Evaluation of the coat protein of the *Tombusviridae* as HR elicitor in *Nicotiana* section

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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Evaluation of the coat protein of the *Tombusviridae* as a HR elicitor in *Nicotiana* section *Alatae*

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Dr. James E. Schoelz, Thesis Supervisor

Plants are able to recognize and respond to virus infection with a hypersensitive response, a plant defense response that triggers a cell death pathway and limits the viral infection to the infected leaf. We have previously shown that the coat protein of *Tomato bushy stunt virus* (TBSV) and *Tobacco necrosis virus* strain DH (TNV-D^H) trigger HR in several species of *Nicotiana*, including *Nicotiana langsdorffii*. To identify structural features in the coat protein recognized by the host, we tested the capacity of eight coat proteins of the *Tombusviridae* to trigger a HR in *N. langsdorffii.* These eight coat proteins represented six genera of the *Tombusviridae*. We found that the coat proteins of TBSV, *Cucumber Necrosis Virus*, *Cymbidium ringspot virus*, *Red Clover necrotic mosaic virus* and TNV-D^H triggered HR, whereas the coat proteins of *Turnip crinkle virus*, *Maize chlorotic mottle virus* and *Panicum mosaic virus* did not. A comparison of the amino acid sequences of all eight coat proteins revealed that only two amino acids (Asp155 and Arg161) were conserved amongst the coat proteins that triggered HR and distinguished them from the coat proteins that did not trigger HR. Computer modeling of the coat protein structure allowed for placement of these two amino acids within the three dimensional structure of the coat protein and suggested how mutations might affect the overall structure. Site directed mutagenesis of these two amino acids in the TNV-D^H coat protein abolished HR, demonstrating that they have an essential role in recognition and elicitation of plant defenses against this virus.

Chapter 1. Literature Review

1.1. Introduction

Viruses are notorious for causing diseases in humans, animals and plants. The vast majority of plant viruses have a small genome size, encoding as few as three proteins up to as many as 10 proteins (Schoelz et al., 1986). The virions of most plant viruses are not associated with a membrane. Consequently, they consist of the genome nucleic acid protected through encapsidation by the coat proteins (CPs). The genomic nucleic acids can be composed on single-stranded RNA, single-stranded DNA, double-stranded RNA, or double stranded DNA, with the genomes of most viruses composed of positive sense, single-stranded RNA (Hull, 2008). The virions of some plant viruses are composed of more than one type of CP, but most plant virus genomes carry the information for only one CP. Encapsidation of the viral nucleic acid into the virion is an essential stage in the life cycle of all plant viruses (Rao, 2006).

The assembly of the CP into a virion is an oligomerization process that occurs in one of these three ways (Mateu, 2013). In the first method, The CP subunits are sufficient for the virion assembly; *Satellite Tobacco mosaic virus* (STMV) and *Satellite Tobacco necrosis virus* (STNV) are two examples of this type. Their CPs are composed of 60 identical CP subunits to form the icosahedral virion. In fact, the minimal icosahedral structure can be formed from 12 pentamers. It is said to have a T (triangulation number) of one. Multiples of 60 CP subunits can actually make larger icosahedral visions. A virion with a T=3 structure is the most common form; it is made from 180 identical coat protein subunits. In the second method, the CP assembly needs scaffolding proteins in addition to CP. This type of capsid assembly has been observed in dsDNA phages &

animal viruses, dsRNA viruses and ssDNA phages. Scaffolding proteins only participate in transient protein-protein interactions and then are removed as the virus mature. In the third method, virion assembly requires an interaction of the nucleic acid and CP subunits. The thermodynamics of this process had shown that assembly has a nucleation phase and a growth phase, which follows a sigmoidal curve. The virion assembles when the concentration of the CP is above a specific level; below this concentration disassembly happens. Furthermore, capsid-ligand binding increased capsid stability. In the case of ssRNA plant viruses such as *Tomato bushy stunt virus* (TBSV), S*outhern bean mosaic virus* (SBMV), STNV and *Cowpea chlorotic mottle virus* (CCMV), divalent cations such as Ca^{2+} are necessary for capsid assembly and maintenance (Mateu, 2013).

1.2. Plant surveillance systems for defense against pathogens

Plants utilize two different types of surveillance systems to recognize and defend against plant pathogens (Dangl and McDowell, 2006). Pathogen-triggered immunity (PTI) occurs when plants are able to recognize pathogen-associated molecular patterns (PAMPS). According to the classic definition of PTI, plant viruses would not be considered to encode any PAMPS or to trigger PTI. The other surveillance system is called effector- triggered immunity (ETI). In this surveillance system, pathogen effector proteins are recognized by host resistance (R) proteins. ETI is a refined version of the Gene-For-Gene Theory, which was proposed by H.H. Flor (1971). According to the Gene-For-Gene Theory, pathogens carry a series of avirulence (Avr) genes and hosts carry resistance (R) genes. Host resistance occurs when a pathogen Avr protein is recognized the by corresponding host R protein.

One form of ETI that is very common in host/viral interactions is the hypersensitive response (HR). HR is triggered after the recognition of a pathogen effector by a host R protein and it is manifested through the activation of a cell death pathway in all of the cells infected with a virus. As a result the pathogen infection is restricted to a specific site and consequently cannot spread to the other parts of the plant (Pontier et al., 1998). One of the most thoroughly studied examples of HR against virus infection involves the recognition of the replicase protein of *Tobacco mosaic virus* (TMV) by the N protein derived from *N. glutinosa* (Whitham et al., 1994).

1.3. The Guard Hypothesis

Although the Gene-For-Gene theory established that host resistance is triggered when a pathogen effector/Avr protein is recognized by a host R protein, there has been a debate about whether this recognition is mediated through a direct interaction or indirect interaction. Depending on the host/pathogen system, either scenario is possible. For example, a yeast two-hybrid experiment showed that the R protein *Pi-ta* in rice interacted directly with the effector AVR-Pita in *Magnaporthe grisea* (DeYoung and Innes, 2006).
On the other hand, the Guard Hypothesis was developed to explain an indirect interaction between an effector/Avr protein and a host R protein (Dangl and McDowell, 2006). In this model, a pathogen effector protein interacts with a target host protein, which causes conformational changes in the structure of the host protein. At this point the R protein, also known as the guard protein, recognizes the conformational change and consequently activates a signaling cascade against pathogen invasion (Gururani et al., 2012). The classic example of the guard hypothesis occurs in the *P. syringae*/tomato pathosystem, in which the AvrPto protein in *P. syringae* with the tomato Pto protein interacting

indirectly. Tomato carries the Prf protein, which has the capacity to recognize the interaction between Pto and AvrPto. Prf is guard against the interaction of Pto and AvrPto.

Similar to the *P. syringe*/tomato interaction, resistance in *Arabidopsis thaliana* to *Turnip crinkle virus* (TCV) involves the interaction of three proteins. Resistance in Arabidopsis ecotype Dijon is triggered by a recognition event between the *HRT* resistance gene and the TCV CP (Dempsey et al., 1997; Oh et al., 1995). A second host protein, designated TIP (for TCV-interacting protein) is required for activation of resistance. The TCV CP physically interacts with TIP, and mutations that block the TIP/CP interaction also abolish resistance (Ren et al., 2000).

1.4. Resistance gene structure

The first plant R genes were cloned in 1994. As plant R genes have been cloned and their nucleotide sequences determined, it has been found that they frequently have several common motifs. The N-terminus has a nuclear binding site (or NBS), whereas the C-terminus consists of Leucine-Rich repeats (LRRs). The N-terminus may also have a Toll/interleukin-1 receptor (TIR), or Coiled-Coil (CC) domain. For example, the tobacco *N* gene belongs to the class of TIR-NBS-LRR genes (Whitham et al., 1994). Other common motifs include a receptor like protein Ser/Thr protein kinase, transmembrane domains, a leucine zipper, and protein kinase motifs (Liu et al., 2007). The size of the known NBS-LRR proteins varies between 860-1900 amino acid residues. The N-terminal NBS is the most studied domain in this group of proteins and it contains several motifs necessary for protein-protein interactions. The LRR domain is involved in ligand-binding and protein-protein interactions. The major secondary structure in LRR

domains is the β-sheet. NBS-LRR proteins are involved in triggering the defense-signaling cascade.

1.5. The genus *Nicotiana* as a source for resistance genes that target viruses

The genus *Nicotiana* has been especially valuable for studying the factors that determine systemic virus infections vs. HR. The genus contains 76 species which are organized into 13 taxonomic sections (Knapp et al., 2004). Some species, such as *N. glutinosa* and its close relative *N. edwardsonii* have played key roles in characterizing the application of the gene-for-gene theory to viruses. For example, the first Avr-determinant identified by recombinant DNA techniques was the P6 protein of *Cauliflower mosaic virus*, which elicits a plant defense response in both species (Schoelz et al., 1986). On the host side, the *N* gene, an R gene derived from *N. glutinosa* that recognizes TMV, was one of the first R genes to be discovered and cloned (Holmes, 1938; Whitham et al., 1994). Schoelz and coworkers (Schoelz et al., 2006) made crosses between *N. clevelandii* and *N. glutinosa* to show that resistance to TBSV segregated independently from resistance to TMV. More importantly, a literature search indicated 67 viruses that could infect *N. clevelandii* but not *N. glutinosa*, suggesting that *N. glutinosa* could be a valuable species for understanding how plants recognize and defend against a broad spectrum of viruses (Schoelz et al., 2006). Another important species in the genus is *N. benthamiana,* which serves as a model plant in plant virology. *N. benthamiana* is readily infected by a wide variety of plant viruses, and also is frequently used in agroinfiltration assays for transient expression assays of foreign genes (Goodin et al., 2008).

1.6. Avr determinants in TBSV and TNV-D^{H}

Previous studies had illustrated that the genus *Nicotiana* could be valuable as a source for R genes that target viruses in the *Tombusviridae*, as a majority of *Nicotiana* species that were tested responded with HR to TBSV and *Tobacco necrosis virus* strain DH (TNV-D^{H}) (Angel and Schoelz, 2013; Rodriguez, 2014). Although several *Nicotiana* species reacted to inoculations of TBSV and TNV-D^{H} with HR, further investigations showed that the species were recognizing distinctly different viral Avr products. For example, the TBSV P22 protein triggered HR in *N. edwardsonii*, *N. glutinosa* and *N. forgetiana*, whereas the TBSV P19 protein triggered HR in *N. sylvestris*, *N. tabacum*, and *N. bonariensis* (Angel et al., 2011; Scholthof et al., 1995). The TNV-D^{H} Avr determinant in those *Nicotiana* species has not yet been identified, in part because TNV-D^{H} does not encode proteins analogous to P19 and P22. By contrast, the TBSV and TNV-D^{H} CPs triggered HR in several species in section *Alatae*, including *N. langsdorffii, N. longiflora, N. forgetiana, N. alata,* and *N. bonariensis* (Angel and Schoelz, 2013; Rodriguez, 2014). The only species in section *Alatae* that does not respond to tombusvirus CPs with HR is *N. plumbaginifolia*. A phylogenetic analysis of representative *Nicotiana* species is presented in Figure 1.1. It summarizes the *Nicotiana* species that respond with HR to virion inoculation of either TNV-D^{H} or TBSV. Furthermore, it lists the viral protein responsible for triggering HR, if known.

Truncated coat proteins were designed to determine which sections of the TNV-D^{H} and TBSV coat proteins are responsible for triggering HR in the section *Alatae* (Angel and Schoelz, 2013; Rodriguez, 2014). Progressively larger deletions were made on the 5' end of the coat protein genes, with each deletion defined by a start codon in the

sequence. For TBSV, it was found that the first 79 amino acids did not contribute to HR development (Angel and Schoelz, 2013). The deletion series for the TNV-D^H coat protein is illustrated in Figure 1.2. The construct pATG2 consisted of a deletion from the beginning of the sequence to the second start codon (Fig. 1.2). The next constructs (pATG3, pATG4 & pATG5) were started from the 3rd, 4th and 5th start codons, respectively. The construct pATG4KO consisted of a deletion up to the fourth start codon, and then the fourth start codon was mutated to TTG. All of the constructs were delimited by the restriction enzyme sites *XhoI* and *SacI*, and cloned into the binary *Agrobacterium* vector pKYLX7 (Schardl et al., 1987). After transformation of the construct into *Agrobacterium*, they were tested by agroinfiltration into *Nicotiana* species. Agroinfiltration of the pATG2, pATG3, and pATG4 yielded an HR in the *Nicotiana* species from section *Alatae* (Rodriguez, 2014), whereas the response to agroinfiltration of pATG5 was negative. Furthermore, mutation of the ATG4 start codon to TTG also abolished HR, indicating that the coat protein sequence rather than the RNA sequence was responsible for triggering HR. We concluded that the first 77 amino acids of the TNV-D^H coat protein could be eliminated without affecting its capacity to trigger HR.

1.7. *Tobamovirus* CPs as avirulence gene products

The first plant virus CP to be shown to act as an Avr determinant was that of TMV. The TMV CP triggers HR in *N. sylvestris* in response to the *N'* resistance gene (Knorr and Dawson, 1988; Saito et al., 1989). The ability of the TMV in triggering HR in the eggplant was investigated by using a full-length TMV cDNA construct.

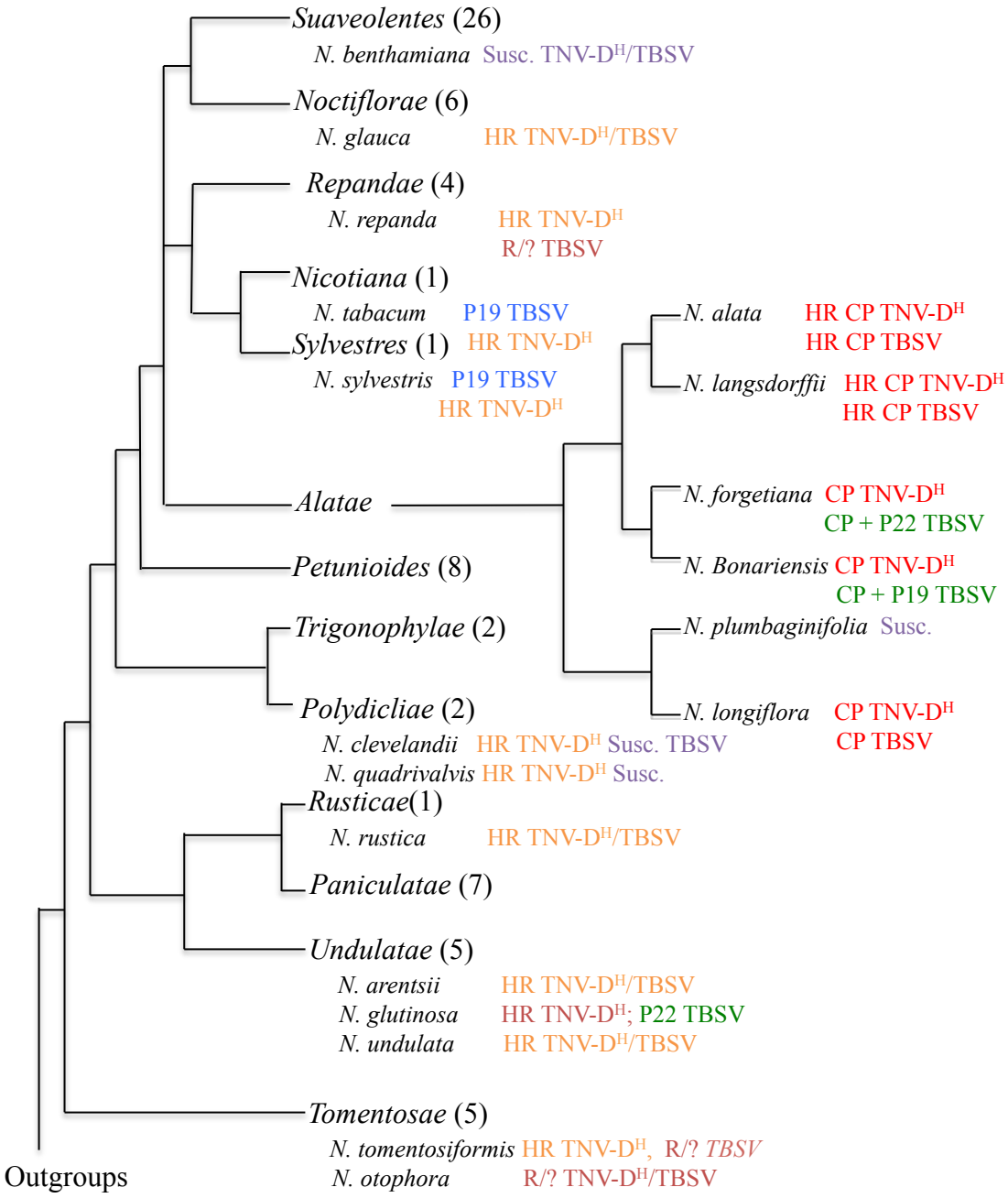

Figure 1-1. Comparison of TNV-D^H and TBSV avirulence determinants within the *Nicotiana* sections. The phylogenetic relationships of the *Nicotiana* sections are summarized from Clarkson et al. (2004). The number in parentheses after the section lists the total number of species in that section, whereas the *Nicotiana* species included in host range tests are listed below each of the sections. *N. edwardsonii* is not included in this figure because it is a species hybrid between *N. glutinosa* and *N. clevelandii* (Christie, 1969). The phylogeny of *Nicotiana* species in the section *Alatae* is derived from Lim et al. (2006) and Lee et al. (2008). The TBSV and TNV-D^H avirulence determinants identified or confirmed in Angel and Schoelz (2013) and Rodriguez (2014) are listed after the species. *Nicotiana* species that responded with nonnecrotic resistance are designated with an "R/?", which also emphasizes that the viral trigger for resistance remains unknown. The three *Nicotiana* species that responded with HR but for which the avirulence determinant was not identified are designated by "HR/?". Species susceptible to TBSV or TNV-D^H are indicated by the abbreviation "Susc.".

