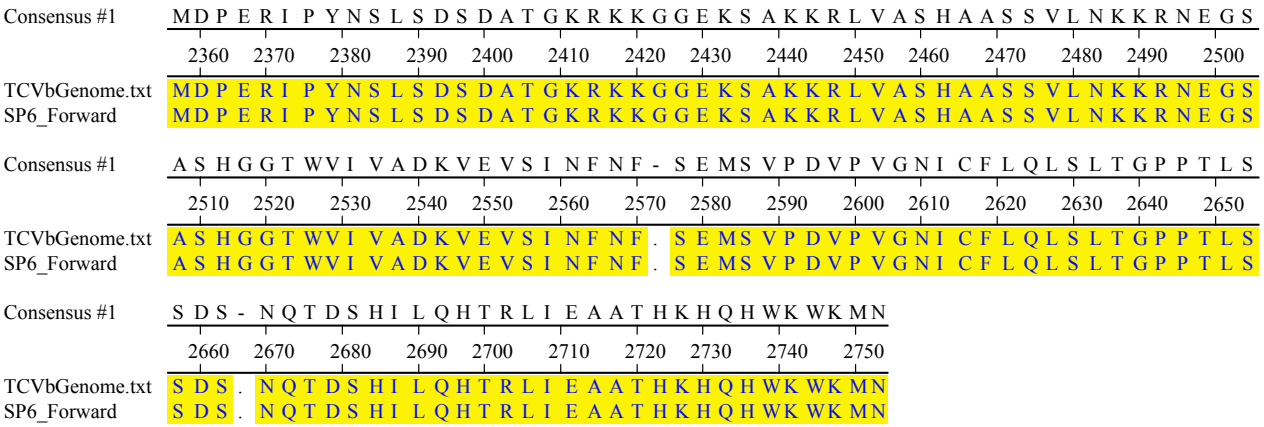

p88MUT Translated
Consensus #1
MP L L HT L NT AL AV GL L GA RY YP E VQ TF L GL P DY VG HM KN VV RS VF QG S GL VV VS S DT VG V
10 20 30 40 50 60
TCVbGenome.txt
SP6_FORWARD
MP L L HT L NT AL AV GL L GA RY YP E VQ TF L GL P DY VG HM KN VV RS VF QG S GL VV VS S DT VG V
Consensus #1
RG TY S NR GQ I GS S L GC I L AV P DS GA DI E I DL DR L VG TE E E AT S CL VE AV GS T AD VP RR RV
70 80 90 100 110 120
TCVbGenome.txt
SP6_FORWARD
RG TY S NR GQ I GS S L GC I L AV P DS GA DI E I DL DR L VG TE E E AT S CL VE AV GS T AD VP RR RV
Consensus #1
RQ KG RF AM HA VN AA KL HF CG VP KP TE AN RL AV S KW L VQ YC KE RH VV DS HI RT I VN TA L P R
130 140 150 160 170 180
TCVbGenome.txt
SP6_FORWARD
RQ KG RF AM HA VN AA KL HF CG VP KP TE AN RL AV S KW L VQ YC KE RH VV DS HI RT I VN TA L P R
Consensus #1
VF T P DA E DI QV VL DL HS VR AH DH RN AL AE AG KV RK WW VN L AM HP MT GR S WS RA W
190 200 210 220 230
TCVbGenome.txt
SP6_FORWARD
VF T P DA E DI QV VL DL HS VR AH DH RN AL AE AG KV RK WW VN L AM HP MT GR S WS RA W
VF T P DA E DI QV VL DL HS VR AH DH RN AL AE AG KV RK WW VN L AM HP MT GR S WS RA
Consensus #1
E R L C R L P D D Q A I S F V R - G C L R E L V G R E T Q I S R G E N P A M R V F P L A N P P K V R
770 780 790 800 810 820 830 840 850 860 870 880 890 900 910
TCVbGenome.txt
Nru_Forward
R R L C R L P D D Q A I S F V R . G C L R E L V G R E T Q I S R G E N P A M R V F P L A N P P K V R
E R L C R L P D D Q A I S F V R . G C L R E L V G R E T Q I S R G E N P A M R V F P L A N P P K V R
Consensus #1
R I F H I C G M G N G L D F G V H N N S L N N L R R G L M E R V F Y V E D A Q K Q L K P A P Q P I P
920 930 940 950 960 970 980 990 1000 1010 1020 1030 1040 1050 1060
TCVbGenome.txt
Nru_Forward
R I F H I C G M G N G L D F G V H N N S L N N L R R G L M E R V F Y V E D A Q K Q L K P A P Q P I P
R I F H I C G M G N G L D F G V H N N S L N N L R R G L M E R V F Y V E D A Q K Q L K P A P Q P I P
Consensus #1
G I F G K L S G I R R R L V R L A G N H T P V P R E K Y P S F Y K G R R A T I Y Q K A L D S L H D S
1070 1080 1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200 1210
TCVbGenome.txt
Nru_Forward
G I F G K L S G I R R R L V R L A G N H T P V P R E K Y P S F Y K G R R A T I Y Q K A L D S L H D S
G I F G K L S G I R R R L V R L A G N H T P V P R E K Y P S F Y K G R R A T I Y Q K A L D S L H D R
Consensus #1
T V S R K D A E L K T F V K A E K I N F T A K K D P A P R V I Q P R D P R Y N I E V G K Y L K P Y E
1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320 1330 1340 1350 1360
TCVbGenome.txt
Nru_Forward
T V S R K D A E L K T F V K A E K I N F T A K K D P A P R V I Q P R D P R Y N I E V G K Y L K P Y E
P V S R K D A E L K T F V K A E K I N F T A K K D P A P R V I Q P R D P R Y N I E V G K Y L K P Y E
Consensus #1
H H L Y R A I D A M W G G P T V L K G Y D V G E L G N I M S N T W D K F R K T C A I G F D M K R F D
1370 1380 1390 1400 1410 1420 1430 1440 1450 1460 1470 1480 1490 1500 1510
TCVbGenome.txt
Nru_Forward
H H L Y R A I D A M W G G P T V L K G Y D V G E L G N I M S N T W D K F R K T C A I G F D M K R F D
H H L Y R A I D A M W G G P T V L K G Y D V G E L G N I M S N T W D K F R K T C A I G F D M K R F D
Consensus #1
Q H V S V D A L R W E H S V Y N A
1520 1530 1540 1550 1560
TCVbGenome.txt
Nru_Forward
Q H V S V D A L R W E H S V Y N A
Q H V S V D A L R W E H S V Y N A


Consensus #1
G F N C P E L A Q L L T W Q L T N K G V G R A S D G F I K Y Q V D G C R M S G D V N T A L G N C L L
1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680 1690 1700 1710
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
A C S I T K Y L M K G I K C K L I N N G D D C V L F F E A D E V D R V R E R L H H W I D F G F Q C I
1720 1730 1740 1750 1760 1770 1780 1790 1800 1810 1820 1830 1840 1850 1860
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
A E E P Q Y E L E K V E F C Q M S P I F D G E G W V M V R N P R V S L S K D S Y S T T Q W A N E K D
1870 1880 1890 1900 1910 1920 1930 1940 1950 1960 1970 1980 1990 2000 2010
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
A A R W L A A I G E C G L A I A G G V P V L Q S Y Y S C L K R N F G P L A G D Y K K K M Q D V S F D
2020 2030 2040 2050 2060 2070 2080 2090 2100 2110 2120 2130 2140 2150 2160
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
S G F Y R L S K N G M R G S K D V S Q D A R F S F Y R G F G Y T P D E Q E A L E E Y Y D N L E L L C
2170 2180 2190 2200 2210 2220 2230 2240 2250 2260 2270 2280 2290 2300 2310
TCVbGenome.txt
T7Promoter_Reverse
Consensus #1
E W D P T G Y K E E L S D R W I L N E F P T T L -
2320 2330 2340 2350 2360 2370 2380 2390
TCVbGenome.txt
T7Promoter_Reverse

