# CHARACTERIZATION OF RESISTANCE TO TOMBUSVIRUS IN *NICOTIANA* SPECIES

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by

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

**CHARACTERIZATION OF RESISTANCE TO TOMBUSVIRUS**

**IN *NICOTIANA* SPECIES**

Presented by: **CARLOS ARIEL ANGEL**

A candidate for the degree of Doctor of Philosophy

And hereby certify that, in their opinion, it is worthy of acceptance.

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## DEDICATION

I dedicate this work to the memory of my father Hector F. Angel, who lived knowing the real value of being a perseverant hard worker with honesty and humility. To my mother Barbara, and my younger brothers Luis Fernando and Ricardo Andrés, who were the reason for keeping me fighting every day, looking for opportunities during difficult times, and now, they make me feel proud for how far we all have gone. This degree is the result of a long process that did not begin when I came to the University of Missouri; it began long time ago and it was based on determination, ethics, and responsibility. Thank you to my close relatives, particularly my grand-uncle Ruben Serna and his family Alba María, Patricia and David, for their continuous and unconditional support, to my uncle Luis Guillermo Angel and his family, to my cousin Cesar A. Osorio and his family, to my grand-aunt Mariela Serna and her family, to my mother's family Calle-Bernal as well. Thanks to Fabio Botero, who listened when it was most needed. Thanks to those who know the meaning of love and friendship and to those who were loved and friends, for teaching me lessons of life.

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**CHARACTERIZATION OF RESISTANCE TO TOMBUSVIRUS IN *NICOTIANA* SPECIES**

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## ABSTRACT

Resistance to plant viruses has been explained by the "gene for gene" model, which requires the recognition between a host resistance(R) gene and a viral avirulence (Avr) gene, resistance that is commonly associated with a hypersensitive response (HR). Our lab has been characterizing resistance to Tombusviruses in *Nicotiana* species following this model. On the host side, we previously showed that dominant resistance to several tombusviruses such as Tomato bushy stunt virus (TBSV), Cymbidium ringspot virus (CymRSV), and Cucumber necrosis virus (CNV) can be introgressed from *N. glutinosa* into the susceptible host *N. clevelandii* through the development of an addition line which we designated *N. clevelandii* Line 36. Recently, we showed that posttranscriptional silencing of the *N* gene not only affected the HR elicited by the tobamovirus Tobacco mosaic virus (TMV), but also the HR to TBSV and CymRSV. We hypothesized that the R gene against tombusviruses could be a member of the *N* family of R genes present in *N. glutinosa* and distinct from it. In this study, I characterized the family of *N* gene homologs (NGHs) in *N. glutinosa*, *N. clevelandii* and Line 36. A

polymerase chain reaction (PCR) approach that amplified a sequence within the nucleotide binding site domain of the *N* gene yielded 143 NGHs, 106 of them translatable, that fell into 15 groups based on phylogenetic analyses. One of the groups was identified as the *N* gene itself. Further work is required to characterize this R gene family to identify the putative tombusvirus R gene sequence.

On the pathogen side, previous studies showed that the TBSV cell to cell movement protein P22 elicited HR in *N. glutinosa* and in its derived species *N. edwardsonii*, and the long distance movement protein P19 elicited HR in *N. tabacum*. We developed an agroinfiltration assay for transient expression of p22 and p19 genes, and found that *Nicotiana* species were able to recognize subtle differences between TBSV, CNV and CymRSV homologous P22 and P19 proteins in their role as Avr determinants. The three P19s were suppressors of gene silencing, but their suppressor function varied in strength and duration in *N. benthamiana*. We traced the resistance elicited by the P19 protein in *N. tabacum* to its ancestor *N. sylvestris*, but *N. tabacum* did not respond with HR to CNV P20.

Furthermore, we explored the genetic diversity in the *Nicotiana* genus against TBSV, CNV and CymRSV by inoculation of virions onto 18 *Nicotiana* species that belong to 10 out of 14 taxonomic sections. We found that 10 species showed HR, 5 were resistant without classical HR, and 3 were susceptible. We subsequently agroinfiltrated the three tombusvirus p22 and p19 genes into leaves of each of the 18 species, and we confirmed that *N. glutinosa* and *N. edwardsonii* responded to all three P22 variants with HR. In addition to *N. tabacum* and *N. sylvestris*, only *N. bonariensis* responded with HR to TBSV P19. Since many of the other *Nicotiana* species responded to TBSV virion

inoculations with HR, we hypothesized that tombusvirus genes other than p22 and p19 could act as Avr determinants. To investigate this hypothesis, we agroinfiltrated the TBSV replicase genes p33 and p92, and the coat protein gene p41 into each of the *Nicotiana* species and found that *N. langsdorfii*, *N. bonariensis* and *N. longiflora*, all species of the Alatae section, responded to p41 with HR. The Alatae section contains an additional four species, so we hypothesized that these other 4 species would also respond to p41 with HR. In fact, we found that *N. alata*, *N. forgetiana*, and *N. mutabilis* were resistant to TBSV virions and recognized the p41 gene as Avr determinant. However, *N. plumbaginifolia* was susceptible to TBSV infection and agroinfiltration of p41 did not trigger an HR. Based on these results we suggest that at least five types of R genes exist in *Nicotiana* species that can recognize TBSV determinants. One R gene recognizes P22 proteins in *N. glutinosa* (Undulatae section) and an accession of *N. forgetiana* (Alatae section). A second is able to recognize the P19 protein in *N. sylvestris* and in some species of Alatae. A third R gene in *N. tabacum* is a variant that can recognize TBSV P19 and CymRSV P19 proteins, but is unable to recognize the CNV P20. A fourth R gene is present in younger species of the Alatae section and recognizes the TBSV p41 gene as avirulence determinant. Finally, there still remains to be discovered at least one more R gene that is present in HR-resistant species that did not respond to individual agroinfiltration of TBSV genes. We showed that agroinfiltration is a powerful technique to screen not only a diversity of plant species as sources for R genes, but also to express viral genes as possible Avr determinants, and this strategy also has value for studying the evolution of resistance genes and their functionality across an entire genus.

# CHAPTER I

# LITERATURE REVIEW

## Resistance to Plant Viruses

Plant resistance to pathogen attack can be divided into two major categories: non-host resistance and host resistance. Non-host resistance is the most common situation in nature because most plants are resistant to most pathogens. This form of resistance is one of the most intriguing events in plant protection, because apparently the infection fails without eliciting any visible reaction in the host (Mysore and Ryu, 2004; Maule et al. 2007). Because of its complexity, the biological and molecular mechanisms of non-host resistance are difficult to evaluate, and are not understood. In contrast, host resistance is more accessible for study, because pathogens may be able to infect to some extent, and there are also variations in the response of the host. Both phenomena, pathogen infection and host response, can be evaluated and linked to genetic mechanisms; consequently, host-type resistance mechanisms are better understood. Plant defenses involve diverse strategies, including preformed structures such as leaf hairs, a thicker cuticle and cell wall, chemical defenses such as the production of reactive oxygen species, phenolic compounds, phytoalexins, and tannins, as well as enzymes such as glucanases and chitinases (Hammond-Kosack and Jones, 1996; Osbourn, 1996). Host resistance requires

the expression of numerous genes for recognition of the aggressor, defense signaling, and mobilization of defenses to halt the infection (Hammond-Kosack and Jones, 1996, 1997; Baker et al. 1997).

One of the pivotal host defense strategies is encompassed in the "gene for gene" theory (Flor, 1971), in which a plant resistance (R) gene product recognizes a specific pathogen avirulence (Avr) gene product to elicit a cascade of defense responses to limit the pathogen infective advance. The most common response associated with the interaction between R and Avr gene product is the hypersensitive response (HR), which is characterized by the rapid death of a limited number of cells in the region of the invading pathogen to restrict its growth (Lam et al. 2001; Torres et al. 2002). There is also a type of non-host resistance that is associated with HR responses (Mysore and Ryu, 2004), but it is not clear if both "gene for gene" and non-host HR are regulated by the same molecular mechanisms. The HR characterized in the gene for gene model has been associated with extracellular changes in pH, efflux of potassium and hydrogen ions, influx of calcium, increases of reactive oxygen species such as hydrogen peroxide, superoxide anions, nitric oxide, and hydroxyl radicals, disruption of membranes, callose and lignin deposition, production of phenolic compounds and antimicrobials, activation of caspase-like proteases, and transcriptional reprogramming, among many other metabolic and cellular processes (Hammond-Kosack and Jones, 1996; Lam et al. 2001; Torres et al. 2002; Hofius et al. 2007).

More recent models have added new details to explain the plant's immune response against pathogens, especially to fungi and bacteria. For instance, the "guard" hypothesis (Van der Biezen and Jones, 1998; Dangl and McDowell, 2006), postulates

that R proteins (guards) can detect and monitor the association of pathogen effectors or avirulence proteins, with plant pathogenicity targets (guardees). The guardee can be affected by the pathogen Avr protein, which then allows the R protein to elicit the defense cascade. A newer explanation, the "zig zag" model (Jones and Dangl, 2006), is based on the capacity of plants to respond to pathogen infections using two branches for immunity. The first one is able to recognize common microbial or pathogen associated molecular patterns (MAMPS or PAMPS) to elicit a basal defense response or PAMP-triggered immunity (PTI), and the second branch depends on the capacity of plant pathogens to deliver effector proteins inside or outside the cell. Some of these effector proteins function as avirulence proteins that are recognized by specific R proteins, resulting in deployment of the defense cascade. This is termed effector –triggered immunity (ETI). Recently, Van der Hoorn and Kamoun (2008) proposed the "decoy" model, in which the role of the guarded host targets depends on the presence or absence of R proteins. Pathogen effectors may have multiple host targets to establish a successful infection for the pathogen. Consequently, a pathogen effector can have different targets in the host, and the host guardee proteins may be dispensable for the virulence/avirulence activities of the effector, acting as a decoy especially in plants without the R gene product.

Plant viruses are responsible for significant economic losses in crops, losses that can be measured in terms of decreased yield and quality. Despite the fact that plant viruses are simple genetic entities, they form obligate and complex relationships with their hosts that we are just beginning to understand. Some plant viral diseases can be managed by cultural and sanitary methods, but genetic resistance is the most cost-

effective and sustainable strategy to control virus diseases (Hull, 2002). Genetic resistance to viruses can be characterized as non-host, polygenic or monogenic, and the mechanisms can vary from immunity, to HR, to tolerance. Among these mechanisms, dominant, monogenic resistance has been attractive for breeders because it can be highly durable and can be readily introgressed into important crop varieties. Furthermore it is amenable to experimental studies, and a practical way to track experimentally on the field, especially when it is associated with a visible phenotype such as HR. Each virus resistance gene acts as part of the plant surveillance system to recognize a specific virus, and this recognition event sets in motion a cascade of plant defense responses that are genetically accessible and measurable (Baker et al. 1997).

Although many studies have focused on dominant resistance to viruses, a high percentage of genes initially characterized by classical genetics and breeding techniques have been recessive. Recessive resistance has been considered to be an impaired susceptibility or an inhibition of the virus' capacity to invade more than a single cell (Hull, 2002; Kang et al. 2005). Viruses use the cell machinery for their replication, and interact with host proteins at many different points in the disease cycle (Maule et al. 2007). Using a candidate approach after conventional and molecular mapping, at least seven different recessive resistance genes and their alleles have been cloned and characterized from pepper, lettuce, pea, tomato, barley, rice, and melon; six additional recessive mutant genes have been characterized from *Arabidopsis thaliana* that condition resistance to viruses. All of these genes encode 4E (eIF4E) and eIF(iso)4E eukaryotic translation initiation factors. These mutations have been shown to inhibit the replication of species of potyviruses, bymoviruses, sobemoviruses, cucumoviruses, and

carmoviruses. In contrast, the dominant forms of these host gene products have been shown to be required for virus multiplication (Robaglia and Caranta, 2006). Approximately 50% of the recessive genes that have been characterized to date confer resistance to potyviruses, and most interact with the VPg viral protein (Kang et al. 2005; Robaglia and Caranta, 2006; Maule et al. 2007).

More than 100 dominant genes for resistance to viruses have been reported; however to date, only a dozen of them have been molecularly cloned and characterized. In contrast to recessive virus R genes, the dominant virus R genes are structurally similar to R genes against other plant pathogens. The dominant R genes that have been cloned have been grouped into five classes depending on the protein structure and their recognition mechanism (Dangl and Jones, 2001; Martin et al. 2003). Regardless of the type of pathogen, the most frequently characterized class is formed by proteins with a nucleotide binding site (NBS) and a variable set of leucines called leucine reach repeats (LRR) at the C terminal region. These NBS-LRR proteins are extremely variable and numerous in plant genomes. For example *A. thaliana* has 149 NBS-LRRs, rice (*Oryza sativa* L.) approximately 600, *Medicago truncatula* and poplar approximately 400 (Meyers et al. 1999, 2003; Cannon et al. 2002; Goff et al. 2002; Richly et al. 2002; Ameline-Torregrosa et al. 2007; Kohler et al. 2008). Depending on their domains at the N-terminal region, the NBS-LRR proteins are divided in two major groups, the TIR-NBS-LRR (TIR for homology with the *Drosophila* Toll and mammalian Interleukin Receptor gene), and the CC-NBS-LRR (CC for alpha-helical Coiled-Coil motif) before the NBS (Meyers et al. 1999; Pan et al. 2000).

The dominant R genes that have been cloned include *N, Tm2-2*, *Rx1*, *Rx2, Y-1*, *Sw5, Rsv1, RT4-4, HRT, RTM1, RTM2,* and *RCY1* (Kang et al. 2005; Soosaar et al. 2005, Maule et al. 2007). As expected for an obligate intracellular parasite, no dominant R genes against plant viruses contain extracellular receptors or transmembrane domains, and most of them are either CC-NBS-LRR or TIR-NBS-LRR proteins. The R genes *Rx1*, *Rx2, Sw5, RCY1, Tm2-2* and *HRT* all have a CC domain, and the *Rsv1* gene is a non-TIR-NBS-LRR gene (Martin et al. 2003; Hayes et al. 2004; Kang et al. 2005; Soosaar et al. 2005, Maule et al. 2007 ). Three virus R genes belong to the TIR-NBS-LRR class of R genes: *N, Y-1,* and *RT4-4* (Whitham et al. 1994; Vidal et al. 2002; Seo et al. 2006). Two R genes, *RTM1* and *RTM2* do not have NBS-LRR domains, and they belong to the jacalin family, which contains genes involved in resistance mechanisms against insects (Chisholm et al. 2000; Whitham et al. 2000). The NBS regions of CC and TIR resistance genes are required for pathogen perception, and are a molecular switch for signaling, which also involves ATP binding and hydrolysis (Tameling and Joosten, 2007). The NBS domain contains several highly conserved motifs such as a P-loop or kinase-1, kinase-2, kinase-3a, and the kinase-3a or "hydrophobic domain GLPL". Both the P-loop or GGVGKTT domain, and the GLPL domain are considered the most important for analysis of diversity of NBS-LRR proteins based on currently characterized R genes against fungi, bacteria, nematodes, and viruses The LRR domain at the C terminal region is the most highly variable, can contain between 10 and 40 short LRR motifs and is under constant selection pressure. The LRR is required for recognition, membrane surface interactions that mediate reversible high affinity protein-protein and protein-RNA

interactions, and also is involved in signaling (Baker et al. 1997; Hammond-Kosack and Jones, 1997; Meyers et al. 1999; Cannon et al. 2002).

**Virus Avirulence Genes**

According to the "gene for gene" model, the activation of the host defense response requires that the plant R gene product recognizes an avirulence (Avr) gene product (Flor, 1971). Studies with bacteria, fungi, and nematodes have led to the concept of effectors, pathogen proteins introduced into plant cells that either enhance pathogen virulence or suppress plant defenses to mediate effector-triggered susceptibility (ETS). Specific recognition of effectors by host R gene products can lead to effector-triggered immunity (ETI) (Jones and Dangl, 2006). In contrast to fungi, bacteria and nematodes, plant viruses are unique amongst pathogen groups, because all proteins in any viral genome can be considered effectors; they are expressed in host cells where they either enhance the virus' fitness through gene expression, replication, or movement, or they suppress plant defenses. Consequently in the case of viruses, it is useful to retain the designation of Avr determinant to distinguish viral effectors that elicit resistance responses from those that promote infection.

Any category of virus protein (e.g. replicase, coat protein, movement protein) can act as an Avr determinant to elicit resistance responses in the host (reviewed by Schoelz, 2006). The first two viral Avr genes to be molecularly characterized were the tobacco mosaic virus (TMV) coat protein which elicits HR in *Nicotiana sylvestris* (Saito et al.

1987; Culver and Dawson, 1991) and the multifunctional CaMV P6 protein which elicits HR in *Datura stramonium* and *N. edwardsonii* (Daubert et al. 1984; Schoelz et al. 1986). Despite the fact that it has been a long time since these two viral Avr genes have been indentified, neither of the corresponding R genes have been isolated. Approximately 50 examples of viral Avr genes have been reported since viral infectious clones were developed (Schoelz, 2006, Kang et al. 2005, Maule et al. 2007), and for 8 out of 12 dominant R genes that have been cloned, the respective viral Avr determinant has been identified. For example, the helicase domain of the TMV replicase protein elicits HR in *N. tabacum* containing the *N* gene derived from *N. glutinosa* (Whitham et al. 1994; Erickson et al. 1999), the coat protein of potato virus X (PVX) elicits resistance responses in potato plants harboring the *Rx1* and *Rx2* genes (Bendahmane et al. 1995, 1999, 2000), the movement proteins of TMV and tomato mosaic virus elicit a resistance response in tomato plants containing the *Tm2-2* gene (Meshi et al. 1989; Lanfermeijer et al. 2003), the movement protein of tomato spotted wilt virus is recognized by the *Sw5* gene in tomato (Brommonschenkel et al. 2000), the coat protein of cucumber mosaic virus elicits resistance responses in *A. thaliana* C24 containing the *RCY1* gene (Takahashi et al. 2001), the coat protein of turnip crinkle virus (TCV) elicits HR in *A. thaliana* ecotype Di-O harboring the *HRT* gene (Oh et al. 1995; Ren et al. 2000), and the 2a protein of cucumber mosaic virus elicits HR in bean plants containing the *RT4-4* gene (Seo et al. 2006). These interactions included examples of different R genes activated by the same category of viral Avr determinant (i.e. coat proteins of different viruses are recognized by different R gene products), and also examples of different Avr proteins from the same virus species activating resistance by different R genes, and in different hosts (i.e the

TMV replicase, movement protein and coat protein all can act as Avr proteins in conjunction with the proper R gene product).

There are two host-virus interactions that may be considered examples of the 'guard hypothesis". There is evidence that the resistance elicited by the coat protein of TCV in *A.thaliana* in response to the R gene *HRT* (for Hypersensitive Response to TCV) is mediated by an interaction with the host transcription factor TIP (for Turnip crinkle virus-Interacting Protein) (Qu et al. 2003; Ren et al. 2005). The second example involves the resistance elicited by the TMV helicase protein in response to the *N* gene; both the N protein and the TMV P50 protein interact with the host chloroplast protein NIP1 (for N – Interacting Protein 1) in the cytoplasm and nucleus (Caplan et al. 2008). However, for both examples with *HRT* and *N* gene mediated resistance, many details remain to be elucidated.

**_N_ gene-Mediated Resistance**

The *N* gene derives its name from the dominant allele responsible for the "Necrotic-type response to infection with TMV" in *Nicotiana* x *digluta*, an interspecific hybrid (*n*=36) created by crossing the resistant female parent *N. glutinosa* (*n*=12) and the susceptible male parent *N. tabacum* var. Purpurea (*n*=24) (Clausen and Goodspeed, 1925 cited by Holmes, 1938). *N. glutinosa* was the original source of the *N* gene. The recessive allele was named n, because of the chlorotic response due to susceptibility to TMV. By using *N.* x *digluta* as a parent, Holmes (1938) made a cross to *N. tabacum* var.

Connecticut Broadleaf (*n*=24) to create a fertile hybrid. Four backcrosses to *N. tabacum* and three rounds of selfings yielded *N. tabacum* var. Holmes Samsoun NN, which had a hypersensitive response to TMV inoculation (Holmes, 1938; Dunnigan et al. 1987). Later, Holmes (1946) inoculated tobacco etch virus (TEV) and TMV to a large number of species, many of them *Nicotiana* species, and most were susceptible to TMV; some developed systemic chlorosis or necrosis, whereas some were susceptible without symptoms, and a few showed local necrotic lesions, which was considered a type of resistance because death of tissues would limit the spread of the virus.

The *N* gene-mediated resistance is temperature sensitive, as TMV is able to move systemically in *N. glutinosa*, and *N. tabacum* NN plants at temperatures above 27-28ºC. Under high temperature conditions, the virus produces symptoms of systemic necrosis, which is possibly associated with faster replication and movement (Samuel, 1931; Weststeijn, 1981). Tobacco plants expressing the *N* gene develop necrotic local lesions within 48 hours at the site of inoculation of TMV at lower temperatures. When *N. edwardsonii* plants, a *Nicotiana* species derived from *N. glutinosa*, are grown at temperatures higher than 32ºC, systemic movement of TMV containing the green fluorecent protein (GFP) is observed without elicitation of systemic necrosis. However, when these plants were moved to lower temperatures around 20 ºC, the resistance response is elicited in all infected tissues, producing collapse of tissues and death of plants (Wright et al. 2000). These results showed the HR elicited by TMV-GFP in *N. edwardsonii* has two phases; the first one is a characteristic rapid cell death, followed by a more extended period in which all infected cells are progressively eliminated.

To characterize the *N* gene, two approaches were followed. Padgett and Beachy (1993) developed an infectious cDNA clone of TMV-Ob, a TMV strain that is able to overcome the *N* gene mediated resistance in *N. tabacum* cv. Xanthi NN. These authors constructed hybrid viruses between virulent and avirulent clones. They found that a mutation in the replicase gene, the TMV gene that encodes a 126 kDa protein, was responsible for elicitation of HR. In a subsequent study, Padgett et al. (1997), made domain exchanges between the TMV-Ob cDNA infectious clone and TMV strain U1 and found that a portion of the helicase domain of the replicase, located between amino acids 692 and 1116, was responsible for activation of the *N* gene-mediated HR. These findings were later confirmed by Erickson et al. (1999) using *Agrobacterium tumefaciens* vectors to express through agroinfiltration several domains of the replicase of TMV U1. They showed that a 50 kDa protein (P50) that contained the helicase domain was sufficient to elicit HR in a temperature-sensitive manner. In addition, they showed that the ATPase activity of P50 was not responsible for triggering HR. The agroinfiltration technique was considered cleaner, because it avoided potential side effects associated with the viral vector such as replication, movement or other viral functions.

The second approach for characterization of the *N* gene was to physically clone it and determine its structure. The *N* gene was one of the first plant R genes cloned, and the first virus R gene to be cloned (Whitham et al. 1994). It was isolated using transposon tagging with the maize *Ac* activator in *N. tabacum* cv. Samsun genotype NN, taking advantage of the temperature-sensitive character of the *N* gene-mediated resistance to perform the mutants screen. Susceptible plants of *N. tabacum* cv. Petite Havana SR1 genotype nn were transgenically complemented with a 10.6 kb DNA fragment containing

the full length *N* gene, demonstrating that *N* is responsible for resistance to TMV by HR activation. Further evidence that the *N* gene was completely functional in other Solanaceous species was obtained by Whitham et al (1996), when they generated transgenic tomato plants that expressed the *N* gene, and found that the *N* gene conferred resistance in tomato to TMV by eliciting HR.

The *N* gene contains 5 exons that can be spliced into an open reading frame (ORF) of 3,432 bp. Two forms of the N protein are produced. The full length N protein is 1,144 amino acids in length and has a molecular weight of 131.4 kDa. A second, truncated protein is produced from a spliced transcript that is 652 amino acids in length, is named $N^{tr}$, and has a molecular weight of 75.3 kDa. The N protein has three domains. At the N terminal region, there is a domain with similarities to the cytoplasmic domain of the *Drosophila* Toll protein and the human IL-1 receptor, a domain called the "TIR". The second domain is a nucleotide-binding site (NBS) containing a P-loop, kinases-2 and 3a, and ATPases. At the C terminal region can be found a tandem array of 14 imperfect leucine-rich repeats (LRR), containing approximately 26 amino acids each. Based on the deduced sequence and organization, the TIR domain could play an important role in virus perception, the NBS could be involved with ATP binding and signal transduction, and the LRR involved in protein-protein interactions (Gao et al. 2007). The authors hypothesized that the N protein could be cytoplasmic (Whitham et al. 1994; Dinesh-Kumar et al. 1995). Konagaya et al (2004) reported that a group of 14-3-3 protein isoforms can interact with the *N* protein at the LRR domain and also interact with the helicase domain P50 of TMV, suggesting a possible role as adaptors to support the interaction between both. Dinesh-Kumar et al (2000) showed that all three domains are essential for

resistance. The *N* gene is one of the first examples of the TIR-NBS-LRR class of R genes, which also includes genes against fungi and bacteria such as *L6, M, RPP5, RPS2,* and *RPS4* (Lawrence et al. 1995; Anderson et al. 1997; Parker et al. 1997; Gassmann et al. 1999).

The ratio of the two proteins, N and $N^{tr}$ plays an essential role in mediating the resistance response. The $N^{tr}$ alternative splicing event occurs by retention of a 70 bp alternative exon between exon 3 and 4 of the *N* gene. Consequently, $N^{tr}$ lacks 13 out of 14 LRRs (Whitham et al. 1994; Dinesh-Kumar and Baker, 2000). The two transcripts, NS for N-Short and NL for N-Long, encode the full-length N protein and the truncated $N^{tr}$ protein, respectively, and both are essential for resistance. The ratio between both transcripts is regulated by TMV signals, and the inability of a susceptible plant to express both transcripts leads to susceptibility to TMV infection. Initially before TMV infection, the NS transcript is prevalent in a ratio 25:1 (NS to NL), and up to 4 hours after infection the NS transcript is still higher, at an 8:1 ratio to NL. However, at 5 to 8 hours after infection, NL is prevalent in ratios from 1:1.5 to 1:20 (NS to NL) respectively. Eight hours after infection, the ratios are again reversed, with the NS transcript predominating by a 12:1 ratio; at 24 hours the ratio is 25:1 (NS:NL). The authors emphasized that a ratio of 1:1 is ineffective to elicit resistance (Dinesh-Kumar and Baker, 2000). Takanabe et al (2006) found discrepancies in the ratios and time frames for accumulation of both NS and NL transcripts using a different TMV strain, the TMV –OM. They also showed that both transcripts were produced at 20ºC, and severely reduced at 30ºC. Alternative splicing is a conserved mechanism that occurs with other TIR-NBS-LRR genes such as the *A.*

*thaliana RPS4* gene (Zhang and Gassmann, 2003), and flax *L6* and *M* genes (Jordan et al. 2002).

Several genetic components of the downstream pathway in the *N*-gene mediated resistance have been identified, and a virus-induced gene silencing (VIGS) platform based on tobacco rattle virus (TRV) vectors to silence defense genes in *N. benthamiana* transgenic for the *N* gene has been developed to identify the role of several genes. Liu et al. (2002ab) demonstrated that silencing *NbRar1*, *NbSGT1*, *NbSKP1*, *EDS1*, and *NPR1/NIM1*, genes that are required for resistance pathways of other TIR-NBS-LRRs genes, abolished the resistance mediated by the *N* gene. Both *NbRar1* and *NbSGT1* associate with the *NbCOP9* signalosome, a multiprotein complex involved in protein degradation via the ubiquitin-proteasome pathway (Liu et al. 2002b), and they are associated with the molecular chaperone Hsp90 (Liu et al. 2004a); consequently these genes are essential. Jin et al. (2003) and Liu et al. (2003), demonstrated that the activation of salicylic acid-induced protein kinase (*SIPK*) and wounding-induced protein kinase (WIPK), two tobacco mitogen-activated protein kinases (MAPKs) by their upstream MAPK kinase (MAPKK) *NtMEK2* leads to HR-like cell death, and that they are required for protein–protein interaction of the MAPK cascade in *N* gene-mediated resistance. Other kinases and transcription factors are also required for the *N* resistance pathway, such as the mitogen-activated protein kinase *NTF6/NRK1*, the MAPK kinase *MEK1/NQK1*, transcription factors WRKY1- WRKY3, and MYB (Liu et al. 2004b).

The *N* gene pathway also appears to recruit other NBS-LRR proteins. Peart et al. (2005) showed that a cofactor identified in a cDNA library of *N. benthamiana* transgenic for the *N* gene, was required for resistance without direct interaction between both.

Silencing of this *N*-requirement gene 1 (*NRG1*) abolished HR, and over-expression of it activated HR-like cell death. *NRG1* encoded a putative leucine- zipper CC-NBS-LRR protein. In addition, Liu et al. (2005) showed that upon silencing the autophagy gene *BECLIN 1* in *N. benthamiana* transgenic for the *N* gene, an HR developed that was not localized to infection sites, resulting in severe collapse of tissues and a spreading systemic necrosis. Not only the infective TMV, but also the avirulent helicase P50 protein was able to initiate the signal for a continuous cell death. The authors suggested that the evolutionarily conserved autophagy pathway plays an essential role in plant innate immunity and negatively regulates HR or programmed cell death.

Many studies have attempted to visualize and confirm a direct interaction between the N protein and the P50 helicase domain of the TMV replicase. Agroinfiltration of both *N* and P50 in *N. tabacum*, followed by yeast two hybrid and pull-down assays, indicated that the N protein directly binds to the P50 protein, requiring ATP as prerequisite, as well as the ATPase activity of the NBS domain of the N protein (Ueda et al. 2006). The authors proposed that the interaction of N with ATP forms a complex that interacts with the TMV P50 protein, changing its conformation to facilitate further interaction with the down-stream signaling factors to elicit HR resistance response. However, Mestre and Baulcombe (2006) contradicted this N – P50 direct interaction. They used agroinfiltation followed by immunoprecipitation to demonstrate that the N protein is oligomerized by the presence of TMV P50, but this is not sufficient to elicit HR. The oligomerization was abolished by a mutation in the P-loop motif; consequently, the *N*-gene mediated defense response depends on an intact P-loop, but loss-of-function mutations in the RNBS-A motif and in the TIR domain retain the ability to oligomerize and associate with

themselves. In addition, oligomerization and stabilization of N was not affected by silencing *NbEDS1* and *NGR1* cofactors, suggesting that oligomerization is an early event in the sequence of events in N-mediated resistance to TMV elicited by P50. Also, silencing of *SGT1* affected the levels of N, suggesting that *SGT1* and associated proteins are part of a system that controls the level of N in a positive or negative manner. Additional evidence about the importance of the TIR domain was provided by Gao et al. (2007), who showed that a substitution of the TIR-NBS domains of two novel N-like proteins (NL-C26 and NL-B69) into the original N protein resulted in a hybrid R protein that could elicit HR when co-expressed with the TMV P50 protein. But neither of the original N-like proteins could trigger HR when co-expressed with P50. An analysis of the putative amino acid sequences showed that the TIR-NBS domains from NL-C26 and NL-B69 are closely related to the TIR-NBS domain of the N protein. In addition, when native LRR domains from these two proteins were assembled with the N's TIR-NBS domains, the HR was severely affected or abolished. The same research group (Haque et al. 2009), recently identified a 20-bp elicitor-responsive sequence upstream of the *N* gene in *N. tabacum* cv. Samsun NN, that was essential and sufficient for promoter stimulation by P50 and *N*. The enhanced gene expression mediated by P50 and/or the N protein seems to require a common *cis*-acting element with three domains to act in a cooperative manner. Regulation of the *N* gene allows keeping expression at low levels when P50 is absent. If there is viral infection, P50 is present, which elicits the defense response, increasing *N* gene expression and N protein levels.

Immunoprecipitation and confocal fluorescence microscopy studies have been used to investigate interactions between N and TMV P50 *in planta*, and also have yielded

host proteins associated with *N* gene mediated resistance. Using agroinfiltration of individual constructs labeled with fluorescence proteins in *N. benthamiana* plants, Burch-Smith et al. (2007) showed that both N and P50 proteins localize in the cytoplasm and nucleus, but nuclear localization of N is essential for it to be functional. The interaction of P50 with the TIR domain of N was observed, confirming that its integrity is critical; the LRR domain is not responsible for this protein-protein interaction. P50 can be also detected in the nucleus, but it is not required for a defense response, suggesting that the interaction of N and P50 occurs in the cytoplasm, which is the natural environment for virus replication and accumulation of P50. The authors suggested that they could not discriminate *in planta* between direct or indirect interaction using bimolecular fluorescence complementation, so host factors might participate in this interaction. To date, the interaction between N and P50 is considered indirect because the TIR domain did not interact directly with P50 in a yeast two- hybrid system and *in vitro* assays (Burch-Smith et al. 2007). In addition, it has been suggested that the TIR and LRR domains interact, and that the TIR also interacts with itself, producing a complex conformation, which can be disrupted by the interaction with P50.

Yeast two-hybrid screens have yielded variable results for identifying potential host proteins that interact with the N protein or with the TMV P50 protein. For example, yeast two-hybrid screens have identified several host proteins that do interact with the P50 helicase, but none of them have been directly associated with *N* gene mediated resistance (reviewed by Schoelz, 2006). Recently, a yeast two-hybrid screen was initiated to look for host factors that interact with the TIR domain of the N protein and the TMV P50 protein. The authors identified a chloroplast protein NIP1 (for N receptor –

Interacting Protein 1) that directly associated with both TIR and P50 in the cytoplasm and nucleus (Caplan et al. 2008). The NIP1 protein is a rhodanese sulfur-transferase protein required for *N* gene mediated resistance. Recognition occurs first with P50 in the cytoplasm and nucleus, and the complex is later recognized by N. This study suggests that the *N* gene mediated resistance mechanism can be explained by the "guard hypothesis" model, in that the host *N* and NIP1 proteins interact only in the presence of the TMV P50 protein.

**Tombusviruses: Genome Structure and Virus Infection Cycle**

The *Tombusvirus* genus belongs within the Tombusviridae family, which also contains the genera *Carmovirus*, *Necrovirus*, *Dianthovirus*, *Machlomovirus*, *Avenavirus*, *Aureusvirus*, and *Panicovirus*; many of these genera are recognized as pathogens of plants. Most tombusviruses are soilborne pathogens, highly stable and transmitted mechanically. Some are transmitted by seed, or by means that do not require a vector; one species is transmitted by a fungus. Tombusviruses can be found in water currents and drainage ditches. Tombusviruses are distributed worldwide, causing severe diseases in edible and ornamental crops. Important diseases caused by tombusviruses include field and greenhouse crops of tomato, eggplant, pepper, carnation, and lettuce (Gerik et al. 1990; Obermeier et al. 2001; Yamamura et al. 2005). The natural host range of individual tombusviruses is relatively restricted; but experimentally, tombusviruses can infect a broad diversity of species belonging to approximately 20 to 30 plant families,

mostly dicotyledonous, including important families such as Compositae, Rosaceae, Fabaceae (Leguminosae), Malvaceae, Cucurbitaceae, Chenopodiaceae, and Solanaceae. The symptoms induced by tombusviruses vary from a very light chlorosis to severe systemic necrosis and death of plants, but this is dependent on host species, virus strain, and environmental conditions.

The *Tombusvirus* genus contains 14 species, including the type virus tomato bushy stunt virus (TBSV). Other confirmed members include carnation italian ringspot virus (CIRV), cucumber necrosis virus (CNV), cymbidium ringspot virus (CymRSV), and artichoke mottle crinkle virus (AMCV). Six species have not yet been classified, including lettuce necrotic stunt virus (LNSV) and maize necrotic streak virus (MNeSV). Tombusviruses have a genome size of approximately 4,700 – 4,800 nucleotides composed of single-stranded RNA in positive sense, form isometric virions (T=3) composed of 180 units of a single coat protein. The virions have a diameter of 30-35 nm that contain a single copy of the genome. The tombusvirus genome is organized into five major ORFs and a sixth small ORF: The first ORF nearest to the 5' end of the RNA encodes a 33 kDa protein (P33). The second ORF encodes a 92 kDa protein (P92) that is translated by a read-through mechanism of the UAG amber stop codon at the end of the P33 ORF. The P92 protein is an RNA dependent RNA polymerase. Both P33 and P92 are required for replication and are present in cells at a ratio of approximately 20 to 1 (P33:P92) (Scholthof et al. 1995c; Yamamura and Scholthof, 2005). The third ORF is translated from a subgenomic RNA (sgRNA1) and encodes the 41 kDa coat protein (P41). The fourth and fifth ORFs are nested, translated from a second single subgenomic RNA (sgRNA2), and encode two proteins, P22 and P19 respectively (Hearne et al. 1990;

Yamamura and Scholthof, 2005). They are involved in cell to cell and long distance movement, host determination, and symptom development (Scholthof et al 1995ab). The P19 protein is also known as a strong suppressor of post-trancriptional gene silencing (Voinnet et al. 1999; Scholthof, 2006). A sixth small ORF at the 3' distal end of the RNA encodes the pX protein; it is not essential for replication, but is required for virus accumulation (Scholthof and Jackson, 1997). The genomic and subgenomic RNAs do not have a 5' cap or 3' poly(A) tail, but there is a translational enhancer at the 3' end around the pX ORF (Yamamura and Scholthof, 2005). Twelve complete tombusvirus genome sequences have been determined from infectious cDNA clones, including CymRSV (Grieco et al. 1989), CNV (Rochon and Johnson, 1991), AMCV (Tavazza et al. 1994), CIRV (Rubino et al. 1995), and MNeSV (Scheets and Redinbaugh, 2006). All species have a genome organization similar to TBSV (Hearne et al. 1990). Interestingly, MNeSV contains sequences with high identity to two different Tombusviridae genera, the *Necrovirus* and *Tombusvirus* genera. A schematic representation of the TBSV genome is illustrated in Figure I.1.