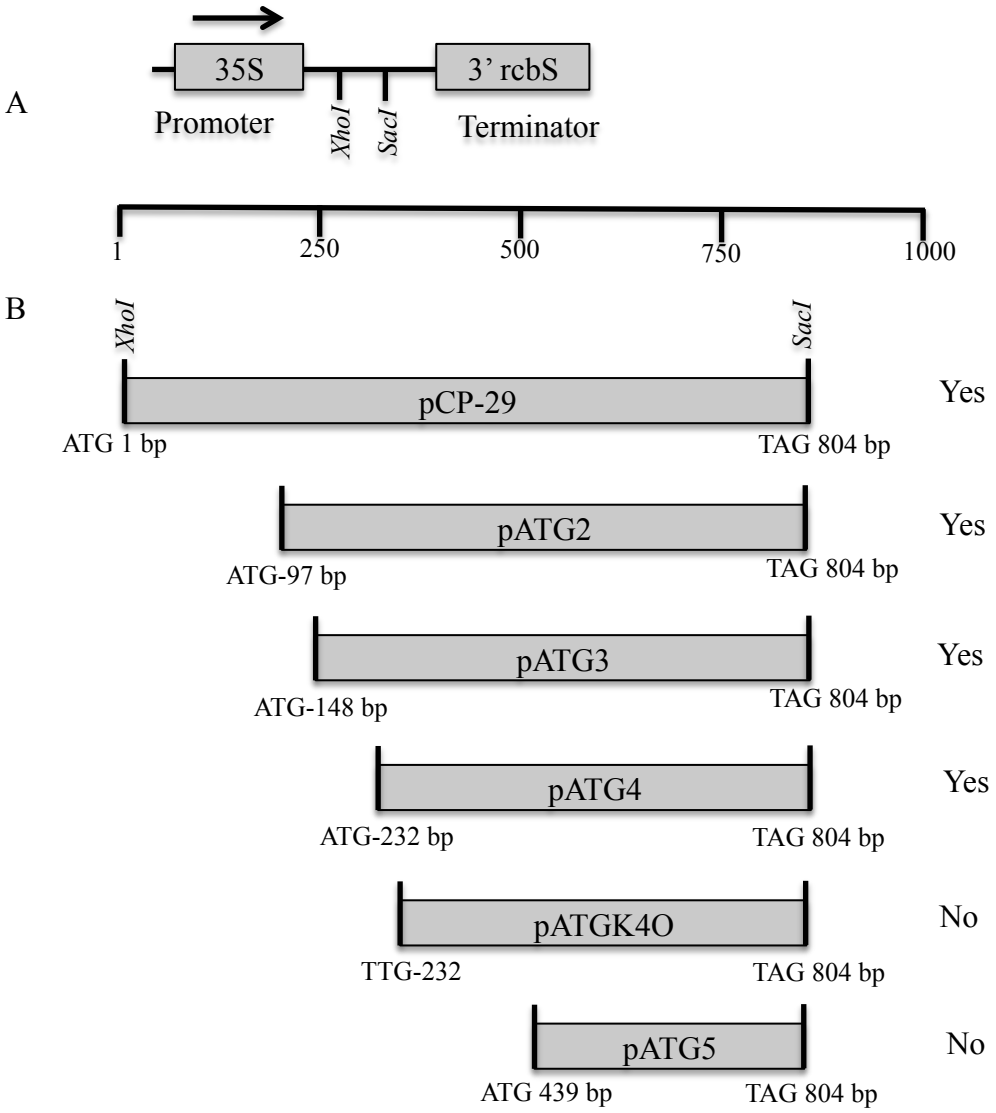

Figure 1.2. TNV-D^{H} coat protein 5' deletions. A) Multiclone site for the binary vector pKYLX7. All CP constructs were inserted into the *Xho*I and *Sac*I sites, placing them under control of the CaMV 35S promoter and *rcb*S terminator. **B)** The first construct (pATG2) consisted of the deletions from the beginning of the sequence to the second start codon. pATG3, pATG4 and pATG5 were started from the 3rd, 4th and 5th start codons. The pATGK4O construct was made to determine if the coat protein is responsible for the HR or it is viral RNA. This construct was made by mutating the pATG4 start codon to TTG.

The cDNA construct did not possess a TMV CP translational start codon and it was used to inoculate eggplant leaves. The result was a virus which replicated and moved cell to cell but was unable to trigger HR in the eggplant. To identify if the coat protein was sufficient to elicit HR, TMV CP was expressed by cloning into a potato X potexvirus (PVX) vector, PVX2C2S. The eggplant leaves that were inoculated with this construct developed a necrotic lesion after 4 dpi while the empty vector did not. These results revealed that the TMV coat protein was sufficient to trigger HR (Dardick and Culver, 1997). The ability of the other *Tobamovirus* coat proteins in triggering HR in eggplant was also investigated. The coat protein of TMV was replaced by Tobacco mosaic virus strain U1 (TMV-U1), Tobacco mosaic virus strain U2 (TMV-U2), *cucumber green mottle mosaic virus* (CGMMV) and *odontoglossum ringspot virus* (ORSV) coat proteins. As a result HR was triggered in eggplant with the constructs that contained TMV-U1, TMV-U2 and CGMMV CPs. In contrast TMV U1 failed to elicit HR in tobacco while pepper showed HR to all chimeric TMV constructs (Dardick and Culver, 1997; Taraporewala and Culver, 1997).

In another research, *Nicotiana* Xanthi-nn plants responded with HR to inoculation with crucifer and garlic infecting TMV (TMV-Cg). To determine which proteins of the TMV-Cg was responsible for the HR, a series of hybrid viruses were made between TMV-U1 and TMV-Cg. These hybrids included U1-RepCg, U1-MPCg, U1-MPCPCg and U1-CPCg. Their analysis showed that hybrid U1-CPCg, which carries the CP of the Cg strain, induced HR in the sensitive *Nicotiana* Xanthi.nn plants (Ehrenfeld et al., 2005).

1.8. The *Tombusviridae*

The *Tombusviridae* is a family of single-stranded, positive-sense RNA viruses that infect plants. The family consists of eight genera as follows: *Dianthovirus, Machlomovirus, Tombusvirus, Panicovirus, Necrovirus, Aureusvirus*, *Avenavirus* and *Carmovirus*. For my research I obtained eight species that represented six of the eight genera in the family (Fauquet et al., 2005). The virus species included TBSV, *Cymbidium Ringspot Virus* (CymRSV) and *Cucumber Necrosis Virus* (CNV) from the genus *Tombusvirus*, *Red Clover Necrosis Mosaic Virus* (RCNMV) from the genus *Dianthovirus*, TCV from the genus *Carmovirus*, *Maize Chlorotic Mottle Virus* (MCMV) from the genus *Machlomovirus*, *Panicum Mosaic Virus* (PMV) from the genus *Panicovirus* and finally TNV strain DH from the genus *Necrovirus*. The genera *Avenavirus* and *Aureusvirus* contain only a few species and it was not possible to locate a cDNA clone of their CP coding sequence. The sections below briefly describe the genomic organization of each of the genera that contributed to my project. The genomic organization of these viruses is illustrated in Figure 1.3.

1.8.1. TBSV, CymRSV and CNV genomic organization

TBSV, CymRSV and CNV belong to the genus *tombusvirus*; TBSV is the type member of this genus. The full-length genomes of TBSV, CymRSV and CNV are 4776, 4733, and 4701 nucleotides, respectively and their genome maps are illustrated in Fig. 1.2 (Grieco et al., 1989; Hearne et al., 1990; Rochon and Tremaine, 1989). All three viruses have the same genome structure, with five ORFs. The first ORF is translated directly from the genomic RNA and terminates at an amber codon, which enables a read through

mechanism for translation of the second ORF. Molecular weigh of the proteins in all the species is same: 33 kDa for ORF1 and 92 kDa for ORF2. Both of these proteins are necessary for viral replication. The other three proteins are expressed through subgenomic RNAs. The third ORF encodes the CP, which for TBSV and CymRSV has a molecular weight (mw) of 41 kDa, whereas for CNV the CP has a mw of 40 kDa. A second sub-genomic RNA encodes ORF's 4 and 5, which are nested proteins. The mw of these two proteins in the TBSV and CymRSV genome is 22 and 19 kDa, respectively, whereas for CNV these two proteins have mw of 21 and 20 kDa. The 19/20 kDa protein, also known as P19/P20, is a strong silencing suppressor, whereas the P21/P22 protein is thought to have a role in movement.

1.8.2. RCNMV genomic organization

RCNMV is the type member of the genus *Dianthovirus*. The genome has a size of 3890 nucleotides, is organized into four ORFs, and is divided onto two RNAs (Xiong et al., 1993). The first and second ORFs on RNA 1 encode proteins for replication, and as with TBSV the larger protein is expressed through a readthrough mechanism. Their sizes are 27 kDa and 88 kDa. The CP is also carried on RNA 1; it has a size of 37-38 kDa. A previous study showed the first 50 residues of the RCNMV CP are engaged in RNA binding that is required for virion formation and movement (Park et al., 2013). The second RNA contains a single RNA that encodes a 34-35 kDa protein. This protein is necessary for cell-to-cell movement.

1.8.3. TCV genomic organization

TCV belongs to the genus *Carmovirus*, of with *Carnation mottle virus* is the type member (Fauquet et al., 2005). The 4.0 kb (4050 nucleotides) genomic RNA is organized into three ORFs. The first ORF is translated into two proteins that have sizes of 28 and 88 kDa. The P88 protein is translates by a readthrough mechanism of the P28 protein. The 2nd and 3rd ORFs are encoded in a subgenomic RNA. The result of the translation of the 2nd is the production of the coat protein with a size of 38 kDa (P38). The third ORF is translated to produce an 8 kDa protein (P8) (Carrington et al., 1989).

1.8.4. MCMV genomic organization

MCMV is the type member of the genus *Machlomovirus*. The 4.4 kb genomic RNA may encode up to six ORFs (Nutter et al., 1989). ORF 1 encodes a 32kDa protein of unknown function. The second ORF encodes a 48 kDa protein, with a readthrough product that has a size of 112 kDa. Both the 48 kDa and 112 kDa proteins form the viral components of the polymerase. The third ORF encodes a protein hat has a size of 7 kDa, but may also form a readthrough product of 33 kDa. ORF 4 encodes the CP, which has a size of 25 kDa proteins.

1.8.5. PMV genomic organization

PMV is the type member of the genus *Panicovirus.* It has a genome size of 4326 nucleotides that is organized into six ORFs (Turina et al., 1998). Similar to other viruses in the *Tombusviridae*, ORF 1 is translated directly from the genomic RNA to produce a 48 kDa protein; a readthrough mechanism of the amber termination codon allows for translation of a 112 kDa protein. A single 1.5 kb sub-genomic RNA is responsible for

translation of the next four ORFs, with protein products of 8kDa, 6.6kDa, 26kDa and 15 kDa proteins, respectively. The PMV CP corresponds to the 26 kDa protein.

1.8.6. TNV strain DH genomic organization

TNV-D^H is the type member of the genus *Necrovirus*. TNV strain DH has a genome size of 3762 nucleotides that is organized into six ORFs (Molnar et al., 1997). The first ORF encodes a 22 kDa protein, and ends at nucleotide 645 with an amber codon to allow for a readthrough for synthesis of a 82 kDa protein. The 22 kDa and 82 kDa proteins are required for replication. All of the other ORFs are translated from one of two subgenomic RNAs. ORF2 and ORF3 encode proteins that each have a size of 7 kDa. The TNV-D^H CP is found on the second subgenomic RNA. For TNV strain DH, it has a size of 29 kDa.

1.9. Structural features of the CPs of the *Tombusviridae*

The virions of the positive sense RNA plant viruses are classified into three major groups: spherical (icosahedral), rigid rods and flexible filaments. Phylogenic studies based on the CP have shown that there are not significant similarities in the sequences among the three groups, but secondary and tertiary structures are very similar and many functions are conserved (Callaway et al., 2001). The CPs usually have four distinct domains: an N-terminal (R domain) which is positively charged and interacts with the RNA, a connecting arm (a domain), a central domain (S domain) which forms the virion shell, and the C-terminal (P domain) (Dolja and Koonin, 1991). In many cases the structure of the virions, as well as individual CPs has been determined. I briefly describe in the following section the virion structure of the tombusviruses.

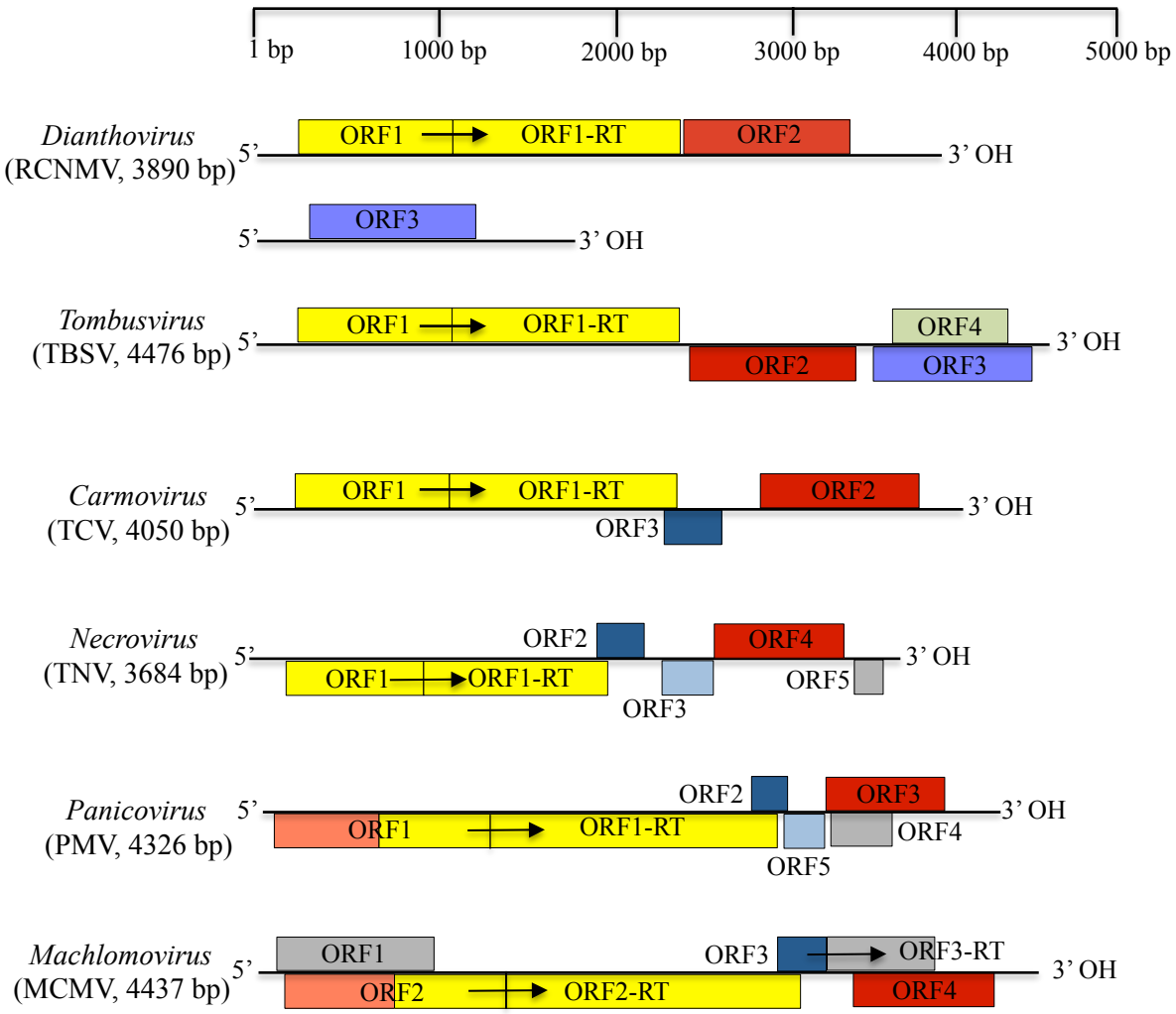

Figure 1.3. *Tombusviridae* genome organization. Yellow boxes are ORFs that encode polymerase proteins. Red boxes represent the coding region for the coat protein. Blue boxes represent a viral movement protein. There is an extra movement protein in *Panicovirus*, *Necrovirus* and *Carmovirus* that is painted in light blue. The green box in the *Tombusvirus* genus is a silencing suppressor. The orange regions in the MCMV and PMV are similar to each other; however they do not have sequence similarity with the other *Tombusviridae*. Gray boxes illustrate unique proteins for the indicated virus.