p8 Sequence

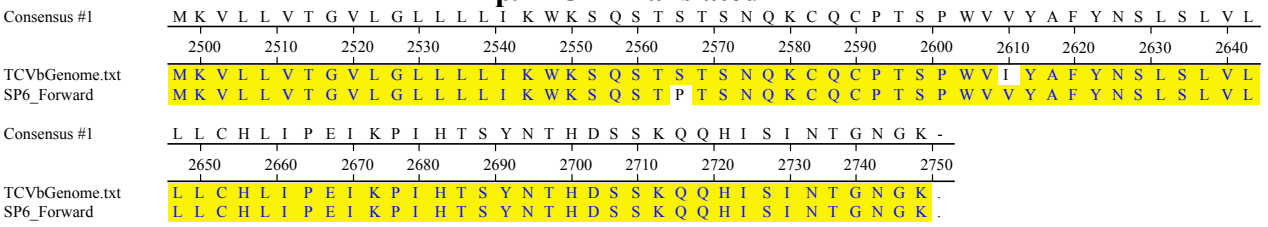


p8 Translated

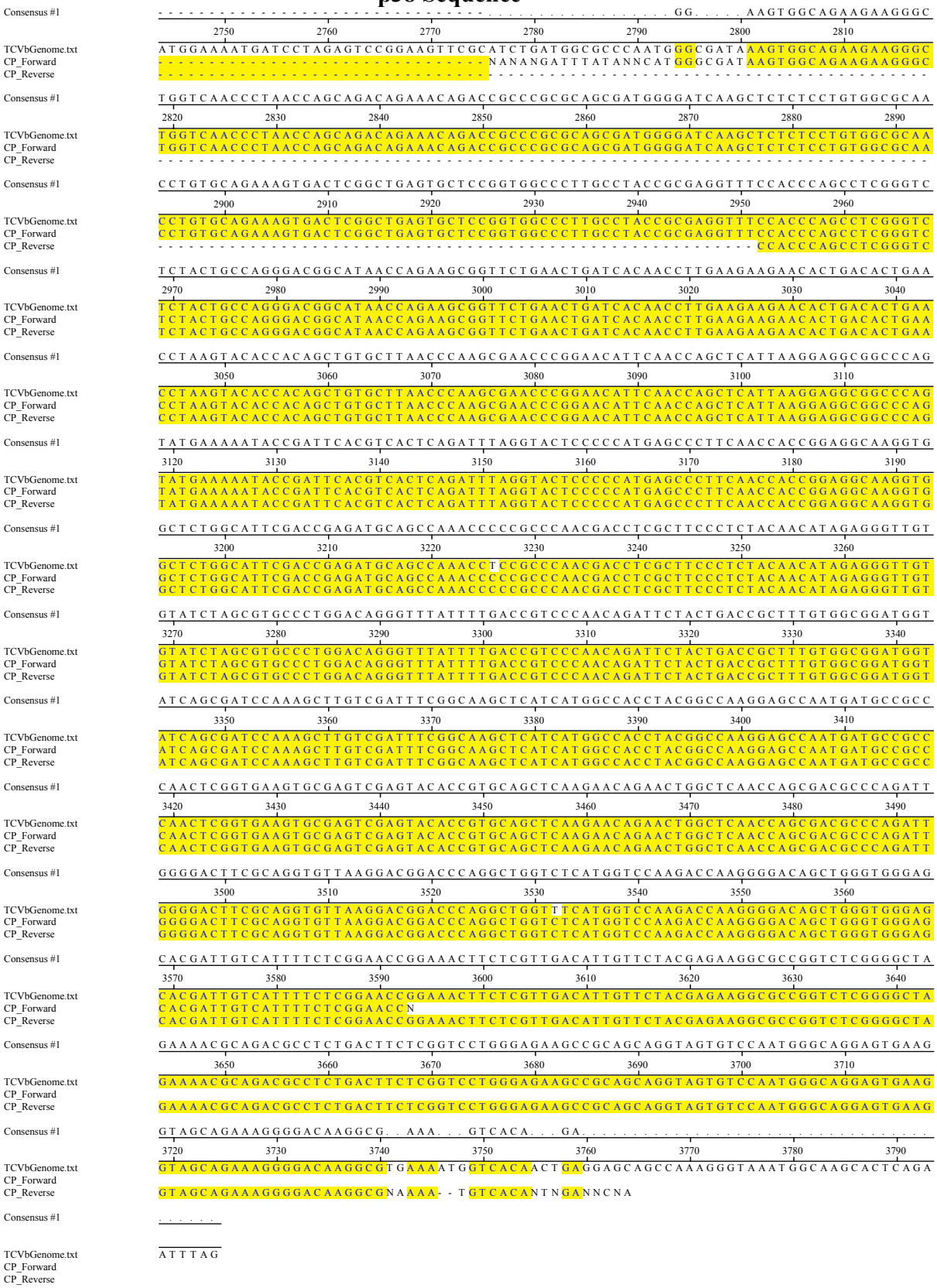


p9MUT Sequence

p9MUT Translated



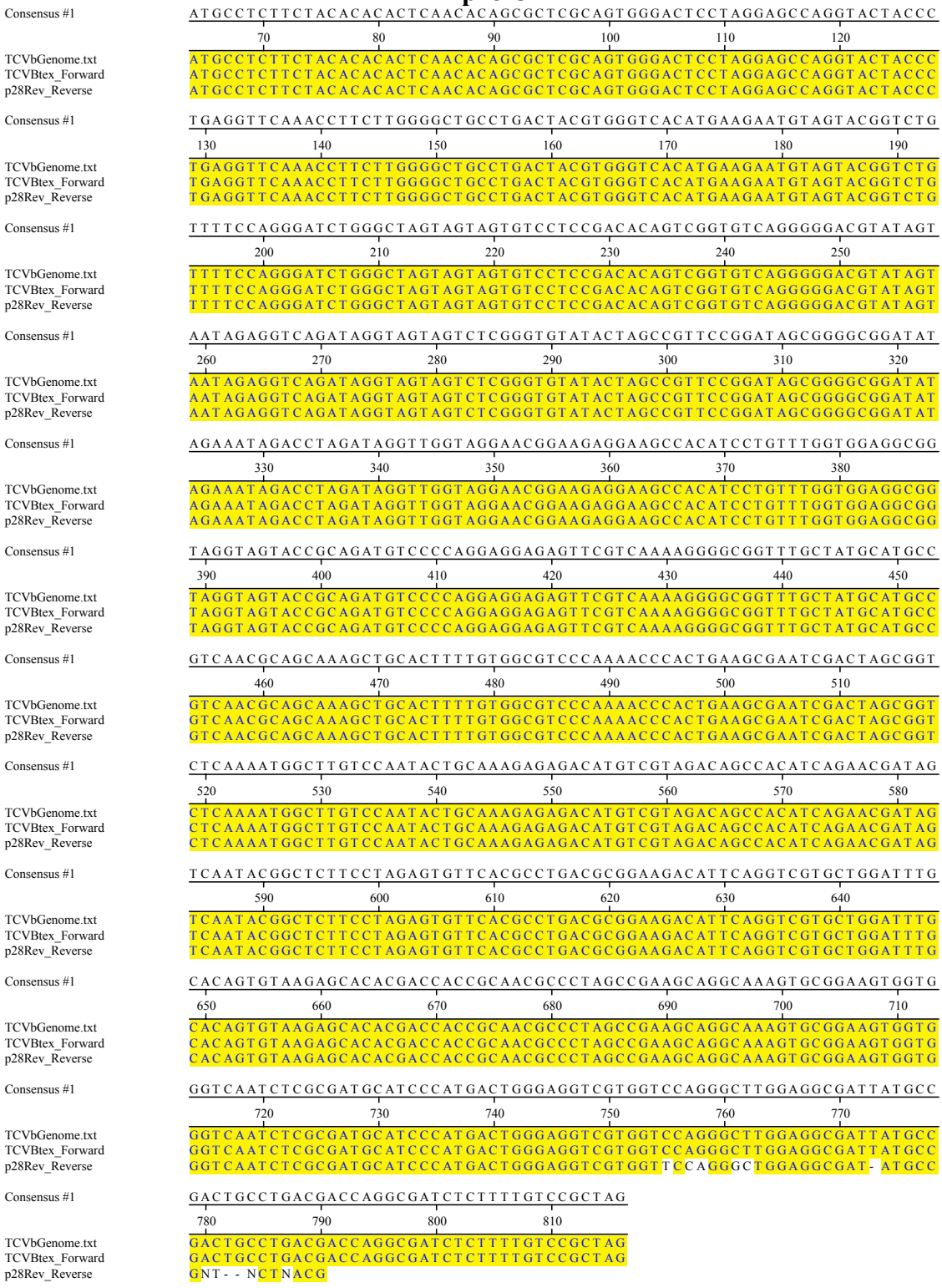
p38 Sequence



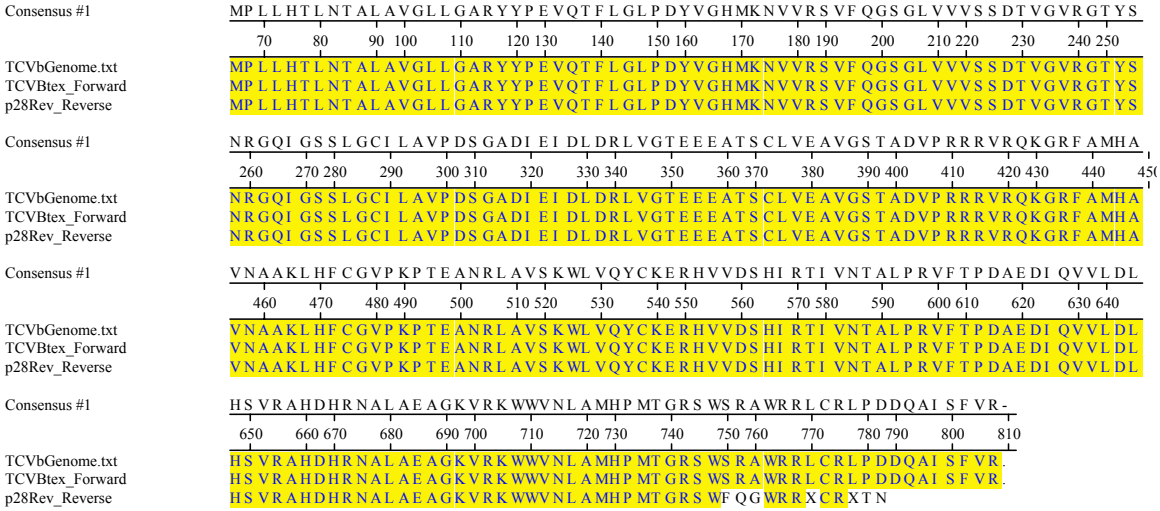
p38 Translated

```
Consensus #1     XXXXXXXXXXXXXXXXXXXXKWQKKGWSTLTSRQKQTARAAMGIKLSPVAQPVQKV
                 2750 2760 2770 2780 2790 2800 2810 2820 2830 2840 2850 2860 2870 2880 2890 2900
TCVbGenome.txt   MENDPRVRKFASDGAQWAI KWQKKGWSTLTSRQKQTARAAMGIKLSPVAQPVQKV
CP_Forward       XXXXXXXXXXXXKRFINMGDKWQKKGWSTLTSRQKQTARAAMGIKLSPVAQPVQKV
CP_Reverse       XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

Consensus #1     TRLSAPVALAYREVSTQPRVSTARDGITRSGSELITTLKKNTDTEPKYTTAVLNP
                 2910 2920 2930 2940 2950 2960 2970 2980 2990 3000 3010 3020 3030 3040 3050 3060 307
TCVbGenome.txt   TRLSAPVALAYREVSTQPRVSTARDGITRSGSELITTLKKNTDTEPKYTTAVLNP
CP_Forward       TRLSAPVALAYREVSTQPRVSTARDGITRSGSELITTLKKNTDTEPKYTTAVLNP
CP_Reverse       XXXXXXXXXXXXXXXTQPRVSTARDGITRSGSELITTLKKNTDTEPKYTTAVLNP

Consensus #1     SEPGTFNQLIKEAAQYEKYRFTSLRFRYSPMSPSTTGGKVALAFDRDAAKPPPND
                 3080 3090 3100 3110 3120 3130 3140 3150 3160 3170 3180 3190 3200 3210 3220 3230
TCVbGenome.txt   SEPGTFNQLIKEAAQYEKYRFTSLRFRYSPMSPSTTGGKVALAFDRDAAKPPPND
CP_Forward       SEPGTFNQLIKEAAQYEKYRFTSLRFRYSPMSPSTTGGKVALAFDRDAAKPPPND