**Expression and Functions of Tombusvirus Proteins**

After penetration and infection of initial host cells, the viral RNA is unencapsidated, and the replication proteins P33 and P92 are translated. Replication of the TBSV, CymRSV and CIRV genome involves the initial targeting of the plus strand RNA genome (+RNA) to membrane-associated replication sites, specifically to

multivesicular bodies, and assembly of the viral replicase occurs at subcellular membrane surfaces within the cytosol of host cells to produce the complementary minus-strand RNA (-RNA) (Scholthof et al. 1995c; Rubino and Russo, 1998). The (–)RNA is produced as an intermediate replicative form that is required as a template for synthesis of new (+)RNA progeny. These new (+)RNAs are released from the site of replication to the cytosol to begin anew the synthesis of the viral genome, assembly into virions, or movement to adjacent cells. Both the P33 and P92 proteins have specific domains for RNA binding, an aspect that could be associated with recruitment of the viral RNA to the replication sites in a cooperative manner between P33 and P92. Several host proteins have been identified in the viral replicase complex by the yeast two-hybrid system. In addition, tombusviruses have been replicated successfully in yeast, a system that has proven valuable for understanding replication of viral RNAs (Scholthof et al. 1995c; Rajendran and Nagy, 2003; Panavas et al. 2005; Pogany and Nagy, 2008).

There is little information about the role of P33 and P92 proteins in symptom determination. Scholthof et al. (1995b) did not observe any symptoms, either chlorotic or necrotic responses, in *Nicotiana* species when they expressed TBSV P33 protein via a potato X virus (PVX)-based vector. In contrast, Burgyán et al. (2000) reported that the CymRSV P33 and the CIRV P19 protein were synergistically involved in eliciting a systemic necrosis symptom in *N. benthamiana*. However, the authors did not test expression of the P33 construct by itself, since they used chimeras made between the two viruses.

Tombusviruses sustain deletions in their genomes during replication that are called defective interfering RNAs (DI RNAs). These DI RNAs usually contain 5’ and 3’

untranscribed terminal regions and noncontiguous coding sequences from the central part of the replicase p92 gene and the 3' end of the p22/p19 ORFs. DI RNAs can interfere with viral accumulation by reducing the level of sgRNAs and the corresponding proteins; consequently, if proteins that determine symptom development and movement are reduced, the infection is severely affected (Scholthof et al 1995c; Yamamura and Scholthof, 2005; Hornyik et al. 2006). The effect of DI RNAs is host dependent and cumulative. Omarov et al. (2004) showed that accumulation of high levels of TBSV DI RNAs reduced symptom development and attenuated systemic infection in *N. benthamiana*, but the effects were significantly weaker in pepper plants. A complete review about plant virus DI RNAs including tombusviruses is presented by Simon et al. (2004).

Two subgenomic RNAs (sgRNAs) are produced during replication (sgRNA1 and sgRNA2). Their production is dependent on premature termination of the (-)RNA synthesis from the full length (+)RNA template. These (-)RNAs subsequently serve as templates for synthesis of the sgRNAs. The sgRNA2 accumulates to a higher level than sgRNA1 (Zhang et al. 1999; Qiu and Scholthof, 2001; Yamamura and Scholthof, 2005). The sgRNA1 serves as mRNA for translation of the coat protein p41 gene; the P41 coat protein is not involved in replication. The coat protein gene of TBSV can be replaced with foreign genes to make TBSV an expression vector (Scholthof et al. 1993, 1996, 2002; Scholthof, 1999). Also, the coat protein of TBSV has been modified for translational fusions with short peptides for epitope presentation in the shell of the coat protein. These modified coat proteins can be assembled into particles that can be used for antibody synthesis (Scholthof et al. 1996; Hsu et al. 2006). Although the TBSV coat

protein is not absolutely required for infection, coat protein mutants containing large substitutions are less efficient in replication than mutants that contain an inactivated protein (Scholthof et al. 1993). Some sequences of the coat protein are necessary for subcellular localization. The arm and shell domains of the CNV coat protein function as transit peptide sequences for importation into the chloroplast and stroma, and virus particles that contained deletions in the arm domain showed a reduced capacity to establish infection (Xiang et al. 2006).

The role of the coat protein in symptom determination and infectivity has been studied. Neither the coat protein nor presence of putative *cis*-acting RNA sequences is indispensable for infectivity in both protoplasts and plants. Furthermore, mutants in the coat protein showed variations in symptom development in systemic hosts, but that was attributed to impaired spread over long distances. Furthermore, elicitation of HR in *Chenopodium amaranticolor* occurred in absence of the coat protein gene (Scholthof et al. 1993). Another study has suggested that TBSV P41, P19, and P22 proteins function cooperatively to facilitate systemic infection and lethal necrosis in *N. benthamaiana*. In this example, the coat protein is required for efficient exit from the vascular tissue, whereas P22 and P19 contribute as movement proteins and silencing suppressor respectively (Qu and Morris, 2002).

The sgRNA2 is the mRNA for P22 and P19, two nested genes organized in different reading frames. Although the start codon for the p19 gene is located downstream of the start codon for the p22 gene, the p19 gene is translated more efficiently. Ribosomes can pass over the AUG of p22 because of a poorer context for

initiation of translation, and they scan downstream until they find the start codon of the p19 gene (Scholthof et al. 1999).

The P22 protein is a cell to cell movement protein. It is able to bind RNA, is cell wall and membrane-associated, and is phosphorylated. In addition, P22 is a very important symptom determinant. Mutations that deleted or changed the ORF abolished cell to cell movement and systemic infection in *C. amaranticolor* (Scholthof et al. 1995b; Desvoyes et al. 2002). Using infective clones of TBSV and a PVX-based vector for expression of P22, Scholthof et al (1995ab) confirmed the membrane localization of P22 and determined that P22 triggered HR in *N. glutinosa* and *N. edwardsonii*. Chu et al. (1999) studied the relationship of TBSV P22 regions that control the cell to cell movement and the regulation of HR in *N. edwardsonii*. The authors showed that amino acids in positions 2-4-6, 31-32-34, and 103 were critical for cell to cell movement, whereas amino acids 14-18, and 59 were critical for elicitation of HR in *N. edwardsonii*.

The P19 protein is considered to have a function in long distance movement, is found in the cytosol, is required for symptom determination in a host specific manner, and is a gene silencing suppressor that acts in a counter defense strategy to allow TBSV to infect systemically many hosts (Scholthof, 2006). Mutations that inactivated the P19 protein attenuated the severe necrotic systemic symptoms elicited by wild-type TBSV in *N. benthamiana* and *N. clevelandii*. However, P19 was not required for systemic movement in these two *Nicotiana* hosts. In addition, P19 elicited the formation of local necrotic lesions in *N. tabacum* (Scholthof et al. 1995ab). The absence of P19 did not affect levels of RNA replication of TBSV in early stages in *N. benthamiana* and in cucumber protoplasts until 24 hours after inoculation (Qiu and Scholthof, 2001).

Similarly, Szittya et al. (2003) found that CymRSV P19 is not required for replication of the viral RNA in protoplasts. Park et al. (2002) found that genomic RNA accumulation is regulated by interdependent *cis*- acting elements within the p22-p19 nested ORFs at the 3' end. Although P19 was not important for systemic movement of TBSV in *N. benthamiana* and *N. clevelandii*, it was essential in other hosts such as pepper and spinach (Scholthof, 1995ab).

In several studies P19 mutants have been created to identify domains of the protein associated with specific functions. Turina et al. (2003) showed that P19 contributes to local lesion expansion in cowpea, and cell-to-cell movement in pepper. They showed that charged amino acids in the central domain of P19, between positions 72 and 78, were responsible. Chu et al. (2000) created 12 site-directed mutants within P19 along amino acid regions that were thought to be exposed on the surface of the protein and could be available for host-specific interactions, taking care to not affect the P22 amino acid sequence. These mutants were not deficient in replication in protoplasts or in cell-to-cell movement in plants. Substitution of amino acids at the extreme N-terminal end or within the carboxyl-terminal 70 amino acids did not produce any observable effect on plants. However, mutations dispersed between positions 43 and 85 on the N-terminal half prevented the development of a systemic lethal necrosis in *N. benthamiana* and *N. clevelandii*. The same mutants elicited mostly chlorotic rather than necrotic local lesions in *N. tabacum*, with the exception of the mutant in amino acid 60. The same central region had a pivotal role on RNA binding and silencing suppression activity (Qiu et al. 2002). Recently, Hsieh et al. (2009) evaluated the effect of 22 TBSV P19 mutants on necrosis severity in *N. benthamiana*, capacity to function as a silencing

suppressor, and stability of the P19 protein. The authors found that all mutants were infectious, symptom differences did not correlate strictly with mutation-associated variation in P19 accumulation, and substitutions affecting a central domain of P19 generally exhibited symptoms more severe than mutations affecting peripheral regions. Also, there was a negative effect on the ability of some mutants to capture short interfering RNAs (siRNA) and a reduction in silencing suppression activity, which led to less severe systemic infections. In particular, changes in domains involving amino acids 40 and 120 produced a reduction in necrosis severity and lower TBSV P19 accumulation.

Many studies have focused on the role of tombusvirus P19 in suppression of RNA interference (RNAi) or RNA silencing. The first report of TBSV P19 as a suppressor of posttranscriptional gene silencing (PTGS) was done by Voinnet et al. (1999) when they noted that P19 reversed the effect of silencing of GFP in *N. benthamiana* transgenic for GFP. Initially, the authors infiltrated GFP transgenic plants with an *A. tumefaciens* based vector expressing the *GFP* gene to induce systemic PTGS. Then, when the silenced plants were inoculated with a PVX-based vector expressing the TBSV p19 gene, suppression of GFP silencing occurred in the newly emerged leaves and the GFP expression was recovered in those tissues. Northern blot analysis of RNAs extracted from these younger leaves showed an increase of the previously reduced levels of GFP RNAs. The silencing suppression activity was also found for CymRSV P19 (Silhavy et al. 2002).

The gene silencing mechanism is a defense strategy of plants against viral infections. When a susceptible plant is infected by a virus, double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA) with hairpin structures are produced during replication. The viral dsRNAs are targets of the nuclease DICER-like protein for

cleavage, resulting in dsRNAs 21–25 bp in size called short interfering RNAs (siRNAs). The siRNA duplex is separated in ssRNAs that are incorporated based on sequence complementarities into the RISC (for RNA-Induced Silencing Complex). Recognition of these RNA sequences allows for targeting of the RNA viral genome and its transcripts for degradation by the RISC complex. Consequently, plant species with a strong and effective RNAi silencing mechanism can recover from initial viral infections or maintain the virus at lower titers. The P19 protein of tombusviruses counteracts the plant's silencing machinery by forming dimers that bind the double stranded siRNAs to prevent their transfer to the RISC complex. This capture prevents the siRNAs' incorporation into the RISC complex and its activation for targeting the corresponding ssRNAs, avoiding degradation and spreading of a systemic signal that primes the PTGS mechanism. As a result, the viral genome and transcripts are not targeted by the RISC, virus replication continues, systemic movement occurs without major limitation, and virus titer increases (Voinnet and Baulcombe, 1997; Voinnet et al. 2003; Baulcombe, 2004; Scholthof, 2006). The TBSV P19 protein also targets ALY/REF proteins, a family of proteins with RNA binding properties, and interacts physically with them, relocalizing some from the cytoplasm into the nucleus. This binding and relocalization interferes with the role of P19 as a silencing suppressor in *N. tabacum* and *N. benthamiana* (Park et al. 2004; Canto et al. 2006). A detailed description of the molecular mechanism of RNA silencing suppression mediated by CymRSV P19 protein is presented by Lakatos et al. (2004).

The success of a tombusvirus to overcome the RNAi silencing machinery and infect a host systemically depends on the balance between the amount of siRNAs that could trigger PTGS and the amount of P19 protein that could suppress the silencing

mechanism. Havelda et al (2005) showed that the presence of DI RNAs increases the level of siRNAs, which leads to saturation of CymRSV P19; consequently, the silencing suppressor function is diminished, resulting in a deficient virus infection. Also, environmental factors play a role in PTGS and suppression. For instance, PTGS is temperature dependent in a progressive manner; at 15ºC it is inhibited and DI RNAs do not affect P19 accumulation; at 24ºC PTGS is activated but wild type virus can infect successfully; at 27ºC plants recover from infection because of a very active antiviral RNAi mechanism, and the P19 protein is saturated with siRNA derived from DI RNAs (Szittya et al. 2002, 2003; Havelda et al. 2003; Takeda et al. 2005).

**Transgenic and Natural Resistance to Tombusviruses**

Transgenic, resistant *N. benthamiana* plants have been developed by expressing sequences from the coat protein gene (p41) and the replicase genes (p33 and p92). However, the results have been variable. Rubino et al. (1993) obtained transgenic *N. benthamiana* plants that expressed the coat protein gene of CymRSV, but these plants were only resistant against virion concentrations less than 0.5 µg/ml. Kollar et al. (1993) produced transgenic *N. benthamiana* plants that expressed DI RNA sequences of CymRSV in sense and antisense orientation. The transgenic plants that expressed the DI RNA in the sense orientation prevented the development of necrosis symptoms when inoculated with CymRSV. Kollar and Burgyan (1994) inoculated infectious transcripts of CymRSV into protoplasts of transgenic *N. benthamiana* plants that expressed the

replicase genes p33 and p92 of CymRSV, and found that replication was inhibited. However, mutant transcripts lacking these p33 and p92 genes were able to replicate autonomously when inoculated into the transgenic protoplasts, due to complementation or recombination with the host sequence. Similarly, Rubino and Russo (1995) developed a *N. benthamiana* plant that expressed the replicase genes p33 and p92 of CymRSV. These transgenic plants were resistant to CymRSV, but susceptible to two other tombusviruses, AMCV and CIRV, showing that the resistance was highly specific. Furthermore, transgenic plants expressing tombusvirus sequences showed risks associated with recombination between transgene sequences and viruses. Borja et al. (1999) produced *N. benthamiana* plants transgenic for the coat protein gene of TBSV that failed to resist virus infection when inoculated with wild type virus. Also, the transgenic plants inoculated with coat protein mutants of TBSV and CNV developed symptoms similar to wild type viruses. These authors showed that wild-type and chimeric viruses had been generated by a double recombination event between the defective virus and the coat protein transgene mRNA expressed in transgenic plants. Rubio et al. (1999) designed a DNA cassette to transcribe DI RNAs of TBSV flanked by ribozymes (RzDI), and investigated their potential to protect transgenic *N. benthamiana* plants from tombusvirus infections. The authors found that when the RzDI RNAs were transcribed *in vitro*, mixed with parental TBSV transcripts and inoculated into protoplasts or plants, they became amplified, reducing the accumulation of the parental TBSV RNA; consequently, infection of TBSV and systemic necrosis symptoms were reduced. The resistance character was inherited to F1 and F2 RzDI transformants, but many of these plants eventually presented severe symptoms. The transgenic lines showed a broad-

spectrum of protection against the tombusviruses CNV, CIRV and CymRSV, but they were susceptible to the unrelated viruses TMV that belongs to the *Tobamovirus* genus, bean dwarf mosaic virus (BDMV), which is a *Begomovirus*, and PVX, a *Potexvirus*.

Many of the examples of transgenic resistance could be explained by RNAi silencing mechanisms, which target exogenous transgenes and dsRNAs by sequence homology, leading to degradation of the infective viral genome. In addition, some might be explained by mechanisms that affect the function of the P19 protein, affecting its silencing suppressor activity, and consequently attenuating viral infections (Baulcombe, 1996; Voinnet et al. 1999; Havelda et al. 2003; Lakatos et al. 2004).

A different approach to obtain tombusvirus resistance plants has involved the use of recombinant antibodies. Tavladoraki et al. (1993) developed transgenic *N. benthamiana* plants that expressed single-chained Fv fragments (scFvs) against the coat protein of AMCV. They demonstrated that the drastic reduction of AMCV accumulation was associated with the AMCV-binding specificity of the scFv[F8] antibody. The resistance character was inherited by progeny. Boonrod et al. (2004) developed transgenic plants that expressed three scFvs with different binding properties that target the viral replicase activity of TBSV and CNV. They found that viral titers of both viruses were severely reduced, and postulated that inhibition was due to binding of the scFvs to the motif E of replicase P92. In addition, the accumulation of two heterologous carmoviruses (TCV and red clover necrotic mosaic virus, RCMNV) was also reduced in the transgenic plants. Interestingly, the resistance was also inherited and the antibodies were active not only to the four plant viruses, but also to a human virus (hepatitis C virus). None of these attempts were pursued on a commercial level.

Limited information is available about natural resistance to tombusviruses. Obermeier et al. (2001) studied a severe soilborne disease and dieback in lettuce in California and Arizona caused by the tombusvirus LNSV, and, as part of their studies, they tested the hosts for susceptibility to CNV and several isolates of TBSV obtained from lettuce, tomato, eggplant, and sugar beet. In this list, the authors reported several hosts that showed necrotic lesions and no systemic symptoms. Based on the HR elicited in *N. glutinosa* and *C. amaranticolor* (Scholthof et al 1993, 1995ab), the following hosts could be considered resistant: *Gomphrena globosa*, *Beta vulgaris*, *Chenopodium capitatum*, *C. murale*, *Cichorium intybus*, *Lactuca sativa*, *Sonchus oleraceus*, *Cucumber sativus*, *Cucumis melo*, *Pisum sativum*, *Physalis alkegen*, and *Ph. wrightii* (Obermeier et al. 2001). In a similar study, Ohki et al. (2005) characterized a new isolate of TBSV from nipplefruit (*Solanum mammosum*) in Japan, and listed the following hosts that developed necrotic lesions: *Cucumis sativus*, *Cucurbita pepo*, *Vigna sinensis*, *Vicia faba*, *Phaseolus vulgaris*, *Pisum sativum*, *Beta vulgaris*, *Gomphrena globosa*, and the resistant control *N. tabacum*. Interestingly, *N. glutinosa* developed chlorotic symptoms and did not respond with HR.

In a survey of more than 200 plant virus R genes that had been identified by conventional or molecular methods, not one had been listed against tombusviruses (Kang et al. 2005). The only R gene effective against tombusviruses identified through conventional breeding has been found in lettuce and is effective against LNSV (Grube et al. 2005). These authors screened primitive and modern cultivars of *Lactuca sativa* as well as some wild relatives for virus-free symptoms, and found that approximately 30% of the genotypes were resistant, but not immune. Selected resistant genotypes showed

some level of virus infection, but they were symptom-free. They identified and mapped the dominant locus *Trv1* for resistance to LNSV in the lettuce cultivar "Salinas" and found that it was linked to the *Dm1/Dm3* cluster of resistance genes.

Two TBSV proteins, the P22 and P19 proteins, were implicated in elicitation of HR in different *Nicotiana* species. Scholthof et al. (1995a) expressed these TBSV proteins by using a PVX-based vector and found that the P22 protein elicited HR in *N. glutinosa* and its derived amphidiploid species *N. edwardsonii*, whereas the P19 protein elicited HR in *N. tabacum*. In addition, the TBSV P19 protein played a role in development of severe systemic necrosis in *N. clevelandii* and *N. benthamiana*, and systemic infection of *Spinacia oleracea* and *Capsicum annuum* (Scholthof et al. 1999). These results suggest that there are at least two R genes against tombusviruses in *Nicotiana* species.

Scholthof et al. (1999) further showed that lesion size is dependent on the ratio of expression of the P22 and P19 proteins expressed from the sgRNA2. The natural situation in the viral genome shows that P19 is produced in significantly higher levels than P22 (Scholthot et al. 1995b, 1999) because the translational context of the start codons is good for p19 and poor for p22. In Scholthof et al. (1999), the authors experimentally altered the ratio by altering the translational context of the start codons. They found that changes in the ratio of P22 to P19 did not affect significantly virus replication and systemic infection in *N. benthamiana*, but they did observe larger lesions in *N. tabacum*. The explanation could be if more P22 is expressed than P19, there will be faster cell to cell movement and expression of P19, which would expand the area of induction of HR in *N. tabacum*. In the case of HR elicited by P22 in *N. glutinosa* or *N. edwardsonii*, a higher

expression of P22 in the cell is going to trigger a faster HR response because the threshold required to start the defense signaling cascade is attained in less time, and P22 does not have time to move to adjacent cells. In this instance, the result is a smaller lesion than that induced by the wild type virus. A detailed analysis of codon context and length in the leader sequences of CNV p21 and p20 genes (homologs to TBSV p22 and p19 respectively), was done by Johnston and Rochon (1996), also showing that changes in the codon context upstream of the p21 start codon affected the expression from the downstream p20 start codon.

Schoelz et al. (2006) introgressed a tombusvirus R gene from the resistant source *N. glutinosa* into the susceptible host *N. clevelandii*, through the bridge plant *N. x edwardsonii* var. Columbia (Cole et al. 2001). After three rounds of backcrosses to *N. clevelandii* and two selfings, Schoelz et al. (2006) obtained an *N. clevelandii* addition line (Line 36), homozygous for resistance to the tombusviruses TBSV, CNV, CymRSV, and CIRV, but susceptible to other virus species such as TMV, cauliflower mosaic virus (CaMV) that belongs to the *Caulimovirus* genus, and watermelon mosaic virus 2 (WMV-2), which is a *Potyvirus*. These results showed that the *N* gene, which confers resistance to TMV, is not directly involved in resistance to tombusviruses. However, we hypothesized that the R gene responsible for resistance to tombusviruses could be related at the nucleotide sequence level to the *N* gene, because silencing of the *N* family of resistance genes compromises the HR to the tombusviruses TBSV and CymRSV (Balaji et al 2007).

Screening for host proteins that interact with TBSV P22 and P19 has yielded two prominent host proteins. Desvoyes et al. (2002) used a yeast two-hybrid system to screen

a cDNA library of *N. tabacum* and found a strong interaction between the TBSV P22 protein and the *N. tabacum* host factor interacting (HFi22). HFi22 was also found to bind TBSV P19. HFi22 is a leucine-zipper homeodomain protein, with homology to transcription factors that could bind plant mRNAs. HFi22 increases with virus accumulation (Desvoyes et al 2002; Yamamura and Scholthof, 2005). Hypothetically, HFi22 would bind the viral p22 RNA to assist its trafficking through plasmodesmata, but no further evidence of this function has been presented. HFi22 has not been evaluated as a potential guardee. P22 does not elicit HR in the TBSV resistant *N. tabacum*, but it is not known if a homolog of HFi22 exists in *N. glutinosa*. TBSV P19 interacts with *N. tabacum* and *N. benthamiana* host interacting protein (Hin19) (Park et al. 2004). Hin19 has a high degree of similarity with a class of RNA-binding proteins because of the presence of a RNA recognition motif. Hin19 is thought to be involved in RNA processing and translocation and it is thought to bind to the central region of P19, a region that is also associated with symptom determination, movement and suppression of gene silencing (Chu et al. 2000; Qiu et al. 2002). The authors initially considered the role of Hin19 in plant defense, hypothesizing that the P19 – Hin19 binding could prevent or stimulate defense responses elicited by P19 as an avirulence protein, but have not yet developed further evidence.

Most models concerning resistance to plant pathogens including viruses have involved protein – protein interactions, such as between an R protein and an Avr protein, interactions that might occur directly or indirectly. However, Szittya and Burgyán (2001) showed that a 860 nt sequence (between nucleotide positions 2,666 - 3,526) of the CymRSV coat protein gene elicited an HR in *D. stramonium*. Their evidence suggested

that the viral RNA rather than the coat protein was the elicitor. This was the first report suggesting that the RNA alone, without the presence of the encoded protein, could trigger HR in a plant.

### *Agrobacterium*-Mediated Transient Expression of Genes in Plants

*Agrobacterium tumefaciens* is a soil-borne, Gram negative bacterium that can enter into the plants through wounds and natural openings, causing the formation of galls in dicotyledonous species. These tumors are induced by expression of genes coded by the Ti (tumor inducer) plasmid DNA, which contains a small segment of DNA (the T-DNA) that is transferred to the plant cell and stably integrated into the plant's chromosomes. The T-DNA is flanked on the left and right borders by 23-25-bp direct repeats, which function as *cis* elements essential for transfer of any DNA sequence located between them. In the disease crown gall, this transfer would include the native genes that encode synthesis of hormones such as auxins and cytokinins, which promote abnormal cell growth and induce tumor formation. Thus, any DNA sequence flanked by the right and left T-DNA direct repeats would be transferred. In addition to the T-DNA, the Ti plasmid also contains a set of *vir* genes (*vir* regions *A* to *G*) responsible for excision and transfer of the T-DNA. This transfer mechanism has been adapted such that *A. tumefaciens* is an excellent biological vehicle to introduce foreign DNA into plants without major mechanical damage. The first major application was to develop transgenic plants that would express a great diversity of transgenes. Excellent detailed reviews about the

biology, genetics, and the molecular mechanisms behind *Agrobacterium* – host cell interactions are published by Zupan and Zambryski (1995), Tzfira and Citovsky (2003), and Gelvin (2008, 2009).

Several advances have been made through the years to improve the use of *A. tumefaciens* for delivery of transgenes into plants. These improvements include the development of binary cloning vectors for non homologous recombination, the use of the 35S constitutive promoter of CaMV in the binary vectors, wider host-range plasmids for replication in *E. coli* and *Agrobacterium*, the insertion of multiple restriction enzyme sites for cloning, and more efficient and less toxic selectable markers. All of these developments have significantly improved transformation techniques using *Agrobacterium* such that it is now routinely used in many areas of plant biology and biotechnology, not only in dicotyledonous, but also monocotyledonous species (An et al. 1985; Lazo et al. 1991; Cheng et al. 1997, Lee and Gelvin, 2008).

The analysis of the molecular mechanisms involved in T-DNA transfer and integration have been identified as a transkingdom capacity. For example, the Type IV Secretion System (TFSS) was originally characterized in *A. tumefaciens*, but it is now known to be present in various human pathogens such as *Legionella pneumophila* (legionnaire s disease), *Bartonella henselae* (cat-scratch disease), *Brucella* species (brucellosis) and *Helicobacter pylori* (peptic ulcer) (Valentine et al. 2003). Several translocated proteins are the effectors that mediate disease advance, and their transfer has similarities with that of the T-DNA. The non-homologous recombination of the T-DNA allows it to be integrated randomly into the genomes of not only plants, but also other organisms such as yeast (*Saccaromyces cerevisiae*). A very complete review about the

activities required for inter-kingdom macromolecular transfer involving *A. tumefaciens* and plant cells is presented by McCullen and Binns (2006).

Although the stable expression of foreign genes was a major advance for plant biology, the production of transgenic plants remains difficult for many species; it can be very slow and costly, and the inserted gene can be subject to positional effects when integrated into the plant's chromosomes. Consequently, there has been a need for good transient gene expression systems because they could be faster and less expensive than stable transformation. Previous studies successfully used isolated protoplasts for the introduction of genes to study gene expression in a transient manner (Leon et al. 1991), but this single cell system had limitations. A second method involved the bombardment into plant tissues of particles covered with binary plasmids containing the gene of interest under the control of a constitutive or inducible promoter. However, mechanical damage, the need for visible markers, high variability between assays, and low efficiency of expression (Schenk et al. 1998), led to the development of a system for transient expression based on *Agrobacterium*, allowing gene expression only in those cells that were colonized by the Agrobacteria.

The development of a transient gene expression system based on *A. tumefaciens* has resulted in a rapid, flexible and reproducible approach to high-level expression of useful proteins. In this system, the disarmed strain of *A. tumefaciens* transformed with the binary plasmid containing the gene of interest is grown in a sugar-rich culture medium at pH 5.4 – 5.6 until an O.D of 0.5 to 2.0. Then, the *vir* genes are induced by acetosyringone, a phenolic compound that simulates phenolics produced from wounds in plants. The transformed *Agrobacterium* is infiltrated inside the intact leaf tissue using a

vacuum or by injecting the suspension using a needleless syringe; bacterial cells will be distributed inside the plant tissue layers in the intercellular spaces. From there, the bacterial transfer machinery specified by the *vir* genes will deliver the transgene on the T-DNA into the plant cell for integration into the chromosome. Transformed cells will be reprogrammed for transcription of the new gene, and translation of the mRNA to obtain the encoded protein (Zupan and Zambrisky, 1995; Escudero and Horn, 1997). One of the first reports using *Agrobacterium* mediated transient expression was done by Rossi et al. (1993), who expressed an *uidA* gene construct in *N. tabacum* and evaluated the ß-glucoronidase (GUS) activity in the infiltrated tissues. Kapila et al. (1996) also evaluated this GUS gene in intact leaves of bean (*Phaseolus vulgaris*) plants.

When using the agroinfiltration technique, it is important to remember that *A. tumefaciens* is a plant pathogen that induces a diverse set of plant genes involved in local and systemic defense responses, such as genes in the salicylic acid pathway, including pathogenesis related proteins; although the *A. tumefaciens* strains are "disarmed", they still are invasive bacteria and deliver effector proteins into the cell, among other side effects (Ditt et al. 2001; Pruss et al. 2008; Santos-Rosa et al. 2008). In addition, *Agrobacterium* requires a large set of genes for mediating transformation mechanisms, and it also is able to suppress some defense responses in its goal to successfully infect the host (Veena et al. 2003; Li et al. 2005).

Nonetheless, agroinfiltration has been very useful for studying the function of several pathogen Avr genes and plant R genes against fungi, bacteria, nematodes, and viruses. Agroinfiltration can be used to express or co-express R and/or Avr genes to characterize their roles in elicitation of defense responses. Based on the "gene for gene"

model, a resistance response such as HR can be obtained in three different ways: agroinfiltration of the Avr gene into a resistant host (either naturally resistant or transgenic for the R gene), agroinfiltration of the R gene into a transgenic plant that expresses the Avr gene, and co-agroinfiltration for expression of both R and Avr genes in a susceptible or neutral host. One of the first applications of agroinfiltration for plant pathology involved Van denAckerveken et al. (1996), who agroinfiltrated a T-DNA construct expressing the *Xanthomonas campestris* pv. *vesicatoria* avirulence gene *avrBs3* into pepper plants carrying the corresponding resistance gene *Bs3*, and found that the infiltrated tissue developed an HR. Erickson et al. (1999) demonstrated that the helicase domain P50 of the TMV replicase protein was responsible for eliciting HR in *N. tabacum* plants containing the *N* gene. They confirmed their results by developing transgenic plants that expressed P50 and crossed them with plants containing the *N* gene; seedlings derived from these crosses developed HR lesions.

A good example of the versatility of the agroinfiltration technique can be seen in Bendahmane et al. (2000), who evaluated candidate R genes for their reaction against PVX. They confirmed the identity of the *Rx2* gene based on the HR that developed upon agroinfiltration of *Rx2* into *N. tabacum* plants transgenic for the PVX coat protein. The system was practical, easy, and fast; it allowed the testing of numerous homologs of *Rx2*, which resulted in the cloning of another R gene that recognized the PVX coat protein, the *Rx1* gene. Similarly, Van der Hoorn et al. (2000) agroinfiltrated the *Cladosporium fulvum* avirulence genes *Avr9* and *Avr4* into leaves of tomato plants carrying the matching R genes *Cf-9* and *Cf-4*, an interaction that resulted in HR response. These authors confirmed the agroinfiltration results by stably expressing the R and Avr genes in

transgenic *N. tabacum*, and demonstrating their functionality after agroinfiltration of their Avr or R counterparts. Interestingly, the authors tested the agroinfiltration of *Avr/Cf* genes pairs in different plant families to identify which would respond with HR. Consequently, it was possible to identify conservation of signal transduction pathways required for defense responses. Palanichelvam et al. (2000) showed by agroinfiltration that the gene VI product (P6) of CaMV strain W260 was responsible for eliciting HR in *N. edwardsonii* and cell death in *N. clevelandii*, whereas the P6 protein of the CaMV strain D4 did not elicit HR. In addition, the authors demonstrated that the CaMV P6 protein elicited the HR, because a frame shift mutation after the start codon of gene VI affected protein synthesis and abolished the HR (Palanichelvam et al. 2000). These results confirmed previous results reported by Schoelz et al. (1986) and Kiraly et al. (1999). In those studies, the authors used gene swaps between virulent and avirulent strains of CaMV to develop chimeric viruses.

Agroinfiltration has also been very useful for understanding gene silencing mechanisms in plants. Agroinfiltration of transgenes was shown to trigger the silencing of homologous sequences in the host, resulting in a decrease in the expression of both the transgene and host homolog due to degradation of the mRNA. Transgene-mediated silencing by numerous vectors containing sense, antisense or hairpin sequences allowed the study of the functions of many genes (Wesley et al. 2001). One of the first examples involving agroinfiltration as a silencing delivery system was described in Schöb et al. (1997), who determined sequence requirements for transcriptional gene silencing of a *N. sylvestris* class I chitinase A gene. The author also confirmed the PTGS effect on

transiently expressed transgenes of *GUS* that is targeted for degradation a few days after expression.

Infectious DNA clones of RNA and DNA viruses have been infiltrated into target hosts in a technique that is called agroinfection or agroinoculation. The infectious clone is inserted into the T-DNA of an *Agrobacterium* binary vector; then, it is delivered into plant cells during the normal *Agrobacterium* T-DNA transfer process. The transferred DNA is subsequently transcribed, and the viral RNA can replicate and spread systemically. Viruses delivered in this manner have also been modified to express a foreign gene of interest such as GFP (Scholthof et al. 1996, 2002; Shivprasad et al. 1999; Komarova et al. 2006; Lindbo, 2007ab). The integration of transcriptional and PTGS concepts, agroinfiltration and agroinoculation, and the development of plant viruses as vectors for expression of foreign genes have all contributed to the concept of virus-induced gene silencing (VIGS). VIGS has been used to study the functional genomics of numerous genes in a rapid manner. The most commonly used VIGS vectors are based on TRV and TMV viruses (Baulcombe, 1999; Ratcliff et al. 2001; Burch-Smith et al. 2004; Robertson, 2004; Lindbo, 2007ab; Zhu and Dinesh-Kumar, 2008).

A modification of agroinfiltration delivery for VIGS was developed by Ryu et al. (2004). In this variation, called "agrodrench", the soil adjacent to the roots of the plants is saturated with a suspension of *Agrobacterium* containing VIGS vectors such as TRV-based vectors. The technique was successfully demonstrated for silencing of the phytoene desaturase gene (PDS) in several species of the Solanaceae. The authors argued that this system would be less laborious for large scale experiments than the infiltration of leaves using either a syringe or vacuum.

Agroinfiltration was also used to characterize plant virus suppressors of gene silencing. Ectopic expression of a gene of interest is limited to 3 - 5 days after agroinfiltration due to PTGS. The plant is able to target foreign transgenes as invasive species of RNA and degrade them in a few days. However, co-expression of the gene of interest with a viral-encoded silencing suppressor such as TBSV P19 can prevent the action of PTGS. Voinnet et al. (2003) showed that co-agroinfiltration of TBSV P19 with GFP could prolong and increase the expression of GFP in *N. benthamiana* plants, enhancing expression 50- fold or more in comparison to plants agroinfiltrated with only GFP.

Some studies have taken advantage of transient expression using vacuum agroinfiltration of flower buds to develop *in planta* transgenic tissues, including seeds that later are germinated to obtain transgenic plants without the use of sterile conditions or plant regeneration (Tague and Mantis, 2006). Zottini et al. (2008) agroinfiltrated leaves with a GFP construct and then developed *in vitro* grapevine plantlets. They were able to regenerate successfully stable transgenic plants from leaf tissues.

Several agroinfiltration protocols have been developed to take into account different promoters, marker genes, *Agrobacterium* strains, and binary vectors. Agroinfiltration has also been demonstrated in a diverse range of plant species, including lettuce, tomato, *Arabidopsis*, *Nicotiana* species, pepper, cotton, grapevine, etc. (Voinnet et al. 2003; Wrobledowski et al. 2005; Joh et al. 2005; Lee and Yang, 2006; Cazzonelli and Velten, 2006; Wydro et al. 2006; Sheludko et al. 2006; Santos-Rosa et al. 2008; Zottini et al. 2008; Ma et al. 2008, D'Aoust et al. 2009). Based on the results of these studies, it is clear that many factors affect the efficacy of agroinfiltration, including plant

species and cultivar, age or developmental stage, organ to be infiltrated, nutrition and growing conditions, temperature, and light. In addition, conditions related to the culturing of *Agrobacterium*, such as strain, media composition, pH, growth rate and optical density, induction period, acetosyringone concentration, presence of osmotic agents and detergents may also affect the success of the agroinfiltration experiment. Finally, physical parameters such as the infiltration method (i.e. the amount of pressure developed in vacuum infiltrations) will also affect the outcome.