1.9.1. The TBSV CP

TBSV forms a spherical virion with a diameter of 35 nm. The TBSV structure has been resolved to 2.9Å resolution (Harrison et al., 1978). The individual CP structure is a consequence of the interaction of two globular domains (P and S domains). The N-terminal P domain and the C-terminal S domain are composed of 110 and 168 amino acid residues, respectively with a hinge connecting them. The CP forms a trimer with three distinct structural subunits: A, B and C. The N-terminal part of the sequence is obscure in the A and B subunits and lacks a specific structure but in subunit C the last 33 amino acids of the P domain are highly ordered. Two antiparallel β-sheets form the P-domain; one of the beta-sheets contains six β-strands, and the other one is composed of four β-strands. The S-domain has two β-sheets; each one has four antiparallel beta strands. The S-domain also has several loops and two short α-helices (Harrison et al., 1978).

1.9.2. The TNV CP

TNV forms a spherical virion with a diameter of 26 nm. The structure of the TNV CP is studied at a resolution of 2.25 Å (Oda et al., 2000). The outer diameter of the CP is 280Å and with an average thickness of 34 Å. The TNV CPs are organized according to icosahedral T=3 symmetry and form the capsid (virion). The capsid is composed of 60 trimers (asymmetric unit) with 3 subunits, A, B and C. Similar to other icosahedral viruses; the TNV coat protein has a jelly role motif. Residues 88-276 forming two β-sheets; each sheet is composed of four anti-parallel β-strands. Residues 120-126 form the longest α-helix. The N-terminal 56 amino acids are rich in basic residues, as 13 out of the 56 residues are Arg and Lys (Oda et al., 2000).

In each icosahedral asymmetric unit five Calcium (Ca^{2+}) ions were presented. Three of these were located at A/B, B/C and C/A interfaces and coordinated by five O_2 atoms. Four out of five O_2 atoms were provided by D 275, T 219, D 163 & D 160, the fifth O_2 atom was from a molecule of water. The rest of the Ca^{2+} ions were located inside chains A and C. These two calcium were coordinated by five O_2 atoms that four of those were provided by S 94, D 93, T 175, S 173 and the fifth atom was donated by a molecule of water. Calcium ions stabilize the capsid by binding to O_2 atoms (Oda et al., 2000).

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1.9.3. The PMV CP

The crystallographic structure of PMV was determined at 2.9Å resolution. As with other viruses in the *Tombusviridae* family, the PMV capsid structure is organized according to T=3 icosahedral symmetry (Makino et al., 2013). It was determined that PMV CPs form trimers composed of A, B and C subunits (Makino et al., 2013). A difference between subunits can be seen in the N-terminus. Subunits A and B have a disordered N-terminus, whereas in subunit C, the N-terminus is ordered. The motif is a jellyroll formed from eight parallel β-strands. The N-terminus in the PMV CP does consists of 36 amino acid residues, of which 30% are positively charged. This part (N-terminus) had a disordered structure in A and B. The PMV CP has 242 amino acids, which is 33 residues less than the TNV coat protein. The percentage identity is close to 20%; however both TNV and PMV have similar secondary and tertiary structures (Makino et al., 2013).

1.10. Statement of Research Problem. The goal of my thesis is to identify the features of *tombusvirus* coat proteins that enable recognition by R proteins in *Nicotiana* species that belong to section *Alatae*.

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CHAPTER 2

1- INTRODUCTION

For more than forty years, the gene-for-gene theory has provided a basic framework for understanding how plants recognize and defend against plant pathogens (Flor, 1971). Working with flax and the fungal pathogen *Melamspora lini*, Flor postulated that plants contain single dominant resistance (*R*) genes and pathogens contain a series of Avirulence (Avr). Plant defenses are mobilized when the pathogen Avr gene product is recognized by the host R protein. The manner in which plant defenses are activated can take many forms, but one of the most common reactions is called the hypersensitive response (HR). In a HR triggered by a plant virus infection, the virus is limited to necrotic primary lesions that form in the inoculated leaf (Schoelz et al., 2006).

The genus *Nicotiana* has been especially valuable for studying the factors that determine systemic virus infections vs. HR. The genus contains 76 species which are organized into 13 taxonomic sections (Knapp et al., 2004). Some species, such as *N. glutinosa* and its close relative *N. edwardsonii* have played key roles in characterizing the application of the gene-for-gene theory to viruses. For example, the first Avr-determinant identified by recombinant DNA techniques was the P6 protein of *Cauliflower mosaic virus*, which elicits a plant defense response in both species (Schoelz et al., 1986). On the host side, the *N* gene, an R gene derived from *N. glutinosa* that recognizes *Tobacco mosaic virus* (TMV), was one of the first R genes to be discovered and cloned (Holmes, 1938; Whitham et al., 1994). Another important species in the genus is *N. benthamiana,* which serves as a model plant in plant virology. *N. benthamiana* is readily infected by a wide variety of plant viruses, and also is frequently

used in agroinfiltration assays for transient expression assays of foreign genes (Goodin et al., 2008).

Previous studies had illustrated that the genus *Nicotiana* could be valuable as a source for R genes that target viruses in the *Tombusviridae*, as a majority of *Nicotiana* species that were tested responded with HR to *Tomato bushy stunt virus* (TBSV) and *Tobacco necrosis virus* strain DH (TNV-D^H) (Angel and Schoelz, 2013; Rodriguez, 2014). Although several *Nicotiana* species reacted to inoculations of TBSV and TNV-D^H with HR, further investigations showed that the species were recognizing distinctly different viral Avr products. For example, the TBSV P22 protein triggered HR in *N. edwardsonii*, *N. glutinosa* and *N. forgetiana*, whereas the TBSV P19 protein triggered HR in *N. sylvestris*, *N. tabacum*, and *N. bonariensis* (Angel et al., 2011; Scholthof et al., 1995). The TNV-D^H Avr determinant in those *Nicotiana* species has not yet been identified, in part because TNV-D^H does not encode proteins analogous to P19 and P22. By contrast, the TBSV and TNV-D^H CPs triggered HR in several species in section *Alatae*, including *N. langsdorffii, N. longiflora, N. forgetiana, N. alata,* and *N. bonariensis* (Angel and Schoelz, 2013; Rodriguez, 2014).

In present study I utilized an agroinfiltration assay to investigate the structural features of *Tombusviridae* CPs that contribute to their recognition by *Nicotiana* species in section *Alatae*. The *Tombusviridae* consists of eight genera. I obtained the CP genes from six of the genera and evaluated their capacity to elicit HR in species of section *Alatae*. This dataset allowed me to narrow potential CP sequences that contribute to recognition to two amino acids and their role in HR elicitation was confirmed by point mutagenesis.

2- RESULTS

2-1. A sequence alignment of the TBSV and TNV-D^{H} CPs reveals conservation at 54 amino acid positions.

Previous studies had shown that the CPs of TBSV and TNV-D^{H} elicit HR in *Nicotiana* species that belong to section *Alatae* (Angel and Schoelz, 2013; Rodriguez, 2014). One hypothesis that might explain why the CPs of TNV-D^{H} and TBSV elicit HR in members of *Nicotiana* section *Alatae* is that their primary amino acid sequences have a common motif that might be recognized by a host R protein. To identify sequences in common within the two tombusvirus CPs, CLUSTAL W2 was used to align their CP sequences, and this analysis revealed 54 fully conserved amino acids (Fig. 2.1). However, a deletion analyses of the N-terminus of the two CPs had earlier shown that the first 77 amino acids of the TNV-D^{H} CP and the first 101 amino acids of the TBSV CP could be eliminated from consideration (Fig. 2.1), as these truncated CP sequences were also capable of eliciting HR in the *Alatae* species. Consequently, the N-terminal deletion analysis indicated that the number of conserved amino acids in the CP that could be responsible for triggering HR could be narrowed from 54 to 40 (Fig. 2.1).

To determine whether any amino acids on the C-terminus of the TNV-D^{H} CP could be eliminated without affecting HR, I used PCR to introduce C-terminal deletions into pATG4, a construct that had been shown previously to elicit HR in several *Nicotiana* species including *N. langsdorffii* (Fig. 1.2, Rodriguez, 2014). The construct pATG4Δ54 contained a 162 bp deletion on the 3' end of the TNV-D^{H} CP gene, which resulted in a deletion of 54 codons (Fig. 2.2).

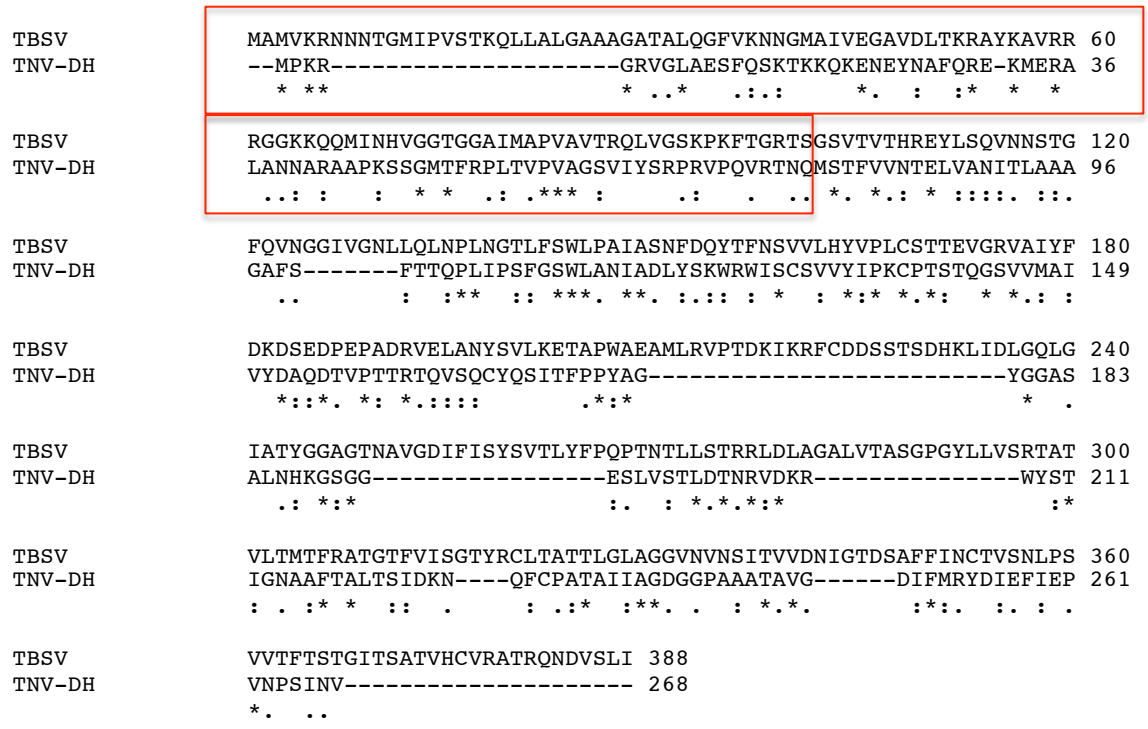


Figure 2.1. TBSV CP and TNV-D^{H} CP sequence alignment. Fully conserved residues are indicated by the (*) symbol. : Conservation between residues with strong similarities indicated by the (:) symbol. Conservation of residues with weak similarities is indicated by (.) symbol. The red box shows the residues from both sequences that were not involved in triggering HR.

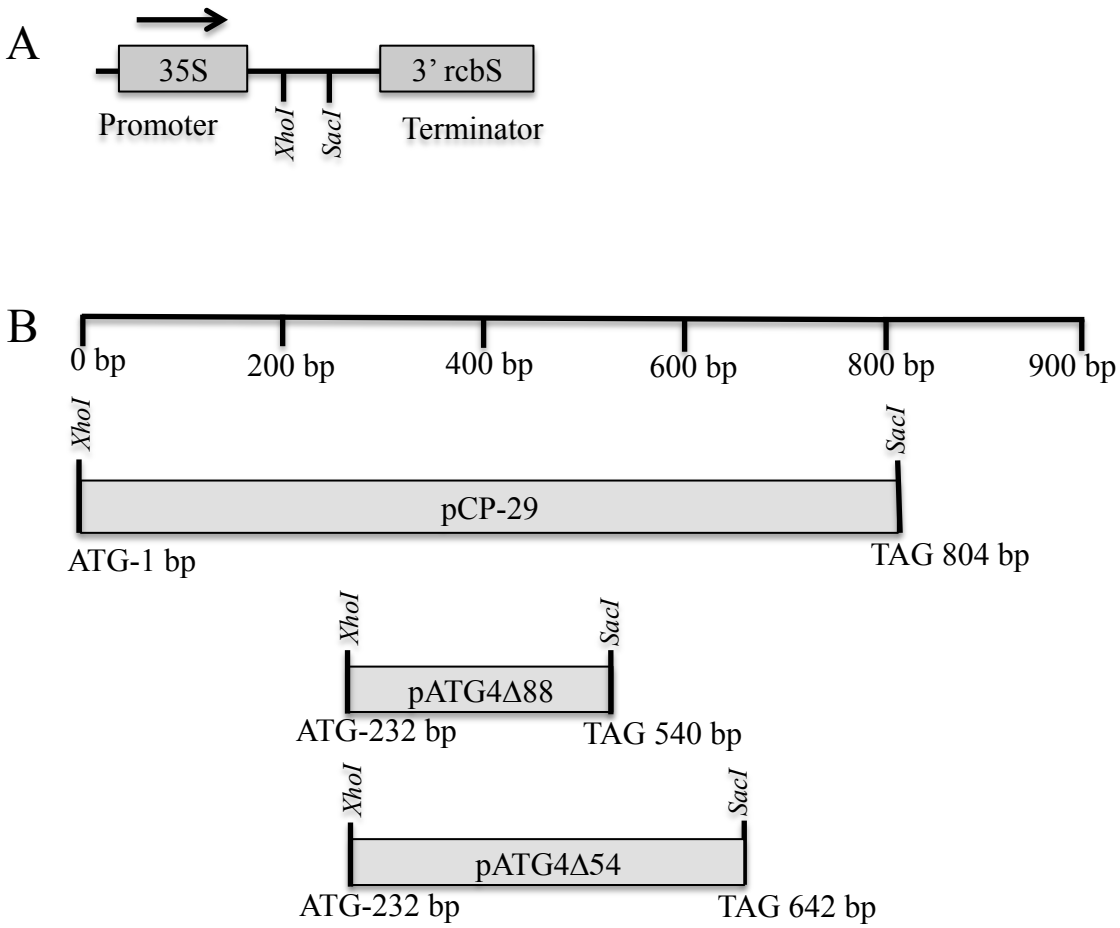


Figure 2.2. TNV-D^{H} CP C-terminal deletion constructs. A) Multiclone site for the binary vector pKYLX7. All CP constructs were inserted into the *Xho*I and *Sac*I sites, placing them under control of the CaMV 35S promoter and *rcb*S terminator. B) Structure of C-terminal TNV-D^{H} CP deletion mutants. The pATG4Δ124 construct contains a C-terminal deletion of 264 bp, or 88 codons. The pATG4Δ55 construct contains a deletion of 162 bp, or 54 codons. Both constructs contained a 231 bp deletion on the 5' end of the CP sequence. The sequenced deleted from the 5' end had previously been shown to have no influence on initiation of HR in *Nicotiana* species (Rodriguez 2014). Translation of both constructs was initiated by a methionine codon that begins at nucleotide 232.