CP_Reverse       SEPGTFNQLIKEAAQYEKYRFTSLRFRYSPMSPSTTGGKVALAFDRDAAKPPPND

Consensus #1     LASLYNIEGCVSSVPWTGFILTVPTDSTDRFVADGISDPKLVDFGKLIMATYGQG
                 3240 3250 3260 3270 3280 3290 3300 3310 3320 3330 3340 3350 3360 3370 3380 3390 340
TCVbGenome.txt   LASLYNIEGCVSSVPWTGFILTVPTDSTDRFVADGISDPKLVDFGKLIMATYGQG
CP_Forward       LASLYNIEGCVSSVPWTGFILTVPTDSTDRFVADGISDPKLVDFGKLIMATYGQG
CP_Reverse       LASLYNIEGCVSSVPWTGFILTVPTDSTDRFVADGISDPKLVDFGKLIMATYGQG

Consensus #1     ANDAAQLGEVRVEYTVQLKNRTGSTSDAQIGDFAGVKDGPRLVSWSKTKGTAGWE
                 3410 3420 3430 3440 3450 3460 3470 3480 3490 3500 3510 3520 3530 3540 3550 3560
TCVbGenome.txt   ANDAAQLGEVRVEYTVQLKNRTGSTSDAQIGDFAGVKDGPRLVSWSKTKGTAGWE
CP_Forward       ANDAAQLGEVRVEYTVQLKNRTGSTSDAQIGDFAGVKDGPRLVSWSKTKGTAGWE
CP_Reverse       ANDAAQLGEVRVEYTVQLKNRTGSTSDAQIGDFAGVKDGPRLVSWSKTKGTAGWE

Consensus #1     HDCHFLGTGNFSLTLFYEKAPVSGLENADASDFSVLGEAAAGSVQWAGVKVAERG
                 3570 3580 3590 3600 3610 3620 3630 3640 3650 3660 3670 3680 3690 3700 3710 3720 373
TCVbGenome.txt   HDCHFLGTGNFSLTLFYEKAPVSGLENADASDFSVLGEAAAGSVQWAGVKVAERG
CP_Forward       HDCHFLGT
CP_Reverse       HDCHFLGTGNFSLTLFYEKAPVSGLENADASDFSVLGEAAAGSVQWAGVKVAERG

Consensus #1     QGXKXVTXEX------------
                 3740 3750 3760 3770 3780 3790
TCVbGenome.txt   QGVKMVTTEEQPKGKWQALRI.
CP_Forward
CP_Reverse       QGEKXVTIET
```