Plants can be considered biofactories, and agroinfitration has the potential to be used as a delivery system for massive production of recombinant proteins such as vaccines for the pharmaceutical industry. However, many issues still are being debated about the reliability and efficiency of this process. On the positive side, plants are cheap to produce, most technology to grow plants is available, and the expression systems are fast and amenable to the extraction of the product. In addition, it is possible to scale production to industrial levels and there is less of a risk of contamination with plants than with animal or yeast systems (Joh and Vander Gheynst, 2006; Mett et al. 2008; Rybicki, 2009). For example, Joh et al. (2005) evaluated the capacity to produce GUS in lettuce after vacuum infiltration, and demonstrated that GUS represented 1.1% of total soluble protein after 72 hours. Negrouk et al. (2005) agroinfiltrated and expressed in lettuce the recombinant antibody IgG1 k anti-tissue factor (hOAT) to a level of 20-80 mg per gram of tissue in less than a week. Similarly, Li et al. (2007) produced in lettuce the active human interferon-beta, a type of interferon that is associated with responses to viral infections in humans and is used for clinical treatment against multiple sclerosis. The authors claimed that it was the first report of transient expression and production of a

biologically active therapeutic protein in lettuce. A complete evaluation and mathematical modeling of the biological and physical variables for vacuum agroinfiltration in lettuce was done by Simmons et al. (2007).

Another agroinfiltration approach for production of pharmaceuticals in plants that has been successful uses viral replicons such as TMV for expression of the target gene, taking advantage of the large area that can be infiltrated with the bacteria. A virus replicon can lead to faster and higher expression than delivery of just a transgene under the control of a strong promoter such as the 35S promoter of CaMV (Marillonet et al. 2004, 2005; Gleba et al. 2007). For example, Sheludko et al. (2006) showed that a 35S-GFP construct yielded a level of GFP protein that was 3.8% and 2.0% of the total soluble protein in *N. benthamiana* and *N. excelsior*, respectively. A TMV vector was able to produce an amount of GFP in *N. benthamiana* that was 16% of the total soluble protein and an amount of GFP in *N. excelsior* that was 63% of the total soluble protein. This study illustrates the effect that a viral vector can have on target protein expression, but also the effect that a host can have on target protein expression, as *N. excelsior* was a better host for expression than *N. benthamiana* when a TMV-based system delivered by agroinfiltration was used.

**General Aspects about *Nicotiana* species**

The *Nicotiana* genus is the fifth largest of the Solanaceae family, containing approximately 75-77 species grouped into 14 taxonomic sections. The origin of the

genus has been considered to be located in the central part of South America, a large region covering the countries of Ecuador, Bolivia, Peru, Chile, Argentina, Paraguay, Uruguay, and Brazil. However, there are native species from North and Central America, as well as some species from Australia. Interestingly, no species has been reported from the northern area of South America or the southern area of Central America. Overall, 75% of the species are native to the Americas and 25% are native to the Australasian continent, including Polynesia in the south pacific. There is one unique species that has been found in Africa, *N. africana*, which is closely related to the Australasian species. All of the Australasian species, a total of approximately 20 species, have been grouped into the Suaveolentes section. The Australasian species are thought to have been derived from an ancient ancestor in South America. This theory is based on the number of their chromosomes.

There is considerable morphological and cytogenetic diversity within the genus, which is reflected in the number of chromosome pairs or ploidy level ($n$), including 40 diploids ($2n=2x$) and 35 allopolyploids ($2n=4x$), where $n$ may equal 9, 10, 12, 16, 18, 19, 20, 21, 22, and 24 chromosomes. It has been hypothesized that the basic number of chromosomes ($x$) for the ancestor of *Nicotiana* is 12 chromosomes, ($2n=2x=24$). This is based on common duplication and hybridization events between two ancient progenitors with $n$ =6 pairs of chromosomes, and the most frequent number ($2n=2x=24$) present in currently known species. The majority of the species contain $n$=12 and 24 pairs of chromosomes. Excellent monographs about the origin and evolution of the genus, as well as complete phylogenetic studies of the entire *Nicotiana* genus, were published by

Goodspeed (1947, 1954), Aoki and Ito (2000), Chase et al. (2003), Knapp et al. (2004), and Clarkson et al. (2004).

During the last two decades, phylogenetic studies of *Nicotiana* evolved as well, incorporating physiological and molecular analyses to update the evolution and genetic relationships among species. For instance, Bogani et al. (1996) compared morphogenetic studies and random amplified polymorphisms in DNA (RAPDs) to differentiate 43 species representative of 13 sections, confirming most of the phylogeny proposed by Goodspeed (1954), including the hypothesis about divergent Petunioid and Cestroid ancestors of *Nicotiana* complexes. Molecular markers of both nuclear or organellar genes were used to examine the phylogeny of *Nicotiana* species. Aoki and Ito (2000) used the chloroplast maturase K gene (*mat*K) to resolve phylogenetic relationships within the genus. They analyzed 39 species from 13 sections. By using a chloroplast gene instead of a nuclear gene, the authors avoided confusion due to the presence of parental sequences in those amphidiploid species. The authors also uncovered some interesting hypotheses concerning the donation of maternal genomes from one species to another including *N. sylvestris* to *N. tabacum*, *N. paniculata* to *N. rustica*, and *N. trigonophylla* to *N. bigelovii*. Aoki and Ito (2000) suggested that the North American species resulted from three independent transcontinental migrations, in contrast to the Australasian species that could have derived from a unique migration of a South American ancestor through Antarctica or through the southern pacific islands.

An example of the power of cytogenetic and molecular tools for reevaluating the phylogeny of almost the entire genus is presented by Chase et al. (2003), who used the internal transcribed spacers (ITS) of nuclear ribosomal DNA and genomic *in situ*

hybridization (GISH) to analyze 66 out of 77 *Nicotiana* species. The authors confirmed most of the previously discovered phylogenetic relationships and organizations; however, some differences arose, especially in some of the hybridizations that lead to the creation of amphydiploid species. Clarkson et al. (2004) published the best supported and complete phylogenetic analysis currently available for *Nicotiana* species, a phylogeny that is illustrated in Figure I.2 according to the authors. They used plastid DNA regions from all 75 species, analyzing the loci *trnL* intron and *trnL*-F spacer, as well as the *trnS-G* spacer, and they included data from the *mat*K and *ndh*F genes as well. They also compared the sequences with outgroups and related genera within the Solanaceae family. The authors found that most of the phylogeny is consistent with previous studies for the diploid species. In addition, their results traced ancestors or donors for most of the allopolyploids, also finding relationships among progenitors of entire tribes. Finally, these authors linked the historical, morphological, and molecular analysis with the biogeographical origin of *Nicotiana* species.

Studies have tried to determine the age of the *Nicotiana* genus based on a molecular clock, and estimated the time when *Nicotiana* separated from the genera *Symonanthus* and *Petunia*. According to Clarkson et al. (2005), 15.3 million years (Myr) would be the appropriate estimation. In addition, the age of the section Repandae is estimated at 4.5 Myr, and the ages of the individual species *N. repanda* and *N. sylvetris* are estimated at 1.1 Myr and 4.5 Myr, respectively. Also, it is clear that the formation of allotetraploids such as *N. tabacum*, *N. rustica*, and *N arentsii* is recent, approximately 0.2 Myr based on *N tabacum*. Lim et al. (2007) adjusted the molecular clock by performing genomic *in situ* hybridization (GISH) and analyzing the tandem repeats NNES, HRS60

and NPAL to suggest a sequence of events that led to a near complete genome turnover in allotetraploids within 5 Myrs. Some of their estimations confirmed the results of others; in particular, they estimated ages of approximately 0.2 Myr for *N. tabacum*, 1.0 Myr for *N. quadrivalvis* (ancestors *N. obtusifolia* and *N. attenuata*), and 4.5 Myr for *N. nesophila* (ancestors *N. sylvestris* and *N. obtusifolia*). Older allotetraploids were estimated to be 4 to 5 Myr old.

Other studies examined the relationships and ancestors or donors of *Nicotiana* species. Confirmation of the ancestor of *N. rustica* (2*n*=4*x*=48) was obtained by Matyasek et al. (2003) and Lim et al (2005), after an analysis of ribosomal DNA subunits by GISH and FISH. They found that *N. paniculata* (2*n*=2*x*=24) contributes to *N. rustica* with the P subgenome and *N. undulata* (2*n*=2*x*=24) with the U subgenome. An RFLP analysis of the intergenic spacer sequence revealed that 80% of the sequences in *N. rustica* are derived from *N. undulata*. Repetitive sequences NPAMBE, NPAMBO, and NUNSSP confirmed the genome distribution. Kitamura et al. (2001) analyzed the chromosomal location of the 5S rDNA spacer sequence in 17 species from 7 sections, and suggested that *N. glauca* is closely related to species within the Alatae section that have 2*n*=18 chromosomes, a result that was not inferred previously. The comparative genomics of the section Alatae was also examined based on divergence of repetitive sequences such as HRS60, NP3R, and NP4R, in 35S and 5S nuclear ribosomal DNA, as well as an analysis of plastid DNA (Lim et al. 2006). The results supported a well-resolved phylogeny based on chromosome numbers, with clades grouping species with *n*=10 and *n*=9 separately.

Other studies examined the co-evolutionary relationships between *Nicotiana* species and their pollinators who are attracted by morphological characters such as floral fragrances, and nectar composition. Most of these studies were done with *Nicotiana* species within the Alatae and Suaveolentes sections (Kaczorowski et al. 2005; Raguso et al. 2006). Intriguingly, not only there is a phylogenetic explanation that links some of the sugars and volatile compounds found in these *Nicotiana* species with their specific pollinators (hummingbirds, moths, bees), but also there is a significant environmental component that contributes to specialization and selection of both plants and pollinators.

Due to the wide use of tobacco by ancient, indigenous civilizations in the Americas and Australasia, and the currently commercial impact of the tobacco industry, a high interest has been placed on studying the origin, genetics, and biochemistry of cultivated tobacco. *N. tabacum* is an allotetraploid ($2n=4x=48$ chromosomes); the subgenome S is derived from *N. sylvestris* ($2n=2x=24$) and subgenome T from *N. tomentosiformis* ($2n=2x=24$). Some researchers suggest that some *N. tabacum* regions are derived from *N. otophora*, but this is controversial. For example, Riechers and Timko (1999) studied genes encoding putrescine N-methyltransferase (PMT), a key enzyme in the pathways of nicotine and related alkaloids. They found that 3 out of 5 PMT members of *N. tabacum* aligned with PMT genes of *N. sylvestris*, and the remaining two with *N. tomentosiformis* and *N. otophora* PMTs respectively. These results about the origin of *N. tabacum*'s genome were supported by AFLP analysis as well (Ren and Timko, 2001), who analyzed genetic polymorphisms and evolutionary relationships among 46 lines of cultivated *N. tabacum* and seven wild species. They found AFLPs associated to the three proposed ancestors, and limited genetic diversity among tobacco lines.

However, several investigators suggested that *N. tabacum* is exclusively derived from *N. sylvestris* and *N. tomentosiformis*. Lim et al. (2000) used fluorescent *in situ* hybridization (FISH) based on sequence repeats, ribosomal DNA and geminivirus-like sequences to discriminate all species within the Tomentosae section, a section that contains *N. tomentosiformis* and *N. otophora*. They did not find strong evidence to support the presence of *N. otophora* genome sequences within the *N. tabacum* genome, contradicting the previous hypothesis about *N. tabacum*'s origin. Fulnecek et al. (2001) agreed on two ancestors, after studying the sequences and structure the 5S rDNA. Results from Kitamura et al. (2001) using the 5S rDNA spacer sequence also supported that *N. sylvestris* and *N. tomentosiformis* are the respective donors of subgenomes S and T of *N. tabacum*. Murad et al. (2002, 2004) traced the subgenome T of *N. tabacum* to a particular lineage, NIC479/84, within *N. tomentosiformis* by using repeated sequences with geminiviral origin from the GRD53 and GRD3 families. Interestingly, other sequences related to endogenous pararetroviruses (EPRVs), transposable elements, and retroelements that were found in *N. tabacum* were also detected in *N. sylvestrtis* and *N. tomentosiformis*; they were almost identical (Matzke et al. 2004).

Limited public information about genetic maps and genomes of tobacco species is available. Bindler et al. (2007) published the first linkage map of *N. tabacum* based on microsatellite markers developed under the Tobacco Genome Initiative (TGI). They studied 16 selected *N. tabacum* lines that were analyzed using 637 pairs of primers. 60% of these primers amplified only one single product from one of the two genomes (S and T), which was good. The map consists of 282 microsatellite markers and 293 mapped SSR loci and one phenotypic trait (flower color) on 24 linkage groups. The map, which is

still incomplete, covers 1,930 cM and is based on the size of both of the *N. tabacum* ancestors' genomes that are estimated to be 1,000 cM each. Similarly, Lewis et al. (2006) mapped quantitative trait loci (QTL) and AFLPs in 21 near isogenic tobacco lines, and found three QTLs that determine leaf number, plant height, and days to flowering; all three traits had been introgressed from *N. tomentosa*. A different type of study was done by Yang et al. (2007), who characterized 118 tobacco accessions using intersimple sequence repeats (ISSR) and inter-retrotransposon amplification polymorphism (IRAP) markers. They found low genetic diversity within and among cultivated tobaccos, but the analysis was sensitive enough to group accessions based on manufacturing quality traits, including flue-cured, sun/-air, burley, and oriental varieties. Moon et al. (2009) reported on the reduction in diversity in the flue-cured tobaccos by using 71 microsatellites and UPGMA analysis. They found that only 48% of potential alleles were detected in breeding materials used in the 1990s – 2000s, a clearly risky tendency towards homogenization as a consequence of very narrow breeding programs that started in the 1940s.

*Nicotiana* species are frequently chosen for study in plant pathology, and the conservation of genetic resources of these species is considered a high priority. The National Plant Germplasm System is responsible for the *Nicotiana* collection. It was transferred from the USDA/ARS to North Carolina State University, preserving seeds of at least 224 accessions representing 59 wild species and 4 created species, as well as more than 2,000 accessions of cultivated tobacco collected worldwide (Lewis and Nicholson, 2007). Species such as *N. benthamiana* and *N. tabacum* are considered plant models and are used in numerous studies (Goodin et al. 2008; Lewis and Nicholson,

2007). For example, Goodin et al. (2008) found 1,743 citations for *N. benthamiana* in the PubMed database in the period 1995 to November 2006, 38% of them registered during the year 2006 alone. Since the early stages of virology, *Nicotiana* species have been widely used as experimental hosts for studies on resistance or susceptibility, and *N. benthamiana* is considered an important model host. In the VIDE database presented by Brunt el al. (1996), approximately 30 *Nicotiana* species are reported for diagnostic purposes. In addition, a large range of *Nicotiana* species have been surveyed for their response to TMV, Tobacco etch virus (TEV), and potato viruses A, X, and Y (Holmes, 1936, 1946, 1951; VanDijk and Cuperus, 1989).

*Nicotiana* species have served as a source of resistance genes in a number of studies. Schoelz et al. (2006) surveyed the literature and found that *N. glutinosa* is resistant to at least 67 virus species from 20 genera, whereas *N. clevelandii* is susceptible to the same viruses. They demonstrated that R genes that target TMV and tombusviruses could be introgressed from *N. glutinosa* to *N. clevelandii*. Gwynn et al. (1986) introgressed resistance to TMV, *Globodera tabacum*, *Meloidogyne incognita*, and *Pseudomonas syringae* pv. *tabaci*, from the resistant parent *N. repanda* to the susceptible *N. tabacum*. The *N* gene was introgressed from *N. glutinosa* into *N. tabacum* using *N. africana* as a bridge plant under the concept of "designer chromosome" (Lewis et al. 2001). In addition, resistance to potato virus Y was transferred from *N. africana* to *N. tabacum* (Lewis, 2005). In other studies, molecular markers were developed to identify R genes in *Nicotiana* species. Julio et al. (2006) developed SCAR markers derived from *N. debneyi* that are linked to resistance to *Peronospora tabacina*, potato virus Y, and *Chalara elegans*. Lewis et al. (2005) characterized *N. glutinosa* chromosome segments in

flue-cured *N. tabacum* in which the *N* gene had been introgressed. Moon and Nicholson (2007) developed AFLP and SCAR markers linked to tomato spotted wilt virus (TSWV) resistance in *N. tabacum*, resistance that had been introgressed from *N. alata*, using as a bridge for genetic compatibility a hybrid between *N. tabacum* x *N. otophora*.

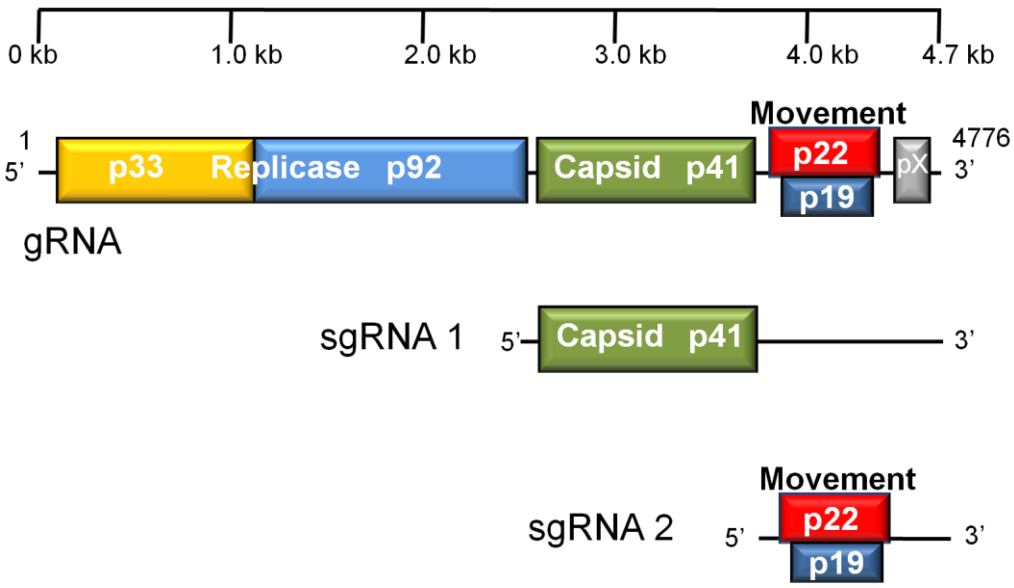


Figure I.1 Representation of the Tomato bushy stunt virus (TBSV) genome organization based on Hearne et al. (1990) and Yamamura and Scholthof (2005). Genomic RNA (gRNA) and the two subgenomic RNAs (sgRNA1 and sgRNA2) are illustrated containing individual genes and the coded proteins.

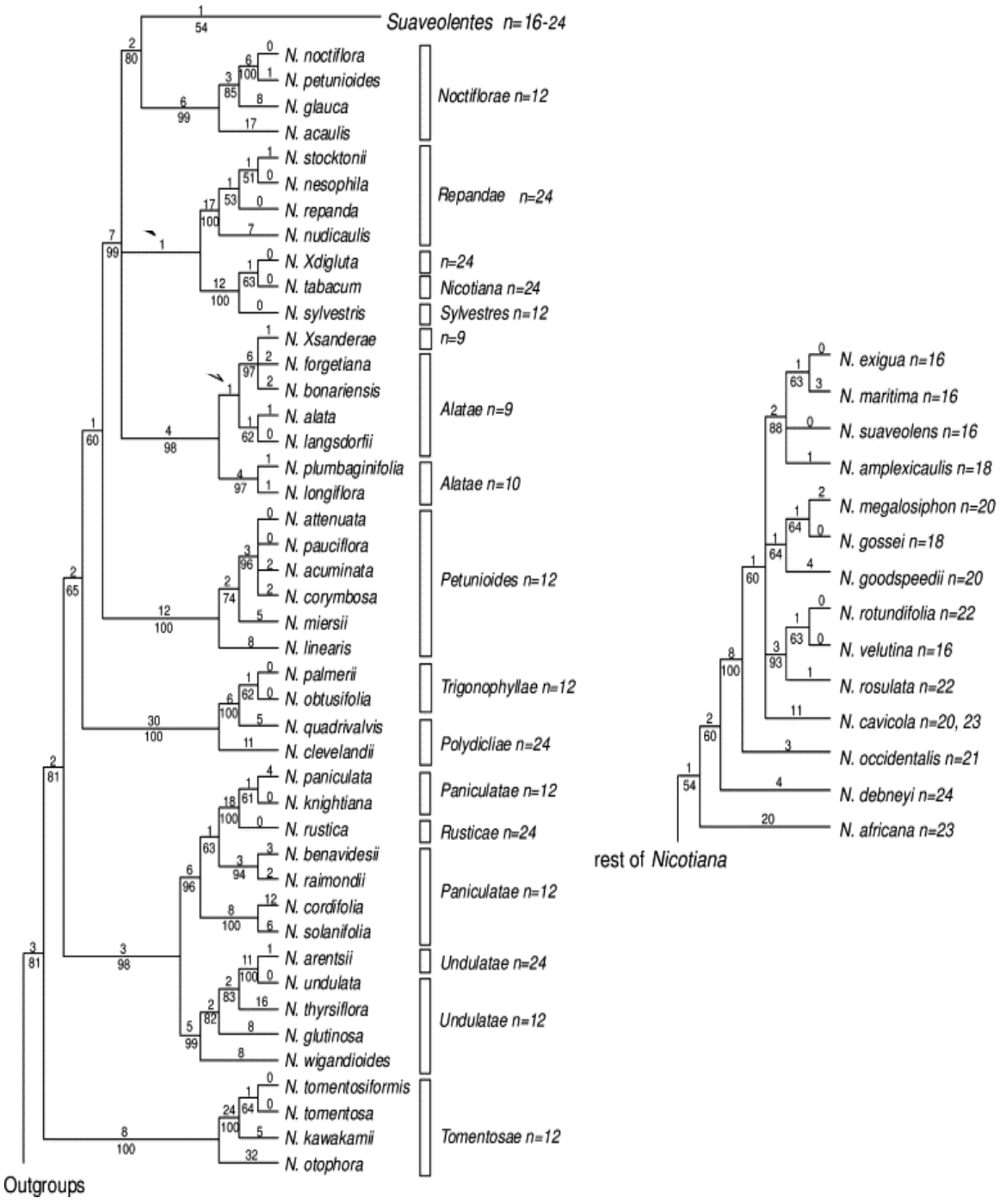


Figure I.2. Phylogeny of the genus *Nicotiana* (Solanaceae) taken from Clarkson et al. (2004), Mol. Phylogenet. Evol. 33: 75–90, with copyright permission from Elsevier. The tree corresponds to one of the most parsimonous trees from all taxon plastid DNA analyses, and the *Nicotiana* sections are grouped according to Knapp et al. (2004), cyted by the authors (Clarkson et al. 2004).

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# CHAPTER II

## CHARACTERIZATION OF THE *N* FAMILY OF RESISTANCE GENES IN *NICOTIANA* SPECIES*

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## ABSTRACT

The *N* resistance gene, a gene that confers resistance to tobacco mosaic virus (TMV), is a representative member of a family of nucleotide binding site (NBS) and leucine-rich repeats (LRR) proteins, which are present in *Nicotiana glutinosa*. Since *N. glutinosa* is resistant to a large number of viruses, we hypothesized that silencing of the entire *N* family of R genes might alter resistance to viruses other than TMV. To test this hypothesis, we constructed transgenic plants that expressed a hairpin RNA based on exon 1 of the *N* gene and found that they exhibited enhanced susceptibility to tomato bushy stunt virus (TBSV) as well as TMV. Consequently, we have evidence that a TBSV resistance gene has sequence similarity to the *N* resistance gene. However, there is limited information about how many *N* gene homologs exist in *N. glutinosa*. To examine the diversity of *N* resistance gene homolog sequences (NGHs), we developed polymerase chain reaction (PCR) primers that amplified a 516 bp DNA segment that matched exactly

the exon 2 of the *N* gene from *N. glutinosa*, the TMV-susceptible host *N. clevelandii*, and *N. clevelandii* Line 36, a homozygous addition line that is resistant to TBSV and susceptible to TMV. These PCR reactions yielded multiple products that flanked the P-loop and GLPL domains of NBS-LRR resistance proteins. We cloned and sequenced 143 NGHs, and found that 106 of them were complete open reading frames without premature stop codons. Based on alignments and phylogenetic analyses we detected a broad range of groups: 11 in *N. glutinosa*, 4 in *N. clevelandii*, and 5 in *N. clevelandii* Line 36. One group from *N. glutinosa* matched precisely the *N* gene sequence as expected, whereas other groups differed from the *N* gene by approximately 2 to 19%. Interestingly, a group of NGHs from *N. clevelandii* matched the *N* gene completely, suggesting that an inactive *N* homolog could be present in this TMV-susceptible species. Of the groups isolated from *N. clevelandii* Line 36, none had greater than 89% identity with the N protein. After bootstrapping analysis we consolidated 15 groups of NGHs including the *N* gene group. We hypothesized that the TBSV resistance gene will be represented in the groups isolated from *N. clevelandii* Line 36, but the evidence suggests that new mining should be done considering that all *N. clevelandii* groups fell into the *N. glutinosa* groups, and the presence of individual outliers in both *N. glutinosa* and *N. clevelandii* Line 36 genotypes.

# INTRODUCTION

One of the cornerstones of plant defenses is encapsulated in the "gene for gene" theory (Flor, 1971), in which a plant resistance (R) gene product recognizes a specific pathogen avirulence (Avr) gene product to trigger a cascade of defense responses to limit the pathogen advance. Frequently, the interaction between R and Avr genes results in a hypersensitive response (HR), which is characterized by the rapid death of a limited number of cells in the region of the invading pathogen (Lam et al. 2001). However, newer theories such as the "guard" hypothesis (Van der Biezen and Jones, 1998), the "zig zag" model (Jones and Dangl, 2006), and the "decoy" model (Van der Hoorn and Kamoun, 2008) have updated and explained in a different and more complex manner the plant's immune response against pathogens, especially fungi and bacteria. However, for all of these models, R genes have a central role.

R genes are shared between monocotyledonous and dicotyledonous plants, are functional against pathogens of different taxa, and are commonly grouped into five classes depending on the protein structure and their recognition mechanism (Dangl and Jones, 2001). However, the most important and frequent class of R gene is comprised of proteins with a nucleotide binding site (NBS) and a variable set of leucines called a leucine reach repeat (LRR), at the C terminal region. These NBS-LRR proteins are extremely variable and numerous in plant genomes. For example *Arabidopsis thaliana* has 149 NBS-LRRs, rice (*Oryza sativa* L.) approximately 600, and *Medicago truncatula* and poplar both have approximately 400 (Meyers et al. 1999, 2003; Cannon et al. 2002;

Goff et al. 2002; Richly et al. 2002; Ameline-Torregrosa et al. 2007; Kohler et al. 2008). Depending on the domains at the N-terminal region of the protein, the NBS-LRR proteins are divided into two groups: the TIR-NBS-LRR (TIR for homology with the *Drosophila* Toll and mammalian Interleukin Receptor gene), and the CC-NBS-LRR (CC for alpha-helical Coiled-Coil motif) before the NBS (Meyers et al. 1999; Pan et al. 2000b). The NBS regions of cloned CC and TIR resistance genes contain several highly conserved motifs: the P-loop or kinase-1, kinase-2, kinase-3a, and the kinase-3 or "hydrophobic domain GLPL". Both the P-loop or GGVGKTT domain and the GLPL domain are considered the most important for the analysis of diversity of NBS-LRR proteins, based on currently characterized R genes against fungi, bacteria, nematodes, and viruses (Baker et al. 1997; Hammond-Kosack and Jones, 1997; Meyers et al. 1999, 2003; Cannon et al. 2002).

Although numerous R genes against plant viruses have been reported by traditional genetic and breeding methods, only a dozen have been physically cloned and their structure elucidated (Kang et al. 2005; Soosaar et al. 2005), and most of them are CC-NBS-LRR or TIR-NBS-LRR proteins. For instance, the R genes *Rx1*, *Rx2*, *Sw5*, *RCY1*, *HRT*, and *Tm2-2* have a CC domain (Martin et al. 2003; Kang et al. 2005; Soosaar et al. 2005), and the *N* and *RT4-4* genes have a TIR domain (Whitham et al. 1994; Seo et al. 2006). The *N* gene is an R gene derived from *N. glutinosa* (Holmes, 1938; Dunigan et al. 1987); its protein product recognizes the helicase domain (P50) of the replicase protein of tobacco mosaic virus (TMV) (Erickson et al. 1999).

The *N* gene was the first virus R gene to be cloned (Whitham et al. 1994), and has been intensely studied. Several other genes in the defense signaling pathway required for

*N*-gene mediated resistance have been characterized (Liu et al. 2002, 2004), and more recently a chloroplast protein NIP1 (for N –Interacting Protein 1) has been identified and shown to be associated with both the N protein and the TMV p50 protein in the cytoplasm and nucleus (Caplan et al. 2008). These last results suggest that the *N* gene mediated resistance mechanism can be explained by the "guard hypothesis" model, involving an indirect interaction between the host N protein, the NIP1 protein, and the viral TMV P50 protein. However, many details remain to be elucidated.

Our lab has been interested in studying resistance to tombusviruses in *Nicotiana* species, and many elements of this interaction conform to the "gene for gene" model. On the pathogen side, previous studies have shown that the tombusvirus tomato bushy stunt virus (TBSV) cell-to-cell movement protein P22 triggers HR in *N. glutinosa* and its related amphiploid species *N. edwardsonii*, whereas the TBSV long distance movement/silencing suppressor protein P19 triggers HR in *N. tabacum* (Scholthof et al. 1995ab). On the host side, we showed that a dominant R gene against tombusviruses coud be introgressed from the resistant species *N. glutinosa* into the susceptible species *N. clevelandii* (Schoelz et al. 2006). We developed *N. edwardsonii* var. Columbia as a bridge (Cole et al. 2001), to move an extra pair of chromosomes from *N. glutinosa* to *N. clevelandii*, and obtained an *N. clevelandii* addition line (Line 36) homozygous for resistance to the tombusviruses TBSV, cucumber necrosis virus (CNV), cymbidium ringspot virus (CymRSV), and carnation italian ringspot virus (CIRV). Although *N. glutinosa* is also the source of the *N* gene, *N. clevelandii* Line 36 was susceptible to TMV, showing that the *N* gene segregated independently from the tombusvirus R gene (Schoelz et al. 2006). Furthermore, we showed that silencing of the *N* family of resistance

genes compromises the HR to the tombusviruses TBSV and CymRSV (Balaji et al. 2007), suggesting that the R gene against the tombusviruses could be a member of the *N* gene family.

We are now interested in cloning the tombusvirus R gene from *N. glutinosa*. However, there is no information available about the size of the *N* family of R genes in *N glutinosa*. Furthermore, very little is known about *N* gene homologs in any *Nicotiana* species. Four genes have been reported to be closely related to the *N* gene. The *NL-25* and *NL-27* genes were isolated from *Solanum tuberosum*, and have been associated with resistance to the soil-borne fungus *Synchytrium endobioticum* (Hehl et al. 1999). They have 76% and 75% nucleotide identity to the *N* gene, respectively. The *Y-1* gene, a TIR-NBS-LRR gene also isolated from *S. tuberosum*, is associated with resistance to potato virus Y (Vidal et al. 2002); it has 79% nucleotide identity to the *N* gene. The fourth sequence to be shown to be closely related to the *N* gene is the *NH* gene, is an NBS-LRR gene isolated from *Nicotiana tabacum* cv. Xanthi (Stange et al. 2004) that is 83% identical to the *N* gene. The *NH* transcript level increased after inoculation of TMV strain Cg, and it was suggested that this implicates NH in the HR-like response, although neither functional evidences were provided nor further genetic studies have been reported to specify resistance to the pathogen. In fact, only the *N* gene has been proven to be a functional R gene.

Resistance gene homologs (RGHs) are portions of R genes that have been recovered from many plant species, and have been used to estimate the number of R genes in that species. Different strategies have been used to clone and analyze RGHs, including sequencing of genomic DNA or sequencing of clones in cDNA libraries.

However, the most commonly used method is a polymerase chain reaction (PCR)-based strategy that utilizes degenerate primers designed against two conserved sequence motifs within the NBS region: the P-loop and the hydrophobic domain GLPL (Kanazin et al. 1996; Leister et al. 1996). In contrast, it was shown that the LRR region seems to be under strong diversifying selection, so it is not attractive for analysis of RGHs (Meyers et al. 1998; Sun et al. 2001). The PCR-based approach targeting the NBS region was successfully applied in numerous plant species, such as soybean (Kanazin et al. 1996), potato (Leister et al. 1996), *Arabidopsis* (Aarts et al. 1998), wheat and barley (Seah et al. 1998), rice and barley (Leister et al. 1998), peanut (Bertioli et al. 2003), apple (Baldi et al. 2004), strawberries (Martinez et al. 2004), sunflower, lettuce, and chicory (Plocik et al. 2004), among many others.

In contrast to other studies in which degenerate primers were used to identify a broad range of RGHs, I chose PCR primer sequences which precisely matched the published *N* gene sequence (Whitham et al. 1994) in an attempt to pull out RGHs that were closely related to the *N* gene. These primer sequences allowed an assessment of the diversity of *N*-related sequences present in *N. glutinosa* and *N. clevelandii*, the two parents of *N. edwardsonii* (Christie, 1969; Cole et al. 2001), as well as *N. clevelandii* addition line 36 (Schoelz et al. 2006). This information allowed an estimate of the number of off-target genes affected when trying to silence the *N* gene in *N. edwardsonii*, as well as providing a list of candidates for the tombusvirus R gene.

## RESULTS

Nucleotide sequence analyses of both CC and TIR-NB-LRR resistance genes revealed several conserved amino acid stretches present within exon 2, including the motifs GGVGKTT and GLPLAL of the NBS domain. Degenerate primers based on these motifs were used successfully in numerous studies to amplify portions from true resistance genes and RGHs (Fig. II.1) (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996). However, to assess the diversity of RGHs related to the *N* gene in *Nicotiana* species, instead of degenerate PCR primers, we developed forward and reverse primers that precisely matched the *N* gene sequence (Whitham et al. 1994) (Fig. II.1). These primers might be expected to amplify only the *N* gene, but instead I found that they amplified a subset of RGHs that were closely related to the *N* gene. To distinguish the RHG sequences isolated in our study from the full range of RGHs isolated by degenerate primers, we designated our sequences as NHGs (for *N* Gene Homologs). We also compared our NGH sequences to other published *N*-related genes such as *NL-25* and *NL-27* (Hehl et al. 1999), *Y-1* (Vidal et al. 2002), and *NH* genes (Stange et al. 2004).

### Characterization of NGHs in *N. glutinosa*

I cloned and sequenced 77 NGHs from *N. glutinosa* genomic DNA. The clones varied in size from 498 to 516 bp, and had nucleotide sequences that were 81% to 100% identical to the *N* gene. Based on their sequence size and lack of stop codons, 45 NHGs

contained complete open reading frames (ORF), and therefore were selected for further analysis. The other 32 clones were considered to be pseudogenes and excluded. The 45 clones that had complete ORFs were aligned into 11 groups (Fig. II.2), each of which showed between 87% to 100% nucleotide identity and 79% to 100% amino acid identity to the *N* gene and protein, respectively. Group 11 contained NHGs that were identical to the *N* gene. Based on this analysis, we propose that *N. glutinosa* contains at least 10 RGHs that are closely related in sequence to the true *N* gene. They constitute the *N* family of RGHs. This number is in agreement with a previous estimate of 8 to 10 *N*-related RGHs in *Nicotiana* spp. (Whitham et al. 1994).

Although the PCR primers precisely matched the *N* gene sequence, the *N* gene itself was represented in only one-fifth of the NGH sequences, as this group contained only eight sequences derived from *N. glutinosa* (Fig. II.2). Interestingly, genes that were described previously as *N* homologs, such as *NL*-25, *NL*-27, and *Y-1* from potato (Hehl et al. 1999; Vidal et al. 2002), and *NH* from *N. tabacum* (Stange et al. 2004) were not closely associated with any of the 11 groups derived from *N. glutinosa* (Fig. II.2; Table II.1).

**Identification and analysis of NGHs in *N. clevelandii***

I cloned and sequenced 30 NGHs from *N. clevelandii*, all were 516 bp in size. An additional analysis revealed that 28 NGHs were complete ORFs and lacked premature stop codons. The nucleotide sequences of the 28 NGHs varied from 87% to 100% identity to the *N* gene, and the amino acid sequences varied from 79% to 100% identity to

the *N* protein. They were aligned into 4 groups, all of which could be placed within previously identified *N. glutinosa* NGH groups (Fig. II.2, Groups 1, 2, 4 and 11). Interestingly, the *N* gene group was one of the four that contained *N. clevelandii* RGHs (Fig. II.2, Group 11). Eleven of the *N. clevelandii* NGH sequences were aligned with the *N* gene group, four of which were identical to the *N* gene sequence, and 6 were identical to the N protein (data not shown). Although *N. clevelandii* is susceptible to TMV, the isolation of an NGH group that matched the *N* gene sequence indicated that this host might contain a form of the *N* gene inactivated through a point mutation at another location or through a premature termination of the downstream coding region, or there are polymorphisms that lead to a different specificity. As occurred with *N. glutinosa,* no NGH clones from *N. clevelandii* were closely associated with the previously published *NL-25, NL-27, Y-1*, and *NH* genes (Fig. II.2).