The construct pATG4Δ88 contained a deletion of 264 bp on the 3' end of the TNV-D^H CP gene, which resulted in a deletion of 88 codons (Fig. 2.2). The two C-terminal deletion mutants were agroinfiltrated *N. langsdorffii*, *N. longiflora* and *N. plumbaginifolia* in order to determine their abilities to triggering HR. Each leaf was also agroinfiltrated with pCP-29 as a positive control and the empty vector pKYLX7 as a negative control. Three plants per species and three leaves per plant were used for the agroinfiltration. Neither pATG4Δ88 nor pATG4Δ54 could elicit HR, whereas pCP-29 elicited HR in *N. langsdorffii* and *N. longiflora* consistently (Fig. 2.3). None of the constructs elicited HR in *N. plumbaginifolia*, in agreement with previous results that showed that this was the one member of section *Alatae* that did not respond to agroinfiltration of the TNV-D^H CP with HR (Rodriguez, 2014).

2-2. Analyzing the potential of the coat proteins of viruses in *Tombusviridae* family to trigger HR in *Nicotiana* section *Alatae*.

The *Tombusviridae* consists of eight genera, and a phylogenetic analysis of the CPs of the type members for each genus reveals that TBSV and TNV CPs are only distantly related within the family (Fig. 2.4). Since both the TBSV and TNV-D^H CPs triggered HR in the same *Nicotiana* species, we hypothesized that other CPs in the family might also trigger HR in *Nicotiana* species belonging to section *Alatae*. To test this hypothesis, we obtained CP genes from representatives of the *Dianthovirus* (*Red clover necrotic mosaic virus* – RCNMV), *Carmovirus* (*Turnip crinkle virus* - TCV) *Machlomovirus* (*Maize chlorotic mottle virus* - MCMV), and *Panicovirus* (*Panicum mosaic virus* – PMV) genera.

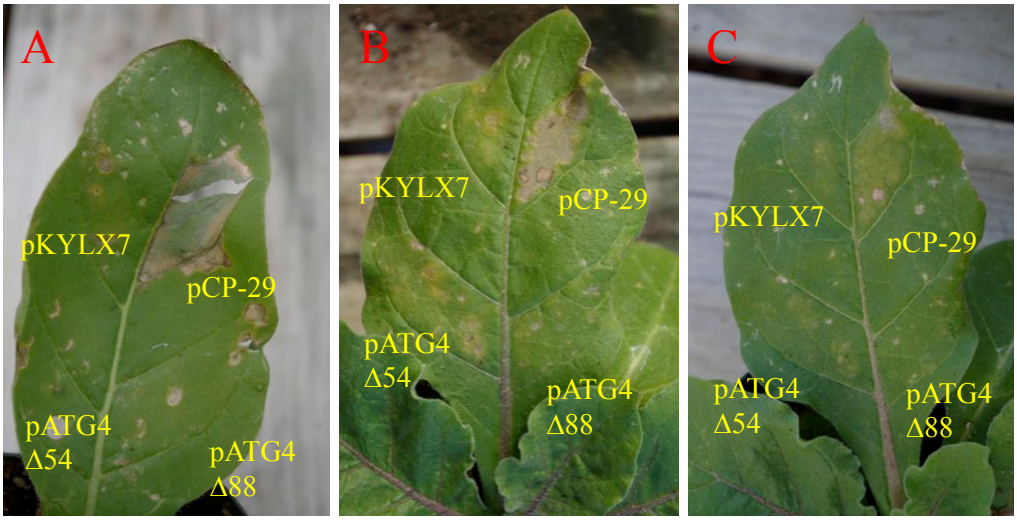


Figure 2.3. Agroinfiltration of TNV-D^{H} CP and C-terminal CP deletions. Panels A-C illustrate the response of *Nicotiana* species to agroinfiltration of TNV-D^{H} CP constructs and pKYLX7 at 7 dpi. A) *N. langsdorffii* B) *N. longiflora* C) *N. plumbaginifolia*

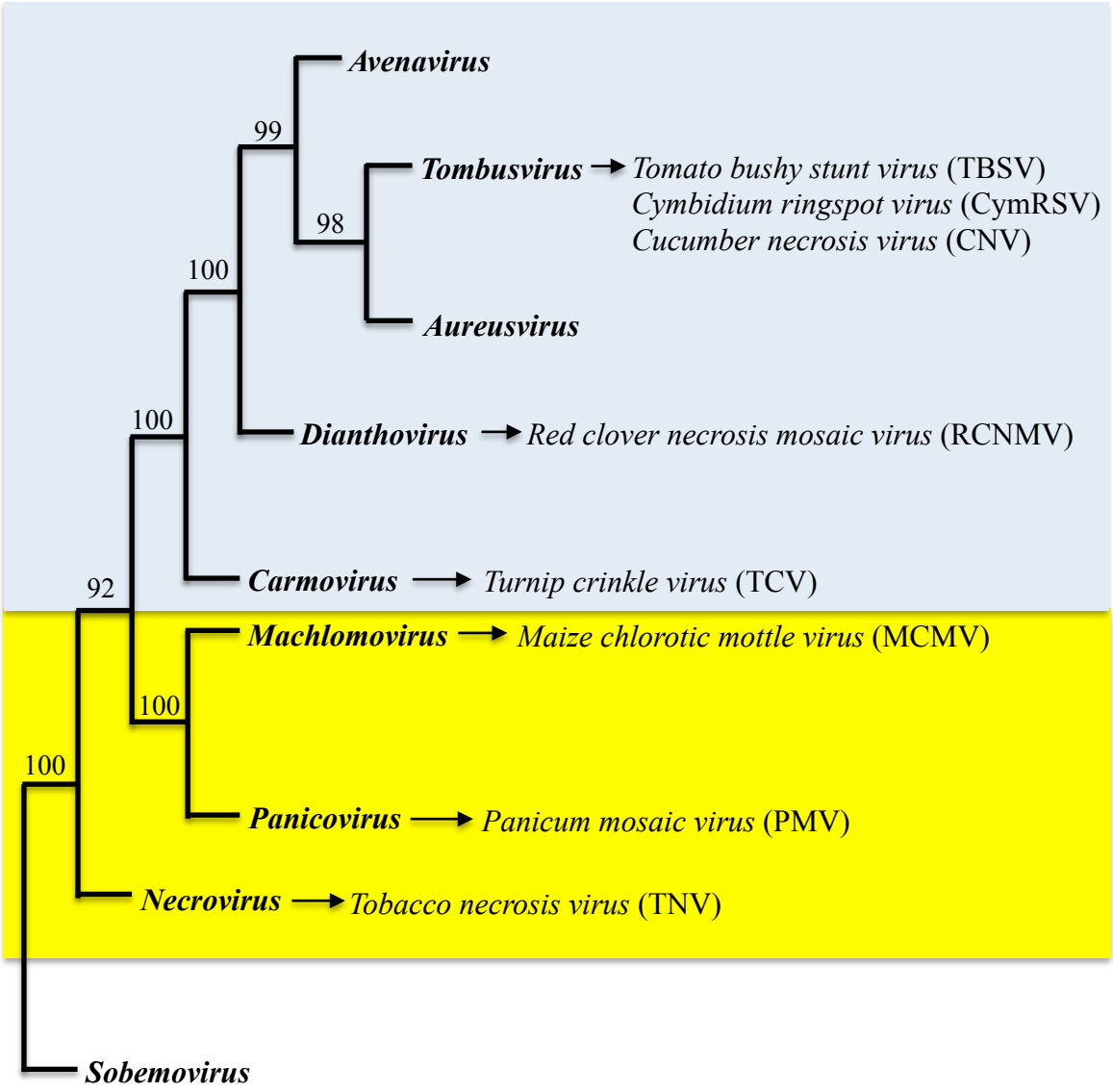


Figure 2.4. *Tombusviridae* Phylogenetic tree. The phylogenetic tree is based on the CPs of the *Tombusviridae* genera. The colored boxes indicate two morphological virion types in the this family. Genera in the blue box have a protruding domain in the CP sequence, whereas genera in the yellow box have a smooth surface. Bootstrap values are shown at the branch nodes in the tree. The *Sobemovirus* genus is not a member of the *Tombusviridae*. This figure is redrawn from Fauquet et al., 2005.

These virus clones were readily available from collaborators in the United States. In addition, we also included two other viruses from the *Tombusvirus* genus: *Cucumber necrosis virus* (CNV) and *Cymbidium ringspot virus* (CymRSV), as these viruses were already present in our collection. The CP sequences for CNV, CymRSV, PMV and MCMV were separately cloned into the Agrobacterium binary vector pKYLX7 (Schardl et al., 1987), the same binary vector used for expression of the TBSV and TNV-D^H CP sequences (Angel and Schoelz, 2013; Rodriguez, 2014). The structure of these clones is illustrated in Figure 2.5. By contrast, the CPs of TCV and RCNMV had already been cloned into *Agrobacterium* binary vectors.

Each of the CP constructs was agroinfiltrated into leaf panels of *Nicotiana* species as indicated in Table 2.1. I typically agroinfiltrated more than one construct into a leaf, but every leaf was also agroinfiltrated with the positive TNV-D^H control pCP-29 and the empty vector pKYLX7. The positive control ensured that a leaf was capable of responding with HR, whereas the empty vector allowed me to test for nonspecific necrosis associated with infiltration of *Agrobacterium*. In each test, a CP construct was agroinfiltrated into three plants and three leaves per plant, for a total of nine infiltrations. Each construct was evaluated in multiple tests. Plants were observed ten successive days after agroinfiltration and results were recorded.

Agroinfiltration tests for *N. langsdorffii* are illustrated in Figure 2.6. HR consistently was elicited in the leaf panel agroinfiltrated with the pCP-29, as well as leaf panels agroinfiltrated with RCNMV CP, CNV CP, and CymRV CP. No HR was observed in leaf panels agroinfiltrated with MCMV CP, TCV CP and PMV CP.

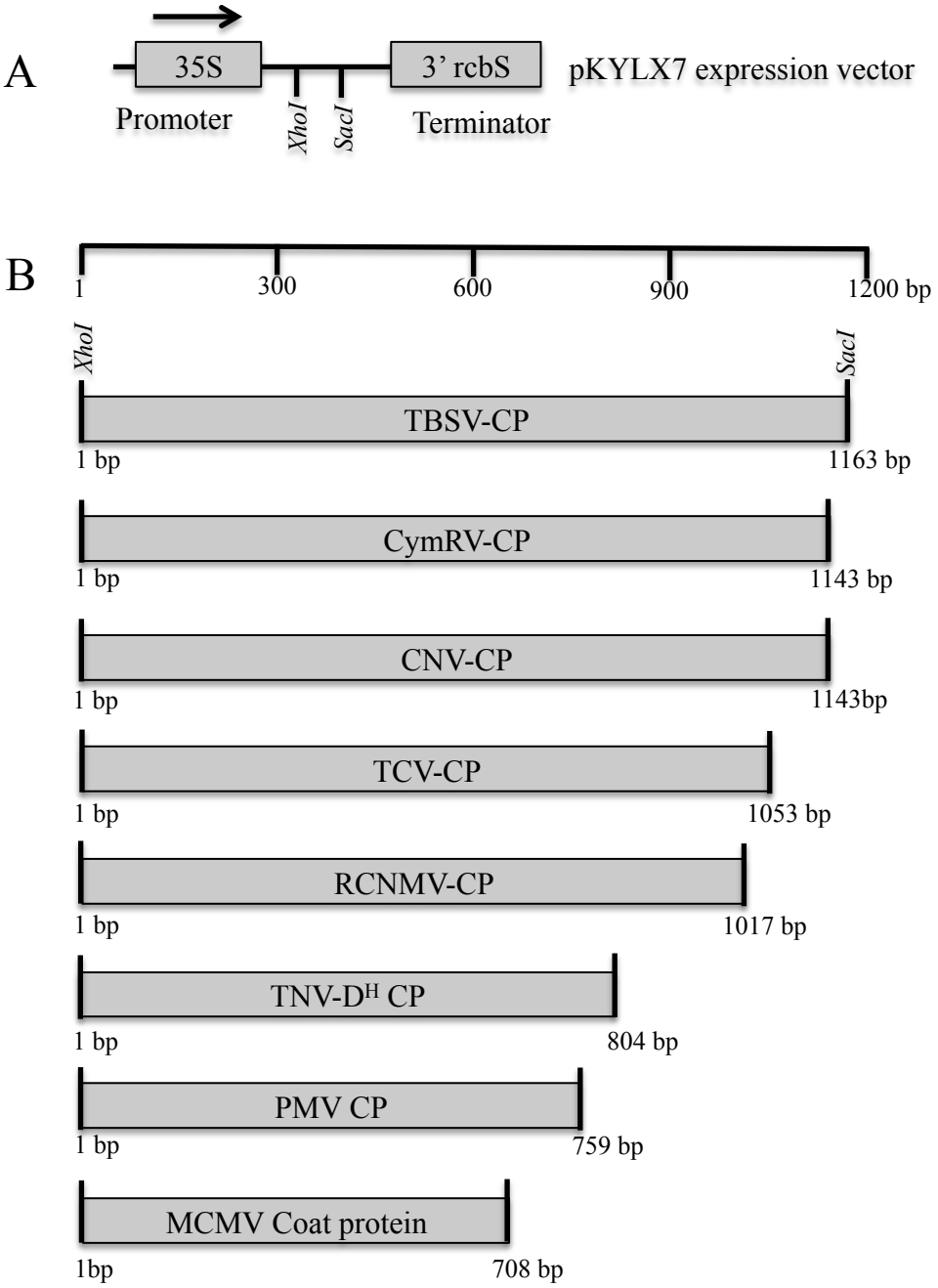


Figure 2.5. *Tombusviridae* coat protein constructs. A) Multiclone site for the binary vector pKYLX7. All CP constructs were inserted into the *Xho*I and *Sac*I sites, placing them under control of the CaMV 35S promoter and *rcb*S terminator. The only exceptions were the CPs of TCV and RCNMV, as they had already been cloned into other binary vectors B) The *Tombusviridae* coat protein constructs are arranged according to their lengths.