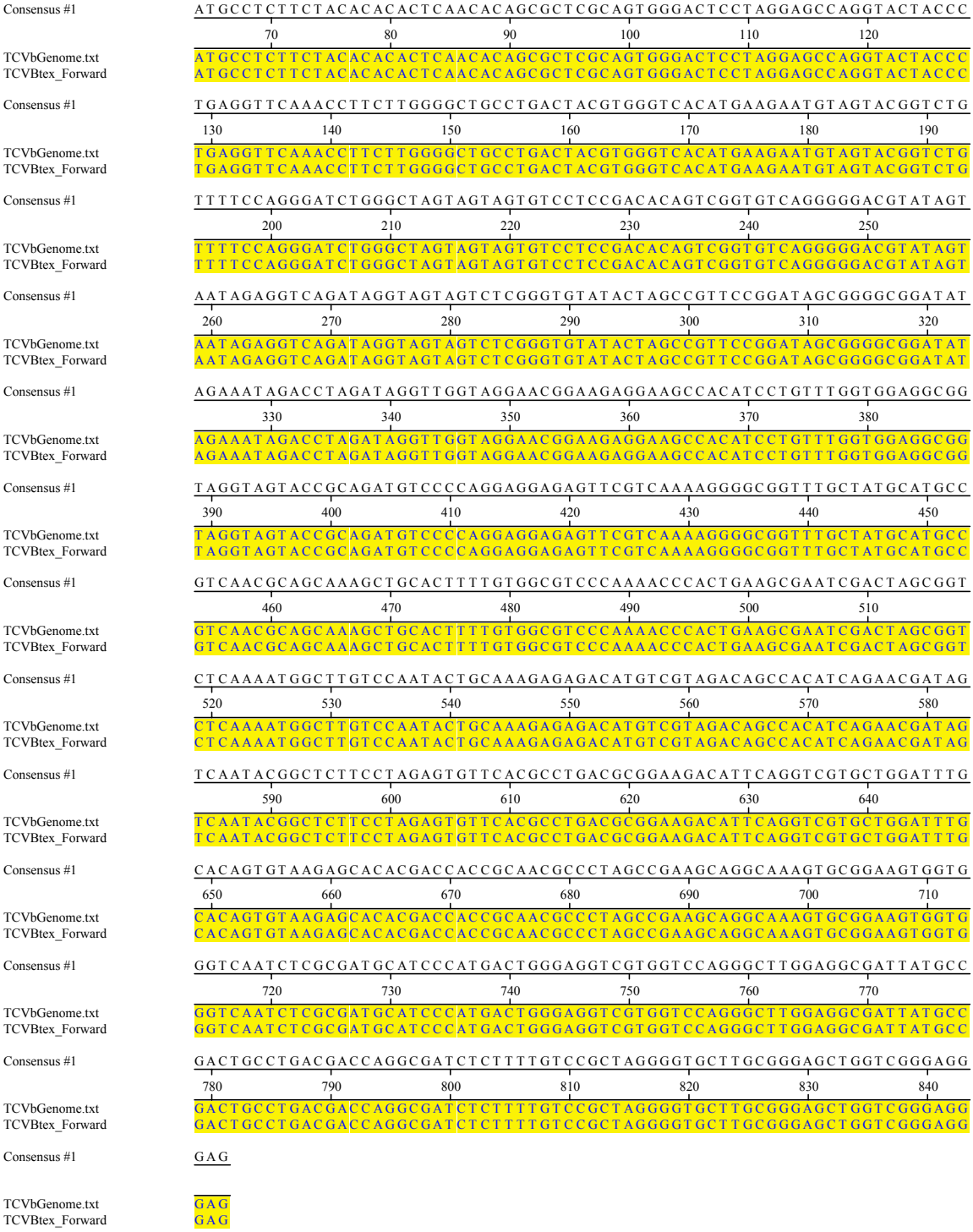
Full-length TCV
p28 ORF

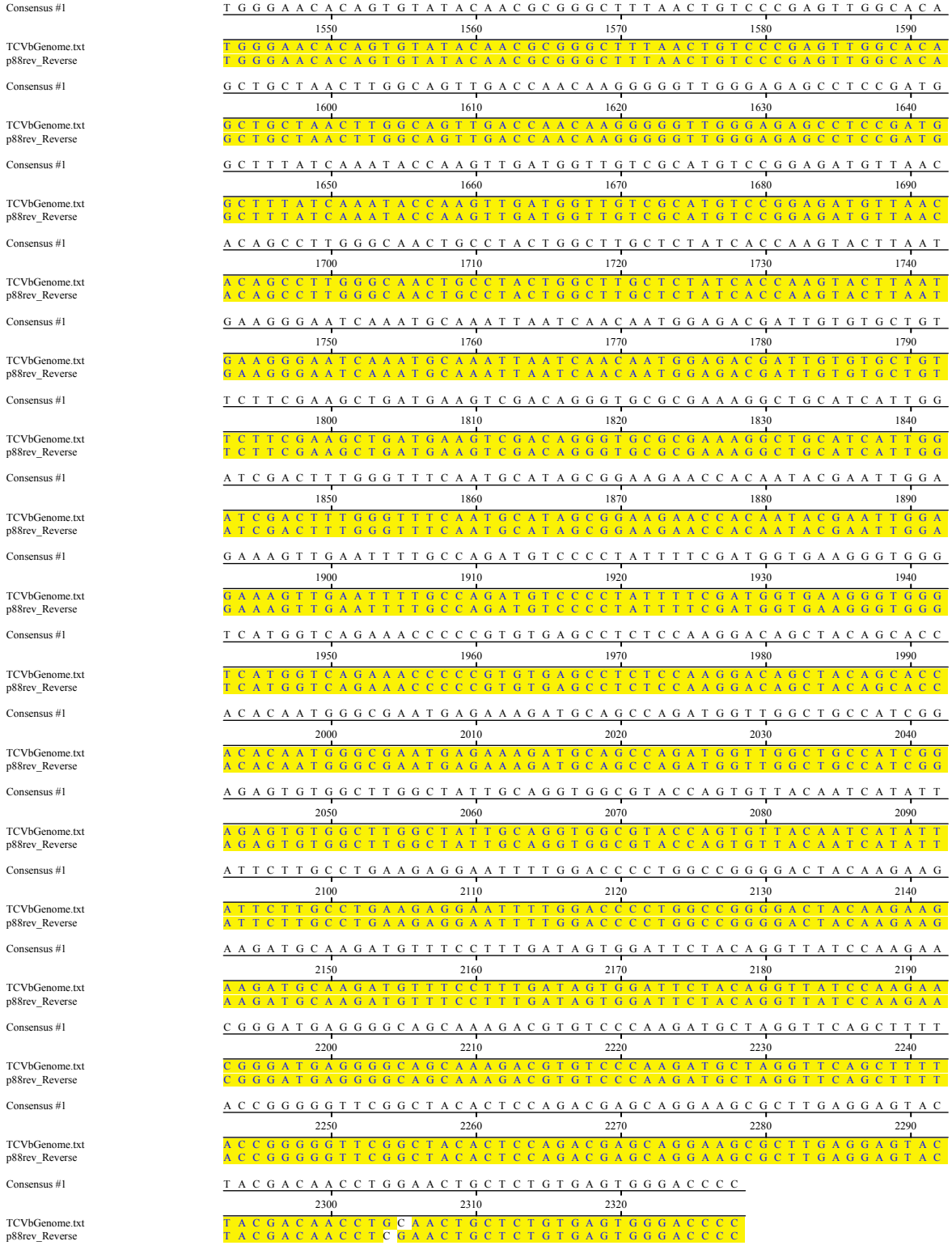


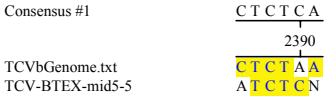
Full-Length TCV
Translated p28 ORF



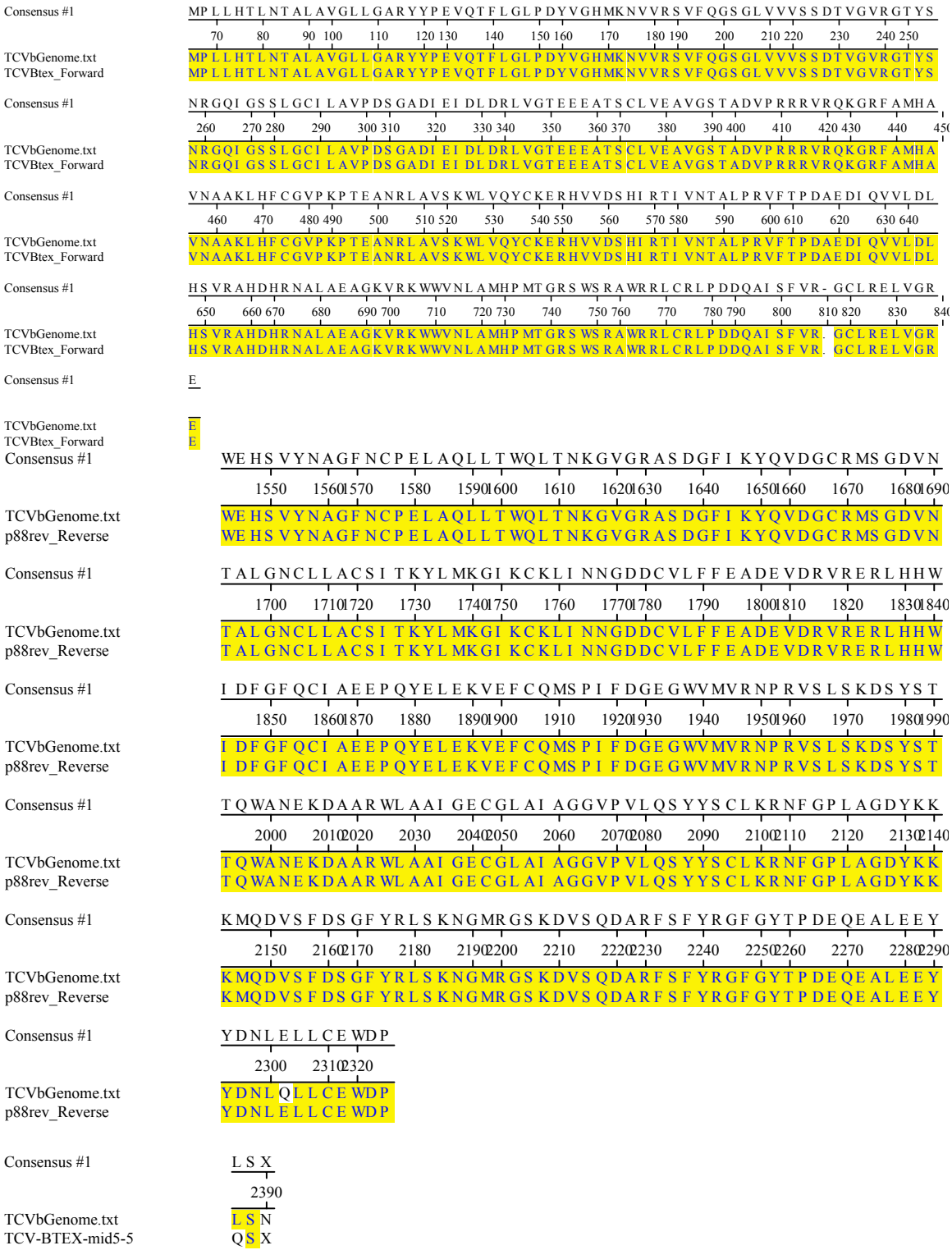