**Characterization of NGHs in the tombusvirus resistant *N. clevelandii* Line 36**

*N. clevelandii* Line 36 is an addition line that contains the full chromosome complement from *N. clevelandii* (2*n*=48) and an extra pair of chromosomes from *N. glutinosa* (Schoelz et al. 2006). Line 36 is homozygous for resistance to a broad range of tombusviruses (Schoelz et al. 2006) and susceptible to TMV. Previous results indicated that the tombusvirus R gene might belong to the *N* family of R genes. We found that *N. edwardsonii* plants in which the *N* gene was targeted for posttranscriptional gene silencing exhibited an abolished or delayed HR after inoculation of the tombusviruses TBSV and CymRSV (Balaji et al. 2007). Because the tombusvirus R gene is derived

from *N. glutinosa*, I hypothesized that an analysis of NGHs from Line 36, in comparison with NGHs from *N. glutinosa* and *N. clevelandii*. This might reveal which NGHs were derived from *N. glutinosa* and which were derived from *N. clevelandii* and might reveal the identity of the tombusvirus R gene.

I cloned and sequenced 36 NGHs from Line 36. The inserts had sizes from 513 to 517 bp and 33 encoded complete ORFs. The amino acid sequences of the 33 NGHs varied from 87% to 89% identity to the *N* protein. The alignment and bootstrapping analyses indicated that 30 out of the 33 Line 36 NGH nucleotide sequences fell within one of three major groups (Fig. II.3, Groups VI, VII and XIV). Groups VI and XIV are clearly derived from *N. clevelandii*, and consisted of Line 36 and *N. clevelandii* NGHs exclusively. Interestingly, the inclusion of Line 36 NGHs in the analysis with *N. clevelandii* and *N. glutinosa* NHGs altered the alignment such that three *N. clevelandii* NGHs, originally associated with *N. glutinosa* NGHs in Fig. II.2 (Group 1), became aligned into their own group (Fig. II.3, Group XIV). Similarly, Group VII was dominated by NGHs from Line 36 and *N. clevelandii*, but did contain one *N. glutinosa* NGH (Ng157). Two NGH clones, Line36-110, and Line36-60, were identified as outliers and formed their own groups (Fig. II.3, Groups V, VI, and XI). The amino acid sequences of Line36-110, and Line36-60, showed 89%, and 86% identity to the *N* protein, respectively (Table II.1). The final Line 36 clone, Line36-101, was aligned within Group XI, a group that contained three *N. clevelandii* NGHs and eight *N. glutinosa* NHGs (Fig. II.3). Similar to *N. glutinosa* and *N. clevelandii*, none of the NGHs isolated from Line 36 were associated with the previously published *NL-25*, *NL-27*, *Y-1*,

and *NH* genes; the closest was the NGH Line36-101, with 86% identity to the *NH* gene at the amino acid level (data not shown).

**Consolidated analysis of NGHs in *Nicotiana* species**

The consolidated analysis illustrated in Fig. II.3 includes all 106 NGHs isolated from *N. glutinosa*, *N. clevelandii*, and *N. clevelandii* Line 36 that coded for complete NBS domains without premature stop codons. It also includes the sequences of the *N* gene, as well as the previously published homologs *NL-25*, *NL-27*, *Y-1*, and *NH* for comparison. The phylogenetic tree and bootstrapping analysis (Fig. II.3) showed that the 106 sequences could be aligned into 15 groups, suggesting that the *N* gene has at least 14 homologs in the two *Nicotiana* species I analyzed. Out of the 106 NHGs, 98 were contained in 7 major groups, representing 93% of the samples. The other 8 NHGs were identified as outliers that formed their own groups (Groups I, II, III, V, VIII, IX, X, and XII).

One of the seven major groups clearly contained the *N* gene, and only 19 clones derived from both *N. glutinosa* and *N. clevelandii* had nucleotide sequences that differed from the *N* gene by less than 2%. The other 6 major groups contained respectively 12 NGHs (Group IV), 6 NGHs (Group VI), 24 NGHs (Group VII), 12 NGHs (Group XI), 12 NGHs (Group XIII), and 13 NGHs (Group XIV). Interestingly, most of these major groups had distinctive compositions. Groups VI, VII, and XIV were formed mainly by sequences from *N. clevelandii* and Line 36, whereas Groups IV, XI, and XIII were comprised almost exclusively of NGHs from *N. glutinosa*. In Groups IV and XIII, the

majority of sequences were derived from *N. glutinosa*, but each group also contained one NGH from *N. clevelandii*. Intriguingly, we did not isolate sequences from *N. glutinosa* or *N. clevelandii* that were closely related to the other published *N* homologs such as *NL-25*, *NL-27*, *Y-1*, and *NH* genes.

To examine differences in NGH composition at the amino acid level, I selected 21 NGHs: the NGH representative of individual groups (Groups I, II, III, V, VIII, IX, X, and XII), one randomly selected from group VI, and two NGHs from each of the other six major groups (Groups N, IV, VII, XI, XIII, and XIV), which represented the top and the bottom of each group, to investigate the differences among sequences contained within the same group. To these NGHs, I added the sequences of the N protein and its four published homologs, to complete a total of 26 representative sequences (Table II.1, Fig. II.4). Most changes are due to single amino acid substitutions, and were conserved across multiple groups. When sequences were analyzed at the amino acid level, I found that the 15 NGH groups were clearly separated, and there was a consistency in groupings between the nucleotide sequence analysis and the amino acid analysis. For example, I observed that groups defined by nucleotide sequences that differed by greater than 1% were also aligned into separate groups at the amino acid level. For instance, Line36-18 (Group VI) and Line36-21 (Group VII) differ from each other by 2% (Table 2.1), confirming the patterns originally developed at the nucleotide sequence level.

# DISCUSSION

A previous study showed that posttranscriptional silencing that targeted the *N* gene also abolished resistance to a tombusvirus R gene, suggesting that the tombusvirus resistance gene was related at the nucleotide sequence level to the *N* gene (Balaji et al. 2007). A second study had shown that the tombusvirus R gene was distinct from the *N* gene, because they segregated independently (Schoelz et al. 2006). Consequently, the goal of the present study was to characterize the diversity of *N*-related sequences in *N. glutinosa*, because it is the source for both the *N* gene and the tombusvirus R gene. In addition, I was also interested in examining the diversity of *N*-related sequences in the susceptible host *N. clevelandii*, because the tombusvirus R gene had been introgressed into *N. clevelandii* and it would be important to know if *N. clevelandii* carried any other *N*-related sequences.

My approach differed from most studies focused on the diversity and evolution of RGHs because we did not use degenerate primers. It might have been expected that I would only recover the *N* gene itself, but instead I isolated a restricted set of RGHs that were closely related to the *N* gene, RGHs which I designated NGHs. After editing and checking for premature stop codons, I found that 106 out of 143 NHGs contained ORFs, which would make them candidates to be true R genes. I found 10 homologs in addition to the *N* gene in *N glutinosa*, a number similar to the 8-10 estimated by Whitham et al. (1994) based on Southern hybridization for *N*-related sequences in *N. glutinosa*. Interestingly, *N. clevelandii*, the TMV-susceptible host, also contained four groups of

NGHs that were related to the NGHs found in *N. glutinosa*. When I added to the analysis the NGHs from Line 36, the total number of *N*-related groups increased to 15, including the *N* gene. There may be additional NGHs to be isolated based on the emergence of individual sequences as outliers from the genomic DNA of *N. glutinosa* and Line 36.

The number of RGHs that I cloned and sequenced approximated the numbers reported by others. For example, Couch et al. (2006) considered 70 sequences as a proper sample size to estimate the homologs of the *I2* gene family in *N. tabacum*, a non TIR-NBS-LRR gene that confers resistance to the fungus *Fusarium oxysporum* f.sp. *lycopersici*. The *I2* gene was originally described in *Solanum pimpinellifolium*. These authors used a PCR-based approach to estimate the number of RGHs at 21, but with Southern blot hybridizations the number was estimated to be 15 RGHs. Similarly, the number of *N* gene homologs reported in other species of the Solanaceae. For instance, Leister et al (1996) showed at least six PCR products with high identity to the *N* gene exist in potato. Trognitz and Trognitz (2005) performed a limited survey of RGHs in *Solanum caripense* (a relative of potato and tomato) using degenerate P-loop and GLPL primers and Southern blot hybridization. They found 3 groups of TIR homologs with an average of 50% (35%–99%) identity, including the *N* gene; and using Southern blot hybridization, they found another 16 homologs.

The alignment of the nucleotide and amino acids of the NGHs in this study illustrated the location of the polymorphisms in the NGH sequences. For example, the sequences were more conserved in the kinase-2 and kinase-3a motifs than at other parts of the sequence, and amino acid substitutions at these locations were mostly with similar amino acids (Fig. II.4). The nucleotide and amino acid variations between groups were

mostly caused by point mutations. An analysis of conserved motifs in the NBS domain are important because it is known that several loss-of-function alleles in R genes are due to point mutations within conserved motifs inside the NBS domain (Pan et al. 2000a). It seems that most of the variation in our NGHs is located between the RNBS-C motif and the GLPL motif, results that agreed with Li et al. (2006) and Yaish et al. (2004), who showed that polymorphisms of RGHs of ryegrass and lentils (*Lens* species) were higher in discrete regions around the Kinase-2 motif and near the GLPL motif respectively. However, it is valid to point out that monocotyledonous species such as ryegrass contain limited numbers or almost no TIR genes. All NGHs that we isolated were TIR genes based on the presence of conserved amino acids in specific motifs, such as F----FL--- at the RNBS-A motif, and the absence of a Tryptophan (W) residue at the end of Kinase-2 motif (KVLIVLDD–), which is characteristic of the TIR classes of NBS-LRR genes (Meyers et al. 1999; Cannon et al. 2002).

We also found well conserved sequences in the P-loop and GLPL motifs, in contrast to high variation reported by Cannon et al. (2002), and Bertioli et al. (2003) for TIR genes in peanut (*Arachis* spp. ) and *Arabidopsis*, likely because we did not use degenerate primers. The discriminatory effect of P-loop – GLPL specific primers is also reported by Trognitz and Trognitz (2005), with emphasis on the GLPL reverse primer. Primers targeting the P-loop and GLPL motifs seem to amplify many more TIR-NBS R genes than non-TIR-NBS R genes (Yu et al. 1996; Bertioli et al. 2003).

It is also important to recognize that not all 15 of the NGH groups may actually specify resistance to a plant pathogen. We do not know how many of these NGHs could be truly expressed, functional R genes or how many might be pseudogenes. Some studies

used NBS profiling of cDNAs to better estimate possible true NBS-LRR genes (Brugmans et al. 2008). This might be revealed by an analysis of nucleotide sequences upstream and downstream of the NBS sequences we characterized in our study. Furthermore, although NBS-LRR proteins have usually been associated with plant innate immunity (Meyers et al. 2003), they also have been associated with other metabolic processes such as drought tolerance, development, and photomorphogenesis, processes which may or may not be related to innate immunity (Tameling and Joosten, 2007).

One of our objectives was to identify possible candidates for a tombusvirus R gene, an R gene that might have emerged from an analysis of Line 36 in conjunction with *N. glutinosa* and *N. clevelandii*. My hypothesis was that if the tombusvirus R gene is truly a member of the *N*-family of R genes, then Line 36 should contain an R gene sequence derived from *N. glutinosa* that is not present in *N. clevelandii*. However, I did not anticipate that all four of the NGH groups present in *N. clevelandii* would also be found within *N. glutinosa*. Just as with the *N* gene, it may be that the tombusvirus R gene might have a nonfunctional homolog in *N. clevelandii*. If this were true, then an analysis of NGHs from Line 36 would not reveal a putative tombusvirus R gene. In addition, the analysis of Line 36 produced two NGHs, Line36-110 (Group V) and Line36-60 (Group XII), that could not be categorized into a previously identified group associated with either *N. glutinosa* or *N. clevelandii*. Consequently, more work will be necessary to determine the derivation of these outliers. However, my study remains valuable because it provides an initial estimate of the sequence diversity of NGHs in both *N. clevelandii* and *N. glutinosa*.

## MATERIALS AND METHODS

### Isolation of *N* gene Homologs.

All genomic DNA extractions from *N. glutinosa*, *N. clevelandii*, and *N. clevelandii* Line 36 leaves were made following the procedure described by Dellaporta et al (1983). To amplify *N* gene homologs by PCR, primers were designed to amplify a 516 bp DNA segment between the P-loop or kinase-1 domain (GGVGKTT) and the hydrophobic domain (GLPLAL) in the NBS region (Li et al. 2006). The primers precisely matched the *N* gene sequence (Whitham et al. 1994; Gene Bank Acc. # U15605). An *Eco*RI restriction site was added to the 5' end in the forward primer (5'-CCCCGAATTCGGGGGAGTCGGTAAAACAACA-3') and a *Kpn*I site added to the 5' end of the reverse primer (5'-CCCCGGTACCGAGGGCTAAAGGAAGGCC-3') to facilitate cloning. These primers were designed to amplify a DNA segment of the *N* gene beginning at nucleotide position 933 and ending at nucleotide position 1448. Primers were synthesized by Integrated DNA technologies (Coralville, IA).

### PCR conditions.

25 µL reactions were done using the following conditions: thermophilic DNA Polymerase buffer 1X, $MgCL_2$ 2.5 mM, dNTPs 0.4 mM, Forward and Reverse primers 0.4 µM each, DNA Taq Polymerase 1.25 units (Promega Corp, Madison, WI), and 200

ng genomic DNA template. The PCR reactions were run according to the following steps: Step 1 (94°C x 5 min; 55°C x 30 sec; 72°C x 2 min; 1 cycle); Step 2 (94°C x 30 sec; 55°C x 30 sec; 72°C x 2 min, 35 cycles), and Step 3 (94°C x 30 sec; 55°C x 30 sec; 72°C x 10 min, 1 cycle).

**Cloning and Sequencing.**

PCR products were size-separated on a 1.0% agarose preparative gel, and the appropriately sized band was extracted and purified using a Qiaquick DNA gel elution kit (Qiagen, Valencia, CA). Purified fragments were cloned into pGEM7ZF and pGEM-T-easy vectors following the manufacturer's instructions (Promega Corp, Madison, WI), and transformed into *E. coli* JM101 or DH5α competent cells (Invitrogen, Carlsbad, CA) for blue/white colony selection on LB agar medium supplemented with Ampicillin at 50 µg/ml. Randomly selected colonies with inserts were grown and screened by two sets of PCR reactions. In the first PCR screen, primers were used that amplified the M13 and T7, or SP6 and T7 borders of the pGEM vector. The second PCR reaction amplified the approximately 516 bp nucleotide sequence of the RGH. Plasmid preparations that contained RGH inserts were made by the STET procedure (Sambrook et al. 1989), and restriction enzyme digestions were used to confirm the insertion of the *N* gene DNA segment. The nucleotide sequence of RGH-positive clones was determined by sequencing the clone in both directions by the DNA Core Facility at the University of Missouri (Columbia, MO).

**Sequence and Phylogenetic Analyses.**

Sequences were manually edited, cleaned of vector and primer sequences, and initially compared using the NCBI BLAST-N (Altschul et al. 1990) and BLAST-2SEQ (Zhang et al. 2000) programs. All values were set to default and the low complexity filter was used. Identical clones were recovered and sequence errors in the homologs due to Taq polymerase were estimated to be equal to or less than 1%, in agreement with previous studies (Couch et al. 2006; Plocik et al. 2004). Verification of this estimation was done by cloning PCR products amplified by a high performance Taq-Plus Precision DNA polymerase (Stratagene, La Jolla, CA) following the manufacturer instructions, with the following PCR conditions for a 25 µl reaction: Step 1 (95ºC x 2 min); Step 2 (95ºC x 1 min, 55ºC x 1 min, and 72ºC x 1 min 30 sec, 30 cycles); Step 3 (72ºC x 10 min). Preliminary nucleotide alignments for complete and incomplete ORFs and phylogenetic analysis were made with Clustal-W (Higgings et al. 1994) using the ClustalW WWW Service at the European Bioinformatics Institute (ECBI) http://www.ebi.ac.uk/Tools/clustalw2/ECBI (Lopez et al. 1997), and/or MEGA software (Tamura et al. 2007). Those sequences with stop codons within the ORF were discarded for further analysis and those that contained complete ORFs were aligned again to construct phylogenetic trees. Final nucleotide sequence alignments were performed using Clustal-W and Clustal-X1.83 (Higgings et al. 1994; Thompson et al. 1997) with a phylip output format and edited in Jalview (Clamp et al. 2004) or MEGA format (Tamura et al. 2007). The phylogenetic trees containing RGHs from two or more *Nicotiana* species were made using the maximum parsimony method, and inferred by bootstrapping

values calculated on the base of 1,000 replicas per data set, using the program "protpars" of Phylip (Felsenstein 2000) and MEGA 4 (Felsenstein, 1985; Tamura et al. 2007). The tree showing *N. glutinosa* and *N. clevelandii* RGHs was viewed and rooted using ATV (Zmasek and Eddy, 2001). The tree showing all *Nicotiana* species RGHs that coded for the protein NBS domain was made using MEGA 4 (Tamura et al. 2007), and branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein, 1985). The maximum parsimony tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 (Felsenstein, 1985; Nei and Kumar, 2000), in which the initial trees were obtained with the random addition of 10 replicas of the sequences. The codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). Final phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). Aligment of the amino acid sequence of 21 representative NGHs of 14 groups, the N protein and four published N homologs was done by Clustal W at the ECBI (Lopez et al. 1997) using Blossum 62 matrix and viewed utilizing the interface to BioEdit 7.0.9.0 software (Hall, 1999).

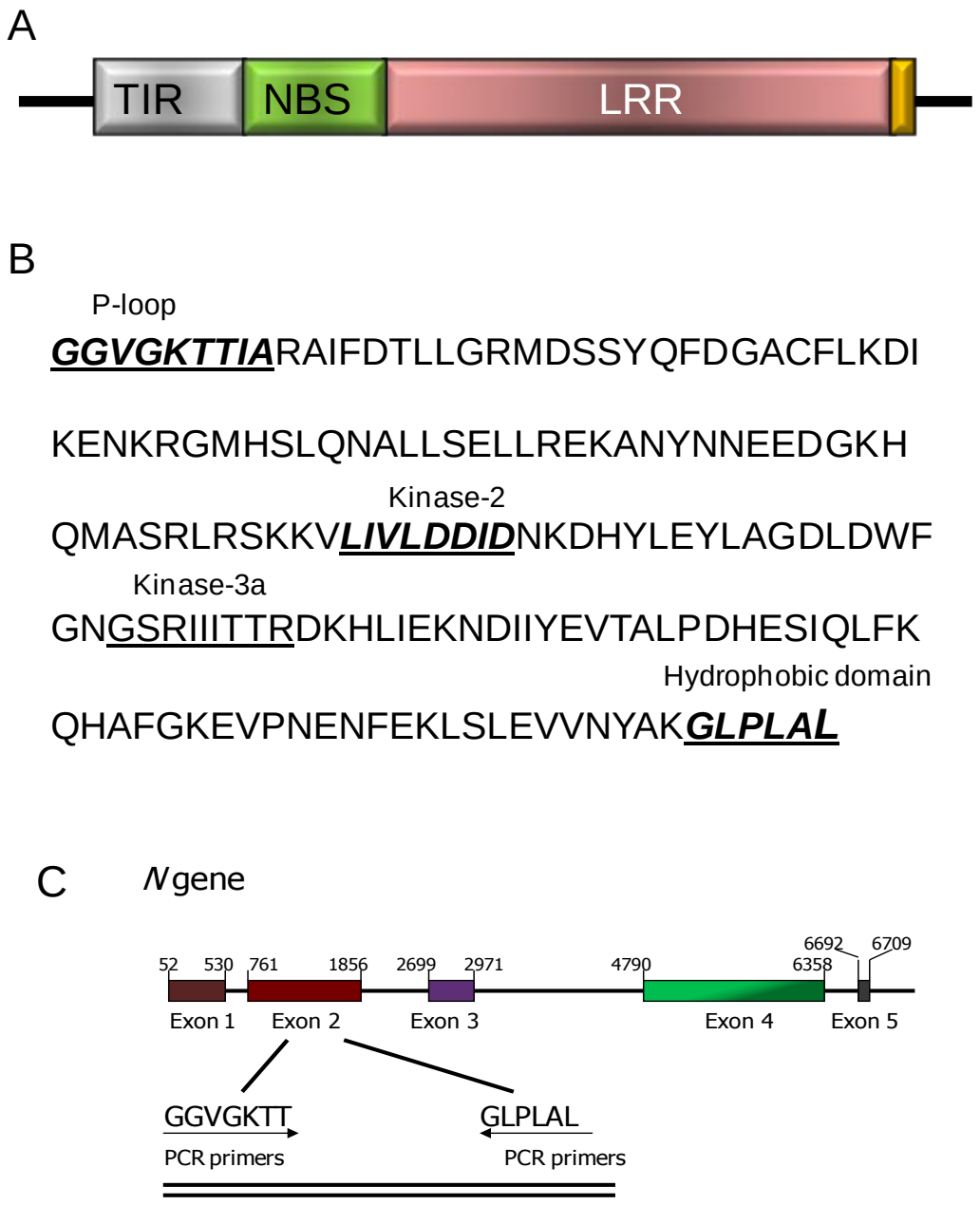


Figure II.1. **A**, Representation of the basic structure of a TIR-NBS-LRR resistance gene. **B**, Amino acid sequence of the NBS domain of the N protein showing the most common motifs used to analyze diversity of NBS-LRR resistance proteins. **C**, Diagram of the *N* gene and localization of P-loop and GLPL hydrophobic motifs in the exon 2 which were selected to amplify and clone RGHs from *Nicotiana* species.

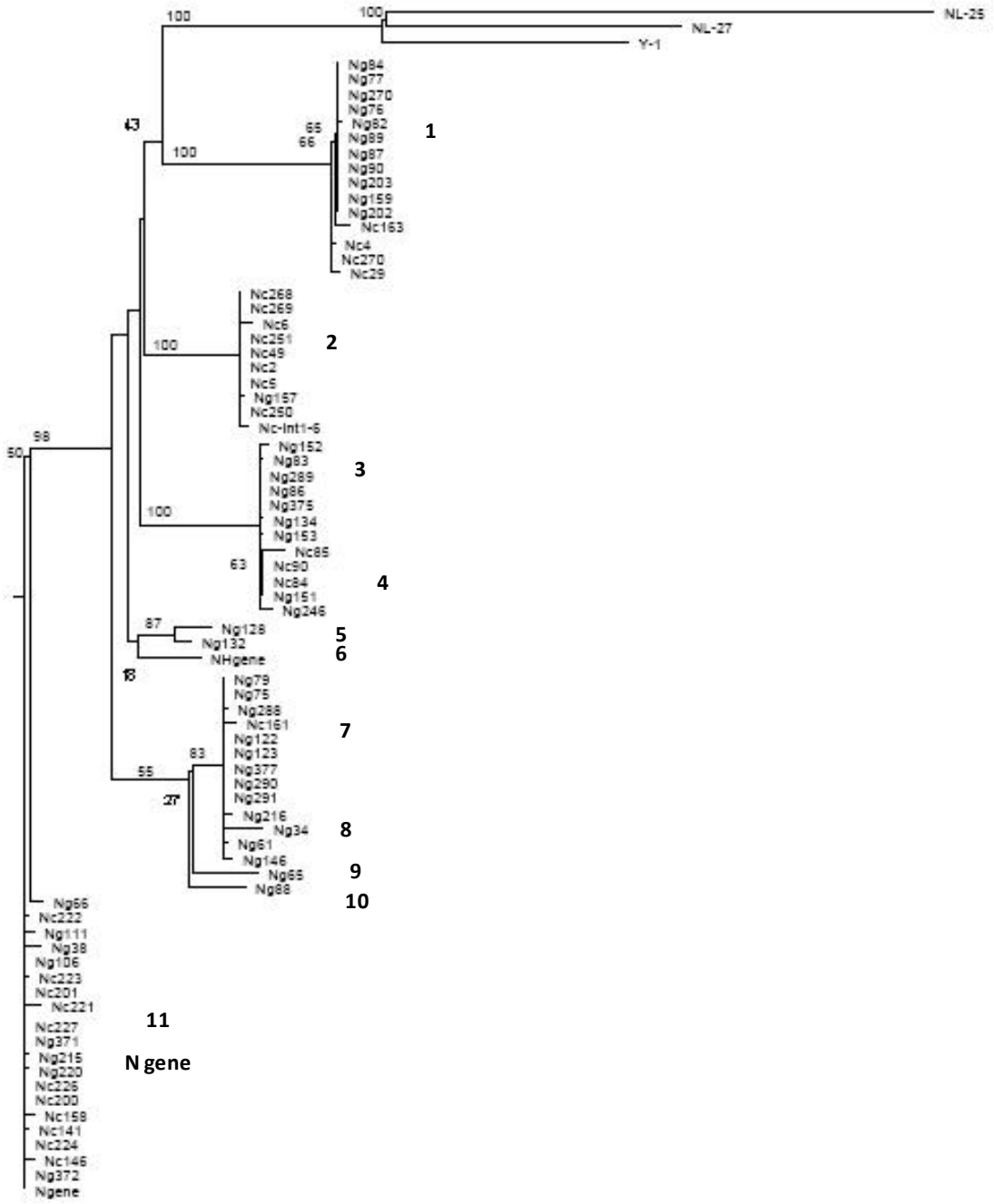


Figure II.2. Phylogenetic analysis of *Nicotiana glutinosa* (Ng) and *N. clevelandii* (Nc) *N* gene homologs (NGHs). The tree is based on the alignment of nucleotide sequences of RGH clones, the *N* gene, and four published *N* homologs (*NH*, *NL*-25, *NL*-27, and *Y-1*). Bootstrapping values on the branches are shown as a percentage based on 1,000 replicates. To account for possible polymerase chain reaction errors, nucleotide sequences that differed by ≤1% were considered to be representative of a single sequence. Modified from Balaji et al. (2007), Mol. Plant-Microbe Interact. 20:1262-1270, with persimission from APS Journals.

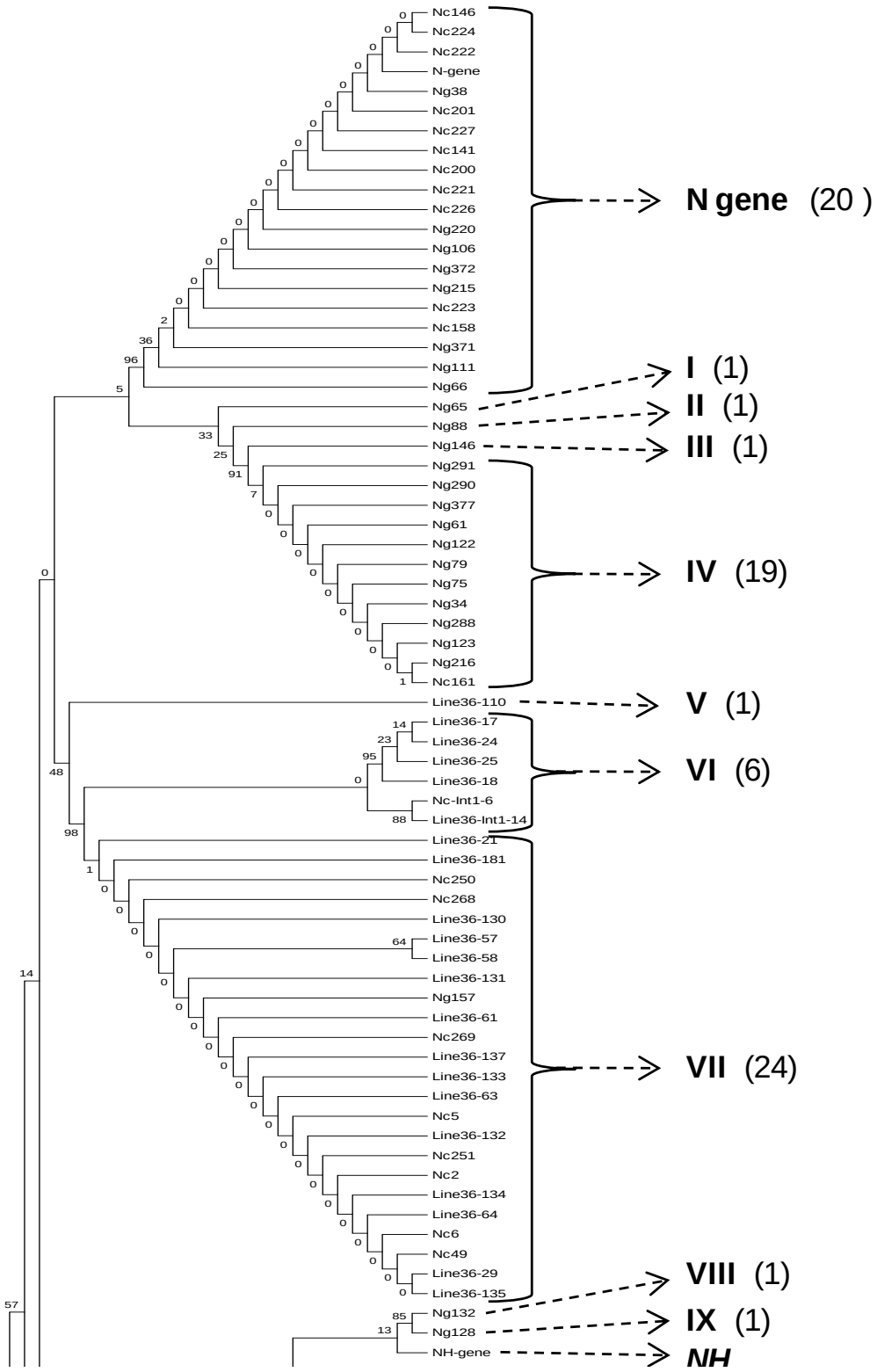


Figure II.3 continues and legend in next page →

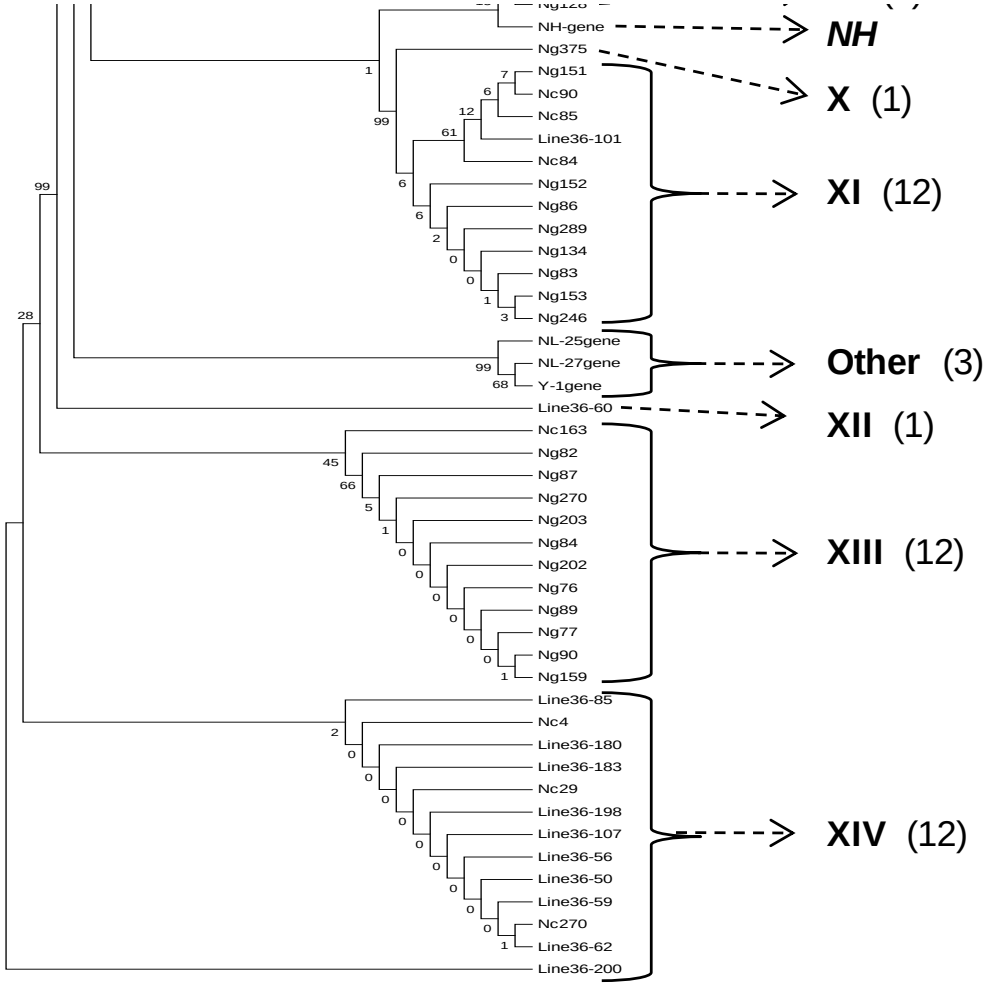


Figure II.3 continued.

Figure II.3. Phylogenetic analysis of *N* gene homologs (NGHs) isolated from *Nicotiana glutinosa* (Ng), *N. clevelandii* (Nc), and *N. clevelandii* Line 36 (L36). The tree is based on the alignment of 106 nucleotide sequences of NGH clones, the *N* gene, and four published *N* homologs (*NH*, *NL-25*, *NL-27*, and *Y-1*). Bootstrapping values on the branches are shown as a percentage based on 1,000 replicas. To account for possible polymerase chain reaction error, nucleotide sequences that differed by ≤1% were considered to be representative of a single sequence. Strongly supported groups are illustrated with Roman numerals, and the number of sequences contained in each group is showed in parenthesis ( ).