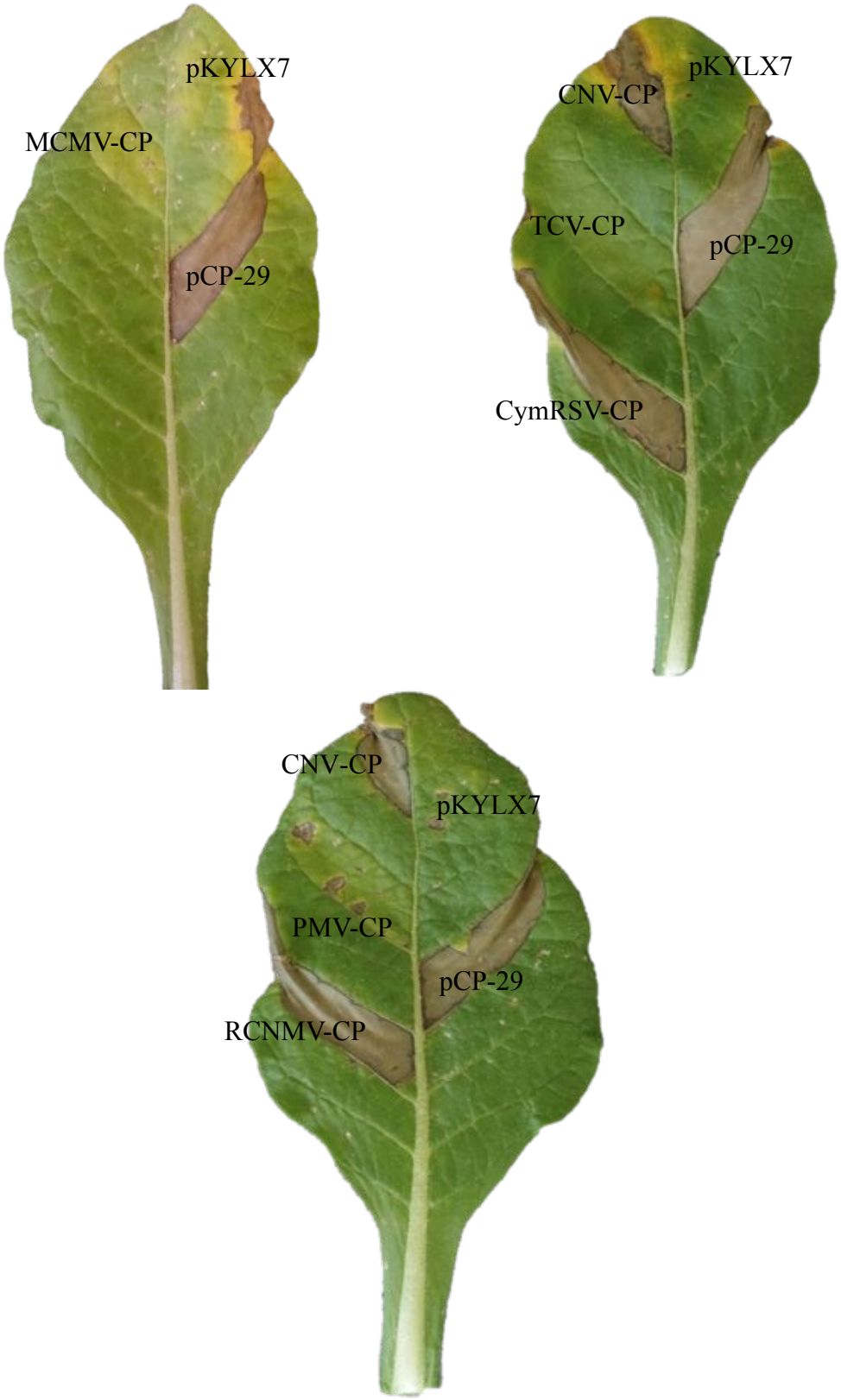


Figure 2.6. *Tombusviridae* coat proteins constructs agroinfiltrated into *N. langsdorffii at* 7dpi. The TNV-D^{H} CP and pKYLX7 were agroinfiltated into every leaf, as they showed that leaf had the capacity to respond with HR to an elicitor (pCP-29), but also would not react with nonspecific necrosis to Agroinfiltration of the empty vector (pKYLX7).

Table 2-1. Reaction of *Nicotiana* spp. to agroinfiltration of the *Tombusviridae* coat proteins

	HR appearance upon agroinfiltration						
Nicotiana spp.	TNV-D^{H}	CNV	CymRSV	RCNMV	TCV	MCMV	PMV
N. langsdorffii	HR	HR	HR	HR	No HR	No HR	No HR
N. longiflora	HR	HR	HR	HR	No HR	No HR	No HR
N. alata tw7	HR	HR	HR	HR	No HR	No HR	No HR
N. alata tw8	HR	HR	HR	HR	No HR	No HR	No HR
N. forgetiana tw50	HR	HR	HR	HR	No HR	No HR	No HR
N. forgetiana tw51	HR	HR	HR	HR	No HR	No HR	No HR
N. plumbaginifolia tw106	No HR	No HR	No HR	No HR	No HR	No HR	No HR
N. plumbaginifolia tw108	No HR	No HR	No HR	No HR	No HR	No HR	No HR

The results of all of the agroinfiltration tests are summarized in Table 2.1. In the case of the *Alatae* species, all were positive for HR except for *N.plumbaginifolia*. HR appeared after 2 to 3 dpi (days past infiltration) in *N.langsdorffii*, *N.forgetiana* and *N.alata* but in the case of *N.longiflora* it usually appeared after 4-6 dpi.

A ClustalW alignment of the eight CPs was used to identify potential HR determinants in the primary amino acid sequence. I sought to identify amino acids common to TNV-D^H, TBSV, CymRSV, CNV and RCNMV. To be considered a potential HR determinant, the CP sequences of TCV, MCMV and PMV must also encode a different amino acid at that site. Only two amino acids were present in the positives (TNV-D^H, TBSV, CNV, CymRV, RCNMV) and absent in the negatives (TCV, PMV, MCMV), at TNV-D^H positions D155 and R161 (Fig. 2.7). The Glycine at position 189 (G189) was eliminated from consideration because it was also present in PMV.

2-3. An exchange of CP amino acid residues between PMV and TNV-D^H fails to identify a potential HR determinant.

Sequence alignment with the ClustalW showed that only two amino acids, aspartate 155 and an arginine 161 distinguished the HR elicitors from non-elicitors (Fig. 2.7). To determine if these two residues in the TNV-D^H CP have a role in triggering HR, their codons were mutated, resulting in substitutions with serine and leucine, respectively (Fig. 2.8). However, upon agroinfiltration of the mutated TNV-D^H CP (D155S/R161L) into *N. longiflora* and *N. langsdorffii,* I found that the HR was not abolished (Fig 2.9).

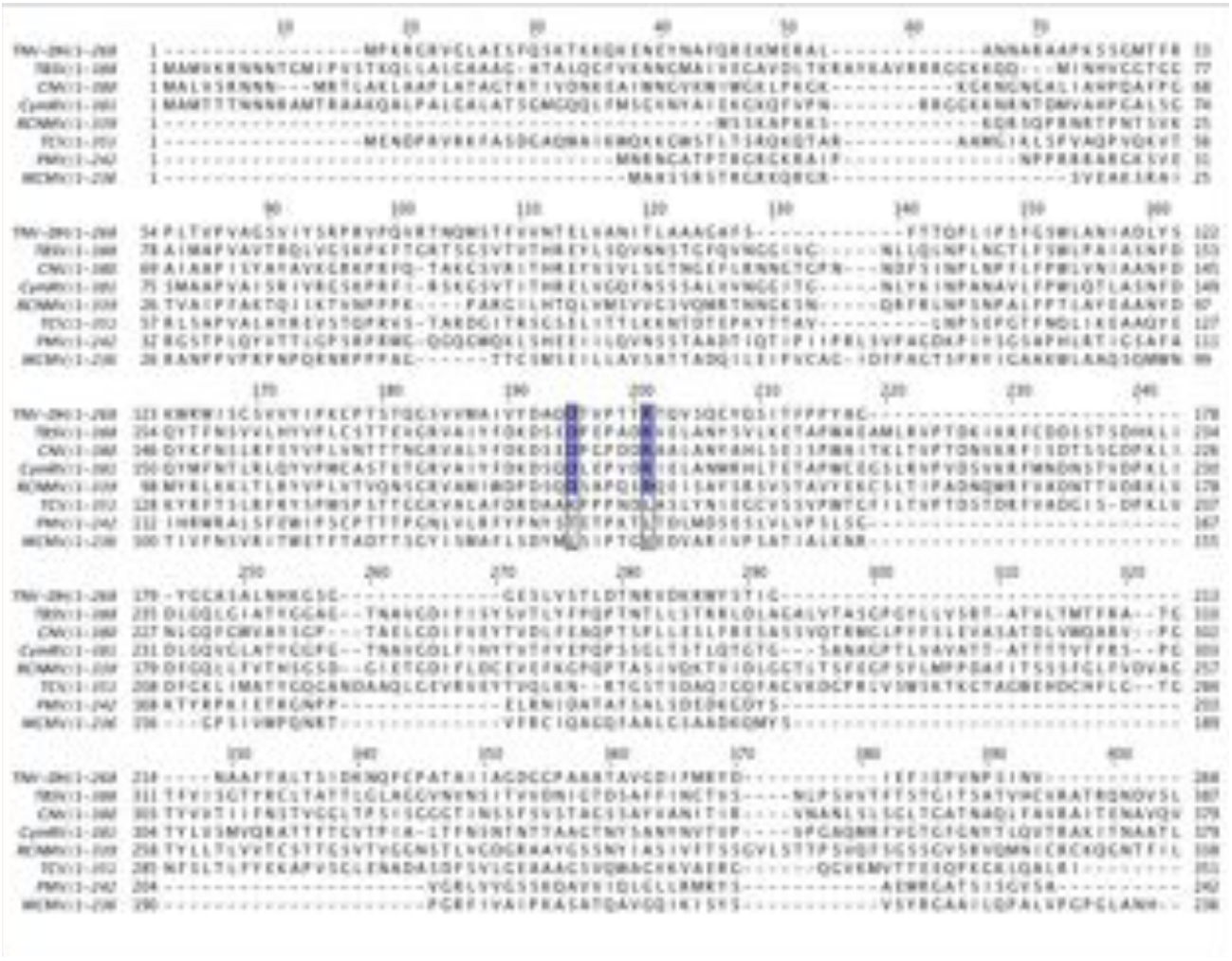


Figure 2.7. Sequence alignment of the *Tombusviridae* CPs. The colored boxes show the two conserved amino acids in the HR-elicitors, which distinguish them from the non-elicitors. These two amino acids were Asp155 and Arg161 in the TNV-D^{H} CP sequence.

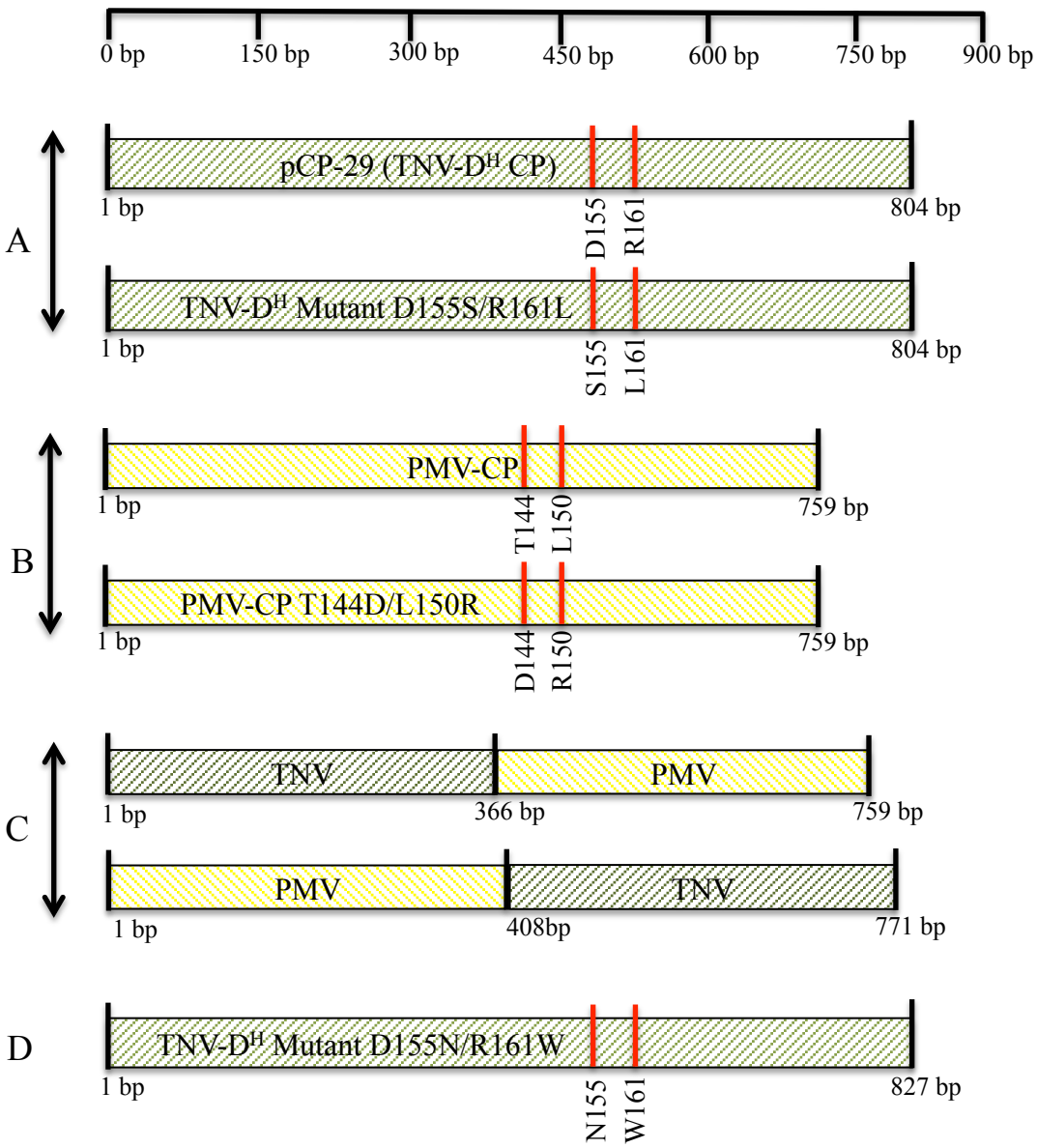


Figure 2.8. CP mutants and chimeric constructs. A) D155 and R161 distinguish HR-elicitors from non-elicitors. In the TNV-D^H CP sequence these two residues were mutated to S155 and L161, respectively. **B)** The PMV CP does not elicit HR in *Nicotiana* section *Alatae*. The residues T144 and L150 were aligned with D155 and R161 in the TNV-D^H CP sequence. These two residues were mutated to D144 and R150 in an attempt to convert the PMV CP into a form that would elicit HR. **C)** Chimeric protein constructs made between TNV-D^H and PMV CPs to determine if a specific secondary structure was responsible for triggering HR. The first construct (TNV-PMV) was predicted to be 759 bp in size and it inherited the N-terminus of TNV-D^H CP and the C-terminus from PMV CP. The construct PMV-TNV was predicted to be 827 bp, with the N-terminus from the PMV CP and the C-terminus from TNV-D^H. **D)** The residues D155 and R161 of TNV-D^H CP were mutated and this time D155 was substituted with N155, and R161 was changed to W161.

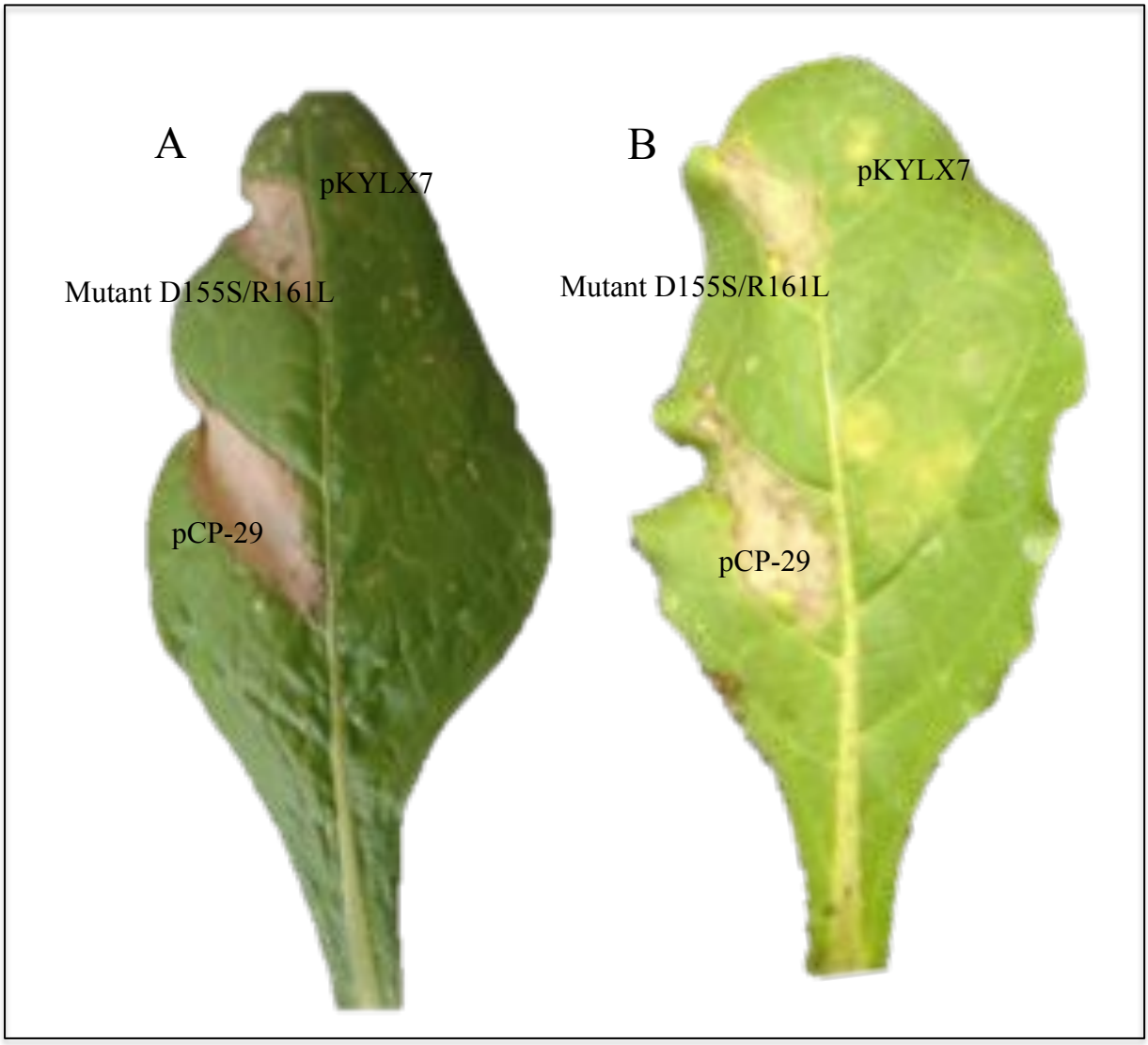


Figure 2.9. Agroinfiltration of TNV-D^{H} the CP mutant D155S/R161L into A) *N. langsdorffii* and B) *N. longiflora.* The TNV-D^{H} CP and pKYLX7 were agroinfiltated into every leaf, as they showed that that leaf had the capacity to respond with HR to an elicitor (pCP-29), but also would not react with nonspecific necrosis to Agroinfiltration of the empty vector (pKYLX7). The leaves were collected and photographed at 7 dpi.