Full-Length TCV
p88 ORF



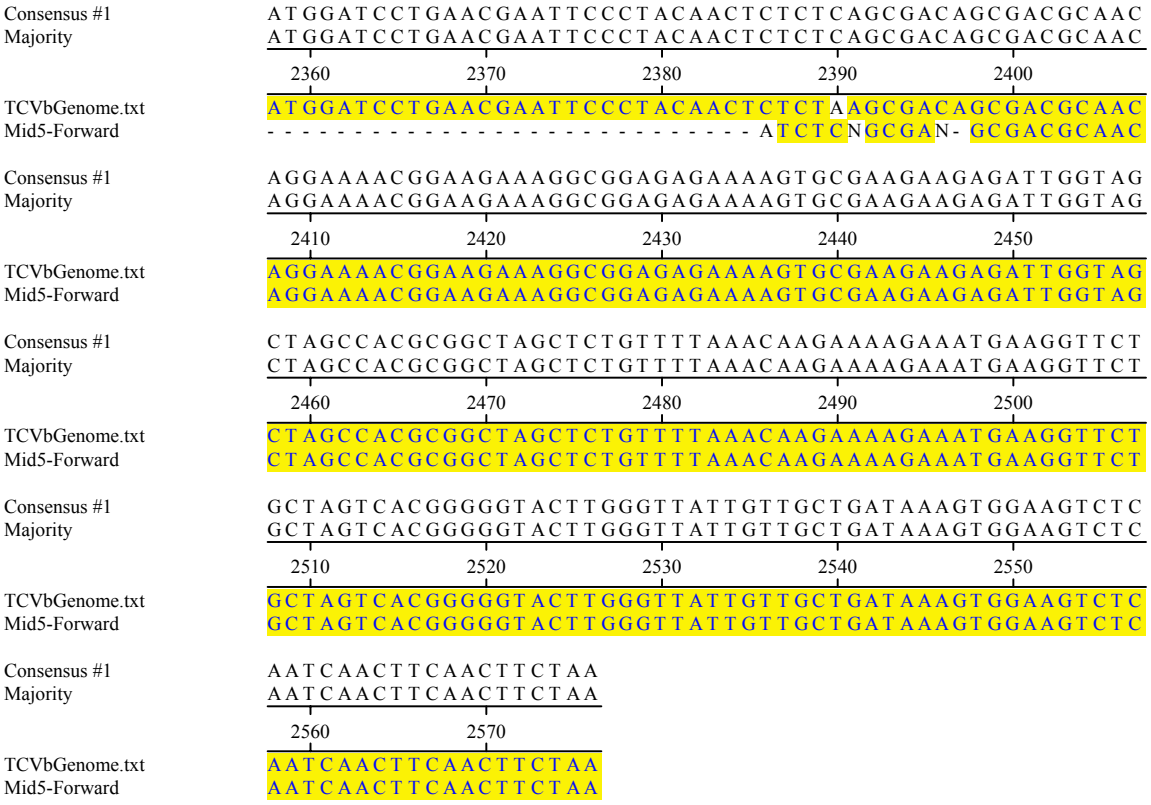

Consensus #1
TCVbGenome.txt
p88rev_Reverse


Consensus #1
C T C T C A
2390
TCVbGenome.txt
C T C T A A
TCV-BTEX-mid5-5
A T C T C N