Table II.1. Multiple comparisons of percentages of identity in the amino acid sequence of the NBS domain (from P-loop to GLPL motifs) of 21 NGHs representative of 14 groups, the *N* gene group, and other four previously published genes (*NH*, *NL*-25, *NL*-27, and *Y-1*) as *N* homologs.

| GROUP | NGH | N gene | NH | NL-25 | NL-27 | Y-1 | Nc226 | Ng66 | Ng65 | Ng88 | Ng146 | Ng291 | Nc161 | Line36-110 | Line36-18 | Line36-21 | Nc49 | Ng132 | Ng128 | Ng375 | Ng151 | Ng246 | Line36-60 | Nc163 | Ng159 | Line36-85 | Line36-200 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | N gene | 100 | | | | | | | | | | | | | | | | | | | | | | | | | |
| NH | NH | 90 | 100 | | | | | | | | | | | | | | | | | | | | | | | | |
| Other | NL-25 | 57 | 59 | 100 | | | | | | | | | | | | | | | | | | | | | | | |
| Other | NL-27 | 69 | 71 | 58 | 100 | | | | | | | | | | | | | | | | | | | | | | |
| Other | Y-1 | 66 | 66 | 57 | 71 | 100 | | | | | | | | | | | | | | | | | | | | | |
| N | Nc226 | 100 | 90 | 57 | 69 | 66 | 100 | | | | | | | | | | | | | | | | | | | | |
| N | Ng66 | 98 | 88 | 57 | 67 | 65 | 98 | 100 | | | | | | | | | | | | | | | | | | | |
| I | Ng65 | 92 | 85 | 56 | 69 | 65 | 92 | 91 | 100 | | | | | | | | | | | | | | | | | | |
| II | Ng88 | 85 | 86 | 58 | 72 | 65 | 85 | 84 | 90 | 100 | | | | | | | | | | | | | | | | | |
| III | Ng146 | 86 | 86 | 55 | 69 | 65 | 86 | 84 | 92 | 90 | 100 | | | | | | | | | | | | | | | | |
| IV | Ng291 | 86 | 87 | 56 | 70 | 64 | 86 | 84 | 92 | 91 | 98 | 100 | | | | | | | | | | | | | | | |
| IV | Nc161 | 84 | 86 | 55 | 69 | 64 | 84 | 83 | 91 | 90 | 97 | 98 | 100 | | | | | | | | | | | | | | |
| V | Line36-110 | 89 | 90 | 58 | 71 | 66 | 89 | 88 | 85 | 88 | 83 | 84 | 83 | 100 | | | | | | | | | | | | | |
| VI | Line36-18 | 87 | 89 | 55 | 69 | 65 | 87 | 86 | 83 | 83 | 83 | 84 | 83 | 94 | 100 | | | | | | | | | | | | |
| VII | Line36-21 | 87 | 90 | 56 | 69 | 66 | 87 | 86 | 83 | 83 | 83 | 84 | 83 | 95 | 98 | 100 | | | | | | | | | | | |
| VII | Nc49 | 87 | 90 | 56 | 69 | 66 | 87 | 86 | 83 | 83 | 83 | 84 | 83 | 95 | 98 | 100 | 100 | | | | | | | | | | |
| VIII | Ng132 | 88 | 90 | 55 | 69 | 64 | 88 | 87 | 84 | 85 | 91 | 92 | 91 | 87 | 87 | 87 | 87 | 100 | | | | | | | | | |
| IX | Ng128 | 87 | 89 | 54 | 67 | 63 | 87 | 86 | 83 | 84 | 90 | 91 | 90 | 86 | 86 | 86 | 86 | 97 | 100 | | | | | | | | |
| X | Ng375 | 87 | 87 | 56 | 70 | 65 | 87 | 86 | 84 | 90 | 82 | 83 | 82 | 90 | 84 | 85 | 85 | 86 | 85 | 100 | | | | | | | |
| XI | Ng151 | 86 | 87 | 56 | 70 | 64 | 86 | 86 | 84 | 90 | 81 | 83 | 81 | 89 | 84 | 84 | 84 | 86 | 84 | 99 | 100 | | | | | | |
| XI | Ng246 | 86 | 87 | 55 | 70 | 64 | 86 | 85 | 84 | 90 | 81 | 83 | 81 | 89 | 84 | 84 | 84 | 86 | 84 | 99 | 98 | 100 | | | | | |
| XII | Line36-60 | 86 | 87 | 56 | 68 | 63 | 86 | 84 | 81 | 83 | 81 | 82 | 81 | 93 | 90 | 91 | 91 | 85 | 84 | 85 | 84 | 84 | 100 | | | | |
| XIII | Nc163 | 79 | 81 | 55 | 65 | 63 | 79 | 77 | 77 | 80 | 79 | 80 | 79 | 80 | 78 | 79 | 79 | 81 | 80 | 80 | 79 | 79 | 86 | 100 | | | |
| XIII | Ng159 | 79 | 82 | 56 | 65 | 64 | 79 | 77 | 77 | 80 | 79 | 80 | 80 | 81 | 79 | 79 | 79 | 81 | 80 | 80 | 80 | 80 | 87 | 98 | 100 | | |
| XIV | Line36-85 | 80 | 83 | 56 | 66 | 65 | 80 | 78 | 78 | 81 | 80 | 81 | 80 | 81 | 79 | 80 | 80 | 82 | 81 | 81 | 80 | 80 | 87 | 98 | 99 | 100 | |
| XIV | Line36-200 | 80 | 83 | 56 | 66 | 65 | 80 | 78 | 78 | 81 | 80 | 81 | 80 | 81 | 79 | 80 | 80 | 82 | 81 | 81 | 80 | 80 | 87 | 98 | 99 | 100 | 100 |

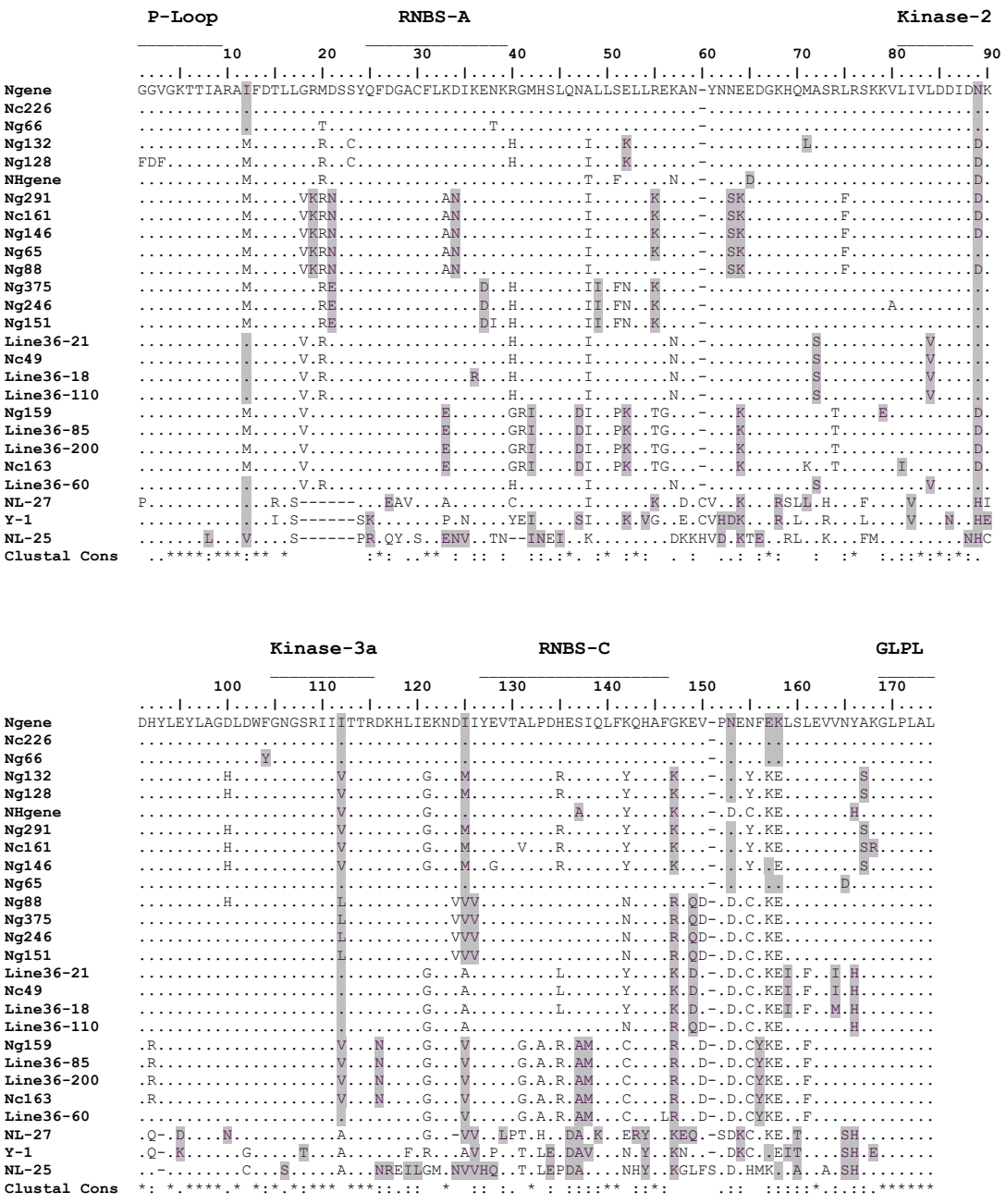


Figure II.4. Amino acid alignment with Clustal W of 21 NGHs representative of 14 groups identified in the bootstrapping analysis. NGHs were isolated from *Nicotiana glutinosa* (Ng), *N. clevelandii* (Nc), and *N. clevelandii* Line 36 (L36), the *N* gene group, and four published *N* homologs (*NH*, *NL*-25, *NL*-27, and *Y-1*). Motifs P-loop, RNBS-A, Kinase-2, Kinase-3a, RNBS-C, and GLPL of the NBS region are indicated on the top. Similar amino acid changes are shaded based on Blossum 62 matrix, and viewed with BioEdit 7.0.9.0 software (Hall, 1999).

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# CHAPTER III

## BIOLOGICAL EFFECTS OF TOMBUSVIRUS P19 AND P22 PROTEINS AGROINFILTRATED IN *NICOTIANA* SPECIES

## ABSTRACT

Previous studies showed that the tomato bushy stunt virus (TBSV) P22 and P19 proteins play an important role in elicitation of symptoms and in triggering plant defenses in *Nicotiana* species, suggesting these two viral proteins function as avirulence determinants in the "gene for gene" resistance model. The TBSV P22 protein elicited a hypersensitive response (HR) in *Nicotiana edwardsonii* and *N. glutinosa*, whereas the TBSV P19 protein triggered an HR in *N. tabacum*, when they were expressed from a potato X virus (PVX)– based vector. In this study, we used *Agrobacterium tumefaciens* – based vectors to express TBSV and cymbidium ringspot virus (CymRSV) P22 and P19 proteins, and cucumber necrosis virus (CNV) P21 and P20 proteins. We found that *Nicotiana* species were able to recognize small differences between these homologous tombusvirus P22 and P19 proteins. The TBSV and CymRSV P22, and the CNV P21 proteins elicited HR in *N. glutinosa* and *N. edwardsonii*, with a slight delay and variation for the CymRSV P22. On the other hand, the TBSV P19 and CymRSV P19 proteins elicited HR in *N. tabacum*, but the CNV P20 did not elicit necrosis in this species.

However, all three tombusvirus P19 homologues did function as silencing suppressors in transient co-agroinfiltration assays with green fluorescent protein expressing constructs in *N. benthamiana*. In contrast to previous studies, we did not obtain systemic necrosis induced by tombusvirus P19 homologues, but we obtained weak local necrosis after 10 dpi conditioned by environmental conditions. In addition, in the current study, we were able to track the source of HR resistance to tombusviruses in *N. tabacum* to its parental genome *N. sylvestris*, which was elicited by three tombusvirus P19 homologues. Consequently, agroinfiltration is a very useful tool not only for testing specific known resistance and avirulence gene interactions, but also can be used for a rapid search across plant species to identify sources of resistance and their pathogen avirulence targets.

# INTRODUCTION

Resistance to plant viruses can frequently be explained by the "gene for gene" model (Flor, 1971, Kang et al. 2005), in which a plant resistance (R) gene product can recognize a pathogen avirulence (Avr) gene product to trigger an active defense response in the host. Through the years the gene for gene model has been modified to include examples in which recognition by the host R protein and pathogen Avr protein is mediated by a third protein, as described in the guard hypothesis (Van der Biezen and Jones, 1998). Furthermore, studies with bacteria, fungi, and nematodes led to the concept of effectors (Martin et al. 2003), pathogen proteins introduced into plant cells that either enhance pathogen virulence or suppress plant defenses to mediate effector-triggered susceptibility (ETS). Specific recognition of effectors by host R gene products can lead to effector-triggered immunity (ETI) (Jones and Dangl, 2006). Plant viruses are unique amongst pathogen groups, because all proteins in any viral genome can be considered effectors. They are expressed in host cells where they either enhance the virus' fitness through gene expression, replication or movement or they suppress plant defenses. Consequently in the case of viruses, it is useful to retain the designation of Avr determinant to distinguish viral effectors that elicit resistance responses from those that promote infection. Any category of virus protein (e.g. replicase, coat protein, movement protein) can act as an Avr determinant to elicit resistance responses in the host (reviewed by Schoelz, 2006). A variety of techniques have been used to identify viral Avr determinants, including gene swaps between virulent and avirulent isolates (Schoelz et al.

1986), expression of viral Avr genes in virus vectors (Scholthof et al. 1995a), stable transformation of plants with viral Avr determinants (Culver and Dawson, 1991), and agroinfiltration of viral Avr determinants in resistant hosts (Erickson et al. 1999; Palanichelvam et al. 2000).

Two proteins of tomato bushy stunt virus (TBSV), the type member of the tombusviridae, were identified as potential Avr determinants in *Nicotiana* species. Most tombusviruses are soilborne pathogens, highly stable, transmitted mechanically or by means that do not require a vector, except one species that is transmitted by a fungus. Also, they can be present in water streams and drainages. They are distributed worldwide, causing severe diseases in edible and ornamental crops. The natural host range is relatively restricted, but experimentally, tombusviruses can infect a broad diversity of species belonging to approximately 20 to 30 plant families, including members of the Compositae, Rosaceae, Fabaceae (Leguminosae), Malvaceae, Cucurbitaceae, Chenopodiaceae, and Solanaceae. However, just a few examples of economically important diseases are associated with tombusviruses, specifically those affecting field and greenhouse crops of tomato, eggplant, pepper, carnation, and lettuce (Gerik et al. 1990; Obermeier et al. 2001; Yamamura and Scholthof, 2005).

Tombusviruses have a genome composed of single-stranded positive sense RNA approximately 4,700 – 4,800 nucleotides in length that is encapsidated into isometric virions 30-35nm in diameter. The TBSV genome is organized into six open reading frames (ORFs) (Hearne et al. 1990; Yamamura and Scholthof, 2005). The first ORF is translated directly from the genomic RNA to produce a 33kDa protein (P33), and a read-through mechanism leads to second protein of a 92 kDa (P92), which is a viral RNA

dependent RNA polymerase. Both P33 and P92 are required for replication in a ratio of approximately 20 to 1. The third ORF is translated from a subgenomic RNA and encodes the 41kDa capsid or coat protein (P41), which is also involved in movement (Scholthof et al. 1993). The fourth and fifth ORFs are nested, translated from a second single subgenomic RNA, and encode two proteins, P22 and P19 (Hearne et al. 1990; Scholthof et al. 1995b). They are involved in cell to cell and long distance movement, host determination, and symptom development (Scholthof et al. 1995ab). The P19 protein is also known by its strong suppressor activity of post-trancriptional gene silencing (Voinnet et al. 1999; Scholthof, 2006). A sixth small ORF at the 3' distal end encodes for a small protein designated pX, which is not essential for replication, but required for virus accumulation (Scholthof and Jackson, 1997).

The two TBSV proteins that were shown to function as Avr determinants, when expressed from a potato virus X (PVX) vector, are P22 and P19. The TBSV P22 protein triggers a hypersensitive response (HR) in *N. glutinosa* and its derived amphiploid species *N. edwardsonii* (Scholthof et al. 1995a), inducing necrotic local lesions and restricting the spread of the PVX vector. The TBSV P19 protein triggers an HR in *N. tabacum* when expressed from the PVX vector (Scholthof et al. 1995a), likewise triggering necrotic local lesions and restricting the spread of PVX. In contrast, the PVX vector with no TBSV insert was able to spread systemically in these three *Nicotiana* species, inducing a mild mosaic symptom. The implication of these studies is that these TBSV proteins are interacting with R proteins, which then lead to the activation of a plant defense response that limits the spread of the virus. In addition, the TBSV P19 protein played a role in development of a severe systemic necrosis symptom in *N. clevelandii* and

*N. benthamiana* when expressed from the PVX vector, a symptom that was similar to the wild type TBSV (Scholthof et al. 1995a). In this instance, the plant defense response was ineffective in limiting the spread of the virus, but the P19 protein did elicit a necrosis response in these hosts.

Previously, we introgressed an R gene against TBSV from the resistant source *N. glutinosa* into the susceptible *N. clevelandii* (Schoelz et al. 2006). We developed a new variety of *N. edwardsonii* (*N. edwardsonii* var. Columbia) that contains the full complement of chromosomes from *N. glutinosa* (Cole et al. 2001), and used it for crosses with *N. clevelandii*. After three rounds of backcrosses to *N. clevelandii* and two selfings, we obtained a *N. clevelandii* addition line (Line 36), which was homozygous for resistance to the tombusviruses TBSV, cucumber necrosis virus (CNV), cymbidium ringspot virus (CymRSV), and carnation italian ringspot virus (CIRV), but susceptible to other viruses such as tobacco mosaic virus (TMV), cauliflower mosaic virus (CaMV), and watermelon mosaic virus 2 (WMMV-2) (Schoelz et al. 2006). These results suggested the existence of an R gene product derived from *N. glutinosa* that was effective against a broad range of tombusviruses. In the present study, we developed an agroinfiltration assay to further characterize the tombusvirus Avr proteins first identified in Scholthof et al (1995a) through the use of a PVX vector. In particular, we investigated whether homologous proteins from the tombusviruses CymRSV and CNV will elicit a similar response in *Nicotiana* species as their TBSV counterparts.

## RESULTS

### Development of HR in *Nicotiana* species after agroinfiltration of TBSV p22 and p19 genes.

To determine if agroinfiltration of TBSV p19 and p22 genes would trigger an HR in selected *Nicotiana* species, we used polymerase chain reaction (PCR) to amplify the DNA segment that encodes the p19 and p22 genes, cloned the DNA segment into the *Agrobacterium* binary vector pKYLX7 (Schardl et al. 1987), and subsequently mobilized the clone into *A. tumefaciens* strain AGL1. Since the p19 gene is nested inside the p22 gene (Hearne et al. 1990), agroinfiltration of a single construct would theoretically express both proteins (Fig. III.1). In fact, agroinfiltration of the nested p19 and p22 gene construct yielded an HR in *N. glutinosa*, *N. edwardsonii*, and *N. tabacum* within 3 - 4 days post infiltration (dpi) (Fig. III.2).

To determine which viral protein triggered HR in which *Nicotiana* species, the start codons of the p19 and p22 genes were mutated to create constructs that would express only the P22 protein, only the P19 protein, or neither protein (Fig. III.1). The construct that expressed only p22 triggered HR in *N. edwardsonii* and *N. glutinosa*, but not in *N. tabacum* (Fig. III.2). Since *N. edwardsonii* is an amphidiploid derived from *N. glutinosa* and *N. clevelandii* (Christie et al. 1969), it is likely that a single R gene product, derived from *N. glutinosa*, is responsible for triggering HR in response to TBSV P22. In contrast, the construct that expressed only P19 triggered HR in *N. tabacum*, but no HR in

*N. edwardsonii* and *N. glutinosa* (Fig. III.2). The construct that contained mutations in the start codons of both p19 and p22 failed to induce HR in any of the three *Nicotiana* species. These agroinfiltration results are in agreement with the expression of P19 and P22 proteins through a PVX vector (Scholthof et al. 1995a), in which it was originally shown that the P22 protein triggers HR in *N. glutinosa* and the P19 protein triggers HR in *N. tabacum*.

Scholthof and coworkers (1995a) also found that a PVX vector that expressed TBSV P19 protein elicited a systemic necrosis symptom in *N. benthamiana* and *N. clevelandii*, two *Nicotiana* species that are also highly susceptible to the TBSV virus. Inoculation of TBSV virions to either of these plants induces an apical wilt, followed by widespread necrosis and death of the plant in as little as 15 – 20 dpi, depending on environmental conditions. Agroinfiltration of TBSV p22 did not elicit any response in either *N. benthamiana* or *N. clevelandii*, whereas agroinfiltration of TBSV p19 resulted in the development of a weak necrosis symptom in both hosts that developed in some infiltrated panels at approximately 10 dpi. The development of this necrosis symptom was enhanced by environmental conditions of low light, but even then not every infiltrated leaf panel developed necrosis. In contrast, p19 infiltrated into *N. tabacum* leaf panels, included in the same test, consistently triggered HR necrosis at 2 – 3 dpi.

In a previous study, the resistance to tombusviruses present in *N. glutinosa* was introgressed into *N. clevelandii* to create an addition line (*N. clevelandii* line 36) that responded with HR to the tombusviruses TBSV, CNV, CymRSV, lettuce necrotic stunt virus and carnation italian ringspot virus (Schoelz et al. 2006). *N. clevelandii* line 36 was agroinfiltrated with the constructs expressing p22 and p19 individually, as well as the

empty vector pKYLX7. Only leaf panels agroinfiltrated with the p22 construct developed HR, usually within 2 – 3 dpi (Fig. III.3). No other symptom was observed in leaf panels agroinfiltrated with p19 or pKYLX7 up to 10 dpi. This experiment showed that *N. clevelandii* containing an introgressed resistance gene from *N. glutinosa* would respond to agroinfiltration of p22 in exactly the same manner as *N. glutinosa* or *N. edwardsonii*.

**The CNV P21 and CymRSV P22 proteins also elicit HR in *N. glutinosa* and *N. edwardsonii*.**

Two lines of evidence suggested that the R gene product that recognized the TBSV P22 protein also recognized the P22 proteins of other tombusviruses. First, *N. clevelandii* line 36 responded to CNV and CymRSV with HR (Schoelz et al. 2006), indicating that, at a minimum, the R gene that recognized these viruses was on the same chromosome as the R gene that recognized TBSV. Second, silencing of the *N* family of resistance genes compromised the resistance response of *N. edwardsonii* to TBSV, CNV, and CymRSV (Balaji et al. 2007), indicating that the R genes effective against these three tombusviruses must share some degree of sequence homology. Taken together, these studies could not prove that a single R gene product recognized the P22 protein of all three tombusviruses, but the R gene or genes must be located on the same chromosome and must be related at the nucleotide sequence level.

To further investigate the HR directed against tombusviruses, we tested whether the P22 proteins of CNV and CymRSV would trigger an HR in *N. glutinosa* and *N.*

*edwardsonii* in an agroinfiltration assay. Four types of constructs were developed for each virus. One construct contained the nested sequences of the p22 and p19 genes, or for CNV the homologs p21 and p20. A pair of constructs was also created for each virus in which the start codons were mutated such that only one or the other of the proteins would be expressed. Finally, we mutated both start codons in a single construct such that neither protein would be expressed.

Agroinfiltration of the CNV p21/p20 nested genes yielded an HR in *N. edwardsonii* and *N. glutinosa* within 3 – 4 dpi, a response that was comparable to that elicited by the TBSV nested construct (Fig. III.2, Table III.1). Agroinfiltration of the construct that expressed only the CNV p21 gene yielded the same responses as the CNV nested construct, whereas the construct that expressed the CNV p20 gene and the double mutant did not elicit any response in *N. edwardsonii* or *N. glutinosa*. Furthermore, agroinfiltration of CNV p21 did not yield an HR in *N. tabacum*, *N. clevelandii*, or *N. benthamiana* (Fig. III.2, Table III.1).

Agroinfiltration of the CymRSV p22/p19 nested genes yielded an HR in *N. edwardsonii* and *N. glutinosa*, but induction time was slower (6 – 7 dpi) than with either of the corresponding TBSV and CNV constructs. In addition, the HR induced by the CymRSV p22/p19 clone was variable, as sometimes only a portion of the infiltrated area became necrotic. With the TBSV and CNV nested clones, the entire infiltrated area consistently became necrotic within 4 dpi (Fig. III.2). Agroinfiltration tests of individual CymRSV genes showed that the CymRSV p22 gene was responsible for triggering HR in *N. edwardsonii* and *N. glutinosa*, but as with the nested construct, HR appeared at 6 – 7 dpi and could vary from only a portion of the infiltration area to the entire infiltrated area.

Agroinfiltration of CymRSV p22 did not yield an HR in *N. tabacum*, *N. clevelandii*, or *N. benthamiana*.

**_N. tabacum_ responds to agroinfiltration of the CymRSV p19 gene with HR, but agroinfiltration of CNV p20 fails to elicit HR.**

Agroinfiltration of the TBSV nested p22/p19 construct elicited an HR in *N. tabacum* due to the expression of the TBSV p19 protein. To determine if the homologous genes of CNV and CymRSV elicit HR in *N. tabacum*, we tested each of the CNV and CymRSV constructs made in the previous section. Interestingly, neither the CNV nested p21/p20 construct nor the CymRSV p22/p19 construct elicited HR in *N. tabacum* up to a period of 10 dpi (Fig. III.4). However, *N. tabacum* responded differently to infiltration of the CymRSV construct that expressed only the p19 protein, as this did elicit HR in *N. tabacum* within 3 dpi (Fig. III.2 panel E). In contrast, the construct that expressed only CNV p20 did not elicit HR (Fig. III.2 panel E; Fig. III.4). We considered that this result might be an artifact due to a potential reduction in the expression of the CNV P20 relative to other constructs. However, CNV P20 did trigger an HR in another species, *N. sylvestris* (Fig. III.4), and an analysis of the silencing suppressor function of CNV P20 indicated that it was comparable to TBSV and CymRSV (see section below). This indicated that the lack of HR might instead be due to a unique feature of the amino acid sequence of CNV P20. A comparison of amino acid sequences of TBSV P19 (Fig. III.5), CymRSV P19, and CNV P20 showed that all three protein sequences were 68% identical. However, CNV P20 and CymRSV P19 were more closely related to each other than to

TBSV P19, and TBSV P19 is just 1% closer related to CymRSV than to CNV P20 (Fig. III.5). Consequently, there are seventeen amino acid positions within the CNV P20 sequence that differ from TBSV P19 and CymRSV P19 that might explain its failure to elicit an HR in *N. tabacum*.

The observation that CNV P20 did not trigger HR in *N. tabacum* suggested that the CNV virus might also not trigger an HR. To investigate whether the CNV virus could elicit an HR in *N. tabacum*, this virus was inoculated to *N. tabacum* in a test that included TBSV and CymRSV virions. All three viruses elicited necrotic lesions by 5 dpi (Fig. III.6) and *N. tabacum* plants were resistant to infection. Agroinfiltration of CNV P21 also did not induce an HR (Fig. III.4), so at this time it is not clear what CNV protein is responsible for this resistance response.

**The HR of *N. tabacum* to TBSV P19 is derived from its *N. sylvestris* parent.**

The *N. tabacum* amphidiploid genome is derived primarily from *N. sylvestris* and *N. tomentosiformis* (Aoki and Ito, 2002; Murad et al. 2002; Kovarik et al. 2004), but some studies have suggested that a portion is also derived from *N. otophora* (Goodspeed, 1954; Riechers and Timko, 1999; Ren and Timko, 2001). Since the parents of *N. tabacum* have been identified, this presented a unique opportunity to identify which parent might have contributed the putative R gene in *N. tabacum* that recognizes the tombusvirus p19 gene to trigger an HR.

To identify which species contributed the putative R gene, we initially inoculated each *N. tabacum* and its three parents with virions of TBSV, CNV, and CymRSV. Only

*N. sylvestris* (Fig. III.6) and *N. tabacum* (Fig. III.7) responded to the three viruses with HR. In contrast, *N. tomentosiformis* and *N. otophora* responded to the three viruses with chlorotic lesions that were most apparent at 5 dpi (Fig. III.7). Interestingly, none of the four *Nicotiana* species developed systemic symptoms in response to the three viruses. Furthermore, DAS-ELISA tests conducted between 21 and 25 dpi showed that no virus could be detected in upper, non-inoculated leaves (data not shown). Consequently, only *N. sylvestris'* response to the tombusviruses was consistent with the HR observed in *N. tabacum*, although *N. tomentosiformis* and *N. otophora* did contain a non-necrotic form of resistance.

Agroinlfiltration of TBSV and CymRSV p19 genes and the CNV p20 gene into the leaves of *N. sylvestris*, *N. tomentosiformis*, and *N. otophora* confirmed that *N. sylvestris* contained the putative R gene. *N. sylvestris* responded to the agroinfiltration of the tombusvirus proteins with HR by 3 dpi (Fig. III.7), whereas no reaction was observed in *N. tomentosiformis* and *N. otophora* (data not shown). Furthermore, no HR was observed in any *Nicotiana* species in response to the TBSV and CymRSV P22 proteins and CNV P21 protein (Fig. III.7), which is consistent with the observation that *N. tabacum* does not contain a R gene product that can recognize these tombusvirus proteins. Significantly, agroinfiltration of CNV p20 into *N. sylvestris* yielded an HR (Fig. III.7), in contrast to results obtained with CNV p20 and *N. tabacum* (Fig. III.2 panel E; Fig. III.4). Consequently, although the plant R gene product that recognizes tombusvirus P19 proteins is derived from *N. sylvestris*, alleles must exist in *N. tabacum* species that can discriminate between CNV P20 and the other two tombusvirus proteins, TBSV P19 and CymRSV P19.

**Evaluation of the gene silencing suppression activity of tombusvirus P19 proteins.**

The P19 proteins of TBSV and CymRSV were shown to have a specific role in suppression of gene silencing (Voinnet et al. 1999; Szittya et al. 2002). For instance, TBSV P19 was shown to enhance the expression of green fluorescent protein (GFP) when co-infiltrated with GFP into *N. benthamiana* leaves (Voinnet et al. 1999, 2003). To investigate whether tombusvirus P19 proteins might differ in the strength of their silencing suppressors, we co-agroinfiltrated TBSV p19, CymRSV p19 and CNV p20 with GFP and then assessed their ability to enhance and extend GFP expression. The treated plants were observed for GFP fluorescence and protein accumulation beginning at 2 dpi. Representative examples are shown at 5 and 7 dpi (Fig. III.8), and at 7 and 17 dpi (Fig. III.9A and III.9B respectively).

As shown in Figure III.8, the maximal GFP expression obtained without co-agroinfiltration of a tombusvirus P19 protein was seen at 5 dpi, and every day afterward the fluorescence was diminished. In contrast, co-infiltration of the constructs expressing TBSV and CymRSV P19, and CNV P20 together with GFP, suppressed RNA silencing, evidenced by an increase of GFP signal with a maximum GFP fluorescence at 7 dpi (Fig. III.8). All three tombusvirus proteins extended and enhanced the levels of GFP expression (Fig. III.8). The CNV P20 was able to maintain GFP expression up to 9 dpi, TBSV P19 could uphold GFP signals for 14 days, and CymRSV P19 preserved the ability to suppress gene silencing and extend expression of fluorescent protein for more than 20 days (data not shown).

The western blot assays of TBSV and CymRSV P19 proteins, CNV P20 protein, and GFP protein expression (Fig. III.9A) showed that compared to the controls with GFP only (lanes 4-5) the levels of GFP protein were increased by co-expression of P19 or P20 proteins (lanes 1-3) at 7 dpi. This effect was even more substantial at 17 dpi (Fig. III.9B). At 7 dpi, TBSV and CymRSV P19 and CNV P20 were detectable, albeit at different levels. However, the antiserum was specific to TBSV P19, and thus quantitative comparisons are not reliable. At 17 dpi, only CymRSV and TBSV P19 proteins were detected, which corresponded with the highest level of GFP for these treatments. We concluded that the CymRSV P19 protein was the strongest silencing suppressor, because in co-agroinfiltration assays with GFP it extended GFP expression for the longest period. The next strongest silencing suppressor was TBSV, whereas CNV P20 was the weakest.

# DISCUSSION

Several studies have contributed to our understanding of the interactions of tombusvirus Avr proteins with putative R proteins in *Nicotiana* species. Using a PVX-based vector, Scholthof et al. (1995a) demonstrated that TBSV P22 protein elicited HR in the TBSV-resistant hosts *N. glutinosa* and *N. edwardsonii*, and the TBSV P19 protein elicited HR in *N. tabacum* and systemic necrosis in *N. clevelandii* and *N. benthamiana*. Burgyán et al. (2000) showed that the P33 protein of CymRSV contributed to the lethal necrosis phenotype in *N. benthamiana* infections, in addition to the P19 protein. On the host side, resistance to TBSV was successfully introgressed from *N. glutinosa* to *N. clevelandii*, and it was shown that the *N. clevelandii* line developed (Line 36) was also resistant to CymRSV and CNV (Schoelz et al. 2006). In addition, we showed that silencing of the resistance to TMV in *N. edwardsonii* also had the effect of silencing resistance to TBSV and CymRSV (Balaji et al. 2007). In the present study we investigated whether the P22 and P19 proteins of CymRSV and CNV would elicit the same response in resistant *Nicotiana* species as their TBSV counterparts.

We initially showed that agroinfiltration and expression of the TBSV P22 protein resulted in an HR at 3-4 dpi in both *N. glutinosa* and *N. edwardsonii* leaves, and did not trigger any visible response in *N. tabacum, N. benthamiana*, or *N. clevelandii*, which is in agreement with Scholthof et al. (1995a). The CymRSV P22 and CNV P21 proteins elicited the same responses as TBSV P22 in these *Nicotiana* species, although we did observe slight delays in HR development in response to the CymRSV P22 protein. An

inspection of the TBSV P22 amino acid sequence revealed that it is 83% identical to CymRSV P22 and 86% to CNV P21 (Fig. III.10). Furthermore, TBSV P22 amino acids shown to be critical for elicitation of HR by Chu et al. (1999), in particular amino acids 14-18 and 59, were conserved in the amino acid sequences of CymRSV P22 and CNV P21. These findings suggest that an R protein derived from *N. glutinosa* is able to recognize conserved features present in tombusvirus P22 proteins.

We found that agroinfiltration and expression of the TBSV P19 protein triggered HR in *N. tabacum*, which also was in agreement with Scholthof et al. (1995a). In agroinfiltration tests of the CymRSV P19 and CNV P20 proteins, only CymRSV P19 triggered HR in *N. tabacum*, whereas CNV P20 did not elicit any response in that host. We also showed that CNV P20 was expressed, because it could elicit HR in *N. sylvestris* and also functioned as a silencing suppressor in *N. benthamiana*, so its inability to trigger HR must be related to differences in its amino acid sequence to those of TBSV and CymRSV. An alignment of CNV P20 with the P19 proteins of TBSV and CymRSV revealed 17 amino acid changes unique to the CNV P20 sequence that might explain its failure to elicit an HR in *N. tabacum* (Fig. III.5). Point mutations have been introduced into the TBSV P19 protein that abolished its capacity to elicit HR in *N. tabacum* (Chu et al. 2000; Hsieh et al. 2009). These authors targeted regions that were exposed on the surface of the protein, and could be available for probable host-specific interactions, as well as siRNA binding, taking care not to alter the TBSV P22 protein sequence present in a different reading frame. In particular, Chu et al. (2000) found that individual mutations at amino acids 43, 71, 72, the double mutant 75/78, and 85, each abolished HR in *N. tabacum* but had no effect on TBSV accumulation in *N. benthamiana*. However, these

amino acids in the P20 protein of CNV are conserved with either TBSV P19, CymRSV P19, or both proteins (Fig. III.5), so they are not responsible for the inability of CNV P20 to trigger HR in *N. tabacum*. It is clear that multiple mutations in P19 can affect recognition by a putative R protein in *N. tabacum*.

We also found that agroinfiltration and expression of the tombusvirus P19 proteins in *N. clevelandii* and *N. benthamiana* leaves did not elicit a necrosis response in a timely manner. The P19 proteins did elicit weak necrosis in *N. benthamiana* leaves after 10 dpi, but this appeared to be conditioned by environmental conditions, especially low light. The late responses of these *Nicotiana* species underscores the fact that these plants are susceptible to tombusvirus infection, in contrast to the relatively rapid HR (within 3 - 5 dpi) that develops in plants that are resistant. Our results differed from Scholthof et al. (1995a), who found that P19 triggered a systemic necrosis symptom in *N. clevelandii* and *N. benthamiana* plants when the protein was expressed from a PVX vector. However, they are in agreement with Burgyán et al. (2000), who found that the systemic necrosis symptom in *N. benthamiana* was dependent on both the P33 protein and P19 proteins. It may be that the systemic necrosis observed by Scholthof et al. (1995a) was due to a synergism between TBSV P19 and one of the PVX proteins. It was shown that suppressors of gene silencing can contribute to synergisms, so the PVX P25 protein (Voinnet et al. 2000) might be one attractive candidate.

Our study also illustrates how agroinfiltration can be used for tracking the origin of R genes. *N. tabacum* is derived mainly from *N. sylvestris* and *N. tomentosiformis* (Aoki and Ito, 2002; Murad et al. 2002; Kovarik et al. 2004), although *N. otophora* is also thought to be a minor contributor (Goodspeed, 1954; Riechers and Timko, 1999; Ren and

Timko, 2001). We used a combination of virion inoculations and agroinfiltration assays to show that the R gene that recognizes the tombusvirus P19 protein is derived from *N. sylvestris*. Interestingly, the CNV P20 protein did not elicit an HR in *N. tabacum*, but did elicit an HR in *N. sylvestris*. It could be that the R gene in the ancestral source of *N. sylvestris* that was incorporated into *N. tabacum* might have subtle differences in structure from the R gene in the *N. sylvestris* that we used in our agroinfiltration studies. Alternatively, the genetic background of *N. tabacum* could exert subtle effects on the R gene's capacity to recognize tombusvirus P19 proteins. This question likely will not be resolved until both R genes are cloned and their structure compared, but we may gain some information in identifying the differences in the tombusvirus P19 protein sequences that led to the differential response in these *Nicotiana* species.

To compare the silencing suppressor function of the tombusvirus P19 proteins, we co-agroinfiltrated into *N. benthamiana* a construct expressing GFP with individual constructs expressing the TBSV and CymRSV p19, and CNV p20 genes, as described by Voinnet et al. (2003). Interestingly, the silencing suppression function of the tombusvirus P19 proteins varied in strength and timing, as assessed by extending the expression of GFP in agroinfitrated *N. benthamiana* leaf panels. Under our assay conditions, expression of GFP alone in *N. benthamiana* lasted for 5 - 7 dpi. We found that CymRSV P19 was the strongest silencing suppressor, as it extended GFP expression up to 18 days, whereas CNV P20 was the weakest, as it could only sustain GFP expression for 9 days. The revelation that P19 proteins differ in silencing suppressor strength may ultimately be valuable for probing their role in silencing suppression.

# MATERIALS AND METHODS

## Viruses and Plants

Seeds of different *Nicotiana* species were obtained from the U.S. Tobacco Germplasm Collection at North Carolina State University (Lewis and Nicholson, 2007) as follows: *Nicotiana clevelandii* (PI 555491), *N. glutinosa* (PI 555507), *N. tabacum* cv. Turkish Samsun and cv. NC95 (PI 552380), *N. benthamiana* (PI 555478), *N. edwardsonii* (PI 555704), *N. sylvestris* (PI 555569), *N. tomentosiformis* (PI 555572), and *N. otophora* (PI 555542). To break dormancy, seeds were treated for 30 min with commercial bleach at 50% strength (2.6 % vol/vol NaOCl) (Burke, 1957).

The tombusviruses TBSV, CNV, and CymRSV were all previously cloned in infectious form (Hearne et al. 1990; Rochon and Johnston, 1991; Grieco et al. 1989). Infectious, uncapped transcripts derived from the clones were made according to Scholthof et al. (1993, 1995b), inoculated to either *N. clevelandii* or *N. benthamiana* to increase inoculum, and infected tissue frozen for further inoculations. For inoculation of test plants, leaves infected with tombusviruses were ground with a mortar and pestle at a dilution of approximately 1:20 (wt/vol) with inoculation buffer (0.05 M phosphate buffer, pH 7.0), then gently rubbed onto *Nicotiana* leaves lightly dusted with 600 mesh carborundum.