A second CP construct was initiated to determine whether the PMV CP could be changed to elicit HR. A sequence alignment between PMV and TNV-D^H coat proteins revealed Asp155 and Arg161 of the TNV-D^H are in alignment with Thr144 and Leu150 in PMV. We used recombinant PCR to mutate Thr144 and Leu150 in PMV CP to Asp and Arg in determine whether we could convert the PMV-CP to a form that would elicit HR (Fig. 2.8). The PMV CP carrying the two mutations was transformed into *Agrobacterium tumefaciens* and agroinfiltrated into the *N. langsdorffii*. However, even after 10 days of observation, no HR was elicited by the PMV construct (Fig. 2.10). The results obtained with the PMV mutant indicated that changes at more than just these two amino acids would be necessary to convert the PMV CP into a structure that could be recognized by a putative R protein present in *Nicotiana* species.

2-4. Creation of chimeric CP sequences between PMV and TNV-D^H fails to identify a potential HR determinant.

Superimposition of the TNV-D^H and PMV CPs structures showed the absence of an arm in the PMV-CP structure (Fig. 2.11). This arm was a combination of an extended loop with an alpha helix and containing 15 residues from Tyr 179 to Gln 193. We hypothesized this arm may be the secondary structural feature that is recognized by the plants and consequently triggers HR. To test this hypothesis we utilized recombinant PCR to create two chimeric proteins between PMV and TNV-D^H.

The first construct consisted of the first 122 residues of the PMV-CP and the last 131 residues from TNV-D^H D CP. The reciprocal construct was composed of the first 136 residues from TNV-D^H CP and the last 121 residues from PMV-CP (Fig. 2.8).

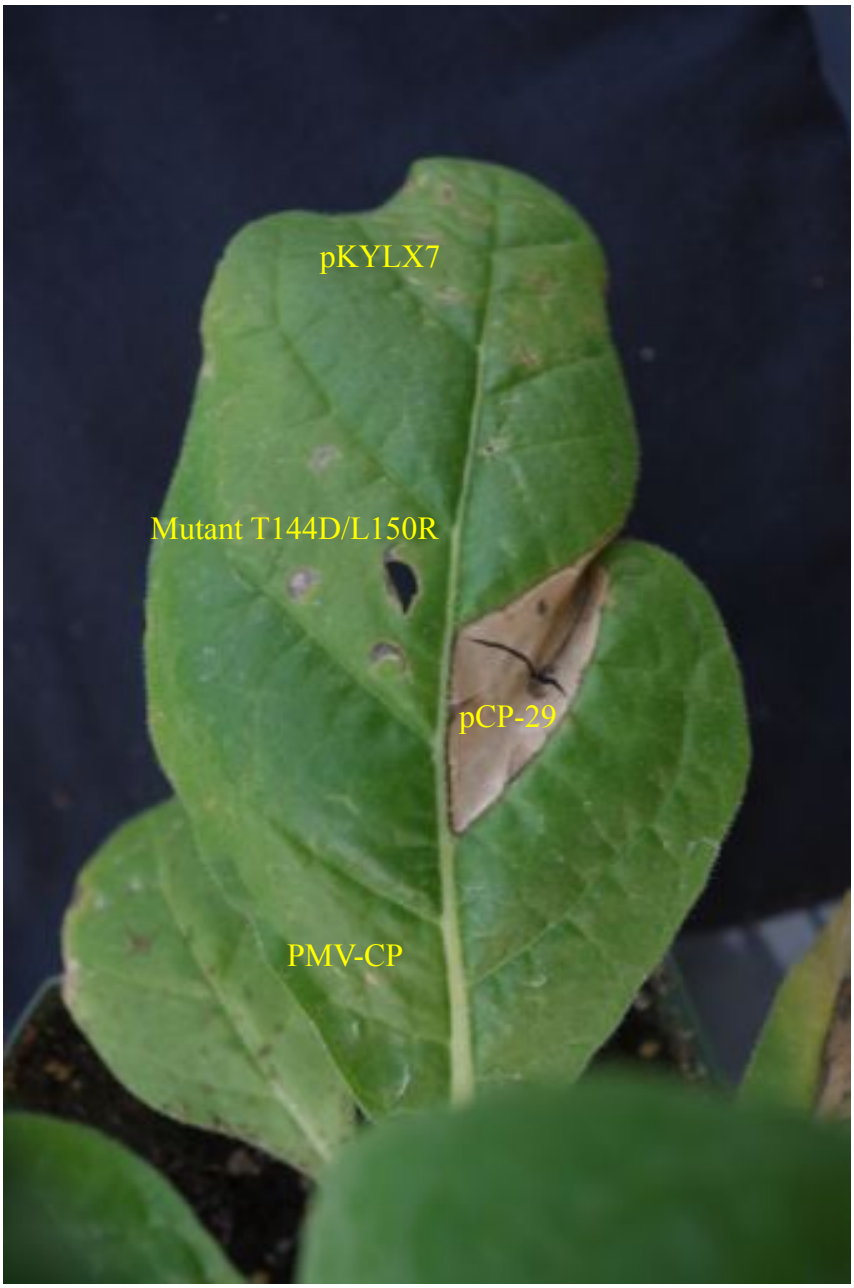
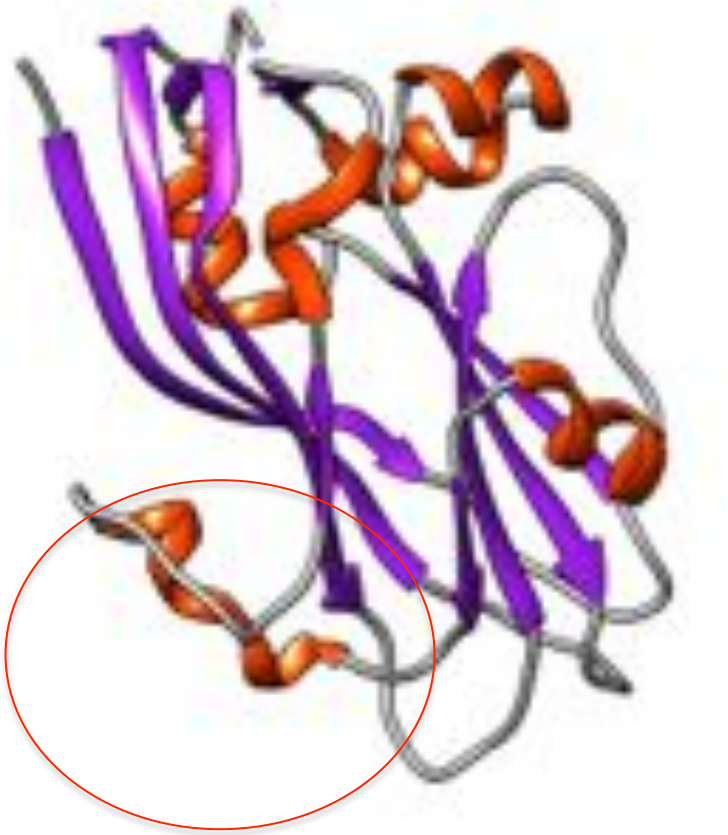


Figure 2.10. Agroinfiltration of the PMV CP mutant construct T144D/L150R into *N. langsdorffii*. The pCP-29 and pKYLX7 plasmids were included, as they showed that that leaf had the capacity to respond with HR to an elicitor (pCP-29), but also would not react with nonspecific necrosis to agroinfiltration of the empty vector (pKYLX7). The photograph was taken at 7 dpi.

TNV CP



PMV CP

Figure 2.11. Secondary structures of wild type TNV CP (1C8N) and PMV-CP (4FYA). TNV and PMV coat proteins share only 15.70 % of similarity but their 3D structure virtually is very similar. One structural difference that distinguishes the TNV CP from the PMV CP is highlighted in the TNV structure by a red circle.

We used MODELLER software package to determine and model the 3D structure of the chimeric proteins. This program uses sequence alignments and crystal structure of the template to make the homologous models for the targeted sequence (Gellert et al., 2006). We used PMV (4FYA) and TNV-D^H (1C8N) as templates for homology modeling. The template data were downloaded from the PDB Data Bank at http://www.pdb.org.

I constructed the chimeric CP genes (Fig. 2.8, TNV-PMV and PMV-TNV) and tested them by agroinfiltration into *N. langsdorffii*, along with the wild type TNV-D^H and pKYLX7. Although the wild type TNV-D^H CP readily elicited HR, I found that neither of the chimeric viruses was able to trigger HR (Fig 2.12). One possible explanation might be that one or both of the chimeric proteins was not expressed properly. To test this possibility, both of the chimeric constructs were agroinfiltrated into *N. benthamiana* leaves along with the pCP-29 (TNV-D^H CP) control. At 3dpi, total proteins were isolated from agroinfiltrated tissues, as well as from healthy, uninfiltrated tissues, and evaluated for expression of the CP construct by ELISA using the polyclonal antiserum directed against the TNV-D^H CP. I found that both the chimeric constructs could be detected by the TNV-D^H antiserum, as the level of expression was significantly higher than the healthy control tissue (Fig. 2.13). However, the wild type TNV-D^H CP was expressed at a much higher level than either of the chimeric constructs. I ultimately concluded that this approach would not be productive, because it would not be easy to determine if differences in CP expression levels observed in the ELISA truly reflected differences in expression or might be due to differences in detection by the TNV-D^H antiserum.

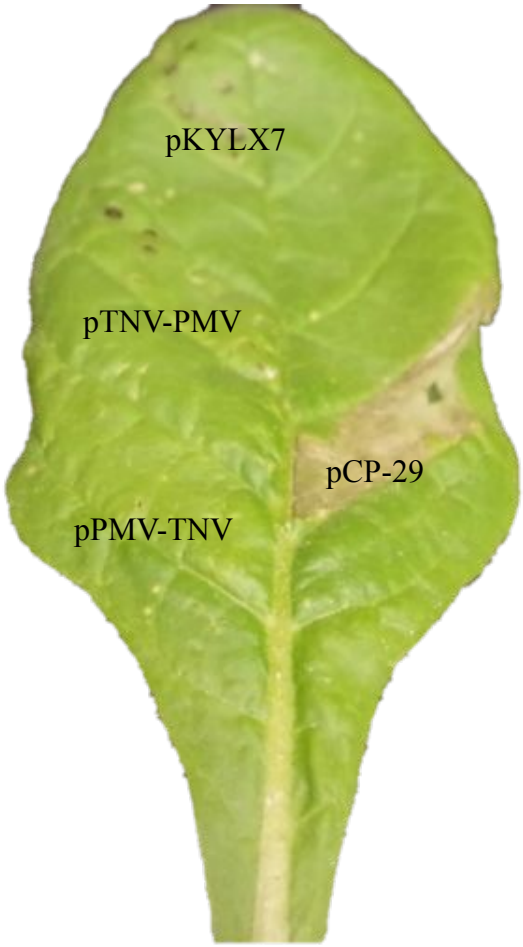


Figure 2.12. Agroinfiltration of the chimeric CP constructs into *N. langsdorffii.* The pCP-29 (TNV-D^H CP) and pKYLX7 plasmids were included, as they showed that that leaf had the capacity to respond with HR to an elicitor (pCP-29), but also would not react with nonspecific necrosis to agroinfiltration of the empty vector (pKYLX7). The photograph was taken at 7 dpi.

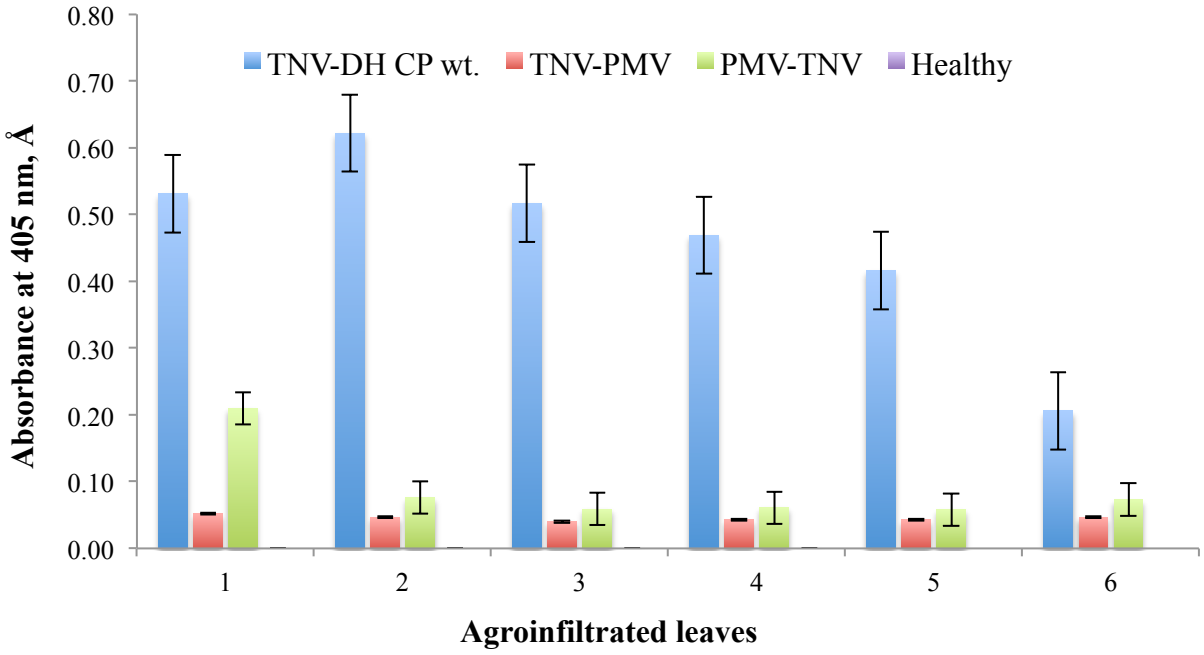


Figure 2.13. Expression of pCP-29 (TNV-D^{H} CP) and chimeric CPs in *N. benthamiana*. To determine the level of protein expression, polyclonal antisera to the TNV-D^{H} CP was used in an ELISA. *N. benthamiana* leaves were agroinfiltrated with all constructs, and leaves were harvested for the ELISA at 3 dpi. The error bars show standard error (SE). Statistical analysis showed there is significant differences in protein expression between TNV-D^{H} CP wt. and chimeric constructs.

2-5. A structural analysis of the TNV-D^H CP, coupled with mutations introduced at D155 and R161, indicates that these two amino acid positions have a role in HR elicitation.