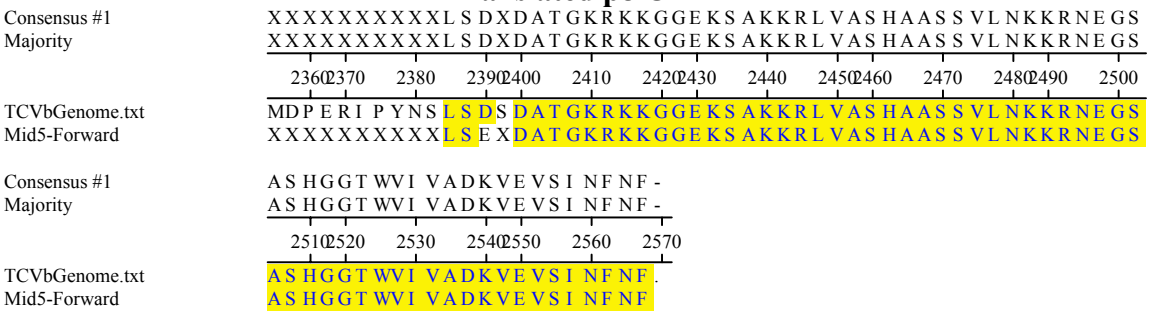
TCV Construct
Translated p88 ORF



TCV Construct
P8 ORF

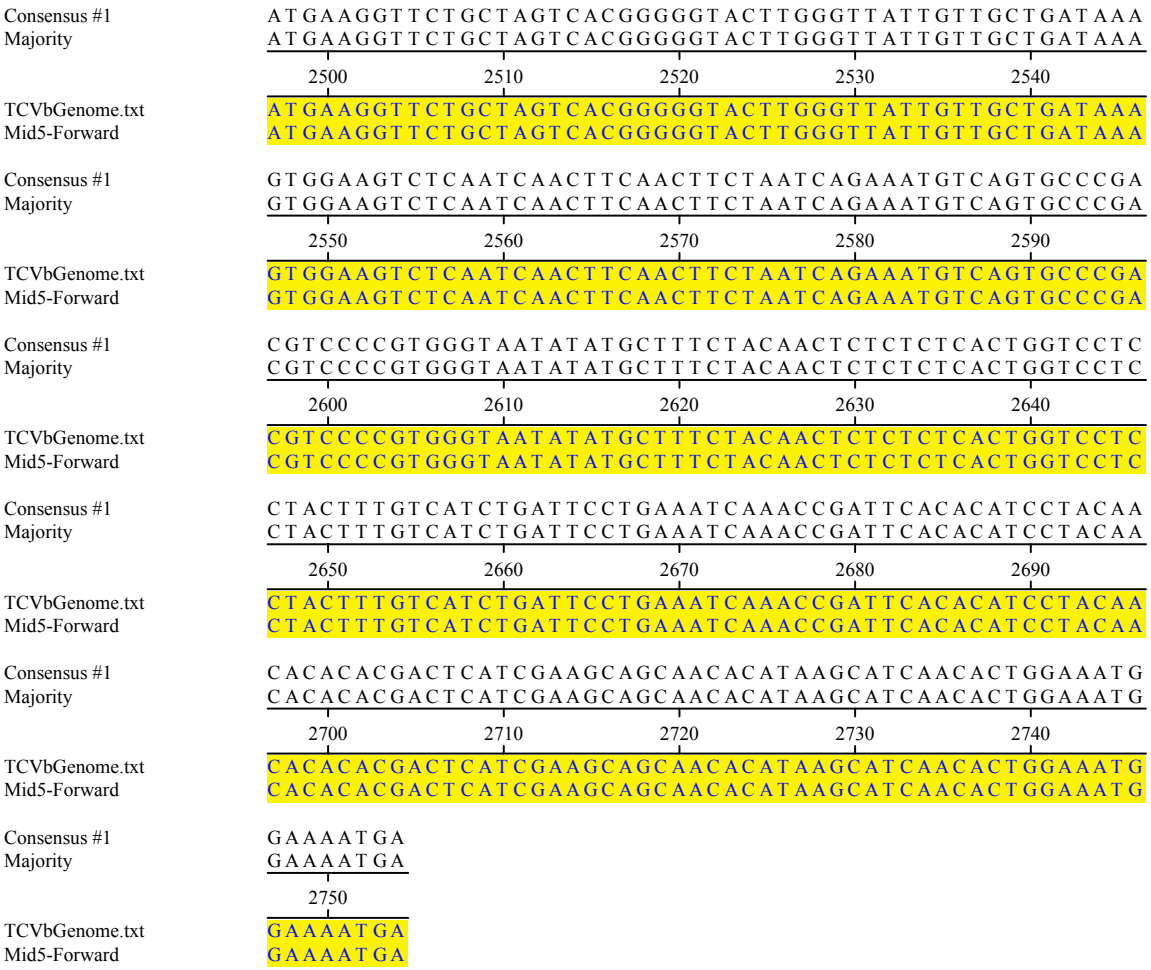


TCV Construct
Translated p8 ORF

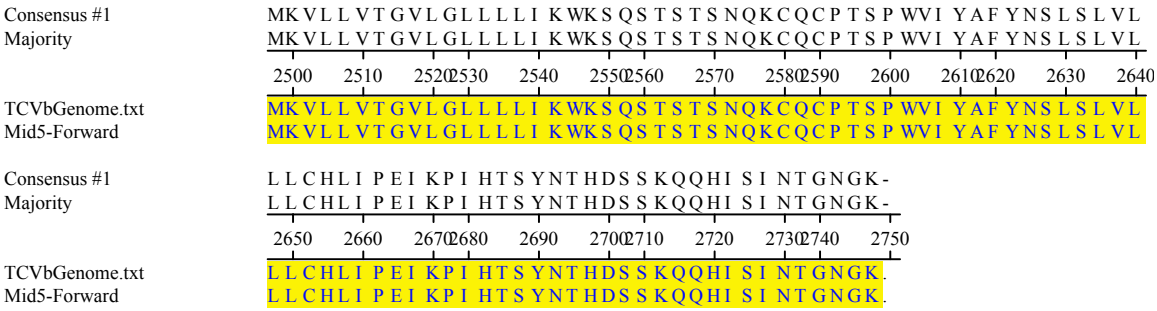


TCV Construct

P9 ORF

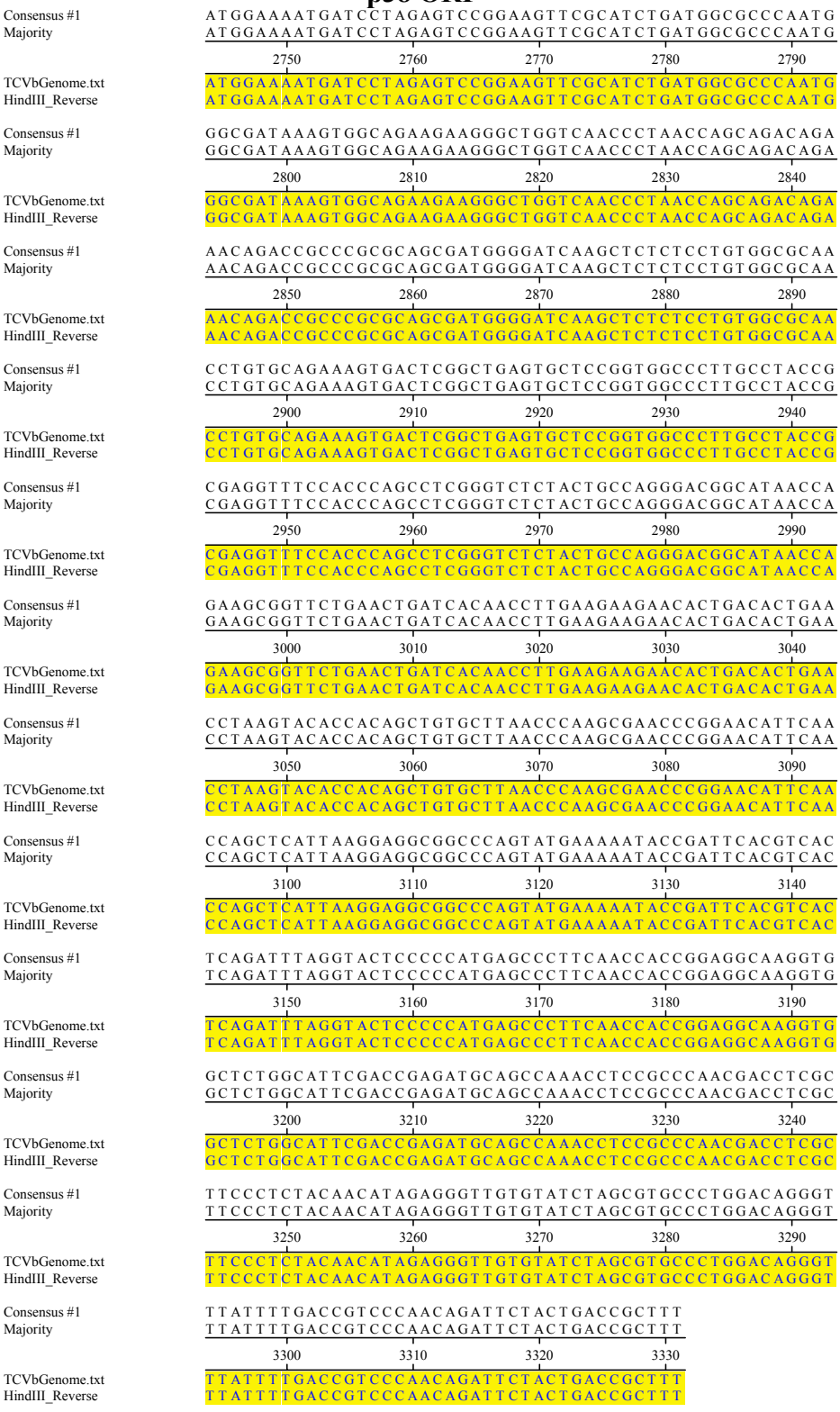


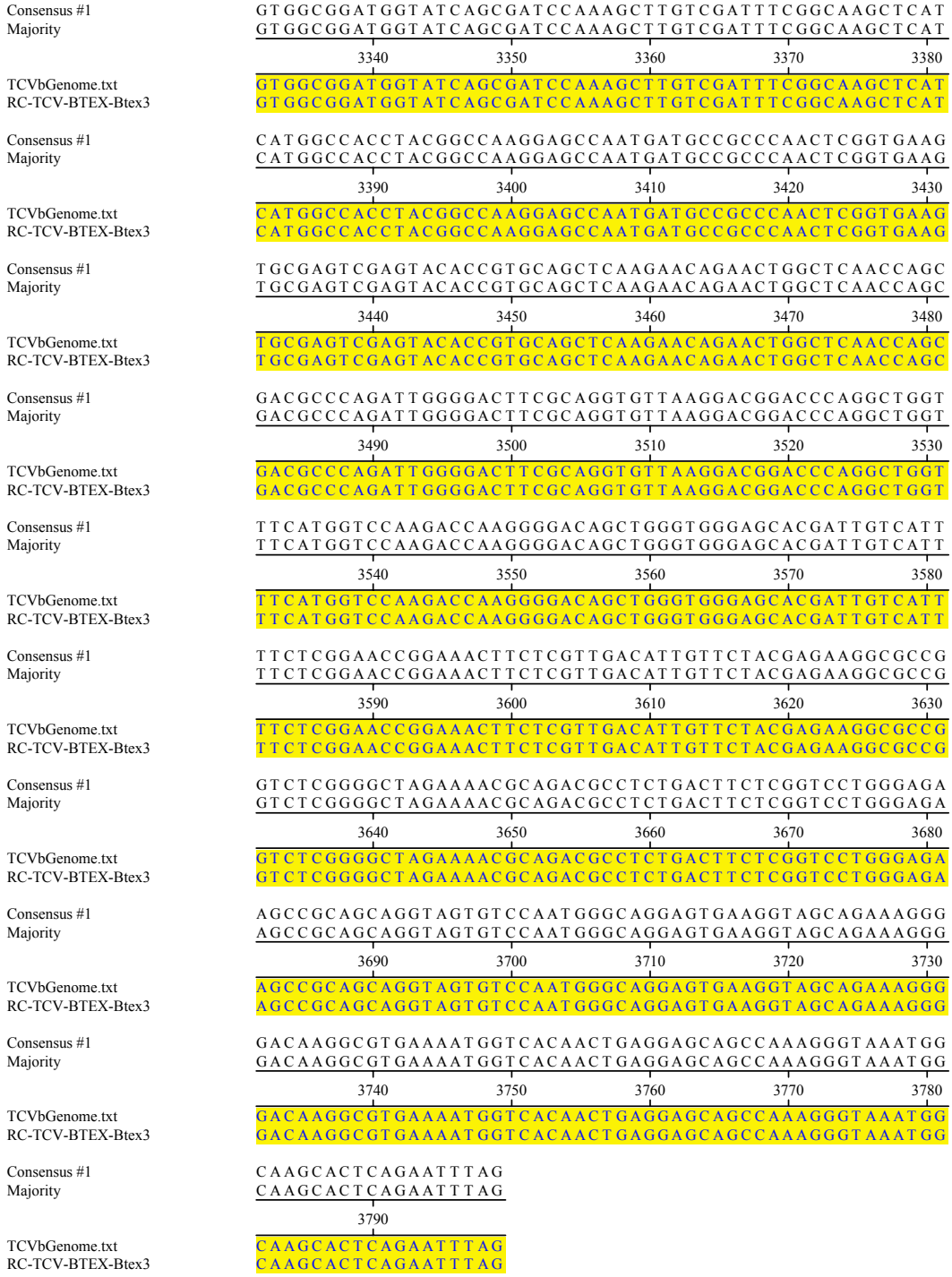
TCV
Translated p9 ORF



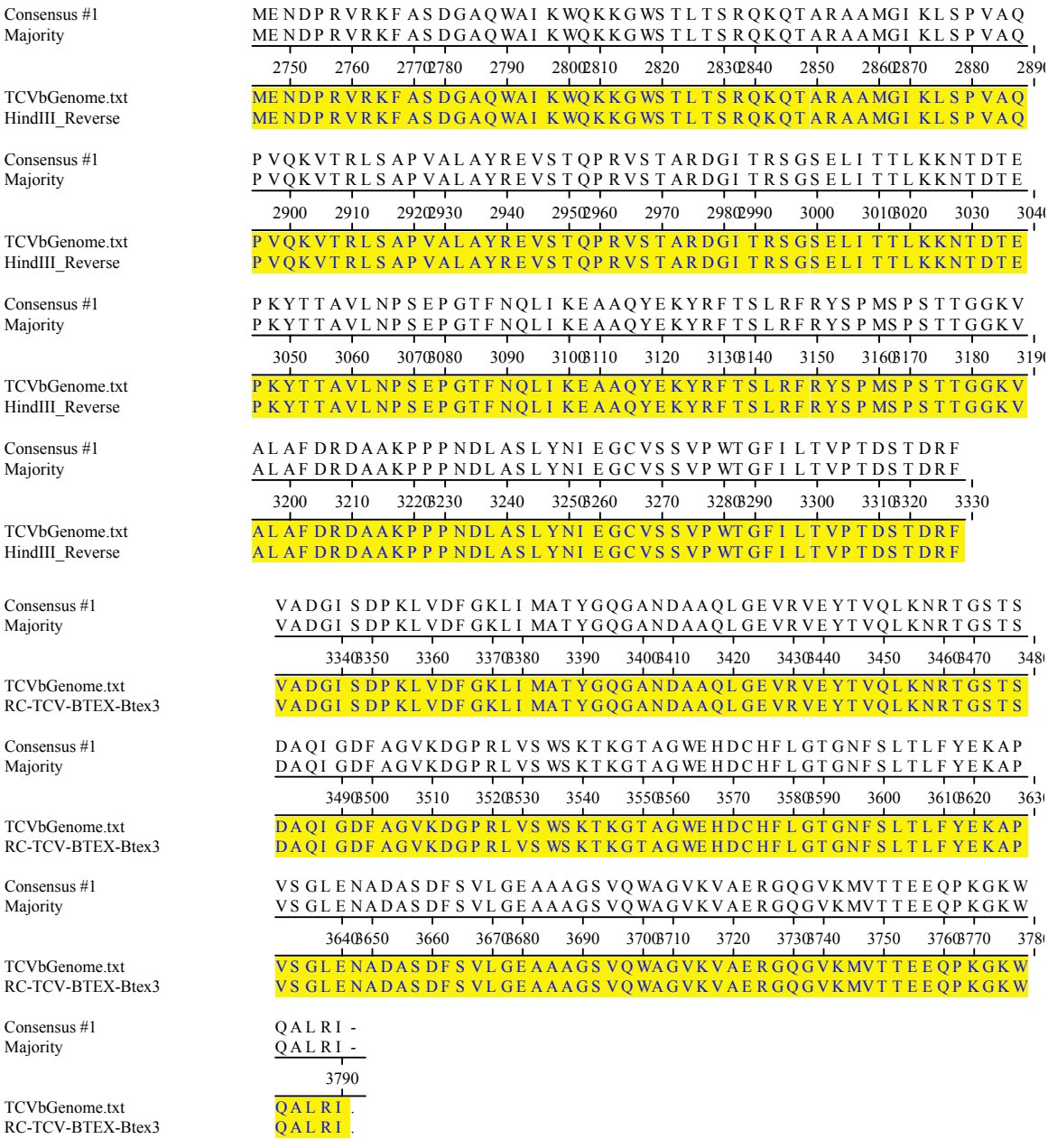
TCV Construct

p38 ORF


Consensus #1
Majority
2750 2760 2770 2780 2790
TCVbGenome.txt
HindIII_Reverse
ATGGAAAATGATCCTAGAGTCCGGAAGTTCGCATCTGATGGCGCCCAATG
Consensus #1
Majority
GGCGATAAAGTGGCAGAAGAAGGGCTGGTCAACCCTAACCAGCAGACAGA
2800 2810 2820 2830 2840
TCVbGenome.txt
HindIII_Reverse
GGCGATAAAGTGGCAGAAGAAGGGCTGGTCAACCCTAACCAGCAGACAGA
Consensus #1
Majority
AACAGACCGCCCGCGCAGCGATGGGGATCAAGCTCTCTCCTGTGGCGCAA
2850 2860 2870 2880 2890
TCVbGenome.txt
HindIII_Reverse
AACAGACCGCCCGCGCAGCGATGGGGATCAAGCTCTCTCCTGTGGCGCAA
Consensus #1
Majority
CCTGTGCAGAAAGTGACTCGGCTGAGTGCTCCGGTGGCCCTTGCCTACCG
2900 2910 2920 2930 2940
TCVbGenome.txt
HindIII_Reverse
CCTGTGCAGAAAGTGACTCGGCTGAGTGCTCCGGTGGCCCTTGCCTACCG
Consensus #1
Majority
CGAGGTTTCCACCCAGCCTCGGGTCTCTACTGCCAGGGACGGCATAACCA
2950 2960 2970 2980 2990
TCVbGenome.txt
HindIII_Reverse
CGAGGTTTCCACCCAGCCTCGGGTCTCTACTGCCAGGGACGGCATAACCA