## ELISA tests for Virus Detection

To evaluate the capacity of tombusviruses to infect *Nicotiana* species, DAS-ELISA (Clark and Adams, 1977) was performed using primary antibodies and alkaline phosphatase conjugates purchased from Agdia ® (Elkhart, IN) for TBSV and CymRSV, and from Acdia Inc. (Fayetteville, AR) for CNV. Plant tissues were collected from upper non-inoculated leaves between 20 to 28 dpi, and samples were ground at a ratio of 1:10 tissue : grinding buffer (1× phosphate buffered saline, 2% polyvinylpyrrolidone MW 40,000, and 0.2% bovine serum albumin). Colorimetric reactions with the substrate p-nitrophenyl phosphate were quantified at 405 nm using a Multiskan MCC/340 microplate reader (Thermofisher Scientific, Cincinnati, OH).

**Amplification and cloning of tombusvirus genes and the GFP coding sequence into the *A. tumefaciens* binary vector pKYLX7.**

PCR primers were developed from the nucleotide sequences of TBSV-cherry isolate (NCBI accession # M21958), CNV (NCBI accession # M25270), and CymRSV (NCBI accession # X15511). Primer sequences for PCR amplification were synthesized by Integrated DNA Technologies (Coralville, IA). The forward primer for the TBSV p22/p19 and CNV p20/p21 nested genes was 5′-CTCGAGATGGATACTGAATACGAACAAGTCAAT-3′ and the reverse primer was 5’- GAGCTCTCAGACTGAAGAGTCTGTCTTACTCG -3’. The forward primer for CymRSV p22/p19 nested genes was 5’-CTCGAGATGGACACTGAATACCAACAAGTT -3’, and the reverse primer was 5’-

GGTACCCTAGACTGAAGAGTCTGTCCTACT -3'. To knock out the p22 genes of TBSV and CymRSV and the p21 gene of CNV, the start codon AUG was mutated to CUC. The forward primer for TBSV and CNV was 5'-CTCGAGTTCCTCGATACTGAATACGAACAA- 3' and the forward primer for CymRSV was 5'-CTCGAGTTCCTCGACACTGAATACCAACAAGTT- 3'.

To knock out the TBSV and CymRSV p19 genes and the CNV p20 gene, the start codon AUG was mutated to UUG, which conserved the amino acid sequence of the TBSV and CymRSV P22 proteins and the CNV P21 protein. The forward primer for TBSV and CNV clones was 5' – CTCGAGATGGATACTGAATACGAACAAGTCAATAAACCTTGGAAC- 3' and for the CymRSV p19 knockout was 5' – CTCGAGATGGACACTGAATACCAACAAGTTAATAAACCTTGGAAC- 3'. To construct the double knockouts, both start codons were changed using forward primers that incorporated the mutations of the single knockouts. Reverse primers for all knockouts were the same used for amplification of tombusvirus nested genes. Polymerase chain reaction (PCR) conditions were: one cycle at 95°C for 2 min, followed by 35 cycles at 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min; and finally one cycle at 72°C for 10 min. The coding sequence of GFP was amplified from the clone pCH32 (Chalfie et al. 1994; Voinnet et al. 1998) with the forward primer 5'-GGCCTCGAGATGAGTAAAGGAGAAGAACTTTTCACT- 3' and the reverse primer 5' – GGCCGGATCCGAGCTCTTATTTGTATAGTTCATCCATGCCATG- 3'.

The amplified DNA was subsequently purified by agarose gel elution using a QIAquick Gel Extraction kit (Qiagen Inc., Valencia, CA) and cloned into the *Xho* I-*Sac* I

or *Xho* I-*Kpn* I restriction enzyme sites of pGEM-7Zf(+) or into pGEM-T easy (Promega Corp., Madison, WI). *Escherichia coli* transformants were selected on LB medium containing kanamycin (50µg/ml) and tetracycline (12.5µg/ml) and tested by colony PCR and restriction enzyme digestions for the presence of the tombusvirus insert. Nucleotide sequences of candidate clones were determined to confirm that the PCR reactions did not introduce errors. Sequencing of clones in both orientations was performed by the DNA Core Facility at the University of Missouri (Columbia, MO).

After confirming the fidelity of all sequences, the tombusvirus and GFP inserts were subsequently cloned into the multiple cloning site of the *Agrobacterium tumefaciens* binary vector pKYLX7 (Schardl et al. 1987). Clones of pKYLX7 containing the tombusvirus and GFP genes were mobilized by electroporation (PG200 Progenetor II, Hoefer Scientific Instruments, San Francisco, CA) into *A. tumefaciens* AGL1 strain (Lazo et al. 1991). Transformants were selected on LB medium supplemented with kanamycin (50 µg/ml), tetracycline (12.5 µg/ml), and carbenicillin (50 µg/ml).

**Agroinfiltrations**

The induction and infiltration medium for experiments involving HR elicitation was 20 mM MES [monohydrate 2-(N-morpholino) ethanesulfonic acid], 60 mM sucrose, 55 mM glucose, pH 5.4, and 2mM acetosyringone dissolved into N-N-dimethylformamide. For experiments involving co-agroinfiltrations of GFP and tombusvirus P19 proteins, the medium for overnight induction contained 200 µM MES, pH 5.85, and 19.5 µM acetosyringone. The infiltration buffer included 10 mM MES (pH

5.85), 10 mM $MgCl_2$, and 2.25 mM acetosyringone. Agroinfiltrations were done on the abaxial surface of fully expanded leaves of 4-8 week old *Nicotiana* spp, using a needleless plastic syringe. Co-agroinfiltrations for silencing suppression experiments were done in *N. benthamiana* only. Every experiment consisted of four agroinfiltrated leaves per plant and two plants per replica. The entire experiment was repeated at least three times.

**Western blots for GFP and tombusvirus P19 detection**

The infiltrated leaves were extracted with Tris-EDTA (TE) buffer, and protein samples were separated by standard SDS-PAGE in 15% polyacrylamide gels and transferred to nitrocellulose membranes (Osmonics, Westborough, MA). The membranes were stained with Ponceau S (Sigma, St. Louis, MO) to verify the efficiency of protein transfer. The antisera against TBSV proteins (Omarov et al. 2006) were applied at the dilution of 1:5,000, respectively. Alkaline phosphatase–conjugated goat anti-mouse or rabbit antiserum (Sigma, St. Louis, MO) was used as the secondary antibody and applied at a dilution of 1:1,000. The immune complexes were visualized by hydrolysis of tetrazolium-5-bromo-4-chloro-3- indolyl phosphate (BCIP) as the substrate in the presence of nitro-blue tetrazolium chloride (NBT). In some experiments, horseradish peroxidase conjugated to goat antimouse antiserum (Bio-Rad, Hercules, CA) was used as the secondary antibody at a dilution of 1:5,000, and the immune complexes were visualized by using the enhanced chemioluminescence detection kit (Pierce, Rockford, IL). Mouse monoclonal IgG2a GFP antibody (GFP (B-2): sc-9996; Santa Cruz

Biotechnology, CA) was used to quantify GFP expression. GFP signals on the inoculated *N. benthamiana* leaves were monitored with a 100 W handheld long-wave ultraviolet (UV) lamp (UV products, Black Ray model B100AP, Upland, CA). An Olympus DP70 camera was used for image acquisition of DIC and wide field fluorescent images.

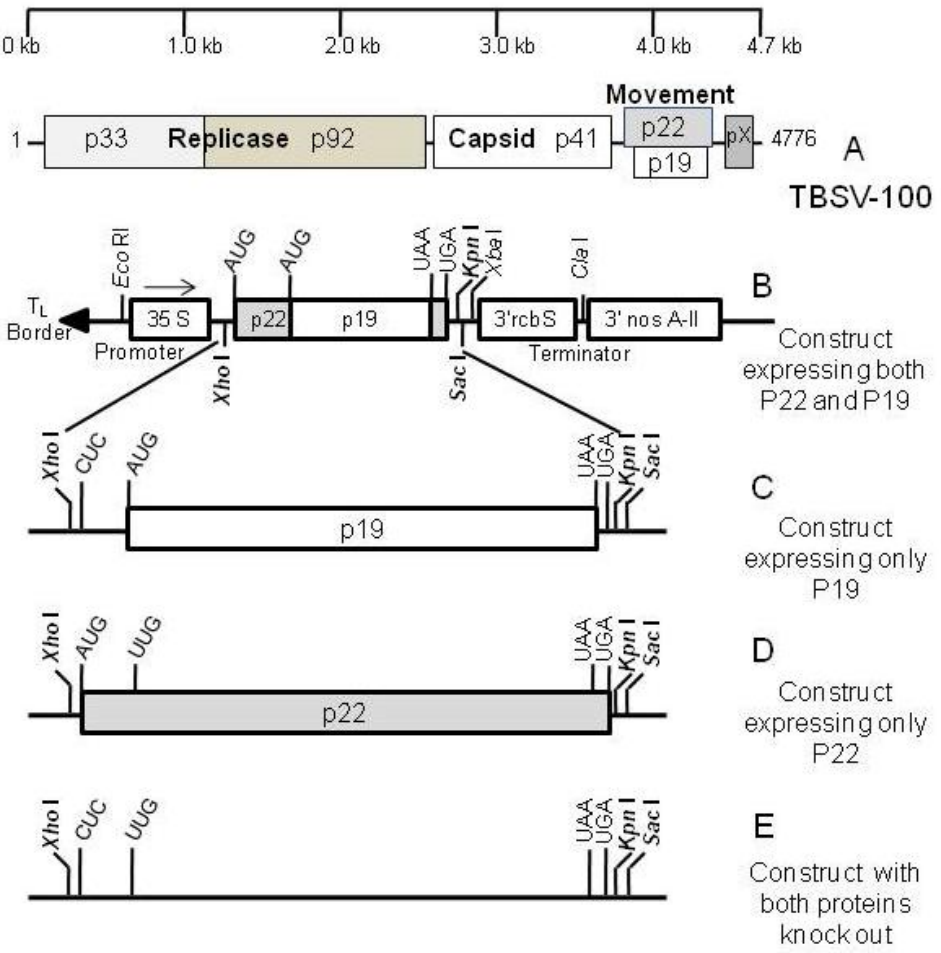



Figure III.1. Organization of the Tombusvirus type TBSV genome, and T-DNA constructs used for transient expression of Tombusvirus p22 and p19 homologous genes in *A. tumefaciens* strain AGL1 vectors. **A**, diagram of TBSV genome in the infectious clone TBSV-100 (Hearne et al. 1990). **B**, pKYLX7 -based construct (~12 kbp; Schardl et al. 1987) for expression of Tombusvirus genes. TBSV and CNV genes were cloned between the *Xho*I and *Sac*I sites, and CymRSV genes were cloned between *Xho* I and *Kpn*I sites in the multiple cloning site. Panels **B** to **E**, Composition of the construct expressing: B, both p22 and p19 nested genes. C, only p19. D, only p22. E, neither p19 or p22 (double knock out).

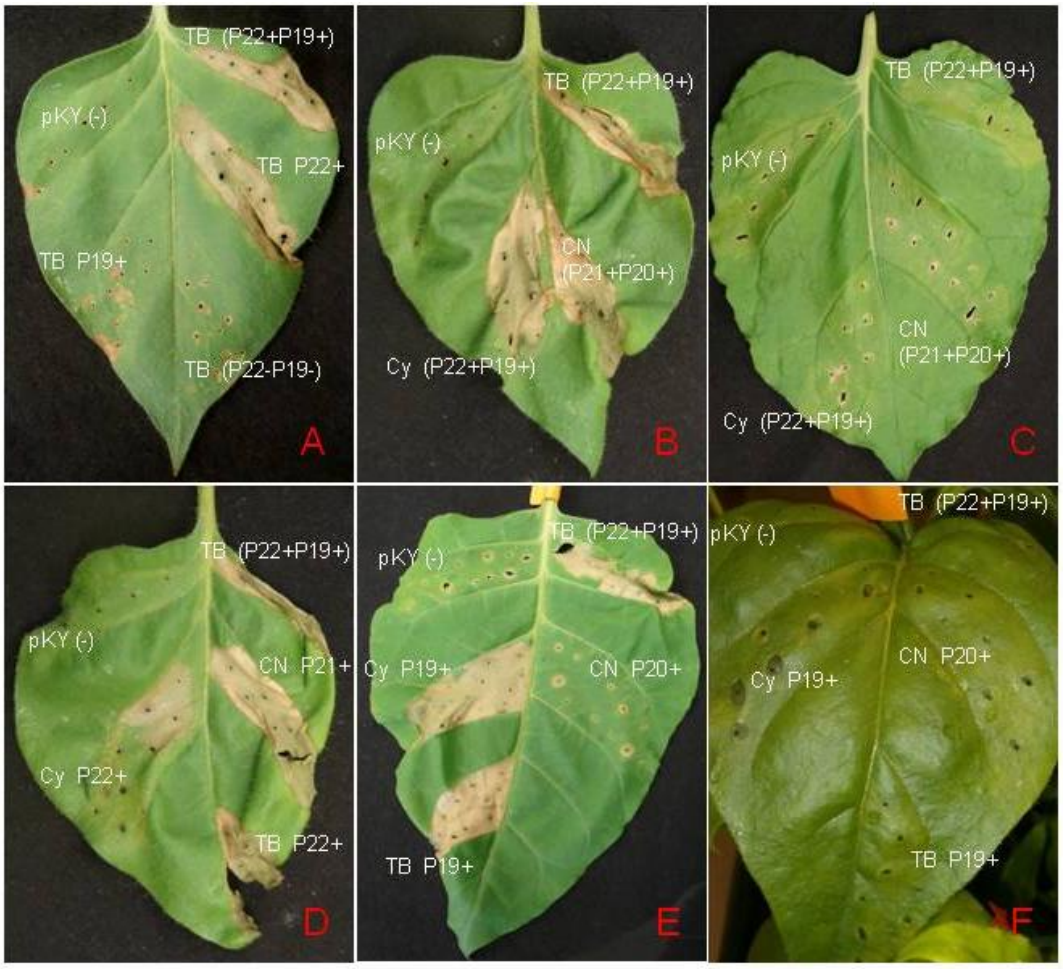



Figure III.2. Response of *Nicotiana* species to agroinfiltration of Tomato bushy stunt virus (TBSV) and Cymbidium ring spot virus (CymRSV) P19 and P22 proteins, and Cucumber necrosis virus (CNV) P21 and P20 proteins: **A**, *N. edwardsonii* at 7 dpi. **B**, *N. edwardsonii* at 7 dpi. **C**, *N. benthamiana* at 7 dpi. **D**, *N. edwardsonii* at 8 dpi. **E**, *N. tabacum* at 8 dpi. **F**, *N. clevelandii* at 5 dpi. pKY(-), pKYLX7 empty vector. TB, TBSV. CN, CNV. Cy, CymRSV. P22+P19+, Construct expressing both P22 and P19 proteins. P21+P20+, Construct expressing both P21 and P20 proteins. P22+, Construct expressing only P22 protein. P19+, Construct expressing only P19 protein. P21+, Construct expressing only P21 protein. P20+, Construct expressing only P20 protein. P22-P19-, Double knock out construct.

Table III.1. Response of five *Nicotiana* species to agroinfiltration of constructs expressing CNV, CymRSV, and TBSV movement proteins.

| | *N. glutinosa* and *N. edwardsonii* | *N. benthamiana* and *N. clevelandii* | *N. tabacum* |
|---|---|---|---|
| TBSV P22-P19 nested | HR | No | HR |
| TBSV P22 Only | HR | No | No |
| TBSV P19 Only | No | No | HR* |
| TBSV P22-P19 double K'O | No | No | No |
| CNV P21-P20 nested | HR | No | No |
| CNV P21 Only | HR | No | No |
| CNV P20 Only | No | No | No |
| CNV P21-P20 double K'O | No | No | No |
| CymRSV P22-P19 nested | HR** | No | No |
| CymRSV P22 Only | HR** | No | No |
| CymRSV P19 Only | No | No | HR |
| CymRSV P22-P19 double K'O | No | No | No |

* 1-2 days faster than TBSV nested genes ** 2-3 days slower than TBSV nested genes

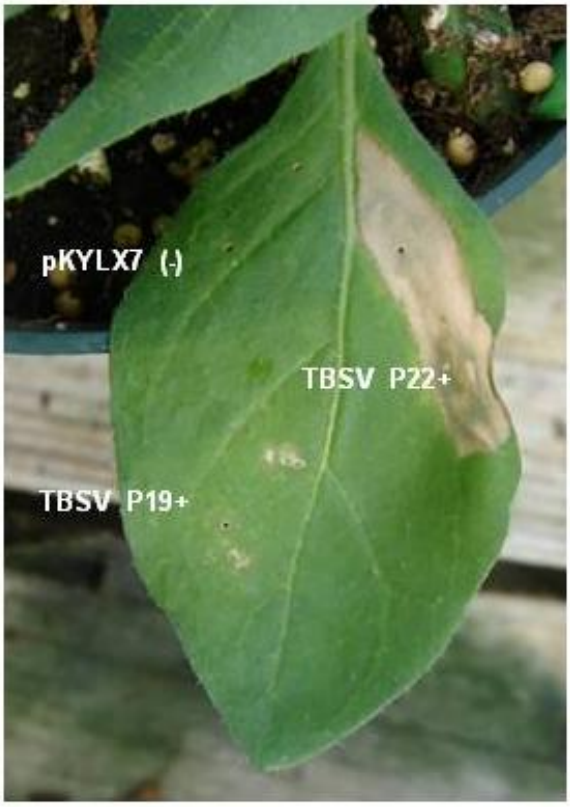



Figure III.3. Agroinfiltration of the TBSV homozygous resistant *N. clevelandii* Line 36 (Schoelz et al. 2006), with constructs expressing the pKYLX7 empty binary vector, and the TBSV P22 and P19 proteins, at 4. dpi.

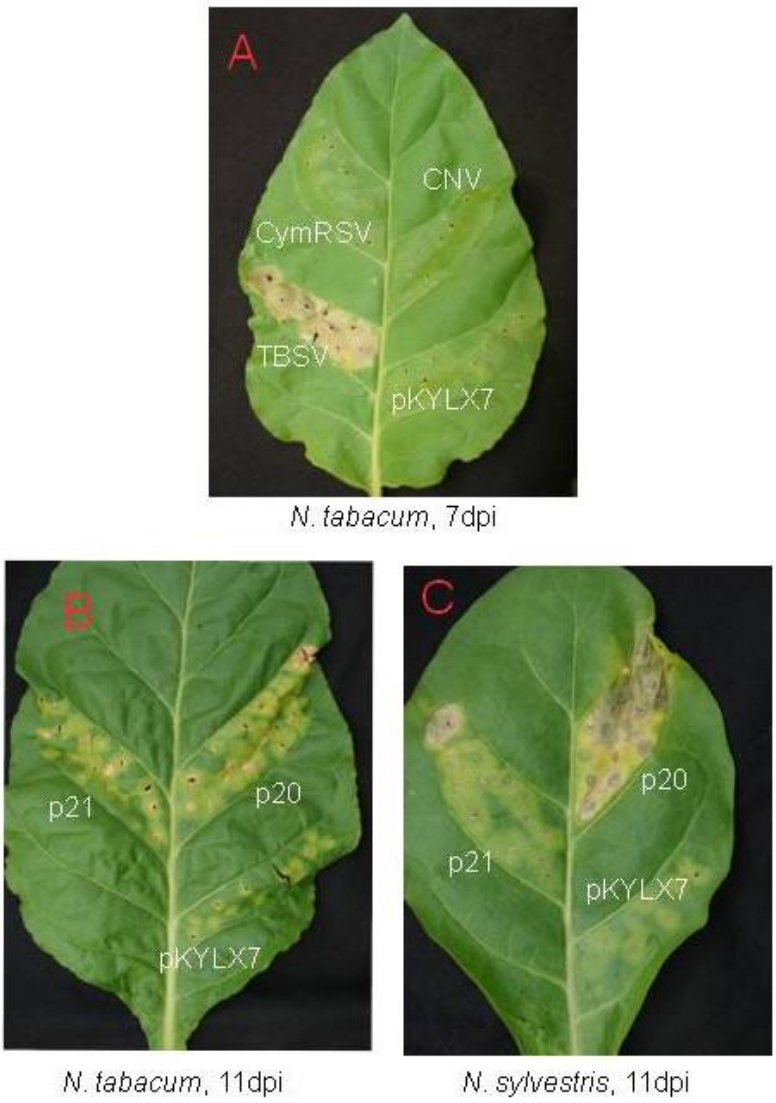
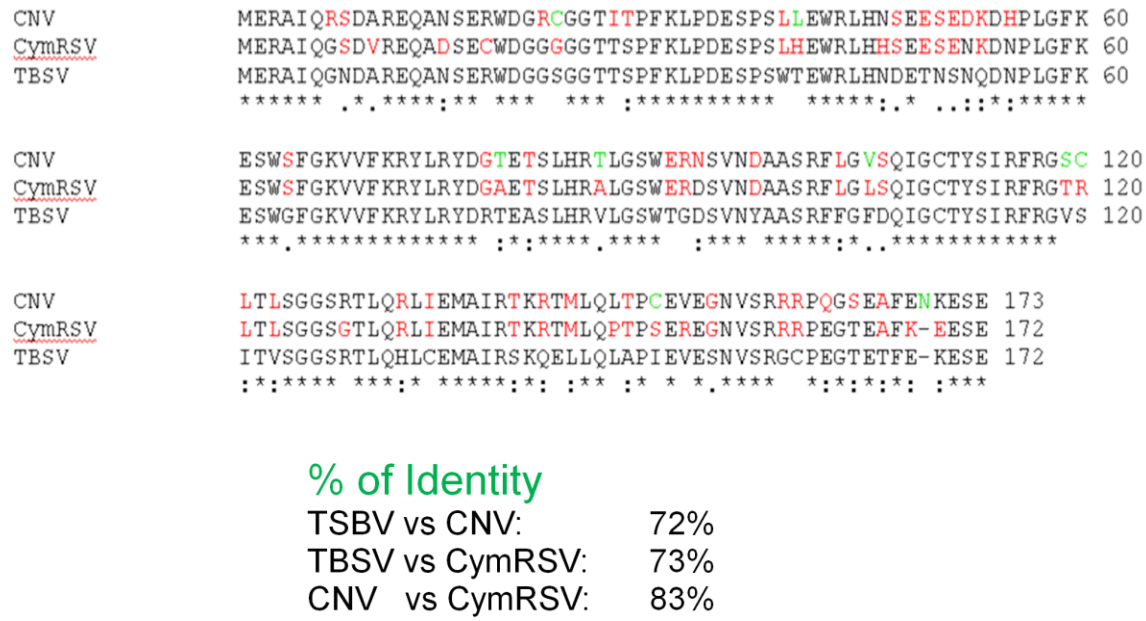



Figure III.4. Agroinfiltration of tombusvirus genes into *Nicotiana tabacum* and *N. sylvestris*. **A**. Agroinfiltration of nested constructs that express both p22 and p19 of TBSV and CymRSV, and both p20 and p21 of CNV in *N. tabacum*. **B**. Agroinfiltration of individual CNV genes into *N. tabacum*. **C**. Agroinfiltration of individual CNV genes into *N. sylvestris*. The panel labeled pKYLX7 was infiltrated with the empty *Agrobacterium* binary vector pKYLX7.




% of Identity

| | |
|---|---|
| TSBV vs CNV: | 72% |
| TBSV vs CymRSV: | 73% |
| CNV vs CymRSV: | 83% |

Figure III.5. Alignment for TBSV and CymRSV P19 proteins, and CNV P20 protein.

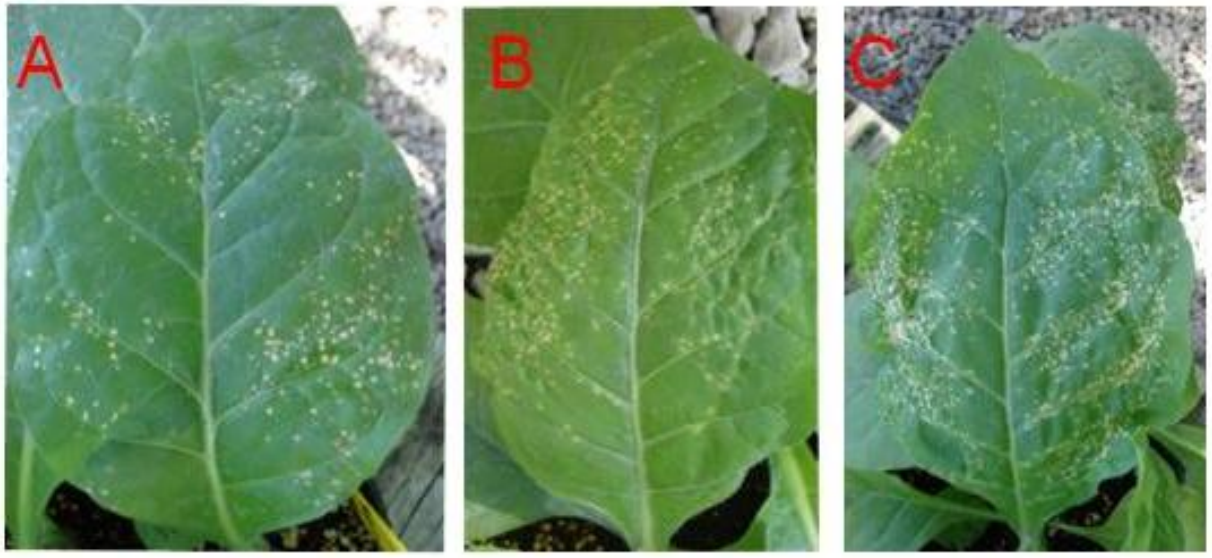



Figure III.6. Inoculation of Tombusviruses to *N. tabacum*. *N. tabacum* leaves were inoculated with **A**. CNV, **B**. CymRSV, and **C**. TBSV. Photos were taken at 5 dpi.

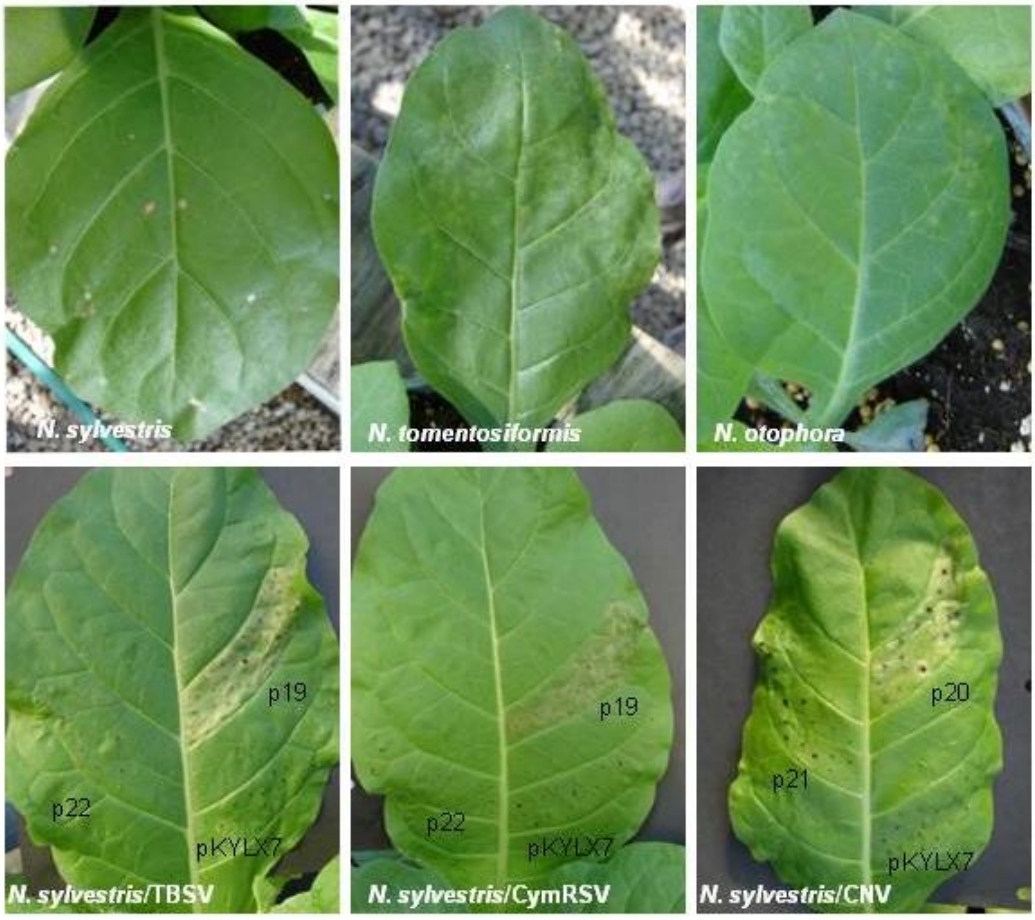



Figure III.7. Response of *N. tabacum* parents to tombusvirus infection and agroinfiltration of tombusvirus proteins. The top row illustrates the response of *Nicotiana* species to inoculation of TBSV at 5 dpi. The bottom row illustrates the response of *N. sylvestris* at 3 dpi to agroinfiltration of TBSV p19 and p22, CymRSV p19 and p22, and CNV p20 and p21. In addition, the empty vector pKYLX7 was also agroinfiltrated in this test.

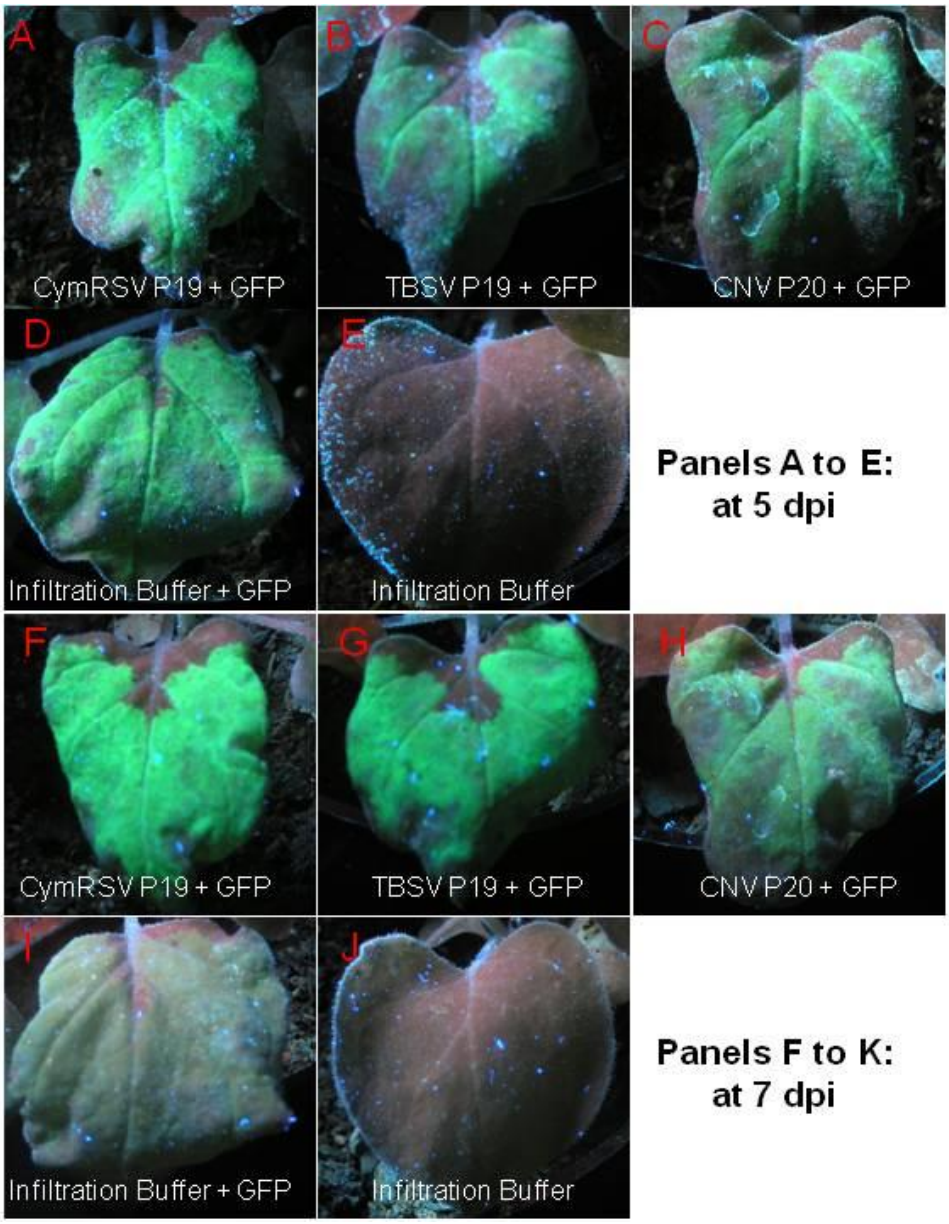



Figure III.8. Transient GFP expression upon co-infiltration of TBSV and CymRSV P19 and CNV P20 expressing constructs. Compared to 5 dpi infiltrated (panels **A** to **E**) and 7 dpi infiltrated *N. benthamiana* leaves (panels **F** to **J**), all samples (except the negative (-) control only infiltrated with infiltration buffer) showed similar amounts of GFP fluorescence at 5 dpi. Nevertheless, different levels of GFP expression were observed at 7 dpi. At 7 dpi, co-infiltration with GFP- and CymRSV P19-expressing agrobacteria showed most intense GFP expression, TBSV P19 displayed intermediate GFP signals, CNV P20 gave much lower GFP expression, while GFP signals faded away on infiltrated leaves only treated with GFP expressing agrobacterium and buffer. Experiment with collaboration of Yi-Cheng Hsieh (Texas A&M University).

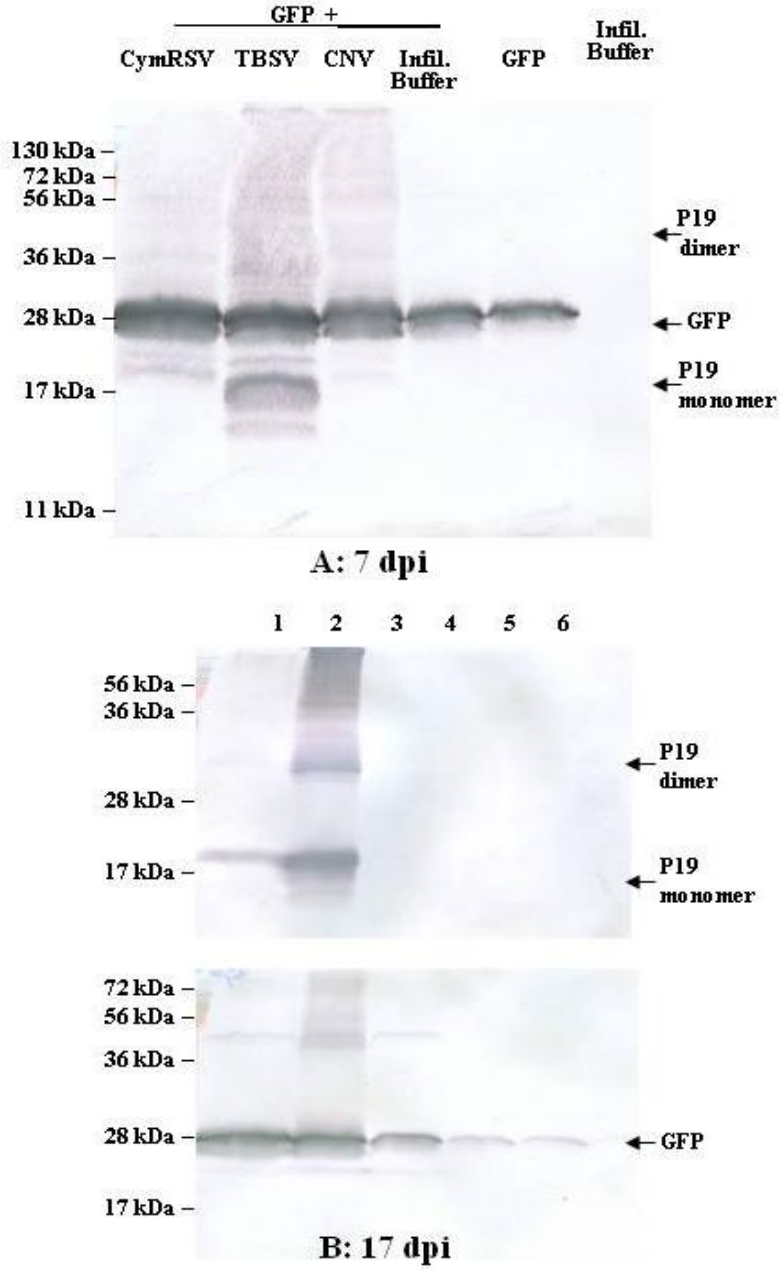



Figure III.9. Western blot for detection of GFP and P19 from infiltrated *N. benthamiana* leaves. **A**, at 7 days after infiltration (dpi). **B**, at 17 dpi. Lanes: 1, CymRSV P19 + GFP co-agroinfiltration. 2, TBSV P19 + GFP co-agroinfiltration. 3, CNV P20 + GFP co-agroinfiltration. 4, GFP agroinfiltration + infiltration buffer (equal volumes). 5, GFP only agroinfiltration. 6, Infiltration buffer only (negative control). Arrows indicate positions of GFP, and of P19 monomer and dimmers. Experiment with collaboration of Yi-Cheng Hsieh (Texas A&M University).