To further investigate whether TNV-D^H residues D155 and R161 play a role in HR elicitation, I chose mutations that would be predicted to influence the interactions with surrounding amino acids. I nullified the negative charge of the Asp155 by mutating it into Asn, whereas the charged Arg161 was mutated to Trp. These mutations were chosen in the program Chimera (http://www.cgl.ucsf.edu/chimera). We used Chimera to have a better understanding of these mutations, and selected the Arg161 to Trp mutation by using the Rotamers program in the structural editing menu. For the Arg to Trp mutation, I used a structural minimization tool to identify Trp local interactions within the TNV-D^H structure. The software found 22 clashes/contacts between Trp161 and local residues. Then structure minimization provided by Molecular Modeling Toolkit in Chimera indicated that Trp would be unable to fit in the CP structure very well, and that this mutation might have a significant effect on the TNV-D^H function. The same procedure was performed to analyze the Asp155 to Asn mutation. The result showed one clashes/contacts between Asn 155 and local residues.

The codons D155 and R161 in the TNV-D^H CP were mutated to N155 and W161, respectively, and the mutant CP sequence (D155N/R161W) was recloned into pKYLX7 for transient expression assays in *N. langsdorffii* (Fig. 2.8). In this assay the wild type TNV-D^H CP and the empty vector pKYLX7 were included on every leaf as positive and negative controls. I found that the mutant TNV-D^H CP (D155N/R161W) was unable to elicit HR in *N. langsdorffii* (Fig. 2.14).

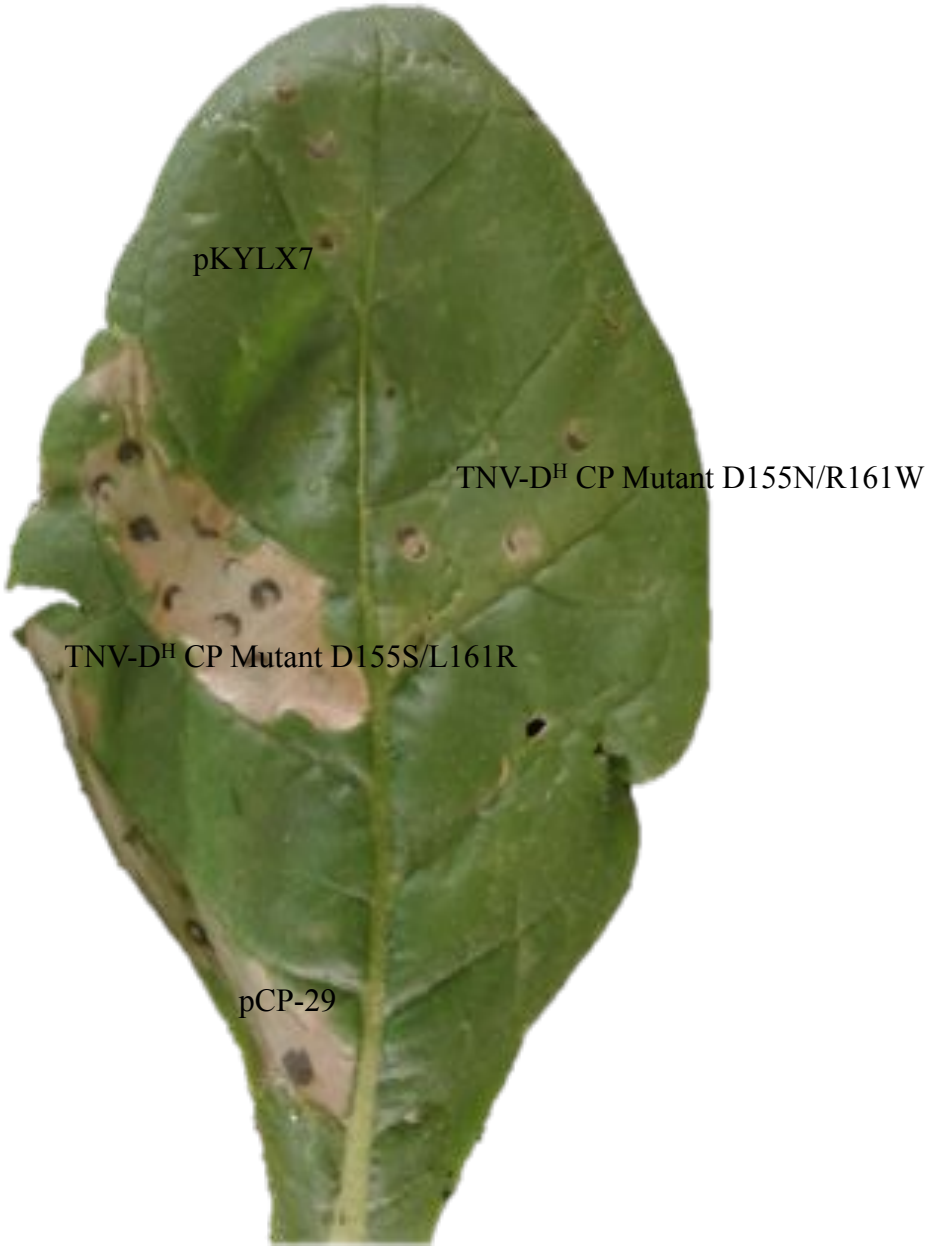


Figure 2.14. *N. langsdorffii* response to the agroinfiltration of A) pCP-29 (TNV-D^{H} CP) and TNV-D^{H} CPs containing point mutations. pKYLX7 empty vector was included as a negative control. As a result HR, was abolished when D and R mutated to N and W in the TNV-D^{H} CP. Leaves were detached and photographed at 7 dpi.

To determine if the mutant (D155N/R161W) was expressed, the construct was agroinfiltrated into *N. benthamiana* leaves, along with the wild type TNV-D^H CP and the TNV-D^H CP mutant D155S/R161L, a construct previously shown to elicit HR (Fig. 2.9). Agroinfiltration of these constructs into four *N. benthamiana* leaves showed that the TNV-D^H CP mutant D155N/R161W was expressed at a level comparable to wild type TNV-D^H CP and the TNV-D^H CP mutant D155S/R161L (Fig. 2.15). I concluded that mutations at residues 155 and 161 had the capacity to disrupt the interaction of the TNV-D^H CP with a putative R protein.The goal of my thesis was to identify the features of *Tombusviridae* coat proteins that enable recognition by putative R proteins in *Nicotiana* species that belong to the section *Alatae*. In this research we investigated the resistance exhibited by *Nicotiana* species section *Alatae* to the coat proteins of eight viruses of the *Tombusviridae* family. I used agroinfiltration to test the *Tombusviridae* coat protein constructs. Agroinfiltration is a reliable and rapid assessment technique, and has been used in several studies for identification of Avr determinants in plants (Angel et al., 2011; Angel and Schoelz, 2013; Erickson et al., 1999; Rodriguez, 2014). We found that the coat proteins of CymRSV, CNV and RCNMV triggered HR in the same species of plants as the TBSV and TNV-D^H CPs. However HR was not elicited by agroinfiltration of the CPs of TCV, MCMV and PMV. The combination of Tombusvirus CPs allowed us to rapidly narrow down potential HR determinants to just two amino acids, and point mutagenesis confirmed their involvement in triggering HR in *Nicotiana* species.

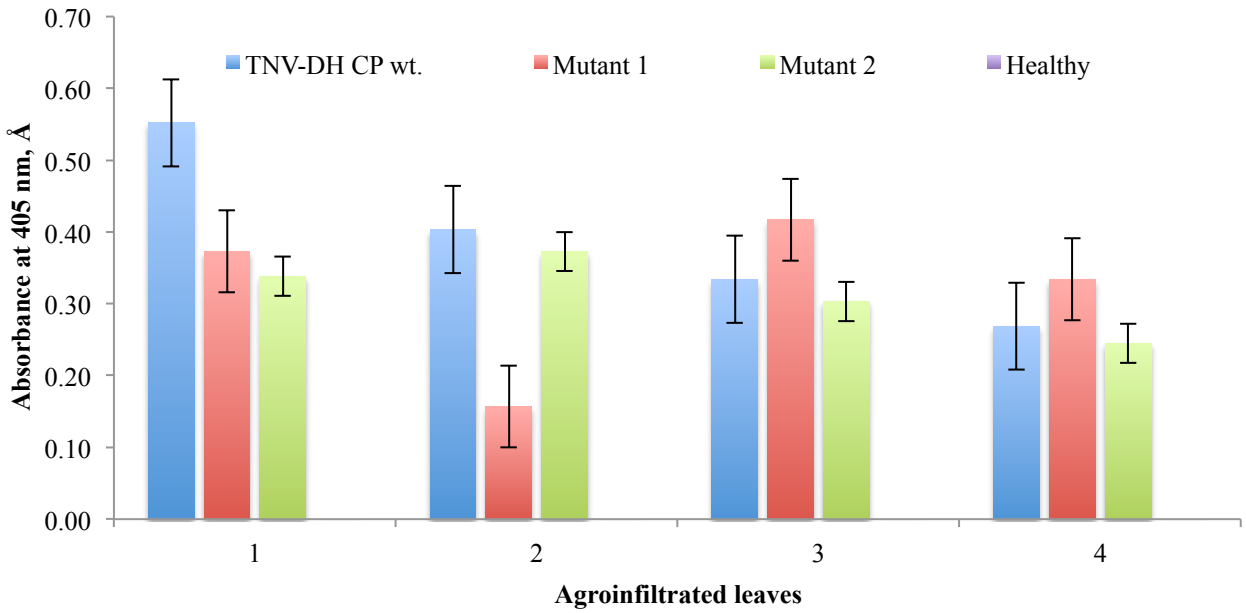


Figure 2.15. Expression of pCP-29 (TNV-D^{H} CP) and mutant CPs in *N. benthamiana*. To determine the level of protein expression, polyclonal antisera to the TNV-D^{H} CP was used in an ELISA. *N. benthamiana* leaves were agroinfiltrated with all constructs, and leaves were harvested for the ELISA at 3 dpi. Mutant 1 (TNV-D^{H} D155S/R161L) elicited HR in *N. longiflora* and *N. langsdorffii*, whereas Mutant 2 (D155N/R161W) did not. The statiscal analysis showed there is no significant differences between TNV-D^{H} CP wt. and mutants proteins expression.

3- Discussion

A previous study of Tobamoviruses showed that the CPs of *Tobacco mosaic virus* strain U1 (TMV-U1), *Tobacco mosaic virus* strain U2 (TMV-U2), *odontoglossum ringspot virus* (ORSV) and *cucumber green mottle mosaic virus* (CGMMV) could trigger HR in pepper. However the U1 and CGMMV CPs failed to elicited HR in tobacco and eggplant, respectively. Thus it is very likely that *R* protein in different plant species may recognize different CP structural features (Dardick and Culver, 1997). However, within a species, an R protein has the capacity to recognize structural features of a broad range of viral CPs within a genus. Taraporewala and Culver (1997) showed the importance of the helical bundle, specially the right face of the bundle of the Tobamovirus CP in host recognition and HR response by the *N'* protein of *N. tabacum*. This part was composed of a central hydrophobic cavity surrounded by charged and polar amino acids, an indication for a binding site (Taraporewala and Culver, 1997). They also showed the residues outside the active site could not contribute to HR (Taraporewala and Culver, 1997). Point mutations within the active site of the TMV CP could affect on coat protein capacity as HR-elicitor. For instance after the W17 and F48 amino acids of the hydrophobic core of the TMV CP mutant P20L (a mutant that triggers HR in *N. sylvestris*) were mutated, the elicitor activity was eliminated (Taraporewala and Culver, 1997).

Similarly, we found that the first 77 and 101 amino acids of the TNV-D^H and TBSV CPs were not involved in the HR (Angel and Schoelz, 2013; Rodriguez, 2014), suggesting that the N-terminus of the Tombusvirus CP does not contribute to HR elicitation. These results were the first indication that the CPs that elicited HR might have a common or specific structural feature that is not present in the non-HR elicitors. We

used a multiple sequence alignment approach to identify the common amino acids between the HR-elicitors that distinguished them from non-elicitors. In the sequence alignment, identical amino acids are expect to serve the same function in the proteins (Barton, 2001). We ultimately identified two amino acids in the TNV-D^H coat protein structure, which we hypothesized had a essential role in inducing HR in *N. langsdorffii*. We confirmed their involvement when the amino acids of the TNV-D^H CP at positions D155 and R161 were substituted to N155 and W161 and HR was abolished.

It has been shown that three dimensional structures of proteins in a family are more conserved than the primary structures (Terradot et al., 2001). In the case of the *Tobamovirus* CPs, HR-elicitors and non-elicitors had similar three dimensional structures (Culver, 2002). Similar to the Tobamoviruses, I found that there were no significant differences in the three dimensional structures of the CPs of the *Tombusviridae* HR-elicitors and non-elicitors showed that. For example, the sequences of the CPs of TNV-D^H and PMV have less than 20% identity but they had essentially the same three dimensional structures (Makino et al., 2013). However, I found that superimposition of PMV and TNV coat proteins structure showed an extra arm in the TNV structure, which was absent in the PMV CP structure. I initially hypothesized that this extra arm might be recognized by *Nicotiana* species to trigger HR, but neither of the chimeric constructs made between the CPs of TNV-D^H and PMV triggered HR in *N. langsdorffii*. An ELISA confirmed that the chimeric proteins were expressed upon agroinfiltration, but their expression levels were very low. It may be that in the process of creating the chimeras, we destabilized the protein structure such that it was either degraded or could not be recognized by the host R protein. For example, it had been shown that maintenance of the

protein's 3D structure was necessary for TMV CP and other Tobamovirus HR-elicitors (Dardick and Culver, 1997).

Having shown that residues D155 and R161 of the TNV-D^H CP contribute to HR in *N. langsdorffii*, we can now address whether their presence in other virus' CPs contribute elicitation. For example, we can mutate the same amino acids in the TBSV CP structure to find out if these mutations will abolish HR induced by the TBSV CP. If HR is abolished, then this would suggest that the same R protein that recognizes TNV-D^H CP also recognizes the TBSV CP. Similarly, we can test other species in section *Alatae* to see if the mutations at amino acids 155 and 161 abolish HR. If the answer is yes, then this would indicate that all the members of the section *Alatae* with the exemption of *N. plumbaginifolia* have inherited the same R protein. Finally, we can incorporate the mutant CP into infectious clones of TNV-D^H and TBSV to determine if they could now evade host defenses in section *Alatae*.

The research on viral CP elicitors provides a unique opportunity to examine how the three dimensional structure of a protein functions as an elicitor of host plant defenses. This work may ultimately contribute to a greater understanding for how host R proteins are able to discriminate between the different pathogen effector proteins they encounter.

4- METHODS

4-1. Coat protein constructs

The CP constructs for TNV-D^H and TBSV were described previously (Angel et al., 2011; Rodriguez, 2014). In both constructs the CP coding sequence was cloned into the binary Agrobacterium vector pKYLX7 (Schardl et al., 1987). The full-length clones of CymRSV and CNV a gift from Dr. Herman Scholthof (Texas A&M University). We

had previously used these virus clones to amplify the p19 and p22 genes (Angel et al., 2011; Angel and Schoelz, 2013). The nucleotide sequence of the CymRSV clone was derived from the infectious clone of Grieco et al. (1989; NCBI accession number X15511). The nucleotide sequence of CNV was derived from the infectious, clone of Rochon and Tremaine (1989; NCBI accession number M25270). The TNV strain DH clone was a gift from Dr. Lorant Kiraly (Hungarian Academy of Sciences); the infectious clone was developed by Molnár and coworkers (1997; NCBI accession number U62546). The MCMV clone was a gift from Dr. Kay Scheets (Oklahoma State University); its nucleotide sequence is described in Nutter et al. (1989; NCBI accession number X14736). The PMV clone was a gift from Dr. Karen Scholthof (Texas A&M University); its nucleotide sequence is described in Turina et al. (1998; NCBI accession number U55002).

The CP clones for RCNMV and TCV were given to us in *Agrobacterium* binary vectors. The RCNMV CP clone was a gift from Dr. Tim Sit (North Carolina State University). The RCNMV CP coding sequence (Xiong et al., 1993; NCBI accession number J04357) was provided to us cloned into the *Agrobacterium* binary vector pPZP212 (Hajdukiewicz et al., 1994). The TCV CP clone was a gift from Dr. Feng Qu (The Ohio State University). The cloning of the TCV CP sequence (Carrington et al., 1989: NCBI accession number M22445) into the *agrobacterium* binary vector is described in Qu et al. (2002).