Consensus #1
Majority
GAAGCGGTTCTGAACTGATCACAACCTTGAAGAAGAACACTGACACTGAA
3000 3010 3020 3030 3040
TCVbGenome.txt
HindIII_Reverse
GAAGCGGTTCTGAACTGATCACAACCTTGAAGAAGAACACTGACACTGAA
Consensus #1
Majority
CCTAAGTACACCACAGCTGTGCTTAACCCAAGCGAACCCGGAACATTCAA
3050 3060 3070 3080 3090
TCVbGenome.txt
HindIII_Reverse
CCTAAGTACACCACAGCTGTGCTTAACCCAAGCGAACCCGGAACATTCAA
Consensus #1
Majority
CCAGCTCATTAAGGAGGCGGCCCAGTATGAAAAATACCGATTCACGTCAC
3100 3110 3120 3130 3140
TCVbGenome.txt
HindIII_Reverse
CCAGCTCATTAAGGAGGCGGCCCAGTATGAAAAATACCGATTCACGTCAC
Consensus #1
Majority
TCAGATTTAGGTACTCCCCCATGAGCCCTTCAACCACCGGAGGCAAGGTG
3150 3160 3170 3180 3190
TCVbGenome.txt
HindIII_Reverse
TCAGATTTAGGTACTCCCCCATGAGCCCTTCAACCACCGGAGGCAAGGTG
Consensus #1
Majority
GCTCTGGCATTCGACCGAGATGCAGCCAAACCTCCGCCCAACGACCTCGC
3200 3210 3220 3230 3240
TCVbGenome.txt
HindIII_Reverse
GCTCTGGCATTCGACCGAGATGCAGCCAAACCTCCGCCCAACGACCTCGC
Consensus #1
Majority
TTCCCTCTACAACATAGAGGGTTGTGTATCTAGCGTGCCCTGGACAGGGT
3250 3260 3270 3280 3290
TCVbGenome.txt
HindIII_Reverse
TTCCCTCTACAACATAGAGGGTTGTGTATCTAGCGTGCCCTGGACAGGGT
Consensus #1
Majority
TTATTTTGACCGTCCCAACAGATTCTACTGACCGCTTT
3300 3310 3320 3330
TCVbGenome.txt
HindIII_Reverse
TTATTTTGACCGTCCCAACAGATTCTACTGACCGCTTT