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CNV             MDTEYEQVNKPWNELYKEATLGNKLTVNVGMEDVEVPLLPSNFLTKVRVSLSGGYITVRR 60
CymRSV          MDTEYQQVNKPWNELYKEVTLGNKLTVNVGMEEEEVLLLPSNFPTKVRVSMSGGYITVRR 60
TBSV            MDTEYEQVNKPWNELYKETTLGNKLTVNVGMEDQEVPLLPSNFLTKVRVGLSGGYITMRR 60
                *****:***********.*************: ** ****** *****.:******:**

CNV             VRIKIIPLVSRKAGVSGKLYLRDISDTTGRKLHCTELLDLGKEIRLTMQHLDFSVSARSD 120
CymRSV          VRIRIIPLVSRKAGVSGKLYLRDISDTTGQKLHCTELLDLGKEIRLTMPHLDFSVSAKSD 120
TBSV            IRIKIIPLVSRKAGVSGKLYLRDISDTTGRKLHCTESLDLGREIRLTMQHLDFSVSTRSD 120
                :**:*************************:****** ****:****** *******::**

CNV             VPIVFGFEDLVSPYLEGRELFSVSLRWQFGLSAQCYSLPPAKWKVMYQEDALKALKPSKI 180
CymRSV          VPIAFGFEELVSPFREGRELFSVSLRWQLGLSAQCYSLPPANVKVMYQEDALKALKPSK- 179
TBSV            VPIVFGFEELVSPFLEGRELFSISVRWQFGLSKNCYSLPQSKWKVMYQEDALKVLRPSK- 179
                ***.****:****: *******:*:***:*** :***** :: **********.*:***

CNV             KKASKTDSSV 190
CymRSV          KKASRTDSSV 189
TBSV            KKASKTDSSV 189
                ****:*****
```

% of Identity

| | |
|---|---|
| TSBV vs CNV: | 86% |
| TBSV vs CymRSV: | 83% |
| CNV vs CymRSV: | 86% |

Figure III.10. Alignment for TBSV and CymRSV P22 proteins, and CNV P21 protein.

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# CHAPTER IV

# DIVERSITY OF RESISTANCE TO THREE TOMBUSVIRUSES IN *NICOTIANA* SPECIES: EMERGENCE OF THE TBSV COAT PROTEIN P41 GENE AS AVIRULENCE DETERMINANT

## ABSTRACT

Our lab has been working on characterization of resistance to tombusviruses in *Nicotiana* species. For example, we recently confirmed by agroinfiltration that the tomato bushy stunt virus (TBSV) P22 protein elicited hypersensitive response (HR) in *N. glutinosa* and *N. edwardsonii*, and the TBSV P19 protein triggered HR in *N. tabacum*, in agreement with previous studies. These results suggested that *Nicotiana* species have different resistance genes to recognize two avirulence proteins. However, little is known about the diversity in resistance to tombusviruses in the entire *Nicotiana* genus, and the capacity of other species to recognize tombusvirus P19 and P22 proteins as Avr determinants. Of 75 *Nicotiana* species, 40 are diploids and 35 are allopolyploids, whose genomes are derived from interspecific recombination between two diploid species as ancestors. To explore this diversity against tombusviruses, we initially inoculated virions of TBSV, cucumber necrosis virus (CNV), and cymbidium ring spot virus (CymRSV) to 18 *Nicotiana* species representative of 10 of the 14 taxonomic sections in the genus. We

found that 10 species responded with HR, another five *Nicotiana* species were resistant, but this resistance did not involve classical HR, and three species were susceptible. To study the role of TBSV, CymRSV, and CNV P19 and P22 proteins in eliciting HR, we agroinfiltrated constructs to express these proteins into leaves of the resistant *Nicotiana* species, especially those that responded with HR. The three tombusvirus P22 proteins elicited HR only in *N. glutinosa* and *N. edwardsonii*. In contrast, the tombusvirus P19 proteins varied in their role as avirulence factors, as the TBSV P19 protein triggered HR in *N. tabacum*, *N. sylvestris*, and *N. bonariensis*, the CymRSV 19 protein triggered HR in *N. tabacum* and *N. sylvestris*, and CNV P20 elicited HR only in *N. sylvestris*. This indicated that *Nicotiana* species can recognize differences in tombusvirus P19 proteins. Furthermore, other tombusvirus proteins might be responsible for eliciting HR in the other six *Nicotiana* species. To determine if other tombusvirus proteins were capable of triggering HR, we created constructs that would express the TBSV p41 coat protein gene, and the p33 and p92 replicase genes. We found that *N. langsdorfii*, *N. longiflora*, and *N. bonariensis*, all members of the Alatae section, responded to agroinfiltration of the TBSV p41 gene with HR, and no other viral protein/host combination resulted in HR. Thus, we evaluated if the other 4 *Nicotiana* species of the Alatae section that are available could respond with HR to virions and TBSV p41 as well. We found that all species and tested accessions responded with HR to virion inoculation, but only *N. plumbaginifolia* was systemically susceptible. On the other hand, *N. alata* accession TW7 and TW8, *N. forgetiana* accession TW51, and *N. mutabilis* responded with HR to the p41 gene; *N. forgetiana* TW50 responded to both p41 and p22 genes, and *N. plumbaginifolia* TW106 and TW108 responded to P19 only. This study shows that *Nicotiana* species are valuable

sources for at least 5 resistance genes to tombusviruses. In addition to the P22 and P19 proteins, we found that the coat protein gene p41 is also an avirulence determinant, conserved in the species of the section Alatae. Furthermore, we found that *Nicotiana* species are able to recognize small differences in avirulence determinants, and agroinfiltration can be used to rapidly survey a broad cross section of a genus to identify new sources of resistance and new avirulence targets.

# INTRODUCTION

Plants have evolved diverse strategies for defense against pathogen infections, strategies that have been described under different theories through the years. Among these, the "gene for gene" model (Flor, 1971) determined that pathogen recognition is genetically controlled by a host resistance gene (R), which targets pathogen strains carrying an avirulence gene (Avr), preventing the host's infection. Within the "gene for gene" model one of the possibities suggested the direct interaction between the protein products of R and Avr genes, which has been proven in a few situations (Deslandes et al. 2003; Dodds et al. 2006). However, later studies showed that the resistance mechanism can also act through an indirect interaction explained under the "guard hypothesis" (Van der Biezen and Jones, 1998), in which pathogen proteins called effectors modify, debilitate, or eliminate host proteins and defense mechanisms. The role of host R proteins would be to monitor these changes and to deploy a cascade of defense responses to limit pathogen growth. The complexity and time frame of these recognition steps and the triggered defense responses were better described by the "zig-zag" model (Jones and Dangl, 2006), which illustrates different events that contribute to the plant immune system. This model distinguishes between pathogen-associated molecular pattern - triggered immunity (PTI) at a trans-membrane level, and the effector-triggered immunity (ETI) inside the host cell. Recently, the "decoy' model (Van der Hoorn and Kamoun, 2008) has been proposed to explain how, depending on the presence or absence of the R gene product in the host, there is unstability of trarget proteins, and the function of the pathogen effector also varies. It means that the same pathogen effector can have different

functions in the host, and the host guardee proteins can be dispensable without affecting pathogen performance when the corresponding guard protein is absent.

Resistance to plant viruses has been mostly explained by the "gene for gene" model, and current theories have been updated to include the ETI mechanism. In contrast to bacteria and fungi, all proteins in a virus genome can be considered effectors because they are expressed inside the host cell and they interact with a variety of host proteins to contribute to virus gene expression, replication or movement, or they modify plant defense pathways through suppression of the host's gene silencing machinery. A viral protein may be considered an Avr determinant if it elicits a plant defense response in the presence of an R gene (reviewed by Schoelz, 2006). In this context of "gene for gene" resistance, and specifically dominant resistance characterized by the rapid programmed cell death or hypersensitive response (HR) (Lam et al. 2001), different viral proteins from a single virus genome can be Avr determinants, and a single viral Avr determinant can be recognized by different R genes. For example, the coat protein of potato virus X (PVX) triggers defense responses mediated by three potato R genes, *Nx*, *Rx1* and *Rx2*, all derived from different potato varieties. The P6 protein of cauliflower mosaic virus (CaMV) triggers HR in *Datura stramonium* and several *Nicotiana* species, but also triggers a non-necrotic defense response in *Nicotiana bigelovii* and *Arabidopsis thaliana* ecotype Tsu-O (reviewed by Schoelz, 2006). In some instances, virus resistance is mediated through a variation of the guard hypothesis. The best example of this mechanism is the resistance elicited by the coat protein of turnip crinkle virus (TCV) in *A. thaliana* Di-O due to the R gene *HRT* (for Hypersensitive Response to TCV), which is

mediated by the host transcription factor TIP (for Turnip crinkle virus-Interacting Protein) (Qu et al. 2003; Ren et al. 2005).

Understanding evolution and diversity of both plant and viral genomes can contribute to a better knowledge of resistance mechanisms to plant viruses (Kang et al. 2005). On the host side, one of the best examples for diversity of virus resistance genes can be found within the *Nicotiana* genus, which consists of approximately 75-77 species grouped into 14 taxonomic sections (Clarkson et al. 2004) distributed in South, Central, and North America, as well as in Australia, and a single species in Africa. *Nicotiana* species are one of the most studied subjects for genome evolution, cytogenetics, and plant pathology, and since the early stages of virology they have been widely used as experimental hosts for characterizing resistance or susceptibility to numerous viruses. For example, the *N* gene, which specifies resistance to tobacco mosaic virus (TMV) was one of the first virus resistance genes to be discovered and cloned (Holmes, 1938; Dunigan et al. 1987; 1946; Whitham et al. 1994). In particular, *N. benthamiana* and *N. tabacum* have been frequently used as model hosts in the study of plant virology (Lewis and Nicholson, 2007; Goodin et al. 2008). *N. glutinosa* and *N. clevelandii* have also emerged as valuable hosts for the study of resistance to viruses. *N. glutinosa* is resistant to at least 67 virus species from 20 genera that can systemically infect *N. clevelandii*, and it is possible to genetically characterize the inheritance of resistance in *N. glutinosa* through an interespecific cross with *N. clevelandii* (Schoelz et al. 2006).

On the pathogen side, many virus gene products were shown to function as Avr genes in *Nicotiana* species. The first viral protein confirmed to be an Avr determinant was the CaMV P6 protein, which is recognized as an Avr determinant by *N. glutinosa*, *N.*

*edwardsonii*, and *N. bigelovii* (Schoelz et al. 1986, Wintermantel et al. 1993). The coat protein of TMV was shown to function as an Avr determinant in *N. sylvestris* (Saito et al. 1987; Culver and Dawson, 1991), whereas the helicase domain (P50) of the 183 kDa replicase of TMV was shown to trigger HR mediated by the *N* gene in *N. tabacum* plants (Erickson et al. 1999), a virus R gene originally found in *N. glutinosa*.

Our lab has been interested in identifying proteins of tombusviruses that trigger resistance in *Nicotiana* species. Tombusviruses have a genome composed of single-stranded positive sense RNA approximately 4,700 – ~ 4,800 nucleotides in length that is encapsidated into isometric virions 30-35 nm in diameter. Tomato bushy stunt virus (TBSV) is the type member of tombusviruses. The TBSV genome is organized into six open reading frames (ORFs) (Hearne et al. 1990; Yamamura and Scholthof, 2005). The first ORF is translated directly from the genomic RNA to produce a 33kDa protein (P33). A read-through mechanism leads to a second protein of 92 kDa (P92), which is a viral RNA dependent RNA polymerase. Both P33 and P92 are required for replication, in a ratio of approximately 20 to 1. The third ORF is translated from a subgenomic RNA (sgRNA1), and encodes the 41kDa capsid or coat protein (P41), which is also required for long distance transport (Scholthof et al. 1993). The fourth and fifth ORFs are nested, translated from a second single subgenomic RNA (sgRNA2), and encode two proteins, P22 and P19 (Hearne et al. 1990; Scholthof et al. 1995b). The P19 and P22 proteins were shown to be involved in cell- to- cell and long distance movement, host range determination, and symptom development. The P19 protein is also known by its strong suppressor activity on post-trancriptional gene silencing (Voinnet et al. 1999; Scholthof,

2006). The sixth ORF encodes for a small protein designated pX, which is not essential for replication, but required for virus accumulation (Scholthof and Jackson, 1997).

We previously showed that a R gene effective against a broad range of tombusviruses could be introgressed from the resistant *N. glutinosa* into the susceptible *N. clevelandii*, and that this R gene product specifically targets the P22 protein of tombusviruses (Schoelz et al. 2006; see chapter III). Furthermore, a second R gene derived from *N. sylvestris* was found that targets TBSV P19, and, since *N. sylvestris* is one of the parents of *N. tabacum* (Aoki and Ito, 2000, Clarkson et al. 2004), a variation of this gene must also exists in *N. tabacum* (see chapter III).

In the present study, I initially surveyed the response of 18 *Nicotiana* species representative of 10 out of 14 taxonomic sections to inoculations of the three tombusviruses TBSV, cucumber necrosis virus (CNV) and cymbidium ringspot virus (CymRSV) to identify the prevelance of HR-type resistance in the genus *Nicotiana*. Having identified *Nicotiana* species that respond with HR, I then sought to determine whether HR was triggered by either P22 or P19 proteins. In the process of conducting this survey, I identified a third tombusvirus gene capable of triggering HR in members of the *Nicotiana* genus.

## RESULTS

### Survey of *Nicotiana* species for resistance to tombusviruses.

To determine the response of 18 different *Nicotiana* species, which are representative of 10 out of 14 taxonomic sections of the *Nicotiana* genus (Clarkson et al. 2004), we inoculated leaves of 4 - 6 week old plants with sap extracts from plants infected with TBSV, CNV or CymRSV. We found that 10 of 18 species responded with HR-type resistance; four species were resistant without development of an HR, one species showed mixed chlorotic local and necrotic lesions, and three species developed systemic infections (Table IV.1). We observed that the three tombuviruses induced identical responses in individual *Nicotiana* species. *Nicotiana* species that responded with HR developed necrotic local lesions at 2-4 days post inoculation (dpi), although three different HR phenotypes were observed. Type 1 was characterized by dark lesions, 2-3 mm in diameter, and was observed with *N. glutinosa* and *N. edwardsonii* (Fig.IV.1A and C). Type 2 lesions were a lighter brown, were 1-2 mm in diameter, and were observed in the *Nicotiana* species *N. tabacum* (Fig. IV.1B and D) and *N sylvestris*. Type 3 lesions were a pale brown or yellowish color, were smaller than 1 mm in diameter, and were observed in *Nicotiana* species *N. rustica*, *N. undulata*, and *N. bonariensis* (Fig. IV.1E). In all cases, ELISA assays conducted between 21 – 28 dpi confirmed that these *Nicotiana* species were truly resistant to infection, as TBSV, CNV and CymRSV were not detected in upper, non-inoculated leaves. Our results were consistent with Scholthof

et al. (1995ab), who also found that local lesions elicited by TBSV in *N. tabacum* were smaller than those elicited in *N. glutinosa* and *N. edwardsonii*.

Four *Nicotiana* species were resistant, but their response to tombusvirus infection did not fit the classical description of HR as a rapid programmed cell death (Lam et al. 2001). Two species, *N. tomentosiformis* and *N. otophora*, responded to each of the three tombusviruses with chlorotic lesions at 3 – 5 dpi that were 2-3 mm in diameter (Fig. IV.1F). These lesions later turned necrotic at 12 -15 dpi. *N. glauca* plants developed diffuse chlorotic local lesions, but they were not followed by systemic chlorosis. Inoculated plants remained symptomless. ELISA assays revealed that no virus could be detected in the upper, non-inoculated leaves, so these three *Nicotiana* species as being resistant to tombusvirus infection. Interestingly, the fourth species, *N. wigandioides*, did not show any local reaction, and no virus was detected in upper non-inoculated leaves, indicating that the plant may exhibit a form of extreme resistance to tombusviruses (Hull, 2002).

*N. clevelandii*, *N. benthamiana*, and *N. quadrivalvis* (Syn. *N. bigelovii*) were all clearly susceptible. Each *Nicotiana* species responded to each of the three tombusviruses with severe systemic chlorosis and necrosis, with death of the plants 2-5 weeks after inoculation (Fig. IV.1G). DAS-ELISA assays conducted on upper, non-inoculated leaves at 21 – 28 dpi confirmed the systemic movement of tombusviruses in these hosts (data not shown). One species, *N. repanda*, developed a mixed reaction between chlorotic and necrotic lesions. Although upper, non-inoculated *N. repanda* leaves remained symptomless, the ELISA assays for these tissues indicated a weak positive reaction, but only for TBSV infections.

**Identification of *Nicotiana* species that respond to agroinfiltration of tombusvirus P22 and P19 proteins.**

I previously found that agroinfiltration of the tombusvirus protein P22 elicited HR in the resistant hosts *N. glutinosa* and *N. edwardsonii,* and that agroinfiltration of the tombusvirus P19 protein triggered a HR in *N. tabacum* and *N. sylvestris* (see chapter III, Table IV.1), suggesting a role of these two viral proteins as avirulence genes in the "gene for gene" model for resistance (Flor, 1971). In addition, I found that neither P19 nor P22 elicited systemic necrosis upon agroinfiltration into *N. benthamiana* and *N. clevelandii* (Table IV.1, see chapter III), indicating that these plants did not contain R genes that could recognize these proteins in the context of virion inoculations or in agroinfiltration assays. Since I now found that a large number of *Nicotiana* species responded to tombusvirus infections with HR, I sought to determine if these *Nicotiana* species could recognize P22 or P19 proteins as avirulence determinants.

Consequently, I agroinfiltrated leaves of each of the 18 *Nicotiana* species with TBSV p22, CymRSV p22, and CNV p21, and scored plants for the presence of HR over a period of twelve days after infiltration. I found that these proteins triggered HR in only *N. glutinosa* and *N. edwardsonii*. This indicated that none of the ten species that responded with HR to inoculation of tombusvirus virions contained an R gene product that could recognize the tombusvirus P22/P21 protein (Table IV.1). I also evaluated the TBSV and CymRSV P19 proteins as well as the CNV P20 protein for their capacity to elicit HR in the 18 *Nicotiana* species. I confirmed that all three tombusvirus proteins

triggered HR in *N. sylvestris*, and in addition that TBSV and CymRSV P19 proteins triggered HR in *N. tabacum*. Of the ten *Nicotiana* species that responded to virion inoculations with HR, only *N. bonariensis* responded with HR to the TBSV P19 protein (Table IV.1). Interestingly, neither the CymRSV P19 nor the CNV P20 protein triggered HR in *N. bonariensis* (Table IV.1), a pattern that was similar to that of *N. tabacum*. These results showed that neither the tombusvirus P22 nor P19 proteins were responsible for eliciting HR in nine of ten *Nicotiana* species that developed HR – type lesions. Consequently, we hypothesized that other tombusvirus genes, such as p33, p92 or p41, might elicit HR in these other *Nicotiana* species.

**Screening of *Nicotiana* species for HR to agroinfiltration of TBSV genes p41, p33, p92, and the non-overlapping region of p92.**

To determine if other tombusvirus genes in addition to p22 and p19 could elicit HR in *Nicotiana* species, we inserted individual TBSV genes into the *A. tumefaciens* binary vector pKYLX7 (Fig. IV.2). Four constructs were created to express the TBSV coat protein gene p41, the TBSV replicase genes p33 and p92, and the portion of p92 that does not overlap with p33 (Fig. IV.2) (Hearne et al. 1990). The rationale for this non-overlapping region of p92 was based on the TMV P50 helicase protein, the portion of the TMV 183 kDa replicase protein that does not overlap with the 126 kDa replicase protein. A previous study found that the TMV P50 protein was a more effective elicitor of HR than the 183 kDa protein in an agroinfiltration assay of tobacco carrying the *N* gene (Erickson et al. 1999).

Each of the constructs expressing a TBSV gene was agroinfiltrated into the ten *Nicotiana* species that responded to tombusviruses with HR. We found no reaction in any *Nicotiana* species to agroinfiltration of TBSV p33, p92, or the non-overlapping region of the p92 gene. In contrast, the TBSV p41 gene elicited HR in *N. langsdorfii*, *N. longiflora*, and *N. bonariensis*, within 2 to 7 dpi depending on the species and plant age. The fastest HR occurred with *N. langsdorfii* at 2 dpi, whereas the slowest was observed with *N. longiflora* at 7 dpi. Figure IV.3 illustrates the response of *N. langsdorfii* and *N. bonariensis* to agroinfiltration of p41, p33 and p92 at 5 dpi. This variation in timing is interesting because all three species responded to virion inoculations with HR after 2-4 dpi. However, we associated delayed response to the plant age and its capacity to express the infiltrated gene, because agroinfiltrations of p41 in younger plants yielded faster HR reactions in the three species (data not shown)

Burgyán et al. (2000) suggested that a systemic necrosis symptom in *N. benthamiana* was due to the combined effects of the CymRSV P33 and the P19 protein. They were not able to test a P33 construct by itself, since these authors used transcripts of chimeras between CymRSV and CIRV. To determine if a combination of P33 and P19 could trigger necrosis in *N. benthamiana*, I mixed Agrobacteria containing TBSV p19 and TBSV p33 in a 1:1 ratio and agroinfiltrated *N. benthamiana* leaves with this mixture. This combination did not yield any visible necrosis under our assay conditions in *N. benthamiana* leaves (data not shown). Consequently, the co-agroinfiltration and expression of P33 and P19 could not reproduce the effects of a tombusvirus infection in this instance.

**The hypersensitive response elicited by the TBSV p41 gene is conserved amongst *Nicotiana* species within the Alatae section.**

We showed that three *Nicotiana* species belonging to the Alatae section, *N. langsdorfiii*, *N. bonariensis*, and *N. longiflora*, responded with HR to TBSV, CNV and CymRSV virion inoculations, and that all three species responded to TBSV p41 agroinfiltation with HR. Based on my results, I hypothesized that other members of the Alatae section might repond to TBSV p41 with HR. The section Alatae of *Nicotiana* contains 8 species, although *N. azambujae* is known only by ancient herbarium specimens from Brazil (Clarkson et al. 2004; Lim et al. 2006). However, the other four species were readily available from the collection of Dr. Timothy Holtsford at the University of Missouri (Columbia, MO). He obtained most of them from the USDA-ARS *Nicotiana* collection at North Carolina State University.

To elucidate if the HR elicited by the TBSV p41 gene is conserved among the other four available species within this section, I evaluated the response of these species to TBSV virions and found that all responded to TBSV infection with necrotic local lesions. Although all four species responded with necrotic lesions (Table IV.2), there was considerable variation in this response. Both of the accessions of *N. alata*, as well as the single accession of *N. mutabilis*, responded to virion inoculations with low numbers of pale-brown lesions 2-3 mm in diameter and 1-2 mm in diameter, respectively. The two accessions of *N. forgetiana* responded to TBSV inoculation with large, tan lesions that were 2-3 mm in diameter. An ELISA of these three species confirmed that this response was a true HR, as there was no evidence of systemic spread of the virus. The

two accessions of *N. plumbaginifolia* responded with numerous nearly black lesions smaller than 1 mm in diameter. In contrast to the other members of the Alatae, both of the accessions of *N. plumbaginifolia* were susceptible, as evidenced by the symptoms in the upper noninoculated leaves and the confirmation of the ELISA test.

Having shown that all members of the Alatae section responded to TBSV with HR, I sought to determine which TBSV protein was the elicitor. Although I focused on the TBSV p41 gene, I also tested all of the other TBSV genes I developed for agroinfiltration. I found that both of the accessions of *N. alata* and *N. forgetiana*, as well as the unique accession of *N. mutabilis* responded with HR to agroinfiltration of the TBSV p41 gene (Fig. IV.4A, B, and D). Interestingly, I also found that the *N. forgetiana* TW50 accession responded to the TBSV P22 protein with HR, but the *N. forgetiana* accession TW51 did not (Table IV.2, Fig. IV.4C). The TBSV p41 gene failed to elicit HR in both accessions of *N. plumbaginifolia*. Instead, these two accessions responded with HR to the TBSV P19 protein after 6-8 dpi (Table IV.2, Fig. IV.4G). No HR responses or necrotic effects were observed in species utilized as controls such as *N. benthamiana* and *N. clevelandii* (Fig. IV.4H).

# DISCUSSION

In this study, we screened 18 *Nicotiana* species belonging to 10 out of 14 taxonomic sections of the genus (Clarkson et al. 2004) for resistance to the tombusviruses TBSV, CNV and CymRSV. Fifteen out of 18 species were resistant, but resistance was manifested in different ways. Ten species responded with HR-type resistance, although there were differences in the HR phenotypes in terms of lesion size and number. Another five species were resistant, but did not develop a classic HR (Lam et al. 2001) (Table IV.1). Four of these five species, *N. glauca*, *N. wigandiodes*, *N. tomentosiformis*, and *N. otophora*, were previously reported to respond with non-necrotic resistance to tobacco etch virus (TEV) (Holmes, 1946), potato virus A, potato virus Y, and X (Van Dijk and Cuperus). All resistant and susceptible *Nicotiana* species responded in the same way to the three viruses. Tombusvirus-resistant species belonged to sections of *Nicotiana* native to South and Central America, and susceptible species were native to Australia and North America, the sections Suaveolentes and Polydicliae, respectively. This trend in resistance and susceptibility was previously observed by Holmes (1946, 1951) and Van Dijk and Cuperus (1989) for a variety of viruses, including TMV and several potato viruses.

We hypothesized that the *Nicotiana* species that responded with HR to virion inoculations would respond with HR to at least one of the two previously identified avirulence determinants, the P22 or the P19 proteins. In the case of the P22 protein, only *N. glutinosa* and its derived species *N. edwardsonii* responded with HR, as previously described (see chapter III). *N. glutinosa* belongs to the section Undulatae, which also includes *N. arentsii* and *N. undulata*. I hypothesized that both *N. arentsii* and *N. undulata*

might carry an R gene that recognizes the P22 protein. However, although *N. arentsii* and *N. undulata* responded to virion inoculations with HR, the agroinfiltration of the p22 gene did not elicit HR. It might be that neither of these species carries an R gene that recognizes P22, but this variation could also reflect conflicting results in phylogenetic studies for the Undulatae section (Clarkson et al. 2004). I previously showed that agroinfiltration of p19 elicits HR in *N. tabacum* and *N. sylvestris.* The only new *Nicotiana* species to respond with HR to agroinfiltration of p19 in the present study was *N. bonariensis*, and it only responded with HR to the TBSV P19 protein, not to CNV P20 or CymRSV P19 proteins. In some manner, *N. bonariensis* must be able to recognize differences between homologous P19 proteins as did *N. tabacum*. Despite the fact that most *Nicotiana* species responded to virion inoculations with HR, only a few *Nicotiana* species developed HR in response to agroinfiltrations of P22 and P19 proteins. Consequently, I hypothesized that other tombusvirus proteins, in addition to P22 and P19, must act as Avr determinants.

To identify new TBSV genes that might act as Avr determinants, I created new *A. tumefaciens*-based constructs for the coat protein gene p41, the replicase genes p33 and p92, and the region of p92 that does not overlap with p33. I found that only the TBSV p41 gene elicited HR in three species: *N. langsdorfii*, *N. bonariensis*, and *N. longiflora*. Although the TBSV coat protein gene had not been implicated previously as an Avr determinant in *Nicotiana* species, the coat protein gene of CymRSV was previously shown to elicit HR in the solanaceous host *Datura stramonium*. Szittya and Burgyán (2001), created chimeric virus transcripts to show that an 860 nt untranslatable RNA sequence comprising the CymRSV coat protein gene was an elicitor of a very rapid HR

in *D. stramonium*. This was the first report of a viral RNA sequence serving as an Avr determinant rather than the protein sequence. In our study, we did evaluated whether the TBSV coat protein or the corresponding RNA sequence is responsible for triggering HR. This will be a priority for future studies. Interestingly, the coat protein of the Carmovirus TCV, another member of the Tombusviridae family, was able to elicit HR in *A. thaliana* ecotype Di-O containing the *HRT* resistance gene (Qu et al. 2003; Ren et al. 2005).

The TBSV coat protein is dispensable for infectivity in both protoplasts and plants. However, coat protein mutants showed variations in symptom development, but this was attributed to impaired long distance spread of the virus (Scholthof et al. 1993). Qu and Morris (2002) concluded that TBSV P41, P19, and P22 function cooperatively to facilitate systemic infection and lethal necrosis in *N. benthamiana*, and the coat protein is required for efficient exit from the vascular tissue into upper, non-inoculated leaves. Mutations in the coat protein of CymRSV and CNV affected infectivity and systemic movement in *N. clevelandii* and *N. benthamiana* in different ways. A mutant of CymRSV showed restricted long distance movement, eliciting a HR in *N. clevelandii*, but moved systemically in *N. benthamiana* (Dalmay et al. 1992). Other coat protein mutants of CNV were able to move systemically in *N. clevelandii* (Rochon et al. 1991).

*N. langsdorfii*, *N. bonariensis*, and *N. longiflora* are all members of the section Alatae of *Nicotiana*. Consequently, I investigated whether the other four available species of this section might also recognize the TBSV p41 gene as an Avr determinant. I found that *N. alata*, *N. mutabilis*, and *N. forgetiana* responded with HR to virion inoculation and also to agroinfiltration of the TBSV p41 gene. The fourth species, *N. plumbaginifolia* was susceptible to TBSV virion inoculation with the development of

systemic symptoms. Interestingly, the agroinfiltration assay indicated that the TBSV P19 protein was responsible for a local necrosis symptom rather than p41, as *N. plumbaginifolia* responded to agroinfiltration of the p19 gene with a delayed HR.

The members of the *Nicotiana* genus have been the subject of numerous phylogenetic studies. I found that I could utilize this information to investigate the evolution of R genes within the genus. For example, it has been hypothesized that the ancestral basic number of chromosomes for *Nicotiana* should be 12 (2*n*=24). Newer species show a reduction and increase in the chromosome number (Goodspeed, 1954; Aoki and Ito, 2000; Clarkson et al. 2004, 2005; Lin et al. 2001; Lim et al. 2006). With this evolutionary scheme, *N. sylvestris* (2*n*=24 chromosomes) might be considered as one of the ancestors for sections such as Repandae, with 2*n*=24, and Alatae, with 2*n*=20 or 18 (Chase et al. 2003; Clarkson et al. 2004; Lim et al. 2006). I found that *N. sylvestris* did not respond to agroinfiltration of TBSV p41 gene, but the conserved character of resistance elicited by p41 was widely conserved within the species of Alatae, especially in the subclades with the lowest number of chromosomes (2*n*=18). For instance, *N. alata* and *N. langsdorfii* that are considered sister species with 2*n*=18 (Clarkson et al. 2004), only responded with HR to p41 (Fig. IV.5). In contrast, an analysis of the internal transcribed spacer (ITS) and non-transcribed spacer sequences (NTS) of the 5S rDNA suggested that sections Sylvestres (*N. sylvestris*) and Alatae are not sisters, but Sylvestres is the sister of Noctiflorae (i.e. *N. glauca*), and Alatae is the sister of Petunioides (Clarkson et al. 2005). Future studies should focus on whether resistance targeting TBSV p41 can be found within the Petunioides.

Although resistance to p41 predominated in the Alatae, some species in this group also exhibited resistance elicited by TBSV P19 protein. For example, *N. bonariensis* responded to both TBSV p41 and p19 genes, whereas *N. plumbaginifolia* appears to contain a defective version of the R gene that recognizes P19. Lim et al. (2006) noted that despite similarities in rDNA and ITS sequences, substantial chromosome repattering is apparent in the Alatae section, including chromosome numbers, chromosome structure, a high degree of polymorphisms, and the distribution and abundance of repeats. They further suggested that *N. bonariensis* (2*n*=18) is the most highly altered genome, with a higher number of specific repeats. Lin et al. (2001) noted that *N. bonariensis* has a greater amount of nuclear DNA than *N. plumbaginifolia* (2*n*=20), which in turn has a higher nuclear DNA content than *N. sylvestris*. It may be that *N. bonariensis* acquired a copy of the R gene that targets the P19 protein at some point in the past. Another interesting comparison can be seen with *N. plumbaginifolia* and *N. longiflora*, two species that are considered very closely related, with almost identical karyotypes and genetic compatibility (Lin et al. 2001). *N. longiflora* contains the R protein that recognizes the p41 gene, whereas *N. plumbaginifolia* must have lost this gene.

We also found resistance in one member of the Alatae section to TBSV P22. Both TBSV p22 and p41 genes elicited HR in *N. forgetiana* accession TW50. In contrast, HR was elicited by only the p41 gene in the *N. forgetiana* accession TW51. The differential response of these two accessions means that it should be possible to examine the inheritance of resistance triggered by p22. Interestingly, until now, the only *Nicotiana* species that responded to P22 with HR were found in the Undulatae section. Consequently, the origin is of the resistance to tombusvirus P22 is not clear, but it may

have arisen independently in two different hosts after the divergence of *Nicotiana* species.

To conclude, it is possible that during evolution, a series of genetic events involving mutation, translocation, gene duplication, recombination, selection, or deletion, events which are common in the evolution of *Nicotiana* species (Aoki and Ito, 2000; Chase et al. 2003; Clarkson et al. 2004, 2005), played a key role in shaping the resistance to tombusviruses, such that there exists at least five different R genes or alleles. One R gene recognizes P22 proteins in *N glutinosa* (Undulatae section) and an accession of *N. forgetiana* (Alatae section). A second is able to recognize the P19 protein in *N. sylvestris* and in some species of Alatae. A third R gene in *N. tabacum* is a variant that can recognize TBSV P19 and CymRSV P19 proteins, but is unable to recognize the CNV P20. A fourth R gene is present in younger species of the Alatae section and recognizes the TBSV p41 gene as avirulence determinant. Finally, there still remains to be discovered at least one more R gene that is present in HR-resistant species that did not respond to individual agroinfiltration of TBSV genes. We showed that *Nicotiana* species are valuable sources for resistance genes to tombusviruses TBSV, CNV and CymRSV, and that diversity can be explored through agroinfiltration of different viral genes to test known avirulence determinants, such as P22 and P19 proteins, in new hosts, as well as discovering new Avr determinants such as TBSV p41. This strategy also could have value for studying the evolution of resistance genes and their functionality across an entire genus.

## MATERIALS AND METHODS

### Viruses and Plants

Most of the seeds of the *Nicotiana* species were obtained from the U.S. Tobacco Germplasm Collection at North Carolina State University (Lewis and Nicholson, 2007) as follows: *Nicotiana clevelandii* (PI 555491), *N. glutinosa* (PI 555507), *N. tabacum* cv. NC 95 (PI 552380), *N. benthamiana* (PI 555478), *N. edwardsonii* (PI 555704), *N. sylvestris* (PI 555569), *N. tomentosiformis* (PI 555572), and *N. otophora* (PI 555542) *N. repanda* (PI 555552), *N. glauca* (PI 555504), *N. rustica* (PI 55554), *N. arentsii* (PI 555475), *N. undulata* (PI 555574), *N. wigandioides* (PI 302471), *N. langsdorfii* (PI 42337), *N. quadrivalvis* (PI 555485), *N. bonariensis* (PI 555489), *N. longiflora* (PI555531). Seeds of the following *Nicotiana* species belonging to the Alatae section were provided by Dr. Timothy Holtsford from the University of Missouri (Columbia, MO): *N. alata* TW7 (PI 72334) and TW8 (PI 555474 ), *N. forgetiana* TW50 (PI 555501) and TW51 (PI 555502), *N. plumbaginifolia* TW106 (PI 555548) and TW108 (PI 302478), and *N. mutabilis* (T.H. lab 011205-1). To break dormancy, seeds were treated for 30 min with commercial bleach at 50% strength (2.6 % vol/vol NaOCl) (Burke, 1957).

The tombusviruses TBSV, CNV, and CymRSV have all been previously cloned in infectious form (Hearne et al. 1990; Rochon and Johnston, 1991; Grieco et al. 1989). Infectious, uncapped transcripts derived from the clones were made according to Scholthof et al (1993, 1995b), inoculated to either *N. clevelandii* or *N. benthamiana* to

increase inoculum, and infected tissue was frozen for further inoculations. For inoculation of test plants, leaves infected with tombusviruses were ground with a mortar and pestle at a dilution of approximately 1:20 (wt/vol) with inoculation buffer (0.05 M phosphate buffer, pH 7.0), then gently rubbed onto *Nicotiana* leaves lightly dusted with 600 mesh carborundum.

**ELISA tests for Virus Detection**

To evaluate the capacity of tombusviruses to infect *Nicotiana* species, DAS-ELISA (Clark and Adams, 1977) was performed using primary antibodies and alkaline phosphatase conjugates purchased from Agdia ® (Elkhart, IN) for TBSV and CymRSV, and from Acdia Inc. (Fayetteville, AR) for CNV. Plant tissues were collected from upper, non-inoculated leaves between 20 to 28 dpi, and samples were ground and loaded into polystyrene plates at a ratio of 1:10 tissue : grinding buffer (1× phosphate buffered saline, 2% polyvinylpyrrolidone MW 40,000, and 0.2% bovine serum albumin). Colorimetric reactions with the substrate p-nitrophenyl phosphate were quantified at 405 nm using a Multiskan MCC/340 microplate reader (Thermofisher Scientific, Cincinnati, OH).

**Amplification and cloning of tombusvirus genes into the *Agrobacterium tumefaciens* binary vector pKYLX7**

The PCR amplification and cloning of the TBSV and CymRSV p22 and p19 genes, and of the CNV p21 and p20 genes, has been described previously (see chapter III). In addition, each of these genes was cloned into the *Agrobacterium tumefaciens* binary vector pKYLX7 (Schardl et al. 1987) and their expression confirmed (Angel, chapter III).

PCR primers were developed from the nucleotide sequence of the TBSV-cherry isolate (NCBI accession # M21958) to amplify and clone the TBSV p33, p92, and p41 genes, as well as the region of p92 that is not also present in p33. Primer sequences for PCR amplification were synthesized by Integrated DNA Technologies (Coralville, IA). The template used for PCR of the p41 gene was the infectious clone TBSV-100 (Hearne et al. 1990) provided by Herman Scholthof from Texas A&M University (College Station, TX). The p41 gene was cloned using the forward primer 5' – CTCGAGATGGCAATGGTAAAGAGAAACAAC- 3' and the reverse 5' – GAGCTCCTAAATTAGAGAAACATCATTCTG- 3'. To clone the TBSV replicase genes p33 and p92, the template used for PCR was the clone pMal/TBSV/p92 provided by Dr. Peter Nagy from the University of Kentucky (Lexington, KY) (Rajendran and Nagy, 2003), which was based on the mutant TBSV clone pHS-175 (Scholthof et al. 1995c). In the clone pMal/TBSV/p92, the p33 ORF amber termination codon (UAG) has been replaced by a tyrosine codon such that the p92 gene could also be amplified. The p33 gene was amplified using the forward primer 5'-CTCGAGATGGAGACCATCAAGAGAATGATT- 3' and the reverse primer 5' – GAGCTCCTATTTGACACCCAGGGACTCCTG- 3'. The p92 gene was amplified using the same forward primer used for p33 amplification and the reverse primer 5' -

GAGCTCTCAAGCTACGGCGGAGTCGAGGAT- 3'. A construct expressing the region of the p92 gene that does not overlap with p33 was made by developing a forward primer in which the start codon was placed immediately after the amber stop codon of p33. The forward primer for this clone was 5' – CTCGAGATGGGAGGCCTAGTACGTCTACCTGGG- 3' and the reverse primer was the same as that used to amplify the entire p92 gene. This non-overlapping region has the signature motifs of a RNA-dependent RNA polymerases (Rajendran and Nagy, 2003).

Polymerase chain reaction (PCR) conditions were: one cycle at 95°C for 2 min, followed by 35 cycles at 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min; and finally one cycle at 72°C for 10 min. The amplified DNA was subsequently purified by agarose gel elution using a QIAquick Gel Extraction kit (Qiagen Inc., Valencia, CA) and cloned into the *Xho* I-*Sac* I or *Xho* I-*Kpn* I restriction enzyme sites of pGEM-7Zf(+) or into pGEM-T easy (Promega Corp., Madison, WI). Nucleotide sequences of candidate clones were determined to confirm that the PCR reactions did not introduce errors. Sequencing of clones in both orientations was performed by the DNA Core Facility at the University of Missouri (Columbia, MO). After confirming the fidelity of all sequences, the tombusvirus insert was subsequently cloned into the multiple cloning site of the *Agrobacterium tumefaciens* binary vector pKYLX7 (Schardl et al. 1987). *Escherichia coli* transformants were selected on LB media containing kanamycin (50 µg/ml) and tetracycline (12.5 µg/ml) and tested by colony PCR and restriction enzyme digestions for the presence of the tombusvirus insert.

**Agroinfiltrations**

Clones of pKYLX7 containing the tombusvirus genes were mobilized by electroporation (PG200 Progenetor II, Hoefer Scientific Instruments, San Francisco, CA) into *A. tumefaciens* AGL1 strain (Lazo et al. 1991). Transformants were selected on LB medium supplemented with kanamycin (50 µg/ml), tetracycline (12.5 µg/ml), and carbenicillin (50 µg/ml). The induction and infiltration medium was 20 mM MES [monohydrate 2-(N-morpholino) ethanesulfonic acid], 60 mM sucrose, 55 mM glucose, pH 5.4, and 2 mM acetosyringone dissolved into N-N-dimethylformamide. Agroinfiltrations were done when cell growth reached 1.0-1.2 units of optical density at 550 nm. The induced bacterial suspension was infiltrated on the abaxial surface of individual panels of fully expanded leaves of 4-8 week old *Nicotiana* spp, using a needleless plastic syringe. Every experiment was repeated at least three times, four leaves per plant, two plants per replicate.

**Detection of Tombusvirus P19 and P22 proteins**

To confirm that vectors were expressing TBSV genes in plant tissue upon agroinflitration of *Nicotiana* species, western blots were performed for P22 and P19 proteins on agroinfiltrated *N. benthamaniana* leaf panels and compared against a positive control consisting of a TBSV virion-inoculated plant. Proteins from the infiltrated leaves were extracted with 0.05 M phosphate buffer pH 7.0, and total protein concentration in the sample was estimated using the Bradford method (Bradford, 1976) with a Bio-Rad protein assay dye reagent (Hercules, CA). 10 µg of total protein were separated by

standard SDS-PAGE in 12% Tris HCl polyacrylamide ready gels (Bio-Rad, Hercules, CA) in two identical gels; in one gel proteins were visualized by Coomassie stain (Sambrook et al. 1989), whereas the proteins in the second gel were transferred to nitrocellulose and PVDF membranes (Osmonics, Westborough, MA). The antisera against individual TBSV P19 and P22 proteins were kindly provided by Dr. Herman Scholthof from Texas A&M University (College Station, TX), and applied at dilutions of 1:2,000 or 1:5,000. Alkaline phosphatase–conjugated secondary antibodies, either anti-mouse or anti-rabbit (Promega Corp., Madison, WI) were used for tombusvirus protein detection at a dilution of 1:7,500. The immune complexes were visualized by hydrolysis of the substrate tetrazolium-5-bromo-4-chloro-3- indolyl phosphate (BCIP) in the presence of nitro-blue tetrazolium chloride (NBT) (Promega Corp., Madison, WI).

Table IV.1. Response of eighteen *Nicotiana* species to virion inoculation and agroinfiltration of constructs expressing CNV, CymRSV, and TBSV homologous P22 and P19 movement proteins.

| *Nicotiana* spp. | Section | Virion Inoculation | Agroinfiltration | | |
|---|---|---|---|---|---|
| | | | TBSV | CymRSV | CNV |
| *N. glutinosa* | Undulatae | HR | P22 | P22 | P21 |
| *N. quadrivalvis* | Polydicliae | Susceptible | -- | -- | -- |
| *N. clevelandii* | Polydicliae | Susceptible | -- | -- | -- |
| *N. tabacum* | Nicotiana | HR | P19 | P19 | -- |
| *N. edwardsonii* | Undulatae | HR | P22 | P22 | P21 |
| *N. langsdorfii* | Alatae | HR | -- | -- | -- |
| *N. longiflora* | Alatae | HR | -- | -- | -- |
| *N. bonariensis* | Alatae | HR | P19 | -- | -- |
| *N. undulata* | Undulatae | HR | -- | -- | -- |
| *N. repanda* | Repandae | M.C.N.L. | -- | -- | -- |
| *N. glauca* | Noctiflorae | C .L. R. | -- | -- | -- |
| *N. rustica* | Rusticae | HR | -- | -- | -- |
| *N. sylvestris* | Sylvestres | HR | P19 | P19 | P20 |
| *N. arentsii* | Undulatae | HR | -- | -- | -- |
| *N. otophora* | Tomentosae | C .L. R. | -- | -- | -- |
| *N. tomentosiformis* | Tomentosae | C .L. R. | -- | -- | -- |
| *N. wigandiodes* | Undulatae | No Reaction | -- | -- | -- |
| *N. benthamiana* | Suaveolentes | Susceptible | -- | -- | -- |

HR: Hypersensitive Resistance Response C.L.R.: Chlorotic Local Lesions and Resistance
M.C.N.L.: Mixed Chlorotic and Necrotic Lesions

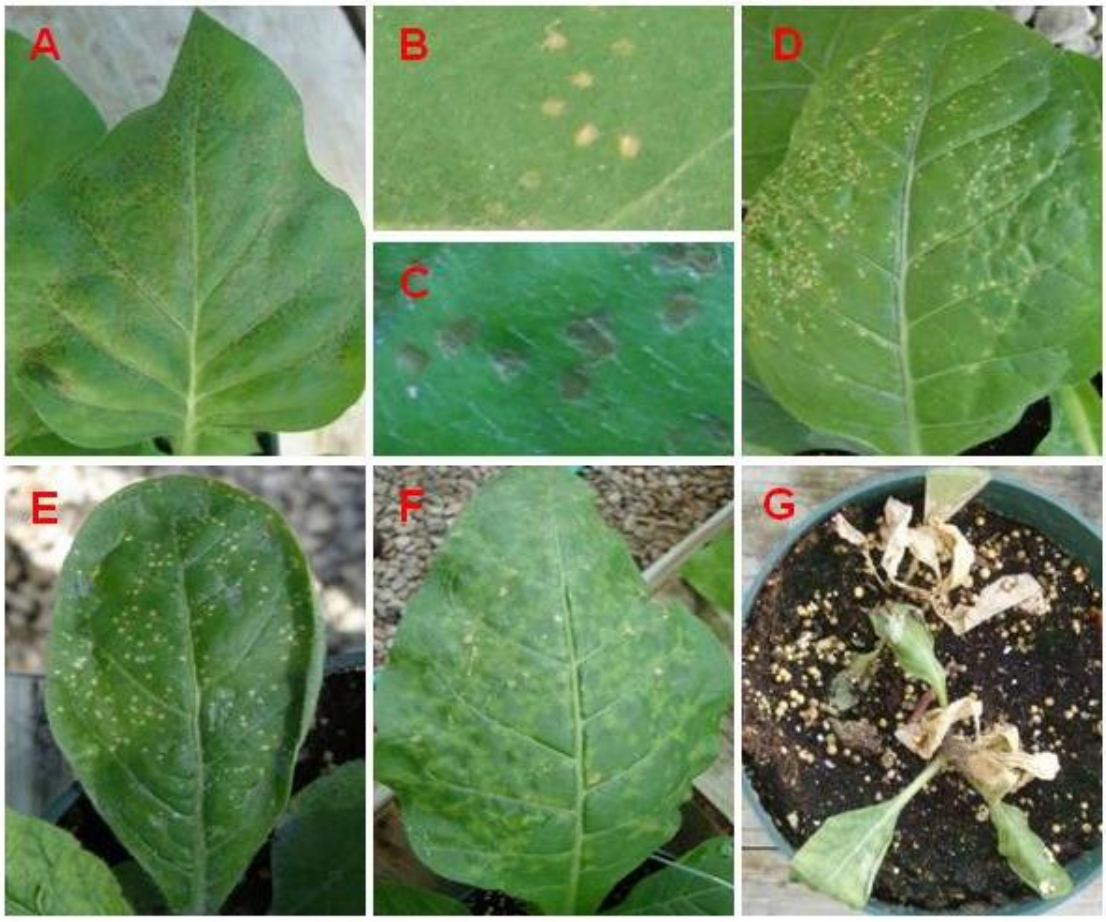



Figure IV.1. Hypersensitive response (HR) and symptoms of *Nicotiana* species to mechanical inoculation of virions of Tomato bushy stunt virus (TBSV), Cymbidium ringspot virus (CymRSV), and Cucumber necrosis virus (CNV): **A**, TBSV in *N. edwardsonii* at 2 dpi. **B**, Close up of HR type 1 lesions elicited by TBSV in *N. edwardsonii* at 2 dpi. **C**, Close up of HR type 2 lesions elicited by TBSV in *N. tabacum* at 3 dpi. **D**, CymRSV in *N. tabacum* at 5 dpi. **E**, TBSV in *N. bonariensis* at 5 dpi. **F**, CNV in *N. tomentosiformis* at 11 dpi. **G**, Systemic necrosis and death of plants produced by CNV in *N. quadrivalvis* (Syn. *N. bigelovii*) at 14 dpi.

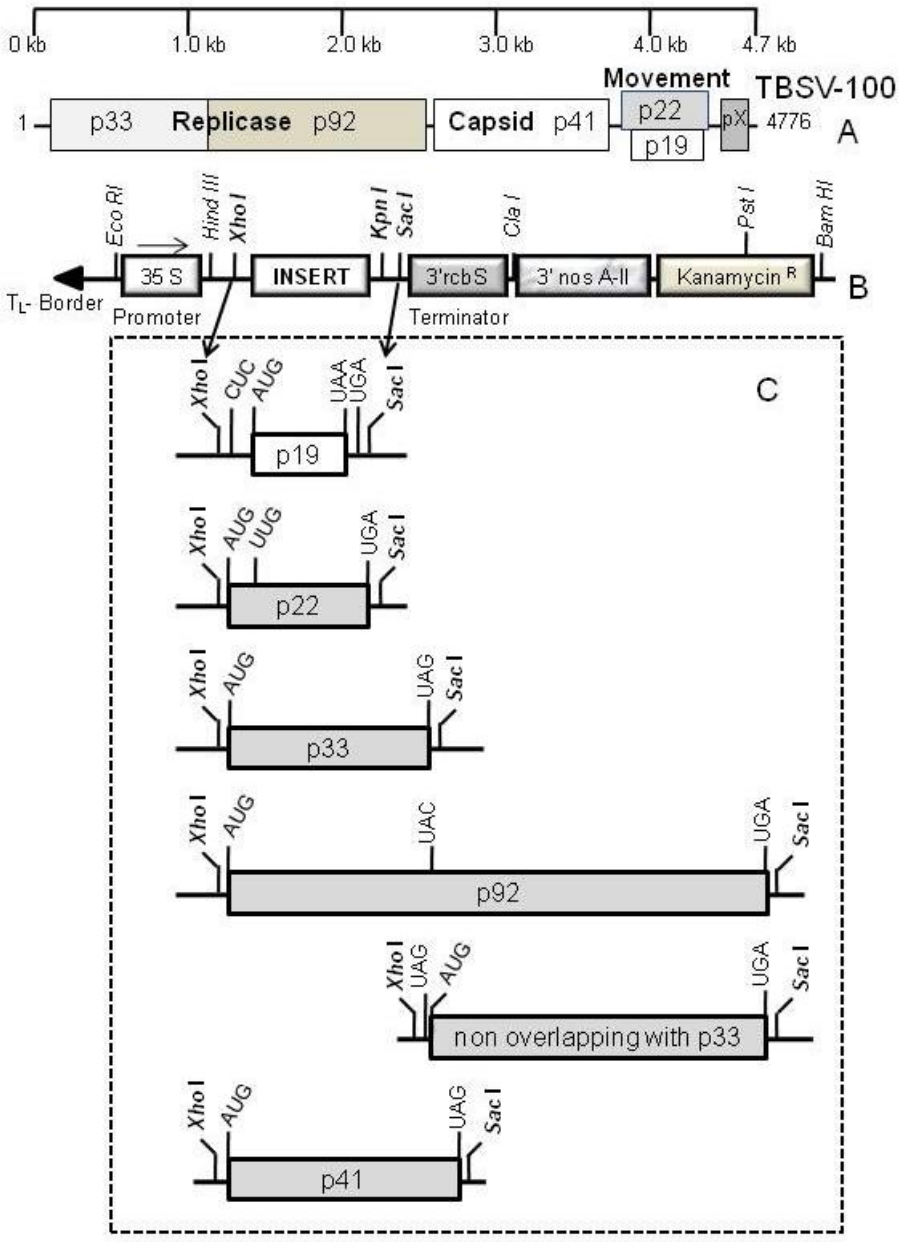



Figure IV.2. Organization of the Tombusvirus type TBSV genome, and T-DNA constructs used for transient expression of TBSV genes in *A. tumefaciens* strain AGL1 vectors. **A**, diagram of TBSV genome in the infectious clone TBSV-100 (Hearne et al. 1990). **B**, pKYLX7 -based construct (~12 kbp; Schardl et al. 1987) for expression of Tombusvirus genes. **C**, Constructs for expression of individual TBSV genes p19, p22, p41, p33, p92, and the non overlapping region of p92 with the p33 genes, which were cloned between the *Xho*I and *Sac*I sites. Cloning of TBSV, CymRSV, and CNV p22 and p19 homolog genes was previously described (see chapter III).

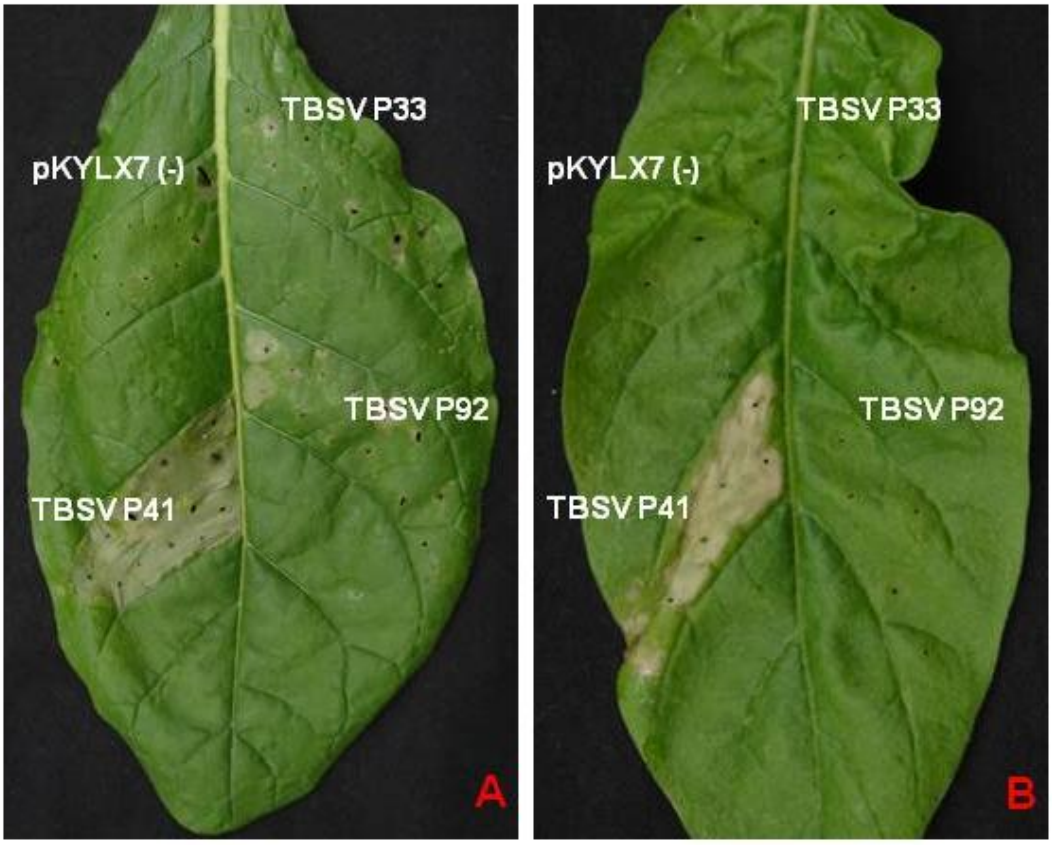



Figure IV.3. Response of **(A)** *N. langsdorfii*, and **(B)** *N. bonariensis* to agroinfiltration of TBSV coat protein gene p41, the replicase genes p33 and p92, and the empty binary vector pKYLX7 (negative control), at 5 dpi.

Table IV.2. Response of *Nicotiana* species within the Alatae section to TBSV virion inoculation and agroinfiltration of individual TBSV genes. *Nicotiana sylvestris* (Sylvestres section) is also included as a relative of Alatae.

| *Nicotiana* spp. Section Alatae | Virion Inoculation | Agro-infiltration |
|---|---|---|
| *N. langsdorfii* | HR | p41 |
| *N. alata* TW 7 | HR | p41 |
| *N. alata* TW8 | HR | p41 |
| *N. forgetiana* TW50 | HR | p41 p22 |
| *N. forgetiana* TW51 | HR | p41 |
| *N. mutabilis* | HR | p41 |
| *N. bonariensis* | HR | p19 p41 |
| *N. longiflora* | HR | p41 |
| *N. plumbaginifolia* TW106 | Susceptible | p19 |
| *N. plumbaginifolia* TW108 | Susceptible | p19 |
| *N. sylvestris* | HR | p19 |

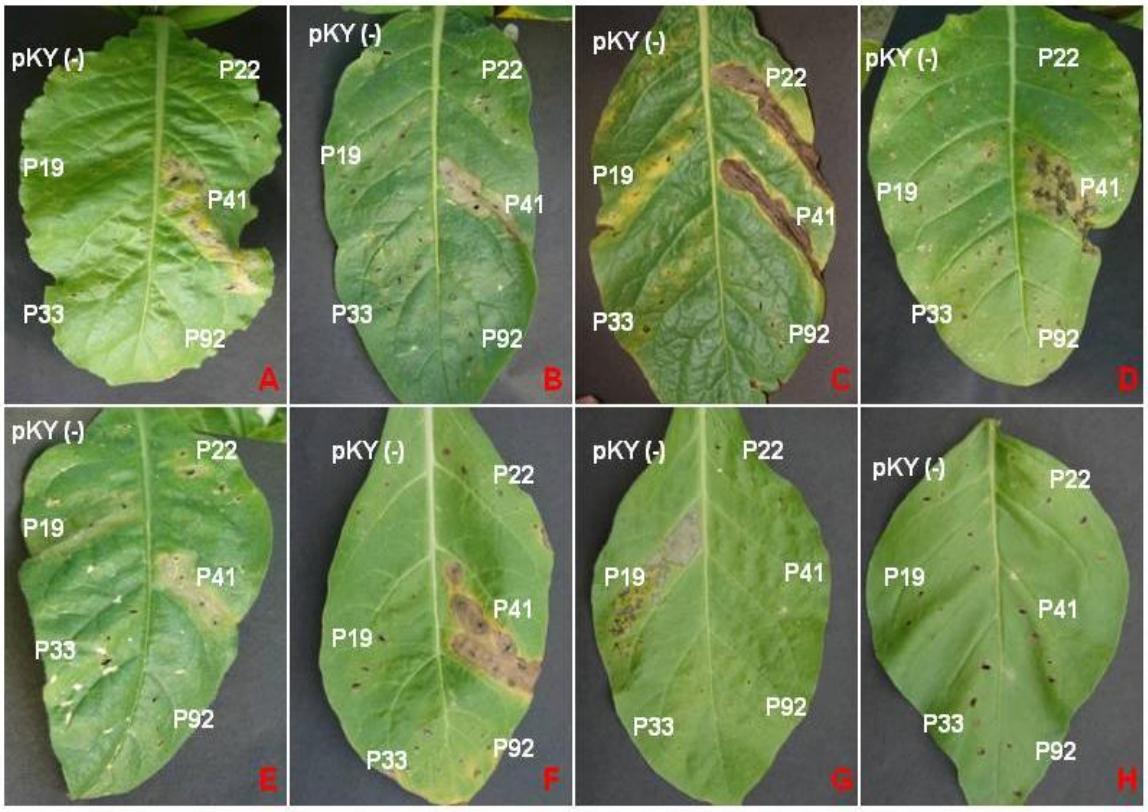
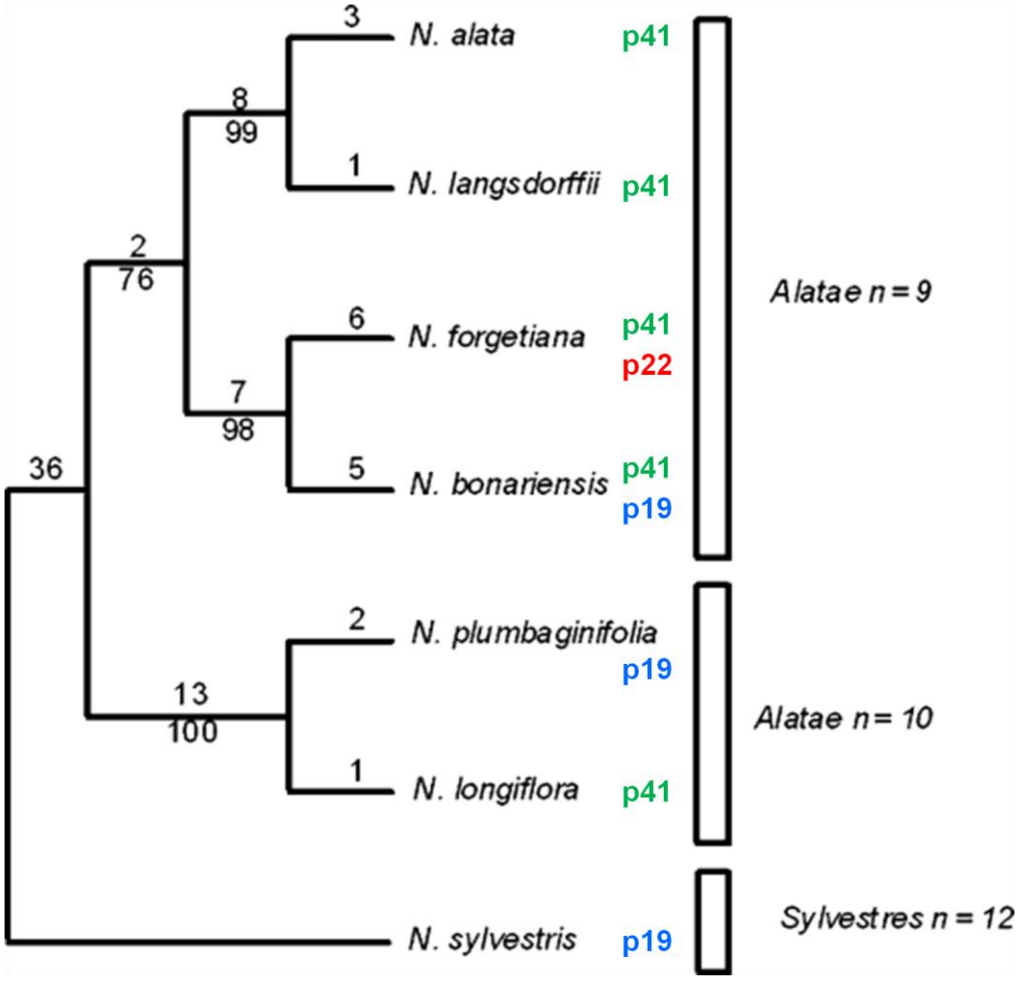



Figure IV.4. Response of *Nicotiana* species within the Alatae section to agroinfiltration of TBSV genes. **A**, *N. alata* TW7 at 5 dpi; **B**, *N. forgetiana* TW51 at 5 dpi; **C**, *N. forgetiana* TW50 at 7 dpi; **D**, *N. mutabilis* at 5 dpi; **E**, *N. bonariensis* at 5 dpi; **F**, *N. longiflora* at 12 dpi; **G**, *N. plumbaginifolia* TW106 at 12 dpi, and **H**, *N. clevelandii* at 12 dpi as negative control. *N. langsdorfii* is illustrated in Fig. IV.3.




Modified from Lim et al. (2006)

Figure IV.5. Phylogeny of *Nicotiana* species section Alatae and its relationship with recognition of TBSV genes as avirulence determinants of HR. Here, we want to illustrate the hypothetical progression in the evolution of resistance to TBSV by recognition of the p19 gene in the putative ancestral species *N. sylvestris*, that is diminished in species with 2*n*=20 chromosomes, and the emergence of resistance elicited by the p41 gene, which is stronger in younger species with 2*n*=18 chromosomes. The basic phylogenetic tree was made by Lim et al. (2006), Plant J. 48: 907–919, as a single most-parsimonius tree of plastid DNA sequence divergence. Used with copyright permission from John Wiley and Sons, Inc.

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## SUMMARY

Resistance to plant viruses has been explained by the "gene for gene" model, which requires the recognition between a resistance (R) gene in the host and an avirulence (Avr) gene in the pathogenic virus, a resistance response that is commonly associated with a programmed cell death or hypersensitive response (HR). Our lab has been studying the resistance to tombusviruses in *Nicotiana* species following this model. On the host side, we previously showed that resistance to tombusviruses can be introgressed from *N. glutinosa* into the susceptible *N. clevelandii* using as a bridge *N. edwardsonii* var. Columbia. The resistance was dominant and effective against a broad range of tombusviruses such as tomato bushy stunt virus (TBSV), cymbidium ring spot virus (CymRSV), cucumber necrosis virus (CNV), and carnation italian ring spot virus (CIRV), but ineffective against other viruses such as tobacco mosaic virus (TMV). In addition, we found that the tombusvirus R gene could be a member of the *N* family of R genes. The *N* gene confers resistance to TMV and is a representative member of the TIR-NBS-LRR resistance proteins (TIR for homology with the *Drosophila* Toll and mammalian Interleukin-1 Receptor gene, NBS for Nucleotide Binding Site, and LRR for Leucine-Rich Repeats), which are present in *Nicotiana glutinosa*. Since *N. glutinosa* is resistant to a large number of viruses, we hypothesized that silencing of the entire *N* family of R genes might alter resistance to viruses other than TMV. To test this hypothesis, we constructed transgenic plants that expressed a hairpin RNA based on exon

1 of the *N* gene and found that they exhibited enhanced susceptibility to TBSV, as well as TMV. Consequently, we have evidence that a TBSV R gene has sequence similarity to the *N* gene. However, there is limited information about how many *N* gene homologs exist in *N. glutinosa*. To examine the diversity of *N* gene homolog sequences (NGHs), we developed polymerase chain reaction (PCR) primers that amplified a 516 bp DNA segment that matched exactly sequences in exon 2 of the *N* gene from *N. glutinosa*, the TMV-susceptible host *N. clevelandii*, and *N. clevelandii* Line 36, a homozygous addition line that is resistant to TBSV and susceptible to TMV. These PCR reactions yielded multiple products that flanked the P-loop and GLPL domains of NBS-LRR resistance proteins. We cloned and sequenced 143 NGHs, and found that 106 encoded complete open reading frames without premature stop codons. Based on alignments and phylogenetic analyses, we detected a broad range of groups: 11 in *N. glutinosa*, 4 in *N. clevelandii*, and 5 in *N. clevelandii* Line 36. One group from *N. glutinosa* matched precisely the *N* gene sequence as expected, whereas other groups differed from the *N* gene by approximately 2 to 19%. After bootstrapping analysis, we consolidated 15 groups of NHGs, including the *N* gene group. Interestingly, a group of NGHs from *N. clevelandii* matched the *N* gene completely, suggesting that an inactive *N* homolog could be present in this TMV-susceptible species. Of the groups isolated from *N. clevelandii* Line 36, none had greater than 89% identity with the N protein. We hypothesized that the R gene against TBSV will be represented in the groups isolated from *N. clevelandii* Line 36, but the evidence suggests that new mining should be done considering that all *N. clevelandii* groups fell into the *N. glutinosa* groups, and the presence of individual outliers in both *N. glutinosa* and *N. clevelandii* Line 36 genotypes.

On the pathogen side, previous studies showed that the TBSV P22 and P19 proteins play an important role in elicitation of symptoms and in triggering plant defenses in *Nicotiana* species, suggesting these two viral movement proteins function as Avr genes in the "gene for gene" resistance model. The TBSV P22 protein elicited a HR in *N. edwardsonii* and *N. glutinosa*, whereas the TBSV P19 protein triggered a HR in *N. tabacum*, when they were expressed from a potato X virus – based vector. In this study, we used *Agrobacterium tumefaciens* – based vectors to express TBSV and CymRSV P22 and P19 proteins, and CNV P21 and P20 proteins. We found that *Nicotiana* species are able to recognize small differences between these tombusvirus P22 and P19 homolog proteins. The TBSV and CymRSV P22, and the CNV P21 proteins elicited HR in *N. glutinosa* and *N. edwardsonii*, with a slight delay and variation for the CymRSV P22. On the other hand, the TBSV P19 and CymRSV P19 proteins elicited HR in *N. tabacum*, but the CNV P20 did not elicit necrosis in this species. However, although all three tombusvirus P19 homologues did function as silencing suppressors in transient co-agroinfiltration assays with green fluorescent protein expressing constructs in *N. benthamiana*. In contrast to previous studies, we did not obtain systemic necrosis induced by tombusvirus P19 homologues, but obtained weak local necrosis after 10 dpi conditioned by environmental conditions. In addition, in the current study, we were able to track the source of HR resistance to tombusviruses in *N. tabacum* to its parental genome *N. sylvestris*, which was elicited by three tombusvirus P19 homologues. Consequently, agroinfiltration is a very useful tool not only for testing specific known R and Avr genes interactions, but it also can be used for a rapid search across plant species to identify sources of resistance and their pathogen avirulence targets.

Based on previous studies and our results using agroinfiltration, we suggested that *Nicotiana* species have different R genes to recognize at least two Avr proteins against TBSV. In addition, we hypothesized that *Nicotiana* species are able to recognize subtle differences between tombusvirus P22 and P19 proteins, resistance that we were able to track to an ancestor genotype. However, little is known about the diversity in resistance to tombusviruses in the entire *Nicotiana* genus, and the capacity of other species to recognize tombusvirus P19 and P22 proteins as Avr determinants. Of 75 *Nicotiana* species, 40 are diploids and 35 are allopolyploids, whose genomes are derived from interspecific recombination between two diploid genomes form ancestral species. To explore this diversity against tombusviruses in a genus with high genetic diversity and evolution events, we initially inoculated virions of TBSV, CNV, and CymRSV to 18 *Nicotiana* species representative of 10 of the 14 taxonomic sections in the genus. We found that 10 species responded with HR, another five *Nicotiana* species were resistant, but this resistance did not involve the classical description of HR, and three species were susceptible. To study the role of TBSV, CymRSV, and CNV P19 and P22 proteins in eliciting HR, we agroinfiltrated constructs to express these proteins into leaves of the resistant *Nicotiana* species, especially those that responded with HR. We confirmed that the three tombusvirus P22 proteins elicited HR only in *N. glutinosa* and *N. edwardsonii*. In contrast, the tombusvirus P19 proteins varied in their role as Avr factors, as the TBSV P19 protein triggered HR in *N. tabacum*, *N. sylvestris*, and *N. bonariensis*, the CymRSV 19 protein triggered HR in *N. tabacum* and *N. sylvestris*, and CNV P20 elicited HR only in *N. sylvestris*. This indicated that *Nicotiana* species can recognize differences in tombusvirus P19 proteins. Furthermore, other tombusvirus proteins might be responsible

for eliciting HR in the other six *Nicotiana* species. To determine if other tombusvirus proteins were capable of triggering HR, we created constructs that would express the TBSV p41 coat protein gene, and the p33 and p92 replicase genes. We found that *N. langsdorfii*, *N. longiflora*, and *N. bonariensis*, all members of the Alatae section, responded to agroinfiltration of the TBSV p41 gene with HR, and no other viral protein/host combination resulted in HR. Thus, we evaluated if the other 4 *Nicotiana* species of the Alatae section that are available could respond with HR to virions and TBSV p41 as well. We found that all species and tested accessions responded with HR to virion inoculation, but only *N. plumbaginifolia* was systemically susceptible. On the other hand, *N. alata* accession TW7 and TW8, *N. forgetiana* accession TW51, and *N. mutabilis* responded with HR to the p41 gene; *N. forgetiana* TW50 responded to both p41 and p22 genes, and *N. plumbaginifolia* TW106 and TW108 responded to p19 only.

To conclude, this study showed that *Nicotiana* species are valuable sources for at least 5 R genes against tombusviruses. In addition to the tombusvirus P22 and P19 homolog proteins, we found that the TBSV coat protein gene p41 is also an avirulence determinant, whose recognition is conserved in most species within the section Alatae. Furthermore, we found that *Nicotiana* species are able to recognize small differences in avirulence determinants, and agroinfiltration can be used to rapidly survey a broad cross section of a genus to identify new sources of resistance and new avirulence targets.

# VITA

Carlos Ariel Angel-Calle was born October 8, 1973 in Samaná (Caldas, Colombia – South America), but lived most of his life in Manizales (Caldas – Colombia); the son of Hector Fabio Angel-Serna, who died in 1987, and Bárbara R. Calle-Bernal., who is still alive. He has two younger brothers, Luis Fernando, who is an industrial engineer, and Ricardo Andres, who is a civil engineer. He graduated from high school in Manizales at the Colegio Mayor de Nuestra Señora in 1989, and attended the Agronomy program at the Universidad de Caldas in Manizales from 1990 to 1995. He began his bachelor research thesis at Colombia's National Coffee Research Center (Cenicafé) in 1995 as an Undergraduate Fellow in Plant Pathology. In 1998 Carlos A. received his Agronomy Engineering bachelor degree and continued working at Cenicafé as a Young Scientist Fellow until 2000 when he joined the faculty as a Research Assistant in the Plant Pathology Department. In 2006 he was promoted to the Scientific Researcher-I position. In August 2004 he began his work as Graduate Research Assistant at the University of Missouri – Columbia, towards his Ph.D. in the Plant Microbiology and Pathology Program Area, under the direction of Dr. James E. Schoelz. Upon completion of his Ph.D. in December 2009, he will stay as a postdoctoral research fellow with Dr. Schoelz in the Division of Plant Sciences at the University of Missouri – Columbia. Carlos A. still holds a faculty position at Cenicafé under a non-paid leave.