Plasmid DNAs were purified as described in Sambrook et al., (1989). The purified plasmids of CymRSV, CNV, PMV and MCMV were used as templates for amplification of the CP gene by PCR. The forward and reverse primers for amplification

of CP sequences are described in (Table 2.2). The restriction enzymes *XhoI* and *SacI* were added to the forward and reverse primers, respectively, to facilitate a later cloning step into the agrobacterium vector pKYLX7.

CP sequences amplified by PCR were initially cloned into plasmid pGEM-T easy (Promega, Madison WI), as described by the supplier. The ligation mixture was incubated at 4°C overnight and then transformed into *E. coli* JM101 competent cells and transformed cells were selected on Luria-Bertani (LB) media supplemented with ampicillin (100 µg/ml). The nucleotide sequences of all CP clones were determined at the DNA Core Facility at the University of Missouri and confirmed to match the published sequence. The CP sequences were subsequently cloned into the *Xho*I–*Sac*I sites of the agrobacterium binary vector pKYLX7 and transformants selected on LB media containing kanamycin (50 µg/ml) and tetracycline (12.5 µg/ml). Plasmids of pKYLX7 were transformed into *A. tumefaciens* strain AGL1 by electroporation as described in Angel and Schoelz (2013).

4-2. Plants

Nicotiana spp. Seeds were planted and grown in the greenhouse. The accessions for all *Nicotiana* species are described in Angel and Schoelz (2013). After 4 weeks individual seedlings were transformed into the pots. Agroinfiltration was performed 2 to 3 weeks after transplanting and plants were utilized for agroinfiltration up to the point of flowering.

Table 2-2. Designed primers for the *Tombusviridae* coat protein expression by PCR

Construct	Primer sequence
MCMV-CP: Fwd. HindIII	5'- GGG AAG CTT ATG GCG GCA AGT AGC CGG TCT- 3'
MCMV-CP: Rev. SacI	5'- GGG GAG CTC TCA ATG ATT TGC CAG CCC TGG- 3'
PMV-CP: Fwd. XhoI	5'- GGG CTC GAG ATG AAT CGC AAT GGA GCT ACC- 3'
PMV-CP: Rev. SacI	5'- GGG GAG CTC TTA TGC GCT AAC CCC ACT GAT- 3'
CymRV-CP: Fwd. XhoI	5'- GGG CTC GAG ATG GCA ATG ACA ACT ACC ACC- 3'
CymRV-CP: Rev. SacI	5'- GGG GAG CTC CTA CAG AAG TGT TGC AGC ATT- 3'
CNV-CP: Fwd. XhoI	5'- GGG CTC GAG ATG GCA CTC GTA AGC AGG ACC- 3'
CNV-CP: Rev. SacI	5'- GGG GAG CTC TTA CAC AAC CTG CAC CGC ATT- 3'

4-3. Agroinfiltration

Three days prior to agroinfiltration, a single colony of *Agrobacterium* containing the desired construct, was transferred to 3 ml LB medium supplemented with kanamycin (50 µg/ml) and then incubated at 27° C for at 24 hrs. A 100 µl aliquot was transferred to 40 ml LB medium containing kanamycin and was grown overnight. Bacterial cells were pelleted by centrifugation and resuspended into 20 ml agroinfiltration media (100 ml agroinfiltration media has MES 0.39 g; sucrose 2 g, glucose 1 g; pH 5.6) supplemented with acetosyringone to a final concentration of 2 mM. The cells were subsequently incubated at 27°C for 8 hours and the OD_{600} adjusted to 1Å before agroinfiltration into individual leaf panels of *Nicotiana* leaves. In each experiment four plants per species and three leaves per plant were infiltrated. Plants were monitored daily for 10 successive days for their reaction to the coat protein.

4-4. Recombinant PCR

Recombinant PCR was used to create chimeric CPs between TNV-D^H and PMV and to make substitutions in residues D155 and R161. Primers used for recombinant PCR are shown in Table 2.3. Primary PCR was performed under standard PCR conditions. The PCR product was gel isolated by using a QIA quick gel extraction kit (Qiagen, Maryland) and purified DNA of each reaction was added along with the primers for secondary PCR. The conditions for secondary PCR are described in (Table 2.4). The secondary PCR product was cloned into the pGEM-T easy vector, sequenced and subsequently cloned into pKYLX7 expression vector, as described in previous sections.

Table 2-3. Designed primers for the TNV-D^H CP mutants, PMV-CP mutant and chimeric constructs

Construct	Primer sequence
TNV-D^H155S/R161L.Fwd.	5'-ATG GAT GCA CAG ACC ACT GTA CCC ACC ACT CTG ACC CAG-3'
TNV-D^H155S/R161L.Rev.	5'-TCA CTG GGT CAG AGT GGT GGG TAC AGT GGA CTG TGC ATC-3'
TNV-D^H155N/R161W.Fwd.	5'-GAT GCA CAG AAC ACT GTA CCC ACC ACT TGG ACC CAG-3'
TNV-D^H155N/R161W.Rev.	5'-CTG GGT CCA AGT GGT GGG TAC AGT GTT CTG TGC ATC-3'
PMV-T144D/L161R.Fwd.	5'-AAC TAC AGC GAC GAA ACA CCT AAG ACC CGC ACA GAC-3'
PMV-T144D/L161R.Rev.	5'-GTC TGT GCG GGT CTT AGG TGT TTC GTC GCT GTA GTT-3'
PMV-TNV chimeric Fwd.	5'-ATC CCC AGC TGT CCT ACG ACA ACT CAA GGG AGT GTG GTT ATG-3'
PMV-TNV chimeric Rev.	5'-CAT AAC CAC ACT CCC TTG AGT TGT CGT AGG ACT GCT GGG GAT-3'
TNV-PMV chimeric Fwd.	5'-ATC CCC AAA TGT CCC ACT TCC ACA CCG GGA AAT CTG GTT TTG-3'
PMV-TNV chimeric Rev.	5'-CAA AAC CAG ATT TCC CGG TGT GGA AGT GGG ACA TTT GGG GAT-3'

Table 2.4. Secondary PCR settings for the recombinant PCR

Temperature	Time (minutes)	Number of Cycles
94° C	3	
37° C	2	1
72° C	3	
94° C	1	
37° C	1	11
72° C	3	
94° C	1	
60° C	2	17
72° C	3	
94° C	1	
60° C	2	1
72° C	10	

4-5. Enzyme-linked immunosorbent assay (ELISA)

DAS-ELISA (Clark and Adams, 1977) was used to determine the level of CP expression. The desired constructs were agroinfiltrated into *N. benthamiana*. Primary antibodies and alkaline phosphatase conjugates were purchased from Agdia (Elkhart, IN). At 3 dpi, agroinfiltrated tissues were harvested, weighed and ground at a ratio of 1:10 tissue/grinding buffer. The grinding buffer was 1X PBS-Tween, with the addition of 2% (v/w) polyvinylpyrrolidone (MW 40,000) and 0.2% (v/w) bovine serum albumin (BSA). Conditions for the ELISA were according to Agdia. Colorimetric reactions with the substrate (p-nitrophenyl phosphate) were measured at 405 nm using a Multiskan MCC/340 micro-plate reader (Thermo Fisher Scientific, Cincinnati, OH).

4-6. Protein homology modeling

The templates for homology modeling (Table 2.5) was obtained and downloaded from protein data bank website (http://www.pdb.org/pdb/home/home.do). The 3D models were built by using basic modeling of the Modeller software (http://salilab.org/modeller). The basic modeling uses only one template to make its homologues model. However advanced modeling was used to build TNV-PMV and PMV-TNV CPs chimeric constructs. Advanced modeling enabled us to use two templates to build the chimeric models (http://salilab.org/modeller/tutorial/).

Table 2.5. The used PDB templates for homology modeling

Coat protein	Template	Template PDB code	Query cover (%)	Identity (%)
TNV-D^H	*Tobacco necrosis virus*	1C8N-A	92	47
CymRSV	*Tomato bushy stunt virus*	2TBV-A	99	46
RCNMV	*Melon necrotic spot virus*	2ZAH-A	82	38
MCMV	*Panicum mosaic virus*	4FY1-A	88	31
TNV-PMV	*Panicum mosaic virus*	4FY1-A	74	69
	Tobacco necrosis virus	1C8N-A	76	33
PMV-TNV	*Panicum mosaic virus*	4FY1-A	96	62
	Tobacco necrosis virus	1C8N-A	99	40

4-7. Protein visualization and amino acids mutation

The software Chimera (http://www.cgl.ucsf.edu/chimera) was used to visualize CPs 3D structures and to perform virtual mutations. This software is downloadable and works on Mac, Windows and Linux platforms. The tutorial for this software is accessible at: http://www.cgl.ucsf.edu/chimera/tutorials.html. The following procedure was used to mutate Asp155 and Arg161 on TNV-D^H CP wt. structure.

Open Chimera, and then in the open menu choose from file and select ok to open the 3D structure from a file on your computer. Alternatively, you can fetch the construct by going to open, "fetch structure by ID". The TNV-D ID number is "1C8N (Table 2.5). To focus on a single chain, go to "Favorites" and choose "Command Line". to delete chains B and C type "delete:.B-C". After the 3D structure is loaded, go to the "Tools" menu and select "Sequence". A window opens that shows the open structure(s) amino acid sequence. From this window select the residue you want to mutate, then go to the "Action" menu, choose "Atom/bonds", and subsequently choose "Show" atoms. With your arrow key select the entire residue, then go to the "Select" menu, choose "zone" and mark "select all atoms/bonds of any residue in the selection zone. Thus software would be able to select the local amino acids within 5 angstroms from currently selected atoms.

Go to the "Action" menu, atoms/bonds and show atoms, deselect all and then select only the residue you want to mutate. This can be accomplished by using the "Control" key, coupled with the mouse and the upper arrow key on the keyboard. After it is selected, go to the "Tools" menu, "Structure editing" and choose "Rotamers". By choosing rotamers you will find access to the rotamers library, which has all the 20 standard amino acids, and you would be able to change your residue to the desired amino

acid. By clicking on “OK”, you will see a window open that shows all the possible directions that new residue can fit in the structure. Choose the one with the highest probability, click on “OK”, then use the upper arrow key on the keyboard to highlight the amino acid. After substitution of a single amino acid go to the “Tools”, “structure/binding analysis” and select “find clashes/contacts”. A new window will open, press “designate” to choose the new amino acid atoms as designated atoms and then “apply”. The clashes include steric and H-bonds.

If Chimera can find any of clashes/contacts then you need to minimize the structure to see if these steric clashes are removable. The structure minimization finds the best energy state for the modeled protein. To minimize structure go to the “tools”, “structure editing” and select “minimize structure”. In the first step of minimization (steepest descent), highly unfavorable clashes are recognized and relieved, then the second step (conjugate gradient) finds the best possible energy state for the modeled construct. A mutation would be neutral if clashes relieve after minimization but if it did not happen then it is very likely that mutation can have significant effect on the protein function. To export to PowerPoint, go to “File” and choose “Save Image”.

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Summary

Plants are able to recognize and respond to virus infection with a hypersensitive response, a plant defense response that triggers a cell death pathway and limits the viral infection to the inoculated leaf. One project in our lab is involves cataloging which species in the genus *Nicotiana* are resistant to members of the *Tombusviridae* family. We previously had shown several *Nicotiana* species respond with HR to the *Tomato bushy stunt virus* (TBSV) virion inoculation. Further analysis of TBSV encoded proteins showed P19, P22 and P41 (coat protein or CP) have the capacity to act as HR elicitors in those *Nicotiana* species. Interestingly we found that all members of the section *Alatae*, except *Nicotiana plumbaginifolia* responded to agroinfiltration of the TBSV CP with HR. Similar analysis with *Tobacco necrosis virus* strain DH (TNV-DH) showed that many of the *Nicotiana* species reacted with HR to virion inoculation. TNV-D^H does not encode protein analogues to the TBSV P19 and P22 proteins. but we could investigate the role of the TNV-D^H CP in HR elicitation.. Interestingly, the result was similar to the agroinfiltration with TBSV CP, as all the species in section *Alatae* responded to agroinfiltration of the TNV-D^H CP with HR except *N. plumbaginifolia*. The deletion analysis showed the first 77 and 79 amino acids of the TNV-D^H and TBSV CPs, respectively were not involved in triggering HR. A sequence alignment of the TNV-D^H and TBSV CPs showed 54 identical amino acids; however when the deletion analysis was taken into consideration, we were able to limit that number to 42 identical amino acids.

TBSV and TNV-D^H CPs were located on opposite sides of the *Tombusviridae* phylogenetic tree; thus we hypothesized that the CPs of the species in between might also trigger HR. To test this hypothesis we obtained at least one representative for each genus.Our colleagues in the other universities provided the CP open reading frames for these *Tombusviridae* members. The new species to be analyzedincluded *Cymbidium ringspot virus* (CymRSV) and *Cucumber necrosis virus* (CNV) of the genus *Tombusvirus*, *Red clover necrosis mosaic virus* (RCNMV) from *Dianthovirus* genus, *Turnip crinkle virus* (TCV) from the *Carmovirus* genus, *Panicum mosaic virus* (PMV) from the genus *Panicovirus*, and *Maize chlorotic mosaic virus* (MCMV) from the *Machlomovirus* genus. After cloning into an *Agrobacterium* expression vector, CP constructs were agroinfiltrated into species of section *Alatae*.

The agroinfiltration test divided the CP constructs into two groups. The first group included CNV, CymRSV and RCNMV CPs which triggered HR in *N. langsdorffii, N. longiflora, N. bonariensis, N. alata* and *N. forgetiana*. The second group included TCV, PMV and MCMV CPs, which failed to elicit HR in the plants. The results revealed HR-elicitor CPs might have some specific amino acids in common, which are absent in the primary sructure of the non-HR elicitors. A comparison of the amino acid sequences of all eight coat proteins revealed that only two amino acids (Asp155 and Arg161) were conserved amongst the coat proteins that triggered HR and distinguished them from the coat proteins that did not trigger HR. We used PCR to mutate Asp 155 to Thr and Arg 161 to Leu; however these mutations were still capable of triggering HR in *N. langsdorffii* and *N. longiflora*. In another effort we used Chimera, a protein 3D visualization software to visualize the mutations. Computer modeling of the coat protein

structure allowed for placement of these two amino acids within the three dimensional structure of the coat protein and suggested how mutations might affect the overall structure. Site directed mutagenesis of these two amino acids in the TNV-D^H coat protein, Asp155 to Asn and Arg161 to Trp, abolished HR, demonstrating that they have an essential role in recognition and elicitation of plant defenses against this virus.

Further research will be directed towards introducing the same mutations into the corresponding amino acids of the TBSV CP. This experiment could confirm the hypothesis that the same amino acids in the TBSV CP abolish HR, an indication that the TNV-D^H and TBSV CPs are recognized by the same R protein. Similarly, other studies could examine whether amino acids 155 and 161 contribute to HR induction in other *Nicotiana* species in section *Alatae*. This analysis could confirm that similar R proteins in members of section *Alatae* recognize the tombusvirus CP for HR elicitation.

VITA

Mohammad Fereidouni was born in April 11, 1977 in Tehran (Iran). He was two years old when the 1979 revolution began and following that the huge changes his country endured with the unwanted war with Iraq, which took eight years. He graduated from high school in 1995, and attended the Environmental Science and Engineering program at the Tehran-Azad University from 1995 to 1999. After graduation, he worked as a tour leader and also as a travel agent for two years and then he was employed as an environmental biologist by a private company (KaniKavan mining Co.) in 2003 for EIA studies. He started his Masters at Tarbiat Modares University in 2006 with KaniKavan financial support. He graduated on October 2008 with a Master's degree in Environmental Science and with an emphasis on biotechnology. He traveled to the U.S. in August 2009, accompanying his wife, Zeinab Chaichi Raghimi who wanted to pursue a Master's of Fine Arts (MFA) degree from the University of Missouri. In 2010 he entered the Department of Biological Engineering and worked for a year on a biofuel production project. His passion for science caused him to switch his major to Biochemistry in 2011; however, this was not a successful transition for him. He started his study as a Master's student in the Division of Plant Sciences on Sep 10, 2012 under Dr. James E. Schoelz supervision.