Consensus #1
Majority
3340
3350
3360
3370
3380
TCVbGenome.txt
RC-TCV-BTEX-Btex3
Consensus #1
Majority
3390
3400
3410
3420
3430
TCVbGenome.txt
RC-TCV-BTEX-Btex3
Consensus #1
Majority
3440
3450
3460
3470
3480
TCVbGenome.txt
RC-TCV-BTEX-Btex3
Consensus #1
Majority
3490
3500
3510
3520
3530
TCVbGenome.txt
RC-TCV-BTEX-Btex3
Consensus #1
Majority
3540
3550
3560
3570
3580
TCVbGenome.txt
RC-TCV-BTEX-Btex3
Consensus #1
Majority
3590
3600
3610
3620
3630
TCVbGenome.txt
RC-TCV-BTEX-Btex3
Consensus #1
Majority
3640
3650
3660
3670
3680
TCVbGenome.txt
RC-TCV-BTEX-Btex3
Consensus #1
Majority
3690
3700
3710
3720
3730
TCVbGenome.txt
RC-TCV-BTEX-Btex3
Consensus #1
Majority
3740
3750
3760
3770
3780
TCVbGenome.txt
RC-TCV-BTEX-Btex3
Consensus #1
Majority
3790
TCVbGenome.txt
RC-TCV-BTEX-Btex3

TCV Construct
Translated p38 ORF



APPENDIX B

HR TRACKING TABLES

Table B-1: Transient Expression Assay in *N. benthamiana*. Eight-week *old N. Benthamiana* plants were infiltrated with *Agrobacterium* carrying a vector expressing each of the TCV ORFs at an A_{600} = 0.3 in combination with *Agrobacterium* carrying vectors expressing AvrPto and Pto, each at an A_{600} = 0.3. Number indicates % of infiltrated site displaying HR lesions (tissue collapse). Three different plants were tested. Leaves 1 and 2 were taken from one plant, while leaf 3 was taken from a second plant and leaves 4 and 5 were taken a third plant.

	Day 4	Day 5	Day 6	Day 7
Leaf 1				
p28MUT	5	10	12	20
p88MUT	30	75	100	100
p88MUT	5	20	45	75
p9	2	15	20	25
p38	20	50	90	90
Leaf 2				
p28MUT	10	10	10	10
p88MUT	5	10	10	10
p88MUT	5	10	10	10
p9	5	10	10	10
p38	5	10	10	10
Leaf 3				
p28MUT	15	90	100	100
p88MUT	25	65	100	100
p88MUT	100	100	100	100
p9	100	100	100	100
p38	100	100	100	100
Leaf 4				
p28MUT	10	10	25	60
p88MUT	65	100	100	100
p88MUT	100	100	100	100
p9	80	100	100	100
p38	85	90	100	100
Leaf 5				
p28MUT	100	100	100	100
p88MUT	100	100	100	100
p88MUT	100	100	100	100
p9	100	100	100	100
p38	100	100	100	100

Table B-2: Transient Expression Assay in *N. benthamiana*. Eight-week old *N. Benthamiana* plants were infiltrated with *Agrobacterium* carrying a vector expressing each of the TCV ORFs at an A_{600} = 0.3 in combination with *Agrobacterium* carrying vectors expressing AvrPto and Pto, each at an A_{600} = 0.3. Number indicates % of infiltrated site displaying HR lesions (tissue collapse). Three different plants were tested. Leaves 1 and 2 were taken from one plant, while leaf 3 was taken from a second plant and leaves 4 and 5 were taken a third plant.

	Day 4	Day 5	Day 6	Day 7
Leaf 1				
p28MUT,p88MUT	8	50	90	90
p28MUT,p8	5	12	15	15
p28MUT,p9	5	5	5	5
p88MUT,p8	5	8	10	10
p88MUT,p9	10	15	35	70
p88MUT,p38	55	80	95	100
p28MUT,p38	20	30	40	50
p8,p9	65	75	85	95
p8,p38	10	35	75	80
p9,p38	10	15	20	35
Leaf 2				
p28MUT,p88MUT	5	5	5	5
p28MUT,p8	5	10	10	10
p28MUT,p9	8	10	10	10
p88MUT,p8	10	60	95	100
p88MUT,p9	5	10	50	60
p88MUT,p38	5	10	60	95
p28MUT,p38	5	5	5	5
p8,p9	5	8	10	10
p8,p38	10	10	12	15
p9,p38	2	2	2	2
Leaf 3				
p28MUT,p88MUT	60	80	100	100
p28MUT,p8	100	100	100	100
p28MUT,p9	95	100	100	100
p88MUT,p8	100	100	100	100
p88MUT,p9	50	100	100	100
p88MUT,p38	80	90	95	100
p28MUT,p38	80	100	100	100
p8,p9	90	100	100	100
p8,p38	100	100	100	100
p9,p38	80	100	100	100

Table B-3: Transient Expression Assay in *N. benthamiana*. Eight-week old *N. Benthamiana* plants were infiltrated with *Agrobacterium* carrying a vector expressing each of the TCV ORFs at an A_{600} = 0.3 in combination with *Agrobacterium* carrying vectors expressing AvrPto and Pto, each at an A_{600} = 0.3. Number indicates % of infiltrated site displaying HR lesions (tissue collapse). Three different plants were tested. Leaves 1 and 2 were taken from one plant, while leaf 3 was taken from a second plant and leaves 4 and 5 were taken a third plant.

	Day 4	Day 5	Day 6	Day 7
Leaf 4				
p28MUT,p88MUT	100	100	100	100
p28MUT,p8	100	100	100	100
p28MUT,p9	80	100	100	100
p88MUT,p8	100	100	100	100
p88MUT,p9	100	100	100	100
p88MUT,p38	80	100	100	100
p28MUT,p38	100	100	100	100
p8,p9	95	100	100	100
p8,p38	100	100	100	100
p9,p38	75	100	100	100
Leaf 5				
p28MUT,p88MUT	100	100	100	100
p28MUT,p8	95	100	100	100
p28MUT,p9	60	80	90	100
p88MUT,p8	100	100	100	100
p88MUT,p9	95	100	100	100
p88MUT,p38	100	100	100	100
p28MUT,p38	100	100	100	100
p8,p9	100	100	100	100
p8,p38	100	100	100	100
p9,p38	100	100	100	100

Table B-4: Transient Expression Assay in *N. benthamiana*. Eight-week old *N. Benthamiana* plants were infiltrated with *Agrobacterium* carrying a vector expressing each of the TCV ORFs at an A_{600} = 0.3 in combination with *Agrobacterium* carrying vectors expressing AvrPto and Pto, each at an A_{600} = 0.3. Number indicates % of infiltrated site displaying HR lesions (tissue collapse). Three different plants were tested. Both leaves were taken from the same plant.

	Day 4	Day 5	Day 6	Day 7
Leaf 1				
P8,p9,p38	5	8	10	10
P8,p28MUT,p88MUT	5	15	30	70
P9,p28MUT,p88MUT	5	10	25	35
P28MUT,p38,p88MUT	2	2	2	2
P8,p28MUT,p38	40	60	70	90
P9,p28MUT,p38	5	10	15	50
P8,p38,p88MUT	5	10	15	15
P9,p38,p88MUT	8	15	15	80
P8,p9,p38,p88MUT	10	10	15	25
P8,p9,p28MUT,p38	2	2	5	15
P8,p28MUT,p38,p88MUT	95	100	100	100
P9,p28MUT,p38,p88MUT	10	12	15	30
P8,p9,p28MUT,p88MUT	30	65	75	85
Leaf 2				
P8,p9,p38	15	15	15	20
P8,p28MUT,p88MUT	2	2	5	5
P9,p28MUT,p88MUT	5	10	12	15
P28MUT,p38,p88MUT	5	8	8	10
P8,p28MUT,p38	5	6	10	10
P9,p28MUT,p38	8	20	25	55
P8,p38,p88MUT	15	20	20	30
P9,p38,p88MUT	20	80	95	95
P8,p9,p38,p88MUT	5	5	10	15
P8,p9,p28MUT,p38	20	35	40	45
P8,p28MUT,p38,p88MUT	15	50	65	75
P9,p28MUT,p38,p88MUT	5	10	15	15
P8,p9,p28MUT,p88MUT	15	35	40